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SABRINA TARTU[1*], AURÉLIE GOUTTE[1], PACO BUSTAMANTE[2], FRÉDÉRIC ANGELIER[1], BØRGE MOE[3], CÉLINE CLÉMENT-CHASTEL[1], CLAUS BECH[4], GEIR WING GABRIELSEN[5], JAN OVE BUSTNES[3], OLIVIER CHASTEL[1]

[1]*Centre d'Etudes Biologiques de Chizé (CEBC), UPR 1934-CNRS, F-79360, France*

[2]*Littoral Environnement Société (LIENSs), UMR 7266-CNRS, Université de La Rochelle, F-17000, France*

[3]*Norwegian Institute for Nature Research, Fram center, NO-9296 Tromsø, Norway*

[4]*Department of Biology, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway*

[5]*Norwegian Polar Research Institute, Fram center, NO-9296 Tromsø, Norway*

Short title: Mercury and intermittent reproduction

[*]tartu@cebc.cnrs.fr

Summary

Mercury, a ubiquitous toxic element is known to alter expression of sex steroids and to impair reproduction across vertebrates but the mechanisms underlying these effects are not clearly identified. We examined whether contamination by mercury predicts the probability to skip reproduction in black-legged kittiwakes (*Rissa tridactyla*) from Svalbard. We also manipulated the endocrine system to investigate the mechanism underlying this relationship. During the pre-laying period, we injected exogenous GnRH (gonadotropin-releasing hormone) to test the ability of the pituitary to release luteinizing hormone (LH, a key hormone for the release of sex steroids and hence breeding) in relation to mercury burden. Birds that skipped reproduction had significantly higher mercury concentration in blood than breeders. Endocrine profiles of these birds also varied based on breeding status (breeders vs. non-breeders), mercury contamination and sex. Specifically, in skippers (birds that did not breed), baseline LH decreased with increasing mercury concentration in males, while it increased in females. GnRH-induced LH levels increased with increasing mercury concentration in both sexes. These results suggest that mercury contamination may disrupt GnRH input to the pituitary. Thus, high mercury concentration could affect the ability of long-lived birds to modulate their reproductive effort (skipping or breeding) according to ongoing environmental changes in the Arctic and impact population dynamics.

Key words: Intermittent breeding; Mercury; GnRH challenge; Luteinizing Hormone; Black-legged kittiwake

1. INTRODUCTION

Mercury is a ubiquitous toxic element of both natural and anthropogenic sources. In its methylated form, mercury can impair reproduction and disrupt the expression of estradiol and testosterone across vertebrates [1, 2]. However the mechanisms underlying these effects are not clearly identified [3]. In response to increased day length, Gonadotropin Releasing Hormone (GnRH) is secreted and triggers luteinizing hormone (LH) release from the pituitary gland. LH, in concert with follicle-stimulating hormone (FSH), promotes gonadal maturation, sex steroid secretion and in turn, the onset of reproduction. It is conceivable that mercury acts primarily on the ability of the pituitary to secrete gonadotropin hormones (LH and FSH), altering sex steroids release and ultimately impairing reproductive behaviour. Mercury may also suppress GnRH in the hypothalamus, thereby reducing LH production. Clearly, there is a need for studies investigating the mechanisms of LH suppression by mercury [3] and subsequent repercussion in breeding animals.

Recent investigations have highlighted a major role for LH on skipped breeding behaviour (non-breeding by individuals that previously bred), a common feature in long-lived birds [4, 5]. In some seabirds, skipping individuals show either low levels of baseline LH (Black-legged kittiwake, *Rissa tridactyla* [4]), or fail to maintain elevated LH levels after a GnRH injection (Snow petrel, *Pagodroma nivea* [5]). LH secretion also appears to be sensitive to environmental stressors in kittiwakes [4]. This opens the yet unexplored possibility that some endocrine disruptors like mercury may play a role in skipping behaviour, by altering the release and secretion of LH.

Here, we test whether mercury concentrations 1) are linked to skipping behaviour and 2) affect patterns of LH release during the pre-breeding period in an arctic population of Black-legged kittiwakes. To evaluate LH release, we challenged the pituitary with an exogenous injection of GnRH. Kittiwakes provide an excellent model to address these

questions, as they bear elevated mercury levels in Svalbard [6] and a significant proportion of adults skip breeding each year [4]. We predicted that high mercury concentration in blood would 1) be linked to a high probability to skip breeding 2) impair baseline and/or GnRH-induced LH levels.

2. METHODS

The study was conducted at Kongsfjorden, Svalbard (78°54′N, 12°13′E) from May 20th to June 6th 2008 (52 birds) and from May 21st to June 7th in 2011 (104 birds) during the pre-breeding period. Birds were caught on the nests and a blood sample was collected immediately after capture. In 2008, we performed a GnRH challenge (French veterinary services permit 79-2): immediately after the first blood sampling, kittiwakes were injected 0.1mL of a solution of GnRH (Electronic supplementary material, figure S1). Blood samples were collected from the alar veins 10 and 30 minutes after the GnRH injection to measure baseline and GnRH-induced levels of LH (both sexes) and testosterone (males only) as detailed in electronic supplementary material. Total mercury from all 2008 and 2011 samples was measured at LIENSs from lyophilized red blood cells, by atomic absorption spectrophotometry on 5-10 mg aliquots [7]. Mercury concentrations are expressed in µg/g dry weight. In both years, focal birds were measured as described in [4] to calculate a scaled mass index and observed daily. The nest content was checked every two days, to monitor if birds engaged in breeding (at least one egg laid) or if they skipped (no egg laid). We also monitored fitness, including date of first egg-laying, clutch size and the number of chicks that reached 12 days post-hatch (hereafter breeding success). To test the effects of mercury and scaled mass index on these reproductive parameters, we pooled data from 2008 and 2011 (no birds were sampled twice). Then, for 2008 we tested the effects of mercury concentration and interaction with sex, on baseline and GnRH-induced hormone levels in skipping and breeding birds. All statistical analyses were performed using R 2.13.1 and we used generalized linear models (GLM) with a normal/binomial error distribution and an identity/logit link function to test our biological assumptions. Model selection was performed by a step-down approach starting from the global model including all the independent variables.

3. RESULTS

Sex and year did not explain reproductive decisions (skippers vs. breeders; GLM, all p-values >0.10). Mercury predicted the likelihood to breed, as skippers had significantly elevated mercury levels compared to breeders (χ^2= 14.06, p=0.001, Fig. 1A-B). This was true in both sexes (females: χ^2= 4.41, p=0.036; males: χ^2= 4.61, p=0.032). There was no interaction between mercury and either sex, year or scaled mass index (GLM, all p-values >0.87). Among birds that bred, pre-breeding mercury concentration did not predict first egg-laying date, clutch size or breeding success (all p-values >0.18).

In birds that bred, baseline LH levels were higher in females than in males ($F_{1,34}$=5.9, p=0.02), but were unrelated to mercury levels ($F_{1,33}$=0.08, p=0.783; mercury × sex: $F_{1,32}$=1.5, p=0.23, Fig. 1C-D). GnRH-induced LH levels were not linked to either sex ($F_{1,17}$=3.23, p=0.09) or mercury levels ($F_{1,17}$=0.88, p=0.361; mercury × sex: $F_{1,17}$=2.72, p=0.118) in breeders.

In skipping birds, baseline LH levels were not affected by sex ($F_{1,12}$=0.43, P=0.523) but significantly and negatively correlated to mercury levels in males, and positively in females (mercury × sex: $F_{1,12}$=19, p<0.001; Fig. 1E-F). GnRH-induced LH levels significantly increased with increasing mercury concentration in skipping males and females (mercury: $F_{1,10}$=21.6, p<0.001; mercury × sex: $F_{1,8}$=0.07, p=0.805, Fig.1E-F). Baseline testosterone levels tended to decrease in skipping males ($F_{1,7}$=5.92, p=0.051) but not in breeding males ($F_{1,19}$<0.01, p=0.97). GnRH-induced testosterone levels were not related to mercury levels neither in skipping ($F_{1,5}$=0.1, p=0.761) nor in breeding males ($F_{1,9}$=0.6, p=0.458).

4. DISCUSSION

Long-lived seabirds often skip reproduction in certain years, and our previous investigations in kittiwakes demonstrate that LH levels are lower in the birds which choose to skip breeding (skippers, [4]). Could environmental toxicants influence LH and/or GnRH levels, and therefore reproductive decisions? We evaluated a possible role for mercury contamination in non-breeding black-legged kittiwakes, and investigated the endocrine mechanisms that underlie this decision.

As expected, total blood mercury measured after arrival significantly predicted the likelihood to breed. Although our study was correlational and would require experimental manipulation of contaminants [1, 8], there is support for causal effects since experimental mercury administration can alter pairing in white ibises (*Eudocimus albus*) and suppress spawning in fathead minnows (*Pimephales promelas*) [1, 8]. In our study, the most contaminated males and females were less likely to breed although there was a large overlap in mercury levels between skipping and breeding birds. Some of the skipping birds may be young or low quality individuals [5], and therefore be intrinsically more sensitive to mercury. In birds that did breed, mercury had no effect on egg-laying dates, clutch size and breeding success. In breeders, total blood mercury averaged 1.8 µg/g (range: 0.91-3.08 µg/g) that could be below a threshold level beyond which breeding success is significantly impaired as shown in other bird species [9]. However, mercury-breeding success relationships appear complex in birds since administration of a low mercury dose could even enhance breeding in Mallards *Anas platyrhynchos* [10].

Mercury concentration predicted LH levels in skippers, but patterns were different among males and females, and varied between baseline and GnRH-induced LH. Baseline LH and testosterone decreased with increasing blood mercury concentration in males, as found in mercury-fed laboratory rats [11]. Conversely, in skipping females, LH and mercury were

positively related (although levels are still significantly lower than in breeding females [4]). These data suggest that mercury-LH relationships are complex and LH disruption could also be the result of differential negative feedback from mercury given its estrogen-like effect [2].

To test the functionality of the pituitary, we performed GnRH challenges in breeders and skippers. Based on previous work [4], we assume that the dose of GnRH we used is sufficient to elicit a significant increase of LH 10 minutes after injection, and interpretation of our results is based on this assumption. If mercury is suppressing LH secretion directly at the pituitary, we would expect LH response to GnRH to be suppressed in skippers. Contrary to our predictions, skippers were clearly able to release LH, and increased LH 3-5-fold above baseline (breeders did showed a slight LH increase). Although based on correlations, it suggests that mercury may act at the hypothalamic level, disrupting GnRH synthesis or secretion. Evidence from other vertebrates demonstrates that mercury can accumulate in the hypothalamus and alter GnRH content and signalling [12, 3]. In response to GnRH decline, the pituitary may have up-regulated GnRH receptors, explaining the large increase in LH with GnRH injection. Thus in kittiwakes, mercury contamination could lead to a decline in GnRH release, the subsequent increase of pituitary GnRH receptors explaining why the disrupted pituitary over-releases LH when experimentally challenged by GnRH. Although we originally hypothesized the mercury was targeting the pituitary, the induced LH response also points to a problem at the hypothalamus. It is additionally possible that mercury could bind to LH [13] making LH less able to activate gonadal receptors. Further experiments evaluating LH and GnRH expression in the brain would be necessary to meter out these possibilities.

Long-lived birds often skip breeding when foraging conditions are poor, modulating their reproductive effort according to environmental conditions [14]. The present situation in the arctic is of concern: mercury levels in seabirds are increasing [15] and if combined to

rapid climate change, we are facing a worst-case scenario [16]. Ultimately, the likelihood to skip breeding may become too high to maintain current population levels.

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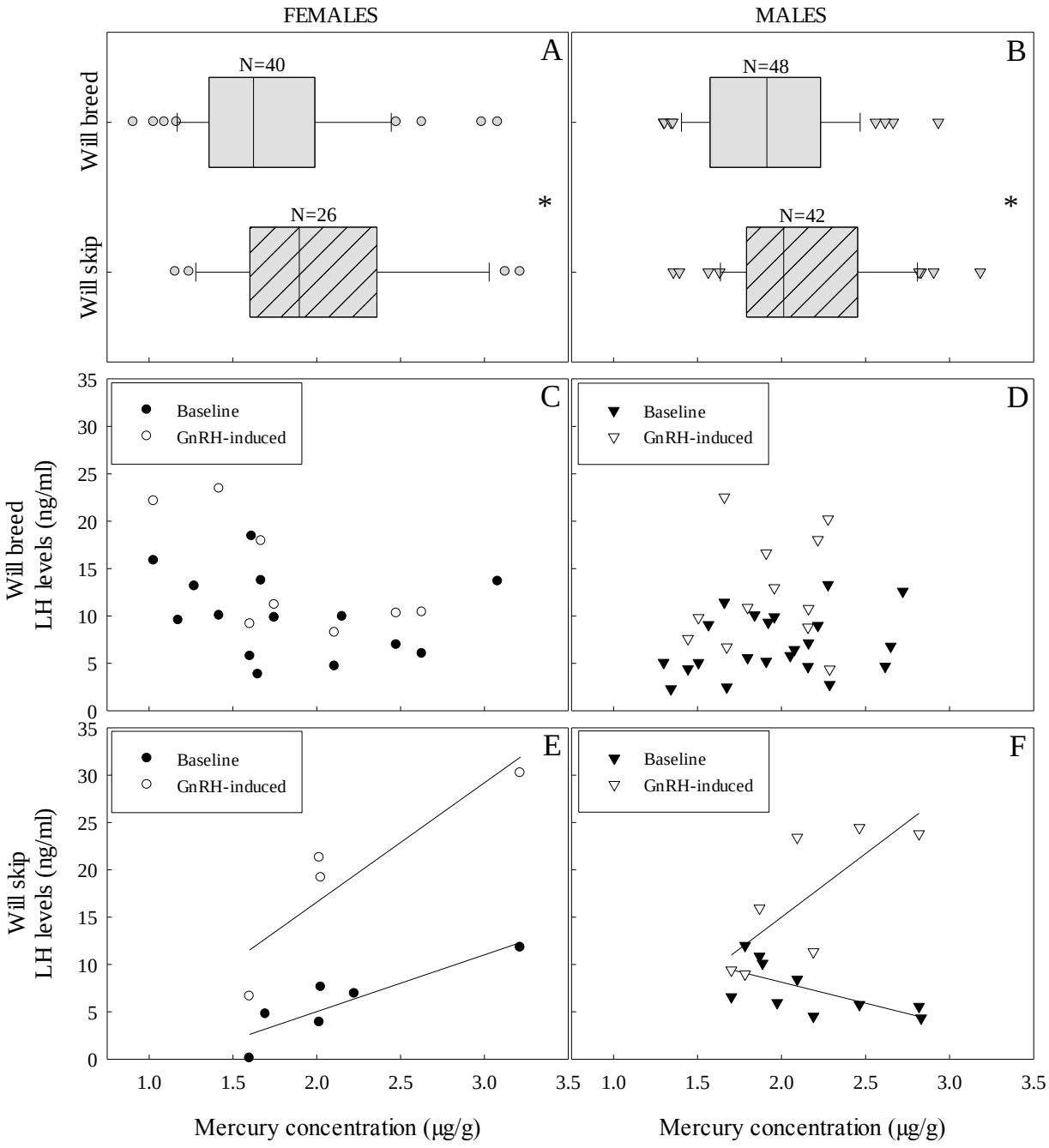

Figure 1: Pre-breeding mercury (µg/g dwt) and LH levels (ng/ml) in breeding and skipping black-legged Kittiwakes. Breeding birds (empty boxes) had lower mercury levels in blood than birds that would skip breeding (striped boxes), both in females (A) and males (B). Boxes represent median, 25th and 75th percentiles and outliers. In breeders (C-D), baseline and GnRH-induced LH levels were not affected by mercury. In skipping females (circles) baseline LH increased with increasing mercury (E), while in skipping males (triangles) baseline LH decreased with increasing mercury (F). GnRH-induced LH levels, in both skipping males and females, increased with increasing mercury levels (E-F).

Supplementary material

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[3]Norwegian Institute for Nature Research, Fram center, NO-9296 Tromsø, Norway

[4]Department of Biology, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway

[5]Norwegian Polar Research Institute, Fram center, NO-9296 Tromsø, Norway

GnRH challenge

Pre-breeding black-legged kittiwakes were caught on the nests and a first blood sample (*ca*. 0.3 mL) was collected within 3 minutes after capture. Immediately after this first blood sampling, birds were injected with 0.1 mL of a solution of GnRH ([Gin^8], Sigma Lot 121H04314) to test the responsiveness of the pituitary gland and the gonads. The GnRH was dissolved in physiological solution to yield a final dosage of 0.6 µg/0.1mL (1.5 µg/kg body mass in 1 mL of 0.9% saline solution). This dose of GnRH has been shown to be sufficient to elicit a significant increase of LH ten minutes after GnRH injection (Fig. S1-B) in several

seabird species including black-legged kittiwakes [1, 2, 3]. We administered 0.1 mL of GnRH solution (GnRH–injected birds, N = 33) or saline solution (control birds, N = 21) directly into the alar vein. Kittiwakes were then placed into cloth bags and subsequent blood samples (*ca*. 0.3 mL) were collected from the alar vein at 10 minutes (to measure GnRH-induced release of LH in males and females) and 30 minutes (to measure GnRH-induced release of testosterone in males only) after the injection.

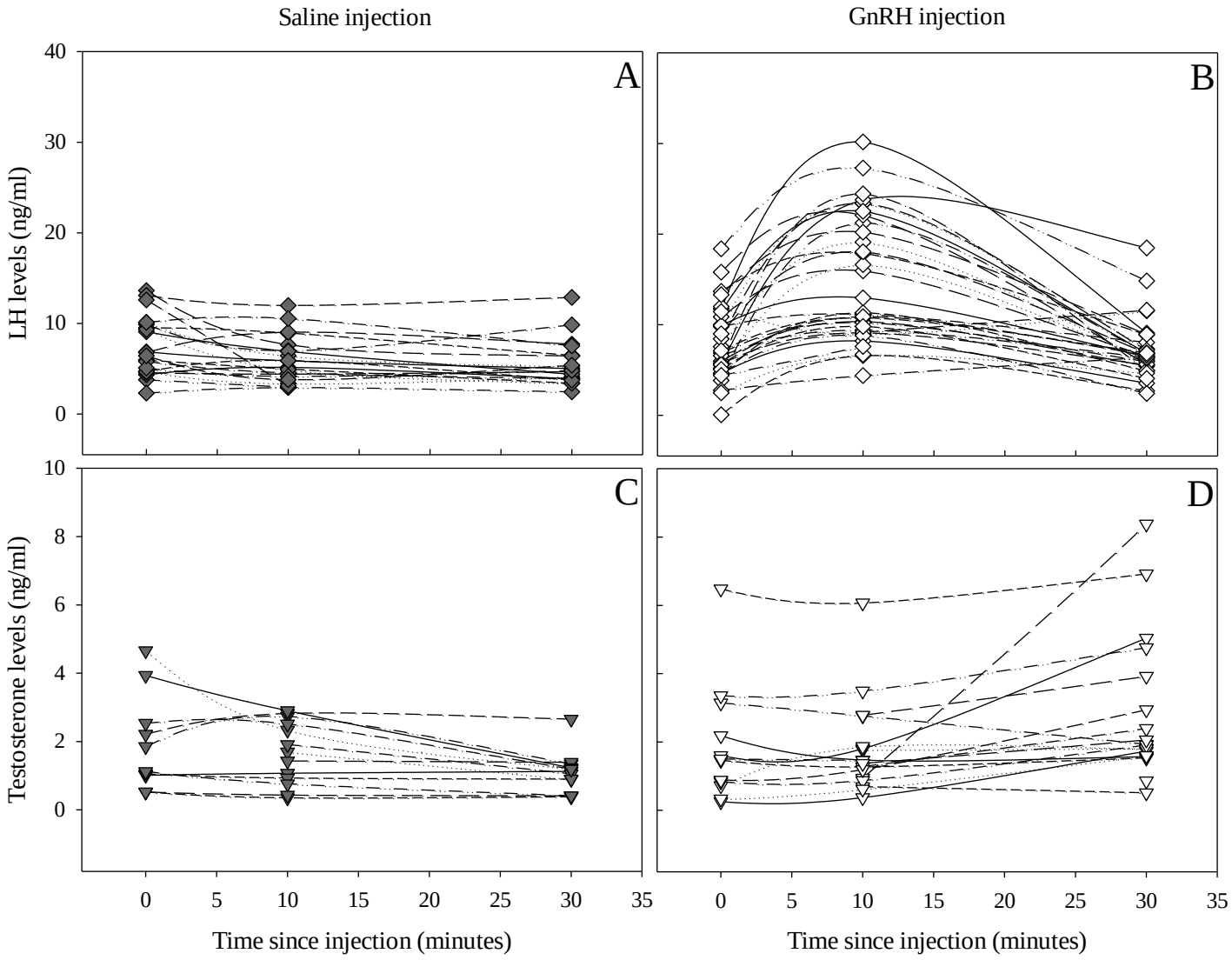
Molecular sexing and hormone assay

Blood samples were centrifuged, and plasma and red blood cells were separated and stored at -20°C until used respectively in hormone assays or molecular sexing, at the Centre d'Etudes Biologiques de Chizé (CEBC). Molecular sexing was performed as detailed in [2]. LH radioimmunoassay was conducted at the CEBC following the methods previously described and validated for Black-legged kittiwake plasma [1]. Pooled plasma samples of kittiwakes produced dose-response curves that paralleled the chicken LH standard curves ("AGM 51122F", sources: LH, Prf. Ishii and Wakabayashi, Wadesa University, Japan, Fig. S1). Parallel curves indicate that the concentration-dependent binding of LH to antibody is similar in kittiwakes and chickens, and that this heterologous RIA can be used to assess relative levels of plasma LH in the Black-legged kittiwakes [1]. The lowest detectable concentration for LH was 0.06 ng/mL and the intra-assay coefficient of variation was 8.7 % (N = 3 duplicates). Plasma concentrations of testosterone were assayed for males only, by radioimmunoassay, at the CEBC as described in [3]. The lowest detectable concentration for testosterone was 0.05 ng/mL and the intra-assay coefficient of variation was 7 % (N = 3 duplicates).

Before GnRH-injection, baseline (i.e. at 0 minutes) hormone levels did not differ between GnRH-injected and control kittiwakes (GLM, LH: $F_{1,51} = 0.04$, $p = 0.842$; testosterone: $F_{1,20} =$

0.0045, p = 0.947, Fig. S1-A,B). Following GnRH injection, LH levels significantly increased over 10 minutes and then declined 30 minutes (GLMM, time as factor, $F_{1,31}$ = 50.808, p <0.001, Fig. S1-B), without sex difference (sex: $F_{1,31}$ = 0.857, p = 0.362; interaction: F1,30 = 1.738, p = 0.109). In control birds, LH levels did not significantly change over 10 and 30 minutes of handling (GLMM, $F_{1,18}$ = 3.203, p = 0.09, Fig. S1-A) and were lower in males than in females at 30 minutes (GLM, $F_{1,15}$ = 6.324, p = 0.024) but not at 10 minutes after the injection of saline solution ($F_{1,16}$ = 2.239, p = 0.154). In GnRH-injected males, testosterone levels significantly increased over 30 minutes after injection (GLMM, $F_{1,16}$ = 8.273, p = 0.011, Fig. S1-D), while in control males, testosterone significantly decreased over 30 minutes (GLMM, $F_{1,12}$ = 11.341, p = 0.006, Fig. S1-C).

Figure S1: Hormonal change after the injection of the GnRH solution B and D panels (open symbols) or of the saline solution A and C panels (control, filled symbols). GnRH-injected birds (B) significantly released LH over the first 10 minutes then LH significantly decreased over 30 minutes, while control levels remained unchanged after injection (A). C and D panels: testosterone levels in males following the injection of saline solution (control, C) or GnRH (D). Testosterone levels of control birds (C) significantly decreased after injection, while GnRH-injected males showed significantly elevated testosterone over 30 minutes.



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