Life with others

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Chapter 4

Synthesis and prospects

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In this final chapter, I aim to give a general synthesis of my thesis and to offer perspectives for future research. I do this based on the findings described in my data chapters, as well as additional data generated during my PhD project that did not quite fit into a complete chapter.

Group formation

Being in a group can confer benefits to individuals, which can ultimately result in increased fitness (Frank, 2011; Krause et al., 2002; Majolo and Huang, 2018). Fruit flies feed, mate and lay eggs together. I found in **Chapter 2** that *D. melanogaster* females actively search others and are attracted to lay their eggs communally, confirming previous reports of communal egg-laying in this species (Amrein, 2004; Bartelt et al., 1985; Billeter and Levine, 2015; Billeter and Wolfner, 2018; Duménil et al., 2016; Wertheim et al., 2002a, 2005). In **Chapter 2**, I used small arenas in which mated females, when given the choice, chose to lay their eggs in groups rather than alone (**Chapter 2**). I extended this finding to a larger setup during a visit to J.F. Ferveur's lab (University of Burgundy, France) at the beginning of my PhD, where I designed a choice assay in a wind tunnel to enable more natural and longer-range behavioural choices (Figure 1A). In this assay, mated flies were released into a large arena where they had to fly against an airflow in search of a substrate to land on. I found that females, when given a choice between fly-exposed and unexposed food patch in a wind tunnel (Figure 1A), preferentially landed on the exposed substrate (73%) over the unexposed patch (27%) (Figure 1B). This finding confirms that *Drosophila* females are attracted to conspecifics and this is at both short- and long-distance.

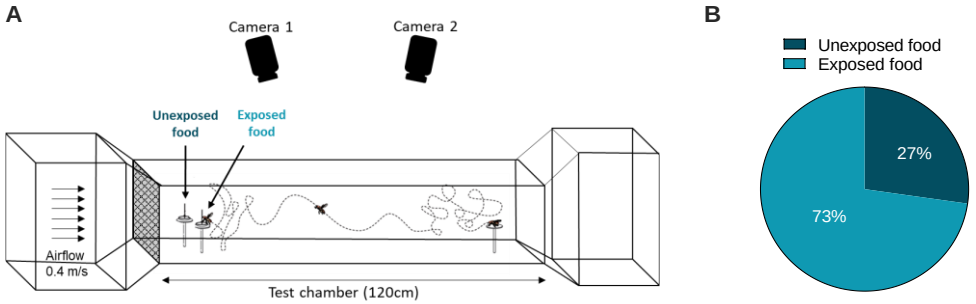

Figure 1. **(A)** Schematic representation of the wind tunnel assay. Mated females were introduced one by one in the wind tunnel and had the choice to land on an exposed or unexposed food patch. The exposed food patch was previously exposed with 12 mated females during 1 hour, allowing the deposition of aggregation pheromones that indicates the presence of a group. **(B)** Proportion of females that preferred to land on the exposed or unexposed food patch (Replicates: 33). Statistical analysis: Chi-squared test ($\chi^2 = 6.818$, df = 1, $p = 0.009$).

Competition

While flies have an inherent tendency to form groups and are attracted to conspecifics, this may also lead to increased competition. In overcrowded situations, individuals in large groups compete for food, space and mates (Clutton-Brock and Huchard, 2013; Clutton-Brock, 1991; Stockley and Bro-Jørgensen, 2011). In **Chapter 2**, I reported a strong competition for food between *D. melanogaster* larvae. I showed that offspring survival decreased with increased group size and that eggs arriving later than others on a substrate of poor nutritional value have reduced chances of survival compared to those who arrived earlier. The offspring thus suffers costs at high density: the individual larvae compete for food and larvae can even become cannibalistic under poor nutritional conditions (Narasimha et al., 2019; Vijendravarma et al., 2013), leading to decreased offspring survival (Courchamp et al., 1999; Etienne et al., 2002; Stephens and Sutherland, 1999; Wertheim et al., 2002b). *D. melanogaster* females have apparently evolved strategies to increase the chance of survival of their own offspring: in the presence of others, females lay eggs faster than when alone to give a competitive advantage to their offspring who can first exploit the limited resources and thus have a better chance of survival (**Chapter 2**). By advancing egg-laying when in a group, females thus avoid inter-individual competition.

As the costs and benefits of group living depend not only on social density but also on available resources (Etienne et al., 2002; Wertheim et al., 2002b), *D. melanogaster* females should assess both nutritional and social context to modulate egg-laying onset. Indeed, probability of an egg developing into a viable adult decreased with the reduction of food quantities (**Chapter 2**). I thus hypothesized that the nutritional quality of egg-laying substrates interacts with group size to modulate egg-laying start-time. To test this hypothesis, I designed an assay where a mated female laid eggs alone or in a group on different nutritional substrates (i.e. high, medium and low quality food). My results reveal that low nutritional substrate decreased the number of eggs a female laid (Figure 2A). Moreover, the start-time of egg-laying reduced as the food quality increased in grouped condition (Figure 2B), showing that females adjust their response to social context depending on food quality. Social environment and food are thus two factors that interact to modulate egg-laying of *D. melanogaster* females. Even though poor nutritional conditions should result in stronger competition among females, grouped females laid their eggs slower when they were in presence of poor food than when they were provided with rich food (Figure 2B). This behaviour could be explained as a delay in egg-laying in the hope to find a better egg-laying substrate. Food availability seems therefore to be a factor that modulates reproduction in *D. melanogaster* females. Further studies will need to investigate the sensory modalities (e.g. olfaction, taste) and neuronal mechanisms involved in this food-dependent egg-laying behaviour.

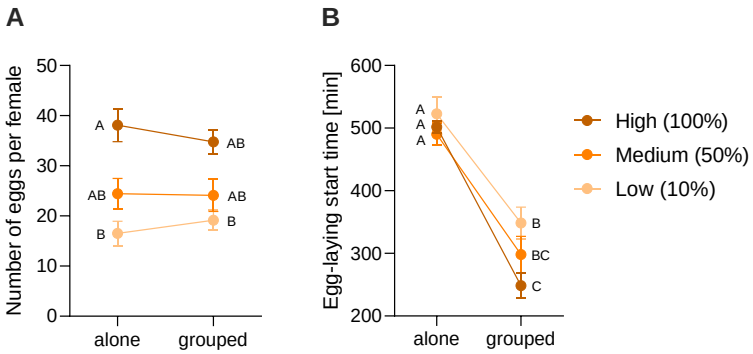

Figure 2. **Interaction of group and food quality in female egg-laying behaviour.** *Oregon-R* mated females were placed alone or in a group with 5 other mated females in a dish containing different food qualities: standard food (high), medium quality food and low quality food, respectively composed of 100%, 50% and 10% of quantities of standard ingredients. **(A)** Number of eggs laid in 24h and **(B)** onset of egg-laying were measured. Replicates per group: 20-35. Egg-laying assays were performed as described in the Materials and methods section of Chapter 2. Different letters indicate differences between conditions. Statistical analysis: Kruskal-Wallis test [(A) $\chi^2 = 16.876$, df = 5, $p = 0.005$; (B) $\chi^2 = 87.132$, df = 5, $p < 0.001$] and post-hoc comparisons [Only p-value < 0.05. (A) alone-low vs alone-high: $p = 0.02$, alone-high vs grouped-low: $p = 0.02$; (B) grouped-low vs grouped-high: $p = 0.01$, alone-low vs grouped-low, -medium and -high: $p < 0.001$, alone-medium vs grouped-low, -medium and -high: $p < 0.001$, alone-high vs grouped-low, -medium and -high: $p < 0.001$].

Competition also seems to be present in different aspects of reproduction, in particular mating. Indeed, competition for mates is a widespread phenomenon affecting individual reproductive success. In *Drosophila*, male-male competition has been well studied and several strategies were highlighted to reduce competition between males. For instance, males prolonged copulation and increased transfer of accessory gland peptides (Fedorka et al., 2011; Wigby et al., 2009) and sperm (Garbaczewska et al., 2013), resulting in the diminution of its mate's receptiveness to rival mates and improving their own fitness (Lizé et al., 2012; Wigby et al., 2009). Females also compete for mates as preliminary assays showed that grouped females mated faster than single females (Figures 3A&B). Mating was, however, not synchronized between group members, because grouped females did not start mating at the same time: the fastest female (ind1) started the mating in average 7 min after the introduction of the males, while the latest (ind6) started the mating after 52 min in average (Figure 3B). Other studies also showed a stimulation of mating in groups and indicated that females mated more frequently in the presence of more numerous and genetically diverse males (Billeter et al., 2012; Gorter et al., 2016; Krupp et al., 2008), and that females remated faster when they perceived the presence of other females (Laturney and Billeter, 2016). The other females may be seen as competitors by focal females, as they are potential sexual partners for males that would deplete their sperm reserves. Fruit fly females thus seem to compete for mates, which leads to a faster mating start-time when they are in a group. Taken

together, my findings revealed that competition play a major role in fruit fly life and affect *D. melanogaster* females' reproduction.

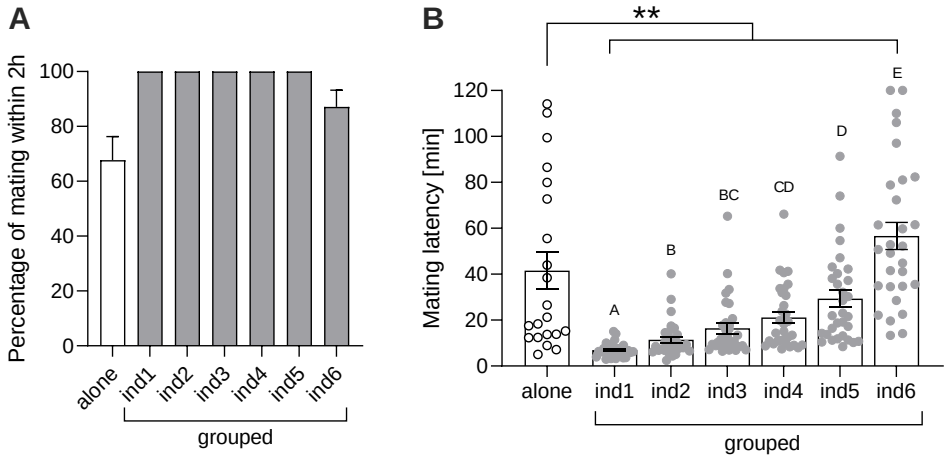

Figure 3. **Effect of group on mating behaviour**. A single virgin female was placed with a single male, or a group of 6 virgin females was placed in mating arenas with 6 males. **(A)** Percentage of mating of each of the 6 grouped and single females which took place within 2 hours after they were placed with males in mating arenas. 31 replicates were made for each social condition (alone, grouped). **(B)** Mating start-time of a female alone or each of the 6 females from the same group. Grouped females were ordered according to their mating latency, i.e. from the fastest to the slowest. Replicates per group: 21-31. Statistical analysis: Wilcoxon-Mann-Whitney (alone vs grouped: $W = 2585$, $p = 0.011$). Kruskal-Wallis test between the mating rank of individuals within the group ($\chi^2 = 104.24$, $df = 5$, $p < 0.001$) with post-hoc comparisons (not detailed here); different letters indicate differences between the mating rank of individuals within the group.

The balance between competition and cooperation

The onset of egg-laying gradually reduced with increasing group size (**Chapter 2**). However, I did not find a limit to the group size for this egg-laying advancement and found that focal females in groups of 50 still laid eggs fastest, despite the reduced space and limited substrate (**Chapter 2**). This was unexpected as negative density-dependence theory (Courchamp et al., 1999) would predict a critical threshold group size above which the overexploitation of resources and competition leads to a reduced egg-laying onset, similar as with the low-quality food (Figure 2B). This lack of group size limit may be due to the egg-laying substrate used in this experiment, which contained rich nutrients (high food quantity, 100%). As shown in **Chapter 2**, offspring survival was high (about 80%) in our lab nutritional substrate, even at large egg group size (i.e. 50), and thus may support the nutritional needs of even a large number of offspring before the substrate becomes overexploited. This could explain why females still laid eggs fastest in large groups despite the high density of flies in the area. It would thus be interesting to test female egg-laying

latency and willingness to lay in different group sizes but with an oviposition substrate containing lower quantity of nutrients (i.e. 10 or 25% as used in survival experiments in **Chapter 2**). This would put females in more drastic or stressful conditions, which may reveal alternate responses to group size. I hypothesize that, under poor food conditions, females would modulate their onset of egg-laying according to the number of flies that are around by stopping laying eggs at high density; hence following the U-shaped curve presented in Figure 4. I predict that females would advance egg-laying with the increase of group size up to an optimal group size (inflection point) above which there is an overcrowding and an overexploitation of the limited resources between group members, leading to an inhibition of female egg-laying (Figure 4). However, an alternative hypothesis is that there is no evolutionary selection for not laying eggs at all when there is no possibility of dispersion, even in drastic conditions. In this scenario, I would predict that females still commence laying eggs rapidly at high density in poor nutritional conditions, but that they would oviposit fewer eggs, or at a lower rate.

Fruit fly females benefit from laying eggs with others when they, or their offspring, can improve the quality of the breeding site (e.g. by inoculating yeasts, or by joint mechanical penetration into the substrate) or when they are in the presence of interspecific competitors such as fungi. Indeed, *D. melanogaster* larvae can cooperate as a group and fend off fungal growth, enhancing their survival (Trienens et al., 2017; Wertheim et al., 2002b). Nonetheless, in my experiments, flies were never in the presence of other competitors (i.e. fungi), which could have masked the beneficial effects of communal egg-laying. By ignoring such natural conditions (i.e. growth of pathogens), I might have failed to observe (the full extent of) cooperation between group members. Hence, I hypothesize that the presence of fungi may be a parameter that modulates female egg-laying timing. It would thus be interesting to add these competitors (naturally present in the wild) to bring another dimension to my study. I predict that in the wild, flies are attracted to conspecifics and benefit from communal egg-laying but would speed-up egg production to avoid competition with the group members, or disperse and lay their eggs somewhere else when resources become overexploited.

To conclude, there is a trade-off between finding a group to increase the chance of offspring survival via cooperation, and avoiding competition within the group as this may dramatically decrease offspring survival. Flies in the wild are likely to experience benefits of communal egg-laying, yet need to speed-up and advance egg-production to avoid competition. Faster egg-laying might thus be seen as a strategy to be competitively superior to other group members, or as a mechanism of competition avoidance in groups.

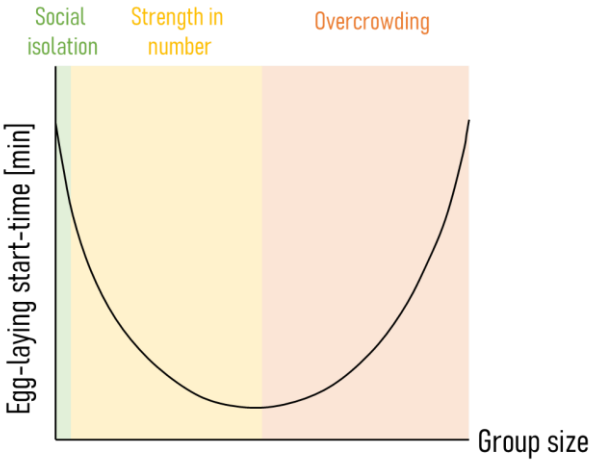

Figure 4. Schematic representation of the hypothesized U-shaped curve of egg-laying timing according to group size under poor food condition in *D. melanogaster*.

Genetic diversity and strain differences

While *Oregon-R* females laid eggs faster in the presence of others, *Canton-S* females did not advance egg-laying in groups (**Box 2**). This difference between *Canton-S* and *Oregon-R* suggests that there is substantial genetic variation for this behaviour. Moreover, while *Canton-S* females did not advance egg-laying when in a group of females, they even postponed egg-laying when in a group of males (**Box 2**). This suggests that they do perceive the presence of others, but respond differently than *Oregon-R* females to this group. The reason or function for this differential behavioural response among different strains/genotypes is currently unknown. However, I hypothesize that egg-laying advancement is a behavioural strategy to reduce the costs of competitive interactions among the offspring, because the offspring of females who laid their eggs later while in a group had decreased chance of survival (**Chapter 2**). Therefore, we would predict that females without this advancement of egg-laying would be outcompeted by females that do display advancement when they would be forming groups together: the offspring survival of females that advance their egg-laying in groups would be higher than the offspring survival of females that do not. Making large groups comprising of both *Canton-S* and *Oregon-R* females, and measuring their relative fitness by genotyping the surviving offspring, could be done to test this prediction.

My findings indicate that there is natural variation in how flies respond to the presence of others. I found variability in egg-laying behaviour in response to a group among several *D. melanogaster* strains, with the onset of egg-laying determined by the genetic

background of females, by the presence of a group, and the interaction between the two (**Box 2, Chapter 3**). This reveals that variability in egg-laying behaviour has a genetic basis, but is also partially a plastic response to social environment. Some females laid their eggs faster in the presence of others (e.g. *Oregon-R*, DGRP line *#25195*), while females from other strains did not advance egg-laying in group and laid their eggs at night (e.g. *Canton-S*, DGRP line *#25200*, *#25198*) (**Box 2**). I hypothesize that this inter-strain variability of egg-laying behaviour in response to a group among *D. melanogaster* could be explained by a variability in sociability between the strains. As described in **Chapter 3**, I assume that strains laying eggs faster in a group than in the alone condition show a strong strength of reaction to others and can be considered as sociable strains.

In **Chapter 3**, I explored sociability in fruit flies by measuring three sociability traits that each captures a different core feature of sociability, through a multidimensional approach. I measured sociability between *Drosophila* Genetic Reference Panel (DGRP) lines and found continuous variation in the strength of response to others in both males and females, indicating genetic variation in sociability among wild-type *D. melanogaster* (**Chapter 3**). These results are in agreement with other studies showing that fruit flies genotypes naturally differ in their degree of sociability (Anderson et al., 2017; Saltz, 2011; Scott, 2018; Wice and Saltz, 2021). My study further revealed that the performances in the three sociability behavioural assays did not correlate, hence indicating that sociability is unlikely to be a unidimensional personality type in fruit flies. Sociability thus does not seem to be a single overarching personality type that influences all the behaviours of a fruit fly, but seems, on the contrary, to affect various behaviours independently (**Chapter 3**). This highlights that sociability of an individual cannot be generalized by measuring one single trait, demonstrating the importance of quantifying multiple aspects of the sociability spectrum to reliably investigate sociability in fruit flies (**Chapter 3**). Our study thus provides an approach to study sociability in fruit flies. By conducting a multidimensional approach, my study therefore highlights the existence of a variation in sociability levels between genetically distinct lines. This natural variation in sociability in *D. melanogaster* and the genetic tractability of this organism, provides the evidence that this species is a valuable model to investigate the pathways of sociability.

My exploration of *Drosophila* sociability paves the way for future research on the genetic underpinnings of sociability and the neuronal mechanisms that drive sociability in various species, including humans. Many biological, physiological, and neurological properties are conserved between humans and fruit flies (Atkinson, 2011; Prüßing et al., 2013; Tolwinski, 2017; van Swinderen and Brembs, 2010). More specifically, similarities can be found between fruit flies and human neurobiology. For instance, about 75% of human disease-related genes have an orthologue in the fruit fly (Reiter, 2001). There are also striking similarities between humans and fruit flies when it comes to the neurotransmitter systems implied in sociability. Namely, one of the genes that is significantly associated with human sociability in a Genome Wide Association Study (GWAS) is the Dopamine receptor D2

(Bralten et al., 2021). Interestingly, lowering dopamine availability in fruit flies resulted in decreased sociability (Fernandez et al., 2017), hinting towards a shared involvement of dopamine in both species. Furthermore, the serotonin reuptake inhibitor MDMA (commonly known as ecstasy) facilitates pro-social behaviour in humans, suggesting a role for serotonin in human sociability (Kamilar-Britt and Bedi, 2015). Along these lines in fruit flies, the silencing of serotoninergic neurons results in decreased social affinity, hinting towards a shared role for serotonin in sociability between both species as well (Sun et al., 2020). Additionally, *Drosophila* has been successfully used to investigate the genetic and neural mechanism underlying human neuropsychiatric diseases, such as attention deficit hyperactivity disorder and autism (Coll-Tané et al., 2019; Hope et al., 2019; van der Voet et al., 2016). This indicates similar functioning of the human and fruit fly brain.

While humans are considered a social species living in complex cooperative societies, *D. melanogaster* is considered a solitary species. However, my research clearly demonstrated that both male and female *D. melanogaster* show behaviours that indicate sociability (**Chapter 3**). This suggests that sociability exists throughout the animal Kingdom. My study (**Chapter 3**) can thus serve as a starting point for other more in-depth studies aiming at identifying genes associated with sociability in fruit flies, and at investigating the genetic and neural substrates of sociability. This information may then also have translational value for understanding human sociability.

To test the hypothesis that sociability genes are conserved across species, we need to show that the same genes are influencing variability in sociability in both humans and flies. 200 DGRP lines will be tested in the three behavioural assays that each captures a different core feature of sociability, as used here in **Chapter 3**. Because sociability is the major similarity between the three measured traits, genes associated with all three traits should play core roles in the mechanism underlying variation in sociability and should pinpoint candidate sociability genes. Expanding the experiments to the 200 DGRP lines will allow to conduct a more reliable GWAS in order to generate a long list of *Drosophila* candidate sociability genes. These sociability genes will be compared with the 19 ones identified in humans (Bralten et al., 2021) in order to find potential conserved sociability genes and pathways between both species and verify the extent of conservation of the genetic architecture of sociability. As a GWAS approach generates false positives, pinpointing genes correlated with sociability will require genetic validation by directly manipulating the gene sequence and observing a sociability phenotype. Thanks to the genetic tractability of *D. melanogaster* model system, null mutants for those candidate sociability genes will thus be tested in the three sociability paradigms (described in **Chapter 3**) and will complement genetic research to functionally validate candidate sociability genes and investigate how these function in the brain. When the ubiquitous sociability in the animal Kingdom derives from evolutionary conservation, these genetic investigations may also allow fundamental research into the origin of social life by delivering validated sociability genes whose function can be studied in all organisms. For instance, validated *Drosophila* sociability genes with human

orthologues will be functionally tested in mice to establish their function in mammalian sociability. Targeted genetic manipulation in the neuronal circuitry of sociability in both mice and flies will help to understand how genes affect the brain to influence sociability and will thus make a start in uncovering the mechanisms in the brain that regulate the drive to interact with others.

Social environment has a pervasive influence on the biology of *Drosophila*

In addition to the genetic differences in reproductive behaviour, social environment also modulates different steps of *D. melanogaster* reproduction. For instance, social context influences female mating by reducing mating latency and increasing mating frequency (Billeter et al., 2012; Gorter et al., 2016; Krupp et al., 2008; Laturney and Billeter, 2016). As shown above, being in a group stimulated mating and reduced mating latency in fruit fly females (Figure 3). Being in a group also stimulated female oogenesis and ovulation under light conditions (**Chapter 2**), showing that social environment can also modulate oogenesis and ovulation in fruit fly females. This resulted in a stimulation of egg-laying and an egg-laying advancement in grouped females (**Chapter 2**). However, social condition did not affect the number of eggs laid by females in 24h, revealing that social environment modulates the onset of egg-laying, but did not affect overall fecundity in *D. melanogaster* (**Chapter 2**). I also showed that the presence of flies from any sex, mating status or species can trigger a fast oviposition in females, indicating that detailed recognition of social context is not required for egg-laying advancement (**Chapter 2**). The presence of others seems therefore to be an important factor that stimulates *D. melanogaster* female reproduction.

D. melanogaster exhibits a variety of social behaviours (e.g. aggregation at food sources, communal egg-laying, collective decisions, female mate copying: copying the behaviour of another female to select mates, social facilitation of long-lasting memory) (Amrein, 2004; Chabaud et al., 2009; Danchin et al., 2018; Duménil et al., 2016; Kacsoh et al., 2015; Mery et al., 2009; Muria et al., 2021; Pasquaretta et al., 2016; Schneider et al., 2012; Wertheim et al., 2002a). *D. melanogaster* is thus social as many aspects of its biology – including reproduction – are modulated by social environment. In addition, although *D. melanogaster* is considered as a 'solitary' species, social isolation or reduced social interactions are known to have important negative impact on health such as a faster tumour progression – as I have shown during my Master's research project (Dawson et al., 2018) – and a reduced lifespan (Ruan and Wu, 2008). This demonstrates the importance of social environment in this species. I moreover found that early social isolation negatively affects *D. melanogaster* reproduction: even though being kept in isolation during rearing did not modulate the onset of female egg-laying (Figure 5A), it decreased female fecundity (Figure 5B). Social environment is therefore a crucial parameter that modulates different steps of reproduction (i.e. mating, oogenesis, ovulation and egg-laying) in female *D. melanogaster*.

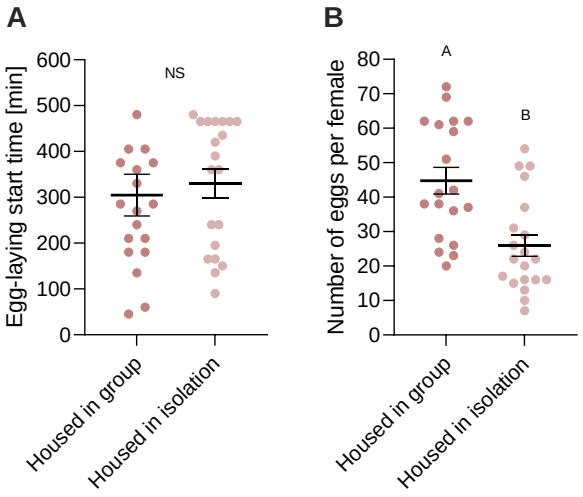

Figure 5. **Effect of social rearing conditions on female egg-laying behaviour.** Females were separated from others after hatching and were housed in isolation or in group of 20 females during 6 days prior to the assay. Females were then mated and placed alone in a dish to measure **(A)** the onset of egg-laying and **(B)** the number of eggs laid per female in 24h. Replicates per group: 19-20. Egg-laying assays were performed as described in the Materials and methods section of Chapter 2. Different letters indicate differences between conditions. Statistical analysis: Wilcoxon-Mann-Whitney [(A) $W = 153$, $p = 0.304$; (B) $W = 310$, $p < 0.001$].

Other factors that modulate fruit fly life such as circadian clock or light are also under social influence. My research revealed that light interacts with social environment to modulate timing of egg-laying (**Chapter 2**). I indeed showed that light inhibited and delayed oogenesis and ovulation in *D. melanogaster* females and led to an inhibition of egg-laying (**Chapter 2**), confirming that egg-laying in *Drosophila* occurs mainly at night (Howlader and Sharma, 2006; Manjunatha et al., 2008; Sheeba et al., 2001). However, the presence of flies lifted these inhibitory effects of light, resulting in grouped females laying eggs during the day while isolated females retained their eggs until darkness. This revealed an interaction between social context and light in female egg-laying behaviour (**Chapter 2**). I propose that females preferentially lay eggs in a group to obtain the advantages of being in a group (i.e. higher offspring survival thanks to a cooperation between larvae in fending off fungal growth) (Trienens et al., 2017; Wertheim et al., 2002b), but do not await darkness to start laying eggs because of a strong competition between the offspring of the females during development. This was supported by the competitive assays I conducted in **Chapter** 2, where the first-laid eggs had a higher survival probability than the later laid eggs.

My experiments indicated that the perception of the group is, at least partially, through visual cues (**Chapter 2**). Photoreceptors from different regions of the retina are responsible for detecting distinct wavelengths and directions of polarized light (e.g. outer photoreceptors R1-R6 cells and inner central cells R7 and R8) (Hardie and Raghu, 2001; Heisenberg and Buchner, 1977; Juusola et al, 2017; Katz and Mink, 2009; Montell, 2012;

Yamaguchi et al., 2008; Zhu, 2013). Further studies could thus be conducted to identify which photoreceptors are involved in light detection, perceiving groups and responsible for advancing egg-laying in grouped mated females.

The circadian clock is controlled by social communication. For instance, circadian clocks may be reset by social communication in fruit flies, showing that in a social context *Drosophila* transmit and receive cues that influence circadian time (Levine, 2004, 2002). Under the influence of genotype, experience and composition of the group, social synchronization of the circadian clock between group members was reported (Levine, 2002). Additionally, my study showed a weak interaction between social context and circadian time (**Chapter 2**). It would be informative to shift circadian patterns and day-night lengths to investigate how it affects fruit fly oviposition, in order to thoroughly study the role of circadian clock in *D. melanogaster* egg-laying behaviour. Mating is known to follow circadian patterns (Beaver and Giebultowicz, 2004; Sakai and Ishida, 2001). Rhythmic mating may thus facilitate rhythmic stimulation of vitellogenesis and hence rhythmic egg-laying (Steel and Vafopoulou, 2002). Loss-of-function mutations in any of the four core circadian clock genes (*period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*)) result in reduced fertility and fecundity in *D. melanogaster* males (Beaver et al., 2002). The *per* gene is also known to be involved in the clock mechanisms governing locomotor activity and egg-laying rhythms in *D. melanogaster* females (Howlader and Sharma, 2006). In addition, juvenile hormone, a key regulator of oogenesis and thus egg-laying, seems to be controlled by circadian rhythm in insects (Elekonich et al., 2001; So et al., 2000). I therefore predict a potential role of circadian clock genes in timing of egg-laying.

Mechanisms underlying social modulation of *D. melanogaster* reproduction

In this section, I will discuss the mechanisms by which social environment modulates oogenesis and the onset of egg-laying in *D. melanogaster*.

The motion pathway to detect group density

In my thesis, I showed that egg-laying gradually started earlier with increasing group size (**Chapter 2**). I further examined whether females modulate egg-laying start-time depending on group size or density, and found that timing of egg-laying of a focal female in a group of 6 females increased with arena size: the lower the density of flies, the slower the egg-laying onset (**Chapter 2**). This finding indicated that egg-laying timing is density-dependent and revealed that *Drosophila* females do not simply 'count' the flies that are around, but take into account the area size and adapt their egg-laying behaviour accordingly.

Moreover, my experiments highlighted that females detect social context through the motion detection pathway, indicating that vision is the main sensory modality necessary

to detect the presence of others in the context of egg-laying advancement (**Chapter 2**). I found that T4/T5 neurons and upstream LC10 neurons are involved in the neuronal circuit that detects motion of other flies leading to a female egg-laying modulation (**Chapter 2**). However, to confirm the involvement of LC10 neurons in the detection of flies in the context of egg-laying advancement, it requires testing more specific LC10 mutants. For instance, one could test more specific LC10 lines such as LC10a, LC10bc, or LC10d (Ribeiro et al., 2018; Wu et al., 2016). Other LC neurons that detect discrete objects could be also involved in group detection in the context of egg-laying advancement and will need to be tested as well, such as LC12, LC13, LC15, LC17 and LC26 (Ribeiro et al., 2018; Städele et al., 2020; Wu et al., 2016). With our current understanding of processing of visual cues, and that visual projection neurons act like filters for ethologically relevant behaviours (Wu et al., 2016), a screening of all LC neurons will thereby give a strongest answer to how females detect visually that they are in group or alone and use that to adapt their egg-laying timing.

Although the motion detection pathway allows females to detect the presence of others (**Chapter 2**), mated females in the presence of dead flies (i.e. unmoving flies) also advanced egg laying (Figure 6A). This result suggests that the motion detection pathway might not be the only pathway involved in group detection in the context of social modulation of egg-laying. I moreover observed that females tend to lay their eggs next to dead bodies (Figure 6B). Studies have described aversive behavioural responses to dead conspecifics across several invertebrate taxa (Sun and Zhou, 2013). For instance, dead bees can be perceived as a signal of danger and thus repel incoming foraging bees (Dukas, 2001; Horna Lowell et al., 2019). Crickets avoid environments treated with alcohol extracts of dead conspecifics (Aksenov and David Rollo, 2017) and display aversive learning in the presence of a dead conspecific (Ebina and Mizunami, 2020). American cockroaches and woodlouse also avoid areas where conspecifics have died (Rollo et al., 2016; Yao et al., 2009). In termites (Chouvenc et al., 2012) and in ants (Wilson et al., 1958), colonies transport and isolate dead conspecifics from the nest to prevent potentially harmful microorganism from proliferating. Surprisingly, in my assay, I saw the opposite: *Drosophila* mated females seemed attracted by these dead flies to lay their eggs. This behaviour has also been observed by other lab members but currently have no explanation. One hypothesis could be that the dead flies may be perceived by the females as potential nutritional resources for their offspring, hence leading the females to lay their eggs near the dead bodies.

The experimental setups that I used may have constrained some of the results that I obtained. All my experiments were performed in small petri dishes, where flies were thus relatively close to each other. In this situation, they used visual cues to detect others. I moreover showed that at a longer distance, flies use other sensory cues, such as olfaction, to detect fly odours and suitable egg-laying substrate (Figure 1), which had also been demonstrated by others (Becher et al., 2010; Duménil et al., 2016; Laissue and Vosshall, 2008; Semaniuk, 2015; van der Goes van Naters and Carlson, 2007). A recent study reported

that an innate aversion to a small visual object in flight can be reversed when approaching an attractive food odour, revealing a multisensory behaviour in *Drosophila* (Cheng et al., 2019). Because environmental factors interact with each other (e.g. social context and food), I expect that *D. melanogaster* females use a combination of different sensory modalities to sense their environment and find the best egg-laying substrate.

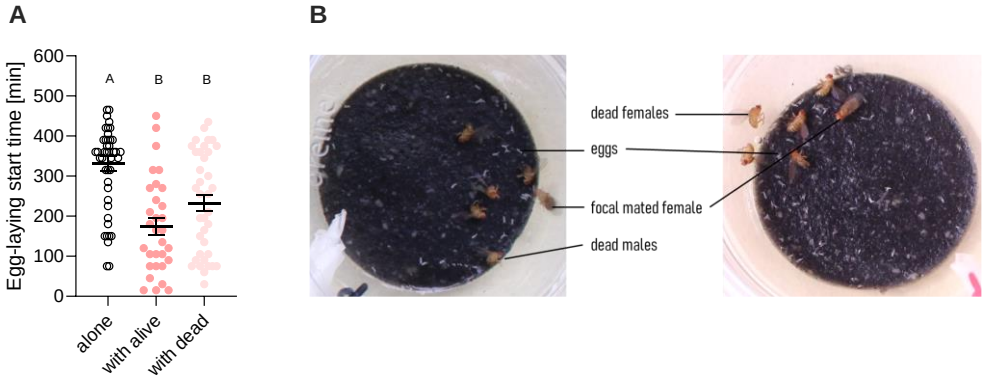

Figure 6. Effect of the presence of dead flies on female egg-laying behaviour. *Oregon-R* mated females were placed alone or in the presence of 5 alive or dead flies. Dead flies were killed 1h before the experiments by heat-shock (45°C during 30 minutes). **(A)** Onset of egg-laying was measured. Replicates per group: 31-45. Egg-laying assays were performed as described in the Materials and methods section of Chapter 2. Different letters indicate differences between conditions. Statistical analysis: Kruskal-Wallis test ($\chi^2 = 23.769$, df = 2, $p < 0.001$) and post-hoc comparisons (alone vs alive: $p < 0.001$, alone vs dead: $p = 0.001$, alive vs dead: $p = 0.085$). **(B)** Photo of the assay. An *Oregon-R* mated female laid her eggs in the presence of 5 dead flies.

Social environment modulates oogenesis via a hormonal pathway

In my thesis, I showed that the presence of flies lifted the inhibition of egg laying during light by stimulating the hormonal pathway involving juvenile hormone. This stimulated oogenesis and led to a faster oviposition in fruit fly females (**Chapter 2**).

Juvenile hormone (JH) is a key hormone that regulates multiple processes in insects. For instance, JH induces metamorphosis and the development of insects (Noriega, 2014; Riddiford, 2012; Smykal et al., 2014). In addition, the transition from nursing to foraging behaviour in the honeybee is accompanied by the onset of circadian rhythm in JH levels in the haemolymph (Elekonich et al., 2001). Connections between JH and worker task specialization have also been found in wasps and ants (Giray et al., 2005; Norman and Hughes, 2016; Shorter and Tibbetts, 2009). Eusociality evolved independently in bees, wasps and ants but JH has a regulatory function in the division of labour in these three species, making JH an extremely interesting hormone for sociality. JH is also a key regulator of female insect reproduction (Bilen et al., 2013; Dubrovsky et al., 2002; Gujar and Palli, 2016; Hernández-Martínez et al., 2019; Kelly et al., 1987; Riddiford, 2012; Santos et al., 2019; Soller et al., 1999). For instance, reproductive dormancy in fruit flies is induced by a

reduction in JH synthesis in the corpus allata in response to winter-specific environmental cues (e.g. low temperatures, short-day length) (Kurogi et al., 2021). In gregarious cockroaches, social facilitation of reproduction is regulated by increased juvenile hormone biosynthesis, which accelerates female oogenesis (Gadot et al., 1989; Uzsák and Schal, 2012). My findings in **Chapter 2** therefore highlight that JH stimulates oogenesis and female reproduction (Dubrovsky et al., 2002; Hernández-Martínez et al., 2019; Kelly et al., 1987; Santos et al., 2019; Soller et al., 1999), and seems to be mediated by social environment in different insect species, including *Drosophila*. In addition, the gene *take-out* (*to*), a clock-controlled gene that is transcribed in a circadian manner in *D. melanogaster*, has been implicated in the regulation of JH (So et al., 2000), showing that JH is also regulated by circadian clock. The juvenile hormone pathway therefore seems to play key role in oogenesis and egg-laying timing in fruit flies, under the control of different factors such as social environment and circadian rhythm.

In addition to the JH pathway, other neuronal and/or hormonal pathways may also take part in social modulation of *D. melanogaster* female reproduction (**Chapter 2**). More specifically, I hypothesize that octopaminergic (OA) neurons are involved at the ovulation level, because these neurons innervate insects' ovary and oviducts allowing contraction of oviducts and the release of mature oocytes (Bloch Qazi et al., 2003; Li et al., 2015; Meiselman et al., 2017; Middleton et al., 2006; Rodríguez-Valentín et al., 2006; Rubinstein and Wolfner, 2013; White et al., 2021). Further studies are warranted to test whether the stimulation of OA neurons can stimulate and restore ovulation in females that are alone. I predict that both stimulation of JH and OA neurons is necessary to restore oogenesis and ovulation respectively in isolated females, and both are therefore necessary to restore egg-laying in these females. These two pathways might work in combination to modulate egg-laying in female *D. melanogaster*.

Furthermore, there is a possible involvement of insulin-like peptides (ILPs), which are analogous to vertebrate insulin. Upon secretion, these peptides serve as hormones, neurotransmitters, and growth factors (Li and Gong, 2015; Wu and Brown, 2006), and regulate various physiological processes such as development (growth and maturation), longevity (aging, lifespan), metabolism, stress resistance and female reproduction (fecundity, oogenesis, vitellogenesis) (Badisco et al., 2013; Claeys et al., 2002; Géminard et al., 2009; Giannakou and Partridge, 2007; Grönke et al., 2010; Ikeya et al., 2002; Karpova et al., 2013; Li and Gong, 2015; Rulifson, 2002; Tatar, 2001; Wigby et al., 2011; Wu and Brown, 2006; Zhang et al., 2017). By the insulin signalling pathway, ILPs mediate nutritional signals and regulate preference for a substrate (Grönke et al., 2010; Ikeya et al., 2002; Karpova et al., 2013; Sheng et al., 2011). ILPs are also crucial players in regulation of insect reproduction, because the injection of insulin promotes ovary development, increased vitellogenin synthesis, elevated productive performance and improved protease (i.e. enzymes that

hydrolyse proteins and polypeptides) activity in female adult insects (Badisco et al., 2013; Zhang et al., 2017).

In ants, larval signals inhibit ovarian activity of adults in a dose-dependent manner by suppressing insulin-like peptide 2 (ILP2) (Chandra et al., 2018). I thus hypothesize that the insulin pathway is involved in social facilitation of oogenesis and egg-laying in *Drosophila* as well. Moreover, both nutrition and JH are necessary for vitellogenesis, and both of these signals actually work through the insulin signalling pathway (Leevers, 2001; Sheng et al., 2011). Indeed, insulin receptor expression has been found in the *corpora allata* (CA) gland, which synthesizes JH (Belgacem and Martin, 2007; Karpova et al., 2013; Tu et al., 2005) and mutations in insulin signalling pathway were shown to alter juvenile hormone synthesis in *D. melanogaster* (Tu et al., 2005). Insulin signalling affects JH synthesis through the control of JH regulatory neuropeptides and thus mediates the JH synthetic activity of mature CA (Nässel, 2002; Tu et al., 2005), stimulates vitellogenesis and controls oogenesis by controlling the development of ovarioles and oocyte maturation (Belgacem and Martin, 2007; Karpova et al., 2013; Sheng et al., 2011; Tu et al., 2005). There is therefore a possible crosstalk between JH and insulin-like peptide signalling pathways that should be explored in future research.

As discussed in **Chapter 2**, light and social isolation can be stressful factors for *D. melanogaster* females. Stress can affect reproductive behaviour and physiology that could thus explain such modulation of oogenesis, ovulation and egg-laying in this species.

Studies found that stressful conditions arrest egg production in *Drosophila* females via a hormonal cascade (Lee et al., 2003; Lim et al., 2014; Meiselman et al., 2017, 2018; Rodríguez-Valentín et al., 2006; Soller et al., 1999; Terashima et al., 2005; Terashima and Bownes, 2004). More precisely, under starvation or thermal stress conditions, ecdysone titers increase. Ecdysone rise acts on inka cells (responsible for the release of ecdysis-triggering hormone, ETH) which creates an ETH deficiency (Meiselman et al., 2017, 2018). This deficiency, in turn, leads to the inhibition of JH synthesis that causes oogenesis arrest, and the reduction of the octopaminergic (OA) neurons activation that innervate the ovary and oviducts, and block ovulation (Meiselman et al., 2018).

Based on these studies, I hypothesize that light and/or social isolation might also be stress factors that trigger this hormonal cascade involving ecdysone, ETH, JH and OA. The hypothetical model is presented in Figure 7. This hormonal cascade initiated by stress could explain the delay of oogenesis, ovulation and egg-laying found in isolated females. I also hypothesize that being in a group reduces the stress provoked by light and isolation and thus restores the female reproductive output by acting directly or indirectly on oogenesis and ovulation respectively via JH and OA neurons. This hypothetical stress pathway (Figure 7) will need to be explored and investigated to see whether stress, induced by light and/or social isolation, can be at the origin of such social modulation of *D. melanogaster* female reproduction.

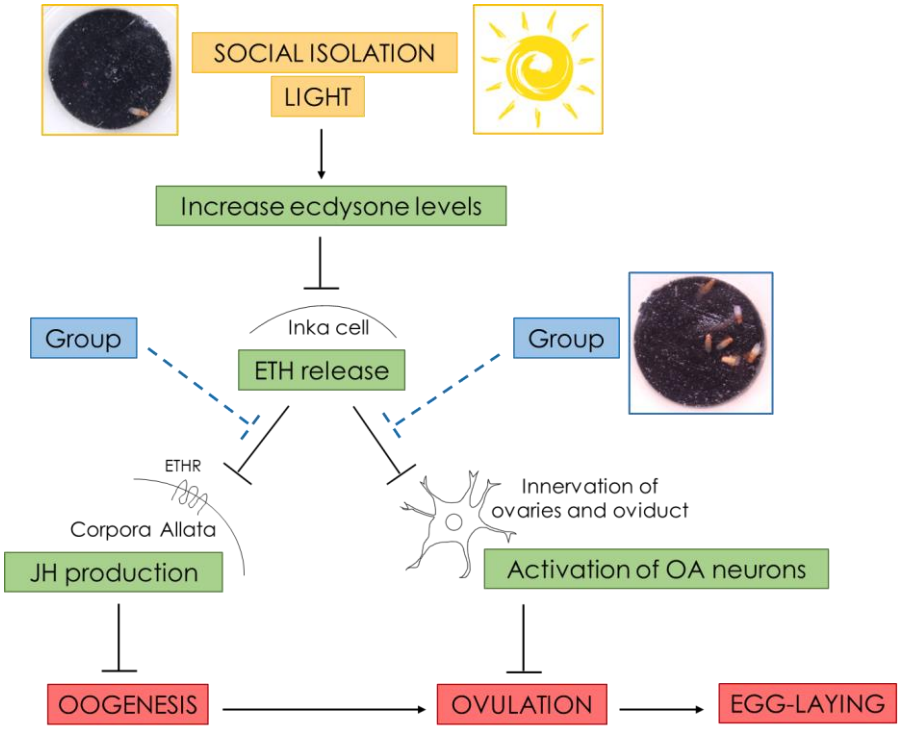

Figure 7. Hypothetical model depicting the *D. melanogaster* female reproductive output under stressful conditions, i.e. under the presence of light and under social isolation condition. Abbreviations: ETH: ecdysis-triggering hormone, ETHR: ETH receptor, JH: juvenile hormone, OA: octopaminergic.

My study hence highlights that an organism as simple and solitary as a fruit fly reacts to social environment which modulates many aspects of its whole life. Overall, my findings indicate that there is not a single dedicated social pathway, but that many neuronal and hormonal pathways seem to be influenced by social environment. I hypothesize that neural and hormonal circuits that regulate an individual's response to non-social environmental stimuli are modulated by neurons sensing social cues, suggesting that many – if not all – pathways are modulated by social input.

Evolution of sociality

Sociality describes the manner in which species associate in social groups and form cooperative societies. Diverse forms of sociality are found across insect taxa and are ranged from solitary (e.g. fruit flies), to subsocial (e.g. burying beetles), to communal colonies (e.g. gregarious caterpillars), to highly eusocial societies (e.g. ants, termites) (Costa, 2018; Leonhardt et al., 2016; Lin and Michener, 1972; Toth and Rehan, 2017; Wong et al., 2013; Zablotny, 2009). *Drosophila* are considered solitary species. However, 'solitary' seems to be

an inadequate word to define these insects as fruit flies suffer from social deprivation which affects their behaviour and physiology (Dawson et al., 2018; Ruan and Wu, 2008) and display a variety of social behaviours as described earlier (Chabaud et al., 2009; Danchin et al., 2018; Duménil et al., 2016; Mery et al., 2009; Muria et al., 2021; Pasquaretta et al., 2016; Schneider et al., 2012).

Social modulation of reproduction is considered a sign of sociality and is most strongly expressed in social hymenopterans, such as ants, some bees and wasps. In their colonies, reproduction is typically monopolized by a single or few individuals (i.e. the queen(s)), while the majority of the colony members (i.e. the workers) remain functionally sterile and do not lay eggs (Wilson, 1971). Worker fertility is suppressed by the queen e.g. through the release of queen pheromones, such as the queen mandibular pheromones in honey bees, or through aggressive interactions (Hoover et al., 2003; Keller and Nonacs, 1993; Traynor et al., 2014). It has been hypothesized that such complex social systems with distinct castes (queens, workers) have evolved from species in which individuals transitioned between a reproductive egg-laying phase and a non-reproductive phase in which they did not produce or lay eggs but instead, provided care for the developing offspring (e.g. through social foraging or nest defence). In such socially primitive species, transitions between reproductive and non-reproductive phases might have been controlled by larval presence. For instance, unlike other ant species, the clonal raider ant *Ooceraea biroi* lacks a queen caste and instead, all workers transition between a reproductive queen-like and a non-reproductive worker-like phase (Oxley et al., 2014; Ravary et al., 2006; Ulrich et al., 2016). These transitions are regulated by the presence of larvae which suppress the release of Insulin-like peptide 2 and by that of ovarian activation and oogenesis (Chandra et al., 2018). Based on these findings, it has been speculated that, during the transition from solitary to socially primitive life, some pathways regulating reproductive physiology might have become responsive to larval social cues. This would have resulted in females that are able to adjust oogenesis and oviposition to offspring requirements (Chandra et al., 2018).

In **Chapter 2**, I demonstrated that a social modulation of reproductive physiology can already be found in rather solitary species such as *D. melanogaster*, questioning the validity of the term "solitary" and making the concept of solitary vs social species a bit artificial. This suggests a scenario in which the mechanisms that adjust reproductive physiology to larval presence evolved by recycling the pathways that adjust competitive reproductive physiology to the presence of other females. In such a model, complex sociality might originate from the ability to adjust oogenesis to the social environment to reduce offspring competition.

Similarities in how social environment modulates female reproduction were found between *D. melanogaster* and German cockroaches. In German cockroaches, social isolation delays oocyte development as well as sexual maturation in isolated cockroach females (Uzsák and Schal, 2012), while the presence of a group stimulates oogenesis and speeds up female

reproduction (Uzsák and Schal, 2012; Uzsák and Schal, 2013) through the increase of JH biosynthesis (Crall et al., 2016; Gadot et al., 1989; Katoh et al., 2017; Uzsák and Schal, 2012; Uzsák and Schal, 2013). I found a comparable case in *D. melanogaster* where isolated females delayed oogenesis, ovulation and egg-laying, while the presence of others stimulated oogenesis through the stimulation of JH synthesis leading to a faster egg-laying in grouped females (**Chapter 2**). These similarities thus suggest that both species evolved from a common ancestor and show the presence of conserved social pathways between social and solitary species.

Sociality is a species tendency to form social associations and is often seen as an evolutionary transition, where species with complex societies differ dramatically from solitary ancestors (Kocher et al., 2014; Liu et al., 2020; Smiseth et al., 2012; Wong et al., 2013). Finding such conserved social pathways between social and solitary species thus raise the possibility that sociality might have evolved more as a gradient than as a transition. From an evolutionary perspective, one scenario can be that social insects could have evolved from a rather competition-driven synchronization of reproduction and became highly collaborative species in part by suppressing this competition.

Most generally, my results highlight the importance of considering group context when investigating reproductive behaviour in animals, even in (so-called) solitary species such as *Drosophila melanogaster*. It would moreover be interesting to do cross-species studies to investigate whether we could find more behavioural or mechanistic similarities as well as shared core mechanisms and genes between social and solitary insects, which would give important insights on how complex social systems evolved. For instance, as explained above, the presence of larvae regulates ovarian activity in ants (Chandra et al., 2018; Ulrich et al., 2016). It would thus be worthwhile to explore whether *D. melanogaster* females also modulate oogenesis and egg-laying in the presence of larvae, because I have only tested adults as group members in my experiments so far. Preliminary assays show that females adjust their egg-laying onset with the presence of eggs deposited on the egg-laying substrate beforehand since I find that start-time of egg-laying reduces as the egg group size increases (Figure 9). This indicates that offspring seems to modulate *D. melanogaster* female reproduction. Other studies will need to be carried out to test whether the presence of larvae affects oogenesis and egg-laying behaviour in this solitary species, as it does in social species.

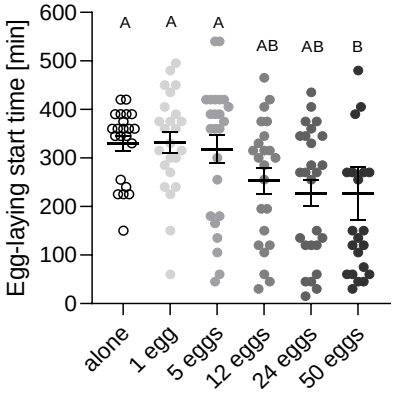

Figure 9. **Effect of the presence of eggs on female egg-laying behaviour.** *Oregon-R* mated females were placed alone or in the presence of different group sizes of eggs (i.e. 1, 5, 12, 24 and 50). Onset of egg-laying was measured in the different conditions. Replicates per group: 22-26. Egg-laying assays were performed as described in the Materials and methods section of Chapter 2. Different letters indicate differences between conditions. Statistical analysis: Kruskal-Wallis test ($\chi^2 = 21.581$, df = 5, $p < 0.001$) and post-hoc comparisons (Only p-value < 0.05. 1egg vs 50eggs: $p = 0.005$, 5eggs vs 50eggs: $p = 0.006$, alone vs 50eggs: $p = 0.009$).

Conclusion

Overall, my thesis reveals that fundamental behavioural and physiological aspects of reproduction are modulated by the presence of others, and provides new information on the mechanisms underlying social modulation of insect female reproduction. I found a social modulation of reproduction in *D. melanogaster*, non-deservedly considered as 'solitary', and thus demonstrated that fruit fly females adapt their behaviour and physiology to the social environment. My study gives new insights on the strong influence of the social environment on an animal's life and may advance research in understanding the origins of sociality and its evolution. My thesis also highlights a variation among *Drosophila* strains in the strength of response to others, showing the existence of a sociability spectrum in this species. *D. melanogaster* is therefore a valuable model to investigate the underpinnings of sociality and sociability.