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Collective exodigestion favours blowfly colonisation and development on fresh carcasses

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Running head

Social strategies in necrophagous blow flies

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Abstract

Necrophagous flies breeding on carcasses face high selection pressures and therefore provide interesting opportunities to study social adaptations. We postulated that blowfly necrophagous larvae gregariousness is an adaptive response to the environmental constraints of fresh carcasses. Cooperation is indeed believed to be a key to the global success of social species. To test this idea, the development of *Lucilia sericata* (Diptera: Calliphoridae) larvae growing on low- or high-digestibility food substrate (control or trypsin-added ground beef muscle, respectively) at different larval densities was monitored. Results showed that larvae developed faster and had decreased mortality at high larval density compared with low larval density. Furthermore, aggregation had no deleterious effect on the morphological characteristics (e.g., size) of post-feeding larvae and flies. We concluded that increased density positively affected population fitness, which is a conclusion consistent with the predictions of the Allee effect. Compared with regular food, larvae fed on high-digestibility food had reduced mortality and faster development on average. According to these results, we postulated that collective exodigestion might be an adaptive response allowing blowflies to colonise fresh carcasses before the arrival of other insects and the multiplication of microbes. This hypothesis is consistent with the idea that cooperation may enable species to expand their niches.

Keywords: Adaptive ecology, Aggregation, Allee effect, exodigestion, niche selection

INTRODUCTION

Collective behaviour is powerful adaptive strategy to cope with a harsh environment, and cooperation is believed to be a key to the global success of social species (Cornwallis et al., 2017). Although eusocial Hymenoptera such as bees and ants are well-known insect societies, gregarious insects also provide interesting examples of efficient collective strategies (Choe & Crespi, 1997; Costa, 2006). Grassé, (1946) defined a part of social behaviour as a *Mass effect*, i.e., a group effect caused by a modification of the medium by the population itself. A striking example of this idea is provided by terrestrial crustacean woodlice, for which desiccation is a primary concern: in response to this environmental stress, aggregation offers group protection against drying (Broly et al., 2014). Deneubourg et al. (1990) also observed that bark beetle larvae aggregate and use communal feeding as a way to overwhelm the reaction of the tree. Similarly, social spiders collectively inject digesting enzymes and then suck up the liquidised prey content, thereby exploiting a common resource that was jointly created (Schneider & Bilde, 2008). In flies, egg aggregations allow larvae to warm and moisten the organic material (Barnard & Geden, 1993; Bryant, 1977), improve larval ability to dig and burrow into the food (Durisko et al., 2014), and limit the growth of competitive fungi (Rohlf et al., 2005; Zvereva, 1986).

Blowflies (Diptera: Calliphoridae) are the most widespread and abundant necrophagous species and are attracted quickly after death (Smith, 1986). Female flies lay eggs on fresh vertebrates carcasses on which their larvae feed until metamorphosis; larvae are confined to the carrion until reaching a sufficient mass/instar. Although some studies report competition among carrion flies larvae (Denno & Cothran, 1975, 1976; Feinberg & Pimentel, 1966; Hanski, 1987; Ives, 1991), Flores et al. (2017) observed that female flies do not preferentially oviposit on carcasses without larvae, and several studies show that ovipositing gravid blowflies

enhance the attractiveness of a carrion (Brodie et al., 2014; Denno & Cothran, 1975; Jiang et al., 2002; Kneidel, 1984; Wertheim, 2005). Therefore, egg aggregation is clearly not avoided and is even sought by adult blow flies. Furthermore, during the three feeding instars, calliphorid larvae actively aggregate (Boulay et al., 2013, 2016): masses of several thousands of larvae are commonly observed in the field (Fenton et al., 1991; Slone & Gruner, 2007). Because females and larvae should avoid competition, whether necrophagous larval aggregation is advantageous is a reasonable question (see Table 1). When they reach a sufficient weight, late third-instar disperse to find sheltered places for pupation, a process known as postfeeding larval dispersal (Gomes et al., 2006). Consequently, postfeeding larvae and pupae are not subject to the same selection pressure than feeding instars.

Table 1: The benefits and costs of gregariousness necrophagous larvae of calliphorids flies.

	BENEFITS	COSTS
Temperature	The mechanisms remain hypothetical (larval metabolism, permanent friction of larvae, microbial activity), but heat production by large larval masses is clearly demonstrated (Charabidze et al., 2011; Gruner et al., 2017; Heaton et al., 2014; Slone & Gruner, 2007). The microclimate inside the mass should increase the developmental speed of maggots.	When the temperature approaches the thermal tolerance range of the larvae, self-regulation processes within the mass must be initiated, which reduce the time allocated to feeding and therefore the growth rate (Charabidze, Hedouin, & Gosset, 2013).
Food availability	Cooperative exodigestion was supposed but not experimentally	After a critical number of individuals are reached, the availability of nutrients per

	demonstrated (Rivers et al., 2011).	individual becomes insufficient, leading to decreases in the speed of development, survival, and weights and sizes (Table A1).
Physico-chemical modifications	Increasing the mass may protect larvae against evaporation and temperature decreases (Broly et al., 2014; Huntington et al., 2007; Pérez et al., 2016).	Excreta rich in ammonia accumulates (Hobson, 1931).
Predators and parasites	High population densities can reduce the risk of predation/parasitism but remains to be demonstrated for necrophagous species (Hunter, 2000; Rohlf & Hoffmeister, 2004).	Chemical signals of the larvae and metabolites increase as a result of feeding (no experimental evidence, see (Frederickx et al., 2014).
Other	Repulsion of scavengers and burying beetles: female burying beetles typically exploit carcasses without blowfly larvae (smith, 1986). Scavengers may avoid carcasses heavily colonised by larvae and/or bacteria (devault et al., 2004).	Increased risk of disease: many pathogens are transmitted in a density-dependent fashion, but this phenomenon has not been demonstrated in necrophagous larvae (Pöppel et al., 2015).

89 Adapted from Rivers et al. (2011).

90

91 Necromass is by nature a discrete and ephemeral food source. Decomposition
92 processes quickly alter flesh (Clark et al., 1997), and the probability of carcass removal
93 by scavengers increase with time and decomposition odour (DeVault et al., 2004). As
94 pioneer species, blowflies strategy depends on their ability to quickly colonize carrions

(Denno & Cothran, 1975). Furthermore, many birds, wasps and necrophagous insects prey on maggots. Voss et al. (2009) report an overall 11.8% parasitism of maggots, and Frederickx et al. (2013) observed that on average, 48% of fly pupae were killed by hymenopteran parasitoids (from 3.5% in May to 90% in September). When the larvae develop rapidly, less time is spent on the cadaver and probability of a food shortage, predation, parasitism and interspecific competition decrease (DeVault et al., 2004; Rivers et al., 2012). That can noticeably be achieved by favouring high-temperatures that increase their development speed (Aubernon et al, 2016, Grassberger & Reiter, 2001). In this respect, a well-known adaptive strategy of blow flies larvae is the so-called *maggot-mass effect*, a local temperature increase attributed to larval metabolism (Charabidze et al., 2011; Slone & Gruner, 2007). Because additional heat gain reduces developmental time, aggregated larvae benefit from this larval-mass effect with faster development than that of isolated individuals (Erzinçlioglu, 1996; Heaton et al., 2014; Johnson & Wallman, 2014). In the same way, aggregation can protect larvae from abrupt decreases in temperature (Huntington et al., 2007; Magni et al., 2016; Rivers et al., 2011). However, these thermal effects mostly occurs during late feeding instars and only within large masses above a thousand larvae (Charabidze et al., 2011; Heaton et al., 2014). Here, we postulated that beside maggot-mass effect, gregariousness favours necrophagous larvae feeding and their early exploitation of fresh carcasses.

According to Disney (1986), the switch to ingesting liquid instead of solid food could be a fundamental revolution in the evolution and diversification of flies. Necrophagous larvae are actually unable to ingest solid particles other than those that are microscopic, and their buccal apparatus is not suitable for mastication (Guyénot, 1907). Therefore, larvae must liquefy the food before ingestion (i.e., exodigestion). The necrophagous larvae of calliphorid flies excrete/secrete various proteolytic enzymes, including trypsin, lipases and amylases (Chambers et al., 2003; Hobson, 1931; Rivers

et al., 2011; Terra & Ferreira, 1994). The sharing of enzymes has been reported as a likely benefit of calliphorid larval aggregation, but this idea has never been demonstrated (Ireland & Turner, 2006; Reis et al., 1999; Rivers et al., 2011). To test the hypothesis of collective benefits through exodigestion, *L. sericata* larvae were bred at different larval densities. Two hypotheses were experimentally tested: (1) larval development speed and survival increases with increasing larval density, and (2) larval development speed and survival increases with increasing food digestibility.

MATERIALS AND METHODS

Ethical note

This research adheres to the ASAB/ABS Guidelines for the Use of Animals in Research, the legal requirements of the country in which the work was conducted, and all institutional guidelines. During the design of the study, a minimum of manipulation with a minimum of used animals was sought.

Insect rearing

Lucilia sericata larvae were obtained from adult flies bred in the laboratory and maintained in tulle cages (50x50x50 cm) at room temperature (23±2°C). Wild flies, 100 to 200, were added every 2 months. The flies were fed caster sugar and water *ad libitum*. After each new generation, minced beef liver was added for 7 days to provide proteins for vitellogenesis and then removed. Laying of eggs was triggered with the placement of 25±5 g of beef liver for 2 h (from 10 a.m. to midday) in cages. Oviposition time (precision ±1 h) was noted as D -1 (see Fig. 1).

Figure 1: Chronological diagram of the experimental process. D_{-1} is the time of oviposition (± 1 h). Larvae were counted and placed on food on the next day (D_0). D_x is the first post-feeding larva and D_y is the first emerging fly.

Larval development

A density-dependent increase in competition is found in many blowfly developmental studies (see Table A1). In some studies, measurements were conducted during the feeding of instars, which likely disrupts aggregates and prevents detection of any social benefit (Martinez Sanchez et al., 2007; Simkiss et al., 1993). Many others suffer from a lack of statistical power (few replications, time between measures too long) (Martinez Sanchez et al., 2007; Reigada & Godoy, 2006; Saunders & Bee, 1995; Serra et al., 2011). Finally, many authors used mixed liver or synthetic medium to feed larvae (Martinez Sanchez et al., 2007; Reis et al., 1999; Simkiss et al., 1993; Smith & Wall, 1997). These food sources are more digestible and less nutritious than muscular tissues and significantly affect larval development (Barnes & Gennard, 2013; Bernhardt et al., 2017; El-Moaty & Kheirallah, 2013; Pérez et al., 2016, 2016; Rueda et al., 2010; Tachibana & Numata, 2001). By contrast, our experiments were performed with relevant larval densities feeding on beef muscle, a realistic food source.

All experiments on development were conducted from January to May 2017 in a thermostatic chamber set at $25\pm 0.1^{\circ}\text{C}$ with constant ventilation (ST4; POL-EKO Aparatura®, Wodzisław Śląski, Poland). A 12:12 h photoperiod cycle was obtained with a fluorescent tube placed inside the incubator (430 lumens, light from 9:30 a.m. to 9:30 p.m.).

Eggs obtained at Day -1 were incubated for 22 h, and then first instars were counted and placed in boxes containing food (D0). Each box was placed in a second box containing 1 cm of sand. All experiments were performed at 25°C . Two independent variables were studied: the initial number of larvae (i.e., larval density) and the digestibility of the food. Six replications for densities d10, d50, d100, and d250 and three replications for density d500 were prepared for each food type.

Two independent variables were studied: the initial larval population (i.e., larval density) and the digestibility of the food. The larval densities (d) tested were 10, 50, 100, 250 and 500 larvae per 50 ± 1 g of food, low enough to prevent maggot-mass effect (i.e. significant local temperature increase, Charabidze et al., 2011). Two types of food were used: the control food (C) was 50 ± 1 g of minced beef muscle at 15% fat mixed with 15 mL of 0.9% NaCl, and pre-digested food (Tr) was obtained with the same meat mixed with 50 ± 0.5 mg of trypsin (Trypsin T4799, porcine pancreas; Sigma-Aldrich®) diluted in 15 mL of 0.9% NaCl. The addition of trypsin changes food digestibility (the size of the molecules) but not the composition or nutritional value of the meat. The beef muscle was thawed for 6h at ambient temperature, mixed according to the two-abovementioned modalities and placed in translucent polypropylene boxes (100x75x63 mm). Six replications for densities d10, d50, d100, and d250 and three replications for density d500 were prepared for each food type.

Experimental procedure

The eggs obtained at D-1 were incubated at 25°C for 22 h (first instar; see Fig. 1) (Grassberger & Reiter, 2001). The next day (D0), young first instars were counted and placed in the boxes containing food. Each box was placed in a second translucent polypropylene box (180x135x195 mm) containing 1 cm of sand. To preserve aggregation and avoid disturbance of feeding larvae, no measurements were performed on feeding instars. Only wandering larvae (also called post-feeding larvae, i.e., third instar larvae that had finished feeding and left the food) were measured. The post-feeding larvae and adult flies were checked three times a day (10:00 a.m., 4:00 p.m., 10:00 p.m.).

Post-feeding larvae found in the sand were quickly rinsed with water and dried on absorbent paper. After weighing (Kern® ALT310-4, Grass Valley, USA), these post-feeding larvae were placed in new boxes (100x75x63 mm) containing 1 cm of sand. The few larvae that left the food before the third instar or weighed less than 25 mg (below the first percentile) were not counted as post-feeding larvae. The mortality rate to post-feeding and the weighted mean of larval weights per replication were calculated.

Newly emerged flies were sampled, left in development for at least 6 h, and then sacrificed by freezing. The flies were sorted by sex using cephalic morphology (Erzinçlioglu, 1996). The area of the right wing was measured to estimate the sizes of males and females (Saunders & Bee, 1995). Data were recorded by taking pictures of dissected and flattened right wings, with the surface area determined using ImageJ software (version 1.51j8, Wayne Rasband, National Institutes of Health, USA, public domain). All female and male flies from densities 10 and 50 were analysed. Random samples of 25 male flies and 25 female flies per replication were analysed for the densities 100, 250 and 500.

Statistical analyses

Statistical tests were conducted bilaterally, with a significance threshold of $\alpha=0.05$. When appropriate, 95% bilateral confidence intervals are presented. All data were analysed with R software via R studio (R version 3.3.2©2016; The R foundation for Statistical Computing | Version 1.0.136©2009-2016 RStudio Inc.).

A logistic regression model (quasibinomial with a link function cauchit) was used for the statistical analysis of larval development times. The variables to explain were (i) the proportion of post-feeding larvae and (ii) the flies present during the respective periods from D2 to D5 and D11 to D15. A step-by-step selection technique was used to improve the model. The AIC criterion (Akaike Information Criterion) was used to determine the quality of a model. For this reason, the model was applied to each food type separately, with time and larval density as explicative factors. Then, the model was applied to compare the effect of food at each density, with time and substrate the explicative factors. ANOVA was used to determine the significance of the model factors.

The odds ratio (OR) of each explicative variable was calculated, which provides an estimate (with confidence interval) for the relationship between two binary variables: when the OR is greater than 1, the two variables are considered associated.

Chi-square tests were used to compare mortality rates by food type, and when significance was detected, pairwise *post hoc* tests were applied.

Two-way ANOVAs (density, food) were used to compare morphometric parameters (size and mass). The data for size of flies from the few individuals of the d10 larval density were insufficient (or even absent for one sex); therefore, we excluded the d10 data from the ANOVAs: only descriptive results are presented.

RESULTS

Effects of larval density

247 The proportions of post-feeding larvae according to development time are shown in
 248 Fig. 2(a) (control food condition). The logistic regression model showed a significant
 249 effect of larval density on the proportion of post-feeding larvae ($P<0.001$). The
 250 development time decreased with larval density above 50 and up to 250 larvae and
 251 then slightly increased at d500 (see Fig. 2(a) for odds ratio of each density condition
 252 versus d10). The logistic regression model also demonstrated a significant effect of
 253 larval density on the development time until emergence, but this effect was less
 254 pronounced ($P<0.001$, see Fig. 3(a)).
 255 Early larval mortality rates (from first instar to post-feeding) were significantly affected
 256 by larval density ($X^2=101.4$, $P<0.001$). The pairwise *post hoc* test highlighted a
 257 decrease in mortality with the increase in larval density (see Fig. 4(a)). Considering
 258 overall mortality (from first instar to emergence), a significant decrease with increasing
 259 larval density was also observed ($P<0.001$; see Fig. 4(b)).

260 Figure 2: Kinetics of transition from larvae to post-feeding instars: (a). control food; (b).
 261 food with addition of trypsin. Percentages $\pm$ SE of post-feeding larvae are given in
 262 relation to larval densities. Odds ratios with 95% confidence interval of the logistic

263 regressions are also given. d = no. of larvae / 50 g of food; NS. = not significant; *
 264 $P < 0.05$, *** $P < 0.001$.

265 Figure 3: Kinetics of imago emergence: (a) control food; (b) food with addition of
 266 trypsin. Percentages $\pm$ SE of imagos are given in relation to larval densities. Odds
 267 ratios with 95% confidence interval of the logistic regressions are also given. d = no. of
 268 larvae / 50 g of food; NS. = not significant; *** $P < 0.001$.

271 Figure 4: Average mortality rate (numbers in bars) $\pm$ SE from first instar (a) to post-
 272 feeding and (b) to emergence. C = control food; Tr = trypsin-added food; densities =
 273 no. of larvae / 50 g food. Columns with the same letters are not significantly different
 274 ($P > 0.05$).

Effects of food digestibility (addition of trypsin)

The addition of trypsin reduced larval development time at low densities, but did not eliminated the benefits of gregariousness at highest densities ($P<0.001$; see Fig. 2(b)). Compared with the control food, the average larval development time on trypsin-added food was shorter at d10 and d50 ($P<0.001$), was similar at d100 ($P=0.27$) and was longer at d250 and d500 ($P<0.001$ and $P=0.01$, respectively) (see Fig. 5). Development time was significantly reduced at d250 versus d10 (OR=23.4[2.1–309.8], $P<0.01$); but with the other increases in larval number, larval development time did not significantly decrease. Regarding development time to fly emergence, a significant effect of density was observed at d250 and d500 ($P<0.001$, OR = 31.4 [8.7–207.4] and 74.9 [20.7–499.8], respectively; see Fig. 3(b) and Fig. A1).

Larval mortality rates on trypsin-added food were also influenced by larval density ($X^2=45.5$, $P<0.001$), with the mortality at d500 significantly lower than that at other densities ($P<0.001$, pairwise *post hoc* tests, see Fig. 4). No significant effect of trypsin on the total development time was detected, with the following p-values: d10: $P=0.08$; d50: $P=0.30$; d100: $P=0.91$; d250: $P=0.97$; and d500, $P=0.17$).

Figure 5: Odds ratios with 95% confidence interval of the effect of trypsin-added food versus control food for larval development time (first instar to post-feeding). d = no. of larvae / 50 g of food; Tr = trypsin-food; C = control food; NS. = not significant; ** $P<0.01$, *** $P<0.001$.

Morphometrics

The mean weight ($\pm$ standard error) of post-feeding larvae was 55 ± 0.4 mg (all conditions combined). Larval density ($F=1.41$, $P=0.24$) or food type ($F=1.93$, $P=0.17$) did not affect the weight (see Fig .A2).

The male to female sex ratio was 0.99 ± 0.003 and was not modified by experimental conditions ($X^2=5.84$, $P=0.76$). Wing surface area of the flies (see Fig A3) was not affected by larval density or by food type for either females ($F=1.81$, $P=0.14$; $F=0.40$, $P=0.53$, respectively) or males ($F=2.24$, $P=0.08$; $F=1.63$, $P=0.20$, respectively). The mean right wing surface area was 15.7 ± 0.1 mm² for females and 13.1 ± 0.1 mm² for males. As expected, males were smaller than females ($F=922.2$, $P<0.001$).

DISCUSSION

Experiments demonstrated (1) a decrease in development time and mortality with increasing larval density and (2) a decrease in development time and mortality on high-digestible food compared with low-digestible food (at low densities of larvae). These results provide evidence of the benefits of collective exodigestion and aggregation and possibly of much larger consequences at the community level such as niche partitioning.

Aggregation of necrophagous larvae is consistent with the Allee effect

Increasing larval density resulted in better development of *L. sericata* larvae on the control fresh meat. With the increase in larval density, (i) *L. sericata* development time was reduced, and (ii) mortality decreased. Due to the selection pressure on the feeding maggots (competition with scavengers, food shortage, parasitism and interspecific/interkingdom competition), their survival notably depends on development time (Benbow et al., 2015, DeVault et al., 2004; Frederickx et al., 2013, Hanski, 1987; Jordan & Tomberlin, 2017). Thus, the observed difference in developmental time with density might have even more dramatic impact on larval survival in the field. Furthermore, larval density did not affect adult fly morphometrics: larvae growing in the large groups assimilated sufficient food fast enough not to affect their metamorphosis

and final size/weight (Charabidze et al., 2013). As the fertility of a female depends on its size (Saunders & Bee, 1995), we reasonably concluded that increased density had no deleterious effect and positively affected population fitness, which is a result consistent with the predictions of the Allee effect (i.e., a positive relationship between fitness and population size) (Allee, 1927; Courchamp et al., 2008).

Inside larval masses, two collective strategies likely act synergistically. Maggot-mass effect, which is a local temperature increase inside large aggregates, was formerly postulated as one of the primary benefits of larval pre-social behaviour (Rivers et al., 2011, 2012). However, significant local warming requires many more larvae than those used for this study (see Fig. A4). As an example, a 24-h decrease in development time as we observed with 250 larvae groups in the present work would require a local temperature shift from 25°C to 34°C (Grassberger & Reiter, 2001), which is warming that is only reached above 5690 aggregated larvae (Charabidze et al., 2011). By contrast, exodigestion occurs at the scale of a single egg-batch (circa 200 larvae) and is fully efficient as soon as a few hundred larvae aggregate and feed together. This initial benefit can be reinforced by the aggregation of additional larvae, resulting in maggot masses sufficiently large to increase local temperature (see Fig. 6 and 7).

This aggregation strategy of necrophagous blowflies may rely on the genetic proximity of feeding larvae, a result previously described in caterpillars (Tsubaki & Shiotsu, 1982). Indeed, the genetic affinity within aggregated larvae is inherently close: each female fly lays egg batches of circa 200 eggs, resulting in hundreds of aggregated offspring (Wall, 1993). Picard & Wells (2010) also found that gravid females ready to oviposit on the same carcass showed a pattern of relatively high relatedness.

Gregariousness may be a blowfly adaptive strategy to breed on fresh carcasses

The results obtained with trypsin-added food also highlight the benefits of aggregation. Compared with those fed fresh control food, larvae fed on high-digestibility food had reduced average mortality and slightly faster development. Therefore, we assumed that the increased development and reduced mortality of aggregated larvae resulted in part from chemical degradation (pooling of enzymes). Consistent with this idea, Wilson et al. (2016) demonstrated that the addition of a trypsin inhibitor drastically reduces exodigestion and slows *L. sericata* larval growth. Green et al. (2002) also showed that feeding activity of groups of *Phormia regina* larvae has a greater effect on the chemical nature of the diet surfaces than the feeding of isolated larvae. However, while trypsin-added food significantly decreased mortality rates at d10, this increased survival was offset by a higher mortality during post-feeding instars (see Fig. 4). This "catching up" effect resulted in a similar global (i.e. first instar to emergence) mortality on both food types. One likely hypothesis is that the addition of trypsin facilitates the ingestion and digestion of the food, and thus allows the weakest larvae (those whom died on control experiments) to survive artificially (Barnard & Geden, 1993). However, many of these larvae were living on borrowed time and they died during post-feeding or pupal development. Furthermore, the addition of trypsin did not completely eliminate the benefits of gregariousness in terms of development time (electronic Table A1). Two cofactors of exodigestion might explain why trypsin does not fully imitate the properties of aggregated larvae. First, larval alkalisation of the medium can increase the pH-dependent enzymatic degradation (Hobson, 1931). Second, a density-dependent selection of competing bacteria may be an important consequence of larval aggregation (Arce, Smiseth, & Rozen, 2013). Indeed, Janzen's "spoiling theory" (Janzen, 1977) hypothesises that bacterial decomposers should produce compounds that cause rich and ephemeral resources to rot, rendering them unpalatable, whereas animals should evolve adaptive strategies of avoidance or detoxification. Thus, carrion

is the site of an intense interkingdom competition between insects and microbial decomposers (Burkepile et al., 2006; Jordan & Tomberlin, 2017; Young et al., 2015). In this context, burying beetles can cooperate to bury a carcass and limit microbial proliferation (Eggert & Müller, 2011; Sun et al., 2014; Trumbo, 2017), and blowfly larvae secrete/excrete efficient antimicrobial peptides, which is a property used in larval debridement therapies (Teh et al., 2017). Therefore, larval aggregation may be an adaptive response that locally limits bacterial proliferation and spoiling (see Fig. 7).

Conclusions

The present work demonstrates that calliphorid larvae pre-social behaviour not only results in significant benefits throughout larval development but also may act as a niche-separation mechanism. It has been demonstrated that fly egg-laying strategies can facilitate coexistence in carrion fly communities (Hanski, 1977; Ives, 1989, 1991; Kouki & Hanski, 1995; Kuusela & Hanski, 1982; Prinkkilä & Hanski, 1995; Spindola et al., 2017). Progressing through the stages of decomposition, a carcass becomes attractive to a relatively predictable sequence of insects (Matuszewski et al., 2011; Megnin, 1894). Although highly nutritional, fresh meat is more difficult to ingest and digest than decayed meat (Benbow et al., 2015). By mutualising exodigestion, calliphorid larvae (i.e., pioneer species) possibly make a food source avoided by other necrophagous species more edible. This hypothesis is consistent with the idea that cooperation may enable species to expand their niches (the so-called 'social conquest hypothesis') (Brooks et al., 2017).

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416

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418

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APPENDIX

Figure A1

Figure A1: Kinetics of imago emergence for the control (solid lines) and trypsin (dashed lines) conditions. (a) Percentages $\pm$ SEs of imagos in relation to larval densities. (b) Logistic regressions.

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751 = control food; Tr = trypsin-added food; densities = no. of larvae / 50 g of food.

= control food; Tr = trypsin-added food; densities = no. of larvae / 50 g of food.

Figure A3

Figure A3: Box plot of the size of the flies, evaluated by the area of the right wing, according to sex: (a) females; (b) males. * = mean area; C = control food; Tr = trypsin-added food; densities = no. of larvae / 50 g of food. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers

Figure A4

Figure A4: Peak temperature recorded inside larval aggregates according to Charabidze et al. (2011) and Heaton et al. (2014). Low peak values were observed for small masses (<500 larvae), whereas large masses produced more heat. A ceiling value ca. 40°C was observed for the largest masses.

772 **Table A1:** Table of literature review

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<u>Species</u>	<u>Larval density</u> (larvae per <i>n</i> gram of substrate)	<u>Substrate</u>	<u>Outcomes</u>	<u>Effects</u>	<u>References</u>
<i>Lucilia sericata</i>	100 ; 200 ; 500 ; 1000 ; 2000 ; 3000 ; 16000 /140g	Beef	Development time Larval weight Weight and size of pupae Fecondity of flies Mortality rate	Increases with increased larval density Decreases with increased larval density Decreases with increased larval density Decreases with increased larval density Increases with increased larval density	Ullyett <i>et al.</i> , 1950
<i>Chrysomya albiceps</i>					
<i>Chrysomya chloropyga</i>					
<i>Chrysomya megacephala</i>	25 ; 50 ; 100 ; 200 ; 250 ; 500 / 25g	Beef liver	Development time Weight and size of pupae and flies Mortality rate of larvae and pupae	Decreases for the intermediate density from 100 to 500/25g Decreases with increased larval density Decreases for the intermediate density from 100 to 200/25g	Goodbrod <i>et al.</i> , 1990
<i>Chrysomya rufifacies</i>					
<i>Lucilia sericata</i>	8 / 4g ; 8g ; 16g ; 32g	Agar + 20 % blood horse meat + 5 % yeast	Weight of pupae Proportion of emergences Weight of flies	Decreases with increased larval density Decreases with increased larval density Decreases with increased larval density	Simkiss <i>et al.</i> , 1992
<i>Lucilia sericata</i>	2 ; 4 ; 8 ; 16 ; 32 ; 64 ; 128 ; 256 ; 512 / 1g	Beef	Mortality rate until emergence Size, fecondity and longevity of flies	Decreases with increased larval density Decreases with increased larval density	Prinkkilä <i>et al.</i> , 1995
<i>Lucilia illustris</i>			Mortality rate until emergence Size, fecondity and longevity of flies	Increases for the intermediate density ~32/1g then strongly decreases Decreases with increased larval density	
<i>Lucialia silvarum</i>			Mortality rate until emergence Size, fecondity and longevity of flies	Decreases with increased larval density Decreases with increased larval density	
<i>Lucilia caesar</i>			Mortality rate until emergence Size, fecondity and longevity of flies	Increases for the intermediate density ~16/1g then strongly decreases Decreases with increased larval density	
<i>Calliphora vicina</i>	1 ; 5 ; 10 ; 20 ; 50 ; 100 ; 150 ; 200 ; 300 / 50g	Beef	Weight of post-feeding larvae Development time Mortality rate Size of flies	Decreases with increased larval density Increases with increased larval density Increases with increased larval density Decreases with increased larval density	Saunders <i>et al.</i> , 1995
<i>Chrysomya putoria</i>	From 100 to 2000 (by 100) / 50g	Whole dried milk and whole dried egg	Weight of pupae Mortality rate	Decreases with increased larval density Decreases with increased larval density	Godoy <i>et al.</i> , 1996
<i>Lucilia sericata</i>	10 ; 20 ; 30 ; 50 ; 100 ; 150 ; 250 ; 300 ; 500 ; 750 ; 1000 ; 1500 / 15g	Lamb liver	Size of flies Proportion of emergences	Decreases with increased larval density Decreases with increased larval density	Smith <i>et al.</i> , 1997
<i>Calliphora vicina</i>	10 ; 20 ; 30 ; 50 ; 100 ; 150 ; 250 ; 300 ; 500 ; 750 ; 1000 ; 1500 / 15g		Size of flies Proportion of emergences	Decreases with increased larval density Decreases with increased larval density	

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<i>Cochliomyia macellaria</i>	200 ; 400 ; 800 ; 1600 / 50g	Whole dried milk and whole dried egg	Mortality rate	Increases with increased larval density	Dos Reis <i>et al.</i> , 1999
<i>Chrysomya putoria</i>					
<i>Lucilia sericata</i>	10 ; 50 ; 100 ; 200 ; 350 ; 500 / (20g renewed every 48 hours)	Agar + 20 % blood horse meat + 5 % yeast	Mortality rate from first instar to fly Weights of pupae and flies Larval development time Longevity of flies Fecundity of flies	Decreases slightly with density from 10 to 100 then increase, particularly from first to third instar. The proportion of emergences decrease with the density. Decreases with increased larval density Increases with increased larval density Decreases with increased larval density Decreases with increased larval density	Moe <i>et al.</i> , 2002
<i>Chrysomya megacephala</i>	200 ; 1000 / 50g	Beef	Mortality rate Fecundity of flies Size of flies	No significant difference Decreases with increased larval density Decreases with increased larval density	Reigada <i>et al.</i> , 2004
<i>Calliphora vomitoria</i>	1 ; 5 ; 10 ; 20 ; 50 ; 75 ; 100 / 10g	Prok liver, brain or meat	Development time from first instar to emergence Mortality rate Size of flies	Decreases with increased larval density Increases with increased larval density Decreases with increased larval density	Ireland <i>et al.</i> , 2005
<i>Lucilia sericata</i>	20-30 ; 50 ; 150 ; 300 ; 650-750 / 15g	Pork liver	Mortality rate Size of flies Development time until emergence	Increases with increased larval density Decreases with increased larval density No significant difference	Martinez-Sánchez <i>et al.</i> , 2007
<i>Protophormia terraenovae</i>	25 ; 50 ; 100 ; 200 ; 500 ; 1000 ; 2000 / 150g	Beef liver	Development time from larvae to pupariation Time for emergence Weight of pupae Proportion of emergence	Decreases with increased larval density No significant difference Decreases with increased larval density Decreases with increased larval density	Rivers <i>et al.</i> , 2010
<i>Lucilia eximia</i>	100 ; 200 ; 400 ; 600 ; 800 / 50g	Whole dried milk and whole dried egg	Mortality rate Fecundity of flies	Increases with increased larval density Decreases with increased larval density	Serra <i>et al.</i> , 2011
<i>Chrysomya albiceps</i>	100 ; 200 ; 400 ; 600 ; 800 ; 1000 / 50g				
<i>Cochliomyia macellaria</i>	from 200 to 2000 by 200 / 50g				
<i>Chrysomya megacephala</i>					
<i>Chrysomya albiceps</i>					
<i>Chrysomya rufifacies</i>	10mg ; 50mg 100mg of first instar larvae / 150g	Kangaroo meat	Development time Weight and size of post-feeding larvae	Decreases with increased larval density Decreases with increased larval density	Johnson <i>et al.</i> , 2014
<i>Calliphora vicina</i>			Development time Weight and size of post-feeding larvae	No significant difference Decreases with increased larval density	
<i>Lucilia sericata</i>	0 ; 2,5 ; 5 ; 7,5 ; 10 ; 12,5 ; 15 (Biobag) / 20g	Pork liver	Mass of tissues digested after 48 h Average mass by larvae after 48 h Larval mortality rate after 48 hours	Increases until 7,5 Decreases from 5 No significant difference	Wilson <i>et al.</i> , 2016