Physiological consequences of exposure to perfluoroalkyl substances, organochlorine compounds and mercury in an Arctic breeding seabird

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Presented by **Pierre BLÉVIN**

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Physiological consequences of exposure to perfluoroalkyl substances, organochlorine compounds and mercury in an Arctic breeding seabird

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THESE DE DOCTORAT DE L'UNIVERSITE DE LA ROCHELLE

Ecole doctorale EUCLIDE
Préparée au Centre d'Etudes Biologiques de Chizé, UMR 7372

Présentée par **Pierre BLÉVIN**

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Pour obtenir le grade de docteur de l'Université de La Rochelle
Spécialité: Biologie de l'Environnement, des Populations, Ecologie

Conséquences physiologiques d'une exposition aux substances perfluoroalkylées, aux composés organochlorés et au mercure chez un oiseau marin Arctique

Directeurs de thèse: **Olivier Chastel** & **Paco Bustamante**

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A - Preface

1 - Acknowledgments

First of all I would like to thank Sophie Bourgeon, Aurélie Goutte, Sandrine Meylan, Heli Routti, Robert Letcher and Jonathan Verrault for accepting to judge my thesis.

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Thank you to the numerous fieldworkers for collecting data and for the nice memories on the field (Olivier Chastel, Frédéric Angelier, Sabrina Tartu, David Costantini, Fabrice Helfenstein, Tomas Taylor, Bruno Michaud, Ségolène Humann-Guilleminot, Hugh Ellis and Scott Shaffer).

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Maxime, Nathan, Candice, Céline), et la team d'Aventures Australes (Florian, Valentin, Elie et Romain).

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To my parents...

2 - Abstract

Due to increasing human activities, a growing number of threats are challenging the fate of biodiversity. Among them, environmental contamination is particularly concerning for living organisms. Used and released in industrialized countries, these highly persistent contaminants can reach remote areas such as the Arctic ecosystem and will biomagnify though food webs and bioaccumulate in organisms. Long-lived seabirds are located in the upper levels of the food chains and thus particularly exposed and sensitive to a chronic contaminants exposure. Through endocrine disruption, these contaminants can impact physiological mechanisms and behavioural traits, inducing *in fine*, long-term fitness consequences on individuals and populations. My thesis focuses on three groups of contaminants: (i) poly- and perfluoroalkyl substances (PFASs), still broadly used in a vast array of industrial processes and increasing in the Arctic; (ii) "legacy" organochlorine contaminants (OCs, pesticides and industrial compounds), banned from use but still well present in the Arctic and (iii) mercury (Hg), a non-essential metal coming of both natural and anthropic origins.

Based on a correlative approach conducted *in natura*, I investigated the physiological and behavioural consequences of exposure to these contaminants during the whole breeding cycle (from pre-laying to chick-rearing period) in an Arctic seabird, the black-legged kittiwake (*Rissa tridactyla*) from Svalbard, Norwegian Arctic. Specifically, I examined the relationships between several PFASs, OCs, Hg and fertility (sperm morphology and motility), sexual signaling (visual: integument coloration and olfactory: chemical signature), parental care behaviors (incubation temperature and egg-turning), ageing (telomere length) and energy expenditure (basal metabolic rate). In addition, some potential underlying mechanisms were also studied to better understand the way through which contaminants can be detrimental for kittiwakes. Furthermore, since physiological mechanisms and behavioral traits investigated here are tightly involved in self maintenance and reproduction, possible effects on fitness are then discussed. This PhD work underlines the major role of certain legacy chlorinated organic compounds (e.g. chlordanes) and significantly contributes at documenting the poorly known toxicological consequences of PFASs exposure in wildlife. Importantly, this PhD shows that PFASs and OCs could impact ageing, energy expenditure and some parental care behaviors in a contrasted manner. Specifically, oxychlordane, a metabolite of a banned organochlorine pesticide was associated with decreased telomere length, lowered metabolic rate and reduced ability to incubate the eggs. Conversely, elongated telomere, increased BMR and enhanced egg rotation were observed in birds bearing the highest concentrations of PFASs. Finally, at least for the considered endpoints, Hg appears as a coming minor threat for kittiwakes. This study highlights the importance of considering several groups of contaminants when investigating the consequences of environmental contaminants exposure in wildlife.

Key-words: Organic contaminant, Trace element, Black-legged kittiwake, Arctic, Ecotoxicology, Ecophysiology

3 - Résumé

A cause d'une anthropisation toujours plus forte des écosystèmes, de plus en plus de menaces pèsent sur la biodiversité. Parmi celles-ci, l'exposition aux contaminants est particulièrement problématique pour les organismes vivants. Emis et utilisés dans les pays industrialisés, ces contaminants hautement persistants dans l'environnement vont gagner les régions polaires puis se bio-accumuler dans les organismes vivants au cours du temps et se bio-amplifier le long du réseau trophique. Ainsi, les oiseaux marins, longévifs et situés dans les maillons supérieurs de la chaine alimentaire, sont particulièrement exposés et vulnérables à une exposition chronique à ces contaminants. A travers une perturbation endocrinienne, ces contaminants vont pouvoir impacter certains mécanismes physiologiques et traits comportementaux, entrainant *in fine* des conséquences à long-terme sur la fitness des individus et populations. Ma thèse s'articule autour de trois grandes familles de contaminants ; i) les composés perfluoroalkylés (PFASs), encore largement utilisés dans plusieurs secteurs industriels et agricoles et en augmentation dans l'environnement ; (ii) les composés organochlorés dits « d'héritage » (OCs), interdits depuis des années mais entrainants toujours des effets délétères sur la biodiversité et (iii) le mercure (Hg), métal lourd non-essentiel ayant une origine à la fois anthropique et naturelle.

Basé sur une approche corrélative *in natura*, je me suis intéressé aux conséquences physiologiques et comportementales d'une exposition chronique à ces trois grandes familles de contaminants présents chez la mouette tridactyle (*Rissa tridactyla*) de l'Arctique Norvégien (Svalbard) au cours de son cycle reproducteur (depuis l'accouplement jusqu'à l'élevage des poussins). Spécifiquement, j'ai étudié les relations entre ces contaminants et la fertilité (morphologie et motilité des spermatozoïdes), l'expression des signaux sexuels (visuel : coloration des téguments, olfactif : signature chimique), les comportements de soins parentaux (température d'incubation et rotation de l'œuf), le vieillissement cellulaire (longueur des télomères) et la dépense énergétique (métabolisme de base). Je me suis également penché sur de potentiels mécanismes sous-jacents permettant d'expliquer ces relations. Puisque ces mécanismes physiologiques et comportementaux sont fortement impliqués dans la valeur sélective des individus, les possibles conséquences à long terme de cette exposition sur la reproduction et survie des individus sont discutées. Ce travail permet de souligner la forte toxicité de certains composés organochlorés « historiques » (en particulier les chlordanes) et d'apporter de toutes nouvelles connaissances sur la toxicité très mal connue des PFASs chez la faune sauvage. Fait important, ce travail de thèse révèle que les PFASs et les OCs pourraient agir de manière contrastée sur plusieurs mécanismes physiologiques et traits comportementaux. Spécifiquement, une forte exposition à l'oxychlordane, un métabolite du chlordane, pesticide interdit depuis des décennies, est associée à des télomères plus courts, une réduction du métabolisme de base et à une moindre capacité à incuber les œufs. A l'inverse, on observe une élongation des télomères, une augmentation du métabolisme de base et une rotation des œufs accrue chez les individus les plus exposés aux PFASs. Le Hg, au moins en ce qui concerne les paramètres étudiés, ne semble pas jouer un rôle majeur. Cette étude souligne l'importance de tenir compte de plusieurs groupes de contaminants lorsqu'on étudie les conséquences de l'exposition aux contaminants environnementaux chez la faune sauvage.

Mots-clés : Contaminants organiques, Métaux lourds, Mouette tridactyle, Arctique, Ecotoxicologie, Ecophysiologie

4 - Publications included in the thesis

This thesis is mainly based on the following papers that are included within the text **in bold and by roman numerals**. These publications are attached at the end of the manuscript.

I. **Blévin, P.**, Angelier, F., Tartu, S., Bustamante, P., Herzke, D., Moe, B., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., 2017. Perfluorinated substances and telomeres in an Arctic seabird: Cross-sectional and longitudinal approaches. **Environmental Pollution** 230, 360–367.

II. **Blévin, P.**, Angelier, F., Tartu, S., Ruault, S., Bustamante, P., Herzke, D., Moe, B., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., 2016. Exposure to oxychlordane is associated with shorter telomeres in arctic breeding kittiwakes. **Science of the Total Environment** 563, 125–130.

III. **Blévin, P.**, Tartu, S., Ellis, H.I., Chastel, O., Bustamante, P., Parenteau, C., Herzke, D., Angelier, F., Gabrielsen, G.W., 2017. Contaminants and energy expenditure in an Arctic seabird: Organochlorine pesticides and perfluoroalkyl substances are associated with metabolic rate in a contrasted manner. **Environmental Research** 157, 118–126.

IV. **Blévin, P.**, Shaffer, S., Bustamante, P., Angelier, F., Picard, B., Herzke, D., Moe, B., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., Organochlorines, perfluoroalkyl substances, mercury and egg incubation temperature in an Arctic seabird: insight from data loggers. **In press in Environmental Toxicology and Chemistry**.

V. **Blévin, P.**, Shaffer, S., Bustamante, P., Angelier, F., Picard, B., Herzke, D., Moe, B., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., Contaminants and parental care in an Arctic seabird: Dissimilar associations of perfluorolkyl substances and organochlorine compounds with egg-turning behaviors. **In preparation for Environmental Science & Technology**.

VI. **Blévin, P.**, Tartu, S., Angelier, F., Leclaire, S., Bustnes, J.O., Moe, B., Herzke, D., Gabrielsen, G.W., Chastel, O., 2014. Integument colouration in relation to persistent

organic pollutants and body condition in arctic breeding black-legged kittiwakes (*Rissa tridactyla*). **Science of the Total Environment** 470, 248–254.

VII. Costantini, D., **Blévin, P**., Herzke, D., Moe, B., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., Higher oxidative damage and lower antioxidant defenses in an Arctic seabird exposed to long-chain perfluorinated carboxylates. **In minor revision in Environmental Research.**

VIII. Angelier, F., Costantini, D., **Blévin, P**., Chastel, O., 2018. Do glucocorticoids mediate the link between environmental conditions and telomere dynamics in wild vertebrates? A review. **General and Comparative Endocrinology** 256, 99–111.

5 - Other publications related to the thesis

Other publications related to the thesis are included within the text in *italic font and underlined*.

1. Sire, J., **Blévin, P**., Bustamante, P., Angelier F., Goutte, A., Tartu, S., Clément-Chastel, C., Moe, B., Bustnes, J.O., Bech, C., Gabrielsen, G.W., Chastel, O., Seasonal variations of blood mercury concentrations in an Arctic seabird, the black-legged kittiwake (*Rissa tridactyla*). **In preparation for Polar Biology.**

2. Guilleminot-Humann, S., **Blévin, P**., Azou-Barré, A., Yacoumas, A., Gabrielsen, G.W., Chastel, O., Helfenstein, F., 2018. Sperm collection in black-legged kittiwakes and characterization of sperm velocity and morphology. **Avian Research** 9:24, 1–12.

3. Haar, A., Hylland, K., Eckbo, N., Gabrielsen, G.W., Herzke, D., Bustnes, J.O., **Blévin, P**., Chastel, O., Moe, B., Hanssen, S.A., Sagerup, K., Borgå, K., 2017. DNA damage in Arctic seabirds: Baseline, sensitivity to a genotoxic stressor and association to organohalogens contaminants. **Environmental Toxicology and Chemistry**, 37, 1084–1091.

4. Tartu, S., Bustamante, P., Angelier, F., Lendvai, Á.Z., Moe, B., **Blévin, P**., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., 2016. Mercury exposure, stress and prolactin secretion in an Arctic seabird: an experimental study. **Functional Ecology** 30, 596–604.

5. Tartu, S., Lendvai, Á.Z., **Blévin, P**., Herzke, D., Bustamante, P., Moe, B., Gabrielsen, G.W., Bustnes, J.O., Chastel, O., 2015. Increased adrenal responsiveness and delayed hatching date in relation to polychlorinated biphenyl exposure in Arctic-breeding black-legged kittiwakes (*Rissa tridactyla*). **General and comparative endocrinology** 219, 165–172.

6. Tartu, S., Gabrielsen, G.W., **Blévin, P**., Ellis, H.I., Bustnes, J.O., Herzke, D., Chastel, O., 2014. Endocrine and fitness correlates of long-chain perfluorinated carboxylates exposure in Arctic breeding black-legged kittiwakes. **Environmental Science & Technology** 48, 13504–13510.

6 - Other scientific involvements & vulgarization during the thesis

a) Conference participation (oral communication)

- Contaminants and telomeres in an Arctic seabird: Organochlorine pesticides and perfluoroalkyl substances are associated with telomere length in a contrasted manner. **Telomere dynamics conference 2017**, Edinburgh, Scotland (3-5 October). Blévin, P., Angelier, F., Tartu, S., Ruault, S., Bustamante, P., Herzke, D., Moe, B., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O.

- Contaminants and energy expenditure in an Arctic seabird: Organochlorine pesticides and perfluoroalkyl substances are associated with basal metabolic rate in a contrasted manner. **SETAC Europe 2017**, Brussels, Belgium (7-11 May). Blévin, P., Tartu, S., Ellis, H.I., Chastel, O., Bustamante, P., Parenteau, C., Herzke, D., Angelier, F., Gabrielsen, G.W.

- Legacy POPs, PFASs, Hg and kittiwakes: a multidisciplinary approach. **Kittiwake meeting 2016**, Chizé, France (19-20 May). Blévin, P., Angelier, F., Tartu, S., Bustamante, P., Herzke, D., Moe, B., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O.

- Exposure to oxychlordane is associated with shorter telomeres in arctic breeding kittiwakes. **Arctic Frontier Conference 2016**, Tromsø, Norway (24-29 January). Blévin, P., Angelier, F., Tartu, S., Ruault, S., Bustamante, P., Herzke, D., Moe, B., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O.

- Exposure to oxychlordane is associated with shorter telomeres in arctic breeding kittiwakes. **Animal Ecophysiology Conference 2015**, La Rochelle, France (4-6 November). Blévin, P., Angelier, F., Tartu, S., Ruault, S., Bustamante, P., Herzke, D., Moe, B., Bech, C., Gabrielsen, G.W., Bustnes, J.O., Chastel, O.

b) Conference organization

- Co-organizer of the **13th international student conference "Ecology and Behavior"**, 2017, Chizé, France (19-23 June).

c) Reviewing activity

- Journal of ornithology

d) Grants

- Young scientist grant (for stay cost) from the telomere dynamic conference, 2017.

- Grant mobility from the University of La Rochelle, 2016, 3 000 euros.

- Arctic Field Grant from the Research Council of Norway, 2016, ~7 000 euros.

e) Teaching & communication

- Co-supervisor of two MSc students (1 & 6 month), 2017.

- Supervisor of a group of MSc students during one week for a research project, 2016.

- French Polar week, APECS France (webinar, recreational activities for kids…), 2015.

7 - A collaborative and multidisciplinary work

a) French-Norwegian collaboration

This PhD is a 3-years project in the framework of a long-term research program (P. 330; Ornitho-Endocrino), scientifically coordinated by Olivier Chastel and supported by the French Polar Institute (IPEV). This multidisciplinary research program, gathering ecotoxicologists, ornithologists, ecologists, ecophysiologists and environmental chemists, aims at investigate physiological and demographic consequences of hazardous chemicals exposure in an Arctic seabird, the black-legged kittiwake. This PhD illustrates well the strong and long-term collaboration between French and Norwegian institutions (Figure 1) including the Centre d'Etudes Biologiques de Chizé (CEBC-CNRS; **Olivier Chastel**, **Frédéric Angelier** and **Sabrina Tartu**), the institute Littoral Environnement et Sociétés (LIENSs-University of La Rochelle; **Paco Bustamante**), the Norwegian Institute for Nature Research (NINA; **Jan Ove Bustnes** and **Børge Moe**), the Norwegian Polar Research Institute (NP; **Geir Wing Gabrielsen**), the Norwegian Institute for Air Research (NILU; **Dorte Herzke**) and the Norwegian University of Science and Technology (NTNU; **Claus Bech**).

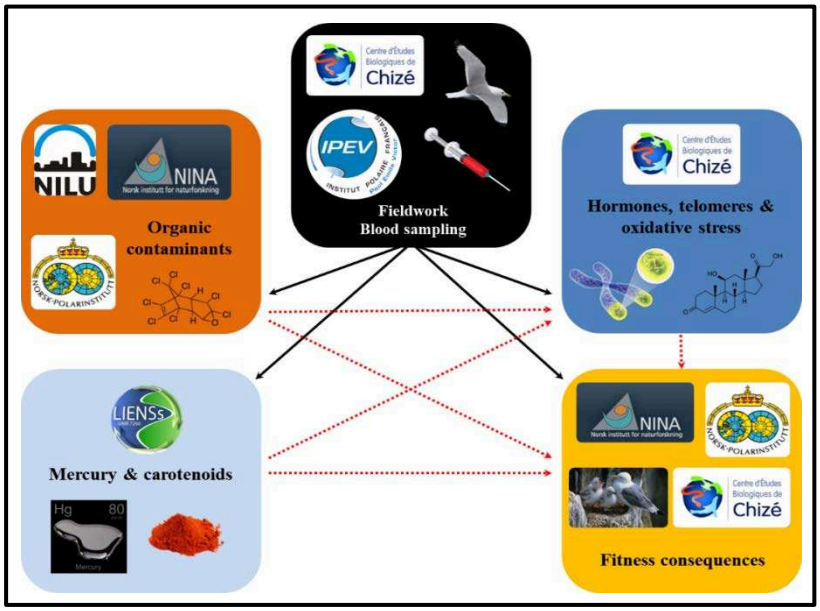


Figure 1: Diagram of the French-Norwegian collaboration during the thesis.

b) Other collaborations

Other fruitful collaborations have been undertaken all along this PhD in order to enlarge the fields of interest and broaden the scientific questions addressed in the thesis (Figure 2). This collaborative work includes **Sarah Leclaire** (Laboratoire Evolution & Diversité Biologiques, CNRS, Toulouse, France) and **Claire Doutrelant** (Centre d'Ecologie Fonctionelle et Evolutive, CNRS, Montpellier, France), **Charlotte Récapet** (LIENSs-University of La Rochelle, France), **Hugh I Ellis** (University of San Diego, USA), **Scott Andrew Shaffer** (San José State University, USA), **Fabrice Helfenstein** (University of Neuchâtel, Switzerland) **and David Costantini** (Natural History Museum of Paris, France).

Figure 2: Other research institutions I have collaborate with during the thesis.

c) Field and lab work contributions

This thesis is a result of a constructive and efficient teamwork that required man-power (e.g. fieldworkers, technicians, students…) all along the PhD. Please find below an overview of the different participants involved in field and lab activities (excluding research funding, team management and coordination, protocol setting-up, fieldwork logistic, statistical analysis and paper writing). It is worth noting that only contributions directly related to the PhD are reported here.

2011	*Fieldwork*	F. Angelier; O. Chastel; S. Tartu
	Contaminant analysis	NILU staff
	Coloration data pre-treatment	**P. Blévin**
2012	*Fieldwork*	F. Angelier; **P. Blévin**; O. Chastel; Hugh. I. Ellis; S. Tartu
	Contaminant analysis	M. Brault-Favrou; C. Churlaud; L. Hanssen
	Molecular sexing & hormone assay	C. Parenteau; S. Ruault; C Trouvé
	Telomere analysis	**P. Blévin**; S. Ruault
2014	*Fieldwork*	A. Ask; F. Angelier; O. Chastel
	Telomere analysis	**P. Blévin**; S. Ruault
	Contaminant analysis	L. Hanssen
2015	*Fieldwork*	F. Angelier; **P. Blévin**; O. Chastel; S. Shaffer; T. Taylor
	Egg logger data processing	B. Picard
	Contaminant analysis	**P. Blévin**; M. Brault-Favrou ; A. Haarr
	Molecular sexing & hormone assay	C. Parenteau; S. Ruault; C Trouvé
2016	*Fieldwork*	**P. Blévin**; O. Chastel; D. Costantini; S. Humann-Guilleminot; B. Michaud
	Coloration data pre-treatment	V. Esteve
	Chemical analysis preen feathers	V. Esteve
	Sperm characterization	A. Azou-Barré; A. Yacoumas
	Contaminant analysis	**P. Blévin**; M. Brault-Favrou
	Molecular sexing & hormone assay	C. Parenteau; C. Ribout; C Trouvé
	Oxidative stress analysis	D. Costantini
	Carotenoid analysis	A. Bonnet; C. Récapet
2017	*Fieldwork*	O. Chastel; F. Helfenstein; S. Humann-Guilleminot

I would like also to mention Julien Sire (Master student). Julien performed numerous mercury and isotope analyses in the lab (with Gaël Guillou, lab tehnician) and worked on the short (within a breeding cycle) and long-term (between years) temporal trends of Hg contamination levels in kittiwakes.

d) Financial supports

P. Blévin was funded by a 3-years PhD grant from **University of La Rochelle**. The project was principally supported by **the French Polar Institute** (IPEV P. 330 to O. Chastel), **the Agence Nationale de la Recherche** (ANR POLARTOP 10-CESA-0016 to O. Chastel; ANR ILETOP 16-CE34-0005 to P. Bustamante), the CNRS (project EC2CO on PFASs) and **the Research Council of Norway** (RCN, AVITOX project n° 234423 to J.O Bustnes; Arctic Field Grant 1786 to S. Tartu in 2012 and Arctic Field Grant 256934 to P. Blévin in 2015; Figure 3). The **"Région Nouvelle Aquitaine"** and the **"Département des Deux-Sèvres"** have financially contributed to the acquisition of some laboratory equipment. P. Blévin received also a mobility grant from **University of La Rochelle**.

Figure 3: Principal funders of the thesis project.

8 - Abbreviations

Institutions

AMAP	Arctic Monitoring Assessment Programme
CEBC	Centre d'Etudes Biologiques de Chizé
IPEV	Institut Polaire Français Paul-Emile Victor
LIENSs	Institut Littoral Environnement et Sociétés
NILU	Norwegian Institute for Air Research
NINA	Norwegian Institute for Nature Research
NIST	National Institute of Standards & Technology
NP	Norwegian Polar Research Institute
NTNU	Norwegian University of Science and Technology
US EPA	United States Environmental Protection Agency

Others

BCI	Body Condition Index
BMR	Basal Metabolic Rate
CBP	Central Brood Patch
CHL	Chlordane
CYP	Cytochrome P_{450}
ITSs	Interstitial Telomere Sequences
LBP	Left Brood Patch
LH	Luteinizing Hormone
PC1	First Principal Component
PCA	Principal Component Analysis
RBCs	Red Blood Cells
RBP	Right Brood Patch
ROS	Reactive Oxygen Species
SMI	Scaled Mass Index
SRM	Standard Reference Material
T3	Triiodothyronine
T4	Thyroxine
T_a	Ambiant temperature
T_b	Body temperature
TH	Thyroid Hormones
T_{inc}	Incubation temperature
T_{min}	Minimal incubation temperature
TRF	Telomere Restriction Fragment
TTR	Transthyretin
WSC	West Spitzbergen Current

Contaminans

c-nona	*Cis*-nonachlor
DDT	Dichlorodiphenyltrichloroethane
FTOH	Fluorotelomer alcohols
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
Hg	Mercury
Hg^0	Elemental mercury
Hg^{II}	Oxydized Hg
Me-Hg	Methylmercury
OCPs	Organochlorine pesticides
OCs	Organochlorines
OXY	Oxychlordane
p,p'-DDE	Dichlorodiphenyldichloroethylene
PCBs	Polychlorinated biphenyls
PFASs	Poly- and perfluoroalkyl substances
PFCAs	Perfluoroalkyl carboxylic acids
PFSAs	Perfluoroalkyl sulfonic acids
PFDcA	Perfluorodecanoate
PFDoA	Perfluorododecanoate
PFHxS	Perfluorohexanesulfonate
PFNA	Perfluorononanoate
PFOA	Perfluorooctanoate
PFOS	Perfluorooctanesulfonate
PFTeA	perfluorotetradecanoate
PFTrA	Perfluorotridecanoate
PFUnA	Perfluoroundecanoate
POPs	Persistent Organic Pollutants
t-chl	*Trans*-chlordane
t-nona	*Trans*-nonachlor

9 - Terminology

"*Ecotoxicology"* is a fairly new discipline first introduced by René Truhaut in 1969 (Truhaut, 1977). Ecotoxicology derived from the words "*ecology"* and *"toxicology"* and can be roughly defined as the study of chemicals in the environment (e.g. origins, fates, effects, levels, trends…; Sparling, 2017). Specifically, ecotoxicology refers in the present thesis to the study of chemicals' effects upon ecosystems including consequences on individual and population levels (Walker et al., 2012).

"Contaminants" and *"pollutants"* are often mixed-up in the literature because the difference between both terms is subtle and highly dependent of the considered scale. Both contaminants and pollutants are chemicals that exist at levels above those that would normally occur in the environment (Walker et al., 2012). However, a pollutant is a contaminant for which there is clear evidence of harmful effects at environmentally realistic concentrations (Walker, 2014). For example, any synthetic chemicals (e.g. pesticides) present in the environment are considered as contaminants. When these contaminants are toxic for wildlife, they are considered as pollutants. Nevertheless, for simplicity reasons, whether or not a contaminant is a pollutant, the term contaminant is used as a general designation in the present thesis.

B - Introduction

1 - Environmental contaminants: a worldwide preoccupation

The industrial revolution at the end of the 18th century marked a start point of an exponentially increasing global-scale human footprint on Earth. Such ability of contemporary human civilization to influence the environment has led to the emergence of the term "Anthropocene" (Crutzen, 2002; Crutzen and Stroemer, 2000). This concept, proposed in 2000s suggests (i) that the Earth is now moving out of its current geological epoch, called the Holocene and (ii) that human activity is largely responsible for this exit, and so, that humankind has become a global geological force in its own right (Steffen et al., 2011). Between 1800 and 2000, energy use increaed by about 40-fold and economic production by 50-fold (Mc Neil, 2001). Similarly to the numerous existing indicators of human activity showing steep and fast growth, the population demographic explosion illustrates well the rate at which human footprint is expanding (Steffen et al., 2006a). Specifically, this explosion increased sharply after World War II, in mid-20th century, a period also called the "Great Acceleration" during which worldwide population increased by more than 50% (Hibbard et al., 2006; Steffen et al., 2011, 2015). Such an exponential rise in human activities logically induces a substantial increase of human needs in a world with limiting resources. Fatally, such overconsumption is likely driving the sixth major extinction event in Earth history (Steffen et al., 2006a, 2015).

Industrialization and burgeoning chemistry during the 20th century, for the Great War first and then toward the civil economy, have concomitantly led the synthesis and release of a huge amount of chemicals into the environment (Steffen et al., 2011, 2015). Such chemicals, broadly used in various business lines (e.g. agriculture, new technologies, cosmetic and textile industries) are ubiquitous in our everyday lives. Empirically suspected as being potentially harmful for living organisms, several chemicals like organochlorine pesticides (OCPs) have been put in the spotlight of toxicological studies. A progressive global awareness has rapidly emerged from the scientific community results highlighting some toxic effects on human health, laboratory animals and wildlife. In that context, environmental contaminants have become a worldwide preoccupation, well-illustrated by the Stockholm convention, adopted in 2001 (amendments in 2009, 2011, 2013, 2015) and today ratified by 182 parties. The Stockholm Convention on persistent organic pollutants (POPs) is a "*global treaty to protect human health and the environment from chemicals that remain intact in the environment for*

long periods, become widely distributed geographically, accumulate in the fatty tissue of humans and wildlife, and have harmful impacts on human health or on the environment" (Stockholm convention, http://www.pops.int/). Initially, twelve POPs, also called "the dirty dozen", have been recognized as causing adverse effects on humans and the ecosystems. Following the Annexes in which chemicals have been listed, ratifying parties are required to take measures to eliminate (Annex A), restrict (Annex B) the production and use of chemicals or reduce (Annex C) unintentional releases of toxicants. These "legacy POPs" include 9 pesticides (aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, HCB, mirex, toxaphene), 1 industrial compounds (PCBs) and 2 non-intentional by-products (dioxins and furans; Stockholm convention, http://www.pops.int). Since 2009, 16 "new POPs" have been added to the convention and 3 additional candidates are currently under review (Stockholm convention, http://www.pops.int). Among them, some poly- and perfluoroalkyl substances (PFASs) are raising a particular interest within the scientific community and this thesis will emphasize on this group of chemicals in order to contribute at filling the gap of knowledge of their toxicological profile.

2 - The Arctic: a sink for environmental contaminants

a) Environmental contaminants: type, origin and fate

In 2015, the European Chemical Agency published information about toxicological profile of 120 000 synthetic chemicals, testifying of the countless toxicants potentially released into the environment (European Chemical Agency, 2017, https://echa.europa.eu/fr/home). Thus, it would be unrealistic for ecotoxicological studies conducted *in natura* to cover the whole range of contaminants present within the ecosystem. In this thesis, we decided to focus on two families of organic halogenated contaminants exclusively produced by human activities: PFASs & organochlorines (OCs); and one non-essential metal: Hg, coming from both anthropogenic and natural sources.

PFASs are manufactured fluorinated chemicals synthetically produced since the 1950s, especially in the northern hemisphere by electrochemical fluorination and telomerization processes (Buck et al., 2011). PFASs are mainly used as surfactants and water repellents in a vast array of industrial and consumer applications (e.g. fire-fighting foam, waterproof clothing, lubricants, food packaging, non-stick coating, insecticides and impregnation agent for carpets, papers and textiles; Figure 5; 3M, 1999; Buck et al., 2012;

Kissa, 2001). Worldwide production of perfluorooctane sulfonyl fluoride, the key building block for PFOS-related compounds was estimated at 96 000 tons (or 122 500 tons, including unusable wastes) during the period 1972–2002 (Paul et al., 2009). PFASs are carbon chains varying in length to which hydrogens are replaced by fluorines (Buck et al., 2011). Chemical bonds between carbon and fluorine atoms are very strong which make the PFASs thermally and chemically stable, resistant to degradation and thus extremely persistent in the environment (Key et al., 1997). PFAS are globally distributed and have been reported in Arctic and Antarctic wildlife (Giesy and Kannan, 2001; Routti et al., 2014, 2015), either released by direct discharge ("direct emissions") or resulting from the degradation of precursor compounds ("indirect emissions"; Figure 4; Butt et al., 2010; Prevedouros et al., 2006). Specifically, the first mechanism involves the transport of directly emitted perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) *via* oceanic currents (Armitage et al., 2006; Wania, 2007). The second pathway corresponds to the transport of volatile precursors through the atmosphere, degradation by atmospheric oxidation to PFCAs and PFSAs, and subsequent wet and dry deposition (Butt et al., 2010; Ellis et al., 2004). In addition, several fluorinated compounds (e.g. volatile fluorotelomer precursors) can be biotransformed into PFCAs and PFSAs through metabolic processes. Thus, the presence of metabolically active "precursors" within the animal body may represent a reservoir of PFASs (Butt et al., 2010; Houde et al., 2011). Finally, other factors like tissue-specific biotransformation ability and protein-binding capacity may also be important, but all these factors are still misunderstood (Butt et al., 2010; Houde et al., 2011). It is worth noting that an overview of PFAS levels in both Arctic biota and abiotic compartments can be found in a recent Arctic Monitoring Assessment Programme (AMAP) report (AMAP, 2017).

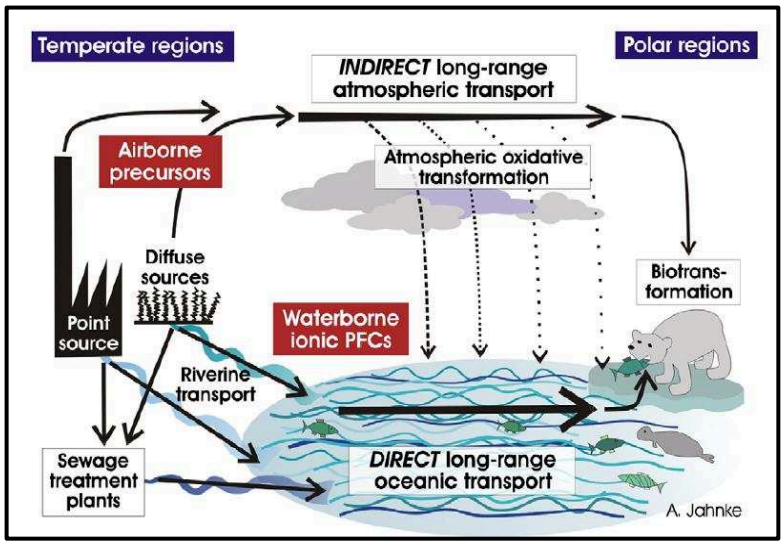


Figure 4: Major transport pathways of PFASs to the Arctic (Schematic by Annika Jahnke in Butt et al., 2010).

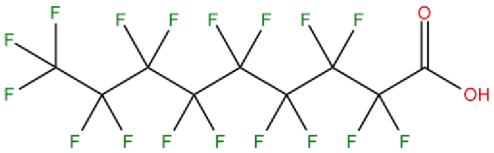
Legacy POPs are synthetically manufactured chlorinated chemicals essentially produced since the beginning of the 20th century. These OCs, banned or regulated by the Stockholm convention, were extensively used in the past in numerous industrial, commercial and agricultural applications (Figure 5). Among them, DDT was widely used during the World War II to protect civilians and soldiers from typhus, malaria, and other diseases transmitted by insects. After the World War II, DDT was still massively employed to control diseases and widely sprayed on agricultural crops, especially cotton (Fisher, 1999; Stockholm convention, http://www.pops.int). Many of these legacy POPs were broadly used as pesticides, like mirex to fight against fire ants or chlordanes, aldrin and dieldrin to kill termites (Fisher, 1999; Stockholm convention, http://www.pops.int). Finally, PCBs, gathering 209 congeners, were used in industry as heat exchange fluids, in electric transformers and capacitors, as additive agents in paint, in carbonless copy paper, and in plastics (Fisher, 1999; Stockholm convention, http://www.pops.int). Due to their physico-chemical properties, legacy POPs undertake long-range transport and can be found in high concentrations in remote places, even exceeding sometimes those reported in areas close to the source (Oehme, 1991; Risebrough et al., 1968; Simonich and Hites, 1995; Wania and Mackay, 1993). Although oceanic currents and river run-offs have been shown to be significant transport pathways (Barrie et al., 1992), the most accepted theory today describes the transport of semi-volatile POPs as a temperature and weather dependent repeated deposition and remobilization process with a final deposition in the cold northern and southern regions (Burkow and Kallenborn, 2000). Specifically, long-life chemicals are transported over long distances according to their degree of volatility (Steffen et al., 2006b). As a result, atmospheric contaminant composition shifts toward more volatile compounds with increasing latitude. In turn, cold temperatures favor the association of POPs to air particles therefore increasing condensation and deposition in the Arctic Ocean (Wania and Mackay, 1993, 1996). This phenomenon is called the "global distillation" or "grasshopper effect". Finally, polar environmental conditions can enhance the persistence of contaminants since winter darkness and low temperatures slow down photochemical and biotic degradation process and because ice formation can entrap POPs for a long time (AMAP, 2016, 2017).

In addition to the numerous synthetic chemicals released into the environment as previously detailed, other contaminants are naturally present on Earth (in crust, atmosphere, and water masses) as is the case for mercury (Hg), a non-essential heavy metal. Fluxes involving natural Hg mobilization are numerous and include volcanic, geothermal and geological activity and soil erosion (Figure 5; Fitzgerald and Lamborg, 2014; UNEP, 2013).

However, despite its natural origin, human activities such as fossil-fuel (coal and petroleum) combustion, artisanal gold mining, industrial activities and biomass burning have considerably increased the global amount of Hg cycling around the world (Figure 5; Mason and Sheu, 2002; Fitzgerald and Lamborg, 2014; Pacyna et al., 2010; Selin et al., 2009; UNEP, 2013). Owing to its high volatility and long atmospheric residence time, elemental Hg (Hg^0) reaches remote areas like the Arctic mainly through the atmospheric transport pathway (AMAP, 2011; Ariya et al., 2004). Over a 1-year cycle, Ariya et al. (2004) have estimated an accumulation of 325 tons of Hg in the Arctic. In spring, when sunlight returns, atmospheric Hg^0 depletes while oxidized Hg (Hg^{II}) levels increase sharply mainly due to photochemical reactions, facilitating dry and wet depositions of Hg^{II} in aquatic ecosystem (AMAP, 2011; Fitzgerald et al., 2007; Mason and Sheu, 2002; Selin et al., 2009; UNEP, 2013). Once deposited in the marine environment, this inorganic Hg can suffer abiotic and biotic reactions (i.e. methylation) carried-out by anaerobic microorganisms (i.e. bacteria) leading to the formation of methylmercury (Me-Hg), the persistent organic and toxic form of Hg (Fitzgerald et al., 2007; Hsu-Kim et al., 2013).

Perfluoroalkyl substances (PFASs)

Carboxylates



Perfluorononanoate (PFNA) - C_9

Perfluorodecanoate (PFDcA) - C_{10}

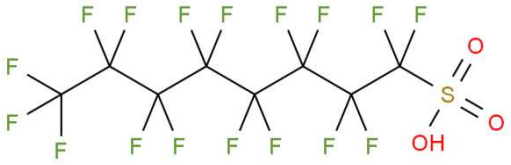
Perfluoroundecanoate (PFUnA) - C_{11}

Perfluorododecanoate (PFDoA) - C_{12}

Perfluorotridecanoate (PFTrA) - C_{13}

Perfluorotetradecanoate (PFTeA) - C_{14}

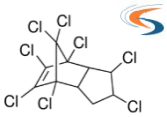
Sulfonates



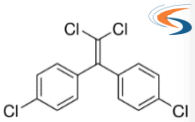
Perfluorooctanesulfonate (PFOS) – C_8

Organochlorines (Legacy POPs*)

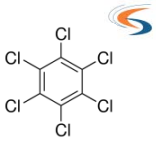
Pesticides



Chlordane*

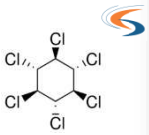


DDT*



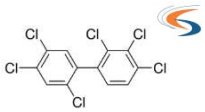
HCB*

Mirex*



β-HCH

Industrial products



PCB*

Non essential metal

$H_3C-Hg^+ X^-$

Methylmercury (Me-Hg)

Figure 5: Molecular structure, uses and sources of the different contaminants considered in this thesis.

b) Environmental contaminants in Arctic biota

Once deposited in the marine ecosystem, contaminants can turn bioavailable for living organisms. Bioavailability corresponds to the fraction of a contaminant that can be assimilated and thus potentially toxic for the organism (Hoffman et al., 2003). Bioavailability is highly dependent on the chemical form of the contaminant as is the case for Hg, more bioavailable under its methylated form (Morel et al., 1998). Once assimilated by living organisms, contaminants can bioaccumulate within individuals and biomagnify along marine food webs (Figure 6; Atwell et al., 1998; Blévin et al., 2013; Borgå et al., 2001; Fisk et al., 2001a, 2001b; Haukås et al., 2007; Hop et al., 2002; Jæger et al., 2009; Kelly et al., 2009; Tomy et al., 2004). Both terms describe the transfer of contaminants from the external environment to the organism. In brief, biomagnification occurs when contaminant levels of the consumer are higher than those found in food sources and bioaccumulation arises when contaminant uptake overcomes elimination rates. Interestingly, the Arctic ecosystem includes long food chains which enhance the biomagnification process (Borgå et al., 2004).

A wide range of physico-chemical (e.g. hydrophobicity, structural complexity) and biological (e.g. biotransformation) factors are known to strongly influence bioaccumulation and biomagnification processes resulting in a substantial variability of contamination levels among marine organisms (Borgå et al., 2004; Fisk et al., 2001a). Among those, hydrophobicity and lipid solubility, usually expressed with the octanol-water partition coefficient (K_{ow}), indicates the compounds likelihood of being efficiently taken up by passive diffusion (Gobas and Morrison, 2000; Thomann, 1989). For OCs, elimination rates decrease and bioaccumulation generally increases with hydrophobicity and lipid solubility (Fisk et al., 2001a). Accordingly, the lipid soluble PCBs better biomagnify compared to the less hydrophobic HCH along a Barents Sea food chain (Borgå et al., 2001). In addition, structuro-chemical properties appears critically important since i) bioaccumulation of PFASs are directly related to the fluorinated carbon chain length, with the highest bioaccumulation potential reported for the longest compounds; and because ii) PFSAs are more bioaccumulative than PFCAs for the same fluorinated carbon chain length (Conder et al., 2008). Finally, biotransformation, defined as *"the process by which chemical substances undergo chemical or biochemical reactions in organisms"*, strongly influences contaminant levels and especially those reported in superior consumers (Boon et al., 1989; Gobas and Morrison, 2000). The cytochrome P_{450} enzyme system (CYP) is involved in the first oxidative step of OCs biotransformation (Walker, 1998), leading to the formation of more or less toxic

metabolites. Biotransformation is particularly well exemplified within Arctic food webs where this process increases with trophic levels and differs greatly among seabird species (Borgå et al., 2005, 2007; Fisk et al., 2001b). Accordingly, relative contribution of the metabolite oxychlordane (from CHLs) is much higher in seabirds than those reported in marine invertebrates and fish (Borgå et al., 2001).

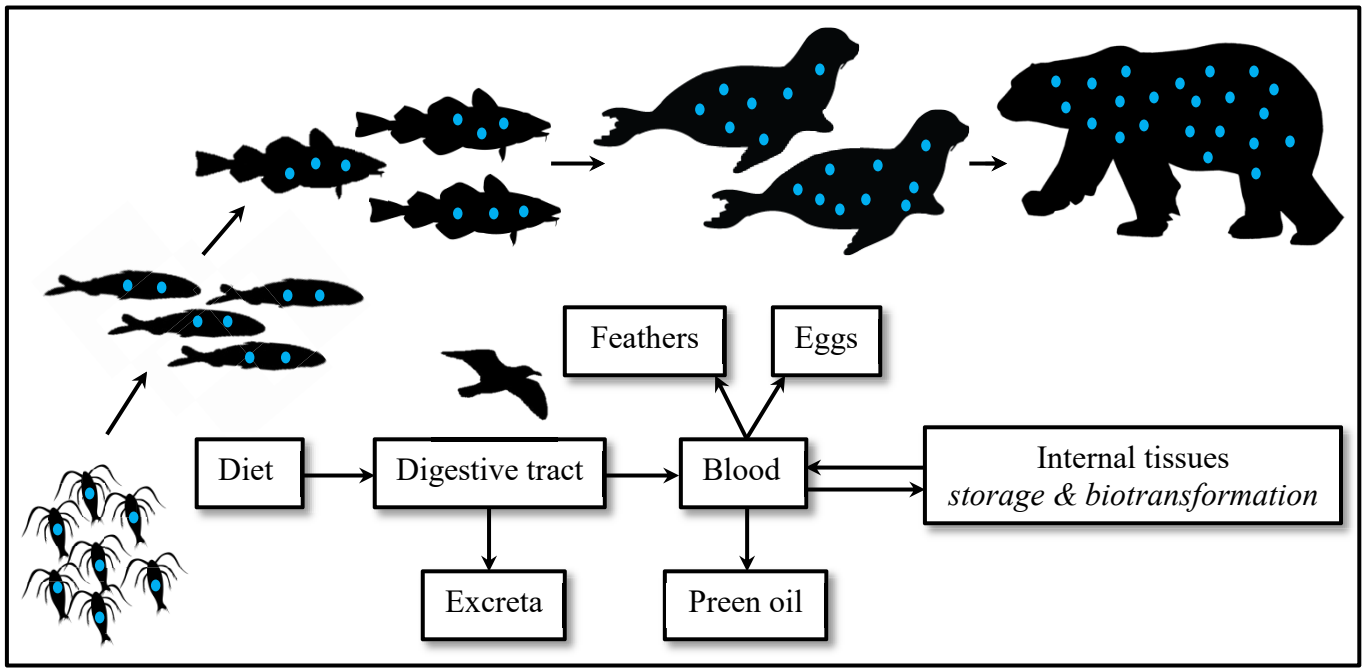


Figure 6: Bioaccumulation and biomagnification in a simplified Arctic food web (above). Simplified model of contaminants dynamic in seabirds (below; Modified from Monteiro and Furness, 1995).

The main route of contaminant exposure for marine predators like seabirds is food ingestion (Figure 6). Contaminant toxicokinetics involve assimilation of contaminants by the digestive tracts, transport into the bloodstream, distribution in internal organs/tissues, remobilization into the blood circulation and excretion in feathers (*via* moult), preen oil (*via* uropygial gland secretions) and eggs (for females). In contrast to the lipophilic OCs associated with lipids and essentially stored in adipose tissues, PFASs bind to proteins and accumulate mainly into the blood and liver; Aas et al., 2014; Jones et al., 2003; Luebker et al., 2002; Verreault et al., 2005). Indeed, a PFASs molecule is divided in two parts, one is hydrophobic and another highly hydrophilic thanks to the carboxylate or sulfonate functional group, thereby making the molecule partly lipophilic and partly hydrophilic.

c) Seabirds as bioindicators of environmental contamination

Investigating environmental contamination is not an easy task and requires a particular attention to accurately select the appropriate indicator. Living organisms appear to be better indicators rather than abiotic matrices (i.e. soil, air, water) which do not reflect the bioavailable and potentially toxic fraction of contaminants. Among those, seabirds have been extensively investigated for decades in numerous ecotoxicological studies and appear as perfect candidates since…

- … many of them are apex predators and thus potentially highly contaminated because of the biomagnification process.
- … they are long-lived species and thus potentially highly contaminated because of the bioaccumulation process.
- … they are tightly linked to the oceanic environment which is considered as the fall-out of many contaminants and therefore chronically exposed to chemicals.
- … they are philopatric, which allows for resampling of individuals from year to year and for investigating some potential carry-over effects.
- … they breed in dense colony, which means it is possible to get a decent sample size for statistical analyses and trustable results.
- … they often breed in sympatry which means it is quite easy to sample several seabird species with contrasted feeding strategies in the same site.
- … they are widely distributed which enables to make consistent geographical comparisons.
- … contaminant concentrations can be determined using non-destructive methods. Samples of blood, feathers, oils and biopsies can provide information of different spatial and time scales. Quantity of collected samples is often suitable to measure a broad panel of contaminants.
- … they are robust and do not appear as highly sensitive to handling.

For all these reasons, seabirds are thus highly relevant to track marine pollution, monitor temporal trends, determine geographical patterns and assess toxicity of contaminants. Consequently, seabirds are relevant bioindicators of environmental contamination (Burger and Gochfeld, 2002, 2004; Elliott and Elliott, 2013; Furness, 1993; Rowe et al., 2008).

3 - Environmental contaminants: a risk for wildlife

Beyond a certain threshold or following a chronic exposure at low doses, contaminants can be toxic for living organisms. Besides, a plethora of studies conducted in birds have provided early warning signals of toxicity. Among them, the most famous example is perhaps the one of DDT contamination in birds of prey (Carson, 1962). This historical study case deserves to be further developed here since it highlights perfectly how contaminants can impact fitness through endocrine disrupting mechanisms.

DDT came rapidly into widespread use in Western countries shortly after the World War II (Walker, 2014). In the early 1950s, abnormal numbers of broken eggs were found in the nests of peregrine falcons (*Falco peregrinus*) and sparrowhawks (*Accipiter nisus*) in several regions of the British Isles (Walker et al., 2012). Based on this observation, Derek Ratcliffe examined the temporal variation of eggshells thickness collected in the UK after 1900 (Figure 7; Ratcliffe, 1967). Interestingly, between 1947 and 1949, a period during which DDT was extensively used in agriculture as an insecticide, eggshells of peregrines and sparrowhawks became markedly thinner in many areas (Ratcliffe, 1967; Walker, 2014). Ratcliffe speculated that this was related to the environmental DDT contamination (Ratcliffe, 1993). This statement was very controversial at the beginning because overall population of these two species did not show declining trends in the UK but only at some localities (Walker, 2014; Walker et al., 2012). However, experimental evidences and further supports from other field studies came forward to confirm this hypothesis (Wiemeyer and Porter, 1970). In North America, where DDT levels were higher than to those found in Europe, the decline of several raptors population like the peregrine falcon or the bald eagle (*Haliaeetus leucocephalus*) during the late 1940s to late 1970s was associated with eggshell thinning caused by *p,p′*-DDE, a toxic and persistent metabolite of DDT (Figure 7; Broley, 1958; Walker, 2014). Later, the endocrine disrupting properties of DDT metabolites have been highlighted and egg-shell thinning is likely the result of this underlying physiological mechanism (Figure 7; Dawson, 2000; Peakall, 1970).

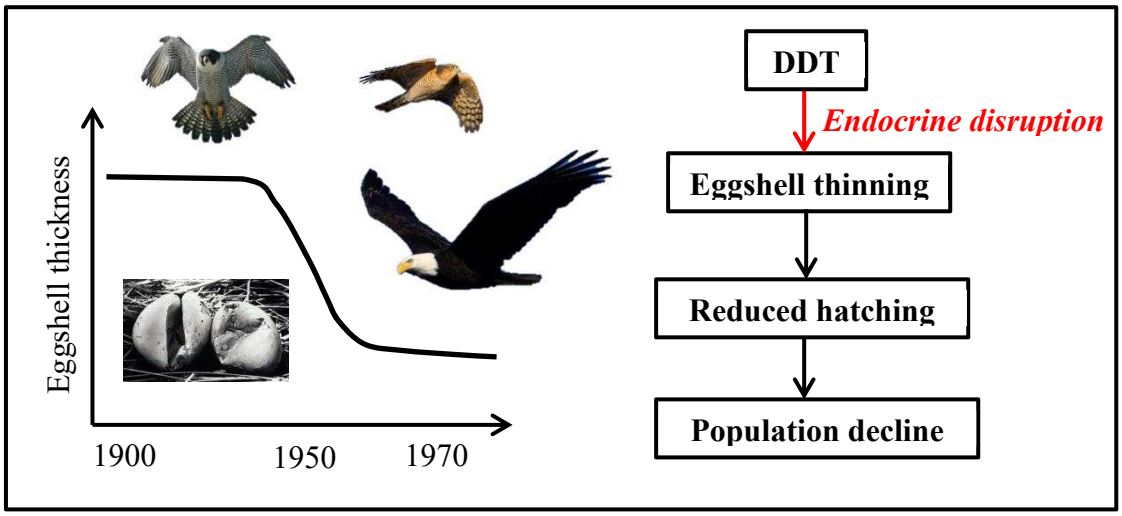


Figure 7: Eggshell thinning in raptors exposed to DDT in 1950s (Ratcliffe, 1967; on the left). Raptor populations decline in North America in response to DDT exposure (on the right).

Among the contaminants of interest in the present thesis, OCs and Hg have received a substantial attention from the scientific community and this is now clearly established that these contaminants are toxic for exposed living organisms. Nevertheless, several questions remained to be addressed and the present thesis will particularly emphasize on physiological consequences still misunderstood or unexplored so far. Current understanding of adverse effects associated with PFASs exposure based on laboratory animal models includes hepatotoxicity, tumor induction, developmental toxicity, immunotoxicity, neurotoxicity and endocrine disruption (DeWitt, 2015). To date, much attention has focused on PFOA and PFOS and on-going research on other PFASs are needed. Specifically, the long-chain PFCAs have been overlooked and deserve particular attention since toxicity appears to increase with the carbon chain length (**Paper VII**; Berntsen et al., 2017). Finally, the consequences of PFASs on wildlife remain largely unexplored and the present thesis contributes at filling the gap of knowledge of toxicological profiles of PFASs.

4 - Thesis objectives

Based on a correlative approach, I investigated the relationships between three groups of contaminants (PFASs, OCs and Hg) and several biomarker endpoints involved in life-history functions (survival, self-maintenance and reproduction) in an Arctic seabird, the black-legged kittiwake from a Norwegian population (Figure 8). By multiplying the number of biological variables of interest, by sampling at different years and by looking at different

stages across the breeding cycle, I hoped to find consistent patterns of toxicity in order to target specific harmful compounds or contaminant families. Specifically, I examined the relationships between several PFASs, OCs, Hg and i) cellular ageing (telomere length), ii) energy expenditure (basal metabolic rate), iii) parental care behaviors (incubation temperature and egg-turning), iii) fertility (sperm morphology and motility) and iv) sexual signaling (visual: integument coloration and olfactory: chemical signature). Since these physiological and behavioral mechanisms are under a hormonal control and because these contaminants are endocrine disruptors, some putative underlying mechanisms were also studied. Specifically, we investigated the relationships between contaminants and several hormones of interest (corticosterone, the stress hormone tighly linked to telomere length; thyroid hormones (THs), involved in energy expenditure and prolactin, involved in the expression of parental care). Such physiological impairments would have, *in fine,* consequences on individual and population fitness.

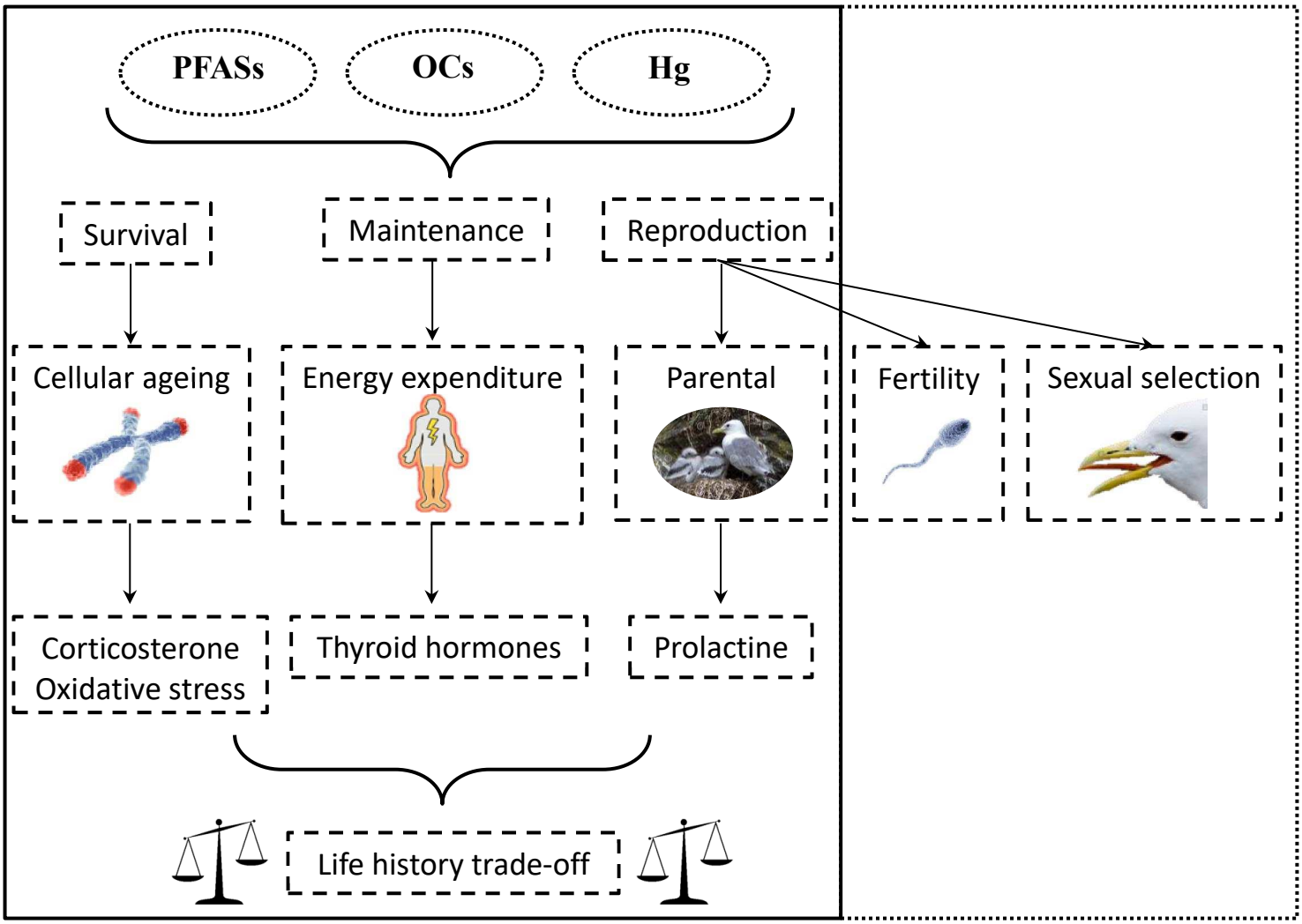


Figure 8 : Overview of the different thesis objectives. The main results presented in this thesis are framed by the solid black line whereas the dashed and black line corresponds to preliminary results and perspectives.

C - General methods

This section is dedicated to general and common methods that you can find within all the papers included in the thesis. Other methods related to specific questions are described in the following chapters.

1 - Study model: the black-legged kittiwake in Svalbard

The black-legged kittiwake *Rissa tridactyla* ("kittiwake" hereafter; Figure 9; Linnaeus, 1758) is a pelagic seabird species that belongs to the order of *Charadriiformes* and to the family of *Laridae*. The kittiwake is the most numerous gull in the world with an overall population estimated around 15 000 000 individuals broadly distributed throughout the northern hemisphere, principally in Arctic and boreal zones (Coulson, 2011; Wetlands International, 2018, https://www.wetlands.org/). They breed in dense colony and nest on coastal cliffs in both the North Pacific and Atlantic Ocean, including the Barents and Greenland Sea up to Svalbard archipelago in the high Arctic (Anker-Nilssen et al., 2000).

Figure 9: Group of adult black-legged kittiwakes (*Rissa tridactyla*) laying on a floating iceberg.

Historically considered as "least concern" by the IUCN, the black-legged kittiwake has been assessed as a "vulnerable" species since 2017 (BirdLife International, 2018, http://www.birdlife.org/). Indeed, global population trends indicate a rapid and alarming

decline since the beginning of the 90s (Barrett et al., 2012; Berglund and Hentati-Sundberg, 2015; BirdLife International, 2018, http://www.birdlife.org/; Descamps et al., 2017; Frederiksen et al., 2004), principally attributed to diverse factors such as climate change, food resources depletion, overfishing and potentially to environmental pollution (Descamps et al., 2017; Frederiksen et al., 2004, 2007; Goutte et al., 2015; Hatch, 2013; Massaro et al., 2000; Sandvik et al., 2014).

Fieldwork was conducted in a kittiwake colony at Kongsfjord in Svalbard archipelago (Krykkjefjellet; 78°54'N, 12°13'E; Figures 10 and 11A), 7 kilometers southeast of Ny-Ålesund village in Norway. Kongsfjord waters are mainly composed and influenced by two different marine currents: (i) the West Spitzbergen Current (WSC), a branch of the North Atlantic Current, bringing large amount of relatively warm and salted water masses and (ii) coastal currents with cold and less salted Arctic waters flowing northward around southern and western Spitzbergen (Cottier et al., 2005; Svendsen et al., 2002). Kittiwake is a medium-size seabird (340 – 500g) feeding mostly on small pelagic fishes, crustaceans and other planktonic invertebrates located near the sea surface (Coulson, 2011; Mehlum and Gabrielsen, 1993). Importantly, water masses carried by the WSC are increasing in Kongsfjord since few years which tend to replace Arctic prey species such as polar cod (*Boreogadus saida*) or the krill (*Thysanoessa libellula*) to a more mixed diet with high contribution of Atlantic fishes like capelin (*Mallotus villosus*) or Atlantic herring (*Clupea harengus;* Vihtakari et al., 2018).

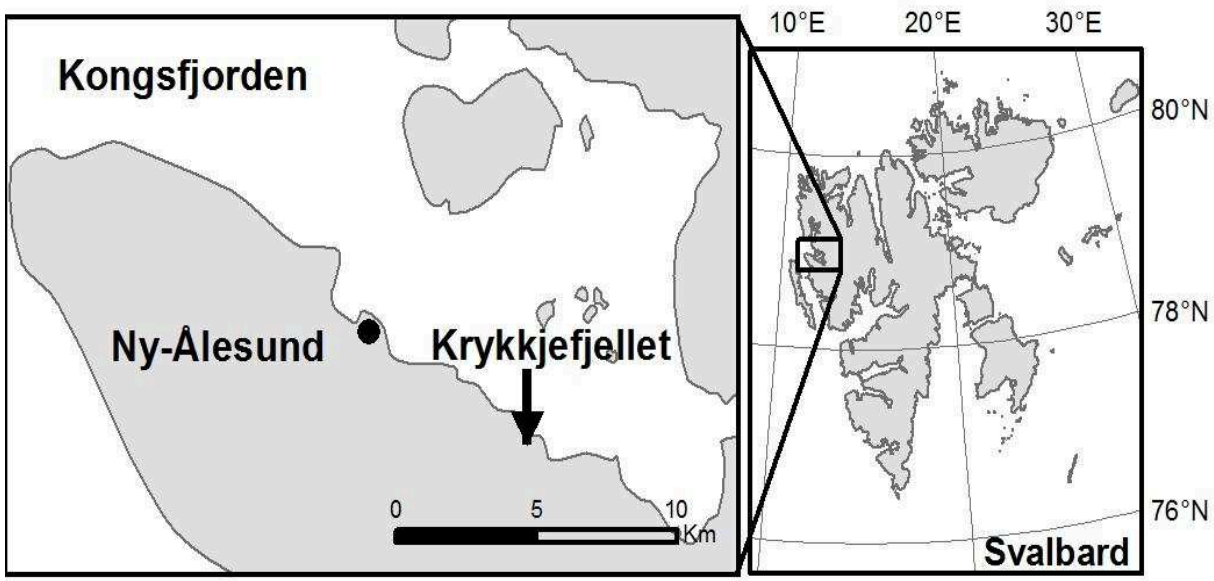


Figure 10: Location of the study area (Kongsfjord, Svalbard) and sampling site (Krykkjefjellet) during the thesis.

Kittiwakes are monogamous and philopatric, meaning that breeders generally come back each year in the same colony, mate with the same partner and breed in the same nest. At Kongsfjord, kittiwakes generally arrive in early April. April and May are then devoted to nest building, nest defense and mating (i.e. the pre-laying period). Egg-laying generally occurs around mid-June and clutch size varies from 1 to 3 eggs even if in most cases, 2 eggs are laid. During approximately 27 days, both partners incubate alternatively the eggs until hatching in early July (i.e. the incubating period; Mehlum, 2006). Once hatched, both parents will alternate nest attendance duties to keep the chicks at an optimal temperature and foraging trips to feed themselves and their offspring. The chick rearing period is the most energy-demanding stage of the breeding cycle, especially the first 15 days until the chicks are thermally emancipated (i.e. the brooding period; Bech et al., 2002; Moe et al., 2002) and fledging occur around 40 days of age (late August – early September; i.e. the fledging period). Once the breeding season is completed, kittiwakes from Kongsfjord will migrate and spend the winter at sea, in South Greenland-Labrador areas (Frederiksen et al., 2012).

2 - Blood sampling, morphometric and reproduction monitoring

Kittiwakes were caught on their nest with a noose at the end of a 5 m fishing rod (Figure 11B). We collected a first blood sample (~1 mL) immediately after capture (i.e. within 3 min) from the alar vein using a heparinized syringe and a 25-gauge needle to assess baseline hormone levels, measure different physiological parameters (depending on the scientific question addressed) and determine the sex of individuals. We then performed a second blood sampling (~2 mL) to assess the concentrations of contaminants. Blood samples were stored on ice in the field. Whole blood and both plasma and red blood cells (RCBs) obtained after centrifugation (10 mn at 7 500 rpm) were kept frozen at -20°C until subsequent laboratory analyses.

Birds were individually marked with a metal band (unique number) and a PVC plastic ring engraved with a three-digit code fixed to the bird's tarsus for identification without perturbation using a telescope. We weighed all birds to the nearest 5 g with a Pesola spring balance to determine the body mass. Skull length (head + bill) was also measured using a sliding caliper with an accuracy of 0.1 mm (Figure 11C). Those morphometric measurements were further used to calculate a proxy of body condition, either by using the residuals of an ordinary least squares regression of body mass against skull length (Body Condition Index (BCI); Jacob et al., 2012; Jakob et al., 1996) or by computing the Scaled Mass Index (SMI)

developed and detailed in Peig and Green (2009). Before release, we marked the birds with small colored spots of dye on the forehead to distinguish them from their partner during subsequent observations from a distance.

At total, the whole kittiwake colony from Krykkjefjellet counts around 350 nests (348 active nests in 2016, 25th June, personal counting) and each year, the reproduction of approximately 150 pairs (reachable nests) is monitored. All these nest contents are thus regularly checked all along the field season with a mirror attached at the end of a long pool to determine reproductive outputs (laying date, egg number, egg presence/ absence, hatching and breeding success). It is worth noting that all these reproductive outputs are not available for each field season depending on our stay duration and period in Svalbard.

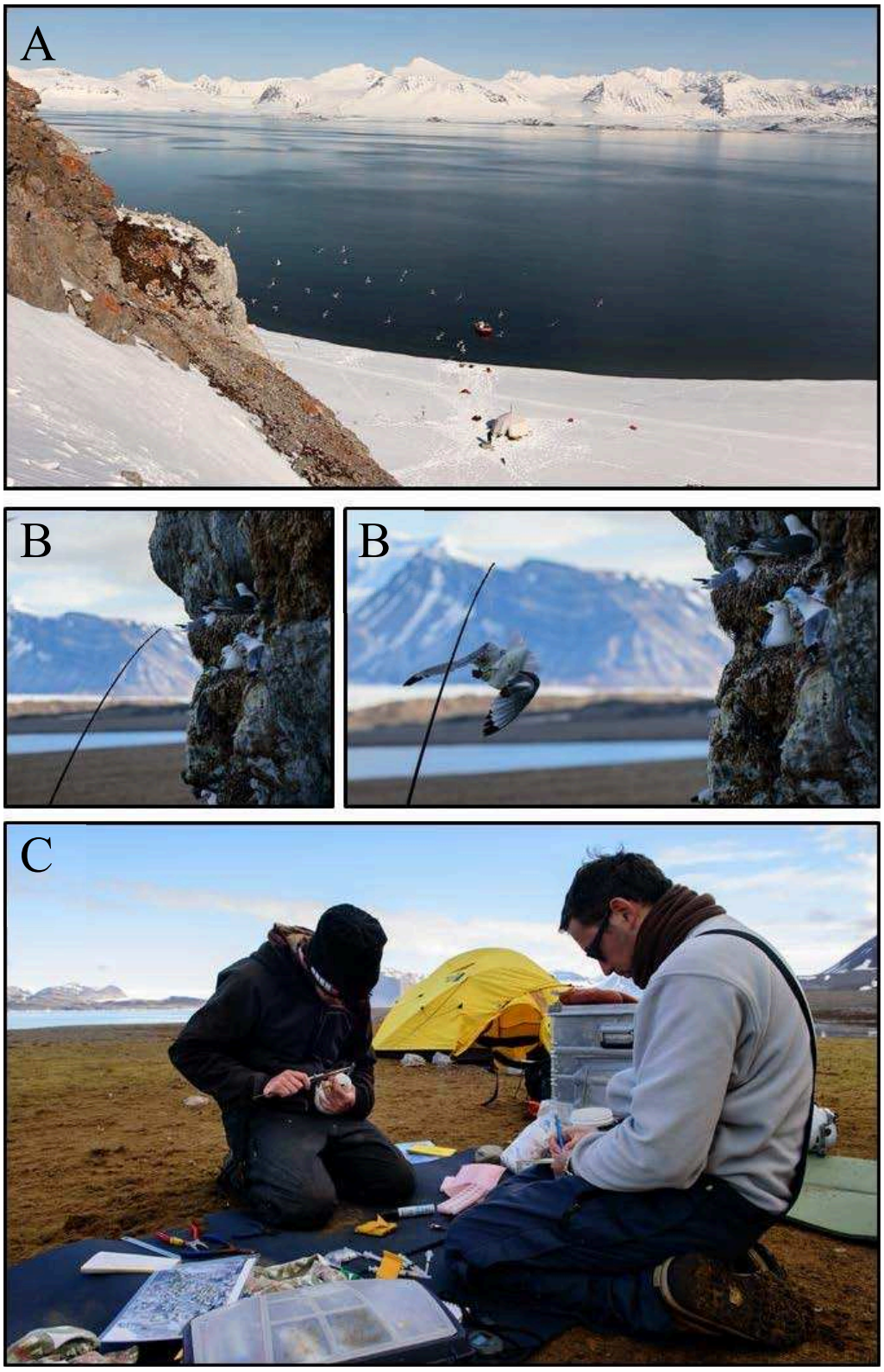


Figure 11: (A) Panoramic view from the cliff edge of the Kongsfjord with our basecamp in the front; (B) A capture of an incubating kittiwake with a loop at the top of a long fishing rod; (C) Field scenery with P. Blévin measuring skull length of a kittiwake and F. Angelier taking notes.

3 - Chemical analyses

a) PFASs

PFASs were analysed from plasma at NILU in Tromsø, Norway. You can find the details of the scanned compounds in the different papers included at the end of the manuscript. In short, a sample (0.2 - 0.5 mL) spiked with internal standards was extracted in methanol or acetonitrile (1 mL) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVICarb graphitized carbon absorbent and glacial acetic acid. Extracts were analysed by UPLC/MS/MS. Recovery of the internal standards was within the laboratory accepted range (see papers for more details). The presented PFAS concentrations were corrected for recovery. Results were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference materials (SRMs; 1957 or 1958 human serum from the National Institute of Standards & Technology (NIST)) run every 10 samples. The deviations of the target concentrations in the SRMs were within the laboratory's accepted range (see papers for more details).

b) OCs

OCs were analyzed from whole blood at NILU in Tromsø, Norway. You can find the details of the scanned compounds in the different papers included at the end of the manuscript. To a whole blood sample of ~1 mL, an internal standard solution was added (^{13}C-labeled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). We first proceeded to the sample denaturation using a mix of ethanol and saturated solution of ammonium sulphate in water. We then ran extraction twice with 6 mL of n-hexane. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975 CMSD were performed following Herzke et al., 2009. Recovery of the internal standards was within the laboratory's accepted range (see papers for more details). The presented OC concentrations were corrected for recovery. Results were validated with blanks (clean and empty glass tubes treated like a sample) and SRMs (1589a or 1958 human serum from NIST) run every 10 samples. The deviation of the target concentrations in the SRMs were within the laboratory accepted range (see papers for more details).

c) Hg

Total Hg was analyzed at LIENSs in La Rochelle, France from freeze-dried and powdered RBCs placed in an Advanced Hg Analyzer Spectrophotometer (ALTEC AMA 254). Hg determination involved evaporation of the metal by progressive heating until 800°C was reached and then held under oxygen atmosphere for 3 min, and subsequent amalgamation on a gold-net. Afterwards, the net was heated to liberate the collected Hg which was then measured by atomic absorption spectrophotometry. All analyses were repeated at least two times until having an acceptable relative standard deviation (see papers for more details). Results were validated with blanks (ran at the beginning of each set of samples) and certified reference material (CRM; Tort-2 Lobster Hepatopancreas, NRC, Canada; certified value 0.27 ± 0.06 (SD) µg/g dw). Measured values were within the accepted range. All blanks contained concentrations below the detection limit (0.005 µg/g dw). Importantly, since Me-Hg is the toxic form of Hg and because Me-Hg accounts for more than 95% of blood Hg burdens in birds (Wolfe et al., 1998), total Hg here was considered to approximate Me-Hg.

4 - Sex determination

Molecular sexing was conducted at CEBC, Chizé, France. Kittiwakes were sexed from RBCs by polymerase chain reaction amplification of part of two highly conserved genes (i.e. CHD) present on sexual chromosomes as described and detailed in Fridolfsson and Ellegren, (1999) and Weimerskirch et al. (2005).

5 - Data used in the thesis: an overview

a) Sampling

For a better comprehension of the PhD project, please find below an overview of the different data exploited in the papers included within the thesis. All these papers are attached at the end of the manuscript.

	Paper I	Paper II	Paper III	Paper IV	Paper V	Paper VI	Paper VII	Paper VIII
Sampling year	**2012 & 2014**	**2012**	**2012**	**2015**	**2015**	**2011**	**2016**	
Stage	**Chick-rearing**	**Chick-rearing**	**Chick-rearing**	**Incubation**	**Incubation**	**Pre-laying**	**Pre-laying**	
Contaminant concentrations								
PFASs								
OCs								
Hg								
Hormone levels								
THs (TT3 & TT4)								
Prolactin								
Physiological parameters								
Telomere length								
Basal Metabolic Rate (BMR)								
Oxidative status								
Behavioral parameters								
Incubation temperature								
Egg turning								
Morphological/ phenotypic traits								
Integument coloration								
Body condition (SMI and BCI)								
Body mass								
Brood patch size								
Reproductive outputs								
Hatching success								

Review

Additional samples have been collected during the 2016 and 2017 field seasons. These data still need to be processed in the lab and/or statistically analyzed. Therefore, we plan to submit further papers for publication in a near future.

Sampling year	**2016**	**2017**
Stage	**Pre-laying**	**Pre-laying**
Contaminant concentrations		
PFASs		
OCs		
Hg		
Hormone levels		
Corticosterone		
Testosterone		
Prolactin		
LH		
Physiological parameters		
Carotenoids levels		
Sperm quality (morphology, motility)		
Chemical signature of preen gland feathers		
Morphological/ phenotypic traits		
Integument coloration		
Body condition (SMI and BCI)		
Body mass		
Reproductive outputs		
Status (breeder/non-breeder)		
Laying date		
Clutch size		
Hatching success		

b) Contaminant levels and relative contributions

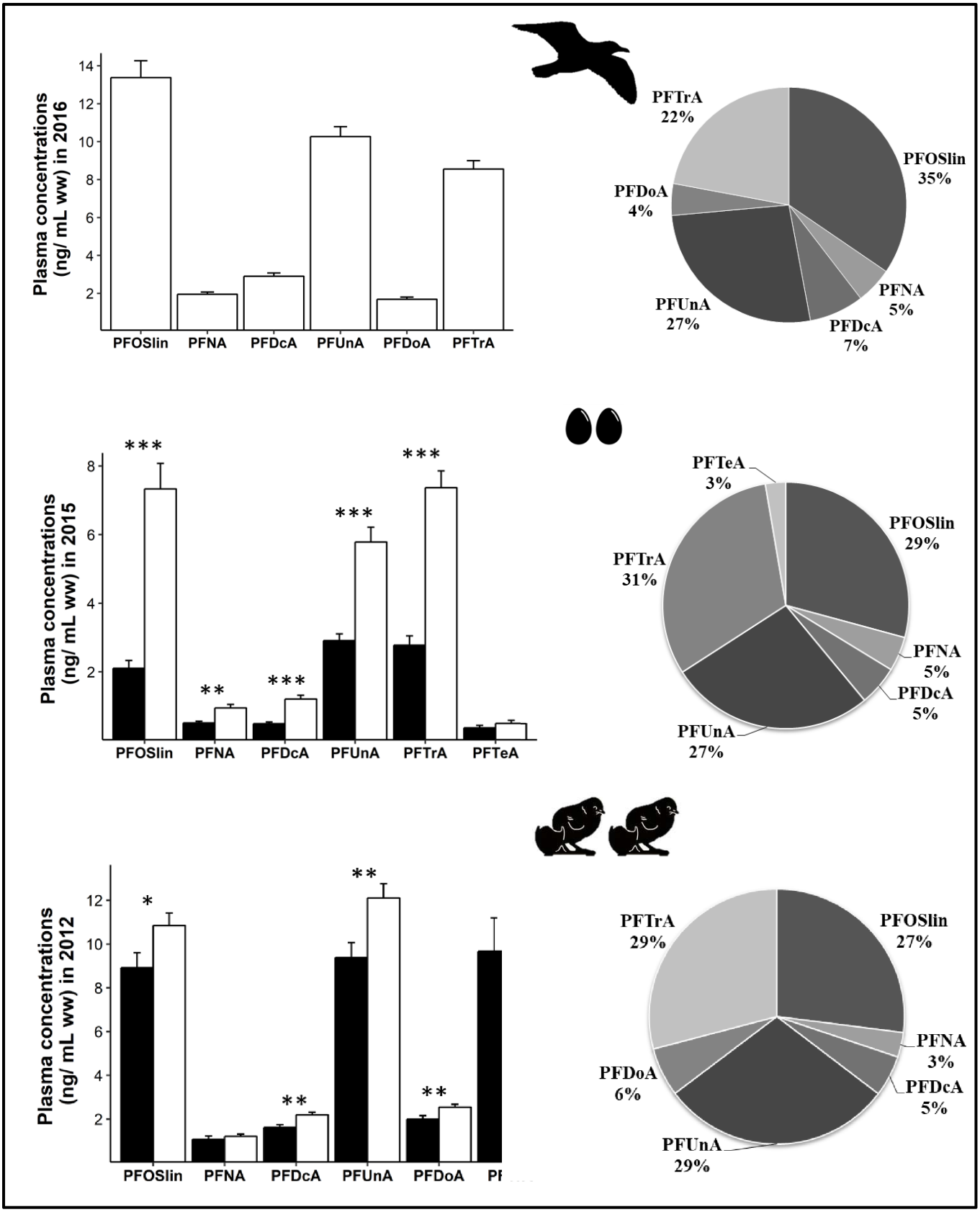


Figure 12: Plasma PFAS concentrations in males (in white) and females (in black) kittiwakes during pre-laying (n = 50 males), incubation (20 males and 20 females) and chick-rearing stages (22 males and 22 females). Relative contributions of each PFAS in plasma of black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

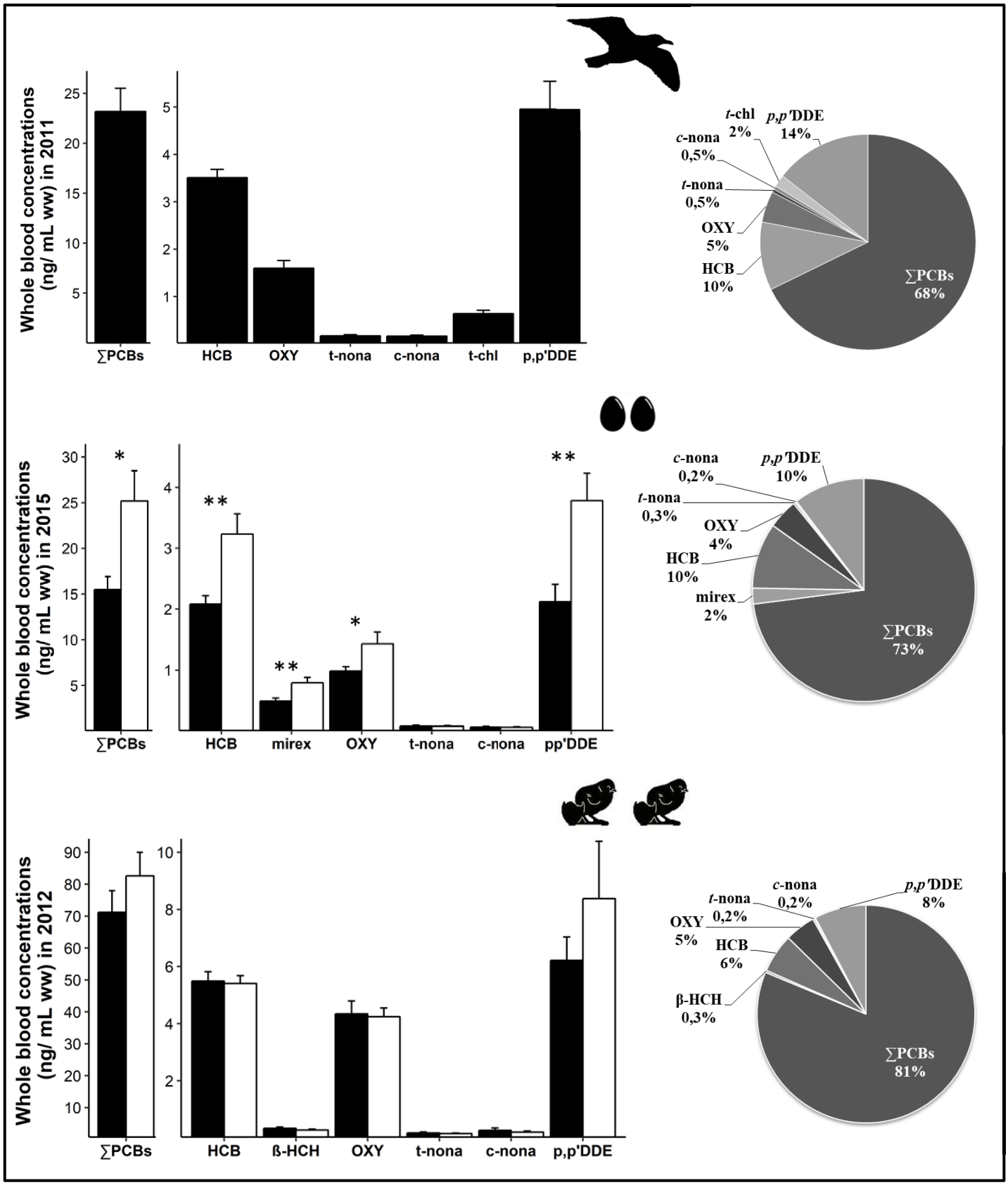


Figure 13: Whole blood OC concentrations in males (in white) and females (in black) kittiwakes during pre-laying (n = 45 females), incubation (20 males and 20 females) and chick-rearing stages (22 males and 22 females). Relative contributions of each OC in whole blood of black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

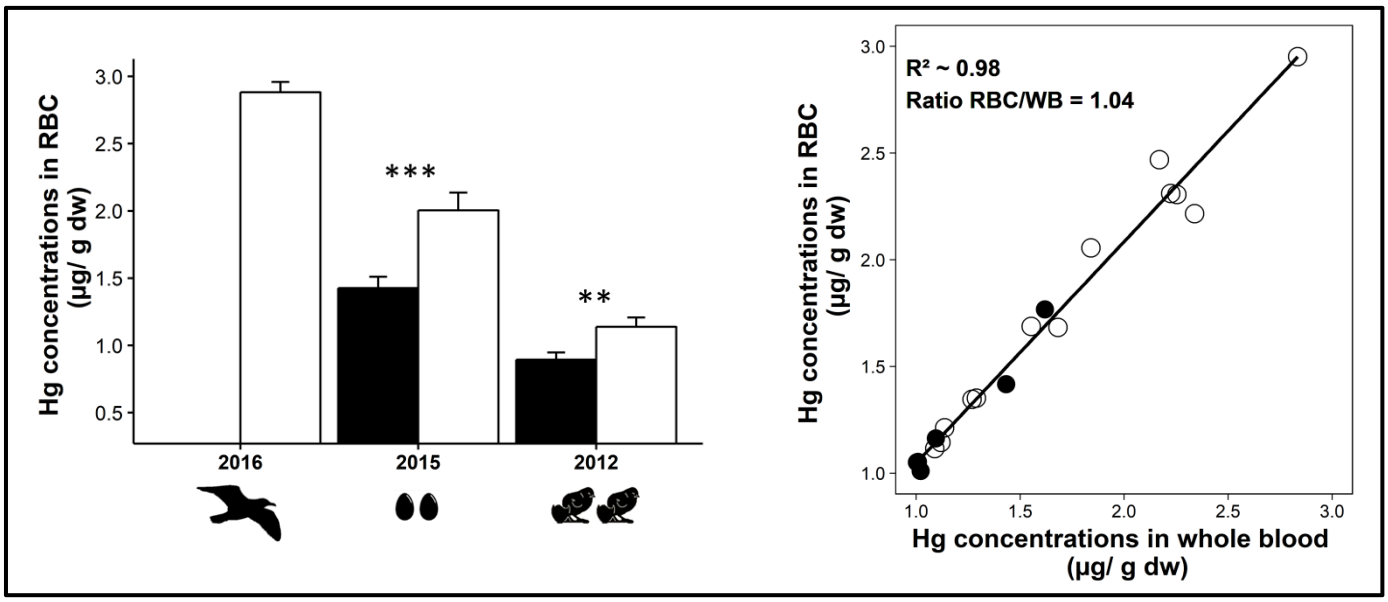


Figure 14: Red blood cells Hg concentrations in males (in white) and females (in black) kittiwakes during pre-laying (2016; n = 50 males), incubation (2015; 20 males and 20 females) and chick-rearing stages (2012; 22 males and 22 females). Within-individual (2015; n =19) correlation between concentrations of Hg in red blood cells and whole blood of black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

Consistently across the different years of sampling and all breeding stages, plasma PFAS profiles (Figure 12) of Kongsfjord kittiwakes were characterized by high concentrations of the sulfonate acid PFOSlin (C_8) and the long and odd numbered carbon-chain-length carboxylates, namely PFUnA (C_{11}) and PFTrA (C_{13}). These three compounds, contributing up to 85-90% (~30% each) of the total PFASs burden in plasma of our kittiwakes (Figure 12), were also found to be predominant in several PFAS profiles of different Arctic seabird species in various tissues (Braune and Letcher, 2012; Butt et al., 2007, 2010; Gebbink and Letcher, 2012; Verreault et al., 2005). This observation is actually consistent with the hypothesis that these long and odd carbon-chain-length PFCAs are more bioaccumulative than the short and even-chain length PFCAs in wildlife (Conder et al., 2008; Martin et al., 2004; Verreault et al., 2005). Furthermore, PFAS profiles were generally shown to be dominated by PFOS in most wildlife but the phase-out by the 3M Company in early 2000s of perfluorooctane sulfonyl fluoride-based products and the regulation taken in 2009 by the Stockholm Convention on POPs tend to decrease the proportion of PFOS in comparison with some increasing PFCAs (e.g. PFUnA & PFTrA) still produced and released in industrialized areas (Butt et al., 2010; Houde et al., 2006, 2011; US Environmental Protection Agency, 2000, 2002; Verreault et al., 2005).

Consistently across the different years of sampling and all breeding stages, whole blood OC profiles (Figure 13) were characterized by high concentrations of ΣPCBs, followed

by *p,p'*-DDE, HCB and oxychlordane in whole blood of our kittiwakes, suggesting selective bioaccumulation and different biomagnification degree of OCs (Borgå et al., 2001, 2005, 2007). Similar blood OC profiles have been shown in several tissues of different Arctic seabird species (Borgå et al., 2001; Buckman et al., 2004; Helgason et al., 2008; Verreault et al., 2004). Interestingly, concentrations of whole blood OCs for Kongsfjord kittiwakes showed a sharp increase between incubation and chick-rearing periods in both sexes (Figure 13). Indeed, the chick-rearing period is an energetically consuming stage leading to a rapid and strong loss of body mass (Moe et al., 2002), which in turn induces a release and mobilization into the blood circulation of lipophilic OCs previously stored in adipose tissues (Bustnes et al., 2005a, 2010; Henriksen, 1995; Henriksen et al., 1998; Nordstad et al., 2012; Routti et al., 2013). Accordingly, the relative abundance of ΣPCBs is increasing all along the breeding season (pre-laying: 68%, incubation: 73%, chick-rearing: 81%) and indication of the high bioaccumulative and remobilization properties of this group of chemicals (Bustnes et al., 2010; Henriksen, 1995).

The 2015 season is characterized by relatively low plasma and blood levels of PFASs and OCs for Kongsfjord kittiwakes (Figures 12 & 13). Interestingly, opportunistic regurgitates collected from handled kittiwakes in our study plot (Krykkjefjellet) were exclusively composed of small marine invertebrates (without occurrence of fish). Accordingly, at a larger geographic scale (Kongsfjord), it has been highlighted that kittiwakes fed almost exclusively on krill (frequency of occurrence about 80%; *Thysanoessa sp*) and almost no fish in summer 2015 (Vihtakari et al., 2018). Consequently, low levels of PFASs and OCs in 2015 can be partly attributed to the relatively low trophic position of their prey.

We measured PFAS concentrations in plasma while OCs were determined from whole blood. Ehresman et al. (2007) showed that PFHxS, PFOS, and PFOA are present in human plasma but not in RBCs, thus with a plasma to whole blood ratio approximating 2:1. Accordingly, Gebbink and Letcher (2012) showed very low PFCA concentrations in RBC and much lower concentrations of PFSAs in RBCs compared to plasma in herring gulls (*Larus argentatus*). I thus investigated contaminants partitioning by considering simultaneously, both PFASs and OCs, taking into account the 2:1 ratio between plasma and whole blood. The profiles were characterized by high proportion ΣPCBs (~60%), followed by *p,p'*-DDE (~7%), HCB (~6%) and then by the 3 predominant PFASs (PFOSlin, PFUnA and PFTrA; ~5.5% each) during both incubation and chick rearing stages (patterns in pre-laying period were not investigated because different sex were sampled for PFASs and OCs).

Hg concentrations in RBC of Svalbard kittiwakes showed a decline over the breeding season (Øverjordet et al., 2015; *Sire et al., in prep*; Figure 14). Interestingly, we found similar Hg concentrations between RBC and whole blood with a ratio approximating 1:1 and a quasi-perfect linear relationship between Hg concentrations in whole blood and RBC ($R^2 = 0.98$; Figure 14). This suggests that Hg in RBC is well representative of Hg in whole blood. However, the order of magnitude is very different from organic contaminants since Hg is expressed in µg/g while OCs and PFAS are in ng/g.

c) Relationships between contaminants

Because correlational studies conducted *in natura* cannot control for potential confounding effects, the relationship among explanatory variables deserves to be examined. Thus, we performed data exploration based on a principal component analysis (PCA) in two years (2012 and 2015, chick-rearing and incubation periods, respectively), where the largest number of contaminants was measured. Interestingly, the PCA correlation circles in figure 15 show that:

- Contaminants considered in this thesis can be divided in two distinct groups: the proteinophilic PFASs and Hg on one side and the lipophilic OCs in the other side.
- Such pattern seems to be consistent across years and breeding periods (at least between incubation and chick-rearing period).

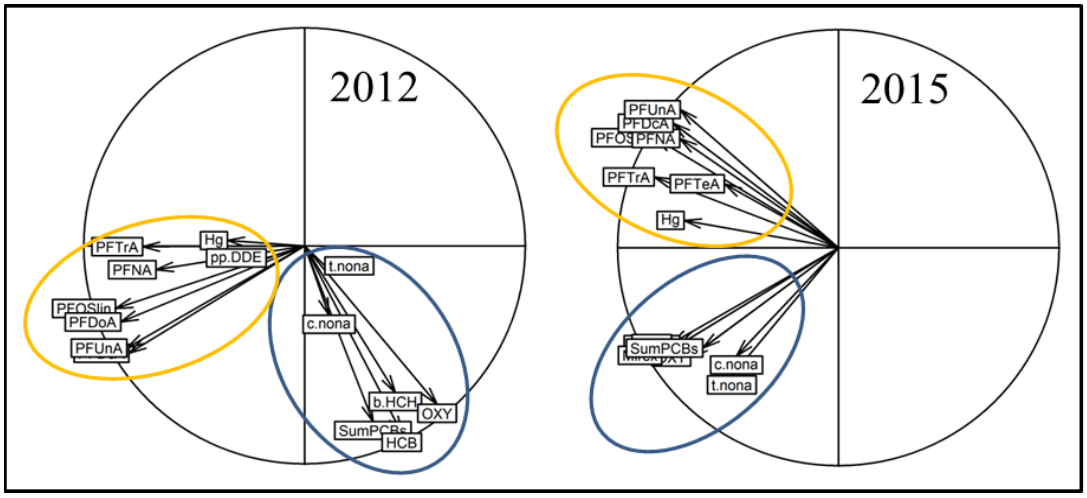


Figure 15: PCA correlation circles showing the relationships between contaminants (PFASs, OCs and Hg) in 2012 (chick-rearing period; 22 males and 22 females) and in 2015 (incubation period; 20 males and 20 females) in black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

D - Contaminants and cellular ageing

This chapter essentially relates, summarizes and discusses the main results from **papers I & II**. The aim of these papers was to investigate the effects of contaminants exposure on ageing. Specifically, we examined the relationships between some PFASs and OCs with telomere length by using both a cross sectional and longitudinal approach in adult breeding kittiwakes during the chick-rearing period. These two publications are attached at the end of the manuscript for further information.

1 - What a telomere is?

Telomeres are non-coding DNA-protein complexes located at the end of linear eukaryotic chromosomes which play a critical role in maintaining and ensuring the genomic integrity and stability (Blackburn, 1991; Monaghan and Haussmann, 2006). Because the DNA polymerase protein complex is unable to fully achieve the chromosomes replication during mitosis (i.e. end-replication problem), telomere length progressively shortens along life as a consequence of repeated cell divisions (Blackburn, 1991; Olovnikov, 1996; Sedivy, 1998). When telomere length reaches a critical lower threshold, cell division can damage coding DNA leading to apoptosis or cellular senescence (Blasco, 2007; Campisi et al., 2001; Harley et al., 1990; Olovnikov, 1996). It was originally thought that telomere loss occurred at a constant rate in individuals through their life, and telomere length could therefore act as an internal 'mitotic clock' to measure the chronological age of organisms into the wild (Haussmann and Vleck, 2002). However, telomere length and telomere dynamics have been shown to be reliable predictors of longevity and survival rate in captive and wild vertebrates, and even promising biomarkers of population extinction in wild lizards (Asghar et al., 2015; Barrett et al., 2013; Bauch et al., 2014; Bize et al., 2009; Boonekamp et al., 2014; Dupoue et al., 2017; Haussmann et al., 2005; Heidinger et al., 2012; Fairlie et al., 2016; Foote et al., 2010; Salomons et al., 2009). Indeed, telomere shortening has been shown to be accelerated by the occurrence of a wide range of environmental stressors (**Paper VIII;** Angelier et al., 2013; Epel et al., 2004; Hau et al., 2015; Meillère et al., 2015; Mizutani et al., 2013; Salmón et al., 2016; Young et al., 2013). Consequently, telomere length is considered as more related to biological age than chronological age *per se* (Barrett et al., 2013; Monaghan and Haussmann, 2006). Importantly, the effect of environmental contaminants on telomere length was until recently almost unexplored for wildlife.

2 - How to measure telomere length?

There are several existing methods available for measuring telomere length (e.g. qPCR, flow-FISH, Telomere Restriction Fragment: TRF) involving varying degrees of technical difficulty and precisions. Telomere length was measured at CEBC using TRF method which is considered as "the golden standard" method providing precise measurements of absolute telomere length. Specifically, TRF was performed by Southern blot and using the TeloTAGGG Telomere Length Assay (Roche, Mannheim, Germany) as previously described and with minor modifications (Foote et al., 2010; Kimura et al., 2010a). We have adjusted the quantity of DNA to allow a correct visualisation of the DNA signal on the gels. Telomere length analysis has already been successfully achieved on the same population of Svalbard kittiwakes (Schultner et al., 2014). Briefly, samples were digested with proteinase K, and DNA was extracted from RBCs by using the DNeasy blood and tissue kit (Qiagen). DNA quality was checked by gel electrophoresis and optical density spectrophotometry. Preliminary tests have been conducted to determine the optimal amount of DNA to be used and, for each sample, 0.7 µg of DNA was digested with the restriction enzymes *HinfI* and *RsaI* for 16 h at 37 °C. Digested DNA samples were then separated using a pulse-field gel electrophoresis (Bio-Rad) on a 0.8% agarose gel. All samples were run in four gels. Samples were randomly assigned to a gel except those used to assess telomere length dynamics which were treated in the same gel. Internal controls were run on each gel to measure inter-gel variations and each gel was run at 3.0 V/cm with an initial switch time of 0.5 s to a final switch time of 7 s for 14 h. Following that step, the gel was depurinated and denaturated in an alkaline solution. The gel was then neutralized and DNA was transferred onto a nitrocellulose membrane by Southern blot (Hybond N+, Amersham Life Science, Amersham, UK). The membrane was incubated at 120°C for 20 min in order to fix the DNA. The DNA was then hybridized with a digoxigenin-labeled probe specific for telomeric sequences and incubated with antidigoxigenin-specific antibody before visualization with a Chemidoc (Bio Rad). Telomere length was then analyzed using ImageJ to extract telomere smear densities. Lane-specific background was subtracted from each density value and telomere length (mean value) was then calculated using a window of 5–30 kb that includes the whole smear (Nussey et al., 2014). Inter-gel CV was 1.40%.

3 - Relationships between contaminant concentrations and telomere length in kittiwakes

We observed no relationships between PFAS concentrations and absolute telomere length when analyzing only one year (cross-sectional approach in 2012; Figure 16A). Because PFASs contamination appears quite repeatable between 2012 and 2014 within the same individual (see **paper I** for more details), the longitudinal approach allows us to relate PFASs contamination in 2012 with telomere dynamics (difference of telomere length between 2012 and 2014). Results from the longitudinal approach indicated PFASs as a good predictor of telomere dynamic. Indeed, There was a significant and positive relationship between the first principal component (PC1) axis and telomere dynamic with the most PFASs-contaminated individuals showing the slowest rate of telomere shortening from 2012 to 2014 (slope = 0.17; p-value = 0.026; R^2 = 0.29; Figure 16B). Additionally, 4 individuals displayed elongated telomeres from 2012 to 2014. PC1 is essentially influenced by high concentrations of PFDcA, PFUnA, PFOSlin and PFDoA. Importantly, all these compounds (not PFNA and PFTrA) were actually positively and significantly (marginally for PFOSlin) related to telomere dynamic. We thus prefer to use a PCA in **paper I** to investigate relationships between PFASs and telomeres.

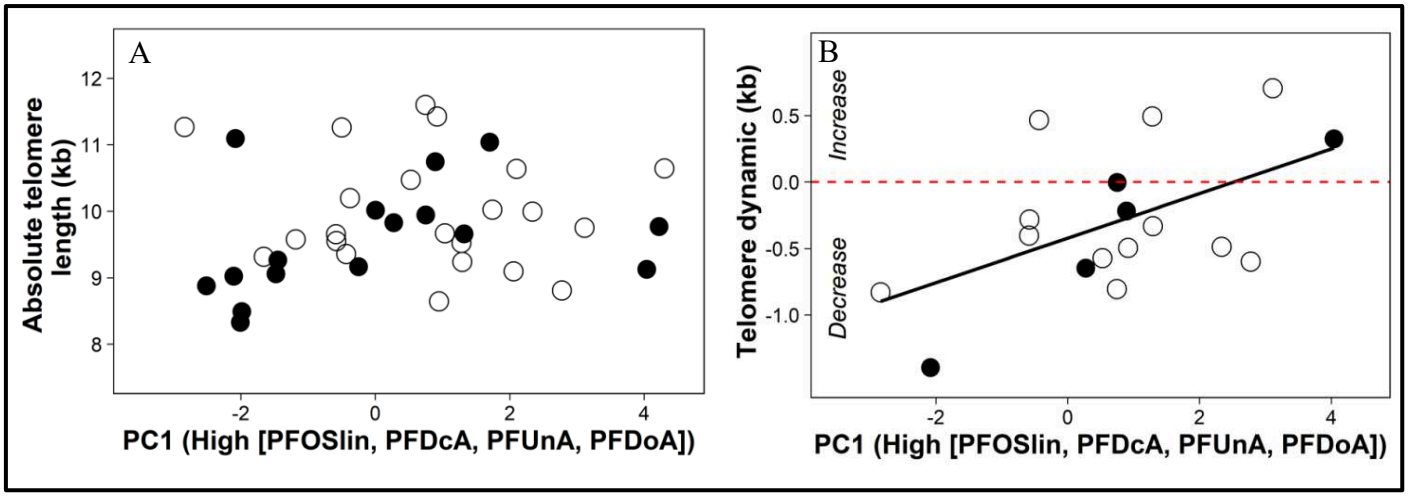


Figure 16: (A) Relationship between absolute telomere length and plasma PFAS concentrations (from PCA) in female (n=16; in black) and male (n=22; in white) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. (B) Relationships between telomere dynamic (difference of telomere length over a time frame of 2 years) and plasma PFAS concentrations (from PCA) in female (n=5) and male (n=12) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

Because telomere length is often tightly linked to survival (Asghar et al., 2015; Barrett et al., 2013; Bauch et al., 2014; Bize et al., 2009; Boonekamp et al., 2014; Haussmann et al., 2005; Heidinger et al., 2012; Fairlie et al., 2016; Foote et al., 2010; Salomons et al., 2009), only chemical compounds known to potentially affect survival of kittiwakes from the same population were considered in **paper II** (Goutte et al., 2015). Thus, we selected the PCBs (CB-99, -118, -138, -153, -180, -183 and -187), and the OCPs (HCB, *p,p'*-DDE, oxychlordane, *trans*- and *cis*-nonachlor). Among all the considered OCs, only blood concentration of oxychlordane, the main metabolite of the chlorinated pesticide "chlordane", is related to telomere length (Figure 17). Indeed, we observed a negative and significant relationship between absolute telomere length and oxychlordane concentrations in females (slope = 4.10^{-4}; p-value = 0.037; R^2 = 0.28), but not in males. Please, see **papers I & II** for more details on statistical analyses and results.

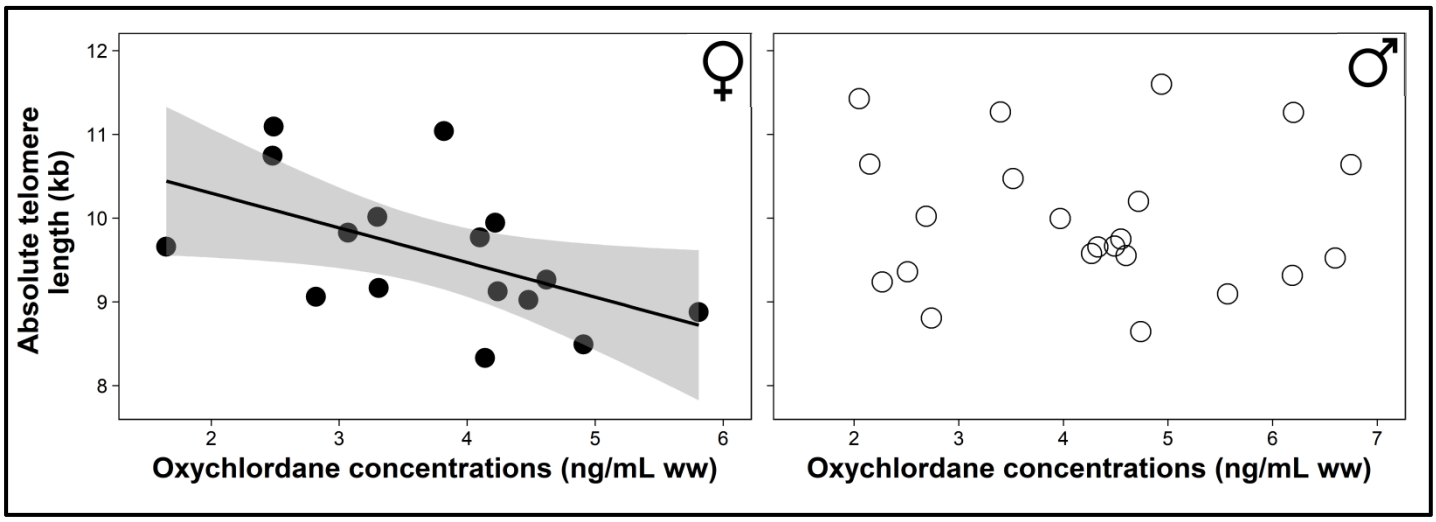


Figure 17: Relationships between absolute telomere length and whole blood oxychlordane concentrations in female (n=16) and male (n=22) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

4 - Discussion

a) Comparison with previous studies

Effects of environmental contaminants on telomere length are almost unknown for wildlife and to date, only two recent studies have addressed this question. The first one reported shorter telomeres in great tit (*Parus major*) nestlings from polluted zones (with a mix of different trace elements) compared to less-polluted control areas (Stauffer et al., 2017). Alternatively, no such relationships were found for pied flycatchers (*Ficedula hypoleuca;*

Stauffer et al., 2017). The second study has been performed on white-tailed eagle (*Haliaeetus albicilla*) chicks in northern Norway (Sletten et al., 2016). Here, the authors did not find any significant relationships between absolute telomere length and organohalogenated contaminants, including PFASs, OCPs and PCBs. Contrary to the results from the longitudinal approach, PFASs did not predict telomere length in eagle chicks. However, this study did not investigate the relationships between PFASs and telomere dynamics, but rather used a cross-sectional approach (i.e. a single measure of telomere length). This could potentially explain the discrepancy between the results of the two studies (this point is further discussed in section D4c). Another potential explanation would rely on a difference in concentration of contaminants between eagle chicks and kittiwake adults. Indeed, oxychlordane concentrations in our kittiwakes (4.02 ± 0.21 (SE) ng/mL ww) were on average, around 3 times higher than those reported for eagles (1.48 ± 0.20 ng/mL ww), which is consistent with the latest hypothesis. However, this explanation does not seem relevant for PFASs since (i) PFOSlin concentrations in adult kittiwakes (9.88 ± 0.46 ng/mL ww) were on average 4 times lower than those in eagle chicks (40.91 ± 5.75 ng/mL ww); (ii) PFUnA concentration in adult kittiwakes (10.75 ± 0.51 ng/mL ww) were on average 2 times higher than those in eagle chicks (5.61 ± 0.53 ng/ mL ww). Given the paucity of available data and the contrasted results between the studies, it appears challenging to make affirmative conclusions and generalizations. However, these inconclusive results highlight the need to increase the number of studies in order to better understand the potential effects of contaminants on telomere length.

Yet, we have some reasons to think that oxychlordane, the principal metabolite of a banned insecticide extensively used during >35 years, could induce telomere shortening. Interestingly, a recent study conducted in elderly people from Helsinki corroborates our results since they reported negative association between PCB-153, *trans*-nonachlor and especially oxychlordane with telomere length in women but not in men (Guzzardi et al., 2016). Moreover, in glaucous gull (*Larus hyperboreus*) from Svalbard and in kittiwakes from the same colony, oxychlordane has been associated with lower survival rate, especially in females (Erikstad et al., 2013; Goutte et al., 2015). Because telomere length appears as a good predictor of survival and longevity (Asghar et al., 2015; Barrett et al., 2013; Bauch et al., 2014; Bize et al., 2009; Boonekamp et al., 2014; Haussmann et al., 2005; Heidinger et al., 2012; Fairlie et al., 2016; Foote et al., 2010; Salomons et al., 2009), female kittiwakes' sensitivity to oxychlordane could affect telomere length, explaining the previously reported lower survival rate in highly contaminated female kittiwakes.

b) Some potential underlying mechanisms

Telomere length adjustment is dynamic with both shortening and maintenance events. Despite a mechanism to slow-down telomere shortening, the end-replication problem leads to progressive attrition of chromosomes, leading to the onset of cellular senescence or apoptosis (Blasco et al., 2007; Campisi et al., 2001; Harley et al., 1990; Olovnikov, 1996). However, telomere restoration based on telomerase activity, an enzyme adding new telomeric sequences onto the ends of chromosomes at each DNA replication, has been shown to be the primary mechanism for telomere maintenance and genomic integrity (Blackburn, 1991, 2005; Greider and Blackburn, 1985). Telomerase is variably active in several proliferative and post-mitotic somatic tissues throughout the lifespan of long-lived seabirds (Haussmann et al., 2007). This latest study highlighted the very high activity of telomerase in bone marrow during the whole lifespan of two seabird species, the common tern (*Sterna hirundo*) and the leach's strom petrel (*Oceanodroma leucorhoa*; Haussmann et al., 2007). The authors stated that "*telomerase activity in bone marrow may be associated with the rate of erythrocyte telomere shortening; birds with lower rates of telomere shortening and longer lifespans have higher bone marrow telomerase activity throughout life*". Indeed, all circulating erythrocytes in birds are produced by the hematopoietic stem cells of the bone marrow (Sturkie and Griminger, 1976), and telomere length measured in blood cells (i.e. erythrocytes and leucocytes) appear to mirror the telomere length of stem cells in bone marrow (Kimura et al., 2010b; Vaziri et al., 1994; but see Reichert et al., 2013). Indeed, several correlational and experimental studies have highlighted a potential role of glucocorticoids in determining telomere dynamics: increased glucocorticoids concentration (i.e. corticosterone and cortisol) were associated with a down-regulation of telomerase activity or/and an accelerated rate of telomere shortening (**Paper VIII**; Bauch et al., 2016; Choi et al., 2008; Haussmann et al., 2012; Quirici et al., 2016; Schultner et al., 2014; Young et al., 2016, 2017; but see Epel et al., 2010). Importantly, we have already shown a negative relationship between baseline corticosterone levels and PFAS concentrations in kittiwakes (*Tartu et al., 2014*). Even if underlying mechanisms are currently unclear, PFASs-induced lower circulating corticosterone levels might potentially result in relatively high telomerase activity in bone-marrow, and therefore in decreased rate of telomere shortening in highly contaminated kittiwakes (Figure 18A). However, we have to be cautious with this proposed mechanism that remains purely speculative and further investigations are needed to confirm this scenario.

Alternatively, one mechanism that could potentially explain telomere shortening in response to oxychlordane exposure is oxidative stress (Figure 18B; Von Ziglinicki et al., 2000; Zhang et al., 2013). Interestingly, several OCs such as PCBs and oxychlordane have been associated to oxidative stress in Svalbard kittiwakes (Lindsøe, 2012). Oxidative stress corresponds to the imbalance between the productions of reactive oxygen species (ROS) and the antioxidant capacity of an organism (Finkel and Holbrook, 2000). When metabolic by-products, such as ROS, are not fully neutralized by antioxidant defenses, they may oxidize cellular macromolecules such as DNA (Houben et al., 2008). As the nucleobase guanine is a major oxidation target of ROS, the (TTAGGG)*n* repeats that constitute vertebrates telomeres are particularly vulnerable to oxidative attacks (Whang et al., 2010). In our study, biomarkers of oxidative stress were not measured. Thus, further investigations measuring at the same time OC levels, proxies of oxidative stress and telomere length are needed to test if oxidative stress induced by oxychlordane exposure could be linked to telomere attrition.

Finally, the reduced telomere shortening in most-PFASs contaminated kittiwakes in inconsistent with the increased oxidative damage and lowered antioxidant reported in the most PFASs-contaminated birds (**Paper VII**). Once again, this is very challenging to conclude about underlying mechanisms triggering telomere length following PFASs exposure and further studies measuring oxidative status, telomerase activity, telomere length and PFAS levels at the same time would help to understand the reported relationships.

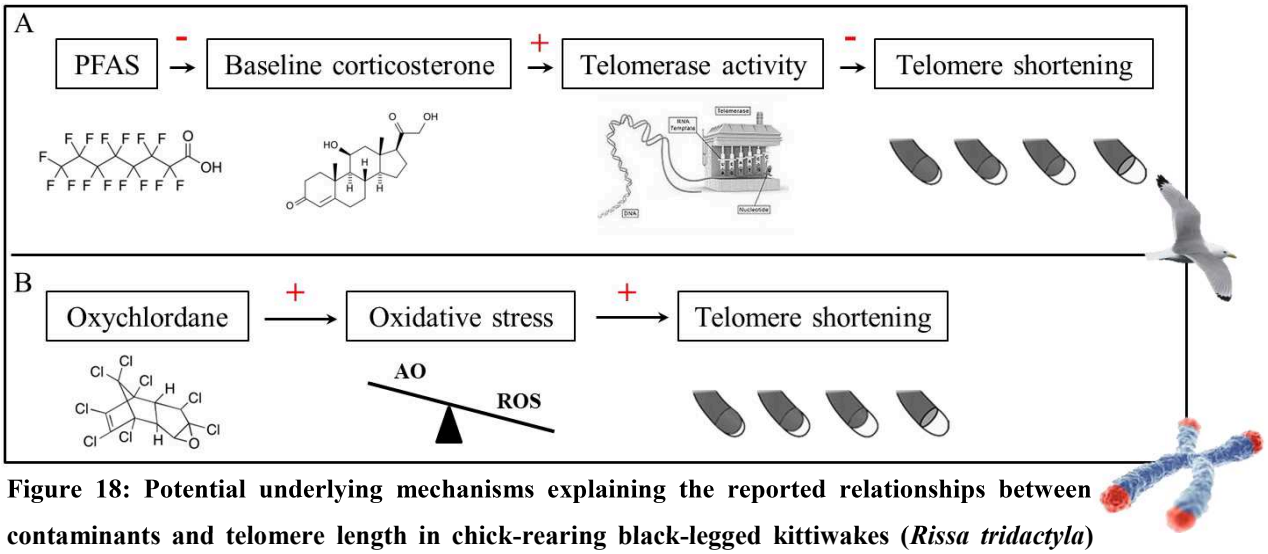


Figure 18: Potential underlying mechanisms explaining the reported relationships between contaminants and telomere length in chick-rearing black-legged kittiwakes (*Rissa tridactyla*) from Kongsfjord, Svalbard. AO means antioxidant.

c) Longitudinal approach versus cross-sectional approach

We observed no relationships between PFASs and absolute telomere length when analyzing only one year (cross-sectional approach in 2012). However, the results from the longitudinal approach indicated PFASs as a good predictor of telomere dynamics. Considering the discrepancy in the findings between the two approaches, our study highlights the need to investigate the effects of PFASs on telomere dynamics with a longitudinal approach, rather than measuring absolute telomere length in a single snapshot. In vertebrates, most of telomere shortening occurs early in life, during growth and developmental stages and this rate of early-life shortening varies to a great extent between individuals (Boonekamp et al., 2014; Hall et al., 2004; Foote et al., 2010; Frenck et al., 1998; Friedrich et al., 2001; Rattiste et al., 2015; Salomons et al., 2009; Zeichner et al., 1999). In addition, telomere length can also be affected later in life, in adults, by variation in stressful experiences (Angelier et al., 2013; Epel et al., 2004; Hau et al., 2015; Mizutani et al., 2013; Young et al., 2013). As a result, there is probably a large inter-individual variability in telomere length in adult kittiwakes and this variability may result from several factors that were not taken into account in our analyses (e.g. age, environmental stressors, etc.). This large inter-individual variability could blur the potential effect of PFASs contamination on telomere length when using a cross-sectional approach, possibly explaining why we were not able to detect any correlations between PFASs contamination and absolute telomere length in 2012. Because PFASs contamination appears quite repeatable over two years within the same individual, the longitudinal approach allows us to relate such PFASs contamination in 2012 with telomere dynamics. Relationships between OCs contamination in 2012 and telomere dynamics have not been investigated here since OCs repeatability cannot be tested (OCs have not been analyzed for field season 2014). Moreover, it seems unlikely that the lipophilic OCs within individual kittiwakes were quite repeatable over such a long period (2 years) given their important dynamic circulation throughout the different organism compartments (storage in adipose tissue, (re)-mobilization into the blood circulation…).

d) Telomere elongation in most PFAS-contaminated kittiwakes

Our study reported some telomere elongation between 2012 and 2014 in 4 kittiwakes (Figure 19A). Telomere elongation has already been associated with nutritional and climatic factors. Recently, Hoezl et al. (2016) showed that food supplementation reduces telomere attrition and is even associated with telomere elongation in a wild mammal species, the dormouse (*Glis glis*). Similarly, Bebbington et al. (2016) reported increased telomere length

with high food availability in a small passerine, the Seychelles warbler (*Acrocephalus sechellensis*). In the same line, kittiwake chicks in nests with low sibling competition (i.e. with less competition for resource), maintained their telomere length across the chick rearing period while chicks in enlarged brood size showed significant telomere shortening (Young et al., 2017). Finally, a study conducted on the black-tailed gull (*Larus crassirostris*) highlighted a potential positive effect of El Niño on telomere dynamics (Mizutani et al., 2013). Interestingly, a recent study investigating the diet of kittiwakes from Kongsfjord seems to indicate a relatively high occurrence of fish, with a probably higher energetic value, compared to other marine invertebrates species with lower nutritional quality during breeding season "2013" (Vihtakari et al., 2018). Therefore, lower rate of telomere shortening in most PFASs contaminated kittiwakes highlighted in our study, in combination with good environmental conditions, could potentially explain why we observed telomere elongation in some kittiwakes between 2012 and 2014.

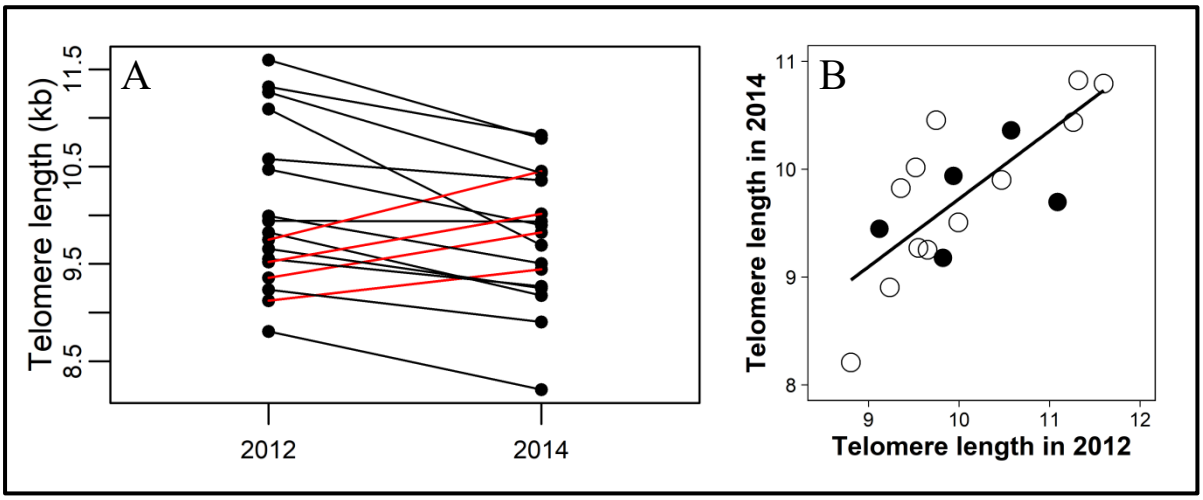


Figure 19: (A) Difference of telomere length between 2012 and 2014 in chick-rearing adult black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. 14 kittiwakes have been sampled twice and 4 birds (indicated with a red line) displayed elongated telomeres. (B) Relationship between telomere length in 2012 and 2014 (males with white dots and females with black dots).

e) Interstitial telomere sequences: a potential bias to the results?

Interstitial telomere sequences (ITSs) are telomeric repeats located within chromosomes which could be an important factor to consider here (Foote et al., 2013). According to the method employed for assessing telomere length (e.g. "denatured TRF method" as in our study), ITSs can be included within the telomere length calculation and could potentially underestimate absolute telomere length because most ITSs are shorter than

most “true telomeres” (Foote et al., 2013). Additionally, number of ITSs can fluctuate between and within individuals of the same species and thus, could bias inter-individual comparisons and longitudinal follow-up (Stier et al., 2017 oral communication during the telomere dynamic workshop). Consequently, it would be appropriate to confirm our results with another method such as the “in gel TRF method” which excludes ITSs from telomere length calculation (Foote et al., 2013). However, given the highly significant and positive relationship between telomere length in 2012 and 2014 ($r_{pearson} = 0.76$; p-value = 4.10^{-4}; Figure 19B), we have some good reason to think that ITSs are relatively constant over this time-window and not a major issue in our study (Nettle et al., 2017 oral communication during the telomere dynamic workshop).

f) Age: a potential confounding factor?

The birds' age was unknown in our kittiwakes and an important point that deserves to be discussed is a potential confounding effect of age which is suggested to negatively affect telomere length (Haussmann and Vleck, 2002; Haussmann et al., 2003). However, this is particularly true for species with shorter lifespans which lose more telomeric repeats with age than species with long lifespans (Haussmann et al., 2003). In long-lived species, telomere loss appears to occur mainly early in life (i.e. between chick and adult stage) rather than during adulthood (Figure 20A; Foote et al., 2010; Hall et al., 2004), as it is the case in other vertebrates (Frenck et al., 1998; Friedrich et al., 2001; Rufer et al., 1998; Zeichner et al., 1999). Consequently, effect of age on telomere length appears to be more complex rather than a simple negative and constant decrease of telomere length with age. Since our study was conducted on breeding adults (i.e. at least 3 - 4 years old; Coulson, 2011) of a long-lived seabird and because we investigated telomere dynamics, with a longitudinal approach, we have some good reasons to think that age in **paper I** is not a major factor influencing telomere length. Additionally, it appears that plasma PFAS and age are positively related in two Antarctic species, the snow petrel (*Pagodroma nivea*) and the South Polar skua (*Stercorarius maccormicki*; Figure 20B; Chastel et al., in prep). In that case, the oldest birds would be the most contaminated ones and thus would display elongated telomeres, which is actually inconsistent with a suggested telomere shortening with age. Finally, it has been reported that in several seabird species, blood level of OCs is unrelated to age in adult birds (Bustnes et al., 2003; Carravieri et al., 2014; Tartu et al., 2015a), and rather reaches a steady-state of equilibrium once adult (Figure 20C; Bustnes et al., 2003; Drouillard, 2002; Henriksen, 1995;

Newton et al., 1981; Robinson et al., 1967). Therefore, it is unlikely that the negative relationship between oxychlordane and telomere length in **paper II** originates from birds with high levels of oxychlordane being the oldest birds with the shortest telomeres.

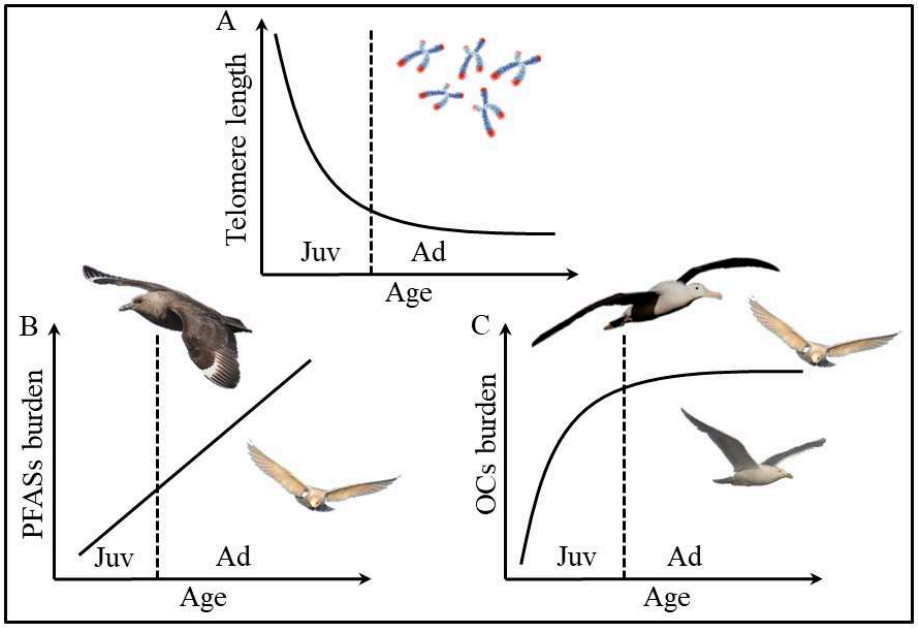


Figure 20: (A) Suggested relationship between telomere length and age in long-lived species. (B) Relationship between plasma PFASs burden and age in the snow petrel (*Pagodroma nivea*) and the South-polar skua (*Stercorarius maccormicki*) from Adelie land, Antarctica. (C) Relationship between blood OCs concentrations and age in the wandering albatross (*Diomedea exulans;* Carravieri et al., 2014), the snow petrel (Tartu et al., 2015a) and the glaucous gull (*Larus hyperboreus;* Bustnes et al., 2003).

E - Contaminants and energy expenditure

This chapter essentially relates, summarizes and discusses main results from **paper III**. The aim of this paper was to investigate the consequences of contaminant exposure on energy expenditure. Specifically, we examined the relationships between some PFASs, OCs and Hg with metabolic rate, considered here as a proxy of basal metabolic rate (BMR) and also with circulating total THs (thyroxine: TT4 and triiodothyronine: TT3) as potential underlying mechanisms in adult breeding kittiwakes during the chick-rearing period. This publication is attached at the end of the manuscript for further information.

1 - What is BMR?

Understanding the concept of energy allocation toward maintenance requirements, activity, growth and reproduction is a central goal which integrates both ecology and physiology. Usually considered as the minimal energetic cost of living, BMR is defined as the lowest rate of energy expenditure in a post-absorptive, adult endotherm at rest in its thermoneutral zone (Bligh and Johnson, 1973; Ellis and Gabrielsen, 2002; McNab, 1997). BMR is by far the most widely assessed parameter in vertebrate energetics (Danforth and Burger, 1984; Ellis, 1984) and has been described for a large variety of species from a wide geographical range (Bryant and Furness, 1995; Ellis, 1984; Ellis and Gabrielsen, 2002; Scholander et al., 1950). Although subject to circadian and seasonal fluctuations (Aschoff and Pohl, 1970; Kendeigh et al., 1977), a significant part of BMR variation within and between species can be attributed to adaptations either to specific environmental conditions or to particular behavioral traits of the species (Bech et al., 1999; Burton et al., 2011; Gabrielsen, 1994; Verreault et al., 2007). However, effects of environmental contaminants on metabolic activity are still poorly known and largely debated in the literature since the few studies that have investigated this topic in adult birds and mammals are limited and somewhat contradictory. Importantly, the consequences of PFASs exposure on energy expenditure were until recently unknown for wildlife.

2 - What are THs?

Thyroid hormones (THs) are involved in a multitude of physiological traits and are known to regulate metabolism. Specifically, T4 and especially T3 are considered as the major

controllers for the regulation of tissue oxygen consumption, thermogenesis and metabolic activity in endotherms (Bobek et al., 1977; Danforth and Burger, 1984; Freake and Oppenheimer, 1995; Hulbert, 2000). The roles of THs in the regulation of metabolism have been well documented in laboratory studies (Hulbert, 2000; Kim, 2008; Silvestri et al., 2005), and several investigations conducted in domestic as well as in free-living animals have highlighted strong and positive associations between total T3 (TT3) levels and BMR (Bobek et al., 1977; Chastel et al., 2003; Elliott et al., 2013; Vézina et al., 2009; Welcker et al., 2013; Zheng et al., 2013). Over the last few years, a significant amount of studies have led to the hypothesis that exposure to environmental contaminants could be the cause of disruption of thyroid function and several studies have reported abnormal TH concentrations and thyroid gland structure in birds exposed to contaminants under controlled laboratory conditions, as well as in free-ranging populations (Cesh et al., 2010; Dawson, 2000; Haugerud, 2011; McNabb, 2005; McNabb and Fox, 2003; Melnes et al., 2017; Nøst et al., 2012; Rolland, 2000; Scanes and McNabb, 2003; Smits et al., 2002; Svendsen et al., 2018; Verreault et al., 2004, 2007, 2013; Wada et al., 2009). Besides, circulating levels of THs appear to be suitable biomarkers as measures of contaminant-associated effects in wildlife (Fox, 1993; McNabb, 2005; Peakall, 1992; Rolland, 2000).

3 - How to measure BMR?

BMR measurements were performed during the chick rearing period on 23 individuals (12 males and 11 females) among the 44 kittiwakes that have been caught in total. After capture and blood sampling, birds were kept in an opaque box and rapidly transported by boat (within 20 min) to the laboratory of Ny Ålesund to measure BMR by open-flow-through respirometry measurements of at least two hours duration. Outside air was drawn into the lab and dried in indicator silica gel before entering a 41 L plexiglass chamber holding the bird. Air was drawn past the bird and into a Sable Systems FoxBox analyzer at a rate of 1.92 ± 0.04 (SD) L/min. CO_2 was measured by the FoxBox, after which the air was scrubbed of CO_2 with indicator soda lime and dried again with indicator silica gel before returning to the FoxBox where O_2 was measured. Prior to and following each BMR measurement, the bird was weighed to the nearest 0.1 g on an electronic balance and its body temperature (T_b) was taken with a Schultheis fast reading reptile mercury thermometer accurate to 0.2°C. During metabolic measurements, the chamber was covered with a towel to allow diffuse light while preventing the bird from observing its surroundings. Chamber temperature (T_a) was

monitored continuously by a probe connected to the FoxBox and averaged 19.2 ± 1.8°C (SD; ranged from 15.1 to 22.7°C). T_b averaged 40.5 ± 0.7°C (SD; ranged from 38.8 to 41.9°C). Readings of all gases, flow rate, and T_a were made every 20 s by the FoxBox and recorded with a time stamp on a laptop computer. Most kittiwakes caught on their nest had been there for an unknown period of time, so it was not known if they were entirely post-absorptive. For that reason, metabolic measurements were not typically begun until about 4 h post-capture. By itself, that did not guarantee a post-absorptive condition, but the time was limited to reduce the duration the bird was away from the nest. Another consideration for BMR is that birds are in their thermoneutral zone when measured. Indeed, two previous studies reported the upper range of that zone to be at least 20°C for kittiwakes (not tested at $T_a \geq 20$°C; Gabrielsen et al., 1988; Humphreys et al., 2007). Because T_a was set by room temperature, seven of the birds were measured at temperatures exceeding 20°C, though in all but two cases (21.6 and 22.7°C) Ta ≤ 21°C. For these reasons, the reported BMR in our study could be slightly different from the strict term's meaning (see **paper III** for more details). Finally, it is worth noting that we used here the mass-specific BMR to control for a potential effect of body mass on BMR (see **paper III** for more details).

4 - How to measure THs?

TT3 and TT4 analyses were performed by radioimmunoassay on 35 and 33 kittiwakes, respectively (TT3: 15 males and 20 females; TT4: 15 males and 18 females). Total TH levels were assessed in duplicates without extraction. 25 µL of plasma were incubated during 24 h with 10 000 cpm of the appropriate ^{125}I-hormone (Perkin Elmer, US) and polyclonal rabbit antiserum supplied by Sigma company (US). The bound fraction (hormone linked to antibody) was then separated from the free fraction by addition of a sheep anti-rabbit antibody and centrifugation. After overnight incubation and centrifugation, bound fraction activity was counted on a wizard 2 gamma counter (Perkin Elmer, US). Cross-reactions of T3 antiserum were defined as follows by Sigma: triiodoD-thyroacetic acid 6%, L-thyroxine 0.2%, diiodo-L-thyrosine < 0.01%, monoiodo-L-thyrosine < 0.01%. Cross-reactions of T4 antiserum were defined as follows by Sigma: triiodothyronine 4%, diiodo-L-thyrosine < 0.01%, monoiodo-L-thyrosine < 0.01%. Inter- and intra-assay variations were respectively 15.9% and 7.5% for TT3, 19.4% and 12.2% for TT4. The lowest TT3 detectable concentration was 0.34 ng/mL and 0.51 ng/mL for TT4. Two samples were serially diluted in the assay buffer and their displacement curves were parallel to the standard curve.

5 - Relationships between contaminant concentrations and BMR in kittiwakes

Among all the PFASs considered in our study, only the long-chained PFTrA significantly explained BMR variations in females, but it did not in males where no valuable models were retained. We found a highly significant and positive relationship between plasma PFTrA concentrations and BMR in female kittiwakes (slope = $7.61*10^{-5}$; $r_{pearson}$ = 0.75; p-value = 0.008; Figure 21A). Considering all the OCs investigated in our study, the model including ΣCHLs as an explanatory variable of BMR showed the best fit to the data. We observed a significant and negative relationship between blood ΣCHLs concentrations and BMR in both sexes (slope = $-4.05*10^{-5}$; $r_{pearson}$ = −0.51; p-value = 0.012; Figure 21B). Finally, Hg was not related to BMR neither in males nor in females. Please, see **paper III** for more details on statistical analyses and results.

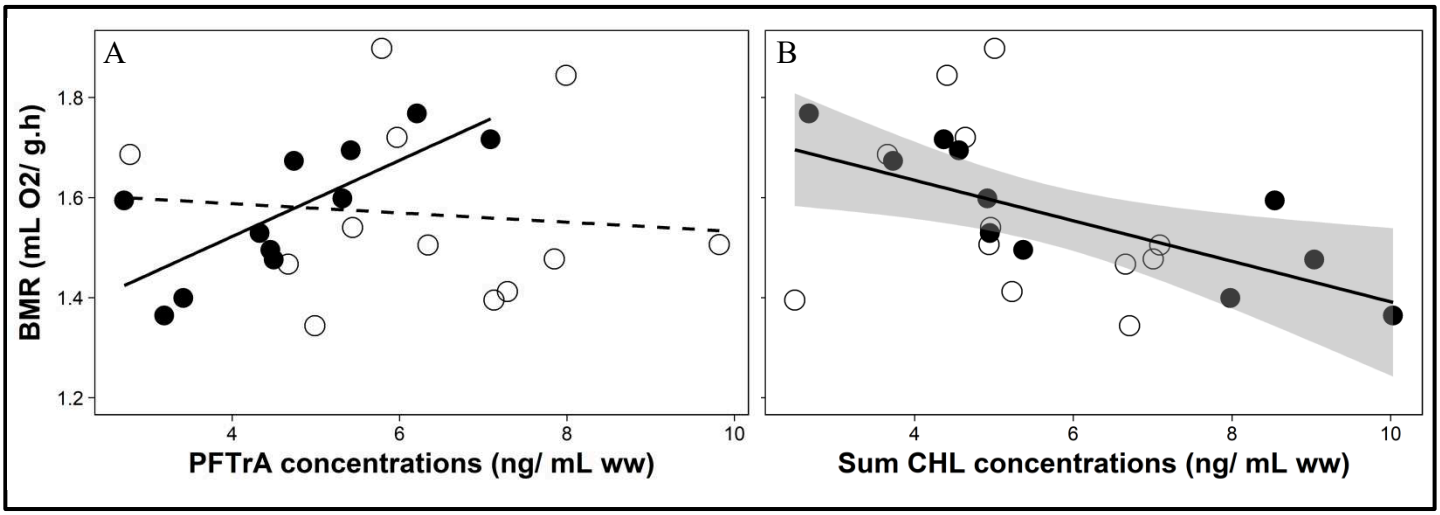


Figure 21: (A) Relationship between BMR and plasma PFTrA concentrations in female (n=11; black dots and solid line) and male (n=12; white dots and dashed line) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. (B) Relationship between BMR and blood ∑CHLs concentrations in female (n=11; in black) and male (n=12; in white) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

6 - Relationships between contaminant concentrations and THs in kittiwakes

Because THs are known to exert a strong control on the regulation of metabolic functions in kittiwakes (Elliott et al., 2013; Welcker et al., 2013), individuals exposed to high concentrations of PFTrA and ∑CHLs may experience altered metabolic activity in response to disrupted thyroid function (see section E7b for more explanations). Surprisingly, plasma concentrations of PFTrA and total TH concentrations were not related (i.e. TT3 and TT4;

Figure 22A). However, ΣCHLs was negatively and significantly related to circulating TT3 ($r_{pearson}$ = −0.38; p-value = 0.027; Figure 22B) but not to TT4 (Figure not shown). Please, see **paper III** for more details on statistical analyses and results.

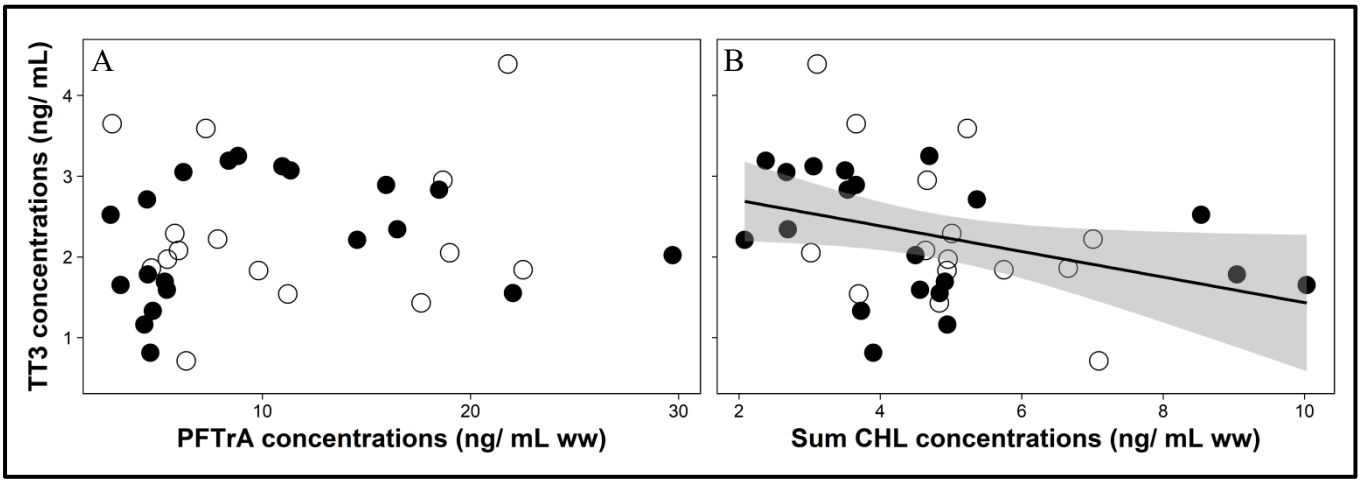


Figure 22: (A) Relationship between TT3 and plasma PFTrA concentrations in female (n=20; in black) and male (n=15; in white) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. (B) Relationship between TT3 and blood ∑CHLs concentrations in female (n=20; in black) and male (n=15; in white) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

7 - Discussion

a) Comparison with previous studies

To date and to the best of our knowledge, our study is the first one to investigate relationships between PFASs and BMR in vertebrates. Therefore, no comparisons with previous studies are possible and our findings can only suggest a possible positive effect of PFTrA on BMR in female kittiwakes (Figure 23A). Moreover, effects of PFASs on THs have received little attention and are somewhat contradictory (DeWitt, 2015). While some studies conducted in murine and primate models indicated negative associations between PFASs and TH levels (Lau et al., 2003; Seacat et al., 2002), Liu et al. (2011) reported increased TT3 levels in response to experimental PFASs exposure in the zebrafish (*Danio rerio*). Additionally, recent studies conducted on Arctic seabirds, the glaucous gull (Haugerud, 2011; Melnes et al., 2017), the northern fulmar (*Fulmarus glacialis*; Braune et al., 2011; Nøst et al., 2012), the Arctic skua (*Stercorarius parasiticus*), and kittiwakes (Ask, 2015; Nøst et al., 2012) have shown significant and positive associations between PFASs and total TH levels. It is worth noting that the study of Ask (2015) was conducted on the same population as ours (but in a different year) and indicated a positive relationship between PFTrA and TT3 only in

females. Although there are several reports showing a positive association between PFASs and total TH levels in seabirds, we did not observe such relationships. Therefore, our study does not confirm the potential thyroid-disrupting properties of PFTrA previously reported in Arctic seabirds and further investigations conducted on a larger sample size and with an experimental approach are thus needed.

Effects of OCs on metabolic activity are still poorly investigated and largely debated in literature. Although some experimental designs conducted in adult mammals reported conflicting observations with increased (Braham and Neal, 1974; Voltura and French, 2000) or unchanged (French et al., 2001; Gordon et al., 1995) metabolic rate in response to OCs exposure (PCBs, DDTs and 2, 3,7, 8-tetra-chlorodibenzo-p-dioxin), other studies conducted in birds corroborate our results. Indeed, decreased metabolic rate was observed in captive doves and pigeons exposed to Aroclor 1254 and DDTs, respectively (Jefferies and French, 1971; Tori and Mayer, 1981). Similar to our findings, a study conducted on free-living Arctic-breeding glaucous gulls revealed negative associations between BMR and concentrations of PCBs, DDTs, and especially CHLs (Verreault et al., 2007). Finally, two previous studies conducted in humans also support our findings since they highlighted lower metabolic activity in response to increasing plasma concentration of OCs mixture (including CHLs; Pelletier et al., 2002; Tremblay et al., 2004). Consequently, our study in combination with previous works points out a negative effect of CHLs on BMR (Figure 23B). Besides, a wide body of evidences suggested a possible disruption of THs in response to OCs exposure in vertebrates and several studies conducted in free-living animals have reported reduced TH concentrations with increasing OC levels (Bourgeon et al., 2017; Braathen et al., 2004; Brouwer et al., 1998; Cesh et al., 2010; Dawson, 2000; Jenssen, 2006; McNabb, 2005; McNabb and Fox, 2003; Melnes et al., 2017; Peakall, 1992; Rolland, 2000; Scanes and McNabb, 2003; Svendsen et al., 2018). For example, a previous study conducted in another Arctic seabird, the glaucous gull, stated that among 18 different congeners, oxychlordane (included here in ΣCHLs) and HCB appeared to be the most prominent OCs in terms of their negative effect on both TT4 concentrations and TT4:TT3 ratios (Verreault et al., 2004). Moreover, Smits and Fernie (2013) showed negative relationships between plasma total THs (TT3 and TT4) and several organohalogenated contaminants, including CHLs, in peregrine falcon nestlings (*Falco peregrinus*). Similarly, an experimental study conducted in American kestrels (*Falco sparverius*) reported depressed TT3 levels in PCB-exposed birds (Smits et al., 2002). Here, we reported a significant negative relationship between ΣCHLs and TT3. Therefore, our study

highlights the potential endocrine disrupting properties of CHLs on circulating TH levels in an Arctic seabird (Figure 23B).

b) Some potential underlying mechanisms

A hormonal cascade along the hypothalamic-pituitary-thyroid axis will lead to the production and release into the blood of T4 from the thyroid gland. The transport of T4 will then be ensured by serum binding proteins such as albumin and transthyretin (TTR). T4 is then principally converted in the liver by peripheral deiodination to T3 under the control of deiodinase enzymes (McNabb, 2007; Silvestri et al., 2005). Although the detailed pathways of how THs can affect energy expenditure are still unclear (Hulbert, 2000), it is now well established under laboratory conditions that plasma concentrations of THs increase the BMR of birds and mammals (Hulbert, 2000; Kim, 2008; Silvestri et al., 2005). This is particularly true for T3, which is considered as the primary metabolically active THs controlling BMR (Bobek et al., 1977; but see Silvestri et al., 2005). Besides, several studies conducted in domestic as well as in free-living animals, including kittiwakes from the same Svalbard population as in our study, have highlighted strong and positive associations between TT3 concentrations and BMR (Bobek et al., 1977; Chastel et al., 2003; Elliott et al., 2013; Vézina et al., 2009; Welcker et al., 2013; Zheng et al., 2013). Surprisingly, no significant correlations were found between total THs and BMR in our study. However, because we measured BMR few hours after blood sampling used for total THs quantification, plasma THs at the time of BMR measurement might be slightly different (Verreault et al., 2007; but see Welcker et al., 2013). In birds, half-lives of THs (T3 and T4) appear to be relatively short (i.e. 3 - 9 hours) and potentially reduced by cold temperatures (McNabb, 2000). Finally, we only measured total THs and not the metabolically active free fraction of T3 which could be more strongly related to BMR than total TT3.

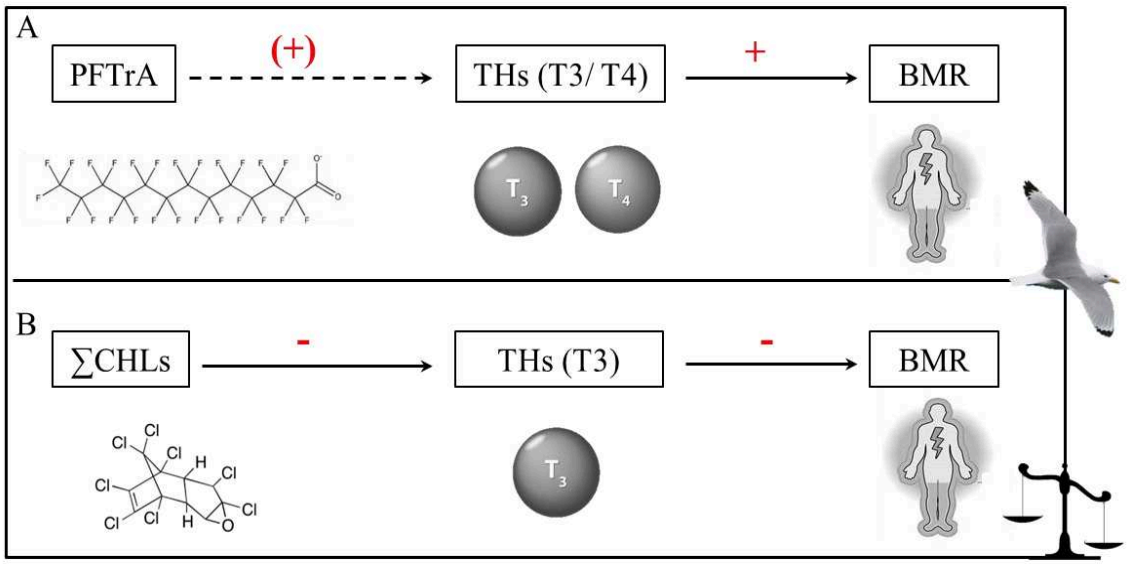


Figure 23: Potential underlying mechanisms explaining the reported relationships between contaminants, THs and BMR in chick-rearing black-legged kittiwakes (*Rissa tridactyla*) from Kongsfjord, Svalbard.

Alteration of endocrine functions triggered by endocrine disrupting chemicals may act through interference with the synthesis, secretion, transport, binding, action, or elimination of endogenous hormones (Damstra et al., 2002). Increased plasma TT3 levels in response to long-term PFASs (i.e. PFNA) exposure in the zebra fish could be explained by (i) an inhibition of the expression of UDP-glucuronosyltransferases and (ii) an up-regulation of the expression of gene transcripts encoding TTR (Liu et al., 2011). Since the metabolic inactivation and elimination of TH mainly occurs in the liver though different pathways such as the glucuronide conjugation thanks to UDP-glycosyltransferase and because PFASs exposure appears to inhibit the UDP-glycosyltransferase, this would lead to reduced TH biliary elimination and may have accounted for the increased plasma TH levels. Furthermore, since TTR is a TH binding protein transporter, an increased expression of TTR should have helped to increase TH levels induced by PFASs exposure (Liu et al., 2011). Moreover, it has been proposed that PFASs increase the expression of transcripts of hepatic transporters in laboratory rats, which in turn, could increase uptake of THs into the liver (Yu et al., 2011). However, potential mechanisms through which PFASs disrupt TH levels are still unclear and other candidate pathways such THs displacing from binding proteins due to structural similarities or direct effects in the thyroid gland itself have been recently suggested (Coperchini et al., 2015, 2017; Mortensen, 2015; Weiss et al., 2009).

Since we reported a significant and negative relationship between ΣCHLs and TT3 levels but not with TT4 in both sexes, we can hypothesize that kittiwakes exposed to high

concentrations of ∑CHLs may experience altered metabolic activity in response to decreased plasma TT3 levels (Figure 23B). TT3 and TT4 were significantly and positively related ($r_{pearson}$ = 0.46; p-value = 0.006), thus ΣCHLs could possibly alter circulating TH levels and specifically T3 levels by affecting either the conversion of T4 to T3 and/or the transport of circulating T3. Indeed, inhibition of mono-deiodinase activity can skew the concentration of the biologically active T3 (Brouwer et al., 1998). This has been reported in white Leghorn chick (*Gallus gallus*) embryos, where administration of PCB mixtures induced decreased hepatic deiodinase activities (Gould et al., 1999). Moreover, given their high degree of structural affinities with THs, some OCs may distort circulating TH levels through competitive interactions with binding sites on their carrier proteins such as the TTR, which is known in birds to have higher affinity with T3 compared to T4 (Brouwer et al., 1998; Chang et al., 1999; Mortensen, 2015; Schreiber, 2002; Yamauchi et al., 2003).

c) Possible fitness consequences

Individual BMR variations may influence fitness because self-maintenance and reproduction are considered as two key life-history components (Burton et al., 2011; Stearns, 1992). In female kittiwakes, adaptive decrease in mass-specific BMR prior to hatching has been suggested as a mean to reduce self-maintenance and to increase allocation of energy to reproduction (i.e. chick food provisioning; Bech et al., 2002; Rønning et al., 2008; Welcker et al., 2015). Exposure to PFTrA may disrupt the ability of female kittiwakes to adaptively decrease BMR, thus might possibly explain the lower hatching success in the most PFASs-contaminated adults (*Tartu et al., 2014*). In female kittiwakes, the BMR during the incubation is a good predictor of the BMR during the chick-rearing period (Bech et al., 1999). Consequently, it is likely that exposure to PFASs during the incubation period could have significant consequences for the metabolic adjustments required for the chick rearing period. In that case, the most PFTrA-contaminated female kittiwakes would allocate more energy for self-maintenance rather than for reproduction. Thus, an indirect positive effect of PFASs exposure might occur on survival rate in adult kittiwakes, at the expense of reproduction. Accordingly, results presented in chapter I (**paper I**) seemed to corroborate this statement since we reported a reduced rate of telomere shortening (i.e. a predictor of survival) over a time frame of 2 years in the most PFASs-contaminated kittiwakes. Moreover, we observed a highly significant and positive relationship between telomere length and BMR in female kittiwakes (females: slope = 8.633, p-value = 0.007; R^2 = 0.46; males: slope = -1.981, p-value

= 0.162; Figure 24) which is consistent (at least for females) with the latest hypothesis. Finally, despite some inconstancies, this has already been demonstrated experimentally and under field conditions in mammals that individuals with high BMR tend to survive more (Jackson et al., 2001; Speakman et al., 2004; but see Burton et al., 2011). However, this hypothesis absolutely needs to be confirmed (i) with capture-mark-recapture (CMR) investigations and (ii) by investigating some potential effects of PFASs on reproductive investment such as parental care behaviors (e.g. incubation temperature, egg-turning behavior).

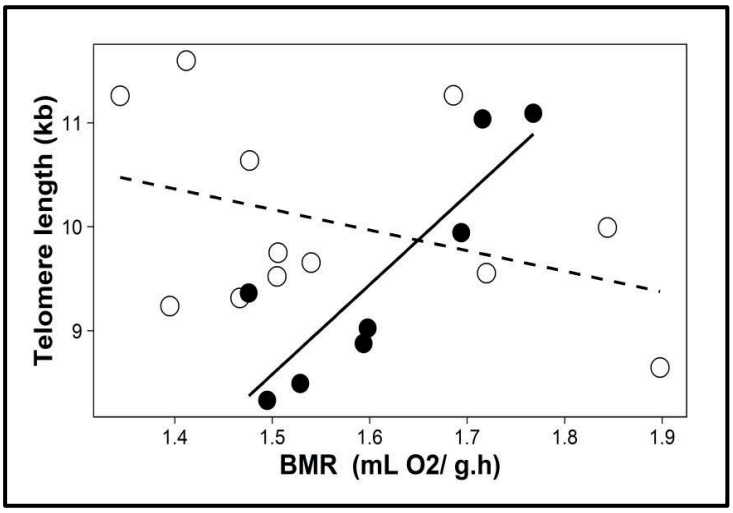


Figure 24: Relationships between telomere length and BMR in female (n=8; in black dots and solid line) and male (n=12; in white dots and dashed line) chick-rearing black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

Energetic balance is a sensitive system and additional costs from CHLs expressed here as a reduction of BMR could have some consequences on fitness. In that case, self-maintenance could be impacted, leading individuals to be less able to cope with reproduction. Besides, one recent study conducted in the same kittiwake population reported decreased adult survival rate and lower breeding probability (Goutte et al., 2015) in response to CHLs contamination. Finally, as presented in chapter I (**paper II**), the most oxychlordane-contaminated female kittiwakes displayed reduced telomere length (i.e. a predictor of survival).

F - Contaminants and parental care

This chapter essentially relates, summarizes and discusses main results from **papers IV & V**. The aim of these papers was to investigate the effects of contaminants exposure on parental care commitment. Specifically, we investigated the relationships between some PFASs, OCs and Hg with egg turning behaviors and incubation temperature but also with prolactin concentrations and brood patch size as potential underlying mechanisms, in incubating kittiwakes. These two publications are attached at the end of the manuscript for further information.

1 - What are the key components of incubation behavior?

Incubation is an essential stage in the life history of most bird species since early life developmental conditions can have long-term fitness consequences (Berntsen and Bech, 2016; Deeming, 2002; Lindström, 1999). Generally, egg attendance patterns involve different parental behaviors such as egg turning and active egg warming; both considered as being key determinants for embryo viability and egg hatchability (Decuypere and Michels, 1992; Elibol and Brake, 2004, 2006a; Funk and Forward, 1953; Poulsen, 1953; Tona et al., 2005a; Tullett and Deeming, 1987; Van Schalkwyk et al., 2000). Specifically, egg turning behavior requires an optimal turning rate with an appropriate angular change to facilitate absorption of albumen by the embryo, to reduce malposition (e.g. head in the small end of the egg) and to prevent the embryo from adhering to the inner shell membrane (Deeming, 1991; Elibol and Brake, 2004, 2006b; Eycleshymer, 1907; New, 1957; Tullett and Deeming, 1987). Therefore, a lack or decrease of egg turning events can retard or prevent albumen absorption and gas exchanges resulting in an abnormal chick development with a lower growth rate, decreasing oxygen consumption and *in fine*, leading to delayed or reduced hatching success (Deeming, 1991; Elibol and Brake, 2006a; Funk and Forward, 1953; New, 1957; Pearson et al., 1996; Robertson, 1961; Tazawa, 1980; Tona et al., 2005b; Van Schalkwyk et al., 2000; Wilson et al., 2003; Yoshizaki and Saito, 2003). In addition, maintaining eggs at an optimal temperature during incubation is a complex process (Turner, 2002) and critically important for complete embryonic development, improved hatchability, offspring's phenotype, and overall survival (Ardia et al., 2010; DuRant et al., 2013; Feast et al., 1998; Hepp et al., 2015; Nilsson et al., 2008; Nord and Nilsson, 2011, 2016; Olson et al., 2006; Webb, 1987).

In birds, set-up and maintenance of parental care behaviors are orchestrated by a cocktail of different hormones acting synergistically such as prolactin, a pituitary hormone considered as the major endocrine controller for incubation behaviors (Angelier et al., 2016; Buntin, 1996; Sockman et al., 2006; Vleck, 2002; Vleck and Vleck, 2011). Specifically, the pectoral skin of incubating birds can become a fleshy, edematous and well-vascularized brood patch, devoid of feathers (Jones, 1971; Lea and Klandorf, 2002). During incubation, this brood patch comes into direct contact with the egg to ensure proper heat transfer between a parent and the developing embryo in the egg (Jones, 1971).

Conditions required for an optimal incubation have been so far extensively investigated in the poultry industry to maximize hatchability of domestic fowl (*Gallus gallus domestica*) for commercial purposes (King'ori, 2011; Lundy, 1969; Tona et al., 2005a). In contrast, environmental conditions such as contaminants influencing incubation of free-ranging birds remained till now poorly investigated (e.g. Hg) and, to our knowledge, the consequences of PFASs exposure on incubation behaviors were until recently unexplored for birds

2 - <u>How to monitor incubation behavior?</u>

All study nests initially contained two natural eggs. We randomly collected and replaced one of these two eggs by an artificial egg containing: i) a triaxial accelerometer and magnetometer to record orientation and angular changes in three dimensions (i.e. roll, pitch and yaw) and ii) a temperature thermistor (as described in Clatterbuck et al., 2017; Kelsey et al., 2016; Shaffer et al., 2014 and Taylor et al., 2018). These loggers have the benefit to provide precise (sensing 1-2° angular changes orientation) and continuous records (~1 sec) of egg turning behaviors. Turning precision of egg-loggers was tested and verified by comparing a video recording of an egg being turned manually with an animation created by post-processing the data collected by the egg-logger (Shaffer et al., 2014). Data loggers recorded core egg temperature with a manufactured accuracy of < 2°C (but testing in the lab in a controlled environment showed the accuracy to be approximately 0.5°C) and precision of 0.125°C based on thermistor component specifications (Shaffer et al., 2014). Artificial eggs were designed according to the kittiwake egg morphology (see **paper IV** for more details). Additionally, in order to mimic the coloration and pattern of the real egg, we painted artificial eggs with a non-toxic water-based paint (Figure 25). Study nests were selected according to

their accessibility and to minimize disturbance to the rest of the colony. Collected eggs were candled and all were determined to be fertile.

Artificial eggs were deployed between 7 and 10 days during the incubation period (Figure 25). All incubating birds readily accepted the dummy egg and did not show abnormal behaviors. Because each partner of a pair was dye marked on the forehead, we could easily identified some incubation bouts of each parent (Figure 25). Thus, we recorded incubation bouts for each individual (excluding data recorded at night because checks were not conducted at night). The day of egg deployments and all records during our presence in the colony (i.e. for blood sampling) were also excluded from the data set in order to avoid any biased data. Once the experiment was completed, the artificial egg was removed and only one egg remained into the nest. Recording duration and incubation stage (i.e. embryo age) were checked for potential confounding effects and included in the analyses if necessary (see **papers IV and V** for more details). Using a mirror at the end of a long pole, we determined hatching success of the experimental nests by conducting regular checks until the end of the field season. Egg-logger data were processed using purpose-built routines in MATLAB (The Mathworks, Natick, MA, USA) following methods detailed in Shaffer et al., 2014 (Figure 25).

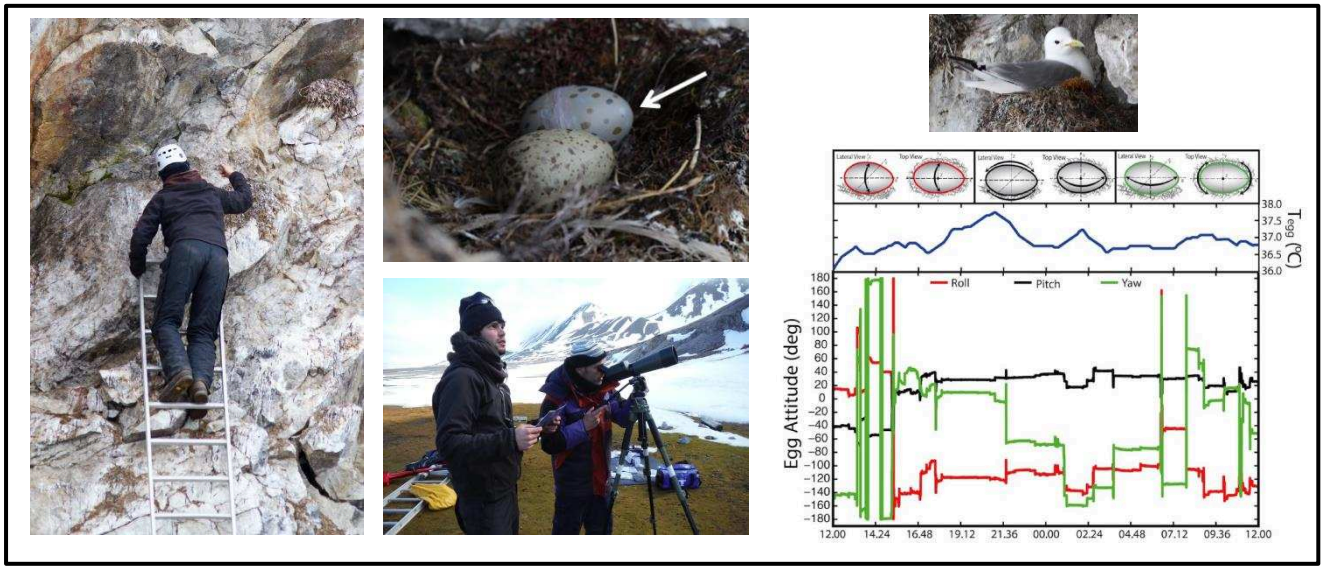


Figure 25: Egg-logger experiment to monitor incubation behavior of breeding black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. The artificial egg is indicated with a white arrow. Example of one time series showing egg turning and incubation temperature once processed with MATLAB (From Shaffer et al., 2014).

3 - <u>How to measure prolactin?</u>

Plasma prolactin concentrations were determined by radioimmunoassay as previously described and validated for this kittiwake population (Chastel et al., 2005). Intra-assay

variation was estimated by including internal standards to the assay ran in duplicates. Intra-assay variation was 7.13%. Bleeding time (i.e. time elapsed from capture to the end of the first blood sampling: 2.48 ± 0.52 min (SD), on average) did not affect baseline prolactin concentrations ($F_{1,19}$ = 0.606, p-value = 0.446).

4 - How to measure brood patch size?

Brood patch size were determined from a photograph, thanks to a ruler placed next to the bird and using Gimp 2.8. Brood patch size was determined in duplicates (all coefficients of variation ≤ 4.06%). Breast feathers were lightly brushed with moistened cotton pad to fully expose the brood patch. All study birds exhibited three brood patches (right: RBP, left: LBP and central: CBP). Thus, to minimize handling time, we only measured the RBP (except for 13 individuals to check for consistencies among brood patches) and assume that results presented here regarding the RBP could also be also relevant for the LBP and CBP (see **paper IV** for more details).

5 - Relationships between contaminant concentrations and incubation behaviors in kittiwakes

Egg-turning frequency was positively and significantly related to PFOSlin concentrations in female kittiwakes only (slope = 3.10^{-4}; p-value = 0.049, R^2 = 0.16). In other words, the most PFOSlin-contaminated females turned their eggs more often compared to the less contaminated ones. OCs and Hg were not significantly related to egg-turning frequency in both males and females. Angular change was positively and significantly associated with PFASs concentrations (i.e. PFOSlin and ∑PFCAs) in females but negatively and significantly related to the ∑PCBs concentrations in female kittiwakes (PFOSlin: slope = 3.10^{-3}, p-value = 0.053, R^2 = 0.19 ; ∑PFCAs: slope = 2.10^{-3}, p-value = 0.007, R^2 = 0.34; ∑PCBs: slope = -5.10^{-4}, p-value = 0.034, R^2 = 0.23; Figure 26). In other words, the most PFASs-contaminated females turned their eggs, in average, with higher amplitude while the most PCBs-contaminated females turned their eggs with lower amplitude. In males, we observed a positive and significant relationship between angular change and ∑PFCAs only (slope = 1.10^{-3}, p-value = 0.044, R^2 = 0.21; Figure 26). Hg was never associated with angular change in both sexes. Please, see **paper IV** for more details on statistical analyses and results.

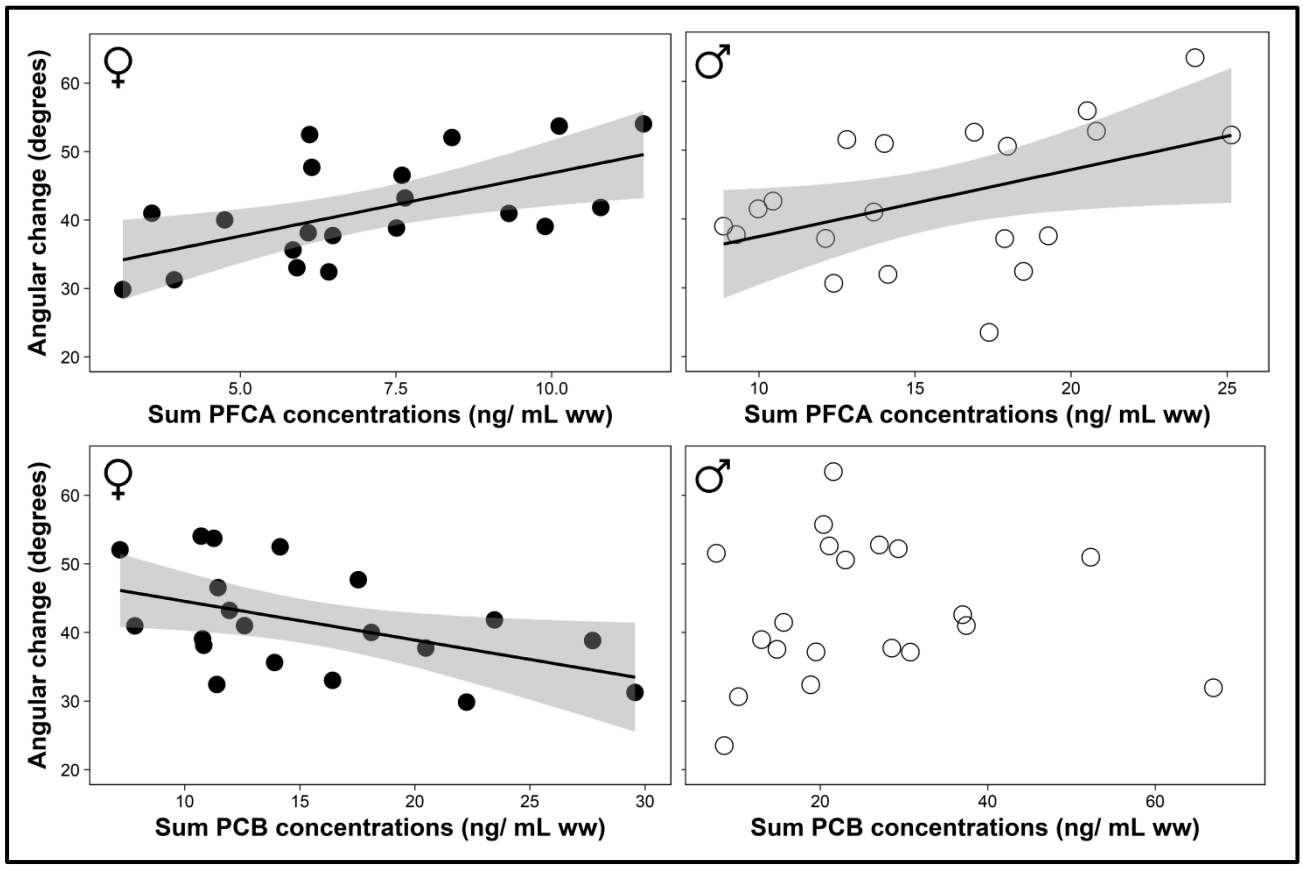


Figure 26: Relationships between plasma ∑PFCAs, blood ∑PCBs concentrations and egg angular change in female (n =20) and male (n =20) incubating kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard.

In addition, we observed a negative and significant relationship between oxychlordane concentrations in blood and the minimal incubation temperature (T_{min}) in males only (slope = $-3*10^{-3}$, p-value = 0.001, R^2 = 0.45; Figure 27), indicating a lower T_{min} with increasing oxychlordane concentrations. OCs were not related to other incubation temperature (T_{inc}) parameters in kittiwakes. Finally, PFASs and Hg were never associated with T_{inc} in both sexes. Please, see **paper V** for more details on statistical analyses and results.

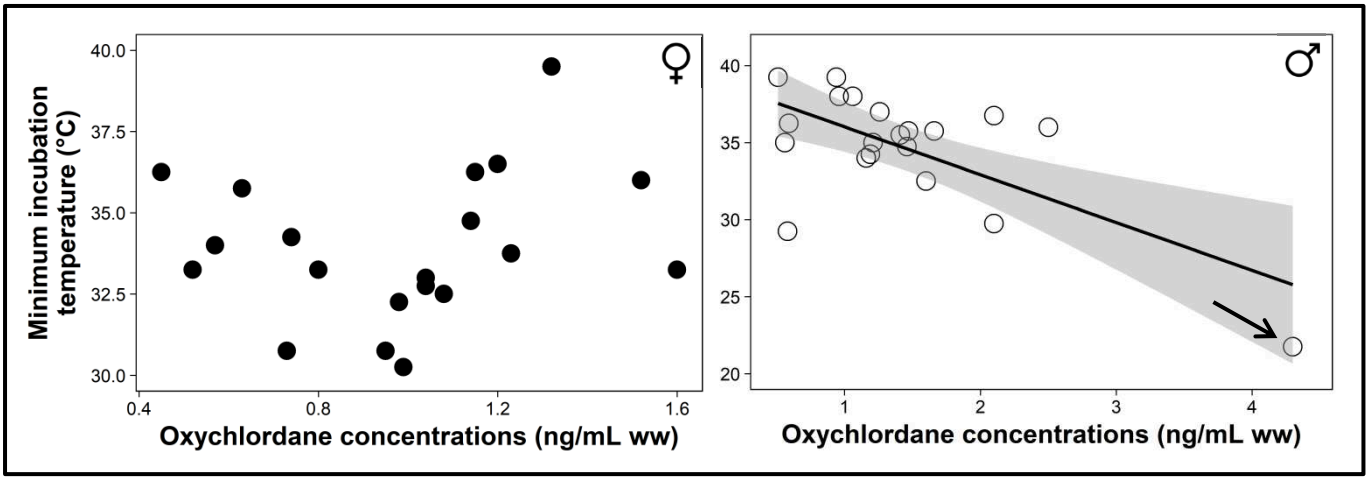


Figure 27: Relationships between blood oxychlordane concentrations and the minimum incubation temperature in female (n=20) and male (n=20) adult kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. The arrow indicates one individual with a fairly strong relative statistical power (see Paper V for more details).

6 - Relationships between contaminant concentrations, prolactin levels and brood patch size in kittiwakes

Because PFASs (i.e. PFOSlin and ∑PFCAs) and ∑PCBs were significantly related to egg-turning behaviors in kittiwakes, we examined relationships between these contaminants and baseline prolactin levels to evaluate potential underlying mechanisms. In females, PFASs were positively and significantly related to the baseline level of prolactin while PCBs were not (PFOSlin: slope = $5*10^{-3}$, p-value = 0.015, R^2 = 0.29; ∑PFCAs: slope = $2*10^{-3}$, p-value = 0.047, R^2 = 0.20). In males, PFASs and PCBs were not related to the baseline level of prolactin.

Because oxychlordane was significantly related to T_{min} in male kittiwakes, we examined relationships between oxychlordane concentrations and baseline prolactin levels, and the size of the brood patch to evaluate potential underlying mechanisms. Baseline prolactin levels were not significantly related to oxychlordane concentrations and brood patch size in both sexes. However, we found a highly significant negative relationship between oxychlordane concentrations and the size of the brood patch in males but not in females (log-10 transformed; slope = $-2*10^{-3}$, p-value = $2*10^{-4}$, R^2 = 0.53). Thus, the most oxychlordane contaminated males had the smallest brood patch.

Importantly, prolactin levels were positively and significantly related to the angular change in females but not in males (females: slope = $3*10^{-1}$, p-value = 0.029, R^2 = 0.24). Furthermore, prolactin levels were not related to the egg-turning frequency and T_{min} in both

sexes. Finally, the size of the brood patch was positively and significantly related to T_{min} in males (slope = 1.178, p-value = $1*10^{-4}$, R^2 = 0.56). Please, see **papers IV & V** for more details on statistical analyses and results.

7 - Relationships between incubation behaviors and hatching probability in kittiwakes

Because we reported some relationships between contaminant concentrations and incubation behaviors, we evaluated the consequences of egg-turning and T_{min} variations on hatching success. There was a positive and marginally significant relationship between egg-turning frequency and the probability that the remaining egg in the experimental nests successfully hatched (Z = 1.678, p-value = 0.093; Figure 28A). In other words, the most frequently turned eggs tend to better hatched. By contrast, angular change was not significantly related to hatching probability. Finally, there was a positive and nearly significant relationship between T_{min} and the probability that the remaining egg in the experimental nests successfully hatched (Z = 1.932, p-value = 0.053; Figure 28B). In other words, the lower T_{min} was, the lower was the hatching success.

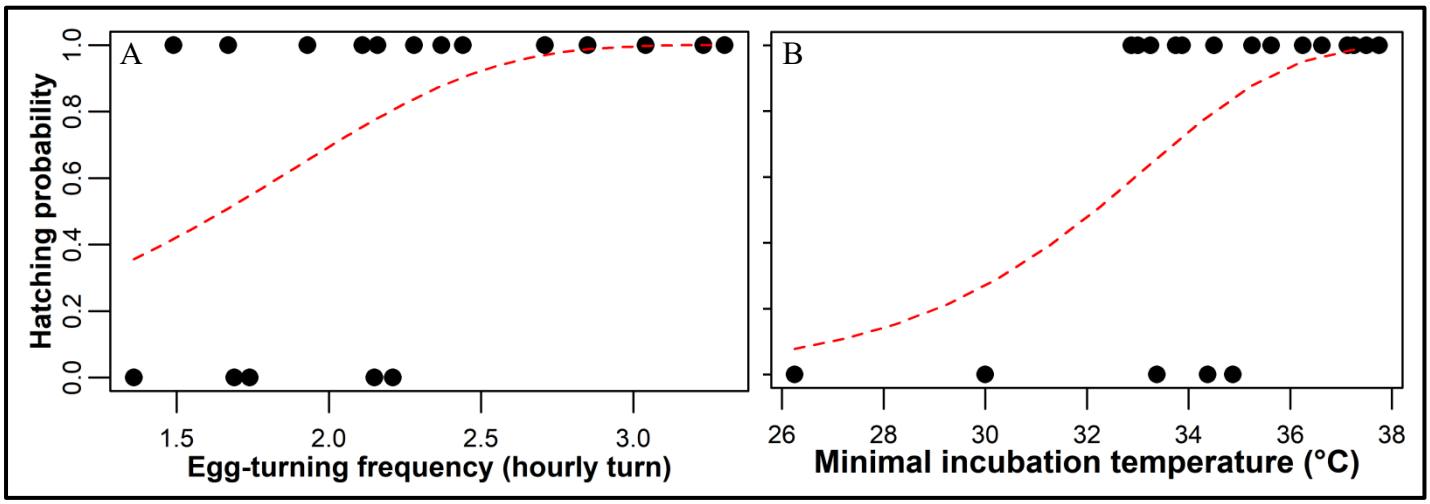


Figure 28: Hatching probability (0 = not hatched; 1 = hatched) of the remaining egg in the experimental nests (n = 20) in relation to A) Egg-turning frequency and B) the minimal incubation temperature (T_{min}). Egg-turning frequency and T_{min} have been calculated by meaning the values of both partners in each nest. Dashed lines indicate nearly (p~0.05) or marginally (p<0.1) significant relationships.

8 - Discussion

a) Comparison with previous studies

Surprisingly, PFASs concentrations were positively related to egg-turning frequency and angular change which suggest a beneficial effect of PFASs on incubation behaviors in kittiwakes. This is an unexpected result since a general and common understanding is that environmental contaminants are associated with adverse effects in living organisms. To the best of our knowledge, the effects of PFASs on reproductive behaviors of birds have never been investigated which make comparison with other works impossible. However, although correlative, previous findings (including the present thesis results) suggested that PFASs could have "beneficial" effects on physiology of Arctic seabirds, including lower stress levels (i.e. baseline corticosterone, *Tartu et al., 2014*), slowed ageing (i.e. telomere length dynamic, **Paper I**) and higher energy allocated for self-maintenance (i.e. basal metabolic rate, **Paper III**; THs, Melnes et al., 2017). Thus, this suggested positive effect of PFASs on parental care behaviors is in line with our findings and one possible explanation could rely on hormesis. In ecotoxicology, hormesis is an adaptive bi-phasic dose response characterized by chemicals inducing stimulation (e.g. beneficial effects) at low doses, but inhibition (e.g. harmful effects) at higher doses (Calabrese, 2008; Calabrese and Baldwin, 2002; Costantini, 2014). Overlooked and ignored for a long time, this historical concept came into focus over the last two decades and an extensive review conducted by Calabrese and Blain (2005, 2011) indicated a relatively high implication of organic chemicals in hormesis phenomenon across numerous taxa. Accordingly, two experimental studies conducted on mallard ducks (*Anas platyrhynchos*) suggested a hormetic response involving a better hatching success of eggs containing low doses of Me-Hg artificially injected or naturally deposited by methylmercury-fed mothers (Heinz et al., 2010, 2012). In addition, experimental exposure to low dose of the synthetic chemical 4-nonylphenol showed a positive effect on fecundity in the fish fathead minnows (*Pimophales promelas*) through possible indirect stimulation of estrogen secretion compared to controls or to high doses (Giesy et al., 2000). However, the suggested beneficial effect of PFASs on egg-turning behaviors and on other physiological mechanisms listed above remains hypothetic and further investigations conducted experimentally and testing for a bi-phasic dose response are needed.

In contrast, OCs have received a greater level of attention and several studies conducted *in natura* or experimentally indicated detrimental effects of CHLs, PCBs and others on an array of reproductive behaviors in birds. Among them, an experimental study where captive American kestrels (*Falco sparverius*) received a mixture of PCBs showed longer incubation periods and altered incubation behaviors like a reduced nest attendance in treated groups (Fisher et al., 2006a). Similarly, exposure to PCBs and oxychlordane was

found to be associated with reduced nest attendance (i.e. longer and/or more frequent absences from the nest site during incubation period; Bustnes et al., 2001, 2005b) and lowered nest temperature (Verboven et al., 2009a). In addition, delayed hatching date in relation to PCBs exposure in male kittiwakes (females were not investigated) from the same colony was also reported (*Tartu et al., 2015b*). As a result, detrimental effects of OCs, including oxychlordane and PCBs on reproductive behaviors in birds appear conclusive. By focusing on parental care behaviors at fine resolution and because my thesis suggests a detrimental effect of both oxychlordane and PCBs on incubation temperature and egg-turning frequency in kittiwakes, our study comes to add a better understanding of the way through which these OCs could impact reproduction of birds.

It is worth noting that our results did not report any relationships between Hg and incubation behaviors which is supported by a recent investigation where no associations were found between egg Hg contamination levels and both egg-turning and incubation temperature in Forster terns (*Sterna forsteri*; Taylor et al., 2018).

b) Prolactin: a key hormone involved in parental care

Prolactin is the major controller of parental behavior and considered as the "parental hormone" (Riddle, 1963). Accordingly, several correlational and experimental studies highlighted the predominant role of prolactin in the set-up and maintenance of incubation behaviors (Angelier et al., 2016; Buntin, 1996; Lynn, 2016; Sockman et al., 2006; Vleck, 2002). For instance, Sockman et al. (2000) showed that a moderate experimental increase in prolactin concentrations induce a better incubation assiduity (i.e. percent day incubating) in American kestrels (even if the higher administrated dose did not show any effects). Furthermore, another work conducted on kittiwakes from the same colony reported that experimentally-induced low prolactin levels were associated with reduced nest attendance during the chick-rearing period (Angelier et al., 2009). Our study suggests a positive effect of prolactin secretion on incubation behavior and specifically on egg angular change in female kittiwakes, adding further and new evidences that prolactin triggers parental behaviors in wild birds. So far, only one study conducted on Adélie penguins (*Pygoscelis adeliae*) have investigated prolactin level and egg-turning behavior. Surprisingly, they found that birds with lowered prolactin levels following implants of self-degradable bromocriptine pellets turned their eggs more often compared to the control group (Thierry et al., 2013). In our study, prolactin concentration is not related to egg-turning frequency. However, the authors stated

that their result was likely attributed to a shift from a down to an upright position of the treated birds, which favor egg-turning events, rather than a direct effect of a prolactin decrease (Thierry et al., 2013).

Since incubation behaviors, including brood patch formation, are triggered by an array of different hormones (Angelier and Chastel, 2009; Buntin, 1996; Lea and Klandorf, 2002; Lynn, 2016; Sockman et al., 2006; Vleck, 2002; Vleck and Vleck, 2011), we can expect to observe a positive relationship between prolactin levels and brood patch size. However, our results do not report such associations in incubating kittiwakes. Several explanations could explain this discrepancy. Firstly, the establishment and maintenance of incubation behaviors, including brood patch formation, is orchestrated by a complex cocktail of different reproductive hormones acting synergistically (Angelier et al., 2016; Buntin, 1996; Lea and Klandorf, 2002; Lynn, 2016; Sockman et al., 2006; Vleck, 2002; Vleck and Vleck, 2011) and further studies focusing on sex steroids (e.g. testosterone, estradiol, progesterone) may provide greater clarity about which endocrine mechanisms are involved in brood patch formation. Then, the timing of blood sampling for prolactin assays could have been conducted too late in the season for comparison to the timing of brood patch formation or the maximum of prolactin secretion. Indeed, brood patch formation is initiated only a few days before egg-laying (Lea and Klandorf, 2002) and our sampling for prolactin assessment was performed several days after egg-laying. This would deserve to be tested by sampling kittiwakes for prolactin assessment at the beginning of brood patch formation.

c) Some potential underlying mechanisms

Given its key role in mediating parental behaviors, we investigated a possible disruption of prolactin secretion originating from the exposure to PFASs and PCBs, both known as endocrine disruptors (DeWitt, 2015; Giesy et al., 2003; Jensen and Leffers, 2008; Khetan, 2014; Tyler et al., 1998). Interestingly, our study revealed a positive and significant relationship between PFASs and prolactin levels in female kittiwakes which is line with the previously suggested positive effect of PFASs on egg-turning behaviors. Consequently, through a possible increase of prolactin secretion, the most PFAS-contaminated kittiwakes (at least females) could better incubate their eggs (at least for angular change; Figure 29A). However, the endocrine disrupting property of PFASs on prolactin secretion remains unknown and has never been explored in birds. As a result, further experimental and correlational studies are needed to confirm this relationship and to understand the endocrine

mechanisms linking PFASs to prolactin in avian models. By contrast, PCBs concentration was not associated with plasma prolactin level in kittiwakes although the presence of a suggested negative trend in females (see Figure 5 in **paper V**). In glaucous gulls, PCBs (and other OCs) have been associated to a decrease plasma prolactin concentration in males only, although several of these associations (including PCBs) did not adhere with the criterion of significance (Verreault et al., 2008). Furthermore, a previous study performed on Antarctic snow petrels (*Pagodroma nivea*) did not report any effects of ΣPOPs, including PCBs, on prolactin levels (Tartu et al., 2015a). Given these inconstancies, it is thus impossible to make affirmative conclusions and we can only speculate that PCBs exposure could decrease prolactin levels in wild birds. Moreover, as mentioned earlier further experimental studies focusing on other hormones and on different doses of PCBs exposure may provide greater clarity about endocrine mechanisms targeted by PCBs and toxicity thresholds.

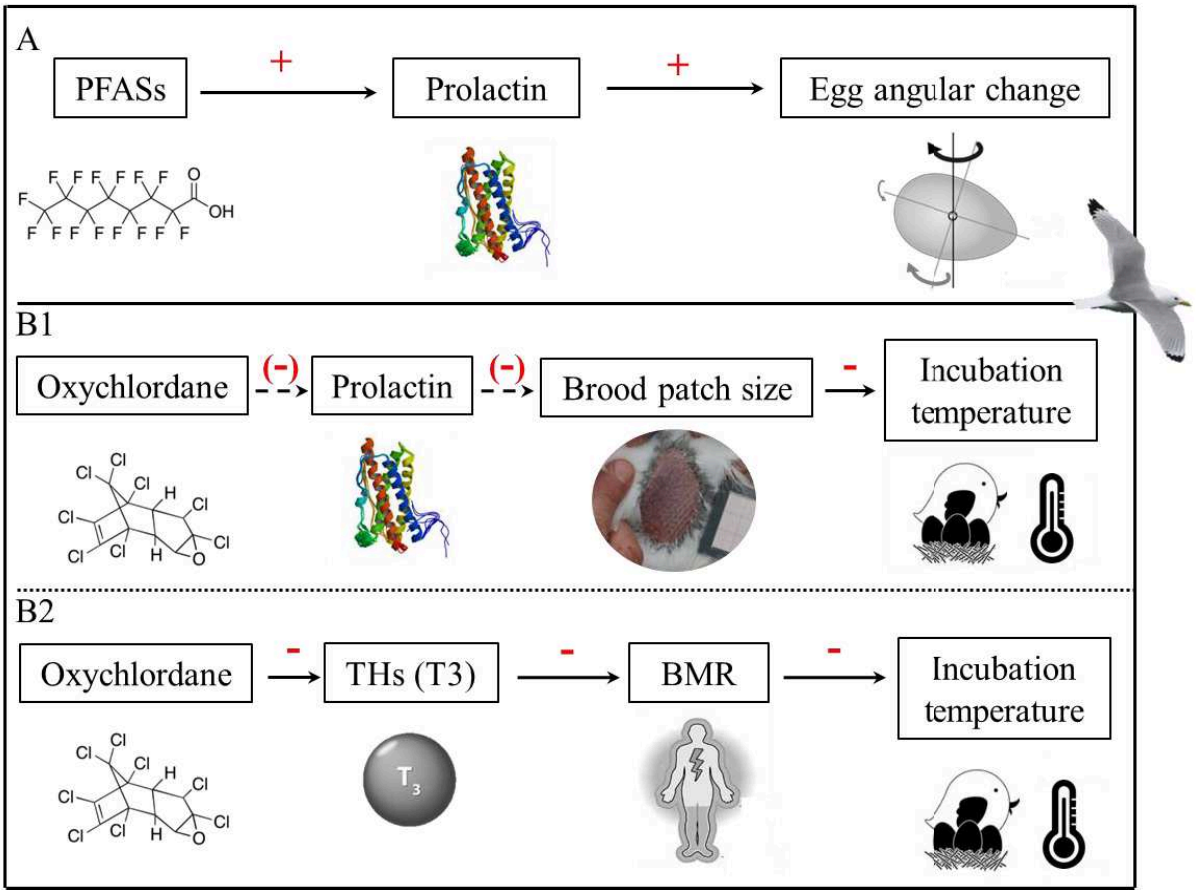


Figure 29: Potential underlying mechanisms explaining the reported relationships between contaminants, prolactin, brood patch and incubation behaviors in breeding black-legged kittiwakes (*Rissa tridactyla*) from Kongsfjord, Svalbard.

Incubation is an energy-consuming phase of the avian reproductive cycle (Nord and Nilsson, 2012; Nord and Williams, 2015; Nord et al., 2010; Tinbergen and Williams, 2002)

and the efficiency with which heat is transferred from an incubating bird to its eggs is related to the energy expenditure of the parent (Gabrielsen and Steen, 1979; Gabrielsen and Unander, 1987). In other words, a higher metabolic rate increases heat production thereby increasing heat transfer from the parent to the embryo, and conversely. Interestingly, lowered TH levels and reduced basal metabolic activity have already been observed in the most chlordane-contaminated individuals, including kittiwakes from the same population and glaucous gulls from Svalbard (**Paper II**; Melnes et al., 2017; Verreault et al., 2004, 2007). In this context, the quantity of heat transferred from parents to the eggs might be reduced in the most contaminated birds thus explaining why we observed a negative relationship between oxychlordane concentrations and T_{inc} of male kittiwakes (Figure 29B2). Another non-mutually exclusive hypothesis could rely directly on the manner in which heat is transferred. Indeed, because contact between the brood patch and egg ensures heat transfer from parents to the embryo (Jones, 1971), investigating relationships between contaminants and the size of the brood patch is relevant. In this context, a reduction in size of the brood patch in the most oxychlordane-contaminated male kittiwakes logically decrease the amount of heat transferred to their eggs (Figure 29B1). This reasoning is consistent with an experimental study on American kestrels (*Falco sparverius)* where smaller brood patches were observed in males exposed to PCBs compared to controls *(*Fisher et al., 2006b).

d) Possible fitness consequences

Since egg-turning behavior is a key determinant for egg hatchability, any effects of contaminants on egg-turning frequency and/or angular change could *in fine*, influence reproductive success of kittiwakes. Thus, the positive effect of egg-turning frequency on hatching success suggested here, although marginally significant, is in line with this statement. As a result, we could hypothesized that the increased egg-turning frequency reported in the most PFOSlin-contaminated female kittiwakes could potentially increase hatchability. Furthermore, a previous study conducted on ring doves (*Streptopelia risoria*) reported a lower hatchability of eggs incubated by birds experimentally exposed to high doses of PCBs (Peakall and Peakall, 1973). Similarly, forster terns had a higher hatching success when eggs laid from OCs contaminated birds (including PCBs) were incubated by less contaminated surrogate parents or with an artificial incubator (Kubiak et al., 1989). Although related to angular change in female kittiwakes, PCBs were not related to egg-turning frequency. Consequently, given this discrepancy, the low statistical power and the small

sample size (only 5 eggs were unhatched), we cannot confirm with certainty that contaminants, through their effects on incubation behaviors, can *in fine*, influence reproductive success of kittiwakes.

In addition, several studies have reported a reduced hatching success of eggs incubated at suboptimal temperatures (Deeming and Ferguson, 1991; Durant et al., 2013; Feast et al., 1998; French, 2000; Moraes et al., 2004; Mortola, 2006; Nord and Nilsson, 2011, 2012; Webb, 1987). Thus, the reduced T_{inc} reported here in the most oxychlordane contaminated kittiwakes could impair hatchability by decreasing hatching probability. However, we cannot completely rule-out another possible non-mutually exclusive hypothesis which relies on a delay of hatching in response to low T_{inc} events. Although kittiwakes displayed a high synchrony in the date of hatching (Melhum, 2006), our fieldwork was completed within a few days after the peak lay date (around 5 days) so it is conceivable that some eggs we considered to be non-viable in fact hatched soon after we stopped monitoring nest contents. This is entirely consistent with previous investigations showing an extended incubation period in eggs incubated below the optimal temperature range (Ardia et al., 2010; Deeming and Ferguson, 1991; Durant et al., 2013; Feast et al., 1998; Martin, 2002; Martin et al., 2007; Mortola, 2006; Nord and Nilsson, 2011, 2012; Webb et al., 1987). An experimental study on wood ducks (*Aix sponsa*) revealed that low T_{inc} resulted in prolonged incubation periods and lower hatching success (Hepp et al., 2006). Consequently, a reduced T_{min} in the most oxychlordane-contaminated kittiwakes could *in fine*, impair egg hatchability, either by lengthening incubation period and/or reducing hatching success.

G - General discussion

1 - Contaminant levels: sex-related differences

As many other studies conducted on different seabird species (e.g. Becker et al., 2002; Bustnes et al., 2007, 2017; Verreault et al., 2004, 2005, 2006a, 2008), the present thesis highlighted some sexual differences of blood contaminant concentrations, with males being more contaminated than females in both incubation and chick-rearing periods (Figures 12, 13 & 14 in section C5b). This pattern appears to be relatively consistent across the 3 groups of contaminants considered in the thesis (PFASs, OCs and Hg). Importantly, the magnitude of the difference of contaminant concentrations between males and females is systematically higher in incubation than in chick-rearing period. This observation corroborates well a maternal egg-transfer and deposition of contaminants into the eggs. Indeed, several correlational and experimental studies have shown that females have the ability to excrete a significant part of their contaminant body burden into their eggs (Bargar et al., 2001; Becker, 1992; Bourgeon et al., 2013; Drouillard and Norstrom, 2001; Gebbink and Letcher, 2012; Verboven et al., 2009b; Verreault et al., 2006b), resulting in a reduced contaminant blood concentrations compared to their mates.

An additional explanation could rely on various foraging strategies between genders. For instance, male kittiwakes may be exposed to higher contaminant loads than females by feeding on larger prey, from higher trophic positions and/or in more contaminated areas. Besides, a study conducted on kittiwakes from the same colony than our study reported some spatial segregation of foraging areas during the pre-laying period, with females foraging inside Kongsfjord while males were tracked both inside and outside the fjord, using the deep waters of the Greenland-Svalbard ridge (Goutte et al., 2014a).

2 - Oxychlordane: one of the most toxic POPs

This PhD work underlines the major role of certain chlorinated organic compounds (e.g. chlordanes) that have been banned for decades and are decreasing in the environment. Indeed, although representing a few % of the total OC burden, oxychlordane, a metabolite of a banned organochlorine pesticide was associated with decreased telomere length, lowered metabolic rate and reduced ability to incubate the eggs in kittiwakes.

a) History of legacy

Chlordane is a man-made substance used in the past during more than 40 years as a pesticide. Technical chlordane (CAS Number 12789-03-6) encompasses a broad mixture of more than 140 related chemicals with *trans*-chlordane and *cis*-chlordane as major components followed by other compounds including *cis*- and *trans*-nonachlor (Koshlukova and Reed, 2014; US department of health and human services, 2018; US EPA, 1997). Chlordane was first registered in the United States in 1948, synthesized hazardously by Julius Hyman, during a study for possible uses of a useless by-product of synthetic rubber manufacturing (Jewkes et al., 1958). Prior to 1978, chlordane was extensively and broadly used as a pesticide on agricultural crops, gardens and lawns; as a termiticide, wood preservative, protective agent on seeds and potatoes; and as a fumigating agent (European Food Safety Authority, 2007; Koshlukova and Reed, 2014; US department of health and human services, 2018). Following the United States Environmental Protection Agency (US EPA), more than 30 million homes were treated with technical chlordane against termites and the estimated annual production peaked around 9 500 tons in the United States and 70 000 tons globally (Dearth and Hites, 1991; Koshlukova and Reed, 2014; WHO, 1988). From 1983 to 1988, chlordane's only approved use was to control termites in homes. Since the end of 1970s, suspicious toxicity on human health, potential threats to wildlife, environmental persistence, bioaccumulation and biomagnification within the food chains have led to its phase-out in many countries by 1998 (Koshlukova and Reed, 2014; US department of health and human services, 2018). In Europe, chlordane has never been produced and its usage was banned since 1981 (European Food Safety Authority, 2007). Two decades after the phase-out, chlordane and its metabolites are still present within the environment, even in remote places such as polar areas, and they are still toxic for living organisms as highlighted by the present PhD. Chlordane is on the United Nations Environment Program list of POPs for which international action is required to reduce risks to humans and the environment. The Stockholm Convention on POPs was adopted on 22 May 2001, entered into force on 17 May 2004 and is now ratified by 182 parties. Initially, twelve POPs (i.e. the dirty dozen) have been recognized as causing adverse effects on humans and ecosystems. Among them, chlordane has been listed under the Annex A which recommends to eliminate the production and stop the use of this chemical (Stockholm convention, http://www.pops.int/).

b) The predominant metabolite of chlordane

Chlordane is classified by the World Health Organization and US EPA as a moderate oral toxicant (Category II, from human and rat studies) and considered as highly toxic for aquatic animals (Koshlukova and Reed, 2014; US EPA, 1997; WHO, 1984). Once incorporated in living organisms, chlordane is readily metabolized by hepatic CYP leading to the production of different metabolites (Walker, 1998). Oxychlordane has been shown to be the predominant metabolite of chlordanes in humans and various animals (Khasawinah, 1989; Saito et al., 1986; Sasaki et al., 1992; Tashiro and Matsumura, 1978). Accordingly, in a 28-days study where *cis*-nonachlor, *trans*-nonachlor and technical chlordane were administered to rats, oxychlordane was the major metabolite accumulated in the adipose tissue (Bondy et al., 2000). Because of this biotransformation process, levels of oxychlordane increase in living organisms during exposure episodes while the mother compound isomers decrease. This statement seems to be in line with the reported levels of chlordanes in Arctic kittiwakes since blood concentrations of oxychlordane were much higher compared to other chlordane-related components (Figure 13 in section C5b). Indeed, the efficiency of kittiwakes to biotransform chlordane isomers and the high biomagnification potential of oxychlordane in marine vertebrates explain well the relatively high levels of this metabolite in this species (Borgå et al., 2001, 2007; Fisk et al., 2001b). Because of its long retention time in adipose tissue and a very low metabolization ability, oxychlordane is believed to be more toxic than its parent isomers (Satoh and Kikawa, 1992). Therefore, oxychlordane may be a major contributor to chlordane toxicity.

c) Experimental evidences of an acute toxicity

Chlordane and its metabolite oxychlordane provide a clear diagnostic criterion related to the lethal and/or sub-lethal poisoning in humans, laboratory models and wildlife (European Food Safety Authority, 2007; Koshlukova and Reed, 2014; Reinke and Deck, 2015; US department of health and human services, 2018). In an experimental study where oxychlordane was administered to female rats at doses ranging from 0.01 to 10 mg/Kg body weight (bw) during 28 days, weight loss, feed refusal and thymic atrophy were major effects at the highest dose, whereas hepatic damages were observed at 2.5 mg/Kg bw and above. Moreover, the dose response curve was steep with 10 mg/Kg bw causing an acute toxicity with 100% morbidity signs (Bondy et al., 2003). Toxicity thresholds vary to a great extent among species. Besides, the lowest observed effect concentration from a life-cycle test

(embryo mortality) has been shown in brook trout (*Salvelinus fontinalis*) exposed to 0.32 µg/L of chlordane (Cardwell et al., 1977). In birds, lethal concentrations of oxychlordane measured in brains of cowbirds (*Molothrus ater*), grackles (*Quiscalus quiscula*), red-winged blackbirds (*Agelaius phoeniceus*) and starlings (*Sturnus vulgaris*) ranged from 5 to 22.1 µg/g (Stickel et al., 1983). In comparison, the reported concentrations of chlordanes and oxychlordane in blood of kittiwakes are higher than exposure toxicity levels of trouts and lower than the lethal concentrations measured in brains of other birds. However, making such comparison in order to extrapolate the degree of threat of which wild animals are exposed does not appear as being relevant here since i) the degree of chlordane bioaccumulation differs among tissues; ii) toxicity thresholds can vary among species and duration of exposure episodes (some wild population are chronically exposed and may have developed a better resistance); iii) metabolic ability differs greatly among species. As a result, no suitable data seems available to get a reliable avian toxicity reference value for chlordanes (Reinke and Deck, 2015). Interestingly, it is suggested that oxychlordane is more toxic than its parent isomers (Ivie, 1973). Besides, an experimental study where oxychlordane was administered to rats indicates an acute toxicity of this metabolite up to 10 times higher than its parent compounds (Bondy et al., 2003). Similarly, purified oxychlordane was 6 times more toxic than technical chlordane in experimentally fed birds (Stickel et al., 1979). Therefore, oxychlordane appears to be highly toxic for living organisms.

d) Toxicological profile in wild birds: a review

Among all considered OCs in this thesis, oxychlordane is suggested to be particularly harmful to several physiological and ecological endpoints in kittiwakes. Thus, our results corroborate well the acute toxicity of oxychlordane, already pointed-out in several experimental studies as detailed above. Yet, the toxicity of chlordanes in free living animals has been overlooked in comparison with other POPs such as DDTs, well mediatized by the book of Rachel Carson "Silent spring", or compared to the famous PCBs extensively used in the past in electric equipment which contribute importantly to the total POPs animal burden (Figure 13 in section C5b). That is the reason why we started to perform an extensive review (work in progress) of the different studies dealing with the consequences of chlordane on wild birds (Appendix A). Interestingly, we noticed the occurrence of many descriptive works compared to less numerous papers dealing with chlordane toxicity in wild birds. Suspicious toxicity of chlordanes occurred in the end of 1970s and several experiments (even some

conducted on birds) highlighted several harmful consequences in different species for a broad panel of biological parameters. Although not still completed, the review indicates that the first field studies conducted in free-living birds were conducted only at the beginning of 2000s, which coincides well with the adoption of the Stockholm convention in 2001 and which results maybe in a strong awareness of the scientific community and policy management priority. Since this period, the number of publications per year have steeply increased and importantly, still highlighting various adverse effects of oxychlordane on wild birds.

Despite its very small relative proportion (4-5%) compared to other OCs considered in this thesis (Figure 13 in section C5b), oxychlordane is of particular concern and has been related to numerous outcome parameters. Importantly, as summarized in Appendix A, several works conducted on various wild bird species strengthen the findings presented in this thesis on kittiwakes. Particularly interesting, studies on another Arctic seabird, the glaucous gull, have also highlighted similar adverse effects of oxychlordane and mother isomers on energy expenditure, parental care behaviors, phenotypic traits and survival rate. Additionally, by comparing the strength of the effects of different OCs (ΣPCBs, *p,p'*-DDE, HCB and oxychlordane) on a number of outcome parameters (immune functions, hormones, reproduction, and survival), Bustnes (2006) pointed-out oxychlordane as a reliable predictor of adverse effects on glaucous gull compared to the other OCs. Although affirmative conclusions are challenging in field observational studies (i.e. correlation among OCs, other potential confounding variables), our results, in combination with previous field investigations indicate an acute toxicity of oxychlordane in wild birds.

3 - PFASs and OCs: some contrasted patterns

My thesis and previous studies conducted on kittiwakes from the same colony indicated dissimilar associations between PFASs and OCs with several physiological endpoints (i.e. ageing, energy expenditure, parental care and stress hormone; Nordstad et al., 2012; *Tartu et al., 2014, 2015b*). Interestingly, such contrasted patterns have already been highlighted when looking at TH levels in another Arctic seabird, the glaucous gull (Melnes et al., 2017). Does that mean that the effects of OCs are compensated by those of PFASs, and/or reciprocally? Is there a confounding effect between PFASs and OCs?

PFASs are known to preferentially accumulate in protein rich tissues (e.g. liver, blood) while OCs are mainly stored in adipose tissues before being released into the blood stream during periods of accelerated lipid mobilization (Aas et al., 2014; Bustnes et al., 2010;

Frindlay and Defretas, 1971; Henriksen, 1995; Jones et al., 2003; Kelly et al., 2009; Luebker et al., 2002; Routti et al., 2013; Vanden Heuvel et al., 1992; Verreault et al., 2005;). Consequently, it appears reasonable to think that the proteinophilic PFASs and lipophilic OCs, because of their different structural and chemical properties, could target physiological functions through very different modes of action without mechanistic interlinkages. Besides, figure 15 in section C5c showing correlations among contaminants clearly divided the contaminants considered in the thesis in two distinct groups: the PFASs and Hg in one side and the OCs in the other side. Furthermore, figure 15 does not suggest negative relationships between PFASs and OCs concentrations in kittiwakes which means that a highly PFASs-contaminated individual is not necessarily poorly OCs-contaminated and inversely. Thus, the contrasted associations between PFASs and OCs highlighted in this thesis suggest a real effect of those two contaminants on studied biomarkers and are probably not the result of confounding effects between PFASs and OCs. In order to disentangle effects of each group, further analyses would deserve to be conducted by splitting individuals in 4 classes (high PFASs/ high OCs, high PFASS/ low OCs, low PFASs/ high OCs, low PFASs/ low OCs) and investigating inter-classes differences. This would maybe enable to indicate predominant effects of one group of chemicals relative to the other one. However, kittiwakes are obviously exposed to a complex cocktail of contaminants which are not measured in this study and future experimental research focusing on structurally opposed chemicals is absolutely required to better understand and clarify the underlying mechanisms though which contaminants and especially PFASs influence health of living organisms.

4 - Contaminants and temporal trends

a) PFASs and POPs: coming and decreasing threats for Arctic kittiwakes?

The present thesis helped to target important chemicals which could disrupt physiology and fitness of kittiwakes. Therefore, these contaminants deserve to be follow-up and an important challenge is to describe long-term temporal trends to predict coming and decreasing threats for kittiwakes.

PFOS in airborne particles were measured at Zeppelin Mountain (a station located at few kilometers from the kittiwake colony) from 2006 to 2012 and concentrations appear constant throughout the monitoring period (AMAP, 2016, 2017). Moreover, a common pattern of PFOS concentrations in Arctic biota showed an increase until about the mid-2000s followed by a decrease, reflecting the voluntary phase-out of the production of PFOS and related products by the largest producer 3M in 2000 (AMAP, 2016; Rigét et al., 2013). By contrast, PFCA are still produced and released into the environment. Unfortunately, PFCA profiles from Zeppelin station do not seem to be available in literature but trends of their precursors 6:2 FTOH, 8:2 FTOH and 10:2 FTOH showed increasing tendencies in air at Alert station (Canadian Arctic) between 2006 and 2012 (AMAP, 2016, 2017). In addition, monitoring of contaminants from Artic biota (including seabirds) are characterized with a relatively high proportion of increasing temporal trends (Figure 30; AMAP, 2016, 2017; Braune, 2015; Braune and Letcher, 2012; Butt et al., 2010; Rigét et al., 2013; Routti et al., 2016, 2017). However, it is worth noting that some slight and recent decreasing patterns of PFCAs have been reported in Arctic biota which could reflect the United Nations Environment Program Strategic Approach for International Chemicals Management initiative to reduce emissions, especially for the long-chain PFCAs (Braune, 2015; UNEP, 2012). The present study, in combination with previous experimental and correlational works suggests some important physiological disruptions and fitness consequences of PFASs exposure on kittiwakes and more generally on living organisms (DeWitt, 2015). Because of their overall increasing trends, particular attention should be given to PFCAs, especially the long-chained ones which seem to be highly toxic for kittiwakes, as well illustrated in **paper VII**, where oxidative damages increase with the carbon chain length.

All the OCs considered in the present thesis are banned from use and listed under the Stockholm convention because of their high toxicity on living organisms (Stockholm convention, http://www.pops.int/). Accordingly, concentrations of these legacy POPs are

generally shown to decrease over years in the atmospheric compartment and Arctic biota (Figure 30; AMAP, 2016; Dietz et al., 2013a). For example, CHL-related isomers and PCBs in airborne particles were measured at Zeppelin Mountain (a station located at few kilometers from the kittiwake colony) and shown declining trends at least during the last two decades (Hung et al., 2016). Similarly, blood concentrations of PCB-153 decrease in kittiwakes from our colony in Kongsfjord from 2007 to 2011 (Bustnes et al., 2017). However, because of their high persistence, such decrease takes time and thus, levels of these POPs can still be of concern for living organisms. This is well illustrated with this PhD, confirming and adding further evidences of detrimental effects of POPs exposure on physiology and fitness of kittiwakes and more generally on living organisms, especially with CHLs and PCBs. Such long-term effects on wildlife, even many years after being banned, illustrate well the important and chronic toxicity of these legacy POPs and would deserve to be considered as a study case.

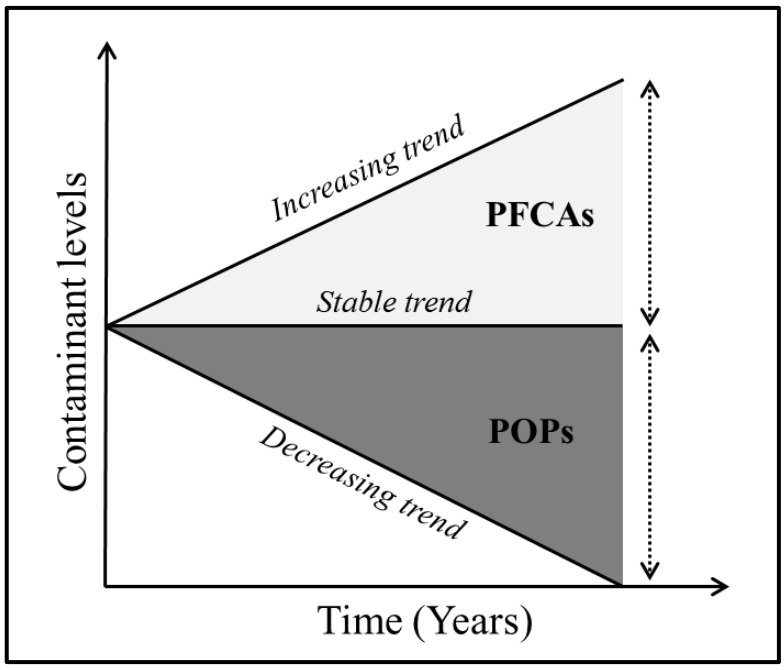


Figure 30: Schematic view of PFCAs and POPs temporal trends in Arctic biota.

b) Hg: a current minor threat for Arctic kittiwakes?

Hg is a well-known toxic chemical for living organisms, well exemplified by the tragedy of Minamata in the 1950s. During this poisoning episode, a chemical factory had released around 150 tons of Hg into Minamata Bay, Japan, resulting in the death of more than 1 500 persons and the paralysis of 10 000 persons through fish consumption (Kudo et al.,

1998). Similarly, Hg is a very well-known threat for biodiversity and several studies conducted under experimental conditions as well as *in natura* reported a vast array of detrimental effects of Hg on numerous health-related functions (Dietz et al., 2013b; Wolfe et al., 1998). Specifically, in seabirds, including Arctic kittiwakes from the same population, Hg has been pointed-out as a potential endocrine disruptors leading to detrimental effects on reproduction (Costantini et al., 2014; Evers et al., 2008; Goutte et al., 2014b,c, 2015; Tan et al., 2009; *Tartu et al.*, 2013, *2014, 2015b, 2016*). Indeed, Hg has been suggested to disrupt the secretion of LH and prolactin in kittiwakes (*Tartu et al.*, 2013, *2016*). Since several endpoints investigated in the present study are directly or indirectly liked to reproduction and under hormonal control, we were originally expected to find relationships with Hg, especially in the chapter dealing with incubation behaviors. However, no relationships involving Hg levels in kittiwakes were evidenced during this thesis.

So why does Hg in kittiwakes appear as a minor threat among all the investigated biological endpoints? Intuitively, one explanation could rely on the levels of Hg which could be potentially too low for being detrimental. Thanks to the long term monitoring programs of the French and Norwegian institutions, we retrospectively analyzed blood samples collected during the chick-rearing period (from early July to early August) since 2000 to get an idea of the Hg temporal trend in Svalbard kittiwakes (Figure 31A). We observed a decrease of Hg levels in kittiwakes from 2000 to 2007 followed with a stable trend until 2016, characterized by relatively low levels of Hg. Importantly, samples used for this PhD project were all collected since 2011, thus during the latest second period with stable and low levels of Hg. Consequently, the lack of reported effects of Hg in the present study could be simply explained by the relatively low levels of Hg contamination. However, we have to be cautious with this statement since Hg has already been detrimental in kittiwakes from the same population exposed to similar levels than those reported in our study (Goutte et al., 2015; *Tartu et al.*, 2013, *2016*). Obviously, inter-annual variability of environmental conditions such as food accessibility or the presence of other stressors can act synergistically, leading *in fine* to harmful effects of Hg, even at relatively low concentrations. Finally, the absence of Hg effects on the investigated parameters does not exclude to find any effects on other physiological and ecological endpoints. Indeed, because Hg is known to be an important neurotoxic (Aschner and Aschner, 1990; Chang, 1977; Dietz et al., 2013b; Wolfe et al., 1998), further studies should be conducted on neurological outputs.

How to explain this temporal trend? Surprisingly, the temporal trend of Hg levels in kittiwakes does not follow the stable trend of atmospheric Hg concentration between 2000

and 2009, recorded in Zeppelin Mountain, a station located at few kilometers to the kittiwake colony (Cole et al., 2013). Thus, Hg levels in kittiwakes do not appear to mirror the local atmospheric Hg contamination of Kongsfjord. Because food ingestion is the main route of Hg exposure in birds (Burger and Gochfeld, 2004), we investigated inter-annual variations of kittiwake foraging ecology. The respective roles of habitats and diets were tested by using the isotopic niche as a proxy of the trophic niche of the species (Newsome et al., 2007), with the ratios of stable isotopes of carbon ($\delta^{13}C$), reflecting the foraging habitats (e.g. offshore *versus* inshore consumers) and nitrogen ($\delta^{15}N$) increasing with trophic levels (Hobson, 1999; Kelly, 2000). Surprisingly, $\delta^{13}C$ and $\delta^{15}N$ trends do not mirror the pattern of Hg levels in kittiwakes (Figure 31B and 31C). While Hg show a first decline from 2000 to 2007 to reach a steady-state with relatively low concentrations, $\delta^{13}C$ and $\delta^{15}N$ appear as being cyclic over the studied period. Thus, foraging habitat and trophic position, despite their clear connection with Hg levels in seabirds (e.g. Atwell et al., 1998; Blévin et al., 2013), do not appear as being the predominant factors explaining the temporal trend of Hg contamination in kittiwakes. Interestingly, a recent study conducted on the same population of kittiwakes indicated a shift from Arctic prey dominance until 2006 to a more mixed diet with high contribution of Atlantic fishes. Capelin (*Mallotus villosus*), an Atlantic species, dominated the diet composition in 2007, marking a shift in the food web (Vihtakari et al., 2018). This breaking point corresponds pretty well to the change in the temporal trend of Hg levels in kittiwakes and the stable trend since 2007 could indicates a dominance of Atlantic prey. However, this statement would deserve to be confirmed by measuring and comparing Hg levels in Atlantic (e.g. capelin) and Arctic prey (e.g. polar cod) from Kongsfjord. In that case, the decrease of Hg levels from 2000 to 2007 could potentially be explained by a progressive establishment of Atlantic prey and/or a reduced oceanic input of Hg in Kongsfjord. At least for the parameters investigated in the present thesis, kittiwakes do not appear as particularly sensitive to Hg levels.

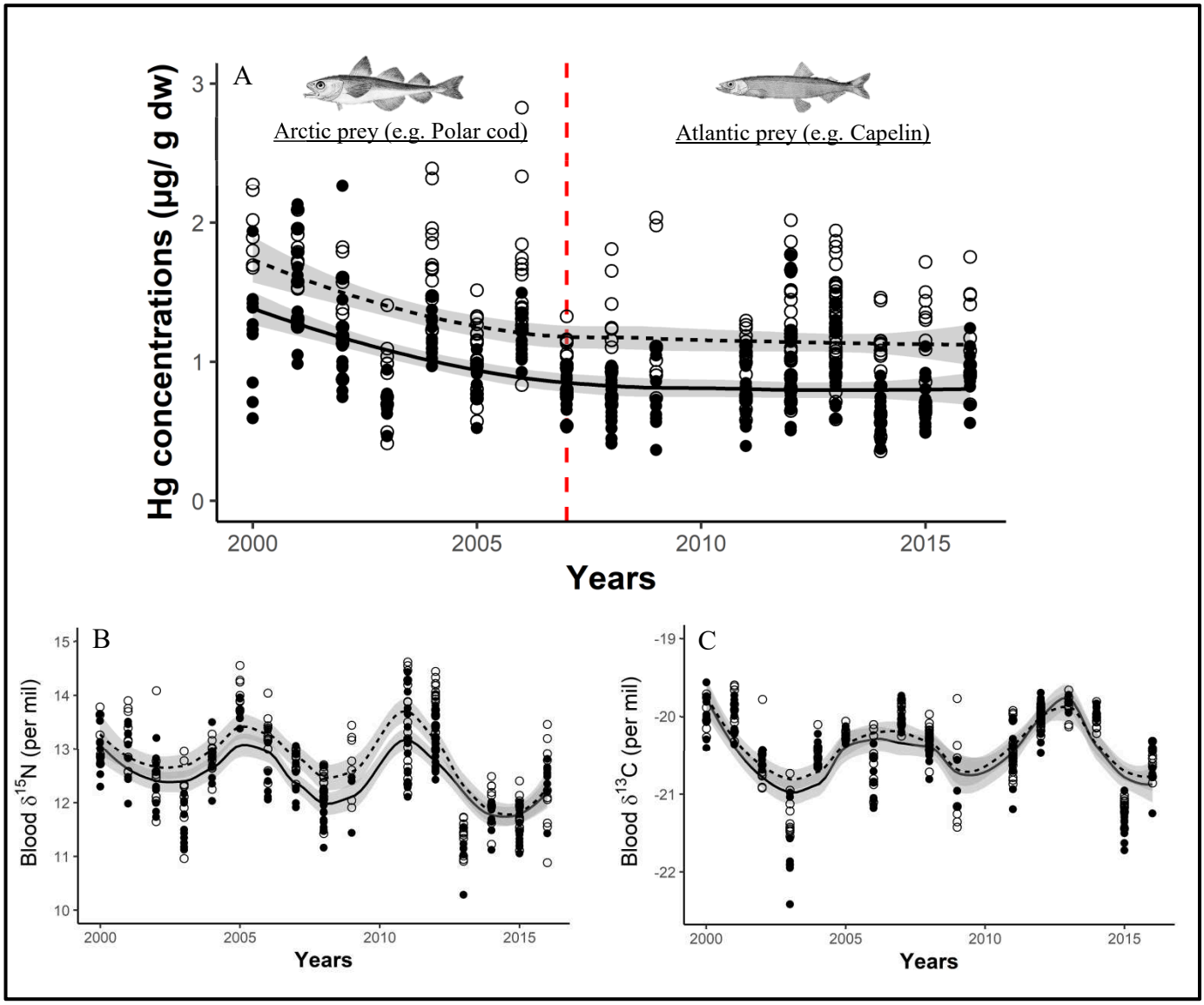


Figure 31: Long-term temporal trend of A) Hg concentrations (RBC, n = 230 females and 261 males) and stable isotopes $\delta^{13}C$ (B) and $\delta^{15}N$ (C) in female (n =185, black points and solid line)) and male (n =183, white points and dashed lines) chick-rearing kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. The vertical dashed and red line represents the shift in the food web; from Arctic prey dominance to a more mixed diet with high contribution of Atlantic fishes (Vihtakari et al., 2018).

5 - Correlational approach versus experimental approach

Under natural conditions, demonstrating causality is often challenging. Perhaps the most commonly employed method consists in correlating individual contaminant levels with biomarkers. However, the presence of some environmental and/or biological confounding factors can potentially skew the toxicological profiles of the targeted contaminants. Apart for ethically reasons, experimental studies conducted *in natura* do not appear either as more convincing since free-living animals are often already contaminated by a complex mixture of contaminants. It is thus difficult to disentangle the effects of the contaminant experimentally administrated from those already present before the experiments. Moreover, experimental studies conducted in laboratory animals, under controlled conditions have also some limitations. First, extrapolating findings from laboratory models (e.g. rodent species) to free-ranging vertebrates like seabirds can be tricky. Second, free-ranging seabirds are exposed to a complex mixture of chemicals with many possible additive, synergetic and antagonistic effects. Third, consequences of an experimental short-term and acute exposure to contaminants can substantially differ from effects of a chronic and low dose exposure *in natura*. As a result, there does not appear to be a "golden standard method" to investigate the effects of contaminants in wildlife and I do believe that a combination of all these approaches is required and would enable to establish relevant toxicological profiles.

H - To be continued…

1) Contaminants and sexual signals

a) Contaminants and integument coloration

This section essentially relates, summarizes and discusses results from **paper VI**. We also present here some very preliminary results which deserve to be confirmed and further analyzed before being submitted for publication.

We first conducted a pilot study in 2011 to explore the effects of contaminants exposure on visual sexual signaling. Specifically, we examined the relationships between some OCs with the coloration of integuments carotenoid-based in adult kittiwakes during the pre-laying period. Since the results suggested an effect of OCs on integument coloration and because the method employed (i.e. by taking numeric photographs) has some important limitations, we performed a second and more extensive study in 2016, based on a more accurate method (i.e. using reflectance spectrometry) and by focusing on a broader panel of contaminants. In addition to OCs, we also examined the effects of PFASs and Hg on coloration parameters of kittiwakes and importantly, we measured the concentrations of several carotenoid components into the plasma in order to better understand the way through which contaminants could disrupt coloration of sexual signals. It is worth noting that OCs analyses remain to be performed for the 2016 samples. Furthermore, since sampling was also designed for other projects, only females were investigated in 2011 and only males were samples in 2016.

- *What does integument coloration mean?*

Many animals exhibit elaborate ornamental traits such as colorful skin, feathers and cuticles that evolved as honest signals of individual quality (Zahavi, 1975). Carotenoids represent one of the central components of color signals used in animal communication and are highly involved in social and sexual behaviors of many species (McGraw, 2006; Møller et al., 2000; Olson and Owen, 1998). In birds, carotenoids are responsible for the yellow to red coloration of many secondary sexual traits (Brush, 1990). For example, in kittiwakes, both sexes show intense carotenoid-based coloration during the breeding season (Doutrelant et al., 2013; Leclaire et al., 2011a), including the red eye-ring, red/orange gapes, orange tongue and

yellow bill. Although the antioxidant property of carotenoids appears to be controversial for birds (Costantini and Møller, 2008; Hartley and Kennedy, 2004; Krinsky, 2001), they are also involved in several physiological functions such as immunity (Blount et al., 2003; Chew and Park, 2004; Faivre et al., 2003; McGraw, 2006; Møller et al., 2000). Thus, in addition to be connected to reproduction through ornamentation, carotenoids appear to play a major role in survival by promoting self-maintenance.

As birds cannot synthetize carotenoids *de novo*, they have to acquire them through their diet, making carotenoids a limiting resource and thus, suggesting the existence of a trade-off between allocating carotenoids towards sexual ornamentation for reproduction or towards health-related functions for self-maintenance (Eraud et al., 2007; Pérez et al., 2010; Von Schantz et al., 1999). Besides, mate choice studies have shown that the most preferred individuals are often those expressing greater carotenoid pigmentation in sexual signals (Hill, 2006; Møller et al., 2000). This implies that healthy individuals should require fewer carotenoids for immune defenses and could therefore allocate more of this limited resource to enhance sexual signals, thereby indicating of a high-quality mate. In kittiwakes, it has been shown that integument carotenoid-based coloration could reflect individual quality (**Paper VI**; Doutrelant et al., 2013; Leclaire, 2010; Leclaire et al., 2011a,b) and as suggested in figure 32, such coloration can vary to a great extent among individuals. Numerous environmental stressors can influence individual quality and thus ornamental traits (Hill, 1995; Lifshitz and St Clair, 2016). Among them, some environmental contaminants, through their detrimental effects on oxidative balance and immune system (Bustnes et al., 2004; Costantini et al., 2014, 2017; Sagerup et al., 2009), could potentially lead to reduced colorful sexual traits (García-Heras et al., 2017; Lifshitz and St Clair, 2016; Marasco and Costantini, 2016).

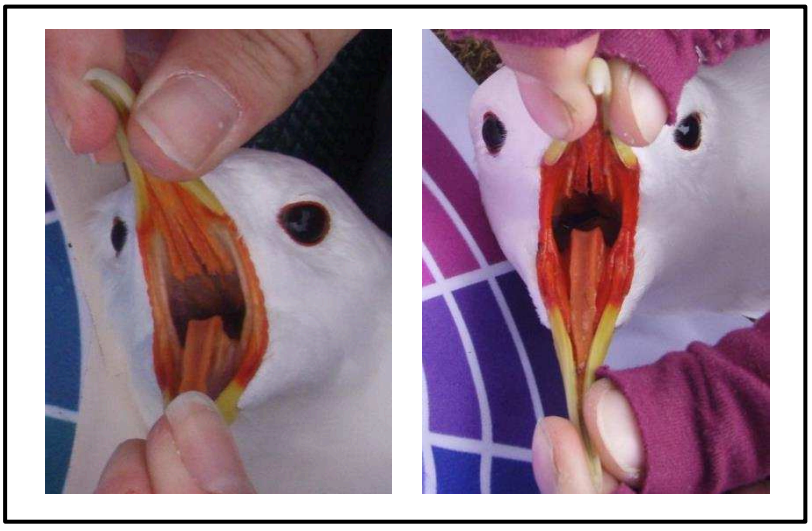


Figure 32: Contrasted coloration intensity between two female black-legged kittiwakes (*Rissa tridactyla*) during the pre-laying period, from Kongsfjord, Svalbard.

- *How to measure integument coloration?*

Regarding the pilot study conducted in 2011, integument coloration was measured from digital photographs as detailed in Montgomerie (2006). Pictures were taken at a standard distance of approximately 40 cm using a digital camera (Olympus U770sw, s770sw) with flash. For each photograph, the same color swatch was placed next to the bird to standardize subsequent measurements. All pictures were analyzed using Adobe Photoshop v.12.0. The average components of red (R), green (G) and blue (B) were recorded within the whole area of the eye-ring and in a standardized selected area for the gapes, tongue and bill. Each component was assessed 3 times to ensure a good repeatability of the measurement (CV < 5%, in all cases). RGB system was then converted into hue (H), saturation (S) and brightness (B). The HSB values of each integument were finally corrected according to the HSB values of the color swatch (Montgomerie, 2006).

Assessing coloration with photography has the benefits to be easily set-up, rapidly processed (i.e. minimizing handling time) and enable to study coloration of tiny and hardly reachable bare parts (e.g. eye-ring). However this method also displays some major limitations that deserve to be pointed-out. First, variations of light conditions can lead to inaccurate and biased measurements and use of flash can create bright areas. Besides, several pictures from the pilot study were unusable and removed from the original data set. Second, the range of color measurements is smaller than the range of colors normally perceived by kittiwakes (Cuthill, 2006; Håstad et al., 2009). Indeed, birds have a different vision compared to humans, with sensitivity to ultraviolet (UV) radiations (Bennett and Cuthill, 1994). For these reasons, we substituted the method based on photography initially used for the pilot study (i.e. 2011) by reflectance spectrometry, well-known for being the golden standard method to accurately and objectively assess bird coloration (i.e. extensive study in 2016; Montgomerie, 2006).

Gape, tongue and bill coloration were measured with a reflectance portable spectrometer, a xenon light source (covering the range 300-700 nm) and a 200-µm fiber optic reflectance probe held at 45° to the integument surface (on a standardized selected area; Figure 34). Reflectance was obtained using SpectraSuite software (Ocean Optics, Inc.). We generated reflectance spectra calibrated with a white standard (WS1 ocean optics) and a dark reference between each bird. The measurements were performed 5 times to ensure a good repeatability and for all patches, hue, chroma and brightness were computed using AVICOL software v.5 (Gomez, 2010). Specifically, hue was computed as the wavelength at which

reflectance was halfway between its minimum and maximum (L_{R50} measured); chroma as the maximum average yellow chroma Abs [$R_{max(500–700\ nm)}$-R_{450}] / R_{AV}; and brightness as the average reflectance over the total range of bird sensitivity 300 – 700 nm.

- *Relationships between OCs and integument coloration*

Saturation of eye-ring and gape decreased significantly with ΣPOPs concentrations (Eye-ring: slope = -2.10^{-6}; p-value = 0.023, Figure 33; Gape: slope = -2.10^{-6}; p-value = 0.047) and a similar relationship, although marginally significant, was found between tongue saturation and ΣPOPs in female kittiwakes (slope = -2.10^{-6}; p-value = 0.069). Bill saturation was not related to ΣPOPs. In other words, the most POPs-contaminated individuals were those displaying a reduced saturation of their labile integuments (Figure 33). Hue of tongue was negatively related to ΣPOPs but the relation seems to be driven by the presence of one influential point (slope = -3.10^{-7}; p-value = 0.030; see Figure 2 in **paper VI**). Hue and brightness were not related to ΣPOPs for all other integuments (see **paper VI** for more details on statistical analyses and results).

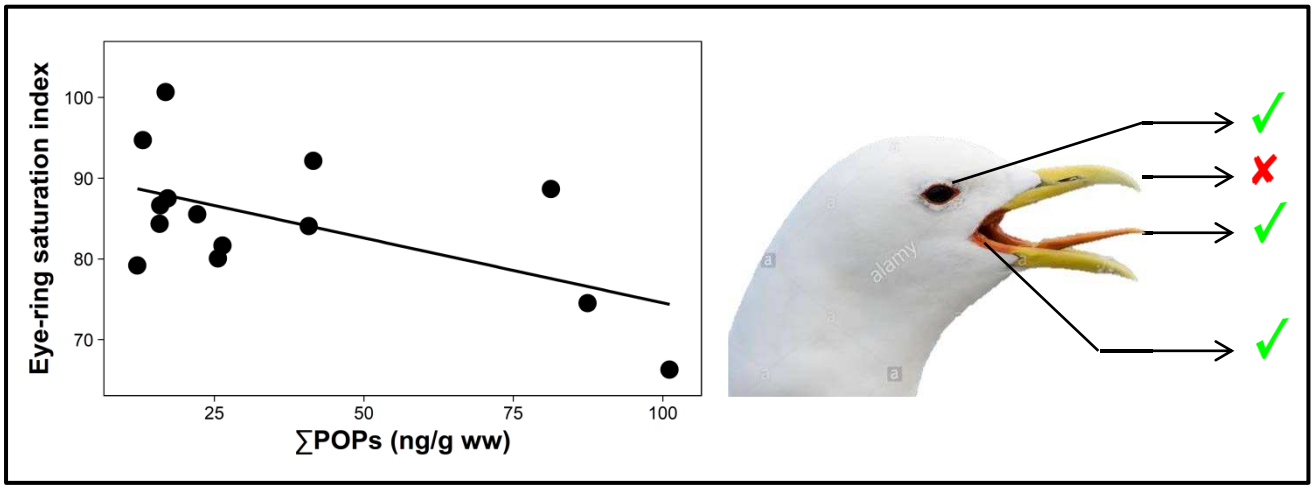


Figure 33: Relationship between eye-ring saturation and blood ΣPOPs concentrations in female (n=14) pre-laying black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard (on the left). Similar relationships were also found for other labile integuments (i.e. tongue and gapes) but not for the bill (on the right).

Saturation is usually assumed as a proxy of the amount of carotenoids present in tissues when color is produced by only one pigment (Montgomerie, 2006). However, integument coloration of kittiwakes results from a mix of different carotenoid species (Doutrelant et al., 2013) and, therefore, a same saturation can be obtained from mix of carotenoid species at different concentrations. This also means that equivalent amounts of

carotenoids may produce different saturations depending on the exact composition of the mix of carotenoids. Consequently, POP levels could affect saturation either by decreasing the amount of pigments and/or by modifying the carotenoid species composition present in integuments. Although the underlying mechanism remains unclear, our study suggests that POPs contamination can affect integument carotenoid-based coloration. This is consistent with previous works since Pérez et al. (2010) showed that organic compounds negatively influence the red bill spot size of adult yellow-legged gulls (*Larus michahellis*) during the courtship period. Moreover, Bortolotti et al. (2003) found that coloration of ceres and lores was disrupted by an enriched-PCB diet in captive American kestrels with exposed males being duller than controls.

Carotenoids are thought to promote health-related functions and might be mobilized to overcome the harmful effects of POPs at the expense of colored sexual signals. Under this scenario, female kittiwakes with the highest POP levels could allocate preferentially their carotenoids towards protective physiological functions whereas female kittiwakes with the lowest POP levels could allocate preferentially the available carotenoids towards sexual signaling. Indeed, POPs have already been known to cause detrimental effects on immunity and oxidative balance in kittiwakes and other seabird species (**Paper VII;** Bustnes et al., 2004; Costantini et al., 2014, 2017; Sagerup et al., 2009). These results are thus consistent with the existence of a trade-off between allocating carotenoids towards sexual ornamentation for reproduction or towards health-related functions for self-maintenance. However, we did not perform any physiological analysis in our study and, thus, the way through which contaminants could impact this suggested trade-off could only be confirmed by coupling both integument coloration and plasma carotenoid measurements in the same study.

- *Relationship between PFASs, Hg and integument coloration: preliminary results*

Very preliminary analyses indicate some significant associations between plasma PFAS concentrations and integument coloration. For example, the carboxylate PFNA (among other PFASs) is significantly and negatively related to bill chroma (i.e. saturation; slope = -0.118; p-value = 0.003; Figure 34). Therefore, this results based on a more accurate method confirm the hypothesis from **paper VI** which states that environmental contaminants can disrupt integument carotenoid-based coloration in kittiwakes.

In order to better understand the way through which contaminants can disrupt coloration of sexual signals, we measured the concentrations of several carotenoid components in the plasma of male kittiwakes. Interestingly, PFNA (among other PFASs) is significantly and positively related to zeaxanthine (slope = 25.811; p-value = 0.011; Figure 34), lutein (slope = 2.649; p-value = 0.008) and β-cryptoxanthine (slope = 0.148; p-value = 0.011) but not to astaxanthine and β-carotene. Lutein and zeaxanthine are pigments responsible for the yellow coloration which make sense since bill of kittiwakes is yellow. Importantly, bill chroma and zeaxhanthine concentrations in plasma are nearly significant and negatively associated (slope = -1.10^{-3}; p-value = 0.057; Figure 34). As a result, these preliminary results corroborate well the existence of a trade-off triggered by contaminants (here by PFASs), between allocating carotenoids towards sexual ornamentation for reproduction or towards health-related functions for self-maintenance. In accordance with this statement, higher protein oxidative damage and lower plasmatic non-enzymatic micro-molecular antioxidants were reported in the most PFASs-contaminated kittiwakes (same data set; **Paper VII**). Moreover, yellow xanthophylls (i.e. zeaxanthin and lutein) have been shown to enhance the innate immune system in kittiwakes (Leclaire et al., 2015). Because this trade-off does not seem to apply for all the measured carotenoid components (e.g. astaxanthine and β-carotene), contaminants could affect coloration by selectively targeting specific carotenoid species present in integuments (e.g. zeaxhantine and lutein in bill).

Finally, Hg concentrations do not appear to be associated with coloration parameters of any measured patches and neither to carotenoid concentrations in plasma.

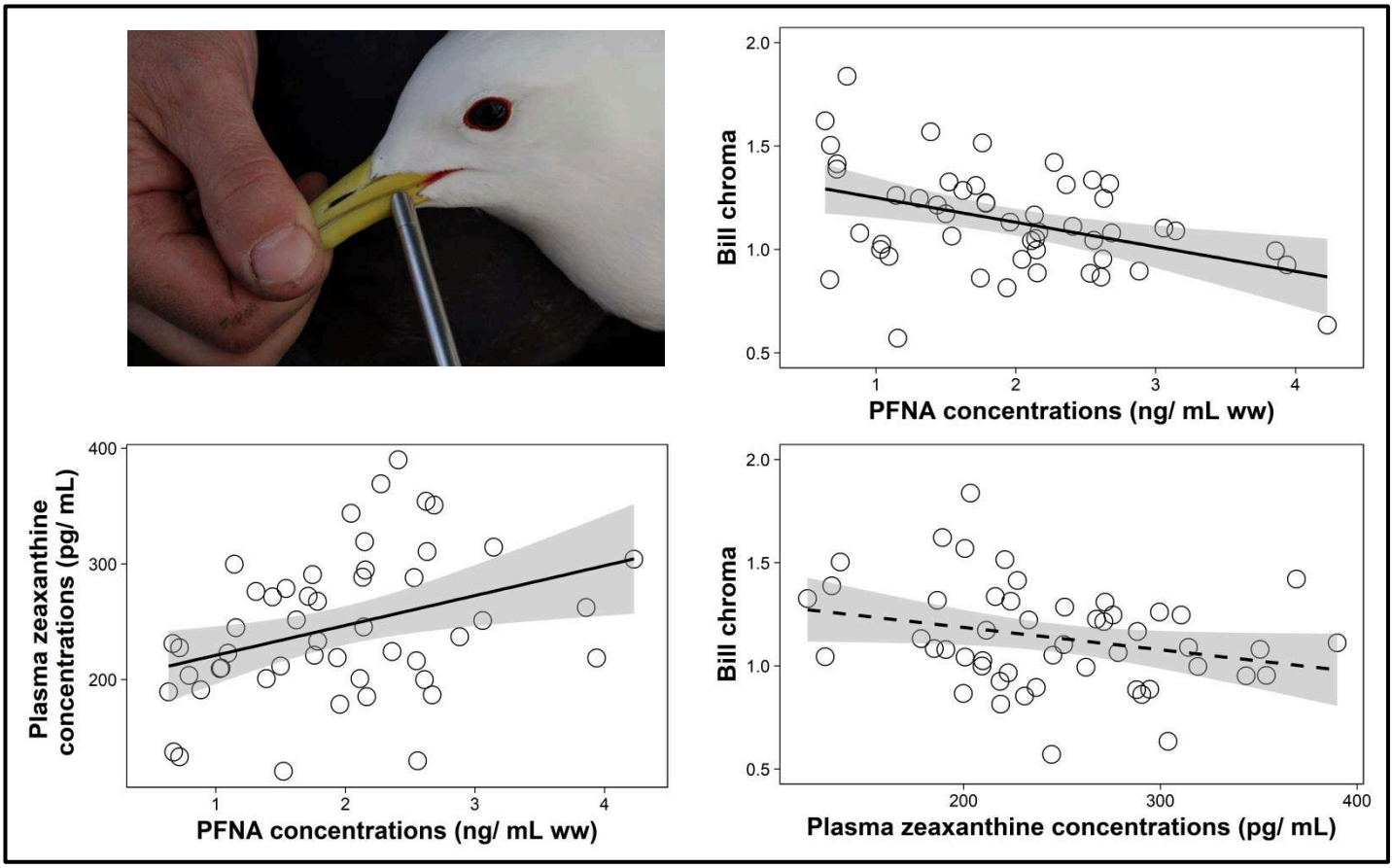


Figure 34: Measurement of bill coloration using reflectance spectrometry and relationships between bill chroma, plasma zeaxanthine and PFNA concentrations illustrating a potential trade-off triggered by PFNA; between allocating zeaxanthine to bill coloration or to health-related parameters in male (n=50) pre-laying black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard. Solid lines indicate significant relationships and dashed line indicates a nearly significant relationship.

b) Contaminants and olfaction

Because birds have long been thought to have a poor sense of smell, avian chemical signaling has been relatively unexplored. The preen gland, also called uropygial gland is located on the back, at the start of the tail and in a vast majority of bird species (Figure 35; Jacob and Ziswiler, 1982; Stettenheim, 1972). This gland produces an oleaginous secretion (i.e. preen oil), largely spread on plumage to protect feathers from degradation and enhance waterproofing (Jacob and Ziswiler, 1982; Moreno-Rueda, 2017; Stettenheim, 1972). In addition to this protective function, preen secretions, characterized by unique chemical signature for each sex and individual, are involved in sexual and social communication (Leclaire et al., 2011c; Moreno-Rueda, 2017). Heterozygosity and genetic dissimilarity between mates are selection criteria in reproduction of many vertebrates (e.g. Mulard et al., 2009). Importantly, it has been shown in kittiwakes that i) chemicals profile of preen secretion

was correlated with heterozygosity and ii) chemical distance was correlated with genetic distance (Leclaire et al., 2012). Since kittiwakes can smell (Leclaire et al., 2009), individual chemical signature can be seen as a signal of genetic mate quality and compatibility, suggesting the existence of a sophisticated odor-based mechanism of mate choice (Leclaire et al., 2012, 2014). As this "body odor" in under hormonal control (Asnani and Ramachandran, 1993; Whittaker et al., 2011) and because PFASs, OCs and Hg are endocrine disruptors, these contaminants could possibly modify the chemical signature of birds, providing an altered signal, potentially leading to some consequences on reproduction. Importantly, such effects of contaminants on olfactory mediated behaviors have already been reported in fish and crustaceans (Olsén, 2011).

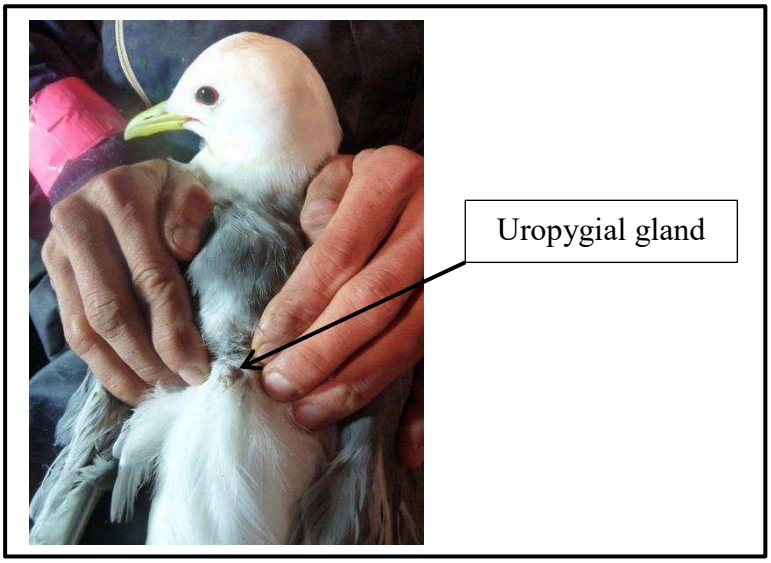


Figure 35: Uropygial gland of black-legged kittiwakes (*Rissa tridactyla*; provided by Sarah Leclaire).

The aim of this study is to investigate the effects of contaminants exposure on olfactive sexual signaling. Specifically, we will examine the relationships between some PFASs, OCs and Hg with chemical composition of preen secretion in pre-laying kittiwakes. We will also focus on hormones involved in reproduction (e.g. LH, corticosterone) as potential underlying mechanisms through which contaminants could disrupt sexual signaling. Finally, we will assess some potential consequences on reproductive parameters. We collected 50 samples of blood and preen feathers in male kittiwakes during the pre-laying period in 2016. To date, lab work is almost completed (OCs analysis has not been conducted yet) and we plan to analyze the data in a near future…

2 - Contaminants and fertility

Underlying mechanisms through which environmental contaminants can impact seabird reproductive success are unequally studied and a better understanding is required. Specifically, the effects of contaminants on seabird fertility parameters such as sperm quality remain totally unexplored. Yet, studying live sperm appears as being a central point in ecotoxicology, especially when assessing the consequences of chemical exposure on reproduction. The reason of this paradox relies on the lack of existing methods to collect sperm in seabird. Therefore, in 2016, we performed a pilot study in order to develop and set-up a non-invasive method to collect viable sperm samples based on a simple massage technique in male kittiwakes (Figure 36; described in *Humann-Guileminot et al., 2018;* attached in Appendix B). We came back in the field with a similar protocol in 2017 to increase the sample size and to get a suitable dataset.

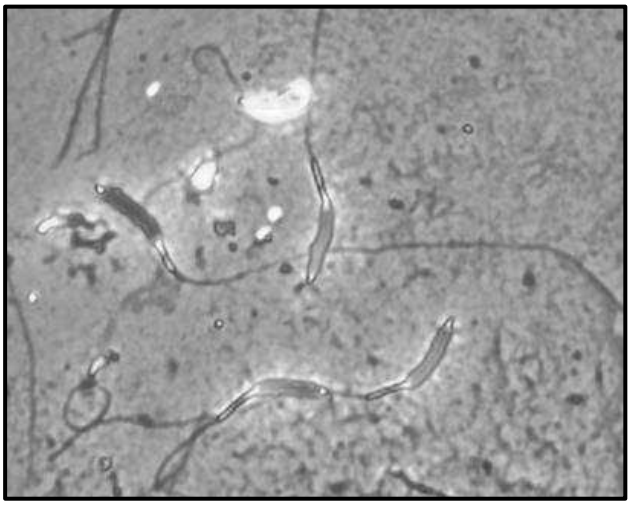


Figure 36: Normal spermatozoa of black-legged kittiwakes (*Rissa tridactyla*), from Kongsfjord, Svalbard (400x magnification and phase contrast; from *Humann-Guileminot et al., 2018*).

The aim of this study is to investigate the effects of contaminants exposure on fertility. Specifically, we will examine the relationships between some PFASs, OCs and Hg with sperm quality (i.e. morphology and motility) in pre-laying male kittiwakes. We will also focus on hormones involved in spermatozoa synthesis (e.g. LH, testosterone) as potential underlying mechanisms through which contaminants could disrupt fertility. Finally, we will assess some potential consequences on reproductive parameters. To date, lab work is in progress and we plan to analyze the data in a near future…

I - Conclusion

My thesis focuses on three groups of contaminants (PFASs, OCs and Hg) and aims at investigating the physiological and behavioural consequences of exposure to these chemicals in an Arctic breeding seabird, the black-legged kittiwake. This PhD work underlines the high toxicity of certain legacy chlorinated organic compounds (e.g. chlordanes) and significantly contributes at documenting the poorly known toxicological consequences of PFASs exposure for wildlife. Importantly, this PhD shows that PFASs and OCs could impact ageing, energy expenditure and some parental care behaviors in a contrasted manner. Specifically, oxychlordane, a metabolite of a banned organochlorine pesticide was associated with decreased telomere length, lowered metabolic rate and reduced ability to incubate the eggs. Conversely, elongated telomere, increased BMR and enhanced egg rotation were observed in birds bearing the highest concentrations of PFASs. Finally, at least for the considered endpoints, Hg appears as a coming minor threat for kittiwakes. This study highlights the importance of considering several groups of contaminants when investigating the consequences of environmental contaminants exposure in wildlife.

Under natural conditions, demonstrating causality is often challenging. Since kittiwakes are exposed to a complex cocktail of environmental contaminants and because of the presence of some potential confounding biological/ environmental factors, my thesis, based on a correlational approach precludes confirming with certainty the contrasted effects between PFASs and OCs reported here. Consequently, it appears necessary to validate the present results, especially for PFASs remaining so far poorly investigated, with an experimental approach. Apart from ethically reasons, experimentally manipulating contaminant burden or raising seabirds in captivity can be logistically challenging and very time consuming. One possible alternative would be to undergo *in vitro* experiments to test for effects of specific contaminants on various tissues and importantly would help to target more precisely the underlying mechanisms proposed in this thesis. In addition, because experimental and correlative studies are in my opinion, an inseparable duo, further works conducted on highly contaminated Arctic seabird species, like the glaucous gull would be particularly relevant to validate the suggested contrasted effects between PFASs and OCs, especially in a context ongoing disturbances affecting the Arctic such as climate change and increasing human activities that may jeopardize seabird populations.

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Paper I

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Perfluorinated substances and telomeres in an Arctic seabird: Cross-sectional and longitudinal approaches☆

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ABSTRACT

Telomeres are non-coding DNA repeats located at the termini of eukaryotic chromosomes, regulated by dynamic processes balancing shortening and maintenance. Despite a mechanism to slow-down telomere shortening, cell division leads to progressive attrition of chromosomes, leading to the onset of cellular senescence or apoptosis. However, telomere restoration based on telomerase activity is the primary mechanism for telomere maintenance. Telomere length is associated to health and survival and can be impacted by a broad panel of environmental factors. However, the effect of contaminants on telomeres is poorly known for living organisms. The aim of this study was to investigate relationships between some poly- and perfluoroalkyl substances (PFASs), body condition and telomere length by using both a cross-sectional and longitudinal approach in adult breeding Black-legged kittiwakes (*Rissa tridactyla*) from Svalbard. First, we examined the associations between absolute telomere length and PFASs contamination in a given year (cross-sectional approach). Second, we investigated the relationships between telomere dynamics and PFASs contamination within a two years' time frame (longitudinal approach). Our results did not show any significant relationships of PFASs and body condition with absolute telomere length in a given year. Surprisingly, we found a positive and significant relationship between PFASs and telomere dynamics in both sexes with elongated telomere in birds bearing the highest concentrations of PFASs. Our study underlines (i) the need to investigate PFAS effects on telomere dynamics with a longitudinal approach and (ii) a potential positive effect of these contaminants on telomere length, with the most contaminated birds showing the slowest rate of telomere shortening or even displaying elongated ones. Our study is the first to report a relationship between PFASs and telomere length in free-living vertebrates. A possible underlying mechanism and other potential confounding factors are discussed.

1. Introduction

Halogenated contaminants such as the poly- and perfluoroalkyl substances (PFASs) are synthetically manufactured chemicals produced since the 1950s. They are mainly used as surfactants and water repellents in numerous industrial and commercial applications because of their unique hydrophobic and oleophobic properties (e.g. fire-fighting foam, waterproof clothing, non-stick coating and impregnation agent for carpets, papers and textiles; Kissa, 2001). PFASs are either released in the environment by direct discharge ("direct emissions") or result from the degradation of precursor compounds ("indirect emissions"; Butt et al., 2010). PFASs are carbon chains varying in length, where hydrogen is replaced by fluorine atoms. Chemical bonds between carbon and fluorine atoms are very strong which make the PFASs thermally and

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chemically stable, resistant to degradation, and thus extremely persistent in the environment (Key et al., 1997; Muir and de Wit, 2010). Because of atmospheric long-range transport and oceanic currents, some PFASs reach remote areas such as the Arctic marine ecosystem, where they are preferentially deposited because of cold climate (AMAP, 2004; reviewed in Butt et al., 2010; Ellis et al., 2004; Giesy and Kannan, 2001; Prevedouros et al., 2006). The Arctic is therefore considered as a sink for environmental contaminants such as the PFASs. Specifically the perfluoroalkyl carboxylic acids (PFCAs), seem to increase in Arctic marine biota, contrary to the PFOS, a compound that belongs to sulfonic acids (PFSAs) which appears to decline since mid-2000s, after the phase-out by the US company 3M (reviewed in AMAP, 2016; Braune and Letcher, 2013; reviewed in Butt et al., 2010; Rotander et al., 2012; Wania, 2003).

Once deposited in the marine ecosystem, PFASs enter in the food chain with phytoplankton uptake, bioaccumulate in living organisms *via* food intake and increase with the trophic position due to biomagnification (Fang et al., 2014; Haukås et al., 2007; Kannan et al., 2005; Kelly et al., 2009; Tomy et al., 2004). There is now strong evidence that (i) PFASs accumulate and persist in protein-rich compartments (e.g. blood, liver, kidneys) and (ii) PFASs biomagnification is enhanced as the carbon chain length increases (Aas et al., 2014; reviewed in Butt et al., 2010; Conder et al., 2008; Kelly et al., 2009; Verreault et al., 2005). Indeed, PFAS profiles in liver and/or plasma of four Arctic seabird species, the Thick-billed murres (*Uria lomvia*), the Northern fulmar (*Fulmarus glacialis*), the Glaucous gull (*Larus hyperboreus*) and the Black-legged kittiwake (*Rissa tridactyla*), were dominated by long-chained PFCAs (Butt et al., 2007; Tartu et al., 2014; Verreault et al., 2005). As top predators, Arctic seabirds are exposed to relatively high concentrations of environmental contaminants; they are thus considered as extremely pertinent biological indicators to investigate the potential hazardous effects of PFASs on wildlife. To date, our knowledge about effects of PFASs exposure is limited (DeWitt, 2015; Jensen and Leffers, 2008; Lau et al., 2007), especially for free-living animals, although few studies have reported interactions between PFASs and physiology. For instance, several studies conducted on fishes and birds reported high concentrations of thyroid hormones and low levels of stress hormones in most PFASs contaminated individuals (Braune et al., 2011; Liu et al., 2011; Nøst et al., 2012; Tartu et al., 2014). More importantly, it has been suggested that PFASs could decrease the hatching success in two avian species, the Black-Legged kittiwake and the Tree swallow (*Tachycineta bicolor*; Custer et al., 2012; Tartu et al., 2014; but see also Bustnes et al., 2008). Further investigations focusing on wildlife and including more physiological and fitness traits are needed to better assess the impact of these contaminants on animals living in natural ecosystems (Kannan, 2011; Lau et al., 2007).

Among potential physiological investigations to be conducted for a better assessment of the toxicological consequences of PFASs exposure, are the telomeres. Telomeres are non-coding DNA repeats located at the termini of eukaryotic chromosomes and play a key role in ensuring the genomic stability (Blackburn, 1991; Monaghan and Haussmann, 2006). Because the DNA polymerase protein complex is unable to fully achieve the chromosomes replication during mitosis (i.e. end-replication problem), telomere length progressively shortens through life as a consequence of repeated cell divisions (Blackburn, 1991; Olovnikov, 1996; Sedivy, 1998). When telomere length is too short, cell division can damage coding DNA inducing cellular senescence or apoptosis (Blasco, 2007; Campisi et al., 2001; Harley et al., 1990; Olovnikov, 1996). Importantly, telomere length and telomere dynamics have been shown to be reliable predictors of longevity and survival in captive and wild vertebrates (Asghar et al., 2015; Barrett et al., 2013; Bauch et al., 2014; Bize et al., 2009; Boonekamp et al., 2014; Haussmann et al., 2005; Heidinger et al., 2012; Fairlie et al., 2016; Foote et al., 2010; Salomons et al., 2009). Moreover, recent studies have demonstrated that the rate of telomere shortening varies to a great extent between individuals. Indeed, telomere shortenning has been shown to be accelerated by the occurrence of a wide range of environmental stressors (Angelier et al., 2013; Epel et al., 2004; Hau et al., 2015; Meillère et al., 2015; Mizutani et al., 2013; Salmón et al., 2016; Young et al., 2013) including heavy metals and persistent organic contaminants (Blévin et al., 2016; Stauffer et al., 2017). However, there is still very few information regarding the effects of contaminants on absolute telomere length in free-living animals and no studies have been conducted so far on telomere dynamics, with a longitudinal approach. To the best of our knowledge, a single study has investigated the influence of PFASs on absolute telomere length (with a cross-sectional approach) in free-living birds but did not report any significant relationships (Sletten et al., 2016). Because of this link with survival and environmental stressors, measuring the effect of specific compounds on telomere length and telomere dynamics appear promising to better assess their impact on wildlife (Bateson, 2015).

In Svalbard, Black-legged kittiwakes (*Rissa tridactyla*, hereafter "kittiwakes"), are exposed to a complex cocktail of organic contaminants and heavy metals which are known to correlate with impaired individual fitness and population dynamics (Goutte et al., 2015; Tartu et al., 2013, 2014, 2015, 2016). Kittiwakes are thus potentially sensitive to a broad mixture of contaminants with many possible additive, synergistic, as well as antagonistic effects. The aim of the present study is to investigate the relationships between several measured PFASs (11 PFCAs and 3 PFSAs), body condition and telomere length by using both a cross-sectional and longitudinal approach in adult breeding kittiwakes from Svalbard. First, we examined the relationships between PFASs contamination and absolute telomere length within a given year (cross-sectional approach in 2012). Second, we investigated the associations between PFASs contamination in 2012 and telomere dynamics by sampling the same kittiwakes twice over a time frame of two years (longitudinal approach, between 2012 and 2014). Predictions are challenging since the impact of PFASs on the survival rate of free-ranging vertebrates remains undocumented with the exception of a study conducted on the glaucous gull where no relationships between PFASs and adult returning rate were found (Bustnes et al., 2008). However, since PFASs are expected to be detrimental for living organisms and appear to disrupt several physiological processes (e.g. endocrine disruption) in wildlife, as well as in laboratory animals (Austin et al., 2003; reviewed in DeWitt, 2015; reviewed in Lau et al., 2007; Liu et al., 2011), we predict that a high PFASs contamination will be associated with a rapid rate of telomere shortening (longitudinal approach), and thus, with short telomeres (cross-sectional approach).

2. Material and methods

Fieldwork was conducted in 2012, from 12th to 27th July and in 2014, from 26th June to 20th July, within a colony of kittiwakes at Kongsfjorden (78°54′N; 12°13′E), Svalbard. In 2012, 44 breeding adults (22 males and 22 females) were trapped while sitting on their nest with a loop at the end of long pole during the chick rearing period. All birds were assigned with a unique three-letter code fixed to the bird's tarsus. We collected a 2 mL blood sample from the alar vein using a heparinized syringe and a 25-gauge needle to assess PFAS concentrations, measure telomere length and determine gender. Then, skull length (head + bill) was measured with an accuracy of 0.1 mm using a calliper and birds were finally weighted to the nearest 2 g with a Pesola spring balance. In 2014, 17 birds (12 males and 5 females) out of the 44

kittiwakes caught in 2012 were recaptured after identification at a distance using a telescope. Indeed, in that colony the adult annual survival rate is 85% and the percentage of birds successfully reaching the chick rearing is about 75% (Goutte et al., 2015). Moreover, some birds were not possible to catch. After capture, these birds were blood sampled to assess PFAS concentrations (only 6 birds) and measure telomere length. Blood samples were stored on ice in the field. Plasma and red blood cells, obtained after centrifugation were kept frozen at −20 °C before subsequent lab work.

Telomere analysis was performed from red blood cells collected in 2012 (n = 38; 22 males and 16 females) and in 2014 (n = 17; 12 males and 5 females) at the Centre d'Etudes Biologiques de Chizé in France (CEBC). Indeed, over the 44 individuals caught in total in 2012, telomeres analysis was conducted on 38 individuals since not enough blood was left for 4 females. Telomere length was measured with the telomere restriction fragment method (TRF) by Southern blot and using the TeloTAGG Telomere Length Assay (Roche, Mannheim, Germany) as previously described and with minor modifications (Foote et al., 2010; Kimura et al., 2010a). Specifically, we have adjusted the quantity of DNA to allow a correct visualisation of the DNA signal on the gels. Briefly, samples were digested with proteinase K and DNA was extracted from red blood cells using the DNeasy blood and tissue kit (Qiagen). Gel electrophoresis and optical density spectrophotometry were used to check for DNA quality. Preliminary tests have been conducted to determine the optimal amount of DNA to be used and, for each sample, 0.7 μg of DNA was digested with the restriction enzymes *HinfI* and *RsaI* for 16 h at 37 °C. Digested DNA samples were then separated with a pulse-field gel electrophoresis (Bio-Rad) on a 0.8% agarose gel. Samples were randomly assigned to a gel except those used to assess telomere length dynamics which were treated in the same gel. At total, all samples were run in 4 gels. Internal controls were run on each gel to measure inter-gel variations and each gel was run at 3.0 V/cm with an initial switch time of 0.5 s to a final switch time of 7 s for 14 h. Following that step, the gel was depurinated and denaturated in an alkaline solution. The gel was then neutralized and DNA was transferred onto a nitrocellulose membrane by Southern blot (Hybond N+, Amersham Life Science, Amersham, UK). The membrane was placed in an incubator and dried at 120 °C for 20 min in order to fix the DNA. The DNA was then hybridized with a digoxigenin-labeled probe specific for telomeric sequences and incubated with antidigoxigenin-specific antibody before visualization with a Chemidoc (Bio Rad). Telomere length was then analyzed using ImageJ software and measured from telomere smear densities. Lane-specific background was subtracted from each density and telomere length (mean value) was then calculated within a window of 5–30 kb that includes the whole smear (Nussey et al., 2014). Inter-gel CV was 1.40. Telomere dynamics relates to the difference of telomere length between 2014 and 2012. Molecular sexing was conducted at the CEBC, from red blood cells of samples collected in 2012 (22 males and 22 females) by polymerase chain reaction (PCR) amplification of part of two highly conserved genes (CHD) present on sexual chromosomes following Fridolfsson and Ellegren (1999).

PFAS concentrations were determined from plasma samples collected in 2012 (n = 44; 22 males and 22 females) and 2014 (n = 6; 4 males and 2 females) at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. We searched for 14 PFASs: perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), linear perfluorooctanesulfonate (PFOSlin), perfluorobutanoate (PFBA), perfluoropentanoate (PFPA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoate (PFUnA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTrA), and perfluorotetradecanoate (PFTeA). Compounds not detected in 100% of the samples were not included in statistical analyses. Thereby, those remaining for further investigations were PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, and PFTrA. Briefly, a sample (0.5 mL) spiked with internal standards was extracted in acetonitrile (1 mL) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVI-Carb graphitized carbon absorbent and glacial acetic acid. Extracts were analyzed by UPLC/MS/MS. Recovery of the internal standards ranged between 50% and 120% and the deviation of the target concentrations in the standard reference materials (NIST Human serum 1958) were within the laboratory's accepted range (76–105%; n = 3). All blanks concentrations were below the instrument detection limits. Limit of detection of each compound is given in Table 1.

Statistical analyses were performed using R 3.3.1 (R Core Team., 2016). We first performed a principal component analysis (PCA; "Ade4 package") with individual PFASs in order to reduce the number of explanatory variables. We preferred this method instead of examining each contaminant separately because, (i) PFAS compounds are highly correlated with each other and (ii) it considerably decreases the number of statistical models since testing many models can potentially increase the type I error. The appropriate use of PCA was tested and confirmed through the Kaiser-Mayer-Olkin measure of sampling adequacy (K-M-O = 0.74) and the Bartlett's test of sphericity ($p < 0.001$). The number of significant principal components was selected according to the Kaiser criterion (i.e. eigenvalue higher than 1; Kaiser, 1960). The PCA resulted in one component (PC1), explaining 71% of the total variance and mainly influenced by high concentrations of PFDcA (factor loading: 0.45), PFUnA (0.45), PFOSlin (0.44), PFDoA (0.44) and to a minor extent PFTrA (0.33) and PFNA (0.32). Body condition was calculated with the residuals of the regression of body mass against skull length. The influence of contaminants and body condition in 2012 on absolute telomere length in 2012 and telomere length dynamics were investigated using linear models. Thus, PC1, body condition and sex were considered as explanatory variables while telomere length in 2012 and telomere dynamics were defined as response variables. Because PFAS concentrations in 2012 were different between sexes (Table 1), including the factor "sex" with the PFASs variable in the same model could induce multicollinearity problems and lead to biased results (Graham, 2003). However, it has been proposed to use the variance inflation factor (VIF) as a statistical tool to assess the extent of dependence between explanatory variables. Several

Table 1
Plasma PFAS mean concentrations ± standard errors (ng/mL ww) in 2012 and limits of detection (LODs) of female and male chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. PFAS gender-related differences have been tested with linear models.

	LODs	Males (n = 22)	Females (n = 22)	$F_{1,42}$	*P*-value
		Mean ± SE	Mean ± SE		
PFOSlin[a]*	$704\ 10^{-3}$	10.85 ± 0.58	8.92 ± 0.68	5.81	**0.02**
PFNA[b]*	$40.9\ 10^{-3}$	1.21 ± 0.1	1.08 ± 0.14	1.92	0.173
PFDcA[c]	$61.9\ 10^{-3}$	2.2 ± 0.12	1.63 ± 0.12	11	**0.002**
PFUnA[d]	$83\ 10^{-3}$	12.11 ± 0.64	9.38 ± 0.69	8.40	**0.006**
PFDoA[e]*	$109\ 10^{-3}$	2.54 ± 0.14	1.99 ± 0.17	8.75	**0.005**
PFTrA[f]*	$360\ 10^{-3}$	11.62 ± 1.41	9.68 ± 1.52	1.57	0.217

Significant p-values are in bold.
*Data were log-transformed to meet the assumption of the linear model.
[a] PFOSlin: Perfluorooctane sulfonate.
[b] PFNA: Perfluorononanoate.
[c] PFDcA: Perfluorodecanoate.
[d] PFUnA: Perfluoroundecanoate.
[e] PFDoA: Perfluorododecanoate.
[f] PFTrA: Perfluorotridecanoate.

studies suggested that below a value of 10, dependence is no longer a major issue (Chatterjee and Price, 1991; Neter et al., 1996), but a more stringent approach is to consider VIF < 3 (Zuur et al., 2009). Because males were more contaminated than females, VIF was then calculated between PC1 and the factor "sex" to ensure that these explanatory variables met independence (VIF = 1.16; calculated with "AED package" developed by Zuur et al., 2009). Biologically relevant models were constructed with PC1, body condition, sex and interactions of PC1 and body condition with sex as predictor variables. The best models were then selected with the bias-adjusted Akaïke's Information Criterion (AICc), defined as a bias adjustment for small-sample size (Burnham and Anderson, 2004). If AICc values differ by more than 2, the lowest AICc is the more accurate, whereas if AICc differ by less than two, models are considered as fairly similar in their ability to describe the data. Additionally, the Akaike weight (*Wi*) was estimated and can be interpreted as approximate probabilities that the model *i* is the best one to predict the data, given the candidate set of models (Burnham and Anderson, 2004; Johnson and Omland, 2004). We finally performed diagnostic plots and Shapiro normality tests on residuals to check if the data sufficiently met the linear model assumptions (Zuur et al., 2009). Data were log-transformed when testing for sex differences of PFAS concentrations and when investigating correlations between each PFAS compounds. A significance level of $\alpha < 0.05$ was used for all tests.

3. Results

3.1. PFAS concentrations

Plasma PFAS mean concentrations ± standard errors for chick-rearing adult kittiwakes in 2012 are listed in Table 1. Linear models to test gender-related differences indicated that all PFASs except PFNA and PFTrA significantly differed between sexes, with males having higher concentrations than females. Such sex-related differences of PFAS concentrations could be attributed either to the ability of females to transfer elevated amounts of contaminants into their eggs (Gebbink and Letcher, 2012) and/or to sexual differences regarding foraging ecology, with males feeding at higher trophic levels or in more contaminated areas than females. All PFASs (log-transformed) were highly and positively correlated with each other (Pearson correlations: $0.49 \leq r \leq 0.93$, all p-values < 0.001; n = 44), indicating similar exposure routes. Finally, PFAS concentrations seem to be repeatable (from 2012 to 2014) within the same individuals (r = 0.59, n = 6; calculated from the repeatability equation developed by Lessells and Boag, 1987). In other words, an individual with relatively high levels of PFASs in 2012 will also show relatively high levels of PFASs in 2014. However, the sample size is low (n = 6) and further studies conducted on a larger sample size would enable to confirm this statement.

3.2. Relationships between PFASs, body condition and telomere length

The model selection to explain absolute telomere length based on PFAS concentrations (PC1) and body condition in 2012 for male and female adult kittiwakes is presented in Table 2. Among the set of candidate models, the null model (parameterized with an intercept only) showed the best fit to the data. None of the other candidate models including sex, PC1 or body condition (as well as the interaction terms with sex) was better than the null model. These variables were therefore not good predictors of absolute telomere length, and PFAS concentrations in 2012 do not appear as good explanatory variables of absolute telomere length in 2012 (PC1, slope: a = 0.06; p = 0.443; Fig. 1).

Table 2
AICc model ranking for absolute telomere length in 2012 based on PFAS concentrations (PC1) and body condition in 2012 in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. (n = 38, 22 males and 16 females). PFASs were measured in plasma.

Models	AICc	ΔAICc	*Wi*
Null	99.6	0	0.35
Sex	99.8	0.3	0.30
PC1	101.3	1.7	0,15
Body condition	101.8	2.2	0,11
PC1 * Sex	102.9	3.3	0,07
Body condition * Sex	104.8	5.3	0,02

AICc, bias-adjusted Akaike's Information Criteria values; *Wi*, AICc weights.

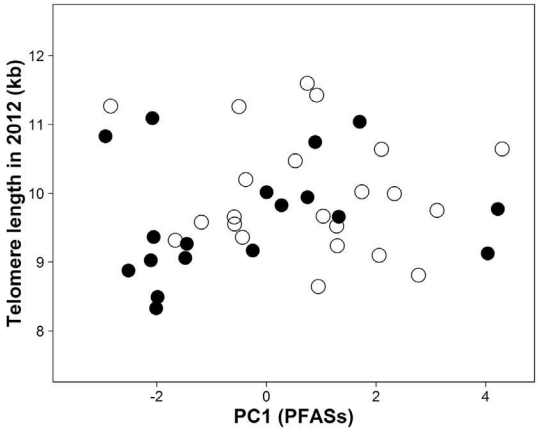

Fig. 1. Relationship between PC1 and absolute telomere length in 2012 in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. The effect of PFAS concentrations in 2012 on telomere length in 2012 was tested with a linear model (slope: a = 0.06, p = 0.443). PC1 is mainly influenced by high concentrations of PFOSlin, PFDcA, PFUnA, PFDoA and to a minor extent PFNA and PFTrA. Males (n = 22) are represented with empty circles and females (n = 16) with filled circles.

The model selection to explain telomere dynamics between 2012 and 2014 based on PFAS concentrations (PC1) and body condition in 2012 for male and female adult kittiwakes is presented in Table 3. Among the set of candidate models, the model including PC1 best fitted the data (ΔAICc = 2.8). PC1 was significantly and positively related to telomere dynamics (slope: a = 0.17, p = 0.026; Fig. 2). In other words, the most PFASs contaminated individuals in 2012 were those showing the slowest rate of telomere shortening from 2012 to 2014. Body condition and the gender were not were not considered as good predictors of telomere dynamics (Table 3).

4. Discussion

We observed no relationships between PFASs, body condition

Table 3
AICc model ranking for telomere dynamics between 2012 and 2014 based on PFAS concentrations (PC1) and body condition in 2012 in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard (n = 17, 12 males and 5 females). PFASs were measured in plasma.

Models	AICc	ΔAICc	*Wi*
PC1	28.8	0	0.7
Null	31.7	2.8	0.17
PC1 * Sex	34	5.2	0.05
Sex	34.4	5.6	0.04
Body condition	34.6	5.8	0.04
Body condition * Sex	41.8	13	0

AICc, bias-adjusted Akaike's Information Criteria values; *Wi*, AICc weights.

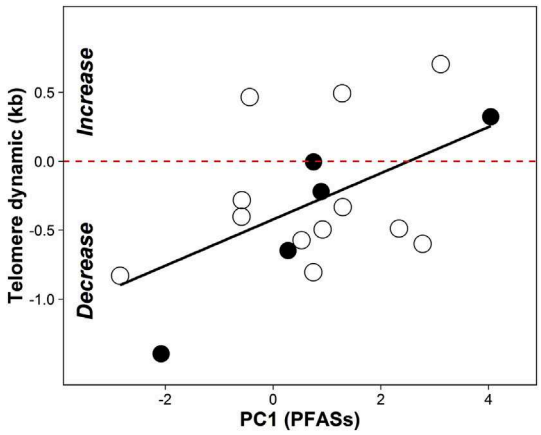

Fig. 2. Relationship between PC1 and telomere dynamics (the difference of telomere length between 2012 and 2014) in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. The effect of PFASs in 2012 on telomere dynamics was tested with a linear model (slope: a = 0.17, p = 0.026). PC1 is mainly influenced by high concentrations of PFOSlin, PFDcA, PFUnA, PFDoA and to a minor extent PFNA and PFTrA. Males (n = 12) are represented with empty circles and females (n = 5) with filled circles. Individuals above red dashed line have increased telomere length whereas the ones bellow showed decreased telomere length between 2012 and 2014. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and absolute telomere length when analyzing only one year (cross-sectional approach in 2012). However, the results from the longitudinal approach indicated PFASs in 2012 as the best predictor of telomere dynamics. There was a significant and positive relationship between PFAS plasma concentrations in 2012 and telomere dynamics with the most PFASs-contaminated individuals showing the slowest rate of telomere shortening from 2012 to 2014. Additionally, among the most PFAS contaminated birds, 4 individuals displayed elongated telomeres from 2012 to 2014. This suggests some potential positive effects of PFASs contamination on telomeres. Considering the discrepancy in the findings between the two approaches, our study highlights the need to investigate the effects of PFASs on telomere dynamics with a longitudinal approach, rather than simply measuring absolute telomere length in a single snapshot. In vertebrates, most of telomere shortening occurs early in life, during growth and developmental stages and this rate of early-life shortening varies to a great extent between individuals (Boonekamp et al., 2014; Hall et al., 2004; Foote et al., 2010; Frenck et al., 1998; Friedrich et al., 2001; Rattiste et al., 2015; Salomons et al., 2009; Zeichner et al., 1999). In addition, telomere length can also be affected later in life, in adults, by variation in stressful experiences (Angelier et al., 2013; Epel et al., 2004; Hau et al., 2015; Mizutani et al., 2013; Young et al., 2013). As a result, there is probably a large inter-individual variability in telomere length in adult kittiwakes and this variability may result from several factors that were not taken into account in our analyses (e.g. age, environmental stressors, etc.). This large inter-individual variability can certainly blur the potential effect of PFASs contamination on telomere length when using a cross-sectional approach, possibly explaining why we were not able to detect any correlations between PFASs contamination and absolute telomere length in 2012. Because PFASs contamination appears quite repeatable over two years within the same individual, the longitudinal approach allows us to relate such PFASs contamination in 2012 with telomere dynamics.

Only three studies have studied the associations between contaminants and telomere length in free-ranging vertebrates (Blévin et al., 2016; Sletten et al., 2016; Stauffer et al., 2017). Thus, this work contributes at filling the gap of knowledge about the potential effects of environmental contaminants on telomere length in wildlife. Contrary to our results from the longitudinal approach, PFASs did not predict telomere length in white-tailed eagle (*Haliaeetus albicilla)* chicks (Sletten et al., 2016). However, this study did not investigate the relationships between contaminants and telomere dynamics, but rather used a cross-sectional approach (i.e. a single measure of telomere length). This could potentially explain the discrepancy between the results of the two studies. Another potential explanation would rely on the difference of concentrations of contaminants between eagle chicks and kittiwake adults but this statement does not seem relevant here. While PFOSlin concentration in kittiwakes (9 884 ± 462 pg/g ww) were on average 4 times lower than those in eagle chicks (40 914 ± 5 746 pg/g ww), PFUnA concentration in kittiwakes (10 746 ± 509 pg/g ww) were on average 2 times higher than those in eagle chicks (5 609 ± 525 pg/g ww). Finally, a recent study conducted on the same kittiwake population showed a negative relationship between telomere length and oxychlordane (Blévin et al., 2016), a metabolite of an organochlorine pesticide considered as very toxic for wildlife (Bustnes, 2006; Erikstad et al., 2013; Goutte et al., 2015). Organochlorines and PFASs are structurally opposed, with organochlorines being lipophilic (Findlay and DeFreitas, 1971) and PFASs having a high affinity with proteins (Heuvel et al., 1992). Moreover, kittiwakes are exposed to an additional mixture of chemicals, which are not included in this study and which could act on telomere length (Stauffer et al., 2017). Consequently, further investigations focusing on various chemicals, structurally different, may enable to clarify such contrasted results.

Telomere length adjustment is dynamic with both shortening and maintenance events. Despite a mechanism to slow-down telomere shortening, the end-replication problem leads to progressive attrition of chromosomes, leading to the onset of cellular senescence or apoptosis (Blasco, 2007; Campisi et al., 2001; Harley et al., 1990; Olovnikov, 1996). However, telomere restoration based on telomerase activity, an enzyme adding new telomeric sequences onto the ends of chromosomes at each DNA replication, has been shown to be the primary mechanism for telomere maintenance and genomic integrity (Blackburn, 1991, 2005; Greider and Blackburn, 1985). Telomerase is variably active in several somatic and post-somatic tissues throughout the lifespan of long-lived seabirds (Haussmann et al., 2007). This latest study highlighted the very high activity of telomerase in bone marrow during the whole lifespan of two seabird species, the Common tern (*Sterna hirundo*) and the Leach's storm petrel (*Oceanodroma leucorhoa;* Haussmann et al., 2007). The authors stated that "*telomerase activity in bone marrow may be associated with the rate of erythrocyte telomere shortening; birds with lower rates of telomere shortening and longer lifespans have higher bone marrow telomerase activity throughout life*". Indeed, all circulating erythrocytes in birds are produced by the hematopoietic stem cells of the bone marrow (Sturkie and Griminger, 1976), and telomere length measured in erythrocytes appear to mirror the telomere length of stem cells in bone marrow (Kimura et al., 2010b; Vaziri et al., 1994; but see Reichert et al., 2013). Thus, which underlying mechanisms could induce a disruption of telomerase activity and how can it be related to PFASs contamination? Indeed, several correlational and experimental studies have highlighted a potential role of glucocorticoids in determining telomere dynamics: increased glucocorticoids concentration (i.e. corticosterone and cortisol) were associated with a down-regulation of telomerase activity or/and an accelerated rate of telomere shortening (Bauch et al., 2016; Choi et al., 2008; Haussmann et al., 2012; Quirici et al., 2016; Schultner et al., 2014; Young et al., 2016, 2016; but see Epel et al., 2010). Importantly, another investigation conducted in the same kittiwake population reported a negative relationship between baseline corticosterone

levels and PFAS concentrations (Tartu et al., 2014). Even if underlying mechanisms are currently unclear, PFASs-induced lower circulating corticosterone levels might potentially result in relatively high telomerase activity in bone-marrow, and therefore in decreased rate of telomere shortening in highly contaminated kittiwakes.

Our study reported some telomere elongation between 2012 and 2014 in 4 kittiwakes. Interestingly, telomere elongation has already been associated with nutritional and climatic factors. Recently, Hoelzl et al. (2016) showed that food supplementation reduces telomere attrition and is even associated with telomere elongation in a wild mammal species, the dormouse (*Glis glis*). Similarly, Bebbington et al. (2016) reported an increased telomere length with high food availability in a small passerine, the Seychelles warbler (*Acrocephalus sechellensis*). Finally, a study conducted on the Black-tailed gull (*Larus crassirostris*) highlighted a potential positive effect of El Niño on telomere dynamics (Mizutani et al., 2013). Therefore, the lower rate of telomere shortenning in most PFASs contaminated kittiwakes highlighted in our study, in combination with good environmental conditions, could potentially explain why we observed telomere elongation in some kittiwakes.

We proposed here one possible underlying physiological mechanism, based on endocrine disruption, potentially explaining the reduced rate of telomere shortening in most PFAS-exposed kittiwakes. Although causality is difficult to assess in correlational studies, the relationships with telomere dynamics reported here may rely on ecological factors, rather than PFASs contamination. Besides, a study conducted in the same kittiwake colony reported a positive relationship between PFASs contamination and body condition in males (Tartu et al., 2014). This could suggest that the apparent positive effect of PFASs on telomere length maybe related to individual quality rather than to PFASs contamination. That is the reason why we included body condition in our analyses as a potential predictor of telomere length. However, body condition in 2012 was not related to absolute telomere length in 2012 and telomere dynamics. Indeed, telomere length does not fluctuate as fast as the body condition does, which is probably too labile compared to the slower rate of change of telomeres. Therefore, further ecological variables directly linked to feeding ecology (e.g. stable isotopes, protein amounts) of kittiwakes should be included as predictors of telomere length. Indeed, since food ingestion is the main route for PFASs exposure, the most contaminated kittiwakes could be the birds feeding at the highest trophic levels and are possibly the individuals of the highest quality.

Another important point that deserves to be discussed is a potential confounding effect of age which is suggested to negatively affect telomere length (Haussmann and Vleck, 2002; Haussmann et al., 2003). However, this is particularly true for species with shorter lifespans which lose more telomeric repeats with age than species with longer lifespans (Haussmann et al., 2003). Indeed, in long-lived species, telomere loss appears to occur mainly early in life (i.e. between chick and adult stage) rather than during adulthood (Hall et al., 2004; Foote et al., 2010), as is the case in other vertebrates (Frenck et al., 1998; Rufer et al., 1998; Zeichner et al., 1999; Friedrich et al., 2001). Since our study was conducted on breeding adults (i.e. at least 3–4 years old; Coulson, 2011) of a long-lived seabird and because we investigated telomere dynamics, with a longitudinal approach, we have some good reasons to think that age in our study is not a major factor influencing telomere length. However, relationships between age and PFASs in seabirds remains undocumented so far and thus, a potential confounding effect of age on PFAS concentrations here cannot be completely ruled out.

Despite some limitations and a moderate sample size, the positive relationship between PFASs contamination and telomere dynamics reported here could suggest a positive effect of PFASs exposure on telomeres and *in fine*, on survival rate of adult kittiwakes. This seems to be corroborated by findings from a recent study about PFASs and self-maintenance metabolism (Basal Metabolic Rate) conducted also on kittiwakes which supports the hypothesis that PFASs may stimulate self-maintenance mechanisms (Blévin et al., 2017). However, only capture-mark-recapture (CMR) investigations would enable to confirm this statement and to fully validate our findings, future experimental investigations focusing on the effects of PFASs on telomere length should be carried out with a laboratory avian model.

Conflict of interest

The authors declare to have no conflicts of interest.

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Paper II

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Exposure to oxychlordane is associated with shorter telomeres in Arctic breeding kittiwakes

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Exposure to oxychlordane is associated with shorter telomeres in arctic breeding kittiwakes

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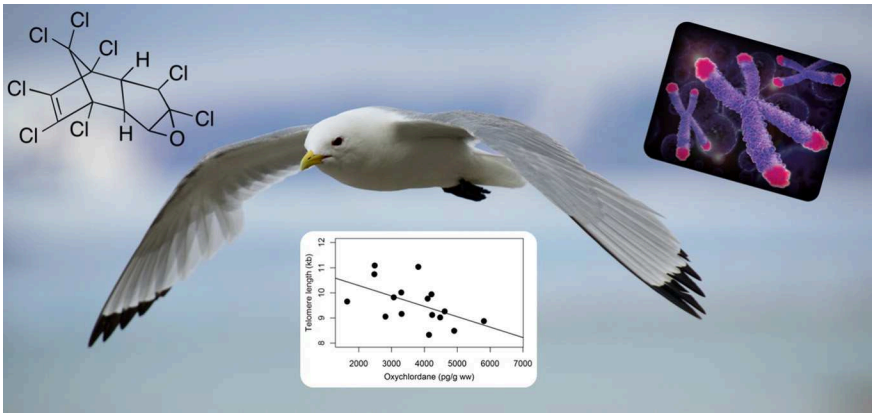
HIGHLIGHTS

- Potential impacts of POPs on telomeres were studied in an arctic seabird.
- No relationship was found between PCBs and telomere length.
- Oxychlordane concentration was associated with shorter telomeres in females.
- This study highlights sex-related sensitivity to banned organochlorine pesticides.

GRAPHICAL ABSTRACT



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ABSTRACT

Telomeres are DNA-protein complexes located at the end of chromosomes, which play an important role in maintaining the genomic integrity. Telomeres shorten at each cell division and previous studies have shown that telomere length is related to health and lifespan and can be affected by a wide range of environmental factors. Among them, some persistent organic pollutants (POPs) have the potential to damage DNA. However, the effect of POPs on telomeres is poorly known for wildlife. Here, we investigated the relationships between some legacy POPs (organochlorine pesticides and polychlorobiphenyls) and telomere length in breeding adult black-legged kittiwakes (*Rissa tridactyla*), an arctic seabird species. Our results show that among legacy POPs, only blood concentration of oxychlordane, the major metabolite of chlordane mixture, is associated with shorter telomere length in females but not in males. This suggests that female kittiwakes could be more sensitive to oxychlordane, potentially explaining the previously reported lower survival rate in most oxychlordane-contaminated kittiwakes from the same population. This study is the first to report a significant and negative

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Organochlorines
PCBs
DNA

relationship between POPs and telomere length in a free-living bird and highlights sex-related susceptibility to banned pesticides.

1. Introduction

Telomeres are DNA-protein complexes located at the end of linear chromosomes which play a critical role in maintaining the genomic integrity (Blackburn, 1991; Monaghan and Haussmann, 2006). The DNA polymerase protein complex cannot replicate the very ends of chromosomes during mitosis, and, consequently telomeres shorten at each cell division (Olovnikov, 1996). When telomeres reach a critical lower threshold, cell division can damage coding DNA leading to apoptosis or replicative senescence (Olovnikov, 1996; Campisi et al., 2001). It was originally thought that telomere loss occurred at a constant rate in individuals through their life, and telomere length could therefore act as an internal 'mitotic clock' to measure the chronological age of organisms into the wild (Haussmann and Vleck, 2002). However, recent studies have shown that telomere length predicts survival (Haussmann et al., 2005; Bize et al., 2009; Salomons et al., 2009; Heidinger et al., 2012; Angelier et al., 2013; Barrett et al., 2013) and is related to a wide range of environmental stressors (Mizutani et al., 2013; Meillère et al., 2015). Consequently, telomere length is considered as more related to biological age than chronological age *per se* (Monaghan and Haussmann, 2006; Barrett et al., 2013).

In humans, telomere erosion can be accelerated by different environmental factors such as exposure to pollutants (Zhang et al., 2013). For instance, it has been reported that outdoor workers exposed to traffic pollution have shorter telomeres than indoor office workers (Hoxha et al., 2009). Similarly, women telomere length decreases as exposure to pollution caused by hazardous wastes increases (De Felice et al., 2012). One underlying mechanism that could potentially explain accelerated telomere shortening is oxidative stress (Von Zglinicki et al., 2000; Zhang et al., 2013). This corresponds to the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of an organism (Finkel and Holbrook, 2000). When metabolic by-products, such as ROS, are not fully neutralized by anti-oxidant defenses, they may oxidize cellular macromolecules such as DNA (Houben et al., 2008). Hence, telomere length may partly reflect oxidative stress history of an individual (Houben et al., 2008). Among contaminants, some persistent organic pollutants (POPs) have the potential to damage DNA by triggering oxidative stress and even decrease survival (Fernie et al., 2005; Isaksson, 2010; Letcher et al., 2010; Erikstad et al., 2013; Costantini et al., 2014; Sletten et al., 2016). However, the effect of POPs on telomere length is poorly known for wildlife. To the best of our knowledge, only one study has addressed this topic in a free-living animal with low contamination levels but failed to find any significant relationships (Sletten et al., 2016).

Due to their high volatility and persistence in time, POPs reach remote areas such as the Arctic (Gabrielsen and Henriksen, 2001). Once deposited in marine ecosystems, living organisms assimilate the POPs *via* food intake. The POP concentrations then increase from the marine environment into the organisms and throughout food webs due to bioaccumulation and biomagnification (Letcher et al., 2010). Seabirds are top predators; consequently, they are particularly exposed to POPs contamination. They therefore appear as highly relevant biological models to investigate the influence of POPs on telomere length. A previous study in a Svalbard population of black-legged kittiwakes *Rissa tridactyla* ("kittiwakes" hereafter) has reported high oxidative stress levels in most POPs contaminated individuals (POPs included polychlorobiphenyls: PCBs and one organochlorine pesticide: OCP; Lindsøe, 2012). Additionally, adult survival rate in the same population of kittiwakes was negatively linked to some OCPs (Goutte et al., 2015). In this study, we investigated the relationships between some legacy POPs (OCPs and PCBs) and telomere length in Svalbard kittiwakes. Because telomere length is classically reduced in response to oxidative stress (Von Zglinicki et al., 2000; Zhang et al., 2013) and often tightly linked to survival (Haussmann et al., 2005; Bize et al., 2009; Salomons et al., 2009; Heidinger et al., 2012; Angelier et al., 2013; Barrett et al., 2013), we predicted that POP levels would be negatively related to telomere length.

2. Materials and methods

2.1. Study area and sampling collection

Fieldwork was carried out in 2012 from July 12th to July 27th in Krykkjefjellet colony of Kongsfjorden, Svalbard (78°54′N, 12°13′E). A total of 38 individuals (22 males and 16 females) were caught on their nest with a noose at the end of a 5 m fishing rod during the chick rearing period. At capture, a 2 mL blood sample was collected from the alar vein using a heparinized syringe and a 25G needle to determine legacy POP levels, telomere length and the sex of individuals. Blood samples were stored at −20 °C until subsequent analyses. The sex of individuals was determined from red blood cells by polymerase chain reaction (PCR) at the Centre d'Etudes Biologiques de Chizé (CEBC) as previously described (Weimerskirch et al., 2005).

2.2. Telomere assay

Telomere length was determined at the CEBC by Southern blot using the TeloTAGGG Telomere Length Assay (Roche, Mannheim, Germany) as previously described and with minor modifications (Foote et al., 2010; Kimura et al., 2010). Telomere length analysis has already been successfully achieved on the same population of Svalbard kittiwakes (Schultner et al., 2014a). Briefly, samples were digested with proteinase K, and DNA was extracted from red blood cells by using the DNeasy blood and tissue kit (Qiagen). DNA quality was checked by gel electrophoresis and optical density spectrophotometry. Preliminary tests have been conducted to determine the optimal amount of DNA to be used and, for each sample, 0.7 μg of DNA was digested with the restriction enzymes *HinfI* and *RsaI* for 16 h at 37 °C. Digested DNA samples were then separated using a pulse-field gel electrophoresis (Bio-Rad) on a 0.8% agarose gel. All samples were run in four gels. Samples were randomly assigned to a gel. Internal controls were run on each gel to measure inter-gel variations. The gels were run at 3.0 V/cm with an initial switch time of 0.5 s to a final switch time of 7 s for 14 h. Following that step, the gel was depurinated and denaturated in an alkaline solution. The gel was then neutralized and DNA was transferred onto a nitrocellulose membrane by Southern blot (Hybond N+, Amersham Life Science, Amersham, UK). The membrane was incubated at 120 °C for 20 min in order to fix the DNA. The DNA was then hybridized with a digoxigenin-labeled probe specific for telomeric sequences and incubated with antidigoxigenin-specific antibody before visualization with a Chemidoc (Bio Rad). Telomere length was then analyzed using ImageJ to extract telomere smear densities. Lane-specific background was subtracted from each density value and telomere length (mean value) was then calculated using a window of 5–30 kb that includes the whole smear (Nussey et al., 2014). Inter-gel CV was 1.40%.

2.3. POPs analyses

POPs were analyzed from whole blood at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. Only compounds that

potentially affect survival of kittiwakes were considered in this study (Goutte et al., 2015). Thus, we selected the ∑PCBs (CB-99, -118, -138, -153, -180, -183 and -187), and the OCPs (HCB, *p,p′*-DDE, oxychlordane, *trans*- and *cis*-nonachlor). To a blood total sample of 0.5 to 1.5 mL, a 100 μL internal standard solution was added (13C-labeled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). The sample was extracted twice with 6 mL of *n*-hexane, after denaturation with ethanol and a saturated solution of ammonium sulphate in water. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975C MSD were performed as previously described (Herzke et al., 2009). For validation of the results, blanks (clean and empty glass tubes treated like a sample, 3 in total) were run for every 10 samples, while standard reference material (3 in total, 1589a human serum from NIST) was run for every 10 samples. The accuracy of the method was within the 70 and 108% range.

2.4. Statistical analyses

Statistical tests were performed using R 2.13.1 (R Development Core Team, 2011). We first checked if telomere length and POP levels differed between sexes using the parametrical test of Welch. The influence of POPs' contamination on telomere length was investigated with Linear Models. Thus, ∑POPs, ∑PCBs, HCB, *p,p′*-DDE, oxychlordane, *cis*- and *trans*-nonachlor were defined as independent variables and telomere length as the dependent variable. Because blood contaminant concentrations differed between males and females (see Results), including the factor "sex" and the variable "∑POPs" in the same model could lead to multicollinearity problems and biased results (Graham, 2003). Additionally, it is now well established that males and females can react in different way to environmental stressors such as POPs contamination. Specifically, previous studies conducted on kittiwakes from Krykkjefjellet colony have reported sex differences regarding body condition, hormones levels, endocrine disruptions, phenology, breeding decision and even survival rate (Goutte et al., 2010, 2015; Schultner et al., 2014b; Tartu et al., 2013, 2014, 2015a). Therefore, male and female kittiwakes were separated in statistical analyses. Diagnostic plots were assessed and Shapiro normality tests were performed on residuals to test whether data sufficiently met the assumption of the linear model. Multiple testing can potentially lead to misleading results, indicating statistical significance in situations where there is none. Consequently, we performed bootstrapping (*i.e.* resampling method) from the data sets of significant relationship and then assessed diagnostic plot to corroborate the results (see Supplementary materials; Chernick and Labudde, 2014). A significance level of $\alpha < 0.05$ was used for all tests.

3. Results

Telomere length was not related to sex ($t = -1.438$, P-value = 0.160) and ∑POPs tended to be higher in male than in female kittiwakes ($t = -1.976$, P-value = 0.056). ∑POPs, ∑PCBs, HCB, *p,p′*-DDE, *cis*- and *trans*-nonachlor were not related to telomere length in male nor in female kittiwakes (All P-values ≥ 0.259; Table 1). In females, we found a significant and negative association between oxychlordane and telomere length (Table 1; Fig. 1a). However, we found no relationship between oxychlordane and telomere length in males (Table 1; Fig. 1b).

4. Discussion

Our results on adult kittiwakes showed that among legacy POPs, only blood concentrations of oxychlordane were negatively associated with telomere length in females but not in males. Oxychlordane and chlordane mixture have already been associated with lower survival in both males and females from the same kittiwake population (Goutte et al., 2015). Our study reports a negative relationship between telomere length only in females, thus providing a possible mechanism linking oxychlordane exposure and female mortality. Regarding males, it is thus likely that oxychlordane exposure may lead to a lower survival rate in another way, independent of telomere attrition. Effects of contaminants on telomere length are still poorly known; most of the studies have focused on humans (Zhang et al., 2013) and to the best of our knowledge, only one study has investigated the relationships between POPs and telomere length in wildlife (Sletten et al., 2016).

So far, 11 of the 14 studies investigating the relationship between telomere length and environmental and occupational chemical exposure in humans, have reported significant negative associations (Rigolin et al., 2004; Hoxha et al., 2009; Bin et al., 2010; McCracken et al., 2010; Pavanello et al., 2010; Li et al., 2011; Rollison et al., 2011; De Felice et al., 2012; Eshkoor et al., 2012; Hou et al., 2012; Wu et al.,

Table 1
Relationships between whole blood ∑POPs, ∑PCBs, OCPs and telomere length in female and male chick-rearing black-legged kittiwakes, *Rissa tridactyla* from Kongsfjorden, Svalbard.

Dependent variables		Independent variables	*df*	*F*	P-value
Females	Telomere length	∑POPs*	1,14	0.802	0.386
		∑PCBs**	1,14	0.833	0.377
		HCB	1,14	0.534	0.477
		p,p′-DDE	1,14	0.066	0.801
		Oxychlordane	1,14	5.343	**0.037**
		cis-Nonachlor	1,14	1.246	0.283
		trans-Nonachlor	1,14	1.301	0.273
Males	Telomere length	∑POPs*	1,20	0.394	0.537
		∑PCBs**	1.20	0.114	0.740
		HCB	1.20	0.002	0.967
		p,p′-DDE	1.20	1.351	0.259
		Oxychlordane	1.20	0.025	0.876
		cis-Nonachlor	1,20	0.077	0.785
		trans-Nonachlor	1,20	0.922	0.348

Significant variables are in bold.
* ∑POPs: CB-99, -118, -138, -153, -180, -183, -187, HCB, *p,p′*-DDE, oxychlordane, *trans*- and *cis*-nonachlor.
** ∑PCBs: CB-99, -118, -138, -153, -180, -183, -187.

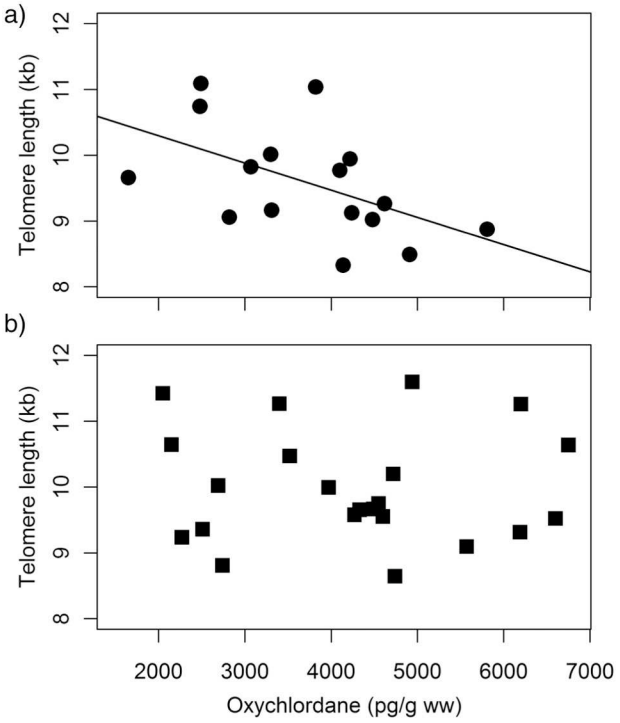

Fig. 1. Relationships between telomere length and whole blood oxychlordane concentrations in female (a) and male (b) chick-rearing black-legged kittiwakes, *Rissa tridactyla* from Kongsfjorden, Svalbard.

2012). For example, shorter telomeres were associated with pesticide exposure in patients with myelodysplastic syndrome (Rigolin et al., 2004; Rollison et al., 2011). Similarly, a study conducted in an apparent healthy Korean population reported a negative association between telomere length and exposure to high levels of POPs, including OCPs (*p,p′*-DDE, *trans*-nonachlor and oxychlordane), PCBs and polybrominated diphenylethers (Shin et al., 2010). The only study investigating relationships between telomere length and contamination in wildlife has been performed on white-tailed eagle (*Haliaeetus albicilla*) chicks in northern Norway (Sletten et al., 2016). In this study, the authors did not find any relationship between telomere length and POPs, including OCPs, PCBs and perfluorinated compounds. However, the relatively low levels of contaminants in white-tailed eagle chicks compared to those measured in adult kittiwakes may explain the discrepancy in the results. Oxychlordane concentrations in kittiwakes measured in the present study (4019 ± 1315 pg/g ww) were on average, around 3 times higher than those reported for eagle chicks (1483 ± 197 pg/g ww). With this exception, our results are thus consistent with previous works and suggest a negative effect of oxychlordane on telomere length in kittiwakes.

Surprisingly, the relationships between oxychlordane and telomere length were sex dependent and a significant relationship was found in females, but not in males. How to explain such a difference? There are some indications that female seabirds may be more sensitive to high levels of OCPs than males. In glaucous gulls (*Larus hyperboreus*) and in kittiwakes, OCPs exposure mostly affected survival rate in females (Erikstad et al., 2013; Goutte et al., 2015). Furthermore, in wandering albatrosses (*Diomedea exulans*), another long-lived seabird species, the relationships between POP levels and oxidative stress were dependent of reproductive effort and breeding females had the highest levels of haptoglobin, a well-known acute phase protein that indicates an ongoing inflammatory response which can limit the spread of oxidative damages, compared to breeding males (Costantini et al., 2014). Thus, the influence of contaminants on oxidative stress may be exacerbated by reproductive effort. Although kittiwakes are considered to share equally incubation and chick rearing duties (Coulson, 2011), egg production represents a significant and important cost for females (Monaghan and Nager, 1997). It is thus possible that the cost of egg production increases the sensitivity of female kittiwakes to oxychlordane. This may therefore explain the sex-dependent relationship between telomere length and oxychlordane burden. Furthermore, although female birds transfer a significant part of their PCBs and DDT burden to their eggs, oxychlordane on the other hand, seemed to be more selectively retained by the female, at least in female glaucous gulls (Verboven et al., 2009). In female kittiwakes, it is thus possible that the energetic cost of clutch production added to the maintenance of significant oxychlordane levels would exacerbate the toxic effects of this chlorinated pesticide.

Our study was conducted on breeding adults (*i.e.* at least 3–4 years old; Coulson, 2011). However, the birds' age was unknown in our study, thus a possible confounding factor could be that birds with high levels of OCPs are the oldest birds with the shortest telomeres. If so, the negative relationship between oxychlordane and telomere length would be induced by the age of individuals rather by a direct effect of oxychlordane on telomere attrition. Although several studies suggested a negative effect of age on telomere length (Haussmann and Vleck, 2002; Haussmann et al., 2003), other reported that telomere loss mainly occurs early in life of long-lived seabird (*i.e.* between chick and adult stage) rather than during adulthood (Hall et al., 2004; Foote et al., 2010), as is the case in other vertebrates (Frenck et al., 1998; Rufer et al., 1998; Zeichner et al., 1999; Friedrich et al., 2001). Additionally, Haussmann et al. (2003) showed a significant and positive relationship between telomere length and age in another long-lived seabird species, the Letch's storm petrel (*Oceanodroma leucorhoa*). Consequently, effect of age on telomere length appears to be more complex rather than a simple negative and constant decrease of telomere length with age. Furthermore, it has been reported that in several seabird species, blood level of POPs is unrelated to age in adult birds (Bustnes et al., 2003; Carravieri et al., 2014; Tartu et al., 2015b), and rather reaches a steady state of equilibrium once adult (Newton et al., 1981; Henriksen, 1995; Drouillard, 2001; Bustnes et al., 2003). Therefore, it is unlikely that the negative relationship between oxychlordane and telomere length originates from birds with high levels of oxychlordane being the oldest birds with the shortest telomeres.

Previous studies have shown decreased antioxidant enzyme activity in relation to contaminants, such as in herring gulls (*Larus argentatus*), where chicks exposed to PCBs combined with dietary restrictions showed negative relationships between catalase, glutathione peroxidase and contaminant levels (Hegseth et al., 2011). Similarly, significant negative associations between oxychlordane, *p,p′*-DDE, PCB-153 and superoxide dismutase enzyme in white-tailed eagle chicks have been reported (Sletten et al., 2016). Finally, a significant positive relationship between oxychlordane, PCBs and oxidative stress was found in a Svalbard population of adult kittiwakes (Lindsøe, 2012). Consequently, oxychlordane could be possibly involved in the generation of oxidative stress through an increase of ROS which are known to reduce telomere length (Von Zglinicki, 2002). As the nucleobase guanine is a major oxidation target for ROS, the (TTAGGG)n repeats, that constitute vertebrate telomeres, are particularly vulnerable to oxidative attacks (Wang et al., 2010). In our study, *in vivo* biomarkers of oxidative stress were not measured. Thus, further studies measuring at the same time POP levels, proxies of oxidative stress and telomere length are thus needed to test if oxidative stress induced by oxychlordane exposure could be linked to telomere attrition. Among other potential mechanisms, oxychlordane could induce a down-regulation of telomerase activity. Indeed, telomere integrity is largely maintained by a telomerase-based mechanism, in which the enzyme telomerase plays a key role by adding hexameric (TTAGGG) repeats to chromosome ends, partially compensating telomere shortening (Greider and Blackburn, 1989; Xin et al., 2008). However, this hypothesis seems unlikely since telomerase is generally inactivated in adult somatic cells of most studied species so far (Monaghan and Haussmann, 2006; Vleck et al., 2007; but see Hatakeyama et al., 2008). Consequently, telomerase down-regulation seems to be more a common feature of large and/or long-lived species (Gomes et al., 2011) rather than being specifically related to contamination levels.

Extensively used during >35 years as a pesticide, usage of chlordane, of which oxychlordane is a major metabolite, tended to decrease in the 80s (U.S. Department of Health and Human Services, 1994). Banned from use and listed as a legacy POP by the Stockholm convention since 2004, oxychlordane provided a clear diagnostic criterion related to the lethal poisoning in several bird species (Blus et al., 1983, 1985; Stickel et al., 1983; Okoniewski and Novesky, 1993; Stansley and Roscoe, 1999; Wiemeyer, 1996). Furthermore, in an experimental study where female rats were gavage with oxychlordane, high administrated doses (10 $mg \cdot kg^{-1}$) revealed acute toxicity, characterized by feed refusal, rapid weight loss and thymic atrophy. At lower doses (2.5 $mg \cdot kg^{-1}$), female rats showed signs of hepatic changes indicative of microsomal enzyme induction (Bondy et al., 2003). Finally, as previously mentioned, in glaucous gulls and in kittiwakes from Svalbard, oxychlordane has been associated with lower survival rates, especially in females (Erikstad et al., 2013; Goutte et al., 2015). In the present study, among legacy POPs, only oxychlordane was negatively associated with telomere length in females. Our results are thus consistent with the idea that oxychlordane is one of the most toxic POPs (Erikstad et al., 2013). Consequently, female kittiwakes' sensitivity to oxychlordane could affect telomere length, explaining the previously reported lower survival rate in highly contaminated female kittiwakes. On the other side, the lack of relationships between oxychlordane and telomere length in males suggest that the lowest survival rate of most oxychlordane contaminated birds is thus probably not mediated by telomere shortening in male kittiwakes.

This study is the first to report a significant and negative relationship between POPs and telomere length in wildlife and therefore partly fills the gap of knowledge about contaminants effect on telomere attrition. However, the present work has some limitations. First, we did not measure oxidative stress or other potential mechanisms. Thus, further studies measuring at the same time POP levels, proxies of oxidative stress and telomere length are thus needed to test if oxidative stress induced by oxychlordane exposure could be linked to telomere attrition. Secondly, among all tested contaminants only blood oxychlordane concentration was negatively associated with telomere length and even though several studies have reported that oxychlordane is highly toxic for birds, an experimental approach would enable to confirm the acute toxicity of this compound on telomere length through oxidative stress. Finally, our study was conducted on a limited number of individuals and to fully validate our finding, future studies investigating effects of POPs on telomere length should be conducted on a larger sample size and other species.

Conflict of interest

The authors declare no competing financial interest.

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Paper III

Blévin, P., Tartu, S., Ellis, H.I., Chastel, O., Bustamante, P., Parenteau, C., Herzke, D., Angelier, F., Gabrielsen, G.W.

Contaminants and energy expenditure in an Arctic seabird: Organochlorine pesticides and perfluoroalkyl substances are associated with metabolic rate in a contrasted manner

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Contaminants and energy expenditure in an Arctic seabird: Organochlorine pesticides and perfluoroalkyl substances are associated with metabolic rate in a contrasted manner

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ABSTRACT

Basal metabolic rate (BMR), the minimal energetic cost of living in endotherms, is known to be influenced by thyroid hormones (THs) which are known to stimulate *in vitro* oxygen consumption of tissues in birds and mammals. Several environmental contaminants may act on energy expenditure through their thyroid hormone-disrupting properties. However, the effect of contaminants on BMR is still poorly documented for wildlife. Here, we investigated the relationships between three groups of contaminants (organochlorines (OCs), perfluoroalkyl substances (PFASs), and mercury) with metabolic rate (MR), considered here as a proxy of BMR and also with circulating total THs (thyroxine (TT4) and triiodothyronine (TT3)) in Arctic breeding adult black-legged kittiwakes (*Rissa tridactyla*) from Svalbard, during the chick rearing period. Our results indicate a negative relationship between the sum of all detected chlordanes (ΣCHLs) and MR in both sexes whereas perfluorotridecanoate (PFTrA) and MR were positively related in females only. MR was not associated with mercury. Additionally, levels of TT3 were negatively related to ΣCHLs but not to PFTrA. The findings from the present study indicate that some OCs (in both sexes) and some PFASs (only in females) could disrupt fine adjustment of BMR during reproduction in adult kittiwakes. Importantly, highly lipophilic OCs and highly proteinophilic PFASs appear, at least in females, to have the ability to disrupt the metabolic rate in an opposite way. Therefore, our study highlights the need for ecotoxicological studies to include a large variety of contaminants which can act in an antagonistic manner.

1. Introduction

Understanding the concept of energy allocation toward maintenance requirements, activity, growth and reproduction is a central goal which integrates both ecology and physiology. Usually considered as the minimal energetic cost of living, basal metabolic rate (BMR) is defined as the lowest rate of energy expenditure in a post-absorptive, adult endotherm at rest in its thermoneutral zone (Bligh and Johnson, 1973; Ellis and Gabrielsen, 2002; McNab, 1997). BMR is by far the most widely assessed parameter in vertebrate energetics (Danforth and Burger, 1984; Ellis, 1984) and has been described for a large variety of species from a wide geographical range (Bryant and Furness, 1995; Ellis, 1984; Ellis and Gabrielsen, 2002; Scholander et al., 1950). Although subject to circadian and seasonal fluctuations (Aschoff and Pohl, 1970; Kendeigh et al., 1977), a significant part of BMR variation within and between species can be attributed to adaptations either to specific environmental conditions or to particular behavioral traits of the species (Bech et al., 1999; Burton et al., 2011; Gabrielsen, 1994; Verreault et al., 2007).

Thyroid hormones (THs) are involved in a multitude of physiological traits and are known to regulate metabolism. Specifically, thyroxine (T4) and especially triiodothyronine (T3) are considered as the major controllers for the regulation of tissue oxygen consumption, thermogenesis and metabolic activity in endotherms (Bobek et al., 1977; Danforth and Burger, 1984; Freake and Oppenheimer, 1995; Hulbert, 2000). The roles of THs in the regulation of metabolism have been well

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documented in laboratory studies (Hulbert, 2000; Kim, 2008; Silvestri et al., 2005), and several investigations conducted in domestic as well as in free-living animals have highlighted strong and positive associations between total T3 (TT3) levels and BMR (Bobek et al., 1977; Chastel et al., 2003; Elliott et al., 2013; Vézina et al., 2009; Welcker et al., 2013; Zheng et al., 2013).

Over the last few years, a significant amount of studies have led to the hypothesis that exposure to environmental contaminants could be the cause of disruption of thyroid function or BMR and several studies have reported abnormal TH concentrations and thyroid gland structure in birds exposed to contaminants under controlled laboratory conditions, as well as in free-ranging populations (Cesh et al., 2010; reviewed in Dawson, 2000; Haugerud, 2011; reviewed in McNabb, 2005; reviewed in McNabb and Fox, 2003; Melnes, 2014; Nøst et al., 2012; reviewed in Rolland, 2000; reviewed in Scanes and McNabb, 2003; Smits et al., 2002; Verreault et al., 2004, 2007, 2013; Wada et al., 2009). Besides, circulating levels of THs appear to be suitable biomarkers as measures of contaminant-associated effects in wildlife (Fox, 1993; McNabb, 2005; Peakall, 1992; Rolland, 2000). Effects of contaminants on metabolic activity are still poorly known and largely debated in the literature, since the few studies that have investigated this topic in adult birds and mammals are limited and somewhat contradictory. Briefly, decreased metabolic rate was observed in mourning doves (*Zenaida macroura*) exposed to the polychlorinated biphenyl mixture (PCB) Aroclor 1254 (Tori and Mayer, 1981) and in pigeons (*Columba livia*) fed with high doses of dichlorodiphenyltrichloroethane (DDT, Jefferies and French, 1971). Similarly, a previous study conducted in an Arctic seabird, the glaucous gull (*Larus hyperboreus*), revealed negative associations between BMR and plasma concentrations of PCBs, DDTs, and particularly chlordane and its metabolites (CHLs, Verreault et al., 2007). In contrast, other studies have reported an increased metabolic rate in response to contamination, as in DDT-exposed short-tailed shrews (*Blarina brevicauda*, Braham and Neal, 1974) and PCB-exposed white-footed mice (*Peromyscus leucopus*) (Voltura and French, 2000).

High latitudes are considered as a sink for environmental pollutants due to atmospheric long-range transport and oceanic currents in combination with a cold climate (Burkow and Kallenborn, 2000). Given their properties (i.e. high volatility and/or persistence), organic pollutants and heavy metals can reach remote areas such as the Arctic (AMAP, 2010, 2011). Once deposited in the marine ecosystem, most of those chemicals bioaccumulate in birds *via* food intake. Due to biomagnification, this exposure will then increase in severity throughout food webs (Atwell et al., 1998; Blévin et al., 2013; Kelly et al., 2009; Letcher et al., 2010). Several Arctic seabirds occupy relative high trophic levels and are consequently particularly exposed and sensitive to high concentrations of environmental contaminants. They are thus relevant biological models to investigate the influence of contaminant exposure on energy expenditure in wildlife (Gabrielsen and Henriksen, 2001; Letcher et al., 2010). In Svalbard, black-legged kittiwakes (*Rissa tridactyla*, hereafter “kittiwakes”), are exposed to a complex mixture of harmful halogenated compounds and heavy metals which correlates with impaired fitness and population dynamic through their endocrine-disrupting properties (Goutte et al., 2015; Tartu et al., 2013, 2014, 2015, 2016). Among them are (1) toxic trace elements of both human and natural origins such as mercury (Hg) which tends to decrease in the Arctic (Cole et al., 2013); (2) the globally decreasing legacy persistent organic pollutants (POPs) which have been extensively used in the past and now banned by the Stockholm convention (Helgason et al., 2008; Rigét et al., 2010); and (3) poly- and perfluoroalkyl substances (PFASs) such as the long-chained perfluoroalkyl carboxylic acids (PFCAs) which currently are the most prevalent PFASs in Arctic biota (Butt et al., 2007, 2010; Tartu et al., 2014). Kittiwakes are thus potentially exposed to a broad cocktail of contaminants with many possible additive, synergistic, as well as antagonist effects.

In kittiwakes, significant relationships between concentrations of OCs, PFASs, Hg and several hormones (e.g. THs, corticosterone) involved in energy metabolism have been observed (Ask, 2015; Tartu et al., 2014, 2015, 2016). Because THs are known to exert a strong control on the regulation of metabolic functions in kittiwakes (Elliott et al., 2013; Welcker et al., 2013), individuals exposed to high concentrations of those chemicals may experience altered metabolic activity in response to disrupted thyroid function. We tested this assumption by investigating the relationships between three groups of contaminants (OCs, PFASs, and Hg) with metabolic rate (MR), considered here as a proxy of BMR and also with circulating concentrations of total THs (TT3 and TT4) in a kittiwake population from Svalbard (Norwegian Arctic). Because BMR is considered as a life-history component (reviewed in Burton et al., 2011), such relationships between contaminants and basal energy expenditure could potentially be related to the decreased survival rate, lower hatching success and breeding probabilities as previously reported in the most contaminated kittiwakes in Svalbard (Blévin et al., 2016; Goutte et al., 2015; Tartu et al., 2014).

2. Material and methods

2.1. Study area and sampling collection

Fieldwork was carried out in 2012, from July 12th to 27th in a colony of black-legged kittiwakes at Kongsfjorden (78°54′N; 12°13′E), Svalbard. A total of 44 individuals (22 males and 22 females) were caught on their nest with a noose at the end of a 5 m fishing rod during the chick rearing period (when raising chicks). At capture, a 2 mL blood sample was collected from the alar vein using a heparinized syringe and a 25-gauge needle to assess contaminant concentrations, TT3 and TT4 levels (when enough blood) and to determine the sex of individuals. Blood samples were stored on ice in the field. Whole blood and both, plasma and red blood cells obtained after centrifugation were kept frozen at −20 °C until subsequent analyses in the lab.

2.2. MR measurements

MR measurements were performed on 23 individuals (12 males and 11 females) among the 44 kittiwakes that have been caught in total. After capture and blood sampling, birds were kept in an opaque box and rapidly transported by boat (within 20 min) to the laboratory in Ny-Ålesund to measure MR by open-flow-through respirometry measurements of at least two hours duration. Outside air was drawn into the lab and dried in indicator silica gel before entering a 41 L plexiglass chamber holding the bird. Air was drawn past the bird and into a Sable Systems FoxBox® analyzer at a rate of 1.92 ± 0.04 (sd) L/min. CO_2 was measured by the FoxBox, after which the air was scrubbed of CO_2 with indicator soda lime and dried again with indicator silica gel before returning to the FoxBox where O_2 was measured. Prior to and following each MR measurement, the bird was weighed to the nearest 0.1 g on an electronic balance and its body temperature was taken with a Schultheis fast-reading reptile mercury thermometer accurate to 0.2 °C. During metabolic measurements, the chamber was covered with a towel to allow diffuse light while preventing the bird from observing its surroundings. This was necessary because the chamber was not in a temperature cabinet but on a lab bench where it was exposed to room temperatures. Chamber temperature (T_a) was monitored continuously by a probe connected to the FoxBox and averaged 19.2 ± 1.8 °C (sd; ranged from 15.1 to 22.7 °C). Body temperature (T_b) averaged 40.5 ± 0.7 °C (sd; ranged from 38.8 to 41.9 °C). Readings of all gases, flow rate, and T_a were made every 20 s by the FoxBox and recorded with a time stamp on a laptop computer. Most kittiwakes caught on their nest had been there for an unknown period of time, so it was not known if they were entirely post-absorptive. For that reason, metabolic measurements were not typically begun until about 4 h post-capture. By itself, that did not guarantee a post-absorptive condition, but the time

was limited to reduce the duration the bird was away from the nest. Another consideration for BMR is that birds are in their thermoneutral zone (TNZ) when measured. Indeed, two previous studies reported the upper end of that zone to be at least 20 °C for kittiwakes (Gabrielsen et al., 1988; Humphreys et al., 2007). Because T_a was set by room temperature, seven of the birds were measured at temperatures exceeding 20 °C, though in all but two cases (21.6 and 22.7 °C) Ta ≤ 21 °C. For those reasons, the measured MR herein could be used as a proxy of BMR. The effect of T_a and T_b is considered below in the Results.

2.3. Chemicals analyses

OCs were analyzed from whole blood at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. The following compounds were scanned: the polychlorinated biphenyls (CB-28, −52, −99, −101, −105, −118, −138, −153, −180, −183, −187 and −194) and the organochlorine pesticides (*o,p′* DDT, *p,p′* DDT, *p,p′* DDE, *o,p′* DDE, *o,p′* DDD, *p,p′* DDD, α-, β-, γ-HCH, HCB, *trans*-, *cis*-chlordane, oxychlordane, *trans*-, *cis*-nonachlor). Compounds detected in less than 70% of the samples were removed from the data set (Noël et al., 2009). Thereby, those remaining for further investigations were the PCBs (CB-28, −99, −105, −118, −138, −153, −180, −183, −187 and −194) hereafter referred as ΣPCBs, the pesticides (*p,p′* DDE, β-HCH, HCB), and the the sum of all detected chlordanes (ΣCHLs: oxychlordane, *trans*-, *cis*-nonachlor). To a blood total sample of 0.5–1.5 mL, a 100 µL of the internal standard solution was added (^{13}C-labeled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). The sample was extracted twice with 6 mL of n-hexane, after denaturation with ethanol and a saturated solution of ammonium sulphate in water. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC, and detection on an Agilent Technology 5975CMSD were performed as previously described (Herzke et al., 2009). For validation of the results, blanks (clean and empty glass tubes treated like a sample) and standard reference material (1589a human serum from NIST; NIST, 2015) were run for every 10 samples. The accuracy of the method was within the 70% and 108% range and the presented OC concentrations were corrected for recovery.

PFASs were analyzed from plasma at NILU. The following compounds were scanned: perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), linear perfluorooctanesulfonate (PFOSlin), perfluorobutanoate (PFBA), perfluoropentanoate (PFPA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoate (PFUnA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTrA), and perfluorotetradecanoate (PFTeA). The compounds detected in > 70% of the samples were PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, and PFTrA. Mean concentrations ± standard errors, ranges and limits of detections (LODs) of the other PFASs are detailed in Supplementary materials. Briefly, a sample (0.5 mL) spiked with internal standards was extracted in acetonitrile (1 mL) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVI-Carb graphitized carbon absorbent and glacial acetic acid. Extracts were analyzed by UPLC/MS/MS. Recovery of the internal standards ranged between 50% and 120%. The deviation of the target concentrations in the SRMs (NIST Human serum 1958; NIST, 2015) were within the laboratory's accepted range (76–105%; n = 3). All blanks contained concentrations below the instrument detection limits. The presented PFAS concentrations were corrected for recovery.

Total Hg analyses were performed at the Littoral Environment et Sociétés laboratory (LIENSs) in La Rochelle, France from freeze-dried and powdered red blood cells placed in an Advanced Hg Analyzer Spectrophotometer (Altec AMA 254, as detailed in Bustamante et al. (2006)). Hg is essentially associated to the red blood cells and thus, represents well the bird Hg exposure. All analyses were repeated at least two times on aliquots ranging from 5 to 10 mg of red blood cells until having a relative standard deviation < 5%. Accuracy was regularly checked using certified reference material (Tort-2 Lobster Hepatopancreas, NRC, Canada; certified value 0.27 ± 0.06 µg/g dw). Blanks were analyzed at the beginning of each set of samples and the detection limit of the method was 0.005 µg/g dw.

2.4. Molecular sexing and total TH assays

Molecular sexing and hormone assays were performed at the Centre d'Etudes Biologiques de Chizé (CEBC), France. The sex of the individuals was determined from red blood cells by polymerase chain reaction amplification of part of two highly conserved genes (CHD) present on sexual chromosomes following Fridolfsson and Ellegren (1999).

TT3 and TT4 analyses were performed by radioimmunoassay (RIA) on 35 and 33 kittiwakes, respectively (TT3: 15 males and 20 females; TT4: 15 males and 18 females). Total TH levels were assessed in duplicates without extraction. 25 µL of plasma were incubated during 24 h with 10000 cpm of the appropriate ^{125}I-hormone (Perkin Elmer, US) and polyclonal rabbit antiserum supplied by Sigma company (US). The bound fraction (hormone linked to antibody) was then separated from the free fraction by addition of a sheep anti-rabbit antibody and centrifugation. After overnight incubation and centrifugation, bound fraction activity was counted on a wizard 2 gamma counter (Perkin Elmer, US). Cross-reactions of T3 antiserum were defined as follows by Sigma: triiodoD-thyroacetic acid 6%, L-thyroxine 0.2%, diiodo-L-thyrosine < 0.01%, monoiodo-L-thyrosine < 0.01%. Cross-reactions of T4 antiserum were defined as follows by Sigma: triiodothyronine 4%, diiodo-L-thyrosine < 0.01%, monoiodo-L-thyrosine < 0.01%. Inter- and intra-assay variations were respectively 15.9% and 7.5% for TT3, 19.4% and 12.2% for TT4. The lowest TT3 detectable concentration was 0.34 ng/mL and it was 0.51 ng/mL for TT4. Two samples were serially diluted in the assay buffer and their displacement curves were parallel to the standard curve.

2.5. Statistical analyses

Statistical tests were performed using R 3.3.1 (R Core Team, 2016). Relationships between continuous variables were tested by the Pearson correlation test. The influence of contaminant levels on MR were investigated with linear models. Biologically relevant models were constructed by incorporating one contaminant and its interaction with the sex when possible. The best models were selected based on the bias-adjusted Akaïke's Information Criterion (AICc), which is a small-sample size bias adjustment (Burnham and Anderson, 2002). As a general guideline, if AICc values differ by more than 2, the lowest AICc is the most accurate, whereas models with AICc values differing by less than 2 have a similar level of support in their ability to describe the data. Additionally, the Akaike weight (*Wi*) was estimated and can be interpreted as the approximate probability that the model *i* is the best one for the observed data, given the candidate set of models (Burnham and Anderson, 2002; Johnson and Omland, 2004). Diagnostic plots were finally assessed to test whether the data sufficiently met the assumption of the linear model and data were log-transformed when necessary (Zuur et al., 2009). A significance level of $\alpha < 0.05$ was used in this study.

3. Results

3.1. OCs, PFASs and Hg

OCs, PFASs and Hg mean concentrations ± standard errors and limits of detection (LODs) in female and male chick-rearing adult kittiwakes are listed in Table 1. OC concentrations were not related to sex ($p \geq 0.245$ for all tests; Table 1) whereas Hg and all PFASs except PFNA and PFTrA significantly differed between sexes, with males

Table 1
OCs (ng/ mL ww), PFASs (ng/ mL ww) and Hg (μg/g dw) mean concentrations ± standard errors and limits of detection (LODs) of female and male chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. OCs have been measured in whole blood, PFASs in plasma and Hg in red blood cells.

		Males (n = 22)			Females (n = 22)				
	LODs	Mean ± SE	Median	Range	Mean ± SE	Median	Range	$F_{1,42}$	P-value
OCs									
ΣPCBs[a]	$18.5\ 10^{-3}$	82.616 ± 7.385	75.618	[23.911; 169.216]	71.176 ± 6.778	59.342	[30.878; 137.262]	1.351	0.252
ΣCHLs[b]	$2.7\ 10^{-3}$	4.585 ± 0.314	4.651	[2.147; 7.091]	4.773 ± 0.470	4.781	[2.082; 10.032]	0.002	0.970
HCB[c]	$0.2\ 10^{-3}$	5.409 ± 0.271	5.280	[3.160; 7.370]	5.491 ± 0.329	5.466	[3.280; 10.000]	0.037	0.848
β-HCH[d]	$53.5\ 10^{-3}$	0.274 ± 0.030	0.251	[0.080; 0.636]	0.331 ± 0.046	0.324	[0.130; 1.190]	1.393	0.245
p,p′-DDE[e]	$23.2\ 10^{-3}$	8.377 ± 2.009	5.960	[1.430; 44.700]	6.216 ± 0.818	6.121	[0.832; 16.000]	0.241	0.626
PFASs									
PFOSlin[f]	$704\ 10^{-3}$	10.847 ± 0.574	10.352	[6.755; 15.191]	8.922 ± 0.676	8.108	[4.440; 14.394]	5.813	**0.020**
PFNA[g]	$40.9\ 10^{-3}$	1.210 ± 0.099	1.210	[0.478; 2.593]	1.081 ± 0.144	0.902	[0.383; 3.047]	1.926	0.173
PFDcA[h]	$61.9\ 10^{-3}$	2.193 ± 0.120	2.269	[1.104; 3.123]	1.625 ± 0.122	1.410	[0.886; 2.894]	11.000	**0.002**
PFUnA[i]	$83\ 10^{-3}$	12.110 ± 0.641	12.110	[6.487; 17.546]	9.383 ± 0.688	8.390	[5.499; 17.313	8.402	**0.006**
PFDoA[j]	$109\ 10^{-3}$	2.541 ± 0.136	2.468	[1.394; 3.815]	1.995 ± 0.160	1.821	[0.926; 4.015]	8.744	**0.005**
PFTrA[k]	$360\ 10^{-3}$	11.618 ± 1.410	9.819	[2.780; 23.055]	9.675 ± 1.521	6.650	[2.711; 29.735]	1.574	0.217
Trace elements									
Hg[l]	$5\ 10^{-3}$	1.14 ± 0.07	1.099	[0.646; 1.771]	0.89 ± 0.05	0.871	[0.505; 1.641]	7.666	**0.008**

Significant p-values are in bold.
[a] ΣPCBs (ΣPolychlorinated biphenyls): CB-28, 99, 105, 118, 138, 153, 180, 183, 187, 194.
[b] ΣCHLs (ΣChlordanes): Oxychlordane, *cis*-nonachlor and *trans*-nonachlor.
[c] HCB: Hexachlorobenzene.
[d] β-HCH: β-hexachlorocyclohexane.
[e] *p,p′*-DDE: Dichlorodiphenyldichloroethylene.
[f] PFOSlin: Perfluorooctane sulfonate.
[g] PFNA: Perfluorononanoate.
[h] PFDcA: Perfluorodecanoate.
[i] PFUnA: Perfluoroundecanoate.
[j] PFDoA: Perfluorododecanoate.
[k] PFTrA: Perfluorotridecanoate.
[l] Hg: Mercury.

having higher concentrations than females (Table 1). Such sex-related differences of PFAS and Hg concentrations could be attributed to the ability of females to transfer elevated amounts of contaminants into their eggs (Becker, 1992; Gebbink et al., 2012; Helgason et al., 2011). Because of the sex-related differences for PFAS and Hg concentrations, including the factor "sex" and the variables PFAS or Hg within the same model could induce multicollinearity problems and lead to biased results (Graham, 2003). Consequently, sexes were analyzed separately in further statistical analyses for Hg and PFASs only.

3.2. MR and total THs

Body mass and MR mean concentrations ± standard errors for male and female chick-rearing adult kittiwakes are presented in Table 2. Since body mass was positively related to non-mass-specific MR ($F_{1,21}$ = 17.01; p < 0.001) and since males were significantly heavier than females (Table 2), non-mass-specific MR tended to be higher in males than in females (Table 2). By contrast, mass-specific MR did not differ between sexes (Table 2) and is not related to body mass ($F_{1,21}$ = 0.99; p = 0.330). Consequently, mass-specific MR ("MR" hereafter) was used in further statistical analyses in order to control for the effect of body mass on MR.

Table 2
Body mass (g), non-mass-specific MR (mL O_2/h), mass-specific MR (mL O_2/g·h), TT3 and TT4 (ng/ mL) mean concentrations ± standard errors for female and male chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. TT3 and TT4 have been measured in plasma.

	Males	Females			
	Mean ± SE	Mean ± SE	*df*	*F*	P-value
Body mass	388.75 ± 4.18	342.08 ± 6.73	1,22	34.72	< **0.001**
Non-mass-specific MR	576.03 ± 24.15	517.21 ± 17.89	1,21	3.831	0.064
Mass-specific MR	1.57 ± 0.05	1.57 ± 0.04	1,21	0.012	0.916
Total T3	2.29 ± 0.25	2.24 ± 0.17	1,33	0.036	0.850
Total T4	17.39 ± 1.57	22.35 ± 1.91	1,31	3.808	0.060

Significant p-values are in bold.

Because some MR measurements were made at T_a exceeding 20 °C, we investigated the effect that such T_a might have. Although T_b was dependent on T_a (R^2 = 0.26, p = 0.009), only one bird had a body temperature exceeding the mean ± 1 sd (> 40.5 ± 0.7 °C). However, we reanalysed all MR tests below, removing any birds whose T_a exceeded 20 °C and we found no change in results. More important was an effect on MR directly. We specifically examined the MR of all birds measured above 20 °C. Three of those birds had MRs above the mean ± 1 sd (> 1.57 ± 0.16 mL O_2/g h). We removed those birds from analyses and again found no differences from the entire data set. Moreover, there does not appear to be relationships between T_a and MR within the whole data set (R^2 = 0.03, p = 0.43, n = 23), nor within individuals above 20 °C (R^2 = < 0.001, p = 0.98, n = 7). Finally, T_b was not related to MR within the whole data set (R^2 = 0.044, p = 0.35). This partly excludes a potential effect of T_a and T_b on metabolic activity in this study and we believe that MR of those individuals closely approximates their BMR. We consequently kept all oxygen measurements in our data set for the statistical analyses below.

Mean concentrations ± standard errors of TT3 and TT4 levels are reported in Table 2. Although TT4 tended to be higher in males than in females, total TH concentrations did not differ between sexes (Table 2). While TT3 was significantly and positively correlated with TT4 when both sexes were pooled (r = 0.46; p = 0.006), no significant correlations were found between total THs and BMR (TT3: r = 0.16; p = 0.530 and TT4: r = 0.38; p = 0.118).

Finally, since BMR is potentially related to individual quality

Table 3
AICc model selection to explain MR variations based on OC concentrations in female and male chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. OCs have been measured in whole blood. Sexes are pooled (n = 23, 12 males and 11 females).

Models[a]	AICc	ΔAICc[b]	*Wi*[c]
ΣCHLs	−21.23	0.00	0.47
ΣCHLs: sex	−18.67	2.56	0.13
ΣPCBs	−18.55	2.68	0.12
HCB	−17.44	3.78	0.07
p,p′-DDE	−17.10	4.13	0.06
null	−16.87	4.36	0.05

Abbreviations: AICc, bias-adjusted Akaike's Information Criteria values; Wi, AICc weights.
[a] Only the five best ranked and the null models are presented.
[b] Scaled ΔAICc; ΔAICc = 0 is interpreted as the best fit to the data among the models.
[c] Weight of evidence interpreted as a proportion. Weights across all models sum to 1.00.

(Blackmer et al., 2005; reviewed in Burton et al., 2011), we checked for a potential confounding effect of the reproductive investment of the kittiwakes (i.e. the number of raised chicks at the time of sampling) on MR and TH levels. However, we did not find any significant relationships between the number of raised chicks at the time of sampling (i.e. 1 or 2 chick size clutch) and MR ($F_{1,20}$ = 1.62; p = 0.22), nor the TH levels (TT3: $F_{1,31}$ = 0.06; p = 0.80, TT4: $F_{1,29}$ = 1.92; p = 0.18).

3.3. Relationships between contaminants, MR and total THs

The AICc model selection to explain MR variations based on OC levels for male and female chick-rearing adult kittiwakes is presented in Table 3. Among the OCs considered in this study, the model including ΣCHLs as an explanatory variable of MR showed the best fit to the data (Table 3). We observed a significant and negative relationship between ΣCHLs and MR in both sexes (Fig. 1; slope = $-4.05*10^{-5}$; r = −0.51; p = 0.012). Additionally, ΣCHLs was negatively and significantly related to circulating TT3 (Fig. 2; r = −0.38; p = 0.027) but not to TT4 concentrations (r = −0.14; p = 0.423). AICc model selection to explain MR variations based on PFASs and Hg levels is presented in Table 4. Considering all the PFASs investigated in the present study, only PFTrA significantly explained MR variations in females, but it did not in males where no valuable models were retained (Table 4). We found a significant and positive relationship between PFTrA and MR (Fig. 3; slope = $7.61*10^{-5}$; r = 0.75; p = 0.008), however, PFTrA and total TH concentrations were not related (TT3: r = 0.32; p = 0.175 and TT4: r = 0.08; p = 0.742). Finally, Hg was not related to MR neither in

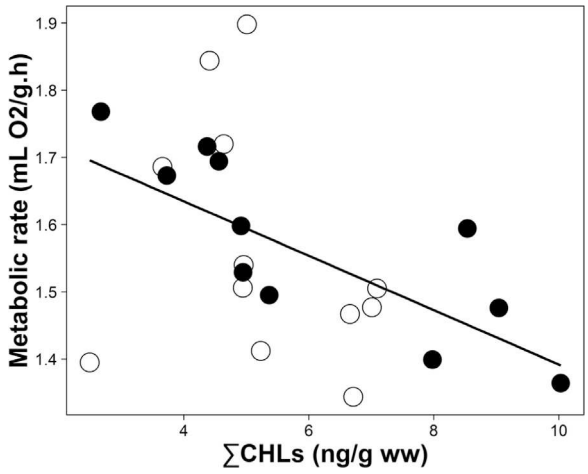

Fig. 1. Relationship between ΣCHLs concentrations and MR in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Males (n = 12) are represented with empty circles and females (n = 11) with filled circles. CHLs have been measured in whole blood.

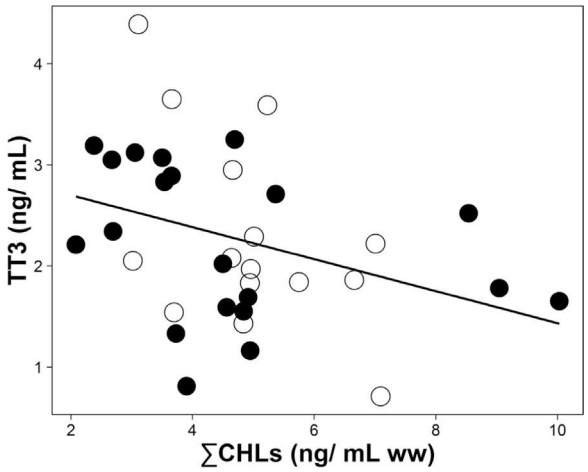

Fig. 2. Relationship between ΣCHLs concentrations and TT3 in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Males (n = 15) are represented with empty circles and females (n = 20) with filled circles. CHLs have been measured in whole blood and TT3 in plasma.

Table 4
AICc model selection to explain MR variations based on PFASs and Hg concentrations in female and male chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. PFASs have been measured in plasma and Hg in red blood cells. Sexes are analyzed separately.

Models[a]	AICc	ΔAICc[b]	*Wi*[c]
Males (n = 12)			
null	−2.91	0.00	0.45
PFNA	0.22	3.13	0.09
PFOSlin	0.33	3.23	0.09
PFDcA	0.45	3.36	0.08
PFTrA	0.65	3.57	0.08
PFDoA	0.74	3.64	0.07
Females (n = 11)			
PFTrA	−13.93	0.00	0.61
PFOSlin	−10.75	3.18	0.12
PFUnA	−9.52	4.41	0.07
PFDcA	−9.22	4.71	0.06
Hg	−8.84	5.09	0.05
null	−8.80	5.13	0.05

Abbreviations: AICc, bias-adjusted Akaike's Information Criteria values; *Wi,* AICc weights.
[a] Only the five best ranked and the null models are presented.
[b] Scaled ΔAICc; ΔAICc = 0 is interpreted as the best fit to the data among the models.
[c] Weight of evidence interpreted as a proportion. Weights across all models sum to 1.00.

males nor in females (Table 4). The two explanatory variables, ΣCHLs and PFTrA were significantly and negatively related (r = −0.51; p = 0.015) in females. In other words, individuals with high concentrations of ΣCHLs had low PFTrA levels, and conversely.

4. Discussion

Our results show that among all tested contaminants, ΣCHLs in both adult males and females (sexes are pooled), and PFTrA only in adult females were the best predictors to explain MR variations in adult breeding kittiwakes, although in opposite ways. We found a significant and negative relationship between ΣCHLs and MR in both sexes whereas PFTrA and MR were significantly and positively related in females only. This suggests a contrasted effect of organochlorine pesticides and PFASs on MR variation, with ΣCHLs possibly inducing a decrease in MR and PFTrA possibly leading to an increase in basal metabolic activity. Additionally, the present study suggests some possible underlying mechanisms linking ΣCHLs and MR in kittiwakes since we reported a significant and negative relationship between ΣCHLs and TT3 levels in both sexes.

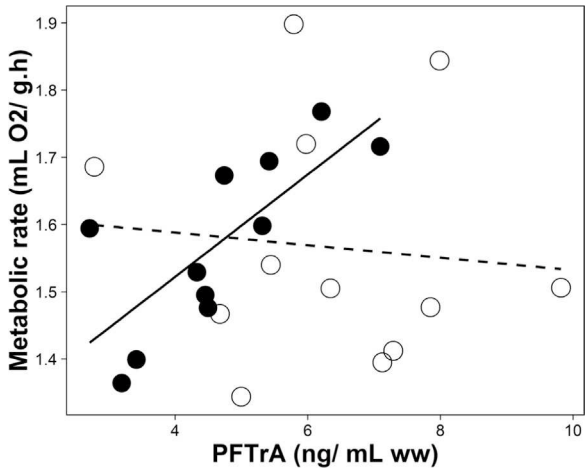

Fig. 3. Relationships between PFTrA concentrations and MR in chick-rearing adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Males (n = 12) are represented with empty circles and dashed line and females (n = 11) with filled circles and solid line. PFTrA has been measured in plasma.

In Svalbard, kittiwakes are exposed to a complex mixture of potential harmful halogenated compounds but only ΣCHLs and PFTrA appear to disrupt their basal energy expenditure. Yet, concentrations of ΣCHLs and PFTrA represent only a small proportion (5% and 29%, respectively) of the total concentrations of ΣOCs and ΣPFASs in our kittiwakes. Consequently, this study highlights a metabolic disruption of those specific contaminants despite their relatively small proportion compared to other more abundant compounds which are not related to MR (e.g. ΣPCBs: 81% of ΣOCs).

4.1. Relationships between contaminants and MR

Effects of contaminants on metabolic activity are still poorly investigated and are largely debated in literature. Thus, this study partly fills the gap of knowledge about the effects of environmental contaminants on energy expenditure in endotherms. Although some experimental designs conducted in adult mammals reported conflicting observations with increased (Braham and Neal, 1974; Voltura and French, 2000) or unchanged (French et al., 2001; Gordon et al., 1995) BMR in response to OCs exposure (PCBs, DDTs and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin), other studies conducted in wild birds corroborate our results. Indeed, decreased metabolic rate was observed in captive doves and pigeons exposed to Aroclor 1254 and DDT, respectively (Jefferies and French, 1971; Tori and Mayer, 1981). Similar to our findings, a study conducted on free-living Arctic-breeding glaucous gulls revealed negative associations between BMR and concentrations of PCBs, DDTs, and especially CHLs (Verreault et al., 2007). Finally, two previous studies conducted in humans also support our findings since they highlighted lower metabolic activity in response to increasing plasma concentration of OCs mixture (including CHLs; Pelletier et al., 2002; Tremblay et al., 2004). Consequently, this study in combination with previous works points-out a possible negative effect of CHLs on BMR.

To date and to the best of our knowledge, our study is the first one to investigate relationships between PFASs and MR in vertebrates. Therefore, no comparisons with previous studies are possible and our findings can only suggest a possible positive effect of PFTrA on MR in female kittiwakes. Regarding the contrasted observations of contaminant effects on metabolic activity reported here and the discrepancy in the results between studies previously conducted, the effects of environmental contaminants on BMR appear to be complex. Such differences could be related to dissimilarities in methodological designs which are known to affect BMR values (Ellis and Gabrielsen, 2002). Moreover, it cannot be excluded that BMR variation in response to contaminant exposure is dependent on the taxa considered (e.g. mammals vs birds; Peakall, 1992) and tightly linked to the level of contamination (dose-dependence effect). Finally, since OCs and PFASs are structurally opposed, with OCs being lipophilic (Frindlay and Defretas, 1971) and PFASs having a high affinity for proteins (Giesy and Kannan, 2002), further investigations focusing on structurally different chemicals may enable to clarify some of our results.

4.2. BMR and THs

A hormonal cascade along the hypothalamic-pituitary-thyroid axis will lead to the production and release in blood of T4 from the thyroid gland. The transport of T4 will then be ensured by serum binding proteins such as albumin and transthyretin (TTR). T4 is then converted by peripheral deiodination to T3 under the control of deiodinase enzymes (McNabb, 2007; Silvestri et al., 2005). Although the detailed pathways of how THs can affect energy expenditure are still unclear (Hulbert, 2000), it is now well established under laboratory conditions that plasma concentrations of THs increase the BMR of birds and mammals (Hulbert, 2000; Kim, 2008; Silvestri et al., 2005). This is particularly true for T3, which is considered as the primary metabolically active THs controlling BMR (Bobek et al., 1977; but see Silvestri et al., 2005). Besides, several studies conducted in domestic as well as in free-living animals, including kittiwakes from the same Svalbard population as in the present study, have highlighted strong and positive associations between TT3 concentrations and BMR (Bobek et al., 1977; Chastel et al., 2003; Elliott et al., 2013; Vézina et al., 2009; Welcker et al., 2013; Zheng et al., 2013). Thus, one possible underlying mechanism that could explain relationships between contaminants and BMR in kittiwakes could be induced by endocrine-disrupting properties of those chemicals. MR and TT3 concentrations were not related in our study, nevertheless we only measured total THs and not the metabolically active free fraction of THs. Furthermore, since MR in the present study was measured few hours after blood sampling used for total THs quantification, plasma THs at the time of MR measurement might be slightly different (Verreault et al., 2007; but see Welcker et al., 2013).

4.3. Relationships between contaminants and total THs

Within the past decades, a wide body of evidence suggested a possible disruption of THs in response to OCs exposure in vertebrates and several studies conducted in free-living animals have reported reduced TH concentrations with increasing OC levels (Braathen et al., 2004; Brouwer et al., 1998; Cesh et al., 2010; Dawson, 2000; Jenssen, 2006; McNabb, 2005; McNabb and Fox, 2003; Peakall, 1992; Rolland, 2000; Scanes and McNabb, 2003). For example, a previous study conducted in another Arctic seabird, the glaucous gull, stated that among 18 different congeners, oxychlordane (included here in ΣCHLs) and HCB appeared to be the most prominent OCs in terms of their negative effect on the variation of the TT4:TT3 ratio (Verreault et al., 2004). Moreover, Smits and Fernie (2013) showed negative relationships between plasma total THs (TT3 and TT4) and several organohalogenated contaminants, including CHLs, in peregrine falcon nestlings (*Falco peregrinus*). Similarly, an experimental study conducted in American kestrels (*Falco sparverius*) reported depressed TT3 levels in PCB-exposed birds (Smits et al., 2002). Here, we reported a significant negative relationship between ΣCHLs and TT3 but not with TT4, while TT3 and TT4 were significantly and positively related. This suggests that ΣCHLs could possibly alter circulating TH levels and specifically T3 levels by affecting either the conversion of T4 to T3 and/ or the transport of circulating T3. Indeed, inhibition of mono-deiodinase activity can skew the concentration of the biologically active T3 (review in Brouwer et al., 1998). This has been reported in white Leghorn chick (*Gallus gallus*) embryos, where administration of PCB mixtures induced decreased hepatic deiodinase activities (Gould et al.,

1999). Therefore, our study highlights the potential endocrine disrupting properties of CHLs on circulating TH levels in an Arctic seabird.

To date, the effects of PFASs on THs have received much less attention and are somewhat contradictory (review in DeWitt, 2015). While some studies conducted in murine and primate models indicated negative associations between PFASs and TH levels (Lau et al., 2003; Seacat et al., 2002), Liu et al. (2011) reported increased TT3 levels in response to experimental PFASs exposure in the zebrafish (*Danio rerio*). Additionally, recent studies conducted on Arctic seabirds, the glaucous gull (Haugerud, 2011; Melnes, 2014), the northern fulmar (*Fulmarus glacialis*; Braune et al., 2011; Nøst et al., 2012), the Arctic skua (*Stercorarius parasiticus*), and kittiwakes (Ask, 2015; Nøst et al., 2012) have shown significant and positive associations between PFASs and total TH levels. Besides, the study of Ask (2015) was conducted on the same population as ours and indicated a positive relationship between PFTrA and TT3 only in females. This could indicate a hormetic response of the thyroid hormone system to PFASs exposure, but underlying mechanisms are still unclear and need to be further investigated (e.g. Videla, 2010). Firstly, it has been proposed that PFASs increase the expression of transcripts of hepatic transporters (e.g. OAPT2) in rats, which in turn, increases uptake of THs into the liver (Yu et al., 2011). Another disruptive effect of PFASs is displacing THs from binding proteins (Mortensen, 2015; Weiss et al., 2009). Finally, PFASs could act directly on the thyroid gland itself (Coperchini et al., 2015). Although there are several reports showing a positive association between PFASs and total TH levels in seabirds, we did not observe a relationship between PFTrA and TT3. Therefore, our study does not confirm the potential thyroid-disrupting properties of PFTrA previously reported in Arctic seabirds and further investigations conducted on a larger sample size in combination with experimental dose-dependence effect of PFASs on TH levels are needed.

4.4. Possible consequences on individual fitness

Individual BMR variations may influence fitness because self-maintenance and reproduction are considered as two key life-history components (review in Burton et al., 2011; Stearns, 1992). In female kittiwakes, adaptive decrease in mass-specific BMR prior to hatching has been suggested as a mean to reduce self-maintenance and to increase chick food provisioning (Bech et al., 2002; Rønning et al., 2008). However, energetic balance is a sensitive system and additional cost from CHLs expressed here as a reduction of BMR could have some consequences on fitness. In that case, self-maintenance could be impacted, leading individuals to be less able to cope with reproduction. Indeed, two recent studies conducted in the same kittiwake population reported decreased adult survival rate, reduced telomere length (a predictor of survival), and lower breeding probability (Blévin et al., 2016; Goutte et al., 2015) in response to CHLs contamination. Despite some inconstancies, this has already been demonstrated experimentally and under field conditions in mammals that individuals with lower BMR tend to survive less (Burton et al., 2011; Speakman et al., 2004; but see Burton et al., 2011). By contrast, exposure to PFTrA may disrupt the ability of female kittiwakes to adaptively decrease BMR, thus might possibly explain the lower hatching success in most PFASs-contaminated adults (Tartu et al., 2014). In female kittiwakes, the BMR during the incubation is a good predictor of the BMR during the chick-rearing period (Bech et al., 1999). Consequently, it is likely that exposure to PFASs during the incubation period could have significant consequences for the metabolic adjustments required for the chick rearing period. In that case, most PFTrA-contaminated female kittiwakes would allocate more energy for self-maintenance rather than for reproduction. Thus, an indirect positive effect of PFASs exposure might occur on survival rate in adult kittiwakes, at the expense of reproduction. However, this statement needs to be confirmed with capture-mark-recapture (CMR) investigations since the present study did not report a relationship between the number of raised chick and MR.

4.5. Limitations of the study and other potential confounding factors

Given the absence of information in literature about PFAS effects on BMR and because our study did not report any relationships between PFTrA and total THs, we cannot conclude with certainty about effects of PFASs on basal energy expenditure. The positive relationship between PFTrA and MR in female kittiwakes may rely on other potential confounding factors. Indeed, since food ingestion is the main route for PFASs exposure, further investigations focusing on ecological variables directly linked to feeding ecology (e.g. *via* stable isotopes, protein amounts analysis) of kittiwakes should be included as predictors of MR. Additionally, the negative relationship between ΣCHLs and PFTrA in female kittiwakes is a crucial point that could lead to misleading interpretations. Similarly, although this study is considering three different groups of contaminants, kittiwakes are obviously exposed to an additional mixture of chemicals which are not included in this study and which could act on energy expenditure too. Finally, our study was conducted on a limited number of individuals and to fully validate our findings, future investigations focusing on the effects of PFASs on BMR should be conducted experimentally, with a laboratory avian model.

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Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2017.05.022.

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Paper IV

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Organochlorines, perfluoroalkyl substances, mercury and egg incubation temperature in an Arctic seabird: insight from data loggers

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Abstract

In birds, incubation-related behaviors and brood patch formation are influenced by hormonal regulation like prolactin secretion. Brood patch provides efficient heat transfer between the incubating parent and the developing embryo in the egg. Importantly, several environmental contaminants are already known to have adverse effects on avian reproduction. However, relatively little is known about the effect of contaminants on incubation temperature (T_{inc}) for wild birds. By using temperature thermistors placed into artificial eggs, we investigated whether the most contaminated parent birds are less able to provide appropriate egg warming and thus less committed in incubating their clutch. Specifically, we investigated the relationships between three groups of contaminants (organochlorines (OCs), perfluoroalkyl substances (PFASs), and mercury (Hg)) with T_{inc} and also with prolactin concentrations and brood patch size in incubating Arctic black-legged kittiwakes (*Rissa tridactyla*). Our results reveal that among the considered OCs, only blood levels of oxychlordane, the main metabolite of "chlordane", a banned pesticide, were negatively related to the minimum incubation temperature in male kittiwakes. PFAS and Hg levels were unrelated to T_{inc} in kittiwakes. Moreover, our study suggests a possible underlying mechanism since we reported a significant and negative association between blood oxychlordane concentrations and the size of the brood patch in males. Finally, this reduced T_{inc} in the most oxychlordane-contaminated kittiwakes was associated with a lower egg hatching probability.

1. Introduction

Egg incubation is an essential stage in the life history of most bird species because developmental conditions for embryos can have long-term fitness consequences (Lindström 1999; Deeming 2002; Berntsen and Bech 2016). Generally, egg attendance patterns involve different parental behaviors such as egg turning and active egg warming; both being considered as key determinants for embryo viability and egg hatchability (Funk and Forward 1953; Decuypere and Michels 1992; Tona et al. 2005a; Elibol and Brake 2006a). Indeed, maintaining eggs at an optimal temperature during incubation is a complex process (Turner 2002) and critically important for complete embryonic development, improved hatchability, offspring's phenotype, and overall survival (Webb 1987; Feast et al. 1998; Olson et al. 2006; Nilsson et al. 2008; Ardia et al. 2010; Nord and Nilsson 2011, 2016; DuRant et al. 2013; Hepp et al. 2015). In birds, incubation behaviors are strongly influenced by hormonal regulation (Vleck and Vleck 2011). Accordingly, a rise in the secretion of the pituitary hormone, prolactin, during egg-laying in combination with a decrease in sex steroid levels facilitate and maintain incubation-related behaviors (Buntin 1996; Vleck 2002; Sockman et al. 2006; Angelier et al. 2016). Concomitantly, the pectoral skin of incubating birds can become a fleshy, edematous and well-vascularized brood patch, devoid of feathers (Jones 1971; Lea and Klandhorf 2002). During incubation, the brood patch comes into direct contact with the egg to ensure proper heat transfer between a parent and the developing embryo in the egg (Jones 1971).

Conditions required for optimal incubation behaviors have been largely investigated in the poultry industry to maximize egg hatchability of domestic fowl (*Gallus gallus domestica;* New 1957; Meijerhof 1992; Tona et al. 2005b; Elibol and Brake 2006a, 2006b). In contrast, the effects of environmental factors like contaminants (i.e. organic contaminants and trace

elements) on incubation behaviors of free-ranging birds *in natura* remain so far poorly investigated. Yet, several environmental contaminants are already known to have adverse effects on avian reproduction (e.g. Fry 1995; Herring et al. 2010; Tartu et al. 2014; Goutte et al. 2015). Through their structural attributes and mode of action potencies, many of these contaminants can disrupt the endocrine system involved in avian reproduction, including prolactin, sex steroid (e.g. testosterone, estradiol, progesterone) and thyroid (e.g. T3, T4) secretions (Rattner et al. 1984; Tyler et al. 1998; Dawson 2000; Giesy et al. 2003; Verreault et al. 2004, 2006a, 2007, 2008; Tartu et al. 2015a; Melnes et al. 2017). Organic contaminants and trace elements have the potential to alter parental behaviors resulting in poor breeding success. For example, different laboratory and field investigations have shown that exposure to organochlorines or mercury (Hg) can be associated with lowered nest or egg temperatures (Peakall and Peakall 1973; Fox et al. 1978; Verboven et al. 2009a), reduced nest attendance (i.e. longer and more frequent absence from the nest site) (Fox et al. 1978; Bustnes et al. 2001, 2005; Fisher et al. 2006a; Tartu et al. 2015a), prolonged incubation period (McArthur et al. 1983; Kubiak et al. 1989; Fisher et al. 2006a) and decreased nest defense /or increased egg predation (Fox et al. 1978; Fox and Donald 1980; Helberg et al. 2005; Goutte et al. 2018). Such detrimental effects of contaminants on incubation behaviors could induce deleterious effects on hatching success. A previous study conducted on ring doves (*Streptopelia risoria*) reported a lower hatchability of eggs incubated by birds experimentally exposed to high doses of polychlorinated biphenyls (PCBs) (Peakall and Peakall 1973). Similarly, Forster terns (*Sterna forsteri)* had a higher hatching success when eggs laid from organochlorine contaminated birds were incubated by less contaminated surrogate parents (Kubiak et al. 1989).

Polar regions are considered a sink for various environmental contaminants due to atmospheric long-range transport and oceanic currents in combination with a cold climate

(Kurkow and Kallenborn 2000). Given their properties (i.e. high volatility and/or persistence), organic contaminants and trace elements such as Hg can reach isolated areas like the Arctic Ocean. Once deposited in the marine ecosystem, contaminants bioaccumulate in living organisms and can biomagnify along the food webs (Borgå et al. 2001; Wania 2003, 2007; Ariya et al. 2004; Tomy et al. 2004; Haukås et al. 2007; Blévin et al. 2013). Long-lived species like many polar seabirds that occupy high trophic levels are exposed to a greater risk of accumulation and sensitivity to high concentrations of contaminants (Letcher et al. 2010; Elliott and Elliott 2013). Consequently, seabirds are considered as highly relevant biological models to investigate the influence of sub-lethal contaminant exposure on reproductive behaviors like incubation temperature (T_{inc}).

In the Norwegian Arctic, black-legged kittiwakes (*Rissa tridactyla*, hereafter 'kittiwakes'), are chronically exposed to a complex mixture of harmful organic compounds and trace elements, which have already been linked to disruption of reproductive hormones and impaired reproductive performance (Tartu et al. 2013, 2014, 2015b, 2016; Goutte et al. 2015; Blévin et al. 2017). Among such complex mixture of chemicals are (i) Hg, a toxic trace element originating from both anthropogenic and natural sources able to disrupt hormones involved in incubation behaviors such as prolactin (AMAP 2007, 2011; Tartu et al. 2016); (ii) legacy organochlorines (OCs; chlorinated pesticides and PCBs), showing decreasing trends in the Arctic, which have been associated with lower incubation temperatures in an Arctic seabird (Helgason et al. 2008; Verboven et al. 2009a; AMAP 2015; Bustnes et al. 2017); and (iii) the globally increasing poly- and perfluoroalkyl substances (PFASs), widely used as surface-active agents (Kissa 2001), especially the perfluoroalkyl carboxylic acids (PFCAs; Braune and Letcher 2013; AMAP 2015). Despite the few studies that have investigated the effects of OCs and Hg on reproductive behaviors, data are still critically lacking and

importantly, to our knowledge, the consequences of PFASs exposure on incubation behaviours in birds are presently unknown.

Using artificial egg loggers, we investigated whether the most contaminated kittiwakes are less committed in incubating their clutch and less able to provide appropriate egg warming. Embedded in artificial eggs, these loggers can provide almost continuous (every second) and precise recording of incubation behaviors (Shaffer et al. 2014; Kelsey et al. 2016; Clatterbuck et al. 2017; Taylor et al. 2018). Specifically, we examined the relationships between blood levels of three groups of contaminants (OCs, PFASs and Hg) and T_{inc} in a kittiwake population from Svalbard in the Norwegian Arctic. Because prolactin secretion and brood patch formation are involved in the onset and maintenance of avian incubation behaviors and thus, tightly linked to T_{inc}, we also investigated relationships between contaminants, plasma prolactin concentrations and brood patch size as potential underlying mechanisms through which contaminant exposure in kittiwakes may influence T_{inc}. Finally, since T_{inc} is considered as a key for egg hatchability, we explored potential effects of T_{inc} on hatching probability.

2. Material and methods

2.1. Fieldwork area and sampling collection

Fieldwork was carried out from 19 June to 12 July 2015, in a colony of black-legged kittiwakes at Kongsfjorden, Svalbard (78°54'N; 12°13'E). We studied 20 incubating pairs because kittiwakes, like other seabirds, share reproduction duties (i.e. incubation and chick rearing) among sexes. A total of 40 individuals (20 males, 20 females) were captured at their nest with a noose fixed at the top of a 6 m fishing rod. We collected the first blood sample

(~0.5 mL) immediately after capture from the alar vein using a heparinized syringe and a 25-gauge needle to assess baseline prolactin concentrations. A second blood sample (~2 mL) was collected to measure the concentrations of contaminants and to determine the sex of individuals using molecular methods. All birds were weighed to the nearest 5 g with a Pesola spring balance to determine the body mass. Finally, a photograph was collected of the whole right brood patch (Fig.1; Canon EOS 1000D, 100 mm, Canon 2018), with a ruler placed next to the bird in order to calculate its brood patch dimensions using Gimp 2.8 (Gimp 2018). Brood patch size was determined in duplicates (all coefficients of variation $\leq$ 4.06%). Breast feathers were lightly brushed with moistened cotton pad to fully expose the brood patch. All study birds exhibited three brood patches (right: RBP, left: LBP and central: CBP). Thus, to minimize handling time, we only measured the RBP of each bird. The size of the LBP and CBP were measured in 13 individuals in order to check whether the RBP measurement can be used to estimate the size of the others brood patches (LBP, CBP). Before release, each bird was marked with colored spots of a non-permanent dye on the forehead to distinguish each bird from its mate (also dyed with a different color) during subsequent observations from a distance. Blood samples were stored on ice in the field. Aliquots of whole blood, plasma and red blood cells were obtained after centrifugation and then kept frozen at -20°C until subsequent laboratory analyses were performed.

2.2. Egg logger experiment and data processing

All study nests initially contained two natural eggs. However, one of these two egg was collected and replaced by an artificial egg containing a temperature thermistor (as described in Shaffer et al. 2014). Artificial eggs were designed and painted to mimic as much as possible the real egg morphology (similar size and shape, approximate mass; Table 1 in

Supplementary Materials) and coloration pattern of kittiwakes using a non-toxic water-based paint (Fig.1). Data loggers recorded core egg temperature every second with a manufacturer-reported accuracy < 2°C (but testing in the lab in a controlled environment showed the accuracy to be approximately 0.5°C) and precision of 0.125°C based on thermistor component specifications (Shaffer et al. 2014). Subsequent tests were also conducted to verify these parameters using a standard poultry incubator with automatic egg turner (Top hatch Incubator, Brower Equipment, Houghton, IA, USA). Study nests were selected according to their accessibility and to minimize disturbance to the rest of the colony. Collected eggs were candled and all were determined to be fertile. Eggs were further dissected to assess the age of the embryo and for use in other contaminants studies (n = 12). To control for potential changes in incubation behaviour that may have occurred across the incubation period, we used the embryo age as a proxy of incubation stage. However, we do not report here any suggested effects of the age of embryo on T_{inc} parameters (Linear mixed effect models (LMMs); T_{min}: $F_{1,10} = 0.14$, $p = 0.72$; T_{max}: $F_{1,10} = 1.13$, $p = 0.31$; T_{mean}: $F_{1,10} = 0.61$, $p = 0.45$; T_{modal}: $F_{1,10} = 1.03$, $p = 0.33$).

Artificial eggs were deployed for 7 and 10 days during the incubation period and all birds readily accepted the artificial egg and exhibited no abnormal incubation behaviours. All loggers recorded data for the entire duration of deployment in the nest. Because each partner of a pair was dye marked on the forehead, we could determine some incubation bouts of each partner at a nest using a spotting scope. Thus, we recorded and kept for further statistical analyses all incubation bouts when we knew which bird was incubating (excluding data recorded at night because checks were not conducted at night). The day of egg deployments and all records during our presence in the colony (i.e. for blood sampling) were also excluded from the data set in order to avoid any biased data. Recording duration (19.83 ± 9.38 (SD) hours, ranging from 4.64 to 43.07 hours) did not influence T_{inc} parameters (LMMs, all $p \geq$

0.169). Upon completion of each deployment, artificial eggs were removed and only one egg was left in the nest. Using a mirror at the end a long pole, we then regularly checked the experimental nest contents to monitor hatching success of the remaining egg until the end of the field season (i.e. 12th July; laying peak from 6th to 9th July).

Logger temperature data were processed using purpose-built routines in MATLAB (The Mathworks, Natick, MA, USA) following methods of Shaffer et al. (2014). Overall, we processed T_{inc} profiles of 40 individuals (Fig.1. in Supplementary Materials) and determine extreme temperature values (minimum temperature: T_{min}; maximum temperature: T_{max}), mean temperature (T_{mean}) and the most frequent incubation temperature within the record period (T_{modal}).

2.3. Contaminant analyses

OCs were analyzed from whole blood at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. We scanned for the following compounds: the organochlorine pesticides (*o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE, *o,p'*-DDE, *o,p'*-DDD, *p,p'*-DDD, HCB, α-, β-, γ-HCH, *trans*-, *cis*-chlordane, oxychlordane, *trans*-, *cis*-nonachlor and mirex) and the polychlorinated biphenyls (CB−28, −52, −99, −101, −105, −118, −138, −153, −180, −183, −187 and −194). Concentrations below the limit of detection (LoD) were assigned by ½ LoD value but only compounds detected in at least 70% of the data set were kept for further statistical analyses. Consequently, compounds remaining for further investigations were the organochlorine pesticides (oxychlordane, *trans*-, *cis*-nonachlor, mirex, HCB and *p,p'*-DDE) and the PCBs (CB−28, −99, −105, −118, −138, −153, −180, −187; expressed here as the ΣPCBs). It is worth noting that *p,p'*-DDE concentrations of 3 males are missing because of injection issues into the GC/ MS. To a whole blood sample of 0.70-1.13 mL, a 100 µL of an

internal standard solution was added (^{13}C-labeled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). We first proceeded to the sample denaturation using a mix of ethanol and saturated solution of ammonium sulphate in water. We then ran extraction twice with 6 mL of n-hexane. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975 CMSD were performed following Herzke et al. (2009). Recovery of the internal standards ranged between 52% and 60%. Results were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference material (1958 human serum from NIST) run every 10 samples. The deviation of the target concentrations in the SRMs were within the laboratory's accepted range (75-111%). All blanks contained concentrations below the instrument detection limits except for HCB (525 pg/ g), PCB-28 (81.8 pg/ g) and -105 (60.8 pg/g).

PFASs were analysed from plasma at NILU. The following compounds were scanned for presence and concentration: perfluorooctanesulfonamide (PFOSA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), linear perfluorooctanesulfonate (PFOSlin), branched perfluorooctanesulfonate (PFOSbr), perfluorodecanesulfonate (PFDcS), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoate (PFUnA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTrA), and perfluorotetradecanoate (PFTeA). Concentrations below LoD were assigned by ½ LoD value but only compounds detected in at least 70% of the data set were kept for further statistical analyses. In short, a sample (0.2 mL) spiked with internal standards (carbon labeled PFAS, Hanssen et al. 2013) was extracted in methanol (1 mL) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVICarb graphitized carbon absorbent and glacial acetic acid. Extracts were analysed by UPLC/MS/MS. Recovery of the internal standards ranged between 74% and 128%. Results

were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference material (1957 human serum from NIST) run every 10 samples. The deviation of the target concentrations in the SRMs were within the laboratory's accepted range (69–130%). All blanks contained concentrations below the instrument detection limits, except for PFCAs, ranging between 5 and 30 pg/ mL.

Total Hg was analyzed at the Littoral Environment et Sociétés laboratory (LIENSs) in La Rochelle, France from freeze-dried and powdered red blood cells placed in an Advanced Hg Analyzer Spectrophotometer (ALTEC AMA 254) as described in Bustamante et al. (2006). Aliquots ranging from 0.44 to 8.59 mg were analysed for each individual, in duplicates (all coefficients of variation $\leq$ 5.42%). Blanks were run at the beginning of each set of samples and certified reference material (CRM; Tort-2 Lobster Hepatopancreas, NRC, Canada; certified value 0.27 ± 0.06 (SD) µg/g dw) were used to validate the accuracy of the analyses. Measured values of the CRM were 0.25 ± 0.01 (SD) µg/g dw, n = 11. All blanks contained concentrations below the instrument detection limit (0.005 µg/g dw).

2.4. Molecular sexing and prolactin assays

Molecular sexing and prolactin assays were conducted at the Centre d'Etudes Biologiques de Chizé (CEBC), France. Kittiwakes were sexed from red blood cells by polymerase chain reaction amplification as part of two highly conserved genes (CHD) present on sexual chromosomes as described in Fridolfsson and Ellegren (1999). Plasma prolactin concentrations were determined by radioimmunoassay as previously described and validated for this kittiwake population (Chastel et al. 2005). Intra-assay (within assay) variation was estimated by including internal standards to the assay. Both samples and internal standards were run in duplicates. CV was 7.13%. Blood collection time (i.e. time elapsed from capture

to the end of the first blood sampling: 2.48 ± 0.52 min (SD), on average) did not affect baseline prolactin concentrations (LMM, $F_{1,19} = 0.606$, $p = 0.446$).

2.5. Statistical analyses

All statistical analyses were performed using R 3.2.3. Linear mixed effect models (LMMs) with the nest identity as a random factor were used to test whether contaminant concentrations, baseline prolactin levels, brood patch size, body mass and T_{inc} differed between sexes. As suggested in Zuur et al. (2009), we used the restricted maximum likelihood estimation (REML) method to avoid any potential biased statistic estimations. Second, we tested the influence of each contaminant concentration on incubation temperatures (T_{min}, T_{max}, T_{mean} and T_{modal}) using linear models (LMs) for each sex separately as males were determined to be more contaminated than females (see Results). Moreover, it is now well established that males and females can react in very different ways to environmental stressors like OCs, PFASs and Hg contamination. Specifically, previous studies conducted on kittiwakes from the same colony reported sex differences regarding effects of contaminants on hormone levels, body condition, breeding decisions, metabolic activity, telomere length and even survival rate (Tartu et al. 2013, 2014, 2016; Goutte et al. 2015; Blévin et al. 2016, 2017). Influence of body mass was also tested since egg temperature is likely warmer as the mass of the incubating bird increases. The best models were selected based on the bias-adjusted Akaïke's Information Criterion (AICc), which is a small sample size adjustment (Burnham and Anderson 2003). As a general guideline, if AICc values differ by more than 2, the lowest AICc is the most accurate, whereas models with AICc values differing by less than 2 have a similar level of support in their ability to describe the data. Additionally, the Akaike weight ($\bar{Wi}$) was estimated and can be interpreted as the approximate probability that the model i is the best

one for the observed data, given the candidate set of models (Burnham and Anderson 2003; Johnson and Omland 2004). Since the concentration of *p,p'-DDE* was missing for 3 males (see Methods) and because model selection based on AICc requires the same number of observations among models, we performed a second run of model selection with removing these 3 individuals from the data set and we found no change in the results. Third, we investigated the relationships between contaminant concentrations, baseline prolactin, brood patch size and body mass with LMs. Finally, we tested whether T_{inc} can affect hatching probability using generalized linear model (GLM) constructed with a "binomial" family and a "cloglog" link function which is consistent with the use of an asymmetric data set (hatched: n = 15; not hatched: n = 5; Zuur et al. 2009). Diagnostic plots and Shapiro normality tests were finally performed on residuals to test whether the data sufficiently met the assumption of the models (i.e. LMM, LM, GLM) and data were log-10 transformed when necessary (Zuur et al. 2009). All data are presented as mean ± SD and we used a significance level of $\alpha < 0.05$.

3. Results

3.1. Sex-related differences

OCs, PFASs and Hg mean concentrations and LODs in female and male incubating adult kittiwakes are listed in Table 1. LMMs with nest identity as a random factor to test sex-related differences indicated that all OCs except *trans*- and *cis*-nonachlor, all PFASs except PFTeA, and Hg concentrations significantly differed between sexes, where males had higher contamination levels than females. LMMS indicated that males incubated the egg at a higher T_{mean} compared to their female partner (LMM, $F_{1,19} = 9.518$, p = 0.006; Fig.2). Mean plasma prolactin concentrations, brood patch size and body mass of female and male incubating adult

kittiwakes are given in Table 2. LMMs with nest identity as a random factor to test sex-related differences indicated no significant differences between sexes for baseline prolactin concentrations, or brood patch size (Table 2). However, as expected, males were significantly heavier than their female partners (Table 2).

3.2. Incubation temperatures and contaminants

According to the model selection, the model including oxychlordane was the best fit model in males (ΔAICc = 5.77; Table 3). Specifically, we observed a negative and highly significant relationship between oxychlordane concentrations in blood and T_{min} in males (LM, slope = $-3*10^{-3}$; p = 0.001; R^2 = 0.45; Fig.3), indicating a lower T_{min} with increasing oxychlordane concentrations. To a lesser extent, both models with HCB or mirex as explanatory variables were also better than the null model (ΔAICc from null model > 2; Table 3). Specifically, we observed a significant negative relationship between blood HCB and mirex concentrations and T_{min} in males (LM, slope = $-1*10^{-3}$; p = 0.023; R^2 = 0.26 for HCB; LM, slope = $-5*10^{-3}$; p = 0.029; R^2 = 0.24 for mirex). PFASs and Hg concentrations were not related to T_{min} in males (Table 3). Finally, model selection also indicated a significant effect of body mass on T_{min} (ΔAICc from null model > 2; Table 3), where heavier males had a higher T_{min} (LM, slope = 0.109; p = 0.021; R^2 = 0.26). It is worth noting that oxychlordane concentrations and body mass were significantly and negatively correlated in males ($r_{pearson}$ = -0.62; p = 0.004; n = 20). Running an additive model including oxychlordane and body mass simultaneously did not improve predictions of T_{min} compared to the model with oxychlordane only (AICc $_{(oxychlordane)}$: 107.08/ AICc $_{(oxychlordane + body\ mass)}$: 109.67). We found no significant relationships between contaminants and body mass on T_{min} in females (Table 3; Fig.3).

The AICc model selection that explained T_{mean} variations based on contaminant concentrations and body mass is presented in Table 4. We found no significant relationships between contaminant concentrations and T_{mean}, in either males, or females. However, the model including body mass was considered as the best predictor in males among the set of candidate models (ΔAICc = 3.65; Table 4), whereas for females no relationship was found. Indeed, there is a significant positive relationship between body mass and T_{mean} in males (LM, slope = 0.049; p = 0.018; R^2 = 0.28).

The AICc model selection that explained T_{max} variations based on contaminant concentrations and body mass is presented in Table 5. There was no significant relationship between contaminant concentrations and T_{max}, either in males, or females. However, the model including body mass was considered as the best predictor in males (ΔAICc = 5.97; Table 5), whereas for females, there was no relationship. There was a significant positive relationship between body mass and T_{max} in males (LM, slope = 0.056; p = 0.006; R^2 = 0.36).

The AICc model selection that explained T_{modal} variations based on contaminant concentrations and body mass is presented in Table 6. There was no significant effect of contaminant concentrations and body mass on T_{modal}, either in males, or females.

3.3. Baseline prolactin, brood patch and contaminants

We observed a relationship between oxychlordane concentrations and T_{min} in males but not in females. Consequently, we examined relationships between oxychlordane concentrations and baseline prolactin levels, and the size of the brood patch to evaluate potential underlying mechanisms. Baseline prolactin levels in males were not significantly related to oxychlordane concentrations (log-10 transformed; LM, slope = -16.21; p = 0.47; Fig.4), to brood patch size (LM, slope = 0.039; p = 0.15), nor to body mass (LM, slope =

0.475; p = 0.07). Baseline prolactin levels in females were not significantly related to oxychlordane concentrations (LM, slope = -6.10^{-3}; p = 0.50), to brood patch size (LM, slope = 0.042; p = 0.23), nor to body mass (LM, slope = 0.044; p = 0.67). However, we found a highly significant negative relationship between oxychlordane concentrations and the size of the brood patch in males but not in females (log-10 transformed; LM, slope = -5.10^{-5}; p = 0.16). Thus, the most oxychlordane contaminated males had the smallest brood patch (LM, slope = $-2*10^{-3}$; p = $2*10^{-4}$; R^2 = 0.53; Fig.4). Body mass and the size of the brood patch were also positively related in males (LM, slope = 0.067; p = 0.029; R^2 = 0.24) but not in females (log-10 transformed; LM, slope = 4.10^{-4}; p = 0.404). Importantly, the size of the brood patch was positively and significantly related to T_{min} in males (LM, slope = 1.178; p = $1*10^{-4}$; R^2 = 0.56; Fig.5).

The size of the LBP and CBP were marginally correlated to the size of the RBP (LBP, $r_{spearman}$ = 0.45; p = 0.13; n = 13 and CBP, $r_{spearman}$ = 0.51; p = 0.078; n =13). We assume that results presented here regarding the RBP could also be relevant for the LBP and CBP.

3.3. Consequences on hatching success

Because there was a relationship between oxychlordane concentrations and T_{min} in males, we evaluated the consequences of T_{min} variations on hatching success. There was a positive and marginally significant relationship between T_{min} and the probability that the remaining egg in the experimental nests successfully hatched (GLM, Z = 1.932; p = 0.053; Fig.6). As a result, the lower T_{min} was, the lower was the hatching success.

4. Discussion

Using temperature thermistors placed into artificial eggs, our results reveal that among the considered OCs, only blood levels of oxychlordane, the main metabolite of the chlorinated pesticides "chlordanes", were negatively related to T_{min} in male kittiwakes. PFAS and Hg levels were unrelated to T_{inc} in kittiwakes. Moreover, our study suggests a possible underlying mechanism between T_{inc} and contaminants since we reported a highly significant and negative association between blood oxychlordane concentrations and the size of the brood patch in males. Such effects on T_{inc} could induce deleterious consequences on egg hatchability.

4.1. Incubation temperature and contaminants

Contaminants such as OCs, PFASs and Hg are ubiquitous and toxic for wildlife. There is now clear evidence about their detrimental effects on the reproductive ecology of birds (e.g. Fry 1995; Herring et al. 2010; Tartu et al. 2014; Goutte et al. 2015). However, little is documented, especially for PFASs, about their potential influence on incubation behaviors and especially on T_{inc}. In the glaucous gull (*Larus hyperboreus*), another polar seabird, a study conducted in Svalbard (Bjørnøya island) showed that ∑PCBs, ∑DDTs and a number of quantitatively minor persistent organic pollutant (POP) classes (total-(α)-HBCD, ∑PBDE, ∑MeO-PBDE, mirex and 3-$MeSO_2$-*p,p'*-DDE) in plasma of incubating birds were negatively correlated with mean nest temperature (Verboven et al. 2009a). Additionally, exposure to ∑PCBs and oxychlordane was found to be associated with reduced nest attendance (i.e. longer and/ or more frequent absences from the nest site during incubation period) in the same species (Bustnes et al. 2001, 2005). Therefore, our results on Svalbard kittiwakes consistently with previous studies seem to highlight some potential associations between some OCs and

their metabolites on T_{inc} in seabird species. However, our results do not report any relationships between PFASs, Hg and T_{inc}. This is supported by a recent investigation by Taylor et al. (2018) where no relationship was found between egg Hg contamination and T_{inc} of Forster terns. Thus, our research contributes to filling the gap in knowledge but additional studies are needed to confirm the generality of our findings in other bird species and importantly, targeting the specific chemicals involved in avian T_{inc} variations.

4.2. What are the possible mechanisms of this relationship?

Incubation is an energy-consuming phase of the avian reproductive cycle (Tinbergen and Williams 2002; Nord et al. 2010; Nord and Nilsson 2012; Nord and Williams 2015) and the efficiency with which heat is transferred from an incubating bird to its egg is related to the energy expenditure of the parent (Gabrielsen and Steen 1979; Gabrielsen and Unander 1987). In other words, a higher metabolic rate increases heat production thereby increasing heat transfer from the parent to embryo, and conversely. Interestingly, lowered thyroid hormone levels and reduced basal metabolic activity have already been observed in the most chlordane-contaminated individuals, including kittiwakes from the same population and glaucous gulls (Verreault et al. 2004, 2007; Blévin et al. 2017; Melnes et al. 2017). In this context, the quantity of heat transferred from parent to eggs might be reduced in the most contaminated birds thus explaining why we observed a negative relationship between oxychlordane concentrations and T_{inc} of male kittiwakes.

Another non-mutually exclusive hypothesis could rely directly on the manner in which heat is transferred. Indeed, because contact between the brood patch and egg ensures heat transfer from parents to embryo (Jones 1971), investigating relationships between contaminants and the size of the brood patch is relevant. In this context, a reduction in size of

the brood patch in the most oxychlordane-contaminated male kittiwakes logically decrease the amount of heat transferred to their eggs. This reasoning is consistent with an experimental study on American kestrels (*Falco sparverius)* where smaller brood patches were observed in males exposed to PCBs compared to controls (Fisher et al. 2006b).

Since incubation behaviors (including brood patch formation) are triggered by an array of different hormones (Buntin 1996; Lea and Klandorf 2002; Vleck 2002; Sockman et al. 2006; Angelier and Chastel 2009; Vleck and Vleck 2011; Lynn 2016) and because of the potential endocrine-disrupting properties of some OCs, reproductive hormones like prolactin could have a key role in explaining why the most oxychlordane-contaminated male kittiwakes exhibited a reduced brood patch and a lowered T_{inc}. However, we did not observe a relationship between prolactin levels and brood patch size, nor to oxychlordane concentrations in male kittiwakes. Several explanations could explain this discrepancy. Firstly, relationships between prolactin and contaminants could be dose-dependent. A previous study on glaucous gulls revealed some negative relationships, although only marginally significant, between blood concentrations of several OCs and plasma prolactin secretions (Verreault et al. 2008). However, levels of chlordanes in glaucous gulls (44.0 ± 7.0 ng/g ww; reported as the sum of heptachlor epoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor and *cis*-nonachlor) were around 28 times higher than those of our kittiwakes (1.569 ± 0.908 ng/g ww; reported as the sum of oxychlordane, *trans*-nonachlor and *cis*-nonachlor). Secondly, the establishment and maintenance of incubation behaviors (including brood patch formation) is orchestrated by a complex cocktail of different reproductive hormones acting synergistically (Buntin 1996; Lea and Klandorf 2002; Vleck 2002; Sockman et al. 2006; Vleck and Vleck 2011; Angelier et al. 2016; Lynn 2016) and further studies focusing on sex steroids (e.g. testosterone, estradiol, progesterone) may provide greater clarity about which endocrine mechanisms are involved in a reduced brood

patch size and lowered T_{inc} in response to oxychlordane contamination. Finally, the timing of blood sampling for prolactin assays could have been conducted too late in the season for comparison to the timing of brood patch formation or the maximum of prolactin secretion. Although brood patch formation is initiated only a few days before egg-laying (Lea and Klandorf 2002), our sampling for prolactin assessment was performed several days after egg-laying. Moreover, it has been suggested that prolactin levels in altricial pelagic seabird species remains high in a relatively steady state throughout incubation and sometimes even during the chick-rearing period, as a strategy to achieve parental care despite parents undertaking prolonged foraging trips at sea (Vleck 1998, 2002; Lormée et al. 2000; Angelier et al. 2016), thus partly excluding this scenario.

4.3. Sex-related differences

Considering each nest separately, our study indicates that male parents generally incubate their eggs at a higher temperature (T_{mean}) compared to their female partners. This observation could rely on a potential difference between sexes regarding energetic expenditure and thus, heat production because males are heavier than females (~8% in the present study). Furthermore, both basal and field metabolic rates have been shown to scale with body mass in kittiwakes from the same colony (Elliott et al. 2013; Welcker et al. 2013; Blévin et al. 2017) and Arctic glaucous gulls (Verreault et al. 2007). Finally, results from the model selection presented in this study indicate a significant contribution of the body mass to several T_{inc} parameters (T_{min}, T_{mean}, T_{max}) in male kittiwakes. Hence, males incubate at a higher temperature than their female partners likely related to difference in body mass.

The relationship between oxychlordane and T_{inc} was sex-dependent and a significant relationship was found in male kittiwakes, but not in females. Interestingly, a previous study

conducted on the glaucous gull showed that males were less able to maintain an optimal nest temperature than females during a costly reproductive event (i.e. induced by clutch enlargement) (Verboven et al. 2009a). This is similar to what was reported in American kestrels, where incubation behaviors of males experimentally exposed to PCBs were more disrupted than that of females of the same treatment (Fisher et al. 2006a). Furthermore, several studies conducted on kittiwakes, snow petrels (*Pagodroma nivea*) and glaucous gulls also reveal a higher susceptibility of males to the effects of contaminant exposure on incubation-related endocrine mechanisms (Verreault et al. 2004, 2006a, 2008; Tartu et al. 2015a, 2016). So, why there is a difference between sexes? Unlike females, males do not have a mechanism to reduce the body burden of contaminants compared to females who can excrete contaminants into their eggs. Indeed, several correlational and experimental studies have shown that females can lower a significant part of their contaminant body burden into their eggs (Becker 1992; Bargar et al. 2001; Drouillard and Nostrom 2001; Verreault et al. 2006b; Verboven et al. 2009b; Gebbink and Letcher 2012; Bustnes et al. 2017). Contaminant levels of incubating males are higher than those reported in females and thus posing a greater challenge for males to cope with costly reproductive tasks.

4.4. What consequences on hatching success?

T_{inc} is critically important for egg hatchability (Funk and Forward 1953; Decuypere and Michels 1992) and several studies have reported a reduced hatching success of eggs incubated at suboptimal temperatures (Webb 1987; Feast et al. 1998; Deeming and Ferguson 1991; French 2000; Moraes et al. 2004; Mortola 2006; Nord and Nilsson 2011, 2012; DuRant et al. 2013). The reduced T_{inc} reported here in the most contaminated kittiwakes could impair hatchability by decreasing hatching probability. However, we cannot completely rule-out

another possible non-mutually exclusive hypothesis which relies on a delay of hatching in response to low T_{inc} events. Although kittiwakes displayed a high synchrony in the date of hatching (Mehlum 2006), our fieldwork was completed within a few days after the peak lay date (around 5 days) so it is conceivable that some eggs we considered to be non-viable in fact hatched soon after we stopped monitoring nest contents. This is entirely consistent with previous investigations showing an extended incubation period in eggs incubated below the optimal temperature range (Webb 1987; Deeming and Ferguson 1991; Feast et al. 1998; Martin 2002; Mortola 2006; Martin et al. 2007; Ardia et al. 2010; Nord and Nilsson 2011, 2012; DuRant et al. 2013). An experimental study on wood ducks (*Aix sponsa*) revealed that low T_{inc} resulted in prolonged incubation periods and lower hatching success (Hepp et al. 2006). Even though further investigations are needed, we assume that a reduced T_{min} in the most oxychlordane-contaminated kittiwakes could *in fine*, impair egg hatchability, either by lengthening incubation period and/ or reducing hatching success.

4.5. Limitations of the study and other potential confounding factors

Our study was conducted on a limited sample size and the reported relationships, although statistically significant, appear to be partly influenced by one individual with a fairly strong relative statistical power (Cook's distance > 1; indicated with an arrow in Fig. 3, 4, 5 and further discussed in Supplementary Materials). However, after removing this bird from the data set, we found similar results (see Supplementary Materials). In addition, there was no valid reason to discard this bird from the data set. Hence, this male kittiwake was the most oxychlordane-contaminated bird of our study. It had the smallest brood patch, exhibited the lowest T_{inc}, failed at hatching, and was observed several times standing on the nest instead of incubating its eggs. Finally, when applying the outlier test of Bonferroni (Hay-Jahans 2011;

Fox 2016), this individual was not considered as an outlier in our data set. Nevertheless, we have to be cautious with our findings and further investigations using a larger sample size of individuals will yield a wider range of contamination levels and thus, will certainly help to confirm or refute the reported relationships.

Among the different T_{inc} parameters considered in this study, only T_{min} was related to contaminant levels. One possible explanation is about the duration of recording periods (19.83 ± 9.38 (SD) hours, ranging from 4.64 to 43.07 hours). A longer duration for each record would ultimately result in more extreme temperature variations including low T_{inc} events that have a stronger impact on T_{mean}. In this case, it would be possible, *in fine*, to find relationships between contaminants and T_{mean}. Nevertheless, our study highlights the importance of focusing on several T_{inc} parameters (such as extreme values) for detecting any subtle effects.

One aspect that is a potential confounding effect is that of body mass which is suggested to positively affect several T_{inc} parameters in males. Body mass and oxychlordane concentrations are negatively related in male kittiwakes. Previous research shows that birds (including kittiwakes), with high OC burdens generally have poor body condition and are lighter in mass than birds with low OC levels (Henriksen 1995; Henriksen et al. 1998, 2000; Helberg et al. 2005; Bustnes et al. 2017). When body mass decreases, the lipophilic OCs such as oxychlordane, previously stored in adipose tissues, are released into the blood circulation and become very toxic to the whole organism (Henriksen 1995; Borgå et al. 2007; Nøst et al. 2012; Routti et al. 2013). It is thus difficult to disentangle a potential confounding effect of body mass or a real impact of contaminants on T_{inc}.

Finally, being a metabolite itself, oxychlordane might not be the direct link in the mechanistic processes, rather than the parent compounds ("chlordanes") which cannot be measured with our sampling design, since they would be metabolized at time of sampling.

Also, the metabolization process itself might be playing a role explaining our observations. However, a causal order of the mechanistic relationships cannot be established here.

5. Conclusion

Chlordane has been listed as a legacy POP by the Stockholm convention since 2004. Its usage as a pesticide was extensive for more than 35 years but decreased in the 1980's (US Department of Health and Human Services 1994). Oxychlordane (primary metabolite of "chlordanes") is considered extremely toxic for wildlife (Wiemeyer 1996; Bondy et al. 2003; Bustnes 2006; Erikstad et al. 2013). Indeed, recent studies reported potential adverse effects of this chemical on thyroid hormones, energy expenditure, nest attendance, reproductive outputs, immune function, morphological traits, telomere length and even survival rate in different seabird species (Bustnes et al. 2002, 2003, 2004, 2005; Verreault et al. 2004, 2007, 2010; Bustnes 2006; Blévin et al. 2016, 2017; Erikstad et al. 2013; Goutte et al. 2015). Our study in combination with previous findings highlights the high toxicity of this compound on wildlife despite its relatively small proportion compared to other OCs (< 5% of ΣOCs considered in this study).

Conflict of interest

The authors declare no conflicts of interest.

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Table 1

OCs, PFASs (ng/g ww) and Hg (µg/g dw) mean concentrations ± standard deviation and limits of detection (LODs) for male and female incubating kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Sex-related differences have been tested using linear mixed effects models with nest identity as a random factor. OCs have been measured in whole blood, PFASs in plasma and Hg in red blood cells.

		Males (n = 20)	Females (n = 20)		
	LODs	**Mean ± SD**	**Mean ± SD**	$F_{1,19}$	**P-value**
Organochlorines					
oxychlordane*	$286\ 10^{-3}$	1.431 ± 0.864	0.983 ± 0.318	5.552	**0.029**
trans-nonachlor*	$18.4\ 10^{-3}$	0.078 ± 0.048	0.079 ± 0.069	0.308	0.585
cis-nonachlor*	$17.6\ 10^{-3}$	0.059 ± 0.033	0.03 ± 0.050	0.032	0.861
mirex*	$31.4\ 10^{-3}$	0.790 ± 0.398	0.491 ± 0.219	12.836	**0.002**
HCB[a]*	$525\ 10^{-3}$	3.230 ± 1.486	2.083 ± 0.610	9.629	**0.006**
p,p'-DDE*	$47\ 10^{-3}$	3.781 ± 1.858	2.122 ± 1.272	10.157	**0.006**
∑PCBs[b]*	$166\ 10^{-3}$	25.179 ± 14.725	15.485 ± 6.345	7.451	**0.013**
PFASs					
PFOSlin[c]*	$270.5\ 10^{-3}$	7.330 ± 3.338	2.102 ± 1.028	100.094	**<0.001**
PFNA[d]*	$20.5\ 10^{-3}$	0.949 ± 0.450	0.511 ± 0.233	18.21	**<0.001**
PFDcA[e]	$36.9\ 10^{-3}$	1.207 ± 0.507	0.489 ± 0.228	42.608	**<0.001**
PFUnA[f]*	$88.5\ 10^{-3}$	5.783 ± 1.933	2.911 ± 0.882	58.694	**<0.001**
PFTrA[g]*	$133.1\ 10^{-3}$	7.367 ± 2.197	2.779 ± 1.200	101.031	**<0.001**
PFTeA[h]	$24.8\ 10^{-3}$	0.497 ± 0.399	0.370 ± 0.305	2.021	0.171
Trace element					
Hg[i]*	$5\ 10^{-3}$	2.004 ± 0.591	1.426 ± 0.377	20.325	**<0.001**

Significant p-values are in bold. (*) indicates a log-10 transformation.
[a] HCB: Hexachlorobenzene
[b] *p,p'*-DDE: Dichlorodiphenyldichloroethylene (17 males)
[b] ∑PCBs (∑Polychlorinated biphenyls): CB-28, -99, -105, -118, -138, -153, -180, -183, -187
[c] PFOSlin: Perfluorooctane sulfonate
[d] PFNA: Perfluorononanoate
[e] PFDcA: Perfluorodecanoate
[f] PFUnA: Perfluoroundecanoate
[g] PFTrA: Perfluorotridecanoate
[h] PFTeA: Perfluorotetradecanoate
[i] Hg: Mercury

Table 2

Plasma baseline prolactin concentrations (ng/ mL), brood patch size (cm^2) and body mass (g) for male and female incubating kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Sex-related differences have been tested using linear mixed effects models with nest identity as a random factor.

	Males (n = 20)	**Females (n = 20)**		
	Mean ± SD	**Mean ± SD**	$F_{1,19}$	**P-value**
Prolactin	94.726 ± 21.915	93.181 ± 10.830	0.084	0.775
Brood patch	12.267 ± 2.565	12.646 ± 1.624	0.313	0.583
Body mass	407.25 ± 18.812	375.75 ± 25.146	34.735	**<0.001**

Significant p-values are in bold.

Table 3

AICc model selection to explain minimum incubation temperature (T_{min}) variations based on OCs, PFASs, Hg concentrations and body mass in male and female kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Effects of contaminants and body mass on T_{min} have been tested using linear models. OCs have been measured in whole blood, PFASs in plasma and Hg in red blood cells.

LMs (T_{min}~)	AICc	ΔAICc[a]	Wi[b]
Males (n = 20)			
oxychlordane	107.08	0.00	0.83
body mass	112.85	5.77	0.05
HCB	113.00	5.92	0.04
mirex	113.42	6.35	0.03
cis-nonachlor	115.57	8.49	0.01
null	116.10	9.02	0.01
Females (n = 20)			
Hg	93.22	0.00	0.16
null	93.47	0.25	0.14
p,p'-DDE	94.47	1.25	0.09
trans-nonachlor	95.03	1.81	0.06
PFTrA	95.18	1.96	0.06
oxychlordane	95.48	2.26	0.05

Abbreviations: AICc, bias-adjusted Akaike's Information Criteria values; *Wi,* AICc weights.
Only the five best ranked and the null models are presented.
(a) Scaled ΔAICc; ΔAICc = 0 is interpreted as the best fit to the data among the models.
(b) Weight of evidence interpreted as a proportion. Weights across all models sum to 1.00.

Table 4

AICc model selection to explain mean incubation temperature (T_{mean}) variations based on OCs, PFASs, Hg concentrations and body mass in male and female kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Effects of contaminants and body mass on T_{mean} have been tested using linear models. OCs have been measured in whole blood, PFASs in plasma and Hg in red blood cells.

LMs (T_{mean}~)	**AICc**	**ΔAICc**[a]	***Wi***[b]
Males (n = 20)			
body mass	79.15	0.00	0.56
null	82.80	3.65	0.09
Hg	84.17	5.02	0.05
HCB	84.63	5.48	0.04
trans-nonachlor	84.92	5.77	0.03
oxychlordane	84.93	5.78	0.03
Females (n = 20)			
null	79.23	0.00	0.18
PFOSlin	80.62	1.39	0.09
Hg	81.22	1.99	0.07
PFNA	81.25	2.02	0.07
PFTrA	81.27	2.04	0.06
p,p'-DDE	81.28	2.05	0.06

Abbreviations: AICc, bias-adjusted Akaike's Information Criteria values; *Wi,* AICc weights.
Only the five best ranked and the null models are presented.
(a) Scaled ΔAICc; ΔAICc = 0 is interpreted as the best fit to the data among the models.
(b) Weight of evidence interpreted as a proportion. Weights across all models sum to 1.00.

Table 5

AICc model selection to explain maximum incubation temperature (T_{max}) variations based on OCs, PFASs, Hg concentrations and body mass in male and female incubating kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Effects of contaminants and body mass on T_{max} have been tested using linear models. OCs have been measured in whole blood, PFASs in plasma and Hg in red blood cells.

LMs (T_{max}~)	AICc	ΔAICc[a]	*Wi*[b]
Males (n = 20)			
body mass	76.78	0.00	0.75
null	82.95	5.97	0.04
ΣPCBs	83.53	6.55	0.03
HCB	83.66	6.68	0.03
trans-nonachlor	83.79	6.81	0.03
mirex	83.85	6.87	0.02
Females (n = 20)			
null	83.69	0.00	0.17
cis-nonachlor	85.24	1.56	0.08
PFOSlin	85.34	1.65	0.08
mirex	85.38	1.69	0.07
PFTrA	85.48	1.79	0.07
PFDcA	86.11	2.42	0.05

Abbreviations: AICc, bias-adjusted Akaike's Information Criteria values; *Wi,* AICc weights.
Only the five best ranked and the null models are presented.
(a) Scaled ΔAICc; ΔAICc = 0 is interpreted as the best fit to the data among the models.
(b) Weight of evidence interpreted as a proportion. Weights across all models sum to 1.00.

Table 6

AICc model selection to explain modal incubation temperature (T_{modal}) variations based on OCs, PFASs, Hg concentrations and body mass in male and female incubating kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Effects of contaminants and body mass on T_{modal} have been tested using linear models. OCs have been measured in whole blood, PFASs in plasma and Hg in red blood cells.

LMs (T_{modal}~)	**AICc**	**ΔAICc**[a]	***Wi***[b]
Males (n = 20)			
mass	84.20	0.00	0.25
null	85.62	1.42	0.12
Hg	85.80	1.60	0.11
trans-nonachlor	86.74	2.54	0.07
oxychlordane	87.10	2.90	0.06
PFTeA	87.23	3.04	0.05
Females (n = 20)			
null	82.78	0.00	0.17
PFNA	84.07	1.29	0.09
PFOSlin	84.20	1.42	0.08
p,p'-DDE	84.47	1.69	0.07
oxychlordane	84.73	1.95	0.06
HCB	84.85	2.07	0.06

Abbreviations: AICc, bias-adjusted Akaike's Information Criteria values; *Wi,* AICc weights.
Only the five best ranked and the null models are presented.
(a) Scaled ΔAICc; ΔAICc = 0 is interpreted as the best fit to the data among the models.
(b) Weight of evidence interpreted as a proportion. Weights across all models sum to 1.00.

Fig.1.

Photograph of the whole right brood patch of an incubating kittiwake (on the left) and deployments of one artificial egg (indicated with an arrow) containing a temperature sensor in a nest of incubating kittiwakes (on the right) *Rissa tridactyla* from Kongsfjorden, Svalbard.

Fig.2.

Mean incubation temperature (T_{mean}) of both partners of adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard in each investigated nest. Solid red lines indicated pairs with males incubating at a higher temperature than females. Dashed black lines indicated pairs with females incubating at a higher than males.

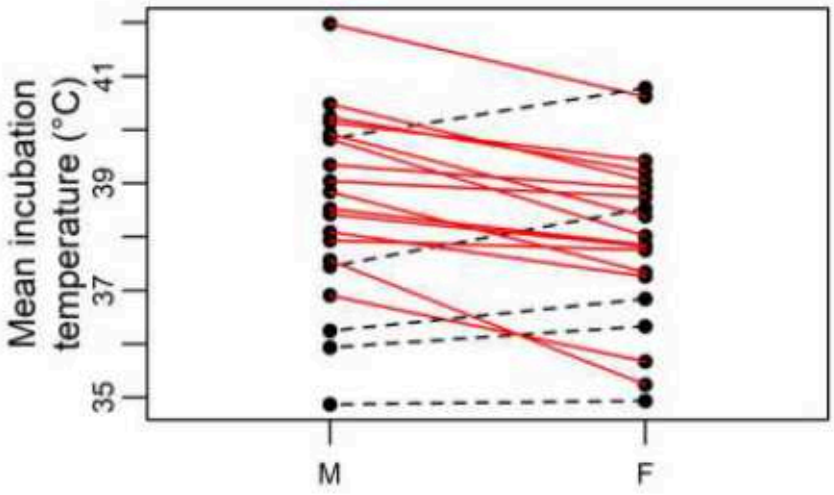


Fig.3.

Relationships between oxychlordane concentrations and the minimum incubation temperature in male and female adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Oxychlordane concentrations have been measured in whole blood. The arrow indicates one individual with a fairly strong relative statistical power (see section 4.5 for more details).

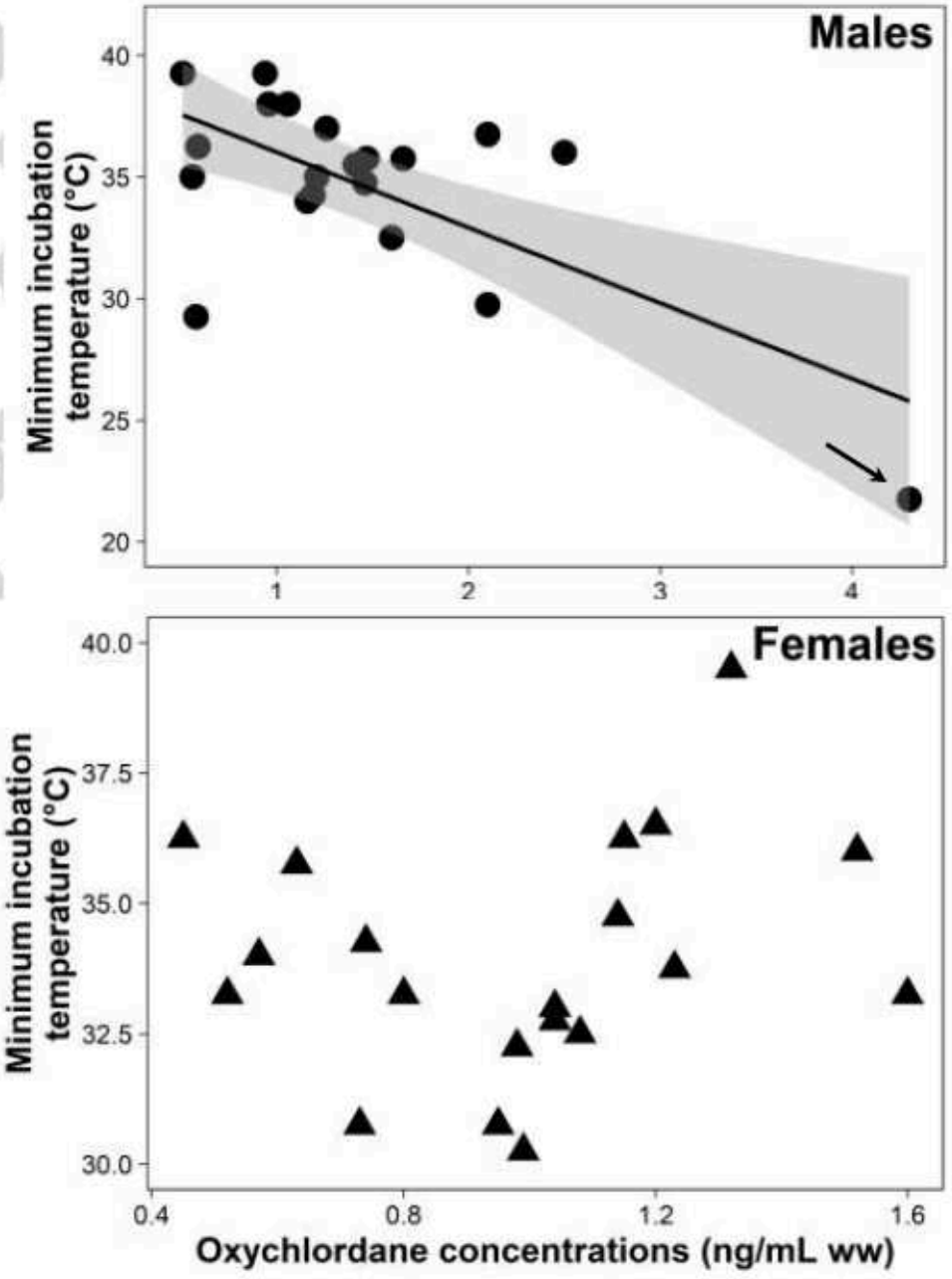


Fig.4.

Relationships between oxychlordane concentrations, baseline prolactin levels and brood patch size in male incubating adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Oxychlordane concentrations have been measured in whole blood and baseline prolactin in plasma. Brood patch size here reflects the size of the right brood patch. The arrow indicates one individual with a fairly strong relative statistical power (see section 4.5 for more details).

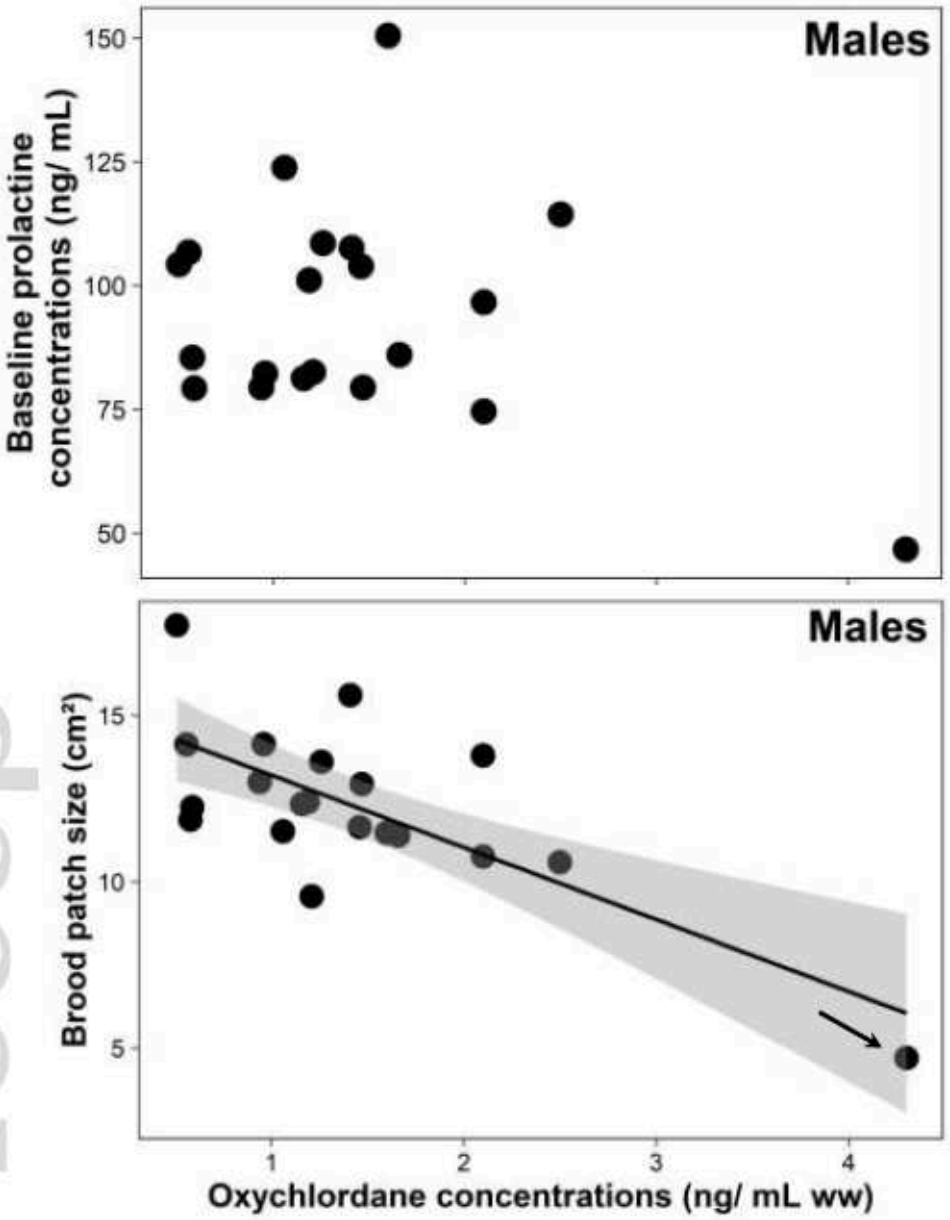


Fig.5.

Relationships between brood patch size and minimum incubation temperature in male incubating adult kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Brood patch size here reflects the size of the right brood patch. The arrow indicates one individual with a fairly strong relative statistical power (see section 4.5 for more details).

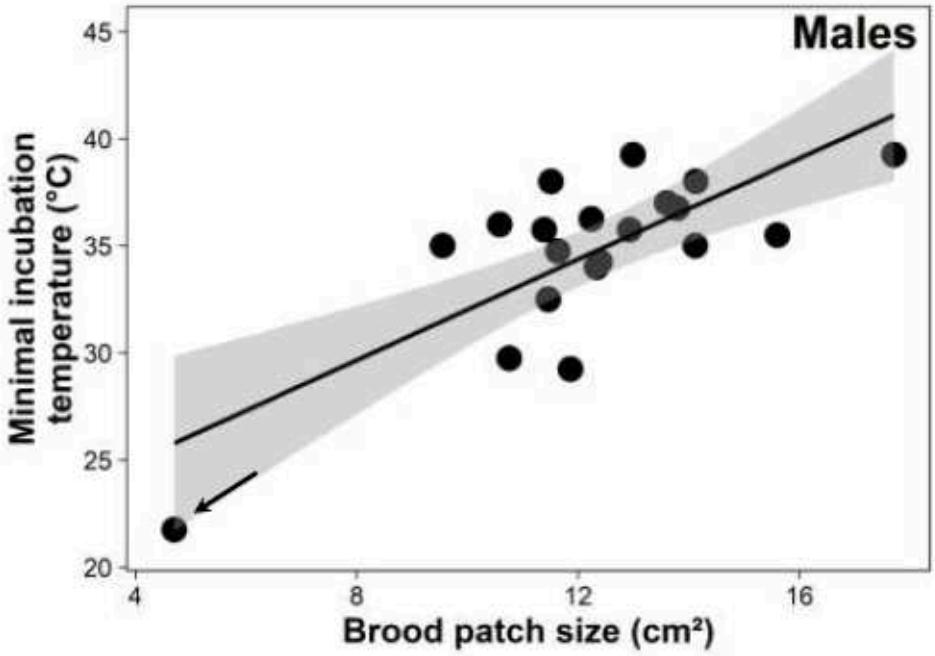
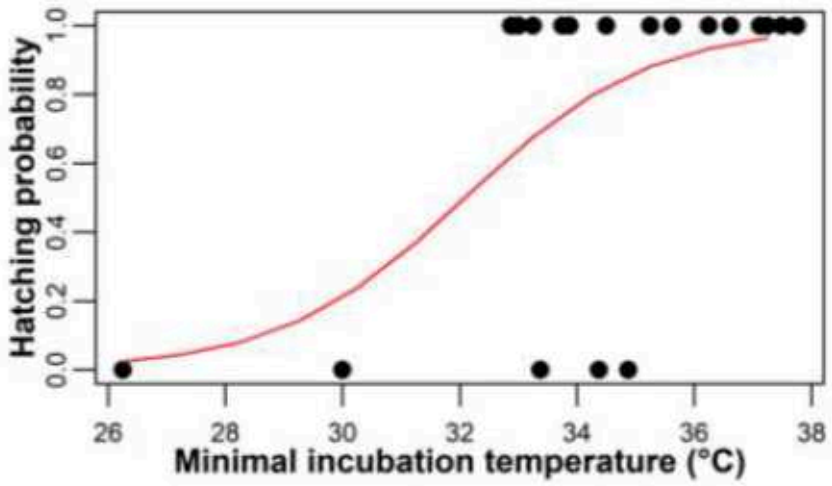


Fig.6.

Hatching probability (0 = not hatched; 1 = hatched) of the remaining egg in the experimental nests in relation to the minimal incubation temperature (T_{min}). T_{min} has been calculated by meaning the minimal incubation temperature of both partners in each nest.



Supplementary material

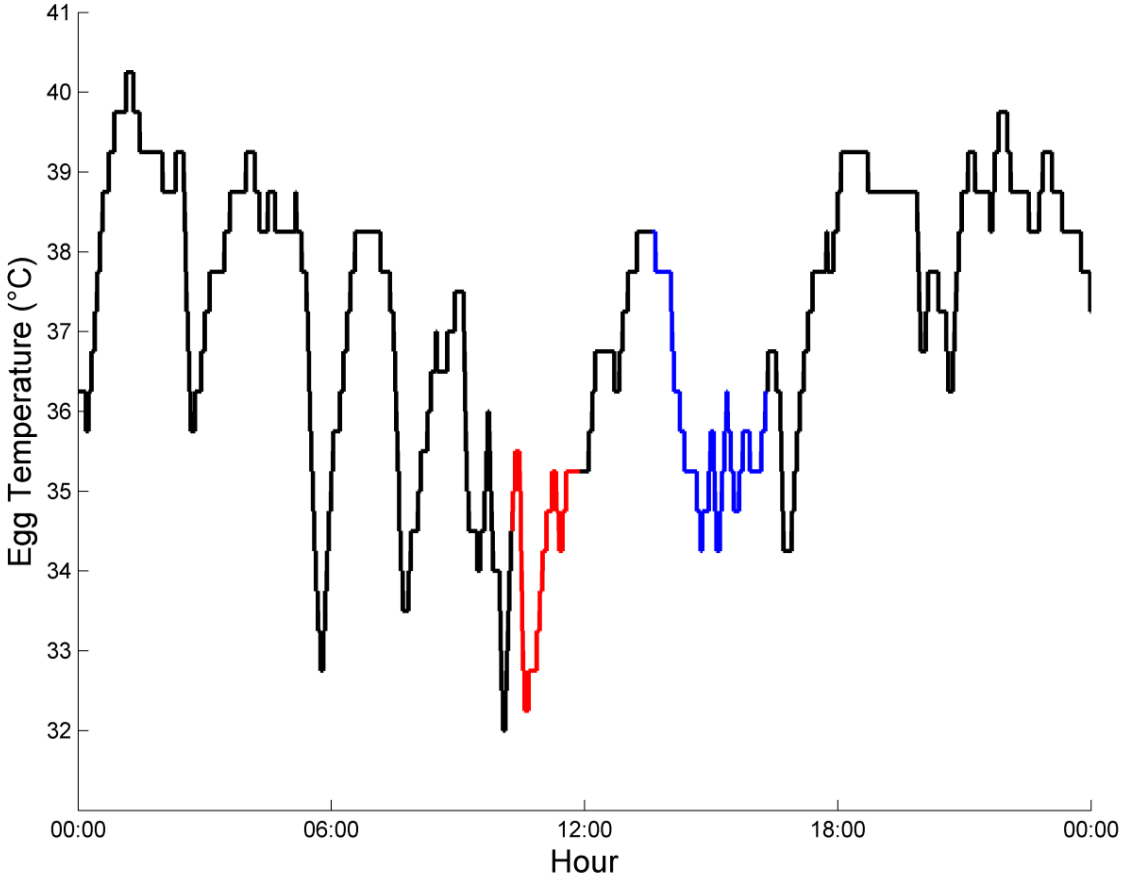
Table 1

Morphological comparison (length, breath and mass) between real eggs (n = 20) collected in nests of incubating kittiwakes *Rissa tridactyla* and artificial eggs (n = 20) deployed for logger experiment. Values represent mean ± SD.

	Length (mm)	**Breath (mm)**	**Mass (g)**
Real eggs	56.1 ± 2.47	40.7 ± 1.26	46.8 ± 3.61
Artificial eggs	56.4 ± 0.25	38.9 ± 0.25	40.3 ± 0.54

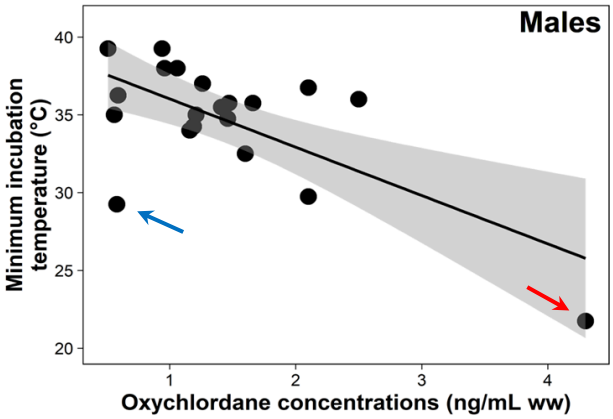
Fig.1.

Representative 24-hour time series showing an incubation temperature profile in kittiwakes *Rissa tridactyla* from Kongsfjorden, Svalbard. Raw data are in black and identified incubation bouts of each partner are colored sections of the profile (male in blue and female in red).

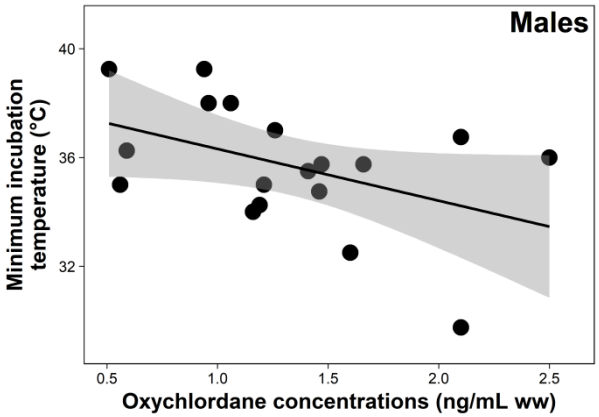


Results and Discussion

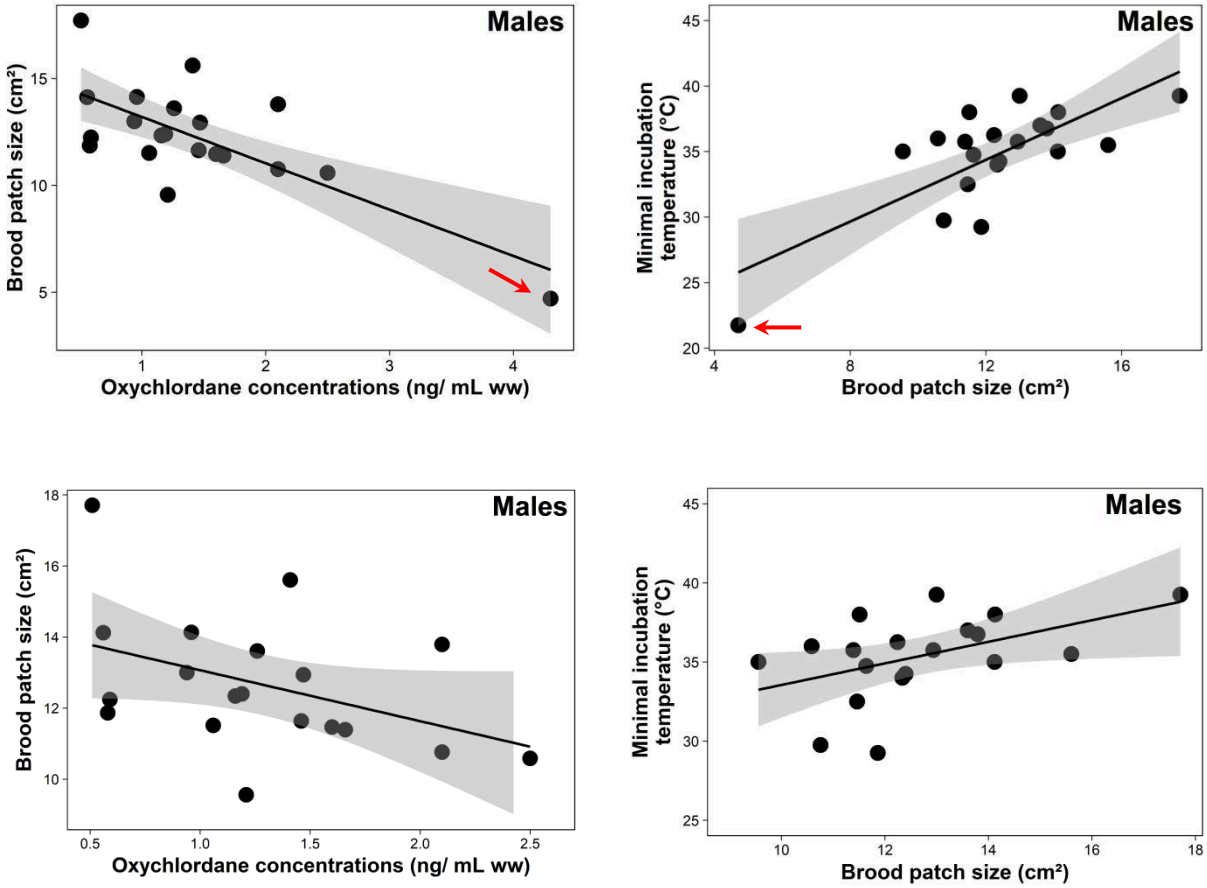
Our study was conducted on a limited sample size and the reported relationship between blood oxychlordane concentrations and T_{min} in incubating male kittiwakes, although statistically significant, appear to be partly influenced by one individual with a relatively high leverage (indicated with a red arrow below).



We thus performed diagnostic tests to assess the presence of potential influential points. The outlier test using the "studentized residuals" revealed one point (indicated with a blue arrow above) as being a potential issue in our data sat even after the Bonferroni adjustment (unadjusted p = 0.002; adjusted p = 0.049). Moreover, the Cook's distance plot indicates another point (indicated with a red arrow above) as being influential (Cook's distance > 1). Consequently, we re-analysed the relationship between oxychlordane concentrations and T_{min} in males without these two points and again found a negative association (although marginally significant) between these two variables which corroborates results presented in the main document (LM, slope = -0.002; p = 0.064; R^2 = 0.20).



We followed the same procedure regarding the reported relationships between (i) oxychlordane concentrations and brood patch size and (ii) brood patch size and T_{min} in incubating male kittiwakes. Diagnostic tests revealed the presence of one influential point in the data set (indicated in red below). We re-analysed both relationships without this individual and again found (i) a negative association (although marginally significant; LM, slope = -0.001; p = 0.074; R^2 = 0.18) between oxychlordane concentrations and brood path size, and (ii) a significant positive relationship between the brood patch size and T_{min} in male kittiwakes (LM, slope = 0.682; p = 0.038; R^2 = 0.23).



Paper V

Blévin, P., Shaffer, S., Bustamante, P., Angelier, F., Picard, B., Herzke, D., Moe, B., Gabrielsen, G.W., Bustnes, J.O., Chastel, O.

Contaminants and parental care in an Arctic seabird: Dissimilar associations of perfluoroalkyl and organochlorine compounds with egg-turning behaviors

In preparation for **Environmental Science & Technology**

Contaminants and parental care in an Arctic seabird: Dissimilar associations of perfluoroalkyl and organochlorine compounds with egg-turning behavior

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Keywords

Perfluoroalkyl substances; Organochlorines; Mercury; Prolactin; Egg-logger; Black-legged kittiwake; Hatching success

1. Introduction

Incubation is a very important stage across avian breeding cycle since early life developmental conditions can have long-term fitness consequences (Berntsen and Bech, 2016; Deeming, 2002a; Lindström, 1999). Generally, egg attendance involves a variety of parental behaviors such as egg turning and active egg-warming; both considered as key determinants for egg hatchability (Decuypere and Michels, 1992; Elibol and Brake, 2004, 2006a; Funk and Forward, 1953; Poulsen, 1953; Tona et al., 2005a; Tullett and Deeming 1987; Van Schalkwyk et al. 2000). Egg turning behavior requires an optimal turning rate with an appropriate angular change to facilitate absorption of albumen by the embryo, to reduce malpositions (e.g. head in the small end of the egg) and to prevent the embryo from adhering to the inner shell membrane (Deeming, 1991, 2002b; Elibol and Brake, 2004, 2006b; Eycleshymer, 1906; New, 1957; Tullett and Deeming, 1987). Accordingly, a lack or decrease of egg turning can retard or prevent albumen absorption and gas exchanges resulting in an abnormal chick development with a lower growth rate, decreasing oxygen consumption, and *in fine*, leading to delayed or reduced hatching success (Deeming, 1991, 2000, 2002b; Elibol and Brake, 2006a; Funk and Forward, 1953; New, 1957; Pearson et al. 1996; Robertson, 1961; Tazawa 1980; Tona et al., 2005b; Van Schalkwyk et al., 2000; Wilson et al., 2003; Yoshizaki and Saito, 2003). In birds, set-up and maintenance of parental care behaviors are orchestrated by a cocktail of different hormones acting synergistically. Among them, a pituitary hormone named prolactin is considered as the major endocrine controller for incubation behaviors (Angelier et al., 2016; Angelier and Chastel, 2009; Buntin, 1996; Sockman et al., 2006; Thierry et al., 2013a; Vleck, 2002; Vleck and Vleck, 2011).

Conditions required for an optimal incubation have been so far extensively investigated in the poultry industry to maximize hatchability of domestic fowl for commercial

purposes (King'ori, 2011, Lundy, 1969; Tona et al., 2005a). In contrast, environmental conditions influencing incubation of free-ranging birds are much less investigated. Among them, some environmental contaminants can modulate avian reproduction through their endocrine disrupting properties and several previous investigations revealed that some chemicals have the potential to alter parental care behaviors. For instance, field and experimental studies have shown that exposure to organochlorines (OCs) and mercury (Hg) can be associated with reduced nest attendance (i.e. longer and more frequent absence from the nest site; Bustnes et al., 2001, 2005; Fisher et al., 2006; Fox et al., 1978: Tartu et al., 2015a), lowered nest or egg temperatures (Fox et al., 1978; Peakall and Peakall, 1973; Verboven et al., 2009), decreased nest defense /or increased egg predation (Fox et al., 1978; Fox and Donald, 1980; Helberg et al., 2005) and prolonged incubation period (Fisher et al., 2006; Kubiak et al., 1989; Mc Arthur et al., 1983). Such detrimental effects of contaminants on incubation behaviors could, *in fine,* induce deleterious consequences on egg hatchability (Kubiak et al., 1989; Peakall and Peakall, 1973).

Polar regions are considered as a sink for environmental contaminants. Through oceanic currents and/ or atmospheric transports in combination with a cold climate, numerous volatile and/ or persistent contaminants can reach isolated areas like the Arctic (Ariya et al., 2004; Burkow and Kallenborn, 2000; Butt et al., 2010; Fitzgerald, 2005; Gabrielsen and Henriksen, 2001; Prevedouros et al., 2006; Selin, 2009; Soerensen et al., 2016; Wania, 2003, 2007). Once deposited in the marine ecosystem, those contaminants turn bioavailable for living organisms, bioaccumulate across their lifespan and biomagnify along trophic webs (Atwell et al., 1998; Blévin et al., 2013; Bustnes et al., 2013; Dietz et al., 2000; Fang et al., 2014; Haukås et al., 2007; Kelly et al., 2009; Tomy et al., 2004). Long lived seabirds feeding on prey from relatively high trophic positions appear therefore as relevant biological models to investigate the effects of contaminants on wildlife (Elliott and Elliott, 2013). In the

Norwegian Arctic, black-legged kittiwakes, *Rissa tridactyla* (hereafter "kittiwakes"), are chronically exposed to a complex mixture of harmful environmental contaminants already known as being detrimental to fitness through their endocrine disrupting properties (Blévin et al., 2016, 2017a, 2017b; Erikstad et al., 2013; Goutte et al., 2015; Tartu et al., 2013, 2014a, 2014b, 2015b, 2016). Among them (i) some poly- and perfluoroalkyl substances (PFASs), especially the most prevalent long chained carboxylate acids (PFCAs), which globally increase in Arctic biota (AMAP, 2015; Braune and Letcher, 2013; Butt et al., 2007, 2010); (ii) some legacy OCs, banned from use and listed in the Stockholm convention, which globally decrease in Arctic wildlife (AMAP, 2015; Bustnes et al., 2017; Helgason et al., 2008; Rigét et al., 2010); and (iii) toxic trace elements of both human and natural origins such as Hg with stable or decreasing trends in the European Arctic (AMAP, 2007, 2011; Cole et al., 2013; Helgason et al., 2008).

Using an egg-logger novel technology recently developed by Shaffer et al. (2014), we want to test if the most contaminated birds are less committed in parental care. By combining a 3-axis accelerometer with a magnetometer embedded in an artificial egg, this logger can provide accurate and continuous records of egg-turning behaviors in a three-dimensional orientation. Specifically, we want to investigate relationships between three groups of contaminants (PFASs, OCs and Hg) with egg turning behaviors (i.e. frequency and angular change) in incubating kittiwakes from Svalbard in the Norwegian Arctic. To the best of our knowledge, effects of environmental contaminants on these egg turning behaviors remain unexplored for PFASs and OCs. So far, only one study conducted on Forster terns (*Sterna forsteri*) has investigated the potential effects of Hg concentrations on egg turning rate but does not report any relationships (Taylor et al., 2018). Our study appears therefore as particularly relevant and partly contributes at filling the gap of knowledge in that topic. Since prolactin secretion is tightly involved in mediating parental care behaviors (Angelier et al.,

2016; Buntin, 1996; Sockman et al., 2006; Thierry et al., 2013a; Vleck, 2002; Vleck and Vleck, 2011), we also examined relationships between contaminant levels and plasma prolactin concentrations as a potential underlying endocrine mechanism through which contaminants exposure in kittiwakes may influence egg turning behaviors. Importantly, effects of PFASs on plasma prolactin levels are still unknown in birds. Finally, since egg tuning is critically important for egg hatchability, we explored some potential effects of egg turning behaviors on hatching probability.

2. Material and methods

2.1. Study site and sampling

Fieldwork was carried-out in 2015, from June 19th to July 12th in a colony of black-legged kittiwakes at Kongsfjord, Svalbard (Figure 1; 78°54'N; 12°13'E). We caught a total of 40 individuals (20 males and 20 females) on their nest with a noose attached to the end of a long pool. Since many seabirds, kittiwakes are bi-parental care breeders and our study was conducted on 20 incubating pairs. We first collected a blood sample (~0.5 mL) from the alar vein immediately after capture to assess baseline prolactin levels for each bird. We then performed an additional blood sampling (~2 mL) in order to measure the concentrations of contaminants and determine gender. All the birds were then weighted to the nearest 5 g using a Pesola spring balance to determine body mass. We also measure the skull length (head + bill) using a sliding calliper with an accuracy of 0.1 mm. These two morphometric measurements were further used to compute the Scaled Mass Index (SMI) for each kittiwake, as a proxy of body condition and following the method developed and detailed in Peig and Green (2009, 2010). Blood samples were stored in ice during the field session. Once back in

the lab, whole blood and both plasma and red blood cells obtained after centrifugation were kept frozen at -20°C until subsequent analyses.

2.2. Experimental design and data processing

All the nests investigated in the present study contained a two natural eggs clutch size. We randomly collected and replaced one of these two eggs by an artificial egg containing both a triaxial accelerometer and magnetometer to record orientation and angular changes in three dimensions (i.e. roll, pitch and yaw; as described in Shaffer et al., 2014; Clatterbuck et al., 2017; Kelsey et al., 2015; Taylor et al., 2018). These loggers have the benefit to provide precise (sensing 1-2° angular changes orientation) and continuous records (~1 sec) of egg turning behaviors. Turning behavior of egg-loggers was tested and verified by comparing a video recording of an egg being turned manually with an animation created by post-processing the data collected by the egg-logger (Shaffer et al., 2014). Artificial eggs were designed according to the kittiwake egg morphology. Additionally, in order to mimic the coloration and pattern of the real egg, we painted artificial eggs with a non-toxic water-based paint. Collected eggs were candled and all of them were fertile. Artificial eggs were deployed between 7 and 10 days during the incubation period. All incubating birds readily accepted the dummy egg and did not show abnormal behaviors. Because each partner was marked with different color of dye spots on the forehead, we could easily identified incubation bouts of each parent. Using a telescope at a distance, we regularly check each day (checks were not conducted at night) which partner was incubating. Once experiment completed, the artificial egg was removed and only one egg remained into the nest. Using a mirror at the end of a long pole, we determined hatching success of the experimental nest by conducting regular checks until the end of the field season (i.e. 12th July; laying peak from 6th to 9th July).

Egg-logger data were processed using purpose-built routines in MATLAB (The Mathworks, Natick, MA, USA) following methods developed and detailed in Shaffer et al., 2014. Raw data accelerometers were converted to 3-2-1 Eulers angles to estimate instantaneous egg movement. According to Shaffer et al. (2014), rotations with an angular change of a minimum of 10° were considered as significant egg-turning events. For all kittiwakes, we finally estimated i) the egg-turning frequency by dividing the sum of all events detected by the duration of the record (expressed on an hourly basis) and ii) the overall angular change, by meaning the value of all egg-tuning events (expressed in degrees). The day of egg deployment and records during our presence in the colony were excluded from the dataset in order to avoid any biased data.

2.3. Contaminant analyses

PFASs were analysed from plasma at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. We scanned for the following compounds: perfluorooctanesulfonamide (PFOSA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), linear perfluorooctanesulfonate (PFOSlin), branched perfluorooctanesulfonate (PFOSbr), perfluorodecanesulfonate (PFDcS), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoate (PFUnA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTrA), and perfluorotetradecanoate (PFTeA). Concentrations below the limit of detection (LoD) were assigned by ½ LoD value but only compounds detected in at least 70% of the data set were kept for further statistical analyses. Consequently, compounds remaining for further investigations were the carboxylates PFNA, PFDcA, PFUnA, PFTrA, PFTeA, expressed as ∑PFCAs and only one sulfonate, PFOSlin. In

short, a sample (0.2 mL) spiked with internal standards (carbon labeled PFAS, Hanssen et al., 2013) was extracted in methanol (1 mL) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVICarb graphitized carbon absorbent and glacial acetic acid. Extracts were analysed by UPLC/MS/MS. Recovery of the internal standards ranged between 74% and 128%. Results were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference material (1957 human serum from NIST) runs every 10 samples. The deviation of the target concentrations in the SRMs were within the laboratory's accepted range (69‑130%). All blanks contained concentrations below the instrument detection limits, except for PFCAs, ranging between 5 and 30 pg/ mL.

OCs were analyzed from whole blood at NILU. We scanned for the following compounds: the organochlorine pesticides (HCB, α-, β-, γ-HCH, *trans*-, *cis*-chlordane, oxychlordane, *trans*-, *cis*-nonachlor, mirex, *p,p'*-DDE) and the polychlorinated biphenyls (CB−28, −52, −99, −101, −105, −118, −138, −153, −180, −183, −187 and −194). Concentrations below LoD were assigned by ½ LoD value but only compounds detected in at least 70% of the data set were kept for further statistical analyses. Consequently, compounds remaining for further investigations were the organochlorine pesticides (mirex, HCB, *p,p'*-DDE and chlordane mixture: oxychlordane, *trans*-, *cis*-nonachlor; expressed as ΣCHLs) and the PCBs (CB−28, −99, −105, −118, −138, −153, −180, −187; expressed here as the ΣPCBs). Concentrations of *p,p'*-DDE are missing for 3 males because of injection issues into the GC/ MS. To a whole blood sample of 0.70-1.13 mL, a 100 µL of an internal standard solution was added (^{13}C-labeled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). We first proceeded to the sample denaturation using a mix of ethanol and saturated solution of ammonium sulphate in water. We then ran extraction twice with 6 mL of n-hexane. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975 CMSD were performed following Herzke et al., 2009. Recovery

of the internal standards ranged between 52% and 60%. Results were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference material (1958 human serum from NIST) runs every 10 samples. The deviation of the target concentrations in the SRMs were within the laboratory's accepted range (75‑111%). All blanks contained concentrations below the instrument detection limits.

Total Hg was analyzed at the Littoral Environment et Sociétés laboratory (LIENSs) in La Rochelle, France from freeze-dried and powdered red blood cells placed in an Advanced Hg Analyzer Spectrophotometer (ALTEC AMA 254) as described in Bustamante et al., 2006. Aliquots ranging from 0.44 to 8.59 mg were analysed for each individual, in duplicates (all coefficients of variation ≤ 5.42%). Results were validated with blanks (ran at the beginning of each set of samples) and standard reference material (Tort-2 Lobster Hepatopancreas, NRC, Canada; certified value 0.27 ± 0.06 (SD) µg/g dw). Measured values were 0.25 ± 0.01 (SD) µg/g dw, n = 11. All blanks contained concentrations below the instrument detection limit (0.005 µg/g dw).

2.4. Prolactin assay and molecular sexing

Prolactin assay and molecular sexing were conducted at the Centre d'Etudes Biologiques de Chizé (CEBC), France. Plasma prolactin concentrations were measured by radioimmunoassay as previously described and validated for this kittiwake population (Chastel et al., 2005). We estimated intra-assay variation (7.13%) by including internal standards to the assay ran in duplicates. It is worth noting that bleeding time (i.e. time elapsed from capture to the end of the first blood sampling: 2.48 ± 0.52 min (SD), on average) did not affect baseline prolactin concentrations (LMM, $F_{1,19} = 0.61$, $p = 0.45$).

2.5. Statistical analyses

All statistical analyses were performed using R 3.3.1 (R core Team, 2016). Linear mixed effect models (LMMs) with the nest identity as a random factor were used to test whether contaminant concentrations, incubation behaviors (i.e. egg-turning frequency and angular change), baseline prolactin levels and body condition differed between sexes. We used the restricted maximum likelihood estimation (REML) method to avoid any potential biased statistic estimations (Zuur et al., 2009). The relationships between contaminants and incubation behaviors (i.e. egg-turning frequency and angular change) were investigated with linear models (LMs) including each contaminants separately (PFOSlin, ∑PFCAs, ∑PCBs, *p,p'*-DDE, HCB, ∑CHLs, mirex and Hg) to avoid any collinearity problems. Similarly, analyses have been conducted for each sex separately as males were more contaminated than females (Figure 2) and because both sexes can react in very different ways to environmental stressors like PFASs, OCs and Hg contamination (Blévin et al., 2016, 2017a; Goutte et al., 2015; Tartu et al., 2013, 2014b, 2016). Then, we examined the relationships between contaminants of interest and baseline prolactin levels to evaluate some potential underlying mechanisms. Finally, we tested whether egg-turning frequency and angular change (calculated by meaning individual values of both sexes in each nest) can affect hatching probability using generalized linear model (GLM) constructed with a "binomial" family and a "cloglog" link function which is consistent with the use of an asymmetric data set (hatched: n = 15; not hatched: n = 5; Zuur et al., 2009). Diagnostic plots and Shapiro normality tests were finally performed on residuals to test whether the data sufficiently met the assumption of the models (i.e. LMM, LM, GLM) and data were log-10 transformed when necessary (Zuur et al., 2009). We used a significance level of $\alpha \leq 0.05$.

3. Results

3.1. Sex-related differences

PFASs, OCs and Hg concentrations are shown in Figure 2. LMMs indicated that all contaminants differ significantly between sexes, with males being more contaminated than females. Egg-turning frequency and angular change are shown in Table 1. LMMs did not indicate any statistical differences of incubation behaviors between males and females. Baseline prolactin levels and body condition are shown in Table 1. LMMs did not report any sexual differences for prolactin concentrations in plasma and females tend to be in better condition than males.

3.2. Egg-turning and contaminants

Recording duration (19.87 ± 9.49 (SD) hours, ranging from 4.65 to 44.63 hours) did not influence angular change (LMM, $F_{1,19}$ = 1.44, p = 0.25) but was positively related to the egg-turning frequency (LMM, $F_{1,19}$ = 4.35, p = 0.05). Consequently, we controlled for an effect of recording duration on egg-turning frequency in further statistical analyses. In addition, to control for potential changes in egg-turning behaviors that may have occurred across the incubation period, we used the age of embryo as a proxy of incubation stage. Embryo age did not influence angular change (LMM, $F_{1,10}$ = 0.77, p = 0.40) but was positively related to the egg-turning frequency (LMM, $F_{1,10}$ = 5.99, p = 0.03). Unfortunately, age of embryo was only available for 12 eggs, thus discarding the possibility to use this variable in statistical analyses conducted on the whole dataset. However, we performed a second run of statistical analysis on a subset (12 males and 12 females), controlling for age of

embryo on egg-turning frequency in the selected model from the first run and found similar results.

Egg-turning frequency was positively and significantly related to PFOSlin concentrations in female kittiwakes only (Table 2). In other words, the most PFOSlin-contaminated females turned their eggs more often compared to the less contaminated ones. OCs, Hg and SMI were not significantly related to egg-turning frequency in both males and females (Table 2). Angular change was positively and significantly associated with PFASs concentrations (i.e. PFOSlin and ∑PFCAs) in females but negatively and significantly related to the ∑PCBs concentrations in female kittiwakes (Table 2; Figure 3). In other words, the most PFASs-contaminated females turned their eggs, in average, with higher amplitude while the most PCBs-contaminated females turned their eggs with lower amplitude (Figure 3). Importantly, concentrations of PFASs and PCBs were not significantly correlated in females (PFOSlin/ ∑PCBs: $r_{pearson}$ = -0.29; p-value = 0.21; ∑PFCAs/ ∑PCBs: $r_{pearson}$ = -0.37; p-value = 0.11). In males, we observed a positive and significant relationship between angular change and ∑PFCAs only (Table 2; Figure 3). Hg and SMI were never associated with angular change in both sexes (Table 2).

3.3. Prolactin, egg-turning and contaminants

Prolactin levels were positively and significantly related to the angular change in females but not in males (Figure 4; LMs, females: $F_{1,18}$ = 5.64, p = 0.03; males: $F_{1,18}$ = 0.20, p = 0.66). Furthemore, prolactin levels were not related to the egg-turning frequency in both sexes (LMs, females: $F_{1,18}$ = 0.49, p = 0.50; males: $F_{1,18}$ = 2.60, p = 0.12).

PFASs (i.e. PFOSlin and ∑PFCAs) and ∑PCBs were significantly related to egg-turning behaviors in kittiwakes. Consequently, we examined relationships between these

contaminants and baseline prolactin levels. In females, PFASs were positively and significantly related to the baseline level of prolactin while PCBs were not (Figure 5; LMs, PFOSlin: $F_{1,18}$ = 7.25, p = 0.01; ∑PFCAs: $F_{1,18}$ = 4.54, p = 0.05; ∑PCBs: $F_{1,18}$ = 1.56, p = 0.23). In males, PFASs and PCBs were not related to the baseline level of prolactin (LMs, PFOSlin: $F_{1,18}$ = 2.53, p = 0.13; ∑PFCAs: $F_{1,18}$ = 2.22, p = 0.15; ∑PCBs: $F_{1,18}$ = 0.76, p = 0.40).

3.4. Consequences on hatching success

We investigated the potential consequences of egg-turning frequency and angular change variations on hatching success. There was a positive and marginally significant relationship between egg-turning frequency and the probability that the remaining egg in the experimental nests successfully hatched (Figure 6; GLM, Z = 1.67; p = 0.09). In other words, the most frequently turned eggs tend to better hatched. By contrast, angular change was not related to hatching probability (GLM, Z = 0.86; p = 0.39).

4. Discussion

Using an egg-logger novel technology recently developed by Shaffer et al. (2014), we investigated the relationships between three groups of contaminants (PFASs, OCs and Hg) with egg-turning behaviors in incubating Arctic kittiwakes. Our results indicated some contrasted associations since PFASs were positively related to egg-turning frequency and angular change in both sexes contrary to ∑PCBs, negatively associated with angular change in females. Hg and SMI were not related to incubation behaviors. Because PFASs and PCBs are endocrine disruptors (DeWitt, 2015; Giesy et al., 2003; Jensen and Leffers, 2008; Khetan,

2014; Tyler et al., 1998), we investigated potential underlying mechanisms by focusing on prolactin, a hormone highly involved in incubation behavior (Angelier and Chastel, 2009; Angelier et al., 2016; Vleck, 1998, 2002). Interestingly, PFASs were positively related to the plasma prolactin levels in female kittiwakes, thus corroborating the previously reported positive relationship between PFASs and egg-turning behaviors (at least for females). Finally, because egg-turning frequency and angular change are key determinants for egg hatchability, we explored some potential consequences on hatching success and found that egg-turning frequency but not angular change was marginally and positively related to the hatching probability. Consequently, PFASs and PCBs, through their endocrine disrupting properties, could modify incubation behaviors of parents, resulting *in fine,* to some effects on hatching success of kittiwakes.

4. 1. Parental care and contaminants

Surprisingly, PFASs concentrations were positively related to egg-turning frequency and angular change which suggest a beneficial effect of PFASs on incubation behaviors in kittiwakes. This is an unexpected result since a general and common understanding is that environmental contaminants are associated with adverse effects in living organisms. To the best of our knowledge, the effects of PFASs on reproductive behaviors of birds have never been investigated which make comparison with other works impossible. However, although correlative, several recent studies suggested that PFASs could have beneficial effects on physiology of Arctic seabirds, including lower stress levels (i.e. baseline corticosterone, Tartu et al., 2014b), reduced ageing (i.e. telomere length dynamic, Blévin et al., 2017b) and higher energy expenditure for self-maintenance (i.e. basal metabolic rate, Blévin et al., 2017a; thyroid hormones, Melnes et al., 2017). Thus, this suggested positive effect of PFASs on

parental care behaviors is in line with previous findings and one possible explanation could rely on hormesis. In ecotoxicology, hormesis is an adaptive bi-phasic dose response characterized by chemicals inducing stimulation (e.g. beneficial effects) at low doses, but inhibition (e.g. harmful effects) at higher doses (Calabrese, 2002, 2008; Costantini, 2014). Overlooked and ignored for a long time, this historical concept came into focus over the last two decades and an extensive review conducted by Calabrese and Blain (2005, 2011) indicated a relatively high implication of organic chemicals in hormesis phenomenon across numerous taxa. Accordingly, two experimental studies conducted on mallard ducks (*Anas platyrhynchos*) suggested a hormetic response involving a better hatching success in eggs containing low doses of methylmercury artificially injected or naturally deposited by methylmercury-fed mothers (Heinz et al., 2010, 2012). In addition, experimental exposure to low dose of the synthetic chemical 4-nonylphenol showed a positive effect on fecundity in the fish fathead minnows (*Pimophales promelas*) through possible indirect stimulation of estrogen secretion compared to controls or to high doses (Giesy et al., 2000). However, the beneficial effect of PFASs on egg-turning behaviors and on other physiological mechanisms listed above remains purely hypothetic and further investigations conducted experimentally and testing for a bi-phasic dose response are needed.

In contrast, PCBs have received a better level of attention and several studies conducted *in natura* or experimentally indicated detrimental effects of PCBs on an array of reproductive behaviors in birds. Among them, an experimental study where captive American kestrels (*Falco sparverius*) received a mixture of PCBs revealed longer incubation periods and altered incubation behaviors like a reduced nest attendance in treated groups (Fisher et al., 2006). In glaucous gulls (*Larus hyperboreus*), another Arctic seabird, exposure to PCBs was found to be associated with reduced nest attendance (i.e. longer and/ or more frequent absences from the nest site during incubation period; Bustnes et al., 2001) and lowered nest

temperature (Verboven et al., 2009). In addition, delayed hatching date in relation to PCBs exposure in male kittiwakes (females were not investigated) from the same colony was also reported (Tartu et al., 2015b). As a result, detrimental effects of PCBs on reproductive behaviors in birds appear conclusive. By focusing on egg-turning behaviors at fine resolution and because we reported a negative association on angular change, our study comes to add a better understanding of the way through which PCBs could impact reproduction of birds.

4. 2. Some contrasted patterns

Importantly, our study indicated dissimilar associations between PFASs and PCBs with angular change in female kittiwakes. Interestingly, such contrasted patterns between PFASs and OCs have already been highlighted for several physiological endpoints, including hormone levels (e.g. corticosterone, thyroid hormone; Melnes et al., 2017; Nordstad et al., 2012; Tartu et al., 2014b, 2015c), ageing (i.e. telomere length; Blévin et al., 2016, 2017b) and energy expenditure (i.e. basal metabolic rate; Blévin et al., 2017a). Besides, the contrasted physico-chemical properties of the proteinophilic PFASs and lipophilic OCs are in line with this observation. Indeed, PFASs are known to preferentially accumulate in protein rich tissues (e.g. liver, blood) while OCs are stored in adipose tissues before being released into the bloodstream during periods of accelerated lipid mobilization (Aas et al., 2014; Jones et al., 2003; Luebker et al., 2002; Verreault et al., 2005; Bustnes et al., 2010; Henriksen, 1995; Routti et al., 2013; Frindlay and Defretas, 1971; Kelly et al., 2009; Vanden Heuvel et al., 1992). Consequently, it appears reasonable to think that PFASs and OCs could target physiological functions through very different modes of action, without mechanistic interlinkage, potentially resulting in contrasted responses. Another potential explanation could rely on a possible confounding effect between contaminants (as suggested in Blévin et al.,

2017a). However PFASs (i.e. PFOSlin, ΣPFCAs) were not significantly related to ΣPCBs in female kittiwakes in the present study, thus partly excluding this hypothesis. However, kittiwakes are obviously exposed to a complex cocktail of contaminants which are not measured in this study and future experimental research focusing on structurally opposed chemicals is absolutely required to better understand and clarify the underlying mechanisms through which contaminants, and especially PFASs, influence health of living organisms.

4. 3. How to explain such relationships?

Prolactin is the major controller of parental behavior and considered as the "parental hormone" (Riddle, 1963). Accordingly, several correlational and experimental studies highlighted the predominant role of prolactin in the set-up and maintenance of incubation behaviors (Angelier et al., 2016; Buntin, 1996; Lynn, 2016; Sockman et al., 2006; Vleck, 2002). For instance, Sockman et al. (2000) showed that a moderate experimental increase in prolactin concentrations induce a better incubation assiduity (i.e. percent day incubating) in American kestrels (even if the higher administrated dose did not show any effects). Furthermore, another work conducted on kittiwakes from the same colony reported that experimentally-induced low prolactin levels were associated with reduced nest attendance during the chick-rearing period (Angelier et al., 2009). Our study suggests a positive effect of prolactin secretion on incubation behavior and specifically on egg angular change in female kittiwakes, adding further and new evidences that prolactin triggers parental behaviors in wild birds. So far, only one study conducted on Adelie penguins (*Pygoscelis adeliae*) have investigated prolactin level and egg-turning behavior. Surprisingly, they found that birds with lowered prolactin levels following implants of self-degradable bromocriptine pellets turned their eggs more often compared to the control group (Thierry et al., 2013a). In our study,

prolactin concentration is not related to egg-turning frequency. However, the authors stated that their result was likely attributed to a shift from a down to an upright position of the treated birds, which favor egg-turning events, rather than a direct effect of a prolactin decrease (Thierry et al., 2013a).

Given its key role in mediating parental behaviors, we investigated a possible disruption of prolactin secretion originating from PFASs and PCBs exposure, both known as endocrine disruptors (DeWitt, 2015; Giesy et al., 2003; Jensen and Leffers, 2008; Khetan, 2014; Tyler et al., 1998). Interestingly, our study revealed a positive and significant relationship between PFASs and prolactin levels in female kittiwakes which is line with the previously suggested positive effect of PFASs on egg-turning behaviors. Consequently, through a possible increase of prolactin secretion, the most PFAS-contaminated kittiwakes (at least females) could better incubate their eggs (at least for angular change). However, the endocrine disrupting property of PFASs on prolactin secretion remains unknown and has never been explored in birds. As a result, further experimental and correlational studies are needed to confirm this relationship and to understand the endocrine mechanisms linking PFASs to prolactin in avian models. By contrast, PCBs concentration was not associated with plasma prolactin level in kittiwakes although Figure 5 suggests a negative trend in females. In glaucous gulls, PCBs (and other OCs) have been associated to a decrease plasma prolactin concentration in males only, although several of these associations (including PCBs) did not adhere with the criterion of significance (Verreault et al., 2008). Furthermore, a previous study performed on Antarctic snow petrels (*Pagodroma nivea*) did not report any effects of ΣPOPs, including PCBs, on prolactin levels (Tartu et al., 2015a). Given these inconstancies, it is thus impossible to make affirmative conclusions and we can only speculate that PCBs exposure could decrease prolactin levels in wild birds. Moreover, because establishment and maintenance of incubation behaviors is orchestrated by a complex cocktail of different

reproductive hormones acting synergistically (Angelier et al., 2016; Buntin, 1996; Lynn, 2016; Sockman et al., 2006; Vleck, 2002; Vleck and Vleck, 2011), further experimental studies i) focusing on sex steroids (e.g. testosterone, estradiol, progesterone) and ii) on different doses of administrated PCBs may provide greater clarity about endocrine mechanisms targeted by PCBs and toxicity thresholds.

4. 4. What consequences on hatching success?

Since egg-turning behavior is a key determinant for egg hatchability, any effects of contaminants on egg-turning frequency and angular change could, *in fine*, influence reproductive success of kittiwakes. Accordingly, a previous study conducted on ring doves (*Streptopelia risoria*) reported a lower hatchability of eggs incubated by birds experimentally exposed to high doses of PCBs (Peakall and Peakall, 1973). Finally, forster terns had a higher hatching success when eggs laid from organochlorine contaminated birds (including PCBs) were incubated by less contaminated surrogate parents or with an artificial incubator (Kubiak et al., 1989). However, the positive effect of egg-turning frequency on hatching success suggested here is only marginally significant and given the low sample size (only 5 eggs were unhatched) we cannot confirm with certainty this effect on reproduction of kittiwakes. Finally, our study is purely correlative and thus, we have to be cautious with putative interpretations since causality is not possible to establish here.

Conflict of interest

The authors declare no conflicts of interest.

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References (work in progress)

Note: most of these references are included in the reference list of the main document of the thesis.

Table 1

Egg-turning frequency (number of hourly turns), angular change (degrees), baseline plasma prolactin concentrations (ng/ mL) and body condition (SMI) for male and female incubating kittiwakes, *Rissa tridactyla*, from Kongsfjord, Svalbard. Sex-related differences were tested with LMMs.

	Females (n = 20)	**Males (n = 20)**		
	Mean ± SD	**Mean ± SD**	$F_{1,19}$	**p-value**
Frequency	2.37 ± 0.71	2.09 ± 0.82	1.45	0.24
Angular change	41.45 ± 7.53	43.08 ± 10.16	0.37	0.55
Prolactin	93.18 ± 10.83	94.73 ± 21.92	0.08	0.78
SMI	398.11 ± 23.12	385.15 ± 19.85	4.17	0.06

Table 2

Output of GLMs examining relationships between contaminants, SMI and egg-turning behaviors in female and male incubating kittiwakes, *Rissa tridactyla*, from Kongsfjord, Svalbard.

	Frequency*		**Angular change**	
Predictor	**Estimate ± SE**	**p-value**	**Estimate ± SE**	**p-value**
Females (n =20)				
PFOSlin	$3.10^{-4} \pm 1.10^{-4}$	**0.05**	$3.10^{-3} \pm 2.10^{-3}$	**0.05**
ΣPFCAs	$1.10^{-4} \pm 7.10^{-5}$	0.16	$2.10^{-3} \pm 6.10^{-4}$	**< 0.01**
ΣPCBs	$-3.10^{-6} \pm 3.10^{-5}$	0.91	$-5.10^{-4} \pm 2.10^{-4}$	**0.03**
ΣCHLs	$3.10^{-4} \pm 4.10^{-4}$	0.57	$-6.10^{-3} \pm 4.10^{-3}$	0.15
p,p'-DDE	$1.10^{-4} \pm 1.10^{-4}$	0.48	$-2.10^{-3} \pm 1.10^{-3}$	0.20
Mirex	$-4.10^{-5} \pm 8.10^{-4}$	0.96	$-8.10^{-3} \pm 8.10^{-3}$	0.31
HCB	$3.10^{-4} \pm 3.10^{-4}$	0.33	$-3.10^{-3} \pm 3.10^{-3}$	0.30
Hg	$-1.10^{-1} \pm 4.10^{-1}$	0.83	-3.98 ± 4.61	0.40
SMI	$2.10^{-3} \pm 7.10^{-3}$	0.80	$3.10^{-2} \pm 8.10^{-2}$	0.69
Males (n =20)				
PFOSlin	$-8.10^{-5} \pm 7.10^{-5}$	0.26	$1.10^{-3} \pm 7.10^{-4}$	0.16
ΣPFCAs	$-2.10^{-5} \pm 5.10^{-5}$	0.61	$1.10^{-3} \pm 5.10^{-4}$	**0.04**
ΣPCBs	$2.10^{-5} \pm 1.10^{-5}$	0.10	$3.10^{-5} \pm 2.10^{-4}$	0.86
ΣCHLs	$2.10^{-4} \pm 2.10^{-4}$	0.49	$3.10^{-3} \pm 3.10^{-3}$	0.31
p,p'-DDE**	$2.10^{-4} \pm 1.10^{-4}$	0.06	$1.10^{-3} \pm 1.10^{-3}$	0.47
Mirex	$6.10^{-4} \pm 5.10^{-4}$	0.25	$9.10^{-3} \pm 6.10^{-3}$	0.14
HCB	$1.10^{-4} \pm 1.10^{-4}$	0.27	$8.10^{-4} \pm 2.10^{-3}$	0.64
Hg	$-5.10^{-1} \pm 3.10^{-1}$	0.13	2.38 ± 4.02	0.56
SMI	$-1.10^{-2} \pm 1.10^{-2}$	0.19	$-8.10^{-2} \pm 1.10^{-1}$	0.53

Significant variables are in bold.
* Controlled for recording duration in each model.
** Sample size = 17 (3 males are missing because of injection issues into the GC/ MS).

Figure 1

Location of the study area (Kongsfjord, Svalbard) and the sampling site (Krykkjefjellet).

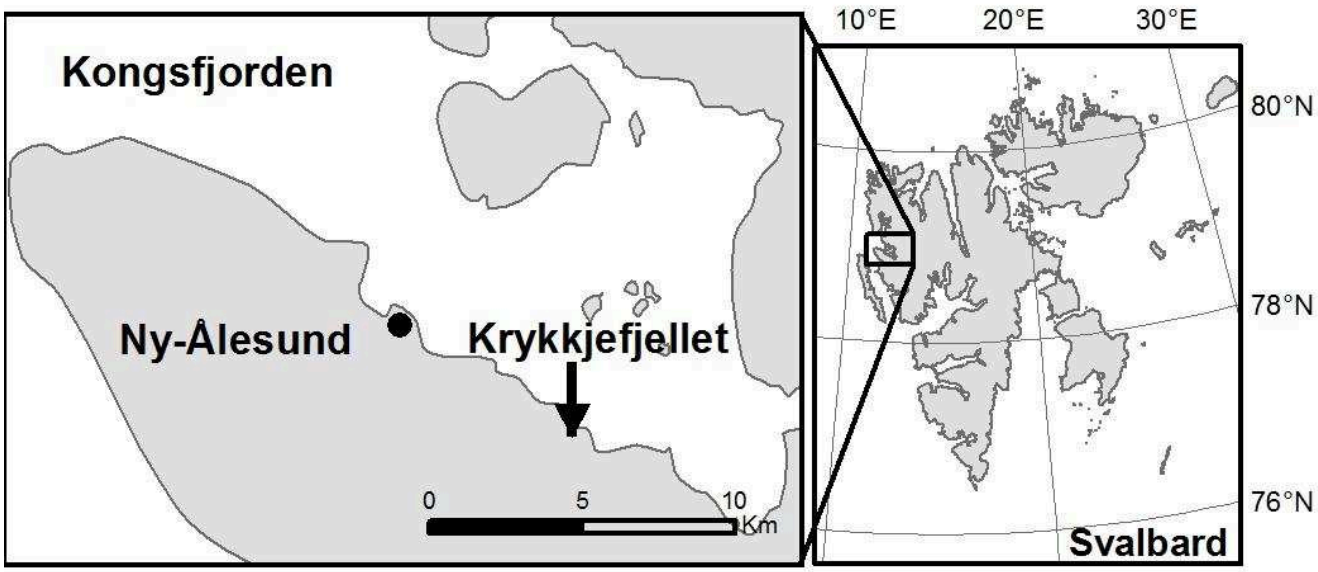


Figure 2

Contaminant concentrations (mean ± SE) in male (in black; n = 20) and female (in white; n =20) incubating kittiwakes, *Rissa tridactyla*, from Kongsfjord, Svalbard. PFASs (expressed in ng/ mL ww) have been measured in plasma, OCs (in ng/ mL ww) in whole blood, and Hg (in µg/ g dw) in red blood cells. Significant differences between males and females are indicated by * (≤ 0.05), ** (≤ 0.01) or *** (≤ 0.001). Sex-related differences were tested with LMMs and all contaminants have been log-10 transformed except for ∑PFCAs.

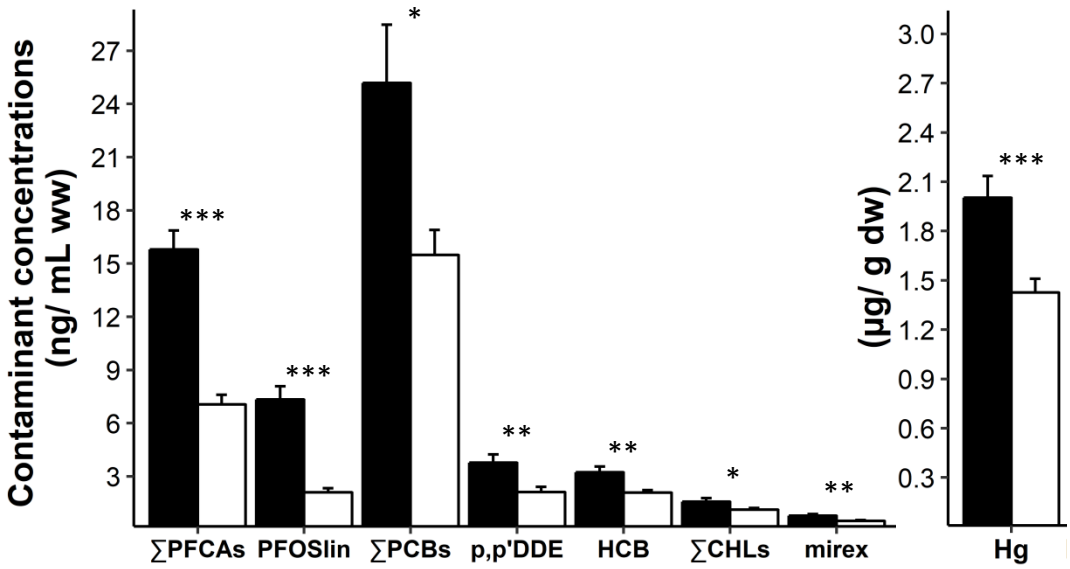


Figure 3

Relationships between plasma ∑PFCAs, blood ∑PCBs concentrations and egg angular change in female (n =20) and male (n =20) incubating kittiwakes, *Rissa tridactyla*, from Kongsfjord, Svalbard. Associations were tested with LMs and significant relationships are indicated by solid regression lines.

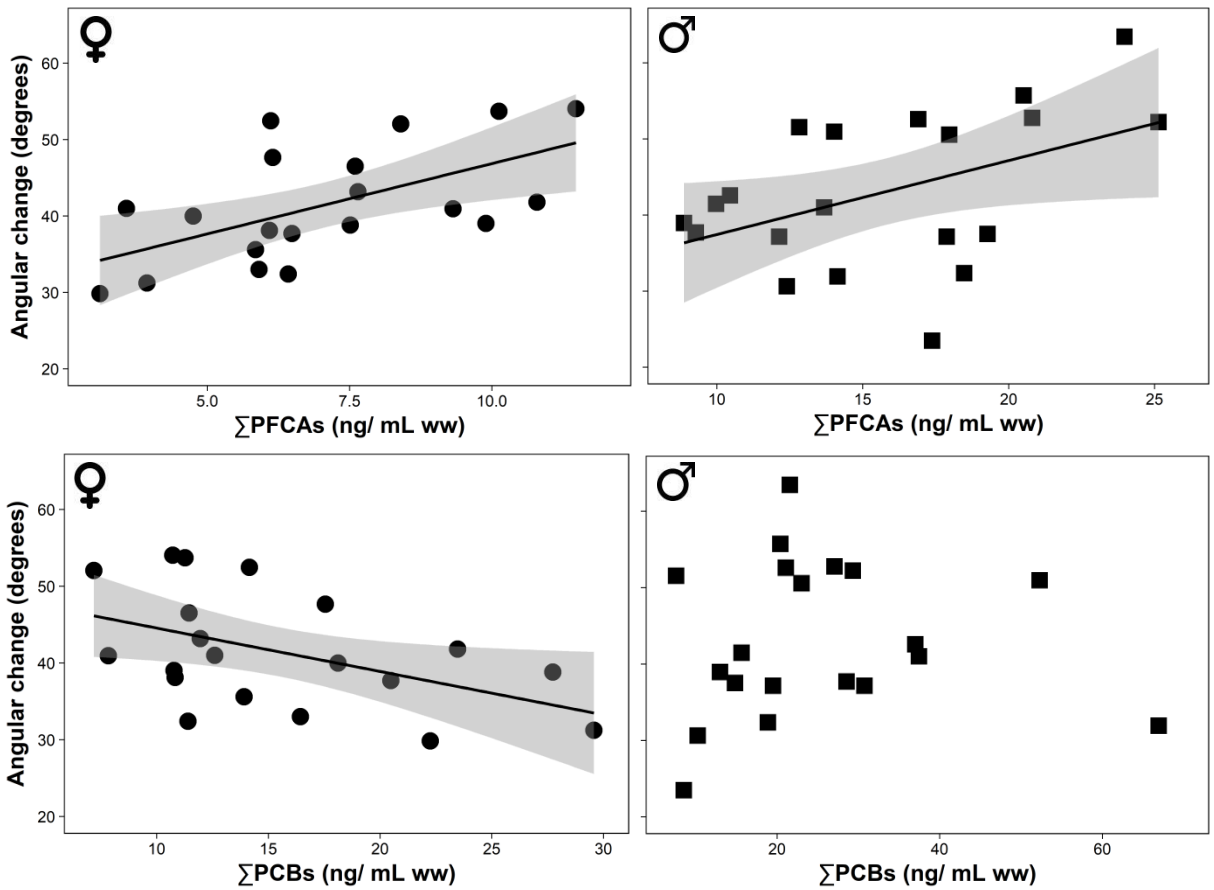


Figure 4

Relationships between baseline plasma prolactin levels and angular change in female (n =20) and male (n = 20) incubating kittiwakes, *Rissa tridactyla*, from Kongsfjord, Svalbard. Associations were tested with LMs and significant relationships are indicated by solid regression lines.

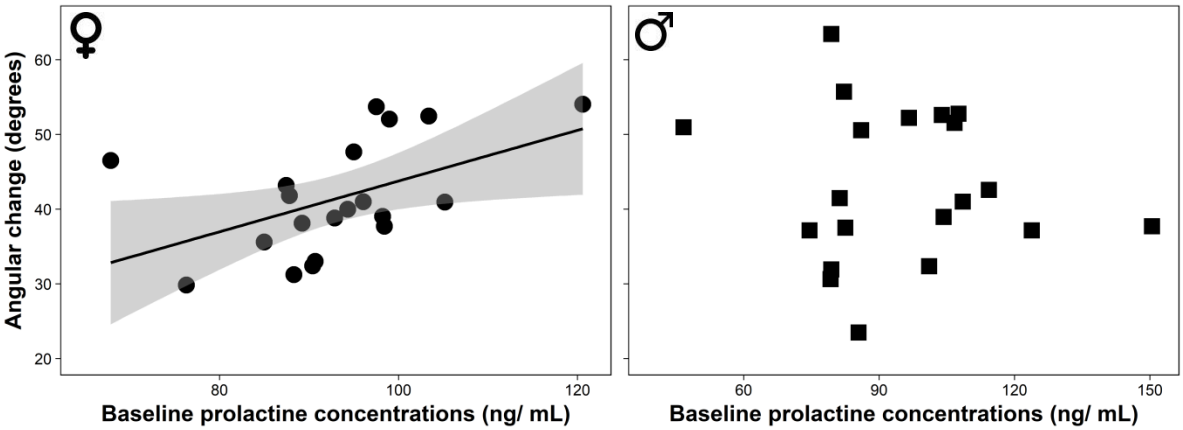


Figure 5

Relationships between plasma ∑PFCAs, blood ∑PCBs concentrations and baseline plasma prolactin levels in female (n =20) incubating kittiwakes, *Rissa tridactyla*, from Kongsfjord, Svalbard. Associations were tested with LMs and significant relationships are indicated by solid regression lines.

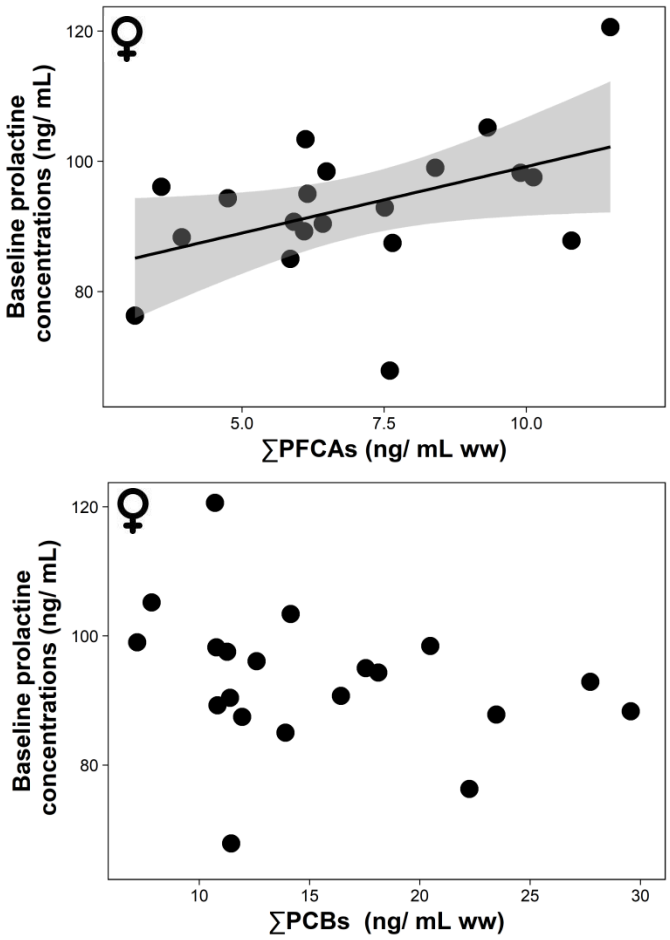
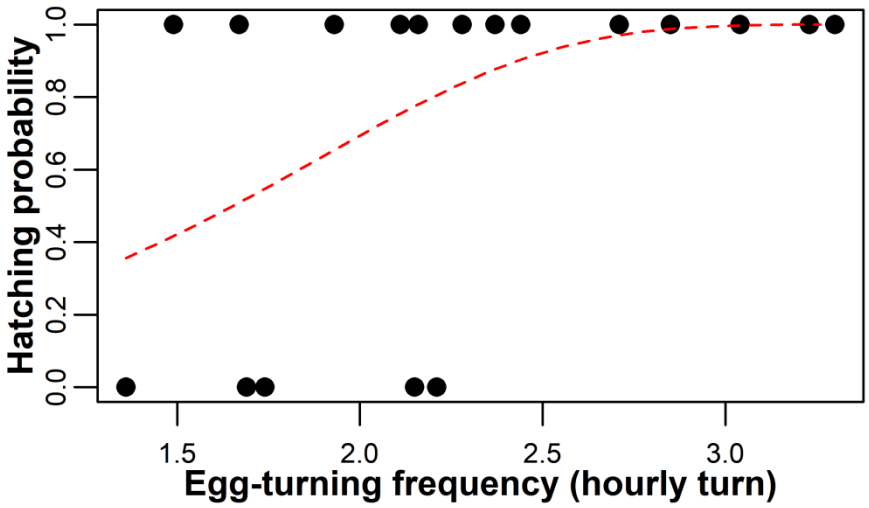


Figure 6

Hatching probability (0 = not hatched; 1 = hatched) of the remaining egg in the experimental nests (n =20) in relation to egg-turning frequency (calculated by meaning the frequency value of both partners in each nest). The association was tested with GLM and the dashed line indicated a marginally significant relationship.



Paper VI

Blévin, P., Tartu, S., Angelier, F., Leclaire, S., Bustnes, J.O., Moe, B., Herzke, D., Gabrielsen, G.W., Chastel, O.

Integument colouration in relation to persistent organic pollutants and body condition in arctic breeding black-legged kittiwakes (*Rissa tridactyla*)

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Integument colouration in relation to persistent organic pollutants and body condition in arctic breeding black-legged kittiwakes (*Rissa tridactyla*)

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HIGHLIGHTS

- We studied the relationships between POPs burden and integument colouration in an artic seabird.
- Saturation of eye-ring, gapes and tongue was negatively related to POPs' burden.
- Individuals with a better body condition displayed more orange gapes and tongue.
- POPs could affect the amount of carotenoid coloration in this arctic seabird.

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ABSTRACT

Vertebrates cannot synthetize carotenoids de novo but have to acquire them through their diet. In birds, carotenoids are responsible for the yellow to red colouration of many secondary sexual traits. They are also involved in physiological functions such as immunostimulation and immunoregulation. Consequently, carotenoid-based colouration is very often considered as a reliable signal for health and foraging abilities. Although a few studies have suggested that carotenoid-based coloured traits could be sensitive to environmental pollution such as persistent organic pollutants (POPs) contamination, the relationships between pollutants and colouration remain unclear. Here, we examined the relationships between the colouration of carotenoid-based integuments and individual POP levels in pre-laying female black-legged kittiwakes from very high latitudes. In this area, these arctic seabirds are exposed to high POPs contamination. Additionally, we investigated the relationships between colouration and body condition, a frequently used index of individual quality. We found a negative relationship between POP levels and several components of integument colouration: saturation of eye-ring, gapes and tongue, suggesting that POPs could disrupt colouration of labile integuments in female kittiwakes. In addition, we found that females in better body condition displayed more orange and brighter gapes and tongue than females in poor body condition. These results demonstrate that hue and brightness are sensitive to the current health and nutritional status of female kittiwakes. Overall, our study shows that carotenoid-based colour integuments can be affected by several environmental-driven variables.

1. Introduction

Many animals exhibit elaborate ornamental traits such as colourful skin, feathers and cuticles that evolved as quality signals. Those signals can have an impact on the fitness of an individual by influencing the behaviour of mates or opponents (Andersson, 1994; Møller et al., 2000). Carotenoids represent one of the central components of colour signals used in animal communication, and thus are highly involved in social behaviours of many species (Møller et al., 2000; Olson and Owen, 1998). In birds, carotenoids are responsible for the yellow to red colouration of many secondary sexual traits (Brush, 1990). Mate choice studies have shown that the most preferred individuals are often those expressing greater carotenoid pigmentation in sexual signals (Amundsen and Forsgren, 2001; review in Hill, 2006). Although the antioxidant property of carotenoids appears to be controversial for birds

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(Costantini and Møller, 2008; Hartley and Kennedy, 2004; Krinsky, 2001), they are involved in other physiological functions such as immunostimulation and immunoregulation (Blount et al., 2003; Chew and Park, 2004; Faivre et al., 2003; review in Møller et al., 2000). Thereby, they enhance T- and B-lymphocyte proliferative responses, stimulate effector T-cell function, enhance macrophage and T-cell capacities, increase the population of specific lymphocyte subpopulations and stimulate the production of various cytokines and interleukins (Bendich, 1989; Chew, 1993). They also maintain the structural integrity of immune cells by removing free radical molecules that are produced through normal cellular activity, and also through environmental stressors (Chew, 1996). Consequently, carotenoids promote survival (immunity, antioxidant capacity) suggesting that a trade-off may exist between allocations of carotenoids towards sexual ornaments signalling versus physiological functions for self-maintenance (Eraud et al., 2007; Pérez et al., 2010a; Von Schantz et al., 1999). It is widely assumed that condition-dependence is a common feature of sexual displays (Kristiansen et al., 2006; Martinez-Padilla et al., 2007; Mougeot et al., 2006, 2007; Pérez-Rodríguez and Viñuela, 2008; Velando et al., 2006). This implies that healthy individuals should require fewer carotenoids for immune defences and could therefore allocate more of this limited resource to enhance sexual signals, thereby indicating of a high-quality mate. Several studies have already highlighted some correlational evidences between carotenoid-based colouration and body condition, a frequently used index of individual quality (Birkhead et al., 1998; Bustnes et al., 2007; Massaro et al., 2003; Mougeot et al., 2006, 2007; Pérez-Rodríguez and Viñuela, 2008; Pérez et al., 2010b). As birds cannot synthetize carotenoids de novo, they have to acquire them through their diet and thus, carotenoid pigmentation depends on the quality and/or quantity of food ingested (Goodwin, 1986). Consequently, carotenoid-based colouration can be considered as a reliable signal of health and foraging abilities (Olson and Owens, 1998).

In addition to this effect of body condition on carotenoid-based colouration, a few studies have suggested that environmental pollution could also affect, and more precisely disrupt, the expression of avian colouration (Eeva et al., 1998; Pérez et al., 2010a). For example, persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and pesticides appear to reduce the expression of carotenoid-based colouration. Thus, captive American kestrels (*Falco sparverius*) exposed to an enriched-PCB diet showed a disruption of both plasma carotenoid concentration and colouration of ceres and lores (Bortolotti et al., 2003). However, this effect of POPs on colouration does not seem equivocal since, Bustnes et al. (2007) did not find any relationships between POP levels and integuments' colouration in free-living great black-backed gulls (*Larus marinus*). Thereby, this discrepancy emphasizes the importance of conducting further studies on the potential deleterious impacts of POPs contamination on carotenoid-based colouration.

The black-legged kittiwake *Rissa tridactyla* is a long-lived and monogamous seabird. Males are bigger than females (Helfenstein et al., 2004; Jodice et al., 2000) but no sexual chromatic dimorphisms are found (Doutrelant et al., 2013; Leclaire et al., 2011a). Both sexes show intense carotenoid-based colouration during the breeding season (Doutrelant et al., 2013; Leclaire et al., 2011a), including the red eye-ring, red/orange gapes, orange tongue and yellow bill. Recent studies have shown that these integuments could reflect individual quality in both sexes (Doutrelant et al., 2013; Leclaire, 2010; Leclaire et al., 2011a,b). In the Arctic, black-legged kittiwakes are exposed to POPs which are known to act as endocrine disruptors and to have a negative impact on reproductive performances (Bustnes et al., 2003, 2008; Helberg et al., 2005; Nordstad et al., 2012). Black-legged kittiwakes are therefore excellent models to investigate the relationships between POPs and carotenoid-based colouration in free-living birds. In that context, the specific aims of the present study were to evaluate the potential correlates of individual POP levels on integument carotenoid-based colouration (eye-ring, gapes, tongue and bill) in pre-laying female kittiwakes from Svalbard. We predicted that females bearing high POP levels would show a reduced expression of integument colouration. In addition, we also examined the correlates between body condition and integument colouration since body condition could reflect individual quality in this species. According to previous studies (Doutrelant et al., 2013; Leclaire, 2010; Leclaire et al., 2011a,b), we predicted that females with a better body condition would display the most colourful integuments.

2. Materials and methods

2.1. Study area and sample collection

Fieldwork was carried out in 2011 from May 21st to June 7th in a colony of black-legged kittiwakes at Kongsfjorden, (Krykkjefjellet, 78°54′ N, 12°13′E), Svalbard. POP analyses were conducted only for females, thus males were not included in this study. Individuals ($n = 28$) were caught on their nest with a noose at the end of a 5 m fishing rod during the pre-laying period (i.e. the courtship and mating period). Females were attending the colony, on their nest on cliffs at a height of 5–10 m during the pre-laying period (i.e. before egg-laying). Birds were individually marked with white PVC plastic bands engraved with a three-letter code and fixed to the bird's tarsus. Thus, kittiwakes could be identified from a distance without perturbation. At capture, blood samples (2.5 mL) were collected from the alar vein using a heparinized syringe and a 25G needle for the determination of blood POP concentrations and molecular sexing. Then, birds were weighted to the nearest 2 g with a Pesola spring balance and skull length (head + bill) was measured with an accuracy of 0.1 mm using a calliper. Kittiwakes were marked with spots of dye on the forehead to distinguish them from their partner during subsequent observations and were released. Using a mirror at the end of an 8 m fishing rod, we checked the whole plot every two days to monitor the subsequent reproductive status of the sampled females (pre-laying breeders were the birds that laid at least one egg after the sampling period). Blood samples were stored at 20 °C until subsequent analyses. Sex was determined at the Centre d'Etudes Biologiques de Chizé (CEBC), by polymerase chain reaction (PCR), as detailed in Weimerskirch et al. (2005).

2.2. POPs' analyses

POPs were analysed from whole blood at the Norwegian Institute for Air Research (NILU) in Tromsø. The following compounds were analysed: the PCBs (CB-28, -52, -99, -101, -105, -118, -128, -138, -153, -180, -183, -187 and -194), and the pesticides (p,p-DDE, α-, β-, γ-HCH, HCB, oxychlordane, trans-, cis-chlordane, trans-, cis-nonachlor). Congeners detected in less than 70% of the samples were removed from the data set (Noël et al., 2009). Thereby, those remaining for further investigations were the PCBs (CB-99, -105, -118, -128, -138, -153, -180, -183, -187 and -194), and the pesticides (p,p-DDE, HCB, oxychlordane, trans-chlordane, trans-, cis-nonachlor). To a blood total sample of 0.5 to 1.5 mL, a 100 μL internal standard solution was added (^{13}C-labelled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). The sample was extracted twice with 6 mL of n-hexane, after denaturation with ethanol and a saturated solution of ammonium sulphate in water. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975C MSD were performed as described by Herzke et al. (2009). The limit for detection was threefold the signal-to-noise ratio, and for the compounds investigated the limit ranged from 0.4 to 122 $pg \cdot g^{-1}$wet weights (ww). For validation of the results, blanks (clean and empty glass tubes treated like a sample, 3 in total) were run for every 10 samples, while standard reference material (3 in total, 1589a human serum from NIST) was run for every 10 samples. The accuracy of the method was within the 70 and 108% range.

2.3. Colour measurements

Integument colouration was measured from digital photographs as detailed in Montgomerie (2006). Pictures were taken at a standard

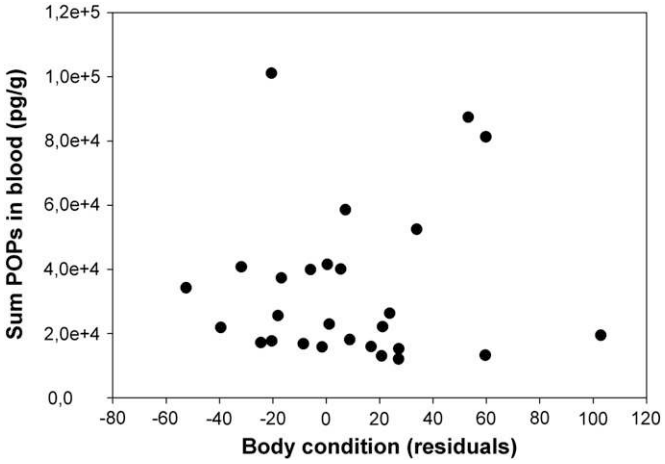

Fig. 1. Relationship between ΣPOPs (pg·g ^{1} ww) in blood and body condition of pre-laying female black-legged kittiwakes.

distance of approximately 40 cm using a digital camera (Olympus U770sw, s770sw) with flash. For each photograph, the same colour swatch was placed next to the bird to standardize subsequent measurements. Prior to photograph analysis, low quality pictures (due to ambient lighting variations) were removed from the data set, thus individuals used for one given integument can partially be different for another one. All pictures were analysed using Adobe Photoshop v 12.0. The average components of red (R), green (G) and blue (B) were recorded within the whole area of the eye-ring and in a standardized selected area for the gapes, tongue and bill. Each component was assessed 3 times to ensure a good repeatability of the measurement (relative standard deviation of <5%, in all cases). RGB system was then converted into hue (H), saturation (S) and brightness (B). The HSB values of each integument were corrected according to the HSB values of the colour swatch. This system is by far the most commonly reported tristimulus colour variables measured in the study of bird colouration and is extensively commented in literature (Montgomerie, 2006).

Such human-oriented model presents some inaccuracies and is only an approximation since gulls and other birds have a tetrachromatic vision and can perceive UV light (Cuthill, 2006; Hastad et al., 2009).

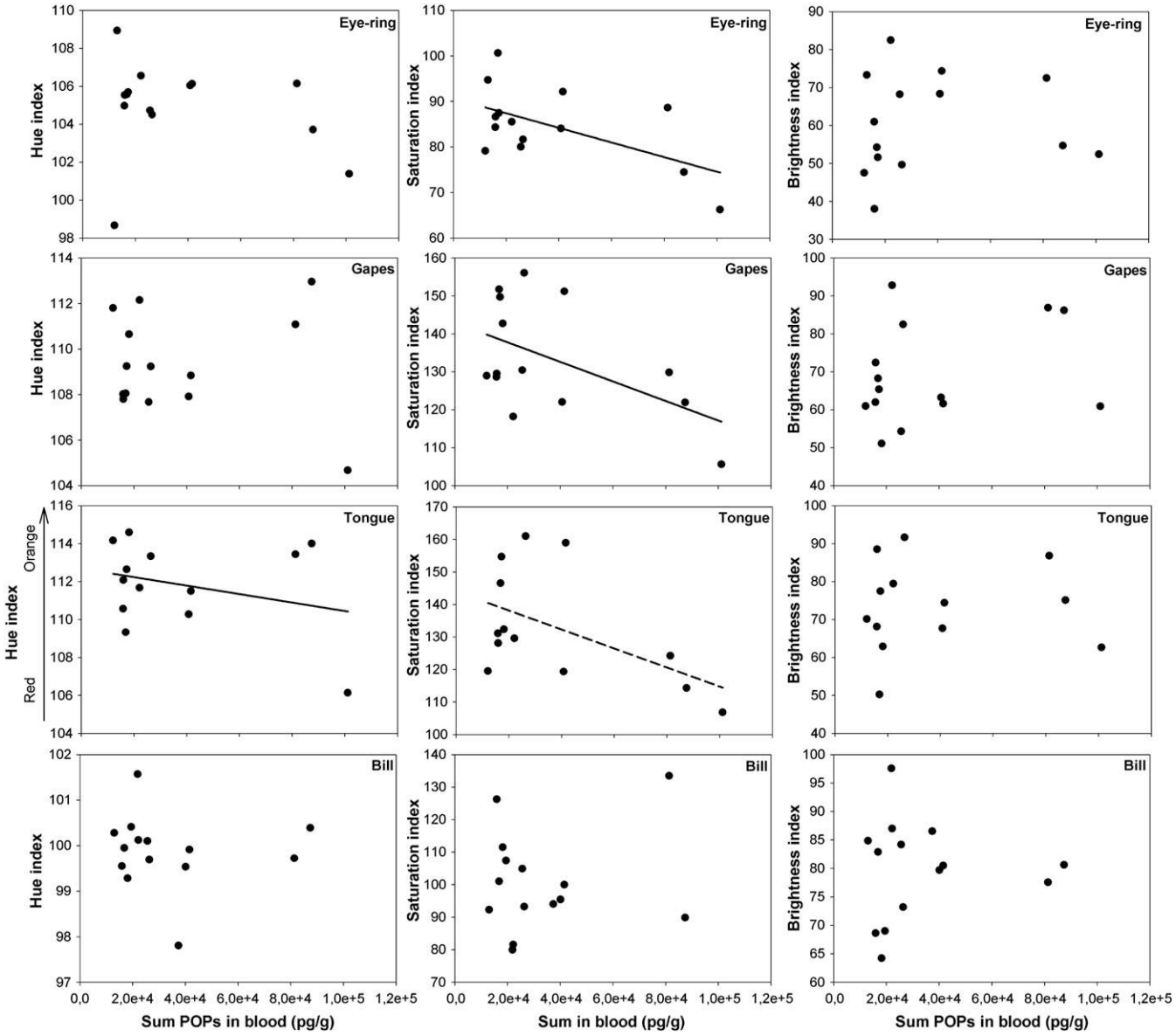

Fig. 2. Relationships between colouration parameters (hue, saturation, brightness) and ΣPOPs (pg·g ^{1} ww) in blood of pre-laying female black-legged kittiwakes for all integuments. Solid line represents significant relationship ($P < 0.05$) and dashed line represents marginally significant relationship ($P < 0.1$).

However, this method has already been investigated on black-legged kittiwakes (Leclaire, 2010; Leclaire et al., 2011a,b) and information obtained from digital pictures is still very useful as it reveals patterns and effects of biological meaning (Alonzo-Alvarez et al., 2004; Bortolotti et al., 2003; Kilner, 1997; Leclaire, 2010; Leclaire et al., 2011a,b; Massaro et al., 2003; Mougeot et al., 2007; Pérez-Rodríguez and Viñuela, 2008; Pérez et al., 2010b). Furthermore, using photography rather than spectrometry might be advantageous because digital photography is a much easier method for quantifying colouration of wet, hard to reach, and irregular surfaces such as gapes, irises and bills (Montgomerie, 2006).

2.4. Statistical analyses

Statistical tests were performed using R 2.14.1 (R Core Team, 2012). ΣPCB and ΣPesticides were highly significant and positively related (Pearson correlation, $r = 0.907$, $t = 10.756$, $P < 0.001$, $n = 27$). Consequently, continuous explanatory variables were defined as follow: body condition (i.e. the residuals of the regression of body mass against skull length) and ΣPOP concentrations (the sum of PCB and pesticides). We first tested the relationship between these two variables using a Pearson correlation. Then, the influence of POPs' contamination and body condition on colouration parameters was investigated with General Linear Models (GLMs). HSB values of each integument as independent variables were log transformed and models were constructed with a normal distribution and an identity link function. Explanatory variables were both included simultaneously in each model. Diagnostic plots were then assessed to test whether the data sufficiently met the assumption of the linear model. A significance level of $\alpha < 0.05$ was used for all tests.

3. Results

Individual POPs' concentration ranged from 1.21×10^4 to 1.01×10^5 pg·g^{-1} ww in whole blood. Body condition was not related to ΣPOP levels (Pearson correlation: $r = 0.06$, $t = 0.306$, $P = 0.766$, $n = 27$, Fig. 1). Saturation of eye-ring and gapes decreased significantly with ΣPOPs and a similar relationship, although not statistically significant was found between tongue's saturation and ΣPOPs (Fig. 2; Table 1), i.e. the most contaminated individuals were those displaying a reduced saturation of their labile integuments. Hue of tongue was negatively related to ΣPOPs but the relation seems to be driven by the presence of an outlier (Fig. 2; Table 1). Besides, hue and brightness were not related to ΣPOPs for all integuments (GLMs: all P-values > 0.315, Fig. 2; Table 1). Hue of gapes and tongue significantly increased with increasing body condition, i.e. individuals with a better condition displayed more orange gapes and tongue (Fig. 3; Table 1). We found a significant increase of brightness of the gapes with increasing body condition, and a similar trend, although not statistically significant was found between the brightness of the tongue and body condition i.e. individuals in better body condition displayed brighter gapes and tongue (Fig. 3; Table 1). By contrast, brightness of the bill decreased in birds with higher body condition (Fig. 3; Table 1). No significant relationships were found between body condition and saturation for all integuments (GLMs: all P-values > 0.116) (Fig. 3; Table 1).

4. Discussion

The results of this study first indicated a negative relationship between POP levels and saturation of labile integuments (i.e. eye-ring, gapes and tongue). This suggests that POPs could affect integument carotenoid-based colouration of kittiwakes. Secondly, body condition was positively related to hue and brightness for gapes and tongue implying that these colour parameters are sensitive to current nutritional conditions and health of individuals, as previously found in this species (Doutrelant et al., 2013; Leclaire, 2010; Leclaire et al., 2011a,b).

Table 1
Effects of ΣPOPs and body condition on colour parameters (hue, saturation and brightness) of carotenoid-based integuments in pre-laying female black-legged kittiwakes.

Independent variables		Dependent variables	*df*	*F*	P-value
Hue	Eye-ring	ΣPOPs	1,11	0.600	0.455
		Body condition	1,11	0.008	0.929
	Gapes	ΣPOPs	1,11	2.678	0.130
		Body condition	1,11	18.681	**0.001**
	Tongue	**ΣPOPs**	1,10	6.431	**0.030**
		Body condition	1,10	15.197	**0.003**
	Bill	ΣPOPs	1,11	0.098	0.761
		Body condition	1,11	0.143	0.713
Saturation	Eye-ring	**ΣPOPs**	1,11	6.978	**0.023**
		Body condition	1,11	0.522	0.485
	Gapes	**ΣPOPs**	1,11	5.019	**0.047**
		Body condition	1,11	0.256	0.623
	Tongue	ΣPOPs	1,10	4.140	0.070
		Body condition	1,10	0.006	0.940
	Bill	ΣPOPs	1,11	0.021	0.888
		Body condition	1,11	1.396	0.262
Brightness	Eye-ring	ΣPOPs	1,11	0.087	0.774
		Body condition	1,11	0.001	0.977
	Gapes	ΣPOPs	1,11	0.233	0.639
		Body condition	1,11	7.080	**0.022**
	Tongue	ΣPOPs	1,10	0.091	0.769
		Body condition	1,10	3.503	0.091
	Bill	ΣPOPs	1,11	1.333	0.273
		Body condition	1,11	6.355	**0.028**

Significant variables are in bold.

4.1. POPs and integument colouration

We found that POP levels negatively affected saturation of labile integuments. This colour parameter is usually assumed as a proxy of the amount of carotenoids present in tissues (Montgomerie, 2006) when colour is produced by only one pigment. However, integuments' colouration of kittiwakes results from a mix of different carotenoid species (Doutrelant et al., 2013) and, therefore, a same saturation can be obtained from mix of carotenoid species at different concentrations. This also means that equivalent amounts of carotenoids may produce different saturations depending on the exact composition of the mix of carotenoids. Consequently, POP levels could affect saturation either by decreasing the amount of pigments and/or by modifying the carotenoid species' composition present in integuments. Thereby, our study provides evidences that POPs' contamination can affect integument carotenoid-based colouration. This is consistent with previous work since Pérez et al. (2010a) showed that organic compounds negatively influence the red bill spot size of adult yellow-legged gulls (*Larus michahellis*) during the courtship period. Moreover, Bortolotti et al. (2003) found that colouration of ceres and lores was disrupted by an enriched-PCB diet in captive American kestrels: exposed males were duller than controls, and juveniles of both sexes were brighter in winter. By contrast, Bustnes et al. (2007) did not find any relationship between POP levels and integuments' colouration in adult breeding great black-backed gulls (*Larus marinus*). Consequently, relationships between POPs and colouration seem to be complex and future studies including further parameters such as period, sex or stage might provide clearer information.

Carotenoids are thought to promote survival (immunity, antioxidant capacity) and might be mobilised to overcome the harmful effects of POPs' ingestion on immunity (Bustnes et al., 2004; e.g. Pérez et al., 2010a; Sagerup et al., 2009) at the expense of coloured sexual signals. Under this scenario, female kittiwakes with the highest POP levels could allocate preferentially their carotenoids towards protective physiological functions (immunity, antioxidant capacity) whereas female kittiwakes with the lowest POP levels could allocate preferentially the available carotenoids towards sexual signalling. These results are thus consistent with the existence of a trade-off between allocations of carotenoids towards sexual ornaments signalling versus physiological functions for detoxification processes (Pérez et al., 2010a). However, we did not

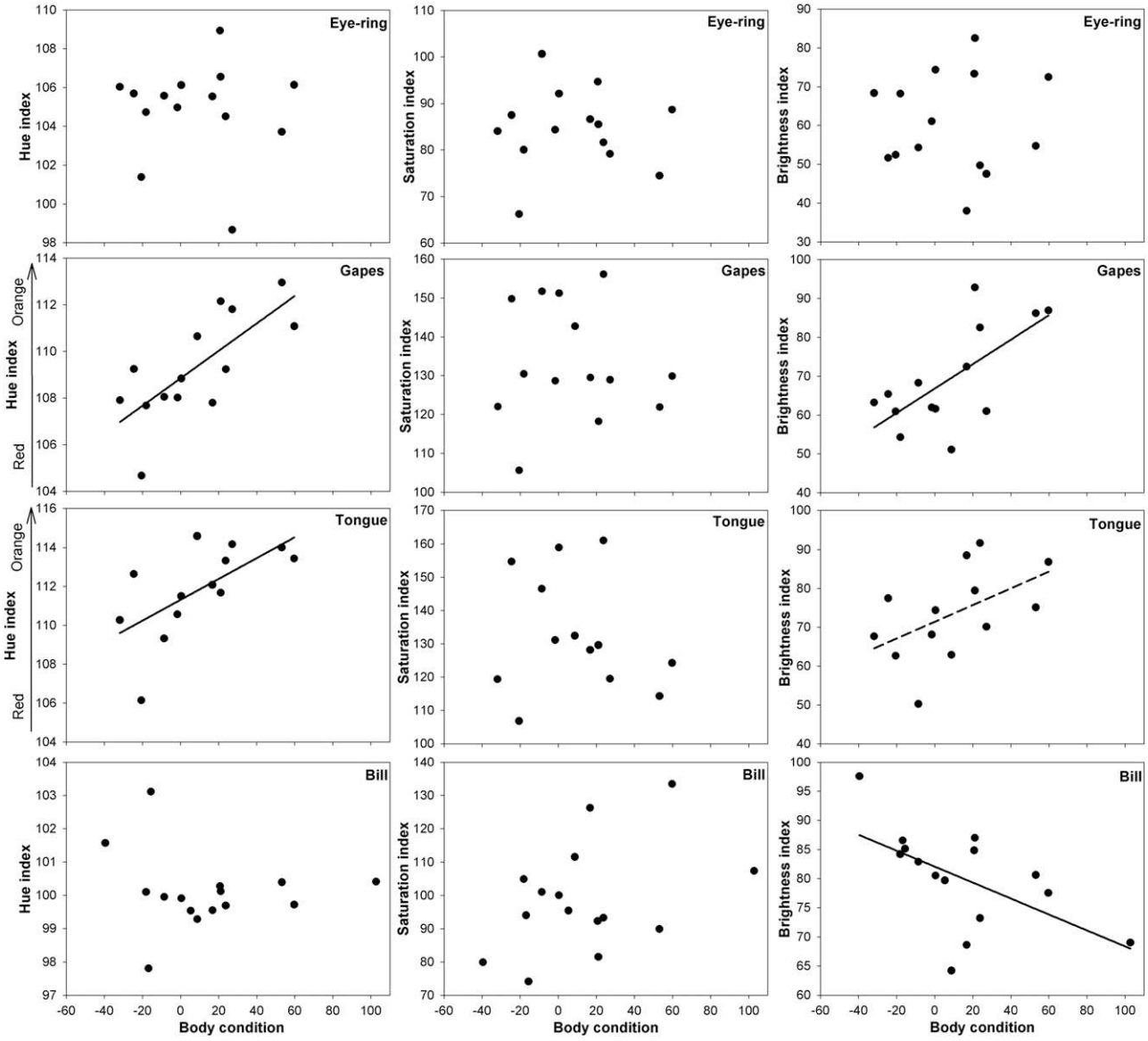

Fig. 3. Relationships between colouration parameters (hue, saturation, brightness) and body condition (residuals) of pre-laying female black-legged kittiwakes for all integuments. Solid line represents significant relationship ($P < 0.05$) and dashed line represents marginally significant relationship ($P < 0.1$).

perform any physiological analysis in our study and, thus, the existence of this trade-off could only be confirmed by coupling integument and plasma carotenoid measurements and POP levels in future studies.

4.2. POPs and body condition

Inter-individual variations in POP levels potentially originated from different foraging behaviours. Indeed, variations in POP levels between birds could be related to the foraging areas used by kittiwakes, i.e. during the pre-laying period, birds forage in oceanic and coastal areas (GPS tracking: Goutte et al., unpublished data). It could also be related to the type of prey ingested, i.e. contaminant levels increase with trophic position according to the biomagnification process (Kelly et al., 2007). Besides, POPs' contamination is usually negatively related to the body condition (Bustnes et al., 2010; Henriksen et al., 1996; Kenntner et al., 2003; Nordstad et al., 2012); organic pollutants are lipophilic and if body fat reserves are low, POPs can be redistributed in internal tissues through the bloodstream (Fuglei et al., 2007). The lack of relationship between POP levels and body condition in our study may be related to an overall sufficient body condition of pre-laying females avoiding a redistribution of POPs in internal tissues.

4.3. Body condition and integument colouration

We reported that body condition was positively related to gapes and tongue hue and brightness, suggesting a beneficial effect of the current condition of kittiwakes on colouration. These results are consistent with the literature since Doutrelant et al. (2013) have shown that kittiwakes displayed brighter and more orange gapes when in better body condition. Animals are thought to absorb carotenoids and other dietary lipids (e.g. fats, oil) through the gut lining via passive diffusion (Parker, 1996; but see During et al., 2002). By doing so, they mix carotenoids with bile salts and fatty acids to form micelles that migrate through the intestinal mucosa and are incorporated into chylomicrons to be secreted into lymph. Then, these micelles enter into the blood where they are transported via lipoproteins (Furr and Clack, 1997). During poor nutritional conditions, the amount of lipids and lipoproteins is reduced (Alonzo-Alvarez and Ferrer, 2001) and this may reduce the extraction yield of carotenoids from food (Solomons and Bulux, 1993), which in turn results in a reduction of circulating carotenoids and, ultimately, in a reduced transfer of carotenoids into integuments. Consequently lower hue and brightness may be related either to poor individual foraging efficiencies (birds in poor body condition ingesting less and/or low

quality food), either to poor environmental quality and thus, to nutritional conditions (e.g. Leclaire, 2010). In addition, metabolic pathways may also be condition-dependent. Before being deposited into integuments, ingested carotenoids may be reduced through the activation of metabolic pathways that may depend on the birds' body condition (Hill, 2000; McGraw et al., 2005). Finally, the relationship between body condition and integument colouration might be related to the current physiological condition of individuals, i.e. the most colourful kittiwakes are those with the best immunological status. This implies that healthy individuals should require fewer carotenoids for immune defences and could therefore allocate more carotenoids to enhance colouration (Pérez et al., 2010a; Pérez-Rodríguez and Viñuela, 2008). However, this hypothesis could only be confirmed by measuring carotenoids in integuments and plasma coupled to the measure of immunological parameters.

Each relationship between POP levels and saturation was very similar among labile integuments. Similarly, relationships between body condition and colouration parameters (hue and brightness) for gapes and tongue suggest that POPs and body condition affect in the same way each labile integuments colouration. Contrary to fleshy integuments with rapid colour changes, e.g. 48 h for the skin of the blue-footed booby *Sulax nebouxi* (Velando et al., 2006), the bill is a keratinized structure and the turnover of carotenoids deposited in the bill is obviously slower (Pérez-Rodríguez and Viñuela, 2008). Moreover, body condition and POP levels of kittiwakes from the studied colony are known to vary rapidly through the breeding season (Moe et al., 2002; Nordstad et al., 2012). Therefore, the faster turnover of eye-ring, gapes and tongue colouration compared to that of bill may explain why we only observed relationships between the current condition of birds (POPs' level and body condition) and labile integuments colourations.

5. Conclusion

The present study provides the first evidence of a potential effect of individual POP levels on integument carotenoid-based colouration of black-legged kittiwakes. In addition, it also shows that body condition can explain integument colouration in this species. However, and importantly, body condition and POPs' burden do not seem to act on the same component of integument colouration. Consequently, our results suggest that, in female black-legged kittiwakes, carotenoid-based colour integuments may be sensitive to several independent pressures, such as POPs' contamination, nutritional conditions and current health of individuals.

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Paper VII

Costantini, D., **Blévin, P**., Herzke, D., Moe, B., Gabrielsen, G.W., Bustnes, J.O., Chastel, O.

Higher plasma oxidative damage and lower plasma antioxidant defences in an Arctic seabird exposed to longer perfluoroalkyl acids

In minor revision in **Environmental Research**

Higher plasma oxidative damage and lower plasma antioxidant defences in an Arctic seabird exposed to longer perfluoroalkyl acids

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ABSTRACT

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) may cause detrimental effects on physiological function and reproduction of Arctic animals. However, there is a paucity of information on the link between PFASs and oxidative stress, which can have potential detrimental effects on key fitness traits, such as cellular homeostasis or reproduction. We have examined the correlations between multiple blood-based markers of oxidative status and several perfluoroalkyl acids (i.e., with 8 or more carbons) in male Arctic black-legged kittiwakes (*Rissa tridactyla*) during the pre-laying period. Higher protein oxidative damage was found in those birds having higher concentrations of perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA) and perfluorotetradecanoic acid (PFTeA). Lower plasmatic non-enzymatic micro-molecular antioxidants was found in those birds having higher concentrations of perfluoroundecanoic acid (PFUnA), PFDoA and PFTeA. Effect size estimates showed that the significant correlations between PFASs and oxidative status markers were intermediate to strong. The non-enzymatic antioxidant capacity (including antioxidants of protein origin) was significantly lower in those birds having higher plasma concentration of linear perfluorooctanesulfonic acid (PFOSlin). In contrast, the activity of the antioxidant enzyme glutathione peroxidase in erythrocytes was not associated with any PFAS compounds. Our results suggest that increased oxidative stress might be one consequence of long-chain PFAS exposure. Experimental work will be needed to demonstrate whether PFASs cause toxic effects on free-living vertebrates through increased oxidative stress.

Keywords: Antioxidants, Birds, Kittiwake, PFASs, Svalbard

1. Introduction

Ecotoxicological studies have so far extensively directed their attention toward legacy persistent organic pollutants (POPs) like organochlorine pesticides (OCPs) and polychlorobiphenyls (PCBs) (Stockholm Convention, 2009). In contrast, less attention has been given to the environmental toxicity of other organic contaminants in the Arctic, such as chlorinated paraffins, phthalates, siloxanes or the perfluoroalkyl and polyfluoroalkyl substances (PFASs; AMAP, 2017). Among these, PFASs remain comparatively much less investigated (DeWitt, 2015). PFASs are synthetically manufactured chemicals, produced since the 1950s, that are widely used for numerous industrial and commercial purposes as water repellents and surfactants (e.g., impregnation agents for carpets, papers and textiles, fire-fighting foam, non-stick coating and waterproof clothing) (Kissa, 2001; Jensen and Leffers, 2008). Chemically and thermally stable, PFASs are highly persistent in the environment and have been detected globally in both wildlife and humans (Key et al., 1997; Giesy and Kannan, 2001; Lau et al., 2007; Muir and DeWit, 2010). Because of oceanic currents and atmospheric long-range transport, PFASs and their precursors and breakdown products can reach high latitudes, such as the Arctic Ocean (Giesy and Kannan, 2001; Prevedouros et al., 2006; Butt et al., 2010). Once deposited in the Arctic marine ecosystem, PFASs bio-accumulate in living organisms and bio-magnify along the food webs (Tomy et al., 2004; Kannan et al., 2005; Haukås et al., 2007; Kelly et al., 2009; Fang et al., 2014). Importantly, (i) PFASs have long half-lives, which facilitates their biomagnification through the food webs depending on the species and PFAS congener (Muir and DeWit, 2010); (ii) the long and odd carbon-chain-length PFASs appear to be more bio-accumulative and toxic than the

short and even-chain length PFASs in wildlife (Martin et al., 2004; Verreault et al., 2005; Conder et al., 2008; Berntsen et al., 2017).

It is worthwhile to note that while PFASs have been produced for over 50 years, it is only since late 1990s that their occurrence in the environment has come under scientific scrutiny. PFASs have raised recent concerns about their potential physiological disrupting properties and negative impacts on reproductive fitness in wildlife (multiple species in Bossi et al., 2005; lesser black-backed gull in Bustnes et al., 2008; northern fulmar in Braune et al., 2011; zebrafish in Liu et al., 2011; tree swallow in Custer et al., 2012; black-legged kittiwake and northern fulmar in Nøst et al., 2012; black-legged kittiwake in Tartu et al., 2014a,b; black-legged kittiwake in Blévin et al., 2017a,b; glaucous gull in Melnes et al., 2017; common eider, black guillemot, black-legged kittiwake, glaucous gull, arctic skua and great skua in Haarr et al., 2018).

Increased molecular oxidative damage and disruption of antioxidant defences are suspected as important mechanisms through which PFASs could be detrimental for cell function and, possibly, for organism health (e.g., Marasco and Costantini, 2016). Experimental evidence on laboratory models found that PFASs may increase production of reactive oxygen species (ROS), increase molecular oxidative damage and up- or down-regulate antioxidant defences (Yao et al., 2005; Eriksen et al., 2010; Liu et al., 2011). Further work found that the perfluoroundecanoic and perfluorododecanoic acids (PFUnA and PFDoA, respectively) are equally potent inducers of stress response genes relative to perfluorooctane sulfonic acid (PFOS) and perfluorononanoic acid (PFNA) and that the effect of carbon-chain-length was more important than the functional group in determining oxidative stress (Nobels et al., 2010). There is thus good reason to expect that long-chain PFASs might cause dysregulation of the oxidative homeostasis, leading

96 to accumulation of oxidative damage to key biomolecules like proteins or nucleic acids.
97 However, the effect of PFAS exposure on oxidative stress is almost unknown for wildlife.
98 Nakayama et al. (2008) found changes in antioxidant gene expression in cormorants
99 (*Phalacrocorax carbo*) exposed to PFASs. Sletten et al. (2016) did not find any
100 significant relationship between plasma PFAS concentrations and the activity of the
101 antioxidant enzyme superoxide dismutase in plasma of white-tailed eagle (*Haliaeetus*
102 *albicilla*) nestlings, while Haarr et al. (2018) did not find any significant relationship
103 between PFASs and amount of DNA damage in lymphocytes in common eider
104 (*Somateria mollissima*), black guillemot (*Cepphus grylle*), black-legged kittiwake (*Rissa*
105 *tridactyla*), glaucous gull (*Larus hyperboreus*), arctic skua (*Stercorarius parasiticus*), and
106 great skua (*Stercorarius skua*).

107 Long-lived species, like many polar seabirds that occupy high trophic levels, are
108 exposed to a greater risk of accumulation and sensitivity to high concentrations of
109 contaminants. In Svalbard (European Arctic), a number of studies showed that black-
110 legged kittiwakes (*Rissa tridactyla*, hereafter "kittiwake") are chronically exposed to a
111 complex cocktail of organic contaminants and trace elements, which are known to
112 correlate with physiological metrics, impaired individual fitness and population dynamics
113 (Tartu et al., 2013, 2014a,b; Goutte et al., 2015; Blévin et al., 2016, 2017a,b). It is,
114 however, unknown whether exposure of kittiwakes to PFASs is associated with markers
115 of oxidative damage and antioxidant protection.

116 In this study, we have examined the correlations between blood-based markers of
117 oxidative status and several PFAS compounds in adult male kittiwakes during the pre-
118 laying period, while controlling for a number of potential confounding factors that might
119 affect the oxidative status independently from PFASs (i.e., body condition, body size,

120 both time and day of blood sampling, hormonal status; reviewed in Costantini, 2014). As
121 with the hormonal status, we measured plasma levels of testosterone, baseline
122 corticosterone and luteinizing hormone because prior work found large individual
123 variation among kittiwakes (Tartu et al., 2013, 2014a,b) and significant effects on
124 organism's oxidative status (Costantini, 2014), which could affect the relationships
125 between PFAS and oxidative status markers. We focused on males because this
126 investigation on oxidative stress is part of a larger project aiming at assessing the overall
127 consequences (ornament coloration, fecundity, oxidative stress, sexual hormones) of
128 PFASs exposure in males during the pre-laying stage (nest site defence, pair-bonding,
129 copulation, nest building) a period during which males appear to be sensitive to pollutants
130 (Tartu et al., 2013).
131 We have also examined whether the effect size of the association between each
132 oxidative status marker and each PFAS compound varies according to their carbon-chain-
133 length (C_{8-14}) because the toxicity of PFASs may increase with carbon-chain-length.
134
135 **2. Materials and methods**
136 *2.1 Sampling*
137 Fieldwork was conducted in 2016 on a colony of Arctic kittiwakes at Kongsfjord (78° 54'
138 N; 12° 13' E), Svalbard. Blood samples were collected from 50 adult males during the
139 pre-laying period (courtship and mating period), from 25th May to 6th June. Birds were
140 caught on their nest with a loop at the end of a long pole. Within 3 minutes since capture
141 0.5 ml of blood were taken from the brachial vein using a heparinized syringe and a 25-
142 gauge needle. This blood sample was used to measure oxidative status markers and
143 hormones. Straightaway, a second sample of venous blood (ca. 2 ml) was collected using

144 another syringe and this blood sample was used to assess the PFASs burden. We collected
145 blood in two separate phases because the first one should have been collected within 3
146 minutes from capture in order to obtain a correct value of basal corticosterone and, given
147 that analyses of PFASs require a large volume of blood, a second bleeding was necessary.
148 Then, tarsus, skull (head + bill) and wing length were measured using a calliper (nearest
149 0.1 mm) and body mass was taken using a Pesola spring balance (nearest 5 grams). Blood
150 samples were stored on ice in the field. On average, blood samples were stored on ice for
151 3h40min (min: 1h30min; max 9h55min) before being centrifuged and stored at -80°C.
152 Plasma and red blood cells, obtained after centrifugation, were kept frozen separately,
153 either at -80°C for subsequent oxidative status markers or at -20°C for PFAS analyses.
154 All samples were analysed within 4 months since collection.
155
156 *2.2 Hormone assays*
157 Plasma levels of testosterone, baseline corticosterone and luteinizing hormone (LH) were
158 measured by radioimmunoassay at the Centre d'Etudes Biologiques de Chizé following
159 protocols previously validated (e.g., Jouventin and Mauget, 1996; Tanvez et al., 2004;
160 Lormée et al., 2013). Briefly, testosterone and corticosterone were extracted using diethyl
161 ether and ethyl ether, respectively. Plasma concentrations of all three hormones were
162 measured by radioimmunoassay. As with corticosterone, a commercial antiserum against
163 corticosterone-3-(O-carboxy-methyl) oxime bovine serum albumin conjugate
164 (Biogenesis, UK) was used. The lowest detectable quantities significantly different from
165 zero at a 90% confidence level were 0.05 ng/ml for testosterone, 0.4 ng/ml for
166 corticosterone and 1.7 ng/ml for LH. All samples were analysed in duplicate.
167 Corticosterone and LH were analysed in a single run and the mean coefficient of variation

168 was 4.9 and 12.0%, respectively. Testosterone was analysed in two runs and the
169 coefficients of variation were 11.2 and 11.5%.
170
171 *2.3 Oxidative status markers*
172 One marker of plasma oxidative damage (protein carbonyls), one marker of plasma non-
173 enzymatic antioxidant capacity and one red blood cell antioxidant enzyme (glutathione
174 peroxidase) were measured at the Centre d'Etudes Biologiques de Chizé using standard
175 methods (e.g., Costantini et al., 2013, 2017). Protein carbonyls (marker of oxidative
176 protein damage) were measured using the Protein Carbonyl Colorimetric assay (Cayman
177 Chemical Company, Ann Arbor, USA). This assay is based on the colorimetric method
178 proposed by Levine et al. (1990). A same volume of plasma was used for all samples and
179 the amount of carbonyls was standardised by the plasma protein concentration according
180 to manufacturer's instructions. Protein carbonyls were derivatized to 2,4-
181 dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The
182 absorbance was read at 370 nm. The extinction coefficient for DNPH (0.022/µM/cm) was
183 used to calculate the concentration of protein carbonyls, which was expressed as nmol/mg
184 protein (amount of carbonyls generated per unit of protein) or as total nmol obtained by
185 multiplying the concentration of carbonyls by the concentration of plasma proteins (i.e.,
186 total amount of carbonyls in the sample, which is also dependent on the amount of
187 substrates available, i.e., proteins). The mean coefficient of variation of duplicates was
188 11.5%. The metric expressed as nmol/mg indicates the amount of carbonyls that occurs
189 in a same amount of protein, thus this is standardised by the amount of substrates (i.e.,
190 proteins) that can be carbonylated. The second metric expressed as total amount of
191 carbonyls indicates the total amount of carbonyls that occurs in the tissue, which is

192 influenced by the amount of proteins available. This second metric is also important
193 because accumulation of carbonyls is detrimental for the cells (Halliwell and Gutteridge,
194 2015). The OXY-Adsorbent test (Diacron International, Italy) was used to quantify the
195 non-enzymatic antioxidant capacity of plasma against HOCl. Values were expressed as
196 either mM of HOCl neutralised or as mM of HOCl neutralised/mg protein to estimate the
197 antioxidant potential of micromolecular antioxidants (e.g., vitamins, carotenoids,
198 glutathione) without the contribution of proteins (i.e., non-enzymatic micro-molecular
199 antioxidant capacity). The correlation between OXY values and protein concentration
200 was actually high and significant ($r = 0.76$, $p < 0.001$), which is to be expected because
201 plasma proteins, such as albumin, are prone to react with HOCl. Although free-radical
202 trapping properties vary among proteins, standardising OXY values by the concentration
203 of total plasma proteins gave us some kind of control about the contribution of proteins
204 to OXY. The mean coefficient of variation of duplicates was 9.4%. The Ransel assay
205 (RANDOX Laboratories, UK) was used to measure the activity of the antioxidant enzyme
206 glutathione peroxidase (GPX) in haemolysates (red blood cells diluted with distilled
207 water). Values were expressed as Units of GPX/mg of protein of haemolysate. The mean
208 coefficient of variation of duplicates was 8.1%. The Bradford protein assay (Bio-Rad
209 Laboratories, Hercules, USA) with bovine albumin as a reference standard was used to
210 measure the concentration of proteins in both plasma samples and haemolysates.
211
212 *2.4 PFAS analyses*
213 Perfluoroalkyl acids (sulfonic and carboxylic) were analysed in plasma at the Norwegian
214 Institute for Air Research (NILU) in Tromsø, Norway. The following compounds were
215 analysed in each plasma sample. Sulfonic: perfluoropropanesulfonic acid (PFPS),

perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), perfluoroheptanesulfonic acid (PFHpS), perfluorooctanesulfonamide (PFOSA), perfluorooctane sulfonic acid (PFOSlin), branched perfluorooctane sulfonic acid (PFOSbr), perfluorononane sulfonic acid (PFNS), perfluorodecane sulfonic acid (PFDcS); Carboxylic: perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTeA) and two precursor compounds, the fluorotelomer sulfonates (6:2 FTS and 8:2 FTS). PFASs with concentrations below the limit of quantification (LOQ) were replaced with a value equal to (LOQ × detection frequency) when the detection frequency (percentage of detection) was > 50% (e.g., James et al., 2002). A 0.2 ml aliquot of plasma spiked with internal standards (carbon labeled PFAS) was extracted in methanol (1 ml) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVICarb graphitized carbon absorbent and glacial acetic acid. Extracts were analysed by UPLC/MS/MS. Recovery of the internal standards ranged between 86.3% and 120%. Results were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference material (SRM; 1957 human serum from NIST) run with every 10 samples. The deviation of the target concentrations in the SRM were within the laboratory's accepted range (69 - 119%). Blanks varied between concentrations below the instrument detection limits and 30 pg/g and were applied as the LOD in the form of 3 times the average concentration.

2.5 Body size and body condition

240 A body size index was estimated using the PC1 from a PCA of tarsus, skull and wing
241 length (loadings, 0.73, 0.83 and 0.45, respectively). As far as the body condition, we did
242 not use the ratio of body mass onto body size nor the residuals of a linear regression of
243 body mass onto body size because both indices have been criticized (García-Berthou,
244 2001). Rather we included both body mass and body size index as factors in the models.
245 In doing so, the coefficient estimate of body mass is calculated considering the effect of
246 body size, thus the outcome reflects the effect of body condition on the given marker of
247 oxidative status (García-Berthou, 2001).
248
249 *2.6 Statistical analyses*
250 Generalized linear models were performed using the software STATISTICA 10
251 (StatSoft. Inc., Tulsa, OK, USA) to assess relationships between each oxidative status
252 marker (protein carbonylation, non-enzymatic antioxidant capacity, non-enzymatic
253 micro-molecular antioxidant capacity and glutathione peroxidase) and the following
254 predictor variables: PFASs congener (PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, PFTriA
255 or PFTeA), body size index, body mass, hormonal status, day of blood sampling and time
256 of blood sampling. Correlations among PFASs were very variable (from small 0.32 to
257 high 0.92), with smaller correlations between PFASs of different chain length and
258 stronger correlations between PFASs of similar chain length. Thus, we did not rely on
259 Principal Components Analysis because it would not allow us to (i) capture all the
260 information associated with single congeners and (ii) test whether connections between
261 PFASs and oxidative status markers were dependent on carbon chain length. All predictor
262 variables were scaled to mean of 0 and standard deviation of 1. These predictor variables
263 were included in all models because prior work showed that each of them can be

264 significantly associated with oxidative status markers (reviewed in Costantini, 2014). The
265 two metrics of protein oxidative damage (protein carbonyls per mg of protein and total
266 protein carbonyls) were combined using the PC1 from a Principal Components Analysis
267 (PCA) because they were highly correlated ($r = 0.79$, $p < 0.001$). This was not done for
268 the non-enzymatic capacity of plasma because both variables were not correlated ($r =$
269 0.22, $p = 0.13$). The hormonal status was estimated using the PC1 from a PCA of
270 corticosterone, testosterone and luteinizing hormone (loadings, 0.39, 0.61 and 0.84,
271 respectively). A normal error function and an identity-link function were applied to
272 models of non-enzymatic antioxidant capacity and GPX. A gamma error function and an
273 identity-link function were applied to models of protein carbonyls and non-enzymatic
274 micro-molecular antioxidant capacity. These functions were selected because the model
275 had the best fitting to the dataset according to the Akaike Information Criterion.
276 Preliminary analyses showed that the time elapsed from the collection of blood to its
277 storage was not significantly correlated (p-value > 0.45) with any marker of oxidative
278 status nor hormones, thus it was not further considered in the analyses. Visual inspection
279 of residuals, Q-Q plots and Cook's distance did not highlight any violation of normality
280 and homogeneity of variance nor the presence of outliers (all samples were below a 0.5
281 Cook's distance). The variance inflation factor was always below 2, indicating that
282 multicollinearity was low. The multicollinearity is thought to be high and problematic
283 when the variance inflation factor is higher than 5.

284 The "compute.es package" (Del Re, 2013) in R (R Core Team et al., 2013) was
285 used to calculate the standardized effect size Hedges' g from test statistics of oxidative
286 status markers that had significant associations with PFASs (i.e., protein oxidative
287 damage and non-enzymatic micro-molecular antioxidant capacity). The "forestplots

288 function" of the "metafor package" in R was used to visualise boxplots of effect size and
289 95% confidence interval. Effect sizes were considered to be small (Hedges' g = 0.2,
290 explaining 1% of the variance), intermediate (g = 0.5, explaining 9% of the variance) or
291 large (g = 0.8, explaining 25% of the variance) according to Cohen (1988).
292
293 **3. Results**
294 Concentrations of detectable PFASs are reported in Table 1 together with other variables
295 measured in kittiwakes. Six out of 20 PFASs (i.e. PFOSlin, PFNA, PFDcA, PFUnA,
296 PFDoA, PFTriA) were detectable in all individuals, while one PFAS (i.e. PFTeA) was
297 detectable in 33 out of 50 individuals. PFOSlin, PFUnA and PFTriA concentrations were
298 the highest of all PFASs measured in the investigated samples, with a percentage
299 contribution for each kittiwake ranging from 23.4 to 54.5%, from 20.3 to 34.8% and from
300 11.8 to 35.2%, respectively. The percentage contribution of all other detected PFASs
301 ranged between 0.1 and 10.6%. PFOSA, PFBS, PFPS, PFHxS, PFHpS, PFOSbr, PFNS,
302 PFDcS, PFHxA, PFHpA, PFOA, and the two precursor fluorotelomer sulfonates (6:2 FTS
303 and 8:2 FTS) were below the detection limit in all the investigated samples.
304 Protein oxidative damage was significantly higher in those birds having higher
305 plasma concentration of PFDoA, PFTriA or PFTeA (Table 2). Effect size estimates
306 increased with chain length of PFASs (indicating an increase of protein damage with
307 chain length) and were significantly different from zero) for PFDoA (95% confidence
308 interval: 0.06 to 1.25), PFTriA (95% confidence interval: 0.01 to 1.20) and PFTeA (95%
309 confidence interval: 0.29 to 1.54; Fig. 1). The non-enzymatic micro-molecular
310 antioxidant capacity was significantly lower in those birds having higher plasma
311 concentration of PFUnA, PFDoA or PFTeA (Table 2). There was also a near-significance

312 tendency of the non-enzymatic micro-molecular antioxidant capacity to be lower in birds
313 with higher plasma PFTriA (Table 2). Effect size estimates were larger for longer PFASs
314 (indicating a decrease of micro-molecular antioxidants with chain length) and were
315 significantly different from zero for PFUnA (95% confidence interval: -1.20 to -0.02),
316 PFDoA (95% confidence interval: -1.35 to -0.14) and PFTeA (95% confidence interval:
317 -1.23 to -0.04; Fig. 2). The non-enzymatic antioxidant capacity including the contribution
318 of antioxidant of protein origin was significantly lower in those birds having higher
319 plasma concentration of PFOSlin, but it was not associated with any other PFAS congener
320 (Table 2). The activity of GPX was not associated with any PFAS compounds (Table 2).
321 Finally, our models showed that (i) kittiwakes in poorer body condition had more
322 plasma protein carbonyls, (ii) the non-enzymatic antioxidant capacity was higher in
323 kittiwakes sampled later in the day, and (iii) the non-enzymatic micro-molecular
324 antioxidant capacity was higher in kittiwakes having lower concentrations of hormones.
325
326 **4. Discussion**
327 Our results provide the first evidence in wild vertebrates that the correlation between
328 oxidative status markers and PFASs is stronger for long-chain congeners. We found that
329 male kittiwakes having higher plasma concentrations of long-chain PFASs had more
330 protein oxidative damage and less plasma antioxidants after controlling statistically for
331 potentially confounding variables. The non-enzymatic antioxidant capacity (including
332 antioxidants of protein origin) was significantly lower in kittiwakes having higher plasma
333 concentration of PFOSlin. On the other hand, the activity of glutathione peroxidase in
334 erythrocytes was not related to any PFAS congener. Effect size estimates were
335 intermediate to large, indicating that PFASs explained from 9 to more than 25% of the

variance in protein oxidative damage and non-enzymatic micro-molecular antioxidant capacity of plasma (Cohen, 1988). Intermediate effect sizes are suggested to be biologically meaningful because average proportions of variance explained in ecological, evolutionary and physiological studies is usually below 7% (Møller and Jennions, 2002). Our effect size estimates were also larger than those found in the comparison of oxidative status markers between animals living in polluted (e.g., air pollution, heavy metals) and unpolluted sites (Isaksson, 2010).

PFOSlin, PFUnA and PFTriA concentrations were the highest of all PFASs measured in the investigated samples, with a percentage contribution for each kittiwake ranging from 11.8 to 54.5%. The concentration of PFOSlin was higher than that previously recorded in males from the same kittiwake population in 2012 (13.4 vs. 10.6 pg/g ww in Blévin et al., 2017a). In contrast, the average concentrations of PFUnA (10.3 vs. 12.1 pg/g ww in Blévin et al., 2017a) and of PFTriA (8.6 vs. 11.6 pg/g ww in Blévin et al., 2017a) were both lower in our study than in prior work (Blévin et al., 2017a). One reason for such differences might be because Blévin et al. (2017a) measured PFASs of male kittiwakes caught during the chick rearing phase. Work on male glaucous gulls during the incubation period in Svalbard found levels of PFOSlin similar to ours, while those of PFUnA (4.4 pg/g ww) and of PFTriA (3.9 pg/g ww) were much lower than those we recorded (Melnes et al., 2017).

The strength of the correlation between oxidative status markers and PFASs increased with the chain length of the congener. While persistent in the environment, PFASs with fewer than eight carbons, such as PFHxA, and PFSAs with fewer than six carbons, such as PFBS, are generally less bioaccumulative in wildlife and humans. However, it is still unclear whether chain length affects toxicity. For example,

360 Vongphachan et al. (2011) experimentally found that SC-PFASs altered the expression
361 of TH-responsive genes in chicken embryonic neuronal cells to a greater extent than LC-
362 PFASs. On the other hand, exposure of laboratory animals to long-chain congeners
363 produces detrimental reproductive, developmental, and systemic effects (Lau et al., 2007;
364 Conder et al., 2008; Jensen and Leffers, 2008; Concawe, 2016) and the toxic effects of
365 PFASs on rat brain cells increased with increasing carbon chain length (Berntsen et al.,
366 2017). Interestingly, the toxic effects of PFASs were attenuated by the antioxidant
367 vitamin E, indicating a possible involvement of oxidative stress in the reduction of cell
368 viability (Concawe, 2016). Further work showed that, compared to short-chain PFASs,
369 long-chain PFASs are stronger inducers of the response of genes regulating the cell
370 oxidative status (Nobels et al., 2010), suggesting that they might be able of causing a
371 stronger oxidative insult to the cells, which need to upregulate their antioxidant response.
372 Effects of PFAS exposure on oxidative status have been moderately investigated
373 in laboratory models and almost unexplored in wild animals. Thus, limited information
374 is available for a comparison with our results and interpretation. Protein carbonylation
375 arises from overproduction of ROS by metabolic reactions that use oxygen and shift the
376 balance between oxidant/antioxidant statuses in favour of the oxidants (Halliwell and
377 Gutteridge, 2015). Protein carbonylation also occurs when carbonyls are introduced into
378 proteins through the reactions with lipid oxidative damage products (malondialdehyde
379 and hydroxynonenal; Halliwell and Gutteridge, 2015). Carbonylation is mostly
380 irreversible and results in alteration of protein structure and function. Only a small
381 fraction of carbonylated proteins can be removed through proteasome-dependent
382 proteolysis. Work carried out on laboratory models found evidence that exposure to
383 PFASs may change expression of genes regulating proteasome activation and proteolysis

(Lau et al., 2007; Nilsen et al., 2008; Zhang et al., 2012). It is, however, unclear, whether such changes in gene expression make proteins one important target of the pro-oxidant effects of PFASs. This is important because when protein carbonyls accumulate, they tend to aggregate leading to cell death, tissue injury and development of disorders. Several studies found higher amounts of plasma protein carbonyls in individuals affected by a given disease (Winterbourn et al., 2003; Hlaváčková et al., 2017), suggesting a potential role of protein carbonylation in disease progression.

Depletion of circulating non-enzymatic antioxidants might reflect increased oxidation due to reaction with ROS, reduced intake from diet or mobilisation of antioxidants from blood to other target tissues. Irrespective of the reason, prior work on other bird species found evidence that circulating antioxidants may be linked to important individual or population fitness-related traits. For example, Saino et al. (2011) found that barn swallows (*Hirundo rustica*) with lower plasma non-enzymatic antioxidants had reduced probability of survival. Beaulieu et al. (2013) found that Gentoo (*Pygoscelis papua*) and Adélie (*Pygoscelis adeliae*) penguins from increasing populations had higher plasma non-enzymatic antioxidant capacity than those from decreasing populations.

Metabolic activity is one important source of ROS production. Thus the association we found between PFASs and oxidative status markers might mirror an effect of PFASs on metabolism. Prior work on the same kittiwake population found a positive association between the long-chain PFTriA and resting metabolic rate in females but not in males (Blévin et al., 2017b). Thus, the association between PFASs and oxidative status markers does not appear, at least for males, to be due to a dysregulation of general body metabolism. The lack of an effect on metabolic rate does not reject the hypothesis that ROS production might have been higher in the more contaminated birds. For example,

PFASs might have localised effects on important ROS generators (e.g., mitochondria of red blood cells or of other target tissues) without compromising the metabolism of the whole organism. *In vitro* studies found that PFAS exposure may impair mitochondrial activity and lead to increased rates of reactive oxygen species production (O'Brien and Wallace, 2004).

It is unclear why the activity of glutathione peroxidase was not associated with any PFAS compound. It might be that up-regulation of this enzyme might have been too costly for the birds given the imminent start of breeding activity or that any effects of PFASs on oxidative status did not come through the pathways involving glutathione peroxidase. The biochemical function of glutathione peroxidase is to reduce hydrogen peroxide to water and organic hydroperoxides to their corresponding alcohols (Halliwell and Gutteridge, 2015). Thus, we cannot exclude that results would have been different if another antioxidant enzyme with a different biochemical function would have been measured. There are, however, many discrepancies in the literature about the response of antioxidant enzymes to PFAS exposure. For example, prior work did not find any association between PFASs exposure and whole-body catalase activity in the planktonic crustacean *Daphnia magna* (Li, 2010) or plasma superoxide dismutase activity in white-tailed eagle (*Haliaeetus albicilla*) nestlings (Sletten et al., 2016). In contrast, exposure to PFASs caused induction of antioxidant enzymes in response to oxidative stress and the suppression of molecular chaperones, leading to reduction in protein stability, in cormorants (Nakayama et al., 2008), affected catalase activity in hepatocytes of freshwater tilapia *Oreochromis niloticus* (Liu et al., 2011), expression of antioxidant genes Sod1, Sod2, Gpx2 and Nqo1 in mouse pancreas (Kamendulis et al., 2014). Irrespective of the mechanisms involved, the activity of GPX in erythrocytes does not

appear to be an informative marker about the impact on oxidative status of the PFASs we have measured in this work.

In conclusion, our work shows that higher protein oxidative damage was found in those birds having higher concentrations of PFDoA, PFTriA and PFTeA. Lower plasmatic non-enzymatic micro-molecular antioxidants was found in those birds having higher concentrations of PFUnA, PFDoA and PFTeA. Experimental work will be needed to ascertain whether the correlation between individual PFAS burden and oxidative status markers reflects a direct toxic effect of PFASs on oxidative homeostasis. It will also be important to determine whether increased oxidative damage or decreased antioxidant defences turn into a reduction in survival probability or lifetime reproductive success. Resistance against oxidative stress may also decrease with chronological age (Marasco et al., 2017). Thus, we highlight the importance of assessing in future studies whether the effects of PFASs on oxidative status markers are stronger in older individuals.

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Conflict of interest

The authors declare no competing financial interests.

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677

678 Table 1. Descriptive statistics of all perfluoroalkyl substances, oxidative status markers
679 (i.e., responde variables) and several individual metrics (potential confounding factors)
680 measured in 50 male black-legged kittiwakes from Svalbard.

Variable	**Mean ± Standard deviation**
CONTAMINANT	
PFOSlin - Linear perfluorooctane sulfonic acid (ng/g ww)	13.4 ± 6.2
PFNA - Perfluorononanoic acid (ng/g ww)	2.0 ± 0.9
PFDcA - Perfluorodecanoic acid (ng/g ww)	2.9 ± 1.2
PFUnA - Perfluoroundecanoic acid (ng/g ww)	10.3 ± 3.7
PFDoA - Perfluorododecanoic acid (ng/g ww)	1.7 ± 0.8
PFTriA - Perfluorotridecanoic acid (ng/g ww)	8.6 ± 3.1
PFTeA - Perfluorotetradecanoic acid (ng/g ww)	1.0 ± 0.8
OXIDATIVE STATUS MARKERS – RESPONSE VARIABLES	
Protein carbonyls (nmol/mg protein)	1.8 ± 0.6
Total protein carbonyls (nmol)	22.5 ± 8.2
Glutathione peroxidase (Units/mg protein)	0.4 ± 0.2
Non-enzymatic antioxidant capacity (mM HOCl neutralised)	169.9 ± 37.1
Non-enzymatic micro-molecular antioxidant capacity (mM HOCl neutralised/mg protein)	13.0 ± 2.0
INDIVIDUAL TRAITS	
Body mass (g)	416.2 ± 23.1
Skull length (mm)	93.9 ± 1.6
Tarsus length (mm)	34.9 ± 1.0
Wing length (mm)	317.1 ± 6.3
Baseline corticosterone (ng/ml)	9.6 ± 7.4
Testosterone (ng/ml)	1.7 ± 1.4
Luteinizing hormone (ng/ml)	6.2 ± 2.4

681

Table 2. The table shows the outcomes of generalized linear models performed to test the effect of each PFASs congener on oxidative status markers. Each model also included a number of potential confounding factors that may affect oxidative status markers independently from PFASs. Significant effects are shown in bold type. Note that the coefficient estimate for the relationship between body mass and the dependent variable (i.e., oxidative status marker) is calculated considering the effect of body size, thus it actually indicates the covariation between a given marker and the individual body condition (García-Berthou, 2011). GPX = glutathione peroxidase; N-E Antioxs = non-enzymatic antioxidant capacity of plasma; N-E Micromol Antioxs = non-enzymatic micro-molecular antioxidant capacity of plasma; ce ± se = coefficient estimate ± standard error.

	Protein Oxidative Damage			**GPX**			**N-E Antioxs**			**N-E Micromol Antioxs**	
Main effect included in the model	ce ± se	P		ce ± se	P		ce ± se	P		ce ± se	p
PFOSlin	0.03±0.12	0.817		0.02±0.02	0.462		-13.7±5.0	**0.006**		-0.20±0.28	0.484
sampling date	0.11±0.13	0.406		0.03±0.03	0.309		-16.6±5.0	**0.001**		0.18±0.31	0.546
sampling time	-0.20±0.11	0.074		0.03±0.02	0.196		14.3±4.6	**0.002**		0.22±0.28	0.425
body mass	-0.29±0.12	**0.013**		-0.02±0.03	0.407		4.0±5.0	0.424		0.00±0.29	1.000
body size	-0.02±0.11	0.840		0.02±0.02	0.403		-5.5±4.7	0.236		0.10±0.27	0.704
hormonal status	-0.10±0.11	0.391		0.02±0.02	0.348		4.0±4.7	0.391		-0.94±0.28	**0.001**
PFNA	-0.03±0.13	0.852		0.01±0.03	0.764		-1.0±6.0	0.872		-0.09±0.34	0.804
sampling date	0.08±0.15	0.580		0.02±0.03	0.433		-10.7±6.0	0.077		0.22±0.35	0.534
sampling time	-0.20±0.11	0.074		0.03±0.02	0.208		14.2±5.0	**0.004**		0.22±0.28	0.423
body mass	-0.30±0.12	**0.011**		-0.02±0.03	0.395		5.2±5.4	0.336		0.01±0.29	0.977
body size	-0.01±0.11	0.896		0.02±0.02	0.353		-7.4±5.0	0.137		0.08±0.27	0.768
hormonal status	-0.08±0.11	0.460		0.03±0.02	0.262		0.28±4.9	0.953		-0.98±0.27	**<0.001**
PFDcA	0.22±0.13	0.106		0.00±0.03	0.904		-0.65±5.8	0.910		-0.49±0.31	0.116
sampling date	0.22±0.14	0.104		0.02±0.03	0.493		-10.5±5.0	0.075		-0.02±0.33	0.950
sampling time	-0.18±0.11	0.088		0.03±0.02	0.195		14.1±4.9	**0.004**		0.21±0.27	0.438
body mass	-0.22±0.12	0.056		-0.02±0.03	0.407		5.1±5.6	0.357		-0.07±0.29	0.809
body size	-0.03±0.11	0.795		0.02±0.02	0.352		-7.4±5.0	0.140		0.09±0.27	0.723
hormonal status	-0.14±0.11	0.204		0.03±0.02	0.249		0.24±4.9	0.960		-0.92±0.27	**0.001**
PFUnA	0.14±0.13	0.268		0.00±0.03	0.970		-3.3±5.6	0.562		-0.62±0.29	**0.035**
sampling date	0.19±0.14	0.181		0.02±0.03	0.517		-12.0±5.8	**0.038**		-0.07±0.32	0.822
sampling time	-0.18±0.11	0.098		0.03±0.02	0.196		13.9±4.9	**0.005**		0.19±0.27	0.483
body mass	-0.24±0.12	**0.045**		-0.02±0.03	0.403		4.2±5.7	0.453		-0.13±0.29	0.659
body size	-0.03±0.11	0.807		0.02±0.02	0.348		-7.1±5.0	0.154		0.12±0.26	0.644
hormonal status	-0.13±0.11	0.237		0.03±0.02	0.247		0.86±4.9	0.861		-0.87±0.26	**0.001**
PFDoA	0.29±0.13	**0.024**		-0.01±0.03	0.761		-1.5±5.4	0.779		-0.73±0.29	**0.012**
sampling date	0.26±0.14	0.066		0.01±0.03	0.625		-10.9±5.6	0.052		-0.10±0.30	0.736
sampling time	-0.18±0.10	0.088		0.03±0.02	0.199		14.1±4.9	**0.004**		0.21±0.26	0.421
body mass	-0.20±0.11	0.076		-0.02±0.03	0.343		4.9±5.6	0.378		-0.16±0.28	0.563
body size	-0.02±0.11	0.833		0.02±0.02	0.325		-7.2±5.0	0.148		0.14±0.25	0.574
hormonal status	-0.14±0.10	0.176		0.03±0.02	0.220		0.21±4.8	0.965		-0.95±0.25	**<0.001**
PFTriA	0.30±0.14	**0.036**		0.00±0.03	0.923		2.0±5.9	0.735		-0.58±0.32	0.064
sampling date	0.26±0.15	0.075		0.02±0.03	0.597		-8.8±6.1	0.153		-0.12±0.34	0.725
sampling time	-0.18±0.11	0.094		0.03±0.02	0.214		14.5±5.1	**0.004**		0.10±0.27	0.725
body mass	-0.26±0.11	**0.026**		-0.02±0.03	0.368		5.7±5.5	0.299		-0.08±0.28	0.763
body size	-0.06±0.12	0.632		0.02±0.02	0.348		-7.9±5.2	0.124		0.20±0.27	0.454
hormonal status	-0.14±0.11	0.181		0.03±0.02	0.226		-0.13±4.9	0.979		-0.92±0.26	**<0.001**
PFTeA	0.33±0.11	**0.003**		-0.02±0.03	0.482		7.4±5.4	0.171		-0.65±0.29	**0.028**
sampling date	0.22±0.12	0.070		0.01±0.02	0.664		-7.4±5.1	0.145		0.05±0.29	0.852
sampling time	-0.27±0.11	**0.013**		0.03±0.02	0.149		12.3±5.0	**0.015**		0.40±0.27	0.144
body mass	-0.25±0.11	**0.018**		-0.03±0.03	0.317		6.4±5.3	0.227		-0.05±0.28	0.858
body size	-0.05±0.11	0.668		0.03±0.02	0.275		-9.0±5.0	0.072		0.19±0.26	0.461
hormonal status	-0.19±0.10	0.062		0.03±0.02	0.171		-1.9±4.9	0.704		-0.84±0.27	**0.001**

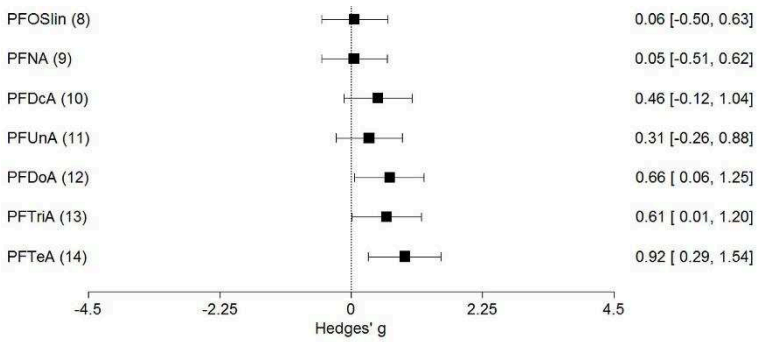
694 **Figure captions**

695 Figure 1. Mean estimates of effect size and 95% confidence interval are shown. These
696 were calculated from the statistical outcomes showing the effect of each PFAS congener
697 on protein oxidative damage. Estimates are positive when values of damage are higher in
698 birds having higher plasma concentration of a given PFAS congener. Note that effect size
699 estimates are significant when the 95% confidence interval does not include zero.
700 Numbers in bracket indicate carbon-chain-length of each PFAS congener.

701

702 Figure 2. Mean estimates of effect size and 95% confidence interval were calculated from
703 the statistical outcomes showing the effect of each PFAS congener on plasma non-
704 enzymatic micromolecular antioxidant capacity. Estimates are negative when values of
705 antioxidants are lower in birds having higher plasma concentration of a given PFAS
706 congener. Note that effect size estimates are significant when the 95% confidence interval
707 does not include zero. Numbers in bracket indicate carbon-chain-length of each PFAS
708 congener.

709



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712 Figure 1

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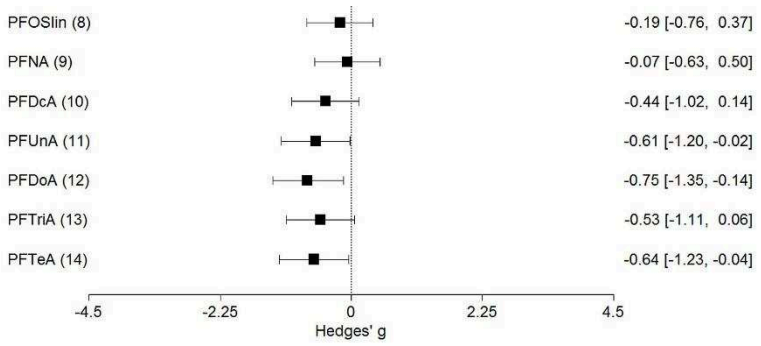

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715 Figure 2

716

Paper VIII

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Do glucocorticoids mediate the link between environmental conditions and telomere dynamics in wild vertebrates? A review

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Review

Do glucocorticoids mediate the link between environmental conditions and telomere dynamics in wild vertebrates? A review

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ABSTRACT

Following the discoveries of telomeres and of their implications in terms of health and ageing, there has been a growing interest into the study of telomere dynamics in wild vertebrates. Telomeres are repeated sequences of non-coding DNA located at the terminal ends of chromosomes and they play a major role in maintaining chromosome stability. Importantly, telomeres shorten over time and shorter telomeres seem to be related with lower survival in vertebrates. Because of this potential link with longevity, it is crucial to understand not only the ecological determinants of telomere dynamics but also the regulatory endocrine mechanisms that may mediate the effect of the environment on telomeres. In this paper, we review the relationships that link environmental conditions, glucocorticoids (GC, the main hormonal mediator of allostasis) and telomere length in vertebrates. First, we review current knowledge about the determinants of inter-individual variations in telomere length. We emphasize the potential strong impact of environmental stressors and predictable life-history events on telomere dynamics. Despite recent progress, we still lack crucial basic data to fully understand the costs of several life-history stages and biotic and abiotic factors on telomere length. Second, we review the link that exists between GCs, oxidative stress and telomere dynamics in vertebrates. Although circulating GC levels may be closely and functionally linked with telomere dynamics, data are still scarce and somewhat contradictory. Further laboratory and eld studies are therefore needed not only to better assess the proximate link between GC levels and telomere dynamics, but also to ultimately understand to what extent GCs and telomere length could be informative to measure the tness costs of speci c life-history stages and environmental conditions. Finally, we highlight the importance of exploring the functional links that may exist between coping styles, the GC stress response, and telomere dynamics in a life-history framework. To conclude, we raise new hypotheses regarding the potential of the GC stress response to drive the trade-off between immediate survival and telomere protection.

1. Introduction: The relevance of studying telomeres in an ecological and evolutionary context

In 2009, Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostack received the Nobel Prize in Physiology and Medicine for the discovery of how chromosomes are protected by telomeres. These discoveries have had an important impact on biomedicine because telomeres and telomere dysfunctions have been closely associated with cancer, ageing and hereditary disease syndromes (Blackburn et al., 2006). Telomeres are well-conserved repeated sequences of non-coding DNA (TTAGGG) located at the terminal ends of chromosomes (Blackburn and Gall, 1978). They play a major role in maintaining chromosome stability during replication processes (Blackburn, 2005). Although telomeres can be restored by the enzyme telomerase, a ribonucleoprotein that adds new sequences onto the ends of chromosomes at each DNA replication (Greider and Blackburn, 1989), telomeres shorten over time and are therefore considered as a molecular marker of cellular ageing (Harley et al., 1990). Importantly, this shortening has been associated with the occurrence of diseases and with increased mortality in humans (Cawthon et al., 2003; Lansdorp, 2009).

Following these discoveries, there has been a growing interest into the study of telomeres in wild animals (Fig. 1). In the early 2000's, Haussmann and his collaborators have initiated the study of telomere biology in an ecological and evolutionary context (Haussmann and Vleck, 2002; Haussmann et al., 2003a; Vleck

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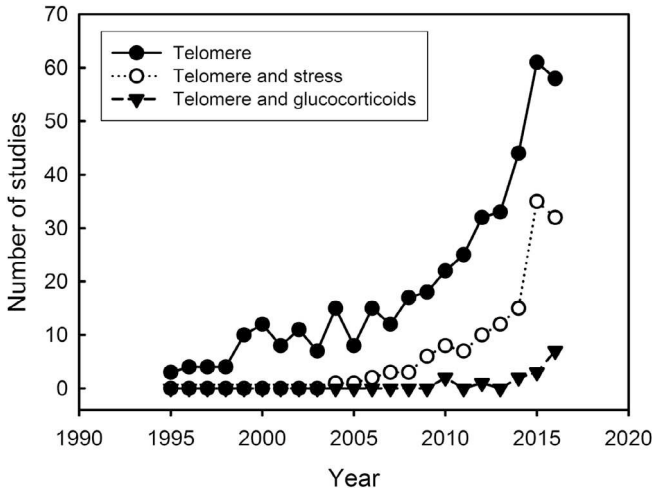

Fig. 1. Trends in the number of studies published each year on different topics from 1995 to 2016. These numbers were found by conducting a search in ISI web of knowledge (search terms, lled circles: telomere, empty circles: telomere and stress, triangles: telomere and glucocorticoids). For all searches, the results were limited to the following categories: ecology, zoology, behavioral sciences, environmental sciences and evolutionary biology.

et al., 2003). By studying telomeres in wild vertebrates, they were the rst to emphasize the relevance of this molecular proxy of ageing to explore life-history trade-offs and selection processes (Haussmann et al., 2003b, 2005; Monaghan and Haussmann, 2006; Vleck et al., 2007; Haussmann and Mauck, 2008). Telomere length was initially thought to be a reliable proxy of age (i.e., "mitotic clock", Haussmann and Vleck, 2002), leading to the idea that it could be a useful tool to obtain detailed information on wild populations. Although the relationship between chronological age and telomere length appears more complex than initially thought (Monaghan, 2010; Dunshea et al., 2011; Boonekamp et al., 2013), there is now strong evidence that telomere length and telomere dynamics are tightly linked to tness (Haussmann and Marchetto, 2010; Bauch et al., 2013; Monaghan 2010, 2014; Ouyang et al., 2016), and especially to longevity and survival in captive and wild vertebrates (e.g. Haussmann et al., 2005; Pauliny et al., 2006; Bize et al., 2009; Salomons et al., 2009; Heidinger et al., 2012; Bauch et al., 2013; Barrett et al., 2013; Asghar et al., 2015a; Fairlie et al., 2016).

These ndings have raised a huge interest in measuring telomere length and telomere dynamics in wild animals (Nussey et al., 2014). Speci cally, several studies have aimed to understand the ecological and life-history determinants of telomere dynamics. Such studies have led to the idea that telomere dynamics are tightly linked to the occurrence of stressors (reviewed in Haussmann and Marchetto, 2010; Monaghan, 2014; Haussmann and Heidinger, 2015; Bateson, 2016; Fig. 1), and more generally to allostasis (*sensu* McEwen and Wing eld, 2003, i.e. maintaining stability through change). As a consequence, there has recently been a growing interest in understanding the links that may exist between the endocrine regulation of allostasis and telomere dynamics in wild vertebrates (reviewed in Haussmann and Marchetto, 2010; Monaghan, 2014; Fig. 1). In that context, glucocorticoids (GCs) obviously deserve a speci c attention because of their involvement in allostasis and the stress response (McEwen and Wing eld, 2003; Wing eld, 2003, 2013; Romero et al., 2009; Angelier and Wing eld, 2013; Wing eld et al., 2015; Romero and Wing eld, 2016). Because of the link between telomeres and survival, integrating telomere dynamics into the framework linking environmental conditions, GCs and life-history decisions may contribute to a better understanding of the life-history/physiology nexus (Zera and Harshman, 2001; Ricklefs and Wikelski, 2002).

In this review, our aim is to emphasize the links that exist among telomere dynamics, environmental conditions and endocrine stress mechanisms in wild vertebrates. We review current knowledge about (1) the determinants of inter-individual variations in telomere length by emphasizing the role of environmental conditions and life-history stages on telomere dynamics; (2) the link that exists between GCs (i.e. the main hormonal mediator of allostasis), oxidative stress and telomere dynamics in wild vertebrates. Finally, we highlight the importance of exploring the functional link that may exist between the GC stress response and telomere dynamics in a life-history framework.

2. Determinants of inter-individual variation in telomere length

2.1. A large inter-individual variability in telomere dynamics through life

In vertebrates, individual telomere length seems to be determined mainly through three steps: pre-natal telomere dynamics, developmental telomere dynamics, and adult telomere dynamics (Fig. 2). Firstly, telomere length is heritable and also affected by environmental, maternal and epigenetic effects (h^2 varying from 0.18 to 1.23 depending on the species, reviewed in Reichert et al., 2015), meaning that all individuals do not start their life with similar telomere length (Shalev et al., 2013). Thus, biomedical studies have rst explored the potential genetic and environmental mechanisms affecting telomere length determination (Njajou et al., 2007; Broer et al., 2013) and, for example, have reported a strong positive in uence of paternal age on offspring telomere length in humans (Unryn et al., 2005; Broer et al., 2013). More recently, ecological studies have con rmed that offspring telomere length is

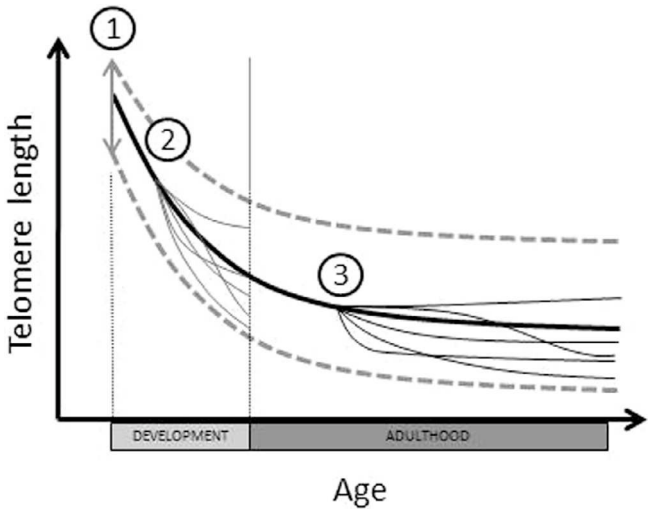

Fig. 2. Schematic representation of individual telomere dynamics through life in wild vertebrates (thick black line): (1) initial telomere length is partly heritable and determined by epigenetic/trans-generational effects; (2) telomeres shorten very quickly during the developmental phase; (3) telomere shortening is much slower during adulthood. For each of these steps, there is an important inter-individual variation in telomere dynamics: (1) Inter-individual variability in initial telomere length is represented by a grey vertical arrow; (2) inter-individual variability in developmental telomere dynamics is represented by a few examples of possible telomere trajectories (thin grey lines); (3) inter-individual variability in adult telomere dynamics is represented by a few examples of possible telomere trajectories (thin black lines). This variability in telomere dynamics translate into large inter-individual differences in telomere length (the theoretical possible range of telomere length is represented by thick grey dashed lines).

related to parents' telomere length through heritability, parental effect (Olsson et al., 2011; Reichert et al., 2015; Asghar et al., 2015b; Atema et al., 2015; Becker et al., 2015), and even transgenerational effects in wild vertebrates (Haussmann and Heidinger, 2015; Bebbington et al., 2016). Secondly, apart from the first steps of the development when the telomeres of the oocytes and early embryo elongate (Liu et al., 2007), growth and development are classically associated with strong telomere attrition in humans (Frenck et al., 1998; Zeichner et al., 1999). Accordingly, most longitudinal studies have found that telomere shortens through growth and development in wild vertebrates (e.g. Pauliny et al., 2006, 2012; Salomons et al., 2009; Boonekamp et al., 2014; but see Ujvari and Madsen, 2009; Ujvari et al., 2016), confirming the idea that development and growth are associated with accelerated cellular ageing in birds (Ricklefs, 2010). Thirdly, after the developmental phase, this shortening seems to slow down as an individual ages and it is even difficult to detect in some species during adulthood (e.g. Foote et al., 2010; Pauliny et al., 2012; Rattiste et al., 2015).

Interestingly, longitudinal studies have clearly shown that telomere dynamics vary between individuals during development and adulthood (e.g. Bize et al., 2009; Foote et al., 2011; Salomons et al., 2009; Barrett et al., 2013; Parolini et al., 2015; Bateson et al., 2015; Stier et al., 2016; Fig. 2). The telomeres of some individuals shorten quickly while the telomeres of other individuals remain steady or even elongate with time (e.g. Bize et al., 2009; Parolini et al., 2015). This large inter-individual variation in telomere dynamics is the main reason why telomere length is not really a reliable predictor of chronological age in wild vertebrates (Dunshea et al., 2011; Pauliny et al., 2012; Boonekamp et al., 2013). However, the strong link between telomere length and remaining lifespan is promising because this suggests not only that telomere length could be a molecular marker of biological age or/and "wear and tear", but also that telomere dynamics and telomere length could help us assess the influence of life-history events and/or environmental conditions on wild vertebrates.

2.2. Oxidative stress as a mediator of telomere dynamics

At the proximate level, oxidative stress is thought to be the primary cause of telomere shortening in wild vertebrates (reviewed in Haussmann and Marchetto, 2010). Oxidative stress is a complex biochemical condition of the organism that is dependent on the rate of oxidative damage generation and oxidation of non-protein and protein thiols that regulate the cell oxidative balance (Jones, 2006; Halliwell and Gutteridge, 2015; Costantini and Verhulst, 2009; Sohal and Orr, 2012). Oxidative damage is caused by reactive oxygen species (ROS), such as free radicals, which are mainly produced by metabolic processes and immune cells (Halliwell and Gutteridge, 2015). ROS can oxidize biomolecules, such as lipids, proteins, and DNA, and importantly, telomeres are especially sensitive to ROS because guanine is a dominant site for oxidatively-generated damage (Kawanishi and Oikawa, 2004; Monaghan, 2014). Supporting the importance of oxidative stress in determining telomere dynamics, a few studies have found an association between telomere dynamics and oxidative stress/antioxidant defenses in wild and domestic vertebrates (e.g. Geiger et al., 2012; Haussmann et al., 2012; Stier et al., 2014; Badás et al., 2015; Kim and Velando, 2015; but see Ouyang et al., 2016 and Boonekamp et al., 2017). For example, Geiger et al. (2012) found that small king penguin chicks (*Aptenodytes patagonicus*) that died early during the growth period had the highest level of oxidative damage and the shortest telomere lengths prior to death. Badás et al. (2015) found that supplementation with antioxidants (which protect against oxidative stress) reduced telomere loss a year following treatment in wild blue tits (*Cyanistes caeruleus*). Mechanistic studies have provided stronger evidence for a link between oxidative stress and telomere shortening (reviewed in von Zglinicki, 2002; but see Boonekamp et al., 2017), providing support for a possible causal link of results obtained in wild animals. For example, in cultures of human umbilical vein endothelial cells exposed to oxidative stress, terminal restriction fragment analysis demonstrated faster telomere shortening than in cells not exposed to oxidative stress (Kurz et al., 2004). In addition, telomere dynamics is also certainly dependent on DNA repair mechanisms that include the enzyme telomerase. Here again, most evidence comes from mechanistic studies, which have shown that this enzyme is the main actor of telomere elongation and restoration (Blackburn et al., 2006). Interestingly, oxidative stress induced a rapid and sustained decrease in the activity of this enzyme (Kurz et al., 2004). Moreover, Kurz et al. (2004) also demonstrated a key role for glutathione-dependent redox homeostasis (e.g., glutathione peroxidase) in the preservation of telomere function. Further support for a role of peroxidase activity in the protection of telomeres from oxidation was found in human embryonic kidney 293 (HEK293) cell lines (Aeby et al., 2016). All these mechanisms have rarely been studied in wild vertebrates (but see Haussmann et al., 2007). The connection between oxidative stress and telomere dynamics may also be mediated by glucocorticoids, suggesting one probable route through which environmental stress impacts on cellular ageing. For example, glucocorticoids may affect the oxidative balance through either genomic (Atanasova et al., 2009) or non-genomic (reviewed in Costantini et al., 2011) mechanisms. For example, exposure to dexamethasone enhances the expression of antioxidant genes under some circumstances (Atanasova et al., 2009). Similarly, glucocorticoids can suppress cellular antioxidant defenses though genomic effects in the rat (Kratschmar et al., 2012). Moreover, they may also affect telomere length through a modulation of telomerase activity (Fig. 3).

3. Environmental conditions and telomere shortening

3.1. Telomere dynamics vary between habitats, sites and years

Biomedical studies have first suggested that lifestyle could affect telomere dynamics in humans. For example, obesity and cigarette smoking have been associated with shorter telomeres in humans (Valdes et al., 2005). More recently, ecological studies have explored the links that exist between environmental conditions and telomere dynamics in wild vertebrates (Table 1). A few studies have specifically compared telomere length and telomere dynamics of wild birds living in contrasting habitats. Overall, they found that an *a priori* less suitable habitat is associated with faster telomere attrition, and consequently, shorter telomeres (Angelier et al., 2013; Young et al., 2013; Salmón et al., 2016). Similarly, other studies reported that telomere dynamics or telomere length vary between years or sites that were characterized by contrasting environmental conditions (Watson et al., 2015; Becker et al., 2015; Quirici et al., 2016; Gangoso et al., 2016; Kirby et al., 2017). Moreover, Young et al. (2015) also nicely demonstrated in thick-billed murres that telomere length is linked with spatial habitat use, foraging efficiency and prey selection. Altogether, these studies emphasize that the environment has certainly a strong influence on telomere dynamics. However, all these studies compared some habitats or periods that drastically differed in terms of environmental conditions, and therefore, several biotic and abiotic variables could be responsible for these differences between habitats and periods. We need experimental studies to tease apart the effects of biotic and abiotic variables on telomeres.

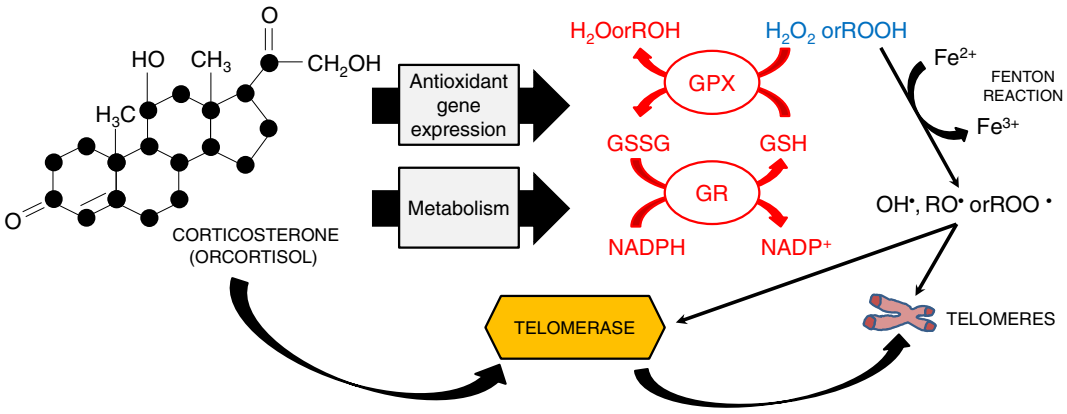

Fig. 3. This illustration shows one hypothetical route through which glucocorticoids may affect telomere dynamics by oxidative stress. Glucocorticoids may in uence expression of antioxidant genes involved in glutathione (GSH) redox-cycle, such as those regulating the antioxidant enzyme GPX (glutathione peroxidase). The effect of glucocorticoids on glutathione metabolism may also be non-genomic, such as through increased metabolism and production of reactive oxygen species. The glutathione redox-cycle is used to detoxify cells from accumulation of hydrogen peroxide (H_2O_2) and organic hydroperoxides (ROOH), whose cleavage (Fenton reaction) induced by metal ions (Fe = iron) can generate very reactive free radicals ($OH^{\cdot}$ = hydroxyl radical; $RO^{\cdot}$ = alkoxyl radical; $ROO^{\cdot}$ = alkylperoxyl radical) that damage telomeres. The impact of glucocorticoids and oxidative stress, respectively, on telomere dynamics may also come through a direct effect on the activity of the enzyme telomerase. GSSG = oxidised glutathione; ROH = alcohol; NADPH = nicotinamide adenine dinucleotide phosphate; GR = glutathione reductase. The information used to make this illustration was taken from Colitz et al., 2004; Kurz et al., 2004; Atanasova et al., 2009; Saretzski, 2009; Costantini et al., 2011.

Table 1
Review of the existing studies that report some variations in telomere length or telomere dynamics between study sites, years, cohorts and habitats.

Variable	Relationship with telomere length/dynamics	Stage	Type of study		Common name	Reference
Cohorts	≠	Development	correlative	Bird	Storm petrel	Watson et al. (2015)
Cohorts	≠	Development	correlative	Bird	White-throated dipper	Becker et al. (2015)
Cohorts	≠	Development	correlative	Mammal	Soay sheep	Fairlie et al. (2016)
Cohorts	≠	Development	correlative	Bird	White-tailed eagle	Sletten et al. (2016)
Years	≠	Adulthood	correlative	Bird	Black-tailed gull	Mizutani et al. (2013)
Sites (longitude)	≠	Adulthood	correlative	Bird	Andean condor	Gangoso et al. (2016)
Sites (latitude)	≠	Adulthood	correlative	Bird	Thorn-tailed rayadito	Quirici et al. (2016)
Sites (elevation)	≠	Development	correlative	Bird	Great tit	Stier et al. (2016)
Sites (elevation)	≠	Development	correlative	Bird	Coal tit	Stier et al. (2016)
Sites (latitude)	≠	Adulthood	correlative	Mammal	Black bear	Kirby et al. (2017)
Sites (elevation)	≠	Adulthood	correlative	Reptile	Common lizard	Dupoué et al. (unpublished)
Habitat of low suitability	–	Adulthood	correlative	Bird	American redstart	Angelier et al. (2013)
Habitat of low suitability	–	Adulthood	correlative	Bird	Thick-billed murre	Young et al. (2013)
Habitat of low suitability	–	Development	experimental/correlative	Bird	Great tit	Salmón et al. (2016)
Habitat of low suitability	=	Development	correlative	Bird	Great tit	Biard et al. (2017)

≠ denotes a signi cant difference in telomere length or telomere dynamics between cohorts, years or sites.
= denotes similar telomere length or telomere dynamics between cohorts, years or sites.
– denotes a negative effect of the variable on telomere length or telomere dynamics.

3.2. Ecological determinants of telomere dynamics

In that context, other correlative and experimental studies have shed some light on the impact of speci c environmental variables on telomere length. Infection and diseases may be associated with reduced telomere length in wild animals (Table 2). For example, Ilmonen et al. (2008) found that repeated exposure to bacteria was associated with rapid telomere attrition in wild-derived mice (*Mus musculus*). More recently, Asghar et al. (2015a) found that chronic malaria infection was associated with signi cantly faster telomere attrition in a wild bird species, the great reed warbler (*Acrocephalus arundinaceus*). However, Sebastiano et al. (2017) did not nd any association between telomere length and herpes viral disease in nestling Magni cent frigatebirds (*Fregata magni cens*).

Although psychological stress and associated disorders have been correlated with reduced telomere length in humans (Epel et al., 2004; Shalev et al., 2013), the in uence of social variables on telomere dynamics has been overlooked in wild vertebrates and we currently lack data on the in uence of social ranks and social bonds on telomere dynamics (Lewin et al., 2015). To our knowledge, a single study has tested the in uence of psychological stress on telomere dynamics in birds and it found that social isolation was associated with fast telomere attrition in African grey parrots (*Psithaccus erithacus erithacus*), a highly sociable species (Aydinonat et al., 2014). In addition to pathogens and social factors, nutritional de cit could also represent a major constraint for wild animals. Several studies have shown that nutritional constraints during the developmental phase are associated with short telomeres in birds (see Table 2). However, the in uence of nutritional constraints (food abundance and food quality) on adult telomere dynamics has rarely been tested in adults, especially in wild vertebrates (Table 2). Recently, Hoelzl et al. (2016) showed that food supplementation reduces telomere attrition and is even associated with telomere elongation in a wild mammal species, the dormouse (*Glis glis*).

Human-induced disturbance may also cause a reduction of telomere length in wild vertebrates. For instance, urban noise has been experimentally shown to reduce telomere length in house sparrow chicks (*Passer domesticus*, Meillère et al., 2015). Similarly,

Table 2
Review of the existing studies that report an in uence of different ecological variables on telomere length or telomere dynamics.

Variable	Relationship with telomere length/dynamics	Stage	Type of study		Common name	Reference
Infection/pathogens/parasites						
Multiple infections	–	Adulthood	experimental	Mammal	House mouse	Ilmonen et al. (2008)
Tuberculosis infection	–	Adulthood	correlative	Mammal	European badger	Beirne et al. (2014)
Malaria infection	0	Adulthood	experimental	Bird	Blue tit	Badás et al. (2015)
Malaria infection	–	Adulthood	experimental	Bird	Great reed warbler	Asghar et al. (2015a)
Immune challenge	–	adulthood	experimental	Bird	European blackbird	Hau et al. (2015)
Herpes virus infection	0	Development	correlative	Bird	Magni cent frigatebird	Sebastiano et al. (2017)
Social factors						
Social isolation	–	Adulthood	correlative	Bird	African grey parrot	Aydinonat et al. (2014)
Dominance rank	+	Adulthood	correlative	Mammal	Spotted hyena	Lewin et al. (2015)
Nutritional factors						
Nutritional antioxidant	–*	Adulthood	experimental	Bird	Blue tit	Badás et al. (2015)
Nutritional micronutrient supplementation	0	Development	experimental	Bird	Zebra nch	Noguera et al. (2015)
Nutritional micronutrient supplementation	+/0**	Adulthood	experimental	Bird	Zebra nch	Noguera et al. (2015)
Nutritional antioxidant	+/0***	Development	experimental	Bird	Yellow-legged gull	Kim and Velando (2015)
Food availability	0	Adulthood	correlative	Mammal	Spotted hyena	Lewin et al. (2015)
Food availability	+	Adulthood	correlative	Bird	Seychelles warbler	Bebbington et al. (2016)
Food supplementation	+	Adulthood	experimental	Mammal	Edible dormouse	Hoelzl et al. (2016)
Developmental factors						
Maternal age	+	Development	correlative	Bird	Great reed warbler	Asghar et al. (2015b)
Maternal age	+	Development	correlative	Bird	White-throated dipper	Becker et al. (2015)
Parental age	0	Development	correlative	Bird	European shag	Heidinger et al. (2016)
Parental age	+	Development	correlative	Bird	Black-browed albatross	Dupont et al. (unpublished)
Brood competition	0	Development	experimental	Bird	Collared ycatcher	Voillemot et al. (2012)
Brood competition	–	Development	experimental	Bird	European starling	Nettle et al. (2013)
Brood competition	–	Development	experimental	Bird	Jackdaw	Boonekamp et al. (2014)
Brood competition	–	Development	experimental	Bird	European starling	Bateson et al. (2015)
Brood competition	–	Development	experimental	Bird	European starling	Nettle et al. (2015)
Brood competition	–	Development	experimental	Bird	Zebra nch	Reichert et al. (2015)
Brood competition	–	Development	correlative	Bird	Magpie/Great spotted cuckoo	Soler et al. (2015)
Brood competition	–	Development	experimental	Bird	European starling	Nettle et al. (2016)
Brood competition	–	Development	correlative	Bird	European shag	Heidinger et al. (2016)
Brood competition	–	Development	correlative	Bird	Black-tailed gull	Mizutani et al. (2016)
Brood competition	–	Development	experimental	Bird	Barn swallow	Costanzo et al. (2017)
Brood competition disadvantage	–	Development	correlative	Bird	Great tit	Stier et al. (2015)
Growth	+	Development	correlative	Bird	Barn swallow	Caprioli et al. (2013)
Growth	0	Development	correlative	Bird	Black-backed gull	Foote et al. (2011)
Growth	+	Development	correlative	Bird	Barn swallow	Parolini et al. (2015)
Growth	+	Development	correlative	Bird	American redstart	Angelier et al. (2015)
Accelerated growth	–	Development	correlative	Bird	King penguin	Geiger et al. (2012)
Accelerated growth	0	Development	experimental	Fish	Brown trout	Näslund et al. (2015)
Slow growth	–	Development	correlative	Bird	Black-tailed gull	Mizutani et al. (2016)
Developmental constraints	–	Development	correlative	Bird	King penguin	Stier et al. (2014)
Reproductive factors						
Reproductive effort	–	Adulthood	experimental	Mammal	House mouse	Kotrschal et al. (2007)
Reproductive effort	0	Adulthood	experimental	Bird	Adélie penguin	Beaulieu et al. (2011)
Reproductive effort	–	Adulthood	correlative	Reptile	Leatherback turtle	Plot et al. (2012)
Reproductive effort	–	Adulthood	correlative	Bird	Common tern	Bauch et al. (2013)
Reproductive effort	–	Adulthood	experimental	Bird	Zebra nch	Reichert et al. (2014)
Reproductive effort	–	Adulthood	experimental	Bird	Blue tit	Sudyka et al. (2014)
Reproductive effort	0	Adulthood	experimental	Bird	Zebra nch	Sudyka et al. (2016)
Climatic factors						
Climate (El Nino)	+	Adulthood	correlative	Bird	Black-tailed gull	Mizutani et al. (2013)
Reduced temperature	–	Development	experimental	Fish	Eastern mosquito sh	Rollings et al. (2014)
Increased temperature	–	Adulthood	experimental	Fish	Siberian sturgeon	Simide et al. (2016)
Increased temperature	–	Adulthood	experimental	Fish	Brown trout	Debes et al. (2016)
Stressors						
Crowding	–	adulthood	experimental	Mammal	House mouse	Kotrschal et al. (2007)
Handling	–	Development	experimental	Bird	European shag	Herborn et al. (2014)
Disturbance	–	adulthood	experimental	Bird	European blackbird	Hau et al. (2015)
Noise	–	Development	experimental	Bird	House sparrow	Meillère et al. (2015)
Contamination						
Heavy metals	–	Development	correlative	Bird	Great tit	Stauffer et al. (2017)
Heavy metals	0	Development	correlative	Bird	Pied ycatcher	Stauffer et al. (2017)
Heavy metals	0	Adulthood	correlative	Bird	Great tit	Stauffer et al. (2017)
Heavy metals	0	Adulthood	correlative	Bird	Pied ycatcher	Stauffer et al. (2017)
Chlordanes	–/0****	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (2016)
Persistent organic pollutants (ΣPOPs)	0	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (2016)

(*continued on next page*)

Table 2 (*continued*)

Variable	Relationship with telomere length/dynamics	Stage	Type of study		Common name	Reference
Polychlorinated biphenyls (ΣPCBs)	0	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (2016)
Hexachlorobenzen (HCBs)	0	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (2016)
Dichlorodiphenyldichloroethylen (pp'-DDE)	0	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (2016)
Per uoroalkylated substances (PFASs)	+	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (2017)
Mercury	+/0**	Adulthood	correlative	Bird	Black-legged kittiwake	Blévin et al. (unpublished)
Organochlorine pesticides (OCPs)	0	Development	correlative	Bird	White-tailed eagle	Sletten et al. (2016)
Per uoroalkylated substances (PFASs)	0	Development	correlative	Bird	White-tailed eagle	Sletten et al. (2016)
Other factors						
Tail regrowth	–	Adulthood	correlative	Reptile	Sand lizard	Olsson et al. (2010)

*This negative effect is indirectly linked to increased reproductive effort in experimental birds.
**The effect was apparent in females only.
***The positive effect of antioxidant supplementation was only apparent for bold chicks.
****The effect was apparent for oxychlordanes in females only.

exposure to handling stress was associated with telomere shortening in European shag nestlings (*Phalacrocorax aristotelis,* Herborn et al., 2014). However, there is currently no direct or experimental data on the in uence of multiple stressors on telomere dynamics that are known to occur frequently in wild vertebrate populations (e.g. predation risk). Work done on captive animals suggests that exposure to multiple stressors may in uence telomere dynamics. Hau et al. (2015) exposed hand-raised adult Eurasian blackbirds (*Turdus merula*) to a combination of repeated immune and disturbance stressors for over one year to determine the effects of chronic stress on telomeres. By the end of the experiment, stress-exposed birds showed greater decreases in telomere lengths as compared to controls, suggesting that repeated exposure to experimental stressors might affect the rate of biological ageing (Hau et al., 2015).

Although a few studies have examined the impact of rising ambient temperature on telomere dynamics in sh (Rollings et al., 2014; Debes et al., 2016; Simide et al., 2016), there is currently little data on the effect of climatic variables on telomere dynamics or telomere length in wild birds or mammals. In black-tailed gulls (*Larus crassirostris*), mild weather and low sea-surface temperature were associated with a slow telomere shortening (Mizutani et al., 2013). This suggests that climate could affect telomere dynamics but additional studies are clearly needed. Finally, a few studies have reported that environmental contaminants can potentially have detrimental effects on telomere dynamics in free-living birds (Sletten et al., 2016; Stauffer et al., 2017). For instance, Blévin et al. (2016) recently found that oxychlordane contamination, an organochlorine pesticide known to be very toxic for wildlife, is associated with shorter telomeres in female black-legged kittiwakes (*Rissa tridacyla*). Our current world is facing multiple sources of contamination and their effects on physiology and tness are often dose-dependent. Therefore, further experimental and correlative studies linking telomere dynamics and contamination are necessary to better understand the impact of anthropogenic pollution on wild vertebrates.

As detailed in the previous paragraphs, many studies have found that telomere dynamics and telomere length are tightly connected to a broad panel of environmental conditions that are experienced by individuals. Importantly, this connection is apparent both during development and adulthood, demonstrating that environmental conditions are probably one of the main drivers of telomere dynamics in wild vertebrates. Further studies are however necessary to better understand not only the link between speci c overlooked environmental conditions and telomere dynamics but also the potential additive, interactive, and antagonistic effects of the occurrence of multiple environmental constraints on telomere dynamics. Overall, all these results demonstrate that studying telomere dynamics or telomere length can be appropriate to assess the survival costs of speci c environmental conditions in wild animals.

4. Telomere shortening through the life cycle

In addition to environmental conditions, predictable life-history events are also likely to have a strong impact on telomere dynamics, especially when they are associated with physiological and metabolic modi cations (Monaghan, 2014). During its life, an organism will face obligatory stages such as development and growth in early life and breeding, wintering, migration, or molt during adulthood (Wing eld, 2008). Although necessary for reproduction and survival, all these stages can be associated with speci c physiological constraints that may affect telomere dynamics.

There is now strong evidence that telomeres shorten very quickly during early life in most wild vertebrates (e.g. Salomons et al., 2009; Foote et al., 2011; Geiger et al., 2012; Heidinger et al., 2012; Reichert et al., 2015; Hammers et al., 2015; Asghar et al., 2015a,b). The pace of early life shortening may differ between males and females (Barrett and Richardson, 2011; Parolini et al., 2015) and seem to be dependent on developmental conditions (Table 2). For instance, the rate of early-life telomere shortening is overall faster when sibling competition is harsher (e.g. Boonekamp et al., 2014; Nettle et al., 2015; Stier et al., 2015; Costanzo et al., 2017, Table 2). Because replication is known to accelerate telomere attrition, such shortening certainly results from the replication processes that are inherent to growth and development of the organism. Moreover, growth is also associated with strong metabolic modi cation and with the production of oxidative stress (Smith et al., 2016) that is known to accelerate telomere attrition (von Zglinicki, 2002; Kawanishi and Oikawa, 2004). In comparison, telomere attrition is much slower during adulthood (e.g. Rattiste et al., 2015), but can still re ect exposure to chronic stress (Hau et al., 2015). Because offspring telomere length could be related to survival probability and lifespan (Pauliny et al., 2006; Heidinger et al., 2012; Barrett et al., 2013; Fairlie et al., 2016), this developmental telomere attrition supports the existence of a trade-off between growth and longevity (Ricklefs, 2006; Lee et al., 2013). Therefore, comparing developmental telomere dynamics among species could help to understand the importance of this trade-off in mediating inter-speci c life-history strategies (e.g. fast vs. slow development). Moreover, at the individual level, developmental telomere dynamics could

help better assessing the in uence of early developmental events on the future performances and the tness of individuals (Monaghan, 2014; Boonekamp et al., 2014).

Several studies have focused on the in uence of reproductive effort on telomere dynamics and they have reported contrasting results (Table 2). Although some of them found that the reproductive phase, and especially the parental phase, is correlated with quick telomere shortening (Bauch et al., 2013; Sudyka et al., 2014; Reichert et al., 2014), others did not nd such an effect (Beaulieu et al., 2011; Sudyka et al., 2016). Interestingly, some of these studies have experimentally shown that parental effort is associated with quick telomere attrition (Sudyka et al., 2014; Reichert et al., 2014). This supports the idea that reproduction is a demanding life-history stage for wild vertebrates and it illustrates perfectly the trade-off that exists between reproduction and survival (or at least organism's "wear and tear"). Surprisingly, the in uence of other life-history stages on telomere dynamics has been much less studied in wild vertebrates. For example, the in uence of molt on telomere dynamics has to our knowledge never been studied. The in uence of migration on telomere dynamics has been studied in a few wild bird species so far. By comparing residents and migratory dark-eyed juncos (*Junco hyemalis*), Bauer et al. (2016) found that migration was associated with shorter telomeres, emphasizing a potential important cost of migration at least in this passerine species. Regarding wintering, studies are also scarce. Quick telomere attrition has been associated with a short time spent on the wintering ground in black-legged kittiwakes (Schultner et al., 2014) and with an increased wintering foraging activity in thick-billed murres (Young et al., 2017). This suggests that this non-breeding stage may actually be associated with telomere preservation, at least when foraging conditions are appropriate. Such telomere preservation probably occurs because wintering individuals may face low energetic demands and may allocate a large part of their resources to protecting processes that limit ageing and telomere shortening. Interestingly and supporting this interpretation, hibernation has also been studied in wild mammals and torpor seems to slow down telomere attrition in dormice (Hoelzl et al., 2016) and even to be associated with telomere elongation in sub-adult djungarian hamsters (*Phodopus sungorus,* Turbill et al., 2012, 2013). Overall, we currently lack crucial basic data to fully understand the costs of several life-history stages on telomere length, and thus organism's longevity. Moreover, to our knowledge, no study has examined the dynamics of telomeres through a whole annual life cycle in wild animals. Such an approach could be especially relevant to assess the relative costs of each life stage (reproduction, migration, wintering, molt, etc.). Finally, studying the impact of speci c life-history stages on the telomere dynamics of individuals living in contrasting environments could shed some light on the costs and bene ts of speci c life-history decisions under contrasting environmental conditions.

5. Glucocorticoids and telomere dynamics

5.1. Are glucocorticoids the link between environmental constraints and telomere dynamics?

In the previous paragraphs, we reviewed the important links that exist between life-history demands, environmental conditions and telomere dynamics in wild vertebrates. Overall, demanding life-history stages and constraining environmental conditions seem to be associated with a rapid rate of telomere attrition and there is also a clear connection between stress and telomere attrition in humans, rodents and wild vertebrates (Epel et al., 2004; Kotrschal et al., 2007; Haussmann and Marchetto, 2010; Shalev et al., 2013; Monaghan, 2014; Hau et al., 2015; Bateson, 2016). This suggests that telomere dynamics could be tightly related to allostatic load (*sensu* McEwen and Wing eld, 2003; Romero et al., 2009) in wild vertebrates. When facing predictable and unpredictable events, organisms develop a suite of behavioral and physiological adjustments to maintain their homeostasis (Wing eld, 2003; Angelier and Wing eld, 2013; Romero and Wing eld, 2016). These adjustments are under control of a few central neurological and endocrine mechanisms (Romero et al., 2009). Among them, the Hypothalamus-Pituitary-Adrenal (HPA) axis appears especially relevant not only because it regulates several physiological functions but also because it is considered as a proxy of allostatic load in wild vertebrates (Romero et al., 2009). Supporting the relevance of this HPA axis when focusing on telomere dynamics, the activation of this axis and the resulting secretion of GCs are functionally linked with the proximate mechanisms regulating telomere dynamics (Fig. 3). Short-term exposure to increased GCs may increase oxidation but also antioxidant protection to limit spread of oxidative damage (reviewed in Costantini et al., 2011). On the other hand, prolonged exposure to high GCs results in increased oxidative stress and reduction of antioxidant defenses in vertebrates (reviewed in Costantini et al., 2011; see also work on wild marmots *Marmota marmota* by Costantini et al. (2012); and work on wild brown trouts *Salmo trutta* by Birnie-Gauvin et al., 2017). Importantly, biomedical studies have also shown that glucocorticoids may affect telomerase activity. Speci cally, exposure to elevated cortisol levels is associated with a down-regulation of telomerase activity (Choi et al., 2008) and glutathione peroxidase (Patel et al., 2002), but mild increases in cortisol levels rather seems to up-regulate telomerase activity (Epel et al., 2010).

In addition, GCs are involved in several physiological systems that are also linked to oxidative stress or/and telomere shortening. For instance, GCs are involved in metabolic processes (reviewed in Landys et al., 2006) and an activation of these processes results in an increased production of ROS (Costantini, 2014). Speci cally, increased circulating GCs levels are associated with protein catabolism and are thought to promote glucose and lipid mobilization (reviewed in Landys et al., 2006). Moreover, GCs are closely connected to immune activation (Martin, 2009) that is known to be associated with the production of ROS and with telomere attrition (Ilmonen et al., 2008; Asghar et al., 2015a). Increased circulating GC levels are known to enhance parts of the innate and adaptive immune responses in the short-term while the pathological maintenance of elevated GC levels during a prolonged period is rather associated with immune suppression (reviewed in Martin, 2009). Similarly, circulating GC levels are associated to disturbance and the occurrence of acute and chronic stressors (Dickens and Romero, 2013; Madliger and Love, 2016) that also seem to accelerate telomere attrition (Epel et al., 2004; Kotrschal et al., 2007; Herborn et al., 2014; Hau et al., 2015). Furthermore, a few studies have also shown that environmental contaminants can both disrupt GC regulation (Nordstad et al., 2012; Tartu et al., 2015; Meillère et al., 2016), and affect telomere dynamics in wild vertebrates (Blévin et al., 2016; Stauffer et al., 2017). Finally, circulating GC levels are known to uctuate during the life-history cycle (reviewed in Romero, 2002), and interestingly, telomere shortening also seems greater during stages when GC levels are elevated. For example, GC levels are elevated during reproduction (Romero, 2002), which is associated with fast telomere attrition (Bauch et al., 2013; Sudyka et al., 2014; Reichert et al., 2014). Moreover, reproductive effort is positively associated with both elevated GCs (e.g. large brood size, Bonier et al., 2011; Love et al., 2014) and rapid telomere attrition (Sudyka et al., 2014; Reichert et al., 2014), emphasizing this potential link between telomere dynamics and GCs.

5.2. Telomere length and circulating glucocorticoids levels: A context-dependent link?

All these results strongly suggest that HPA regulation probably plays a major role in determining telomere dynamics in wild vertebrates. However, only a few studies have examined the link between circulating GCs levels and telomere dynamics in wild vertebrates (Table 3). In thorn-tailed rayaditos (*Aphrastura spinicauda*), telomere length was negatively correlated with circulating corticosterone levels. Similarly, birds with higher corticosterone levels had shorter telomeres in black-browed albatrosses (*Thallassarche melanophrys*, Angelier et al., unpublished data) and in Andean condors (Gangoso et al., 2016). Interestingly, Young et al. (2016) found that the link between telomere length and corticosterone levels was inconsistent in thick-billed murres (*Uria lomvia*). Indeed, corticosterone levels were positively correlated with telomere length in one colony but negatively correlated in another one. Other studies found no correlation between circulating GC levels and telomere length (Young et al., 2016; Ouyang et al., 2016). Similarly, Bauch et al. (2016) found that corticosterone levels were negatively correlated with telomere length in male but not in female Common terns. All these studies suggest that the relationship between GCs and telomere length is complex and context dependent (Young et al., 2016; Bauch et al., 2016). This is not so surprising because GCs can be positively correlated to individual quality in some circumstances (the CORT-adaptation hypothesis, Bonier et al., 2009) and negatively in others (the CORT- tness hypothesis, Bonier et al., 2009). Thus, elevated GC levels can be a proxy of the inability of an individual to cope with its environment, leading to the idea that elevated GC levels should be found in individuals of low quality (Angelier et al., 2010), and thus in individuals with short telomeres (Quirici et al., 2016; Angelier et al., unpublished data; Young et al., 2016; Gangoso et al., 2016). On the other hand, telomere length and GC levels could be positively correlated when GC levels are positively correlated with individual quality. For instance, elevated GC levels are necessary to sustain the energetic demands of reproduction, and therefore, elevated GC levels should be only found in individuals that are able to allocate resources to reproduction, i.e. individuals of high quality with long telomeres (Bauch et al., 2016; Young et al., 2016).

Importantly, a few experimental studies have also examined the link between GCs and telomere dynamics (Monaghan, 2014). In developing offspring, telomere attrition was accelerated by handling stress but an additional experimental increase of circulating GCs levels did not amplify this pattern (Herborn et al., 2014). In domestic chickens (*Gallus gallus*), an embryonic exposure to GCs resulted in shorter telomeres 25 days after hatching (Haussmann et al., 2012). In adults, two experimental studies have to our knowledge examined the impact of corticosterone on telomere dynamics. In black-legged kittiwakes, a temporary increase in corticosterone levels was clearly associated with a faster rate of telomere attrition over a year (Schultner et al., 2014). In captive zebra nches (*Taeniopygia guttata*), injection of corticosterone induced a faster rate of attrition in reproductive females (Tissier et al., 2014). All these studies clearly demonstrate that there is a strong functional link between the HPA axis and telomere dynamics. However, most studies have focused on circulating GC levels without exploring the whole and complex functioning of the HPA axis. The relevance of circulating GC levels as a proxy of physiological stress or tness is currently debated (Bonier et al., 2009; Dickens and Romero, 2013; Love et al., 2014) because the actions of GCs on physiology and behavior are regulated at multiple levels (e.g. receptors, corticosterone-binding globulins, negative feedback, etc. Romero, 2004). Therefore, further laboratory and eld studies are mandatory not only to better assess the proximate link between the HPA axis and telomere dynamics, but also to ultimately understand to what extent GCs and telomere length could be informative to measure the tness costs of speci c life-history stages and environmental conditions.

5.3. Coping styles, the glucocorticoid stress response and telomere dynamics

So far, most studies have focused on baseline circulating GC levels that provide information on the energetic status of individuals (i.e. allostatic load, McEwen and Wing eld, 2003; Romero et al., 2009). However, the link between the physiological sensibility to stressors and telomere dynamics has been overlooked. In wild vertebrates, individuals adopt different strategies to cope with unpredictable events and these coping styles have been linked to the HPA axis (Wing eld, 2003, 2013; Wing eld et al., 2015). In response to an unpredictable event, the HPA axis is activated and this results in the rapid and transitory acute secretion of GCs in the bloodstream (Romero, 2004). These increased circulat-

Table 3
Review of the existing studies that focus on the link between GCs and telomere length.

GC measurement	Relationship with telomere length	Stage	Taxon	Common name	Reference
correlative studies					
Feather corticosterone	+	Adulthood	Bird	Thick-billed murre	Young et al. (2017)
Plasma corticosterone	–	Adulthood	Bird	Thorn-tailed rayadito	Quirici et al. (2016)
Plasma corticosterone	–*	Adulthood	Bird	Common tern	Bauch et al. (2016)
Plasma corticosterone	+	Adulthood	Bird	Thick-billed murre	Young et al. (2016)
Plasma corticosterone	0	Adulthood	Bird	Thick-billed murre	Young et al. (2016)
Plasma corticosterone	–	Adulthood	Bird	Thick-billed murre	Young et al. (2016)
Plasma corticosterone	–	Adulthood	Bird	Black-browed albatross	Angelier et al. (unpublished)
Feather corticosterone	–	Adulthood	Bird	Andean condor	Gangoso et al. (2016)
Plasma corticosterone	0	Adulthood	Bird	Tree swallow	Ouyang et al. (2016)
+ and – respectively denote a positive and negative relationship between GC levels and telomere length					
0 denotes no signi cant relationship between GC levels and telomere length					
* the relationship was apparent in males only					
Experimental studies					
Embryonic injection of corticosterone	–	Development	Bird	Domestic chicken	Haussmann et al. (2012)
Ingestion of corticosterone	–	Development	Bird	European shag	Herborn et al. (2014)
Corticosterone implants	–	Adulthood	Bird	Black-legged kittiwake	Schultner et al. (2014)
Injection of corticosterone	–	Adulthood	Bird	Zebra nch	Tissier et al. (2014)

– denotes a negative effect of the experiment on telomere length.

ing GC levels act on speci c behavioral and physiological systems to activate an emergency life-history stage (ELHS) that aims to promote immediate survival at the expense of other life-history components, such as reproduction (Wing eld et al., 1998). Importantly, there is a large inter-individual variation in this so called GC stress response (Cockrem, 2013) and this variability is associated to different stress sensitivity and coping styles. Although this GC stress response is known to mediate life-history decisions (Wing eld and Sapolsky, 2003; Lendvai et al., 2007; Krause et al., 2016) and to have important tness consequences (Breuner et al., 2008), its link with telomere dynamics has to our knowledge never been examined. Here, we develop a theoretical framework and several hypotheses that may stimulate future studies.

Because elevated GC levels are linked with oxidative stress (Costantini et al., 2011) and reduced telomerase activity (Choi et al., 2008), mounting a GC stress response could be associated with increased DNA damage and telomere shortening. One hidden cost of an increased sensitivity to stress could therefore be an acceleration of telomere shortening, and more generally, ageing. Because telomere length is positively associated with longevity (Pauliny et al., 2006; Bize et al., 2009; Salomons et al., 2009; Heidinger et al., 2012; Barrett et al., 2013; Fairlie et al., 2016), the GC stress response may therefore mediate the trade-off between immediate survival and long-term survival (Hypothesis 1). Thus, a high sensitivity to stress (and a strong GC stress response) could promote immediate survival at the expense of telomere protection, and thus, longevity (Fig. 4A).

Importantly, this trade-off between immediate survival and telomere preservation may be exacerbated or alleviated depending on the environmental context and/or individual quality. Individual quality involves multiple phenotypic traits that are positively correlated with tness (Wilson and Nussey, 2010). These phenotypic traits certainly involve behavior, physiology and morphological components that can bene t to tness under speci c circumstances. Overall, we may expect individuals of higher quality to have a better access to resources and to be able to allocate more resources to multiple competing traits. If individuals of high quality or individuals living in a highly suitable environment have for instance better antioxidant defenses or better DNA repair mechanisms, they may be able to limit the oxidative damages that are associated with elevated GC levels. Therefore, they may bear less telomere shortening when mounting an intense stress response (Hypothesis 2). Under that scenario, the link between telomere dynamics and the intensity of the GC stress response would depend not only on the environmental context, but also on the state of the individual (Fig. 4B).

Finally, the situation could even be more complex when individuals are engaged in a speci c life-history stage. The GC stress response mediates behavioral and physiological adjustments that shift the organism from speci c stages to an ELHS, which prioritizes immediate survival at the expense of other demanding activities (Wing eld et al., 1998). When these demanding activities are associated with oxidative stress and telomere shortening, the disruption of these activities may actually be associated with a slowing down of the rate of telomere attrition. Therefore, a high sensitivity to stress (and a strong GC stress response) could be counter-intuitively associated with reduced telomere attrition under some circumstances (Hypothesis 3, Fig. 4C). Given our current knowledge of the links between GCs, telomere shortening and reproductive effort, the best support for this hypothesis comes from studies that have focused on the reproductive stage. During reproduction, an intense GC stress response is classically associated with a substantial reduction of parental effort (Wing eld and Sapolsky, 2003; Lendvai et al., 2007; Krause et al., 2016), which is known to translate into a reduction of the rate of telomere attrition (Bauch et al., 2013; Sudyka et al., 2014; Reichert et al., 2014).

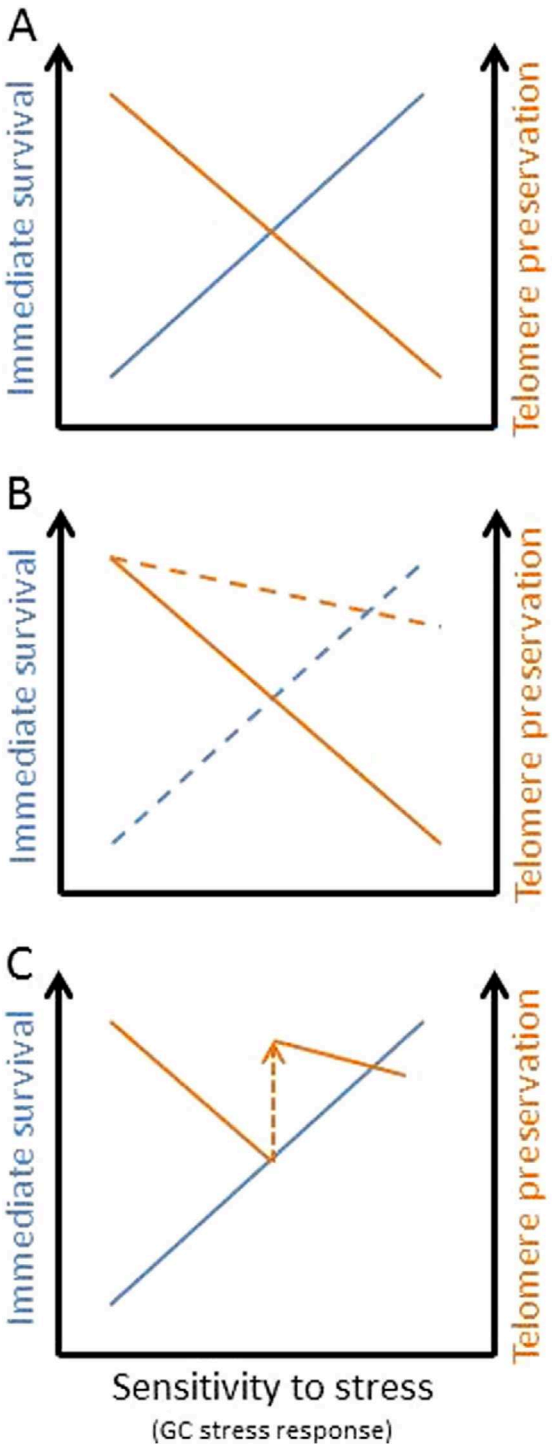

Fig. 4. Theoretical links between the GC stress response, immediate survival and telomere preservation. A: An intense GC stress response is theoretically associated with an improved immediate survival when an acute stressor occurs (blue line) but also with a faster telomere attrition (orange line). B: The relationship between the intensity of the GC stress response and telomere dynamics may be different in high-quality individuals or in individuals living in a highly suitable environment. Because of better antioxidant defenses or DNA repair mechanisms, these individuals may be able to limit the negative effect of elevated GC levels on telomere length (the solid and the dashed line respectively represent individuals of low and high quality). C: Elevated GC levels can switch individual from a speci c life-history stage (e.g. breeding stage) to an emergency life-history stage (e.g. non breeding stage). This switch is associated with reduced demanding activities, and therefore, with limited telomere attrition. This switch from a speci c life-history stage to an emergency life history stage is represented by the orange arrow. (For interpretation of the references to colour in this gure legend, the reader is referred to the web version of this article.)

In contrast, a weak stress response is usually associated with the maintenance of parental effort (Wing eld and Sapolsky, 2003; Lendvai et al., 2007; Krause et al., 2016), and thus with potential signi cant survival costs (“the cost of reproduction”) that would be associated to an important telomere attrition (Bauch et al., 2013; Sudyka et al., 2014; Reichert et al., 2014).

The link between the GC stress response and telomere attrition is however probably not so straightforward and it certainly depends on the costs and bene ts of activating an ELHS that are certainly species and context dependent. Speci cally, these costs and bene ts certainly depend on what aspects of behavior or physiology are up or down-regulated when an ELHS is activated. For instance, high stress sensitivity could be associated with a reduced rate of telomere shortening in breeding individuals if it limits the negative impact of breeding activities on telomere dynamics. However, high stress sensitivity could also be associated with a risk of mounting numerous and repeated GC stress responses that are known to accelerate telomere attrition. Under some circumstances, the negative impact of high stress sensitivity on telomere dynamics may therefore outweigh the bene ts of a reduced breeding effort on telomere dynamics, especially when the costs of reproduction are limited. Therefore, it appears crucial to better assess the costs and bene ts of elevated GC levels and speci c life-history stages on telomere dynamics in multiple species with contrasted life-history strategies and further studies are clearly needed to better understand the functional link between the GC stress response and telomere dynamics in wild vertebrates.

Although the GC stress response is known to be heritable and repeatable (Evans et al., 2006; Cockrem et al., 2009; Angelier et al., 2011; Jenkins et al., 2014), it is also exible because it can be actively modulated by individuals (Wing eld and Sapolsky, 2003; Lendvai et al., 2007; Krause et al., 2016). The ability of individuals to adaptively modulate this GC stress response is thought to be involved in their capacity to cope with a changing world (Angelier and Wing eld, 2013; Wing eld et al., 2015). Exploring the link between the GC stress response, its exibility, and telomere dynamics should further shed some light on the costs and bene ts of the hormonal sensitivity to stress. Therefore, we believe that combining GCs and telomere dynamics measurements in several ecological contexts should open some exciting and promising new research areas in environmental endocrinology.

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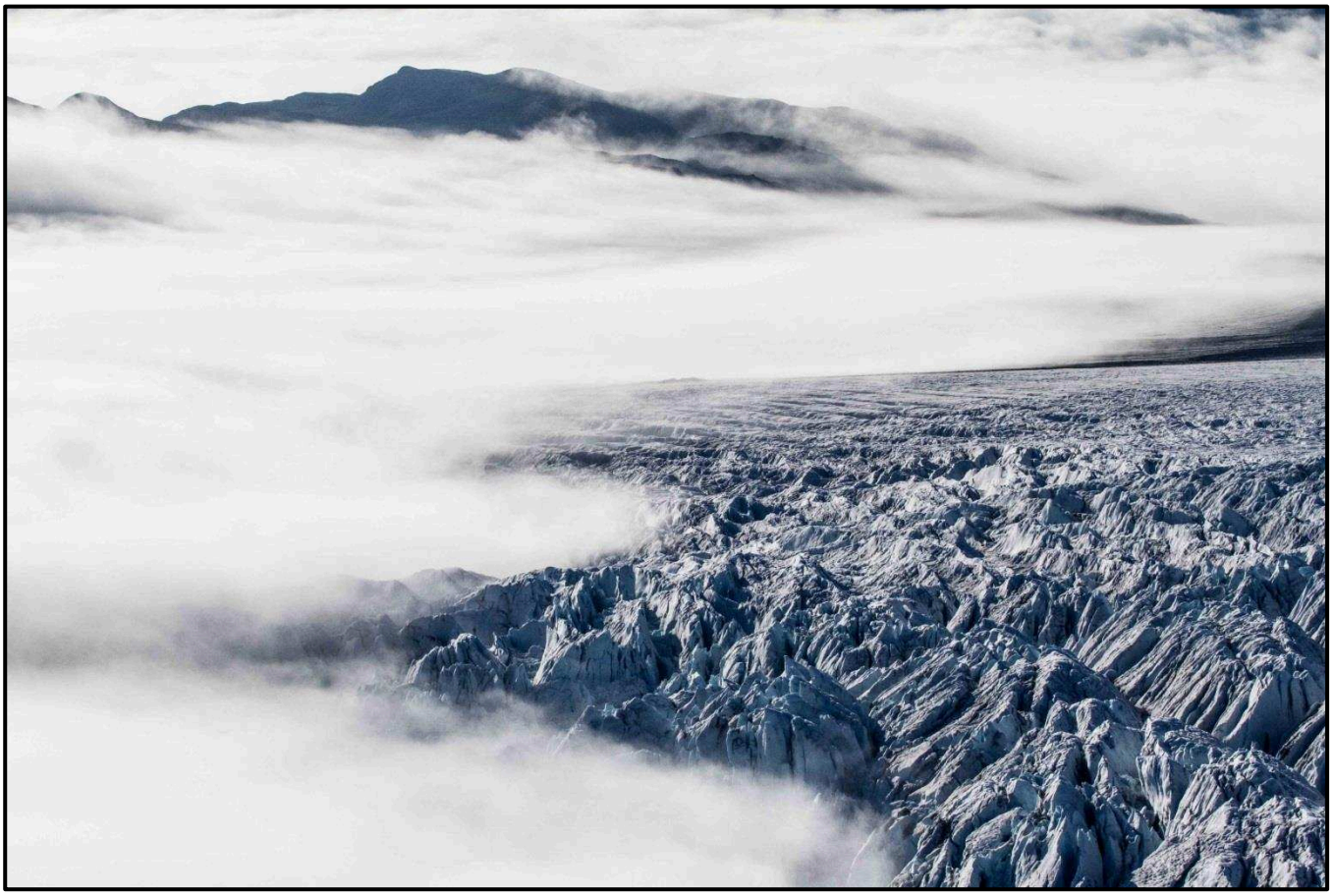
Appendix A

Blévin, P., Chastel, O.

Are legacy POPs still toxic for wild birds? A review

In preparation

Notes: The following table reviews scientific publications investigating the effects of chlordanes (i.e. oxychlordane, *trans*- and *cis*-nonachlor, *trans*- and *cis*-chlordane and heptachlor) in wild birds. **This work is in progress and several relevant references are missing.** We used $\alpha = 0.05$ as a significant threshold value.



Variable	Relationship	Predictor	Tissue	Type of study	Common name	Reference
Endocrine disruption						
Progesterone (plasma)	positive**	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Glaucous gull	Verreault et al., 2006
Testosterone (plasma)	none	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Glaucous gull	Verreault et al., 2006
Baseline corticosterone (plasma)	none	oxychlordane	wb	correlative	Black-legged kittiwake	Nordstad et al., 2012
Baseline and stress-induced corticosterone (plasma)	none**	$\Sigma_{(6)}$ OCPs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	Tartu et al., 2015
Baseline and stress-induced prolactin (plasma)	none	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Glaucous gull	Verreault et al., 2008
Prolactin (plasma)	none	oxychlordane	wb	correlative	Black-legged kittiwake	**Paper IV**
TT4, FT4, TT4:TT3, TT4:FT4 (plasma)	negative**	oxychlordane	wb	correlative	Glaucous gull	Verreault et al., 2004
TT3, FT3 (plasma)	positive**	oxychlordane	wb	correlative	Glaucous gull	Verreault et al., 2004
FT4:FT3, TT3:FT3 (plasma)	none**	oxychlordane	wb	correlative	Glaucous gull	Verreault et al., 2004
TT4:TT3 (plasma)	negative	$\Sigma_{(3)}$ CHLs Including: oxychlordane, *trans*-, *cis*-chlordane	plasma	correlative	Glaucous gull	Verreault et al., 2007
TT4, TT3 (plasma)	none	oxychlordane, *trans*-nonachlor	plasma	correlative	Glaucous gull	Melnes et al, 2017
FT3 (plasma)	negative*	oxychlordane	plasma	correlative	Glaucous gull	Melnes et al., 2017
FT3 (plasma)	none	*trans*-chlordane	plasma	correlative	Glaucous gull	Melnes et al., 2017
FT4 (plasma)	negative*	oxychlordane, *trans*-nonachlor	plasma	correlative	Glaucous gull	Melnes et al., 2017
TT3 (plasma)	negative	$\Sigma_{(3)}$ CHLs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	**Paper III**
TT4 (plasma)	none	$\Sigma_{(3)}$ CHLs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	**Paper III**
TT4:FT4 (plasma)	negative[b]	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Black-legged kittiwake	Nøst et al., 2012

TT4:FT4 (plasma)	negative[b]	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Black-legged kittiwake	Nøst et al., 2012
FT3 (plasma)	positive[b]	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Black-legged kittiwake	Nøst et al., 2012
TT4, TT3, FT4, TT4:TT3, TT3:FT3, FT4:FT3 (plasma)	none[b]	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Black-legged kittiwake	Nøst et al., 2012
TT4, TT3, FT4, FT3, TT4:TT3, TT4:FT4, TT3:FT3, FT4:FT3 (plasma)	none[b]	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	plasma	correlative	Northern fulmar	Nøst et al., 2012
TT4/ TT3/ TT4:TT3 (plasma)	none	$\Sigma_{(2)}$ CHLs Including: oxychlordane, heptachlor	liver	correlative	Northern fulmar	Verreault et al., 2013
TT4 (plasma)	positive	*trans*-nonachlor, $\Sigma_{(4)}$ CHLs Including: oxychlordane, *trans*-, *cis*-chlordane, *cis*-nonachlor	liver	correlative	Ring-billed gull	Técher et al., 2016
TT3, FT3, FT4, TT4:TT3 (plasma)	none	$\Sigma_{(5)}$ CHLs	liver	correlative	Ring-billed gull	Técher et al., 2016
DNA damage						
Telomere length (rbc)	negative*	oxychlordane	wb	correlative	Black-legged kittiwake	**Paper II**
Telomere length (rbc)	none	*trans*-nonachlor *cis*-nonachlor	wb	correlative	Black-legged kittiwake	**Paper II**
Telomere length (rbc)	none[b]	oxychlordane	plasma	correlative	White-tailed eagle	Sletten et al., 2015
Immune and vitamin status						
Heterophile index (blood smears)	positive	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2004
Lymphocyte index (blood smears)	positive	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2004
Antibody response to diphtheria (blood smears)	negative*	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2004
Antibody response to tetanus (blood smears)	none	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2004
White blood cell density (blood smears)	none	oxychlordane	wb	correlative	Great black-backed gull	Bustnes et al., 2007
Immunoglobulin γ (plasma)	none[b]	oxychlordane	plasma	correlative	White-tailed eagle	Sletten et al., 2015
Retinyl palmitate (Vitamin A, liver)	positive	oxychlordane	liver	correlative	Northern fulmar	Verreault et al., 2013
Retinyl palmitate (Vitamin A, liver)	none	heptachlor	liver	correlative	Northern fulmar	Verreault et al., 2013
Retinyl palmitate (Vitamin A, liver)	none[b]	oxychlordane	yolk sac	correlative	Brünnich's guillemot	Murvoll et al., 2007
Retinyl palmitate (Vitamin A, liver)	none[b]	oxychlordane	yolk sac	correlative	Common eider	Murvoll et al., 2007
α-tocopherol (Vitamin E, liver)	negative[b]	oxychlordane	yolk sac	correlative	Brünnich's guillemot	Murvoll et al., 2007
α-tocopherol (Vitamin E, liver)	positive[b]	oxychlordane	yolk sac	correlative	Common eider	Murvoll et al., 2007
α-tocopherol (Vitamin E, plasma)	none[b]	oxychlordane	yolk sac	correlative	Brünnich's guillemot	Murvoll et al., 2007

α-tocopherol (Vitamin E, plasma)	none[b]	oxychlordane	yolk sac	correlative	Common eider	Murvoll et al 2007
Retinol (Vitamin A, plasma and liver)	none	$\Sigma_{(2)}$ CHLs Including: oxychlordane, heptachlor	liver	correlative	Northern fulmar	Verreault et al., 2013
Retinol (Vitamin A, plasma and liver)	none[b]	oxychlordane	yolk sac	correlative	Brünnich's guillemot	Murvoll et al., 2007
Retinol (Vitamin A, plasma and liver)	none[b]	oxychlordane	yolk sac	correlative	Common eider	Murvoll et al., 2007
Retinyl palmitate:Retinol (liver)	none	$\Sigma_{(2)}$ CHLs Including: oxychlordane, heptachlor	liver	correlative	Northern fulmar	Verreault et al., 2013
Oxidative status						
Superoxide dismutase enzyme (SOD, plasma)	negative[b]	oxychlordane	plasma	correlative	White-tailed eagle	Sletten et al., 2015
Parasites						
Intestinal parasite intensity (nematodes)	positive	oxychlordane	liver	correlative	Glaucous gull	Sagerup et al., 2000
Intestinal parasite intensity (trematodes, cestodes, acanthocephalan)	none	oxychlordane	liver	correlative	Glaucous gull	Sagerup et al., 2000
Disease						
Avian vacuolar myelinopathy	none	oxychlordane	composite brain liver fat	comparative	American coot	Dodder et al., 2003
Energy expenditure/ growth						
Basal metabolic rate	negative	$\Sigma_{(3)}$ CHLs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	**Paper III**
Basal metabolic rate	negative	$\Sigma_{(3)}$ CHLs Including: oxychlordane, *trans*-, *cis*-chlordane	plasma	correlative	Glaucous gull	Verreault et al., 2007
Body condition of chick at hatching	negative*	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Daily chick growth	negative*	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2005
Phenotypic traits						
Wing feather asymmetry	positive	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2002
Wing feather asymmetry	positive[a]**	oxychlordane	wb	correlative	Great black-backed gull	Bustnes et al., 2007
Saturation index (eye-ring, gape, tongue)	negative*	$\Sigma_{(7)}$ OCs Including: oxychlordane, *trans*-, *cis*-nonachlor, *trans*-chlordane	wb	correlative	Black-legged kittiwake	**Paper VI**
Saturation index (bill)	none*	$\Sigma_{(7)}$ OCs Including: oxychlordane, *trans*-, *cis*-nonachlor, *trans*-chlordane	wb	correlative	Black-legged kittiwake	**Paper VI**
Brightness index (eye-ring, gape, tongue, bill)	none*	$\Sigma_{(7)}$ OCs Including: oxychlordane, *trans*-, *cis*-nonachlor, *trans*-chlordane	wb	correlative	Black-legged kittiwake	**Paper VI**

Hue index (eye-ring, gape, tongue, bill)	negative[c]*	$\Sigma_{(7)}$ OCs Including: oxychlordane, *trans*-, *cis*-nonachlor, *trans*-chlordane	wb	correlative	Black-legged kittiwake	**Paper VI**
Parental care behaviors						
Time away from the nest	positive	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2005
Nest predation	none	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Nest predation	positive*	oxychlordane	wb	correlative	Great black-backed gull	Helberg et al., 2005
Incubation temperature	negative**	oxychlordane	wb	correlative	Black-legged kittiwake	**Paper IV**
Brood patch size	negative**	oxychlordane	wb	correlative	Black-legged kittiwake	**Paper IV**
Egg turning behaviors (frequency and angular change)	none	$\Sigma_{(3)}$ CHLs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	**Paper V**
Incubation time	none	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Phenology						
Laying date	positive*	oxychlordane	wb	correlative	Great black-backed gull	Helberg et al., 2005
Laying date	negative	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Hatching date	positive**	$\Sigma_{(6)}$ OCPs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	Tartu et al., 2015
Reproductive outputs						
Breeding probability	negative**	$\Sigma_{(4)}$ CHLs Including: oxychlordane, *trans*-chlordane, *trans*-, *cis*-nonachlor	wb	correlative (CMR)	Black-legged kittiwake	Goutte et al., 2015
Clutch size	none	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Clutch size	none	oxychlordane	wb	correlative	Great black-backed gull	Helberg et al., 2005
Clutch size	none**	$\Sigma_{(6)}$ OCPs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	Tartu et al., 2015
Egg volume	none	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Egg volume	none	oxychlordane	wb	correlative	Great black-backed gull	Helberg et al., 2005
Nonviable eggs	positive*	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Eggshell thickness	none	oxychlordane heptachlor *trans*-nonachlor	egg	comparative	Black-crowned night heron	Mats and Parsons, 2004
Eggshell thickness	negative	$\Sigma_{(6)}$ CHLs Including: oxychlordane, heptachlor, *trans*-, *cis*-chlordane, *trans*-, *cis*-nonachlor	egg	comparative	Brown pelican	Vander Pol et al., 2012
Hatching success	negative**	$Mean_{(4)}$ OCs Including: oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2006
Hatching success	none**	$\Sigma_{(6)}$ OCPs Including: oxychlordane, *trans*-, *cis*-nonachlor	wb	correlative	Black-legged kittiwake	Tartu et al., 2015

Probability of raising 2 chicks	none	$\Sigma_{(4)}$ CHLs Including: oxychlordane, *trans*-chlordane, *trans*-, *cis*-nonachlor	wb	correlative (CMR)	Black-legged kittiwake	Goutte et al., 2015
Breeding success	none	$\Sigma_{(4)}$ CHLs Including: oxychlordane, *trans*-chlordane, *trans*-, *cis*-nonachlor	wb	correlative (CMR)	Black-legged kittiwake	Goutte et al., 2015
Early chick survival	none	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Early chick survival	none	$\Sigma_{(4)}$ CHLs Including: oxychlordane, *trans*-chlordane, *trans*-, *cis*-nonachlor	wb	correlative (CMR)	Black-legged kittiwake	Goutte et al., 2015
Survival rate and related parameters						
Return rate	negative	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2005
Return rate	none	$Mean_{(4)}$ OCs Including: oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2006
Survival rate	negative	oxychlordane	wb	correlative	Glaucous gull	Bustnes et al., 2003
Survival rate	negative	$\Sigma_{(4)}$ CHLs Including: oxychlordane, *trans*-chlordane, *trans*-, *cis*-nonachlor	wb	correlative (CMR)	Black-legged kittiwake	Goutte et al., 2015
Survival rate	negative	oxychlordane	wb	correlative (CMR)	Glaucous gull	Erikstad et al., 2013

Studies conducted on gene expression, protein synthesis, enzymatic activity (except for OS) and other blood clinical-chemical parameters have not been included in this review.

*** The effect was apparent (or tested) in females only**

**** The effect was apparent (or tested) in males only**

[a] The effect was marginally significant

[b] Study conducted on chicks

[c] Significant relationship found only for tongue

Appendix B

Guilleminot-Humann, S., **Blévin, P**., Azou-Barré, A., Yacoumas, A., Gabrielsen, G.W., Chastel, O., Helfenstein, F.

Sperm collection in black-legged kittiwakes and characterization of sperm velocity and morphology

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METHODOLOGY

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Sperm collection in Black-legged Kittiwakes and characterization of sperm velocity and morphology

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Abstract

Background: Collecting and studying live sperm is central to many important fields of biology. Yet, a simple method to collect live sperm is lacking in wild seabird species. Here, we describe a non-invasive method to collect viable sperm samples based on a simple massage technique applied to male Black-legged Kittiwakes (*Rissa tridactyla*).

Methods: We studied a colony breeding at Kongsfjorden, Svalbard and successfully obtained sperm samples from 32 males. With a subset of samples ($n = 12$ males), we compared the suitability of several extenders (0.9% NaCl, PBS, Earle's balance salt solution, Dulbecco's modified Eagle medium) in maintaining sperm alive long enough for analyses. With another 18 ejaculates, we conducted computer assisted sperm analyses using the CASA plugin for ImageJ. We provide details about the settings to be used for such analyses. Lastly, droplets from 20 ejaculates were smeared on glass slides and preserved with formalin to characterize sperm morphology in terms of total sperm length, sperm head length, midpiece length and flagellum length, and percentage of abnormal sperm.

Results: With this method and under field conditions, we were able to obtain sufficient amounts of live sperm to assess traits related to sperm quality (e.g. sperm morphology, percentage of motile sperm, sperm velocity). We found that two extenders, Earle's balanced salt solution and Dulbecco modified Eagle's medium, yielded similarly good results. Additionally, we investigated whether specific behaviours were associated with successful sperm collection and whether sperm collection success depended on how long before laying sperm collection was attempted. Finally, we provide mean values for sperm morphology, sperm swimming ability and percentage of motile sperm, which may prove useful for future comparative analyses, and we report high levels of sperm abnormality and within-ejaculate variation in sperm morphology.

Conclusions: We discuss the high percentage of abnormal sperm and high within-ejaculate variation in sperm morphology in light of sperm competition theory and conclude that these figures are likely due to relaxed post-copulatory sexual selection, kittiwakes being strictly monogamous. Finally, we suggest that this method could be applied to other seabird species sharing similar ecology.

Keywords: Black-legged Kittiwakes, Sperm, Spermatozoa, Semen collection, Non-invasive method, Larids, Sperm velocity, Sperm morphology, Abnormal sperm

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Background

There has been a long-time interest in the biology of spermatozoa, undeniably elicited by their large interspecific diversity and their role in transmitting genetic information to the next generation. The vast diversification of sperm morphology was produced in response to the selective pressures imposed by their specific fertilizing environment, i.e. the surrounding environment in external fertilizers and the female reproductive tract in internal fertilizers (Franzén 1956; Alberti 1990; Jamieson 2007). In addition to this selection, post-copulatory sexual selection [sperm competition (Parker 1970) and cryptic female choice (Thornhill 1983; Eberhard 1996)] is also known to drive the evolution of sperm morphology and function (i.e. capacity to reach, penetrate and fertilize the ovum; Fitzpatrick and Lüpold 2014) across and within species. Clearly, strong selective forces act on sperm cells to optimize their fertilizing ability, and the study of the evolutionary patterns and processes underlying inter- and intra-specific variation in sperm quality has elicited much interest in the last two decades (Birkhead and Pizzari 2002; Pitnick et al. 2009; Fitzpatrick and Lüpold 2014; Reinhardt et al. 2015).

Sperm quality is also influenced by environmental factors, like nutrition (Bronson 1989), diseases (Nicopoullos et al. 2004) or pollutants (Jurewicz et al. 2009). For example in humans, there is ample evidence that environmental pollutants affect sperm quality and thus male fertility. Indeed, pesticides, air pollutants and others environmental chemicals have all been shown to decrease sperm quality in human beings (Swan et al. 2003; Sharpe 2010; Lafuente et al. 2016). The impact of pollutants on sperm quality in domestic, livestock or captive animals is also well known (e.g. Hatef et al. 2013; Komsky-Elbaz and Roth 2017), and studies in free-ranging animals would now be timely.

Seabirds are popular models for research in ecology and evolutionary biology because they can be easily captured, individually marked as chicks and adults, equipped with various loggers (Wilson et al. 2007) which allows experimental field studies (e.g. Goutte et al. 2011; Merkling et al. 2017). Additionally, due to their position at the top of the food chain, long-lived seabirds often bio-accumulate many contaminants via food intake and are thus ideal models in eco-toxicological studies (Elliott and Elliott 2013). Surprisingly, although seabirds would be suitable models to study ecological, evolutionary and toxicological questions in relation to sperm quality, no such study exists. The obvious reason being that, with the exception of captive Magellanic Penguins (*Spheniscus magellanicus*) (O'Brien et al. 1999), no method for collecting sperm in seabirds has been described so far, and applying methods originally described for poultry and passerine birds is not as easy as one would intuitively think.

The purpose of this study was to collect live sperm, for the first time, using a non-invasive method in a common seabird with a wide geographical distribution, the Black-legged Kittiwake (*Rissa tridactyla*). In this paper, we also provide information about suitable sperm extenders and relevant timing in relation to the breeding phenology of the kittiwakes, as well as a video showing how the collection was done (Additional file 1: Video S1). Finally, we report values for sperm morphology, percentage of abnormal sperm, and sperm swimming traits, which are valuable information for comparative studies. This method to collect sperm is adapted to field conditions, and we believe that it could be easily adapted to other seabird species with similar ecology (e.g. laridae or alcidae). Because it does not require killing the birds, we believe that this method will appeal to scientists interested in sperm biology in seabirds, and working in a variety of disciplines from veterinary sciences to conservation biology, evolutionary biology and ecotoxicology.

Methods

Model species, study site and bird capture

Black-legged Kittiwakes are pelagic seabirds, which breed in very dense colonies and lay 1–3 eggs (Coulson 2011). They are monogamous, do not engage in extra-pair copulations and exhibit low copulation rates with an average of 14 copulations during the 23 days that precede the laying of the clutch (Helfenstein et al. 2004).

Fieldwork was carried out from May 24th to June 11th 2016, in a colony of Black-legged Kittiwakes at Kongsfjorden (78°54′N; 12°13′E), Svalbard. We caught a total of 50 males directly on their nest using a nylon noose tied at the end of a 5-m fishing rod, during the pre-laying period (i.e. during nest building and copulation). Immediately after capture, birds were first blood sampled for hormones and contaminants. Carotenoid-based ornaments coloration, and biometry (body mass, tarsus length, wing length, skull length) were then measured, which delayed sperm collection for at least 30 min after bird capture. Kittiwakes were individually marked with metal rings and PVC plastic bands engraved with a three-digit code and fixed to the bird's tarsus for identification from a distance.

In order to save time by targetting males who would give us good ejaculates, we noted the behaviour of the male and the female just before capture. Behaviours were categorized as: no specific behaviour observed, female begging for food, male about to mount the female, copulation occurred maximum 1 h ago.

Laying date and temporal optimum for sperm collection

Before release, kittiwakes were marked with spots of dye on the forehead in order to identify individuals and thereby their nest. We checked the nest content of sampled birds every two days to monitor breeding stage (at least one egg is laid or no egg laid) and egg-laying date using a mirror at the end of an 8-m fishing rod. We estimated the most successful time to collect sperm relative to the laying of the first egg of a given pair by plotting a probability of success (number of successful sperm collection divided by the total number of attempts per day) against the number of days before laying. Sperm collection was considered successful when the ejaculate contained at least 10 motile spermatozoa.

Sperm collection

Sperm samples were obtained by firmly massaging the lower back and the base of the tail of the male. For easier handling, we recommend to keep the bird's head in a fabric bag and to maintain the bird on your thighs and on its belly using your forearm, keeping your hands free for massaging and collecting sperm (see Additional file 1: Video S1). In the field, we observed that males wagged their tail during mating, and this observation prompted us to massage the base of the tail while moving it laterally for ca. 5 s. After this massage, the handler lifted the tail, cleared the feathers around the cloaca and gently squeezed the cloaca with two fingers with one hand, while using the other hand to collect the ejaculate directly in a non-heparinized 75 µL capillary with 5 µL graduation (VWR, reference 612-3417). The capillary was placed on the top of the cloaca, closest to the tail (Fig. 1). Males made a series of cloacal contractions before extruding a translucent liquid, which was verified to be sperm under the microscope (see Additional file 2: Video S2). We always avoided pressing too deep under the cloaca or on the belly to avoid contamination by faecal matter.

Assessing sperm survival

For a subset of the males ($n = 12$), we immediately started a stopwatch after the extrusion of an ejaculate in a capillary to assess the survival of spermatozoa. Sperm samples were divided in two 5 µL aliquots and each aliquot was pipetted into 5 µL of either pre-warmed PBS (1× phosphate-buffered saline), physiological saline (0.9% NaCl), DMEM (Dulbecco's modified Eagle medium, 4500 mg glucose/L, 110 mg/L sodium pyruvate and L-glutamine), Earle's balanced salt solution (EBSS) semen extender (including HAS, pyruvate, Hepes, Phenol red, sodium-bicabonate and Gentamicin 10 µg/mL, SpermWash®; Cryos, Aarhus, Denmark) or left undiluted. Three µL of the mix sperm-extender or undiluted sperm were transferred into a 20-µm deep chamber slide (Leja Products B.V., The Netherlands), and the two swimming chambers were continuously visually inspected using an Olympus BX43 microscope (Olympus Co., Japan) with a 10× objective under negative phase contrast (position Ph3 of the annular phase ring). We maintained the temperature of the mix sperm-extender or undiluted sperm at 40 °C (the body temperature of adult kittiwakes ranges between 39.8 and 40.3 °C; Barrett 1978; Brent et al. 1983) using a heating glass plate (MATS-U55S, Olympus Co., Japan) fitted to the microscope stage. Sperm survival was estimated as the time elapsed from the ejaculation until all spermatozoa were immotile.

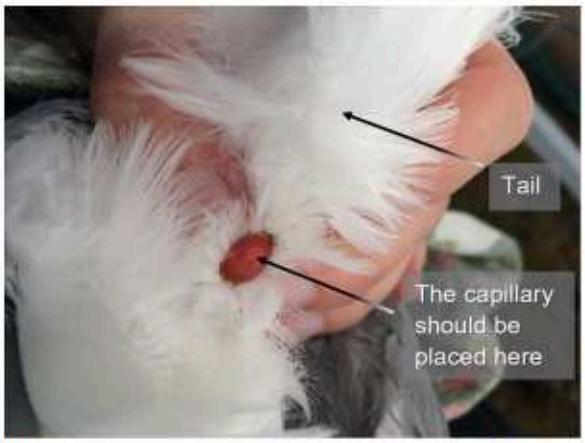


Fig. 1 Picture of a kittiwake's cloaca showing where to apply the capillary to collect the ejaculate

Assessment of percentage of motile sperm, sperm swimming ability and sperm morphology

For another subset of males ($n = 18$), we immediately started a stopwatch after the extrusion of an ejaculate in a capillary and pipetted 5 µL of semen into 5 µL of DMEM. Three µL of this mix were transferred into a 20-µm deep chamber slide (Leja Products B.V., The Netherlands). Sperm swimming ability was monitored using a Toshiba CMOS HD camera (TOSHIBA Corporation, Japan) mounted on an Olympus BX43 microscope (Olympus Co., Japan) with a 10× objective under negative phase contrast (position Ph3 of the annular phase ring). We maintained the temperature of the mix sperm-extender or undiluted sperm at 40 °C (the body temperature of adult kittiwakes ranges between 39.8 and 40.3 °C; Barrett 1978; Brent et al. 1983) using a heating glass plate (MATS-U55S, Olympus Co., Japan) fitted to the microscope stage. We recorded 5-s videos on four to five different fields to maximize the number of tracked spermatozoa. Changing fields to make several short videos of

the same sperm sample was possible because spermatozoa proved to be able to remain motile over 40 min. From the videos, we used the computer-assisted sperm analysis (CASA) plugin (Wilson-Leedy and Ingermann 2007) for ImageJ (Schneider et al. 2012) to assess the mean values for seven traits related to sperm swimming ability: VCL (curvilinear velocity, total distance travelled, µm/s), VAP (average path velocity, smoothed path using roaming average, µm/s), VSL (straight line velocity, distance from origin to end point, µm/s), linearity (LIN: VSL/VAP, path curvature), wobble (WOB: VAP/VCL, side to side movement of the sperm head, also described as the oscillation of the actual trajectory about its average path), BCF (beat cross frequency, the frequency at which VCL crosses VAP, Hz), and progression (PROG: average distance from origin on the average path during all frames analysed). The CASA also assessed the percentage of motile sperm. Our videos were 1280 × 720 in resolution with 25 frames/s. Videos were imported into ImageJ as image stacks and converted to 8-bit images. The "threshold" function was used to discard particles smaller than a sperm cell and create adequate contrast with black sperm cells against a white background. The CASA settings were set as follows: minimum and maximum sperm size were 30 and 150 pixels; search radius (maximal distance in pixels between two frames for moving sperm) was 25 pixels; Low VAP was set to 5 µm/s; the maximum percentage of path with low VAP between frames was 90% and the maximum percentage of path with null VAP was 10%; these last three conditions discarded sperm, which do not show regular motion (sperm stuck or slowed down due to particles or collisions); when examining all the trajectories for a given sample, VCL and VAP always showed a bimodal distribution with a cut-off around 20 µm/s. Therefore, sperm with VAP < 20 µm/s and VCL < 20 µm/s were assumed to be immotile and moved by drift. These estimates were based on 134 ± 121 sperm tracks (mean ± SD; minimum: 20; maximum: 463) per ejaculate.

A small droplet from the ejaculate was immediately smeared with 10% formalin (1:9 v:v; i.e. 4% formaldehyde) on a glass slide. From each slide, we took photos of ten intact sperm cells using the Nikon ACT-1 v2.70 software (Nikon Corporation, Japan) with a Nikon DFC7000T camera (Nikon Corporation, Japan) mounted on a Leica DMR microscope (Leica Microsystems GmbH, Germany) at 400× magnification and phase contrast 2. Seven to 16 sperm cells (mean ± SE: 10.1 ± 0.5) were measured for head, midpiece, flagellum and total length. Additionally, each cell was independently measured twice to assess the amount of variance due to measurement error using random models (Helfenstein et al. 2010). We used a re-sampling procedure to verify that measuring ten sperm cells accurately estimates male means, among-male variation and within-ejaculate variation in total sperm length and length of sperm components (Additional file 3: Fig. S1, S2; Additional file 4: Supplementary dataset). The percentage of measurement error was 4.7% for head length, 12.5% for midpiece length, 1.3% for flagellum length, and 0.2% for total length. The average coefficient of variation [(SD/mean) × 100] for the two measures of the same sperm was 2.5% for head length, 5.2% for midpiece length, 1.1% for flagellum length, and 0.6% for total length. For further analyses the two measures per spermatozoon were averaged.

We estimated the variability in sperm morphology among males by computing an unbiased estimate of among-male coefficient of variation (CV_{am}) using the formula adjusted for small sample sizes (Sokal and Rohlf 1995) based on average sperm measures for each individual. Then, we estimated the percentage of within-ejaculate variance relative to the total variance (within ejaculates + among males/ejaculates) using random models (Helfenstein et al. 2010). Finally, we computed an unbiased within-ejaculate coefficient of variation (CV_{we}) by first calculating an unbiased CV for each male, then averaging the CVs of all individuals (Laskemoen et al. 2007).

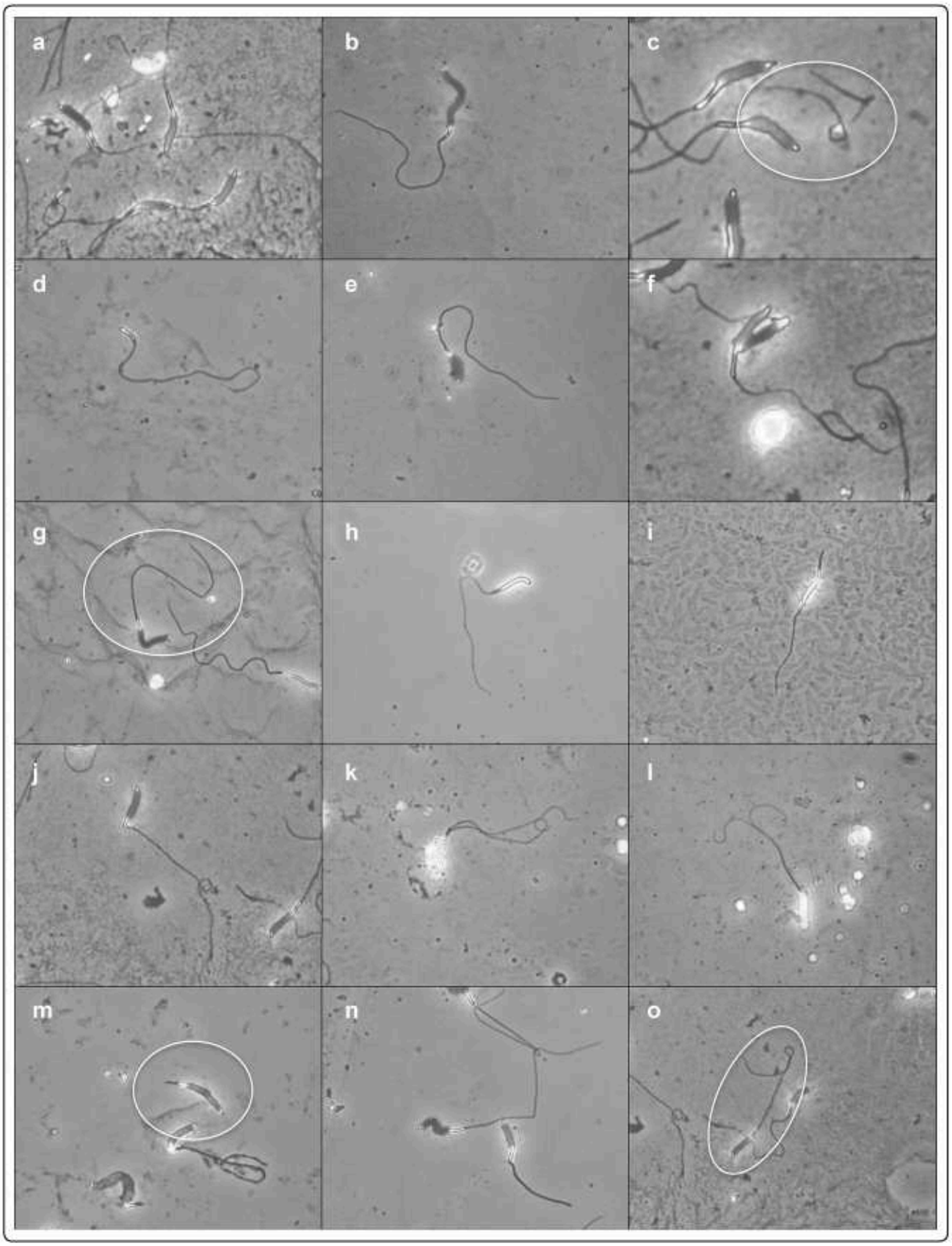
Sperm smears were also used to assess the percentage of abnormal sperm for 19 ejaculates based on 50 spermatozoa per slide. Spermatozoa were classified as morphologically normal, with abnormal head (no head, S-shaped head, bended head, no acrosome, burst head), with abnormal midpiece (no midpiece, broken midpiece) or with abnormal flagellum (no flagellum, broken flagellum, folded flagellum, flagellum with 90° angle, coiled flagellum, double flagellum, split flagellum) (Fig. 2).

Statistics

We investigated whether the date at which a male was trapped relative to the laying of the first egg in the focal nest influenced our success to obtain a spermic ejaculate

(See figure on next page.)

Fig. 2 Pictures of spermatozoa (400× magnification and phase contrast 2) showing: normal sperm (**a**); a sperm with S-shaped head (**b**); a head-less sperm (**c**, **d**); a sperm with burst head (**e**); a double-headed sperm (**f**); a sperm with bended head (**g**); a sperm with no acrosome (**h**); a sperm with abnormally long acrosome (**i**); a sperm with 90°-angle midpiece (**j**); a double-flagellated sperm (**k**); a sperm with split flagellum (**l**); a sperm with broken flagellum (**m**); a sperm with 90°-angle flagellum (**n**); and a sperm with coiled flagellum (**o**)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using a generalized linear model with quasibinomial distribution and logit link function and with number of success/number of attempts as the dependent variable and time relative to egglaying as the explanatory factor.

We tested for an association between sperm collection success and the behaviour of the pair immediately before the male was captured using a Fisher's exact test for 2×4 contingency tables.

Each sperm sample was tested for sperm survival in two conditions (either two different sperm extenders or one sperm extender and undiluted sperm; Table 1). We compared the effect of the various buffers on sperm survival using a Wilcoxon's signed-ranks test for paired samples. To run this test, and because our sample size is modest, we compared sperm survival of a given ejaculate in a given condition (undiluted sperm or sperm diluted in one of our four different extenders) against sperm survival of the same ejaculate in any other condition.

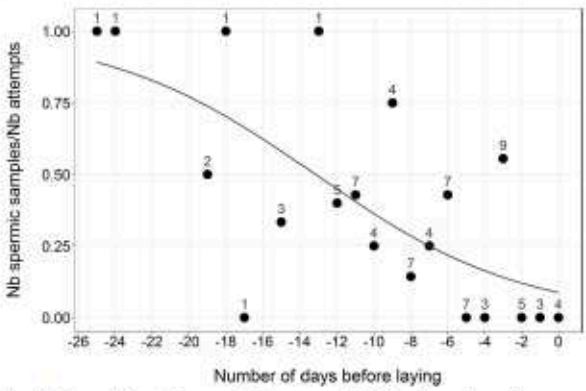

Fig. 3 Proportion of successful sperm collection (numbers of successful attempts divided by the total number of attempts) according to the number of days before laying. The numbers above the dots indicate the number of attempts made on a given day

Results

All trapped individuals were massaged to collect sperm (50 individuals for 85 attempts, some males were trapped twice). All the birds responded to our stimulation and extruded a translucent fluid. This fluid contained spermatozoa in 33% of the cases (28 ejaculates with spermatozoa over 85 attempts). When sperm collection was successful, we always obtained between 5 and 10 µL of sperm per sample (based on capillary graduations). We used sperm samples of at least 10 µL to test the sperm extenders. Thus, only a subsample of all ejaculates ($n = 19$ from 12 individuals) were used to investigate the effect of the sperm extenders on sperm survival.

Temporal optimum for sperm collection and relation to bird behaviour

We collected ejaculates between 0 and 25 days before the first egg was laid in the nest of the focal bird. We could check the nest content of only 20 sampled birds out of the 25 which gave us sperm samples. Figure 3 shows a plot of sperm collection success according to number of days before laying. First, it shows that we trapped only a few birds ($n = 6$) in the period between 25 and 16 days before the laying of the first egg. Second, it shows that the sperm collection success rate declined as birds were trapped closer to the day the first egg was laid in their nest ($\chi^2 = 13.5$, df $= 1$, $p = 0.0002$). Consequently, the period that optimizes sperm collection success seems to be comprised between 15 and 3 days before the laying of the first egg in the focal nest.

We further analyzed whether sperm collection success rate depended on the behaviour of the focal male/pair just before catching the bird. We found that sperm collection success indeed depended on the behaviour of the birds (Fisher's exact test, $n = 85$, $p = 0.022$), with males trapped when they were about to mount their females being more likely to ejaculate semen containing live spermatozoa (Table 1). Other behaviours (female begging for food, after copulation or bird doing nothing) were unpredictive of our success to collect live sperm.

Table 1 Contingency table to test the independence between sperm collection success and the birds' behaviour

	No specific behaviour	Female begging for food	Male about to mount	Copulation occurred recently	Row totals
Successful collection	19 22.4% *24.8%*	5 5.9% *5.4%*	4 4.7% *1.6%*	0 0% *1%*	28
Unsuccessful collection	45 52.9% *50.5%*	9 10.6% *11%*	0 0% *3.2%*	3 3.5% *2.3%*	57
Column totals	64	14	4	3	85

Number, observed percentage and expected percentage (in italic) under H_0 (independence between sperm collection success and the birds' behaviour) for successful (≥ 10 viable sperm in the ejaculate) and unsuccessful sperm collection according to the pair's behaviour immediately before male capture

Sperm survival: comparison of undiluted sperm and extenders

We found that the survival of sperm diluted in PBS was always lower than in any other condition (diluted in extender or undiluted sperm; median survival, mean survival ± SE; PBS: 29 min 20 s, 28 min 46 s ± 3 min 15 s; Alternative condition: 39 min, 40 min 3 s ± 5 min 5 s; $W=0$, $n=6$, $p<0.05$). When sperm were diluted in DMEM and EBSS, spermatozoa survived longer than in any other condition (median, mean ± SE; DMEM: 53 min 1 s, 43 min 40 s ± 5 min 10 s; Alternative buffer: 40 min 40 s, 39 min 20 s ± 4 min 45 s; $W=8$, $n=9$, $p<0.05$ and EBSS: 41 min 40 s, 38 min 38 s ± 4 min 20 s; Alternative buffer: 31 min 10 s, 35 min 39 s ± 4 min 46 s; $W=8$, $n=9$, $p<0.05$). Sperm diluted in NaCl did not survive significantly longer than in any other condition (median, mean ± SE; NaCL: 52 min 13 s, 51 min 15 s ± 2 min 25 s; Alternative buffer: 53 min 25 s, 47 min 29 s ± 6 min 11 s; $W=9$, $n=6$, $p>0.05$). Undiluted sperm did not survive significantly longer than in any extender (median, mean ± SE; No buffer: 27 min 6 s, 29 min 26 s ± 4 min 20 s; Alternative buffer: 28 min 37 s, 32 min 2 s ± 4 min 35 s; $W=10$, $n=8$, $p>0.05$). When excluding samples diluted in PBS, the survival of undiluted sperm or sperm diluted in DMEM, EBSS, or NaCl was on average 40 min 7 s ± 2 min 33 s, and the median survival was 41 min 40 s ($n=32$). Table 2 shows the full data for comparison of sperm survival according to various pairwise combinations of preservation conditions.

Table 2 Sperm survival (seconds) for ejaculates kept under two different conditions

Bird ID	Condition 1	Condition 2	Survival 1	Survival 2
KOC1634	DMEM	Undiluted	1640	2186
KOC1614	DMEM	EBSS	3300	3125
KOC1640	NaCl	DMEM	3210	3568
KOC1637	NaCl	DMEM	3150	3360
KOC1628	Undiluted	PBS	1395	1360
KOC1630	Undiluted	NaCl	3310	3600
KOC1614	Undiluted	DMEM	1565	2411
KOC1635	Undiluted	EBSS	1687	1864
KOC1647	Undiluted	DMEM	1207	1558
KOC1649	Undiluted	EBSS	912	1145
KOC1614	PBS	EBSS	1870	2182
KOC1638	PBS	Undiluted	1794	1866
KOC1640	PBS	DMEM	2500	3360
KOC1640	PBS	NaCl	1104	3116
KOC1630	PBS	EBSS	1725	2500
KOC1640	EBSS	NaCl	2649	2575
KOC1612	EBSS	DMEM	1200	1200
KOC1640	EBSS	DMEM	3100	3181
KOC1624	EBSS	NaCl	3100	2800

Sperm swimming ability and sperm morphology

Mean values for sperm swimming traits and sperm morphological traits are provided in Table 3. Figure 4 illustrates the range of mean (± SD) sperm total length and mean length of sperm components across males.

Discussion

In this study, we demonstrate for the first time that it is possible to collect live sperm in a non-destructive manner and under field conditions from a seabird. We were able to keep the spermatozoa from 19 ejaculates alive for at least 20 min at 40 °C when using the proper extender. Our results suggest that two semen extenders are suitable for maintaining sperm alive: Earle's balanced salt solution (EBSS) and Dulbecco modified Eagle's medium (DMEM). Yet, we recommend using the DMEM extender, because, although it requires to be stored at low temperature until use, it is cheaper than EBSS. Undiluted sperm also performed well in terms of survival, and it could be argued that the seminal fluid alone should be sufficient to maintain sperm alive for long enough to perform sperm quality analyses. It can also be argued that sperm extenders are unlikely to reflect the natural environment that sperm are going to encounter in the female reproductive tract. However, we still rencommend using a sperm extender for two reasons: (1) the use of such an extender is necessary to dilute some highly concentrated ejaculates and (2) in vitro CASA assays may provide valuable information on sperm quality, especially when comparing experimental groups or when investigating how environmental factors (e.g. pollutants) affect sperm production.

Computer assisted sperm analyses (CASA) are very powerful tools to assess parameters linked to sperm quality (Amann and Waberski 2014). Such parameters are usually the percentage of motile sperm, sperm swimming velocity (i.e. straightline velocity VSL, curvilinear velocity VCL or averaged path velocity VAP in µm/s), the linearity of the sperm trajectory, or sperm progressivity (a measure of efficiency in terms of proportion of motion resulting in movement away from the origin; Wilson-Leedy and Ingermann 2007; Amann and Waberski 2014). Several of these parameters, either alone or as a composite index of sperm quality obtained from principal component analyses, have been shown to be good proxies of sperm fertilizing ability in several species (Snook 2005; Simmons and Fitzpatrick 2012). Achieving the appropriate spermatozoa concentration is crucial when conducting CASA. When the sample is too dense, sperm trajectories cross and sperm cells

Table 3 Percentage of motile sperm and sperm swimming ability, and sperm morphology of male black-legged kittiwakes

	% Motile	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	LIN (%)	WOB (%)	PROG (μm)	BCF (Hz)	Head (μm)	Midpiece (μm)	Flagellum (μm)	Total (μm)	Abnormal (%)
Mean	0.31	133.38	121.91	103.86	84.3	90.3	878.98	8.44	10.88	4.23	55.36	70.57	73.18
SD	0.15	49.99	50.51	45.37	0.1	0.1	362.81	2.23	1.11	0.77	4.67	5.07	16.75
N	18	18	18	18	18	18	18	18	20	20	20	20	19
CV_{am}	–	–	–	–	–	–	–	–	10.7	18.9	8.5	7.4	–
% Within-ejaculate variance	–	–	–	–	–	–	–	–	91.5	55.3	92.5	92.0	–
CV_{we}	–	–	–	–	–	–	–	–	20.9	19.5	17.2	14.2	–

Mean, standard deviation (SD) and sample size (*N*) of sperm swimming traits measured in DMEM, sperm morphological traits measured from smears preserved in formalin, and percentage of abnormal spermatozoa per ejaculate. The variability in sperm morphology across males is estimated as the coefficient of variation CV_{am}. Within-ejaculate variability in sperm morphology is estimated as the percentage of within-ejaculate variance $[\sigma^2_{\text{within-ejaculate}}/(\sigma^2_{\text{among-male}} + \sigma^2_{\text{within-ejaculate}})]$ and within-ejaculate coefficients of variation (CV_{we}) (see text for details)

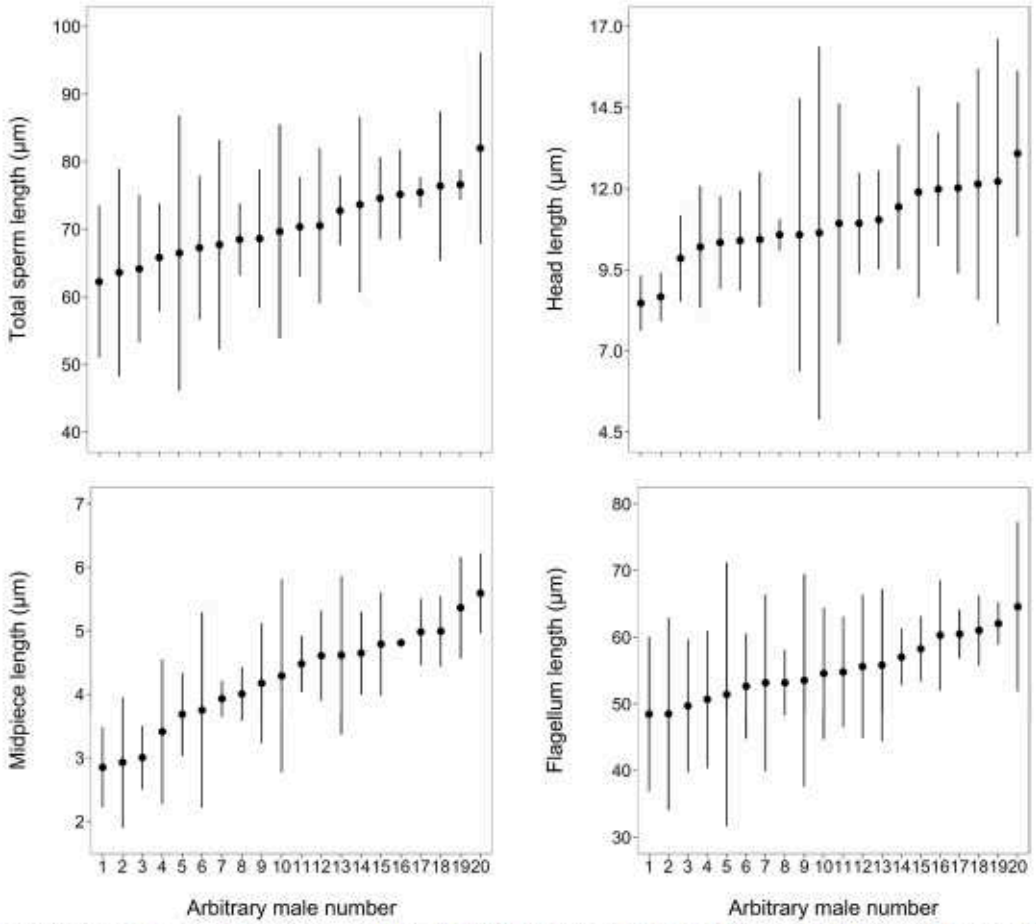

Fig. 4 Within- and between-male variance in sperm morphological traits based on 7–16 sperm cells (mean ± SE: 10.1 ± 0.5) for each of 20 males. Dots represent individual mean lengths (± SD) ranked in order of magnitude

bump into each other, resulting in biased estimations of the aforementioned parameters (Wilson-Leedy and Ingermann 2007). We thus recommend always using a sperm extender to control, and if necessary adjust, the density of the sample to be analysed.

Apart from the addition of a sperm extender, field conditions preclude any sample preparation, and sperm samples cannot be washed and cleaned from unwanted cells or debris. The presence of such cells or debris in sperm samples may compromise the accuracy of the measures performed by the CASA plugin. Here, most particles were removed using the threshold function in ImageJ and by setting a range of size (in pixels) for sperm cells. Yet, we are aware that not all particles could be removed this way. However, this should not affect the estimation of sperm speed or rectitude in sperm trajectory, because immotile particles or particles moved by drift having a size within that of a sperm cell were excluded from the analysis by our criteria on VCL and VAP (see "Methods" section). Nevertheless, it should be noted that such particles lead to underestimating the percentage of motile sperm.

Interestingly, the period when our success rate in collecting sperm was highest, 25 to 6 days before laying, only partly overlaps the peak of copulations reached within the 15 days that precede laying (Helfenstein et al. 2004). Since higher copulation frequency during the 15 days preceding laying may result in lower sperm collection success (see Table 2) due to sperm depletion, we recommend starting sperm collection three weeks before the first egg is usually laid in the colony. This should provide enough time to the researchers to repeatedly capture and massage a large number of males. Nevertheless, one has to keep in mind that sperm quality may vary seasonally, and we recommend to statistically account for this effect using the date relative to laying. In addition to targeting a specific time window, we suggest to target birds about to copulate, i.e. when

the male is about to mount the female, and avoid catching birds that already copulated within the day.

We found that ejaculates contain on average 73.18% of abnormal sperm and between 55 and 92% of the total variance in sperm size and size of sperm components are attributable to variation in morphology within ejaculates rather than between males (Table 3; Fig. 4). These figures are much higher than results of previous studies in wild birds (e.g. Calhim et al. 2007; Laskemoen et al. 2007; Helfenstein et al. 2010; Calhim et al. 2011; Rakha et al. 2015), and they deserve an explanation. First, it could be argued that smearing the spermatozoa on a glass slide may damage the cells and artificially inflate the percentage of abnormal sperm and within-ejaculate variation in sperm morphology. Yet, this method has been used by the authors and other researchers in a variety of bird species (e.g. Calhim et al. 2007; Laskemoen et al. 2007; Helfenstein et al. 2010; Rakha et al. 2015), and such high values of sperm abnormality or within-ejaculate variation in sperm morphology in a wild, free-ranging bird are unusual. Studies conducted on populations of several bird species living around Chernobyl have reported high levels of sperm abnormality, but those values are likely caused by high levels of ionizing radiations increasing mutation rates (Møller et al. 2005; Hermosell et al. 2013). Alternatively, it has been suggested that both inter-male and intra-male/ejaculate variation in sperm morphology may be explained by variation in the strength of post-copulatory sexual selection, particularly sperm competition (Calhim et al. 2007; Kleven et al. 2008). Indeed, van der Horst and Maree (2014) examined the literature and showed that vertebrate species with no sperm competition or extremely low risk of sperm competition exhibit high within-ejaculate variation in sperm morphology and high percentage of abnormal sperm. In birds, Eurasian (*Pyrrhula pyrrhula*) and Azores Bullfinches (*Pyrrhula murina*) produce spermatozoa with an atypical morphology compared to other Passeriformes and their ejaculates are characterized by great variation in sperm mophology and high percentage of abnormal sperm (Birkhead et al. 2006, 2007; Lifjeld et al. 2013). Such characteristics have been interpreted as a consequence of an absence of sperm competition (Birkhead et al. 2006, 2007; Lifjeld et al. 2013). Here, we found a high percentage of abnormal sperm, large proportions of within-ejaculate variance and large within-ejaculate coefficients of variation in sperm morphology (Table 3). These results accord well with the hypothesis of relaxed selection and lack of sperm competition, as Black-legged Kittiwakes are known to be strictly monogamous (Helfenstein et al. 2004).

Conclusions

Unlike passerines birds who store their semen in the seminal glomera (Lake 1981), Black-legged Kittiwakes store their semen inside their body (Lake 1981), thus preventing direct stimulation of the storage organs. Furthermore, these birds are strictly monogamous (Helfenstein et al. 2004), and are likely selected to produce small quantities of sperm during a short period. Nevertheless, we have been able to trap 25 males and obtain as many ejaculates within 2 weeks. We thus believe that larger samples can be aimed for not only in kittiwakes, but in seabirds with similar ecology and exhibiting some levels of extrapair paternity such as other larids (e.g. the Black-headed Gull *Larus ridibundus*; Ležalová-Piálková 2011, or Common Gull *Larus canus*; Bukacińska et al. 1998) or alcids (Wagner 1992) where males are expected to produce larger amounts of sperm (Møller and Briskie 1995).

Additional files

Additional file 1. Video showing sperm collection on a Black-legged Kittiwake.

Additional file 2. Video of spermatozoa in motion.

Additional file 3. We checked the minimum number of sperm cells per ejaculate required for accurate estimates of ejaculate mean and standard deviation using a resampling procedure. We randomly sampled 1 to 15 sperm cells per ejaculate without replacement and computed the associated mean (Fig. S1) and standard deviation (Fig. S2). A visual assessment of the plots reveals that a minimum of 10 sperm cells per ejaculate allows accurate estimates of mean and SD.

Additional file 4. Additional dataset.

Authors' contributions

SHG, OC, PB, and GWG participated in the design of the study and its coordination. SHG, PB and OC collected the data in the field. AY and AAB analysed sperm samples. SHG and FH carried out the statistical analyses. SHG and FH wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare they have no competing interests.

Availability of supporting data

All data generated or analysed during this study are included in this published article and its supplementary information files.

Consent for publication

Not applicable.

Ethics approval
This study was examined and approved by the Norwegian Animal Ethics Committee and the governor of Svalbard, and was conducted under permission FOTS ID 273 8679.

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