Social behaviours and public goods in fruit flies

Effect of egg-laying substrate and social environment on oviposition decisions and the expression of 'public goods' related genes in Drosophila melanogaster fruit flies

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Table of Contents

List of Figures

List of Tables

Abstract

Cooperative behaviours are common across various taxa, but not always well understood. For example, the idea of the 'tragedy of the commons' shows that, among groups of non-relatives, there is expected to be strong selection for non-cooperative cheats. Fruit flies (*Drosophila melanogaster)* have been observed to oviposit their eggs in clusters of mixed maternity. I hypothesised that this could potentially be due to the production of 'public goods', in which mothers could coat the surface of the eggs they lay with beneficial, diffusible protective compounds which could thus also benefit adjoining eggs. Two types of potential public goods were identified. The first was the sex pheromone 7,11-HD, which is present around the outside of *D. melanogaster* eggs and has been found to protect eggs from cannibalism. The second was antimicrobial compounds. For example, female medflies (*Ceratitis capitata)* are known to provision their egg surfaces with anti-microbial peptides (Marchini *et al.*, 1997). It is not yet known though whether *D. melanogaster* has any equivalent antimicrobials. Such pheromones and peptides could potentially act as public goods if they diffuse into the medium and protect the eggs of other females that are laid nearby within egg clusters. This idea sets up the following predictions: that (i) females housed together in groups have the potential to gain public goods benefits by clustering their eggs with those of others, (ii) some, but not all females housed in groups would activate potential egg surface public good genes. I investigated these predictions by measuring the egg clustering decisions and potential 'public goods' gene expression patterns of females held in groups versus those that were socially isolated. I simultaneously tested the effect of two egg-laying substrates, of good and poor nutrient quality, on the basis that eggs laid on a poor food substrate are more likely to be cannibalised. Through this, how diet and social environment affected oviposition behaviour (egg clusters) and the expression of 'public good' related genes, was investigated. In contrast to the prediction, I found that there was no effect of the social environment on the proportion of eggs laid in clusters and that females were even less likely to cluster their eggs on the poor oviposition substrate. Therefore, there was no evidence that females clustered their eggs in a manner that would be predicted by public goods benefits. The effects on gene expression of potential public good genes showed variable support for the public goods hypothesis. Females laying eggs on a poor

food substrate had higher levels of gene expression in anti-microbial genes *Dif* and *Mtk,* compared to females laying on the standard food substrate. Thus, the detection of poor egg deposition substrates activated the expression of antimicrobial protective molecules. Females kept in the grouped environments also showed significantly increased expression of all the tested genes related to 7,11-HD production (*Fad2, fatp1, desat1*) over those maintained alone. However, there was minimal evidence that females showed significant heterogeneity in gene expression as would be predicted if group females were comprised of cooperators and cheaters. These results provide an insight into the nature of egg clustering decisions and how 7,11-HD and anti-microbial peptides are affected by substrate quality and social environment. The results suggest that the expression of antimicrobial and sex pheromone genes is responsive to egg-laying substrate and social groupings, respectively. However, there was no evidence that potential public goods could be dispersed through the mechanism of egg clustering. Further work is needed to determine whether these compounds are 'public goods' and to explore alternative explanations.

Keywords: Cooperation, Public goods, Oviposition, Gene expression, Social Behaviour, *Drosophila.*

Attributions

The experiments included in this thesis were carried out by me.

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Chapter 1: Introduction

1.1 Cooperative behaviours

At first glance, cooperative and altruistic behaviour appears to contradict the perceived competitive force of natural selection, as the competition between individuals in a population suggests selfishness would be beneficial. Despite this, there are many examples of cooperation among species of microbes, fungi, animals and plants. At a fundamental level, biological entities rely upon the cooperation of genes within them, but with genes also having goals that are purely selfish (Nowak, 2006). Cooperative behaviours exist both within kin groups (Bourke, 2013), but can also occur between unrelated individuals when certain conditions are met (Axelrod & Hamilton, 1981) and there are many ways in which a cooperative relationship between individuals can be established.

Altruism often occurs within kin groups and indeed, kin selection is proposed to be the primary route by which altruism can evolve (Hamilton, 1964). Hamilton showed that altruism could come about when the benefits accrued via the transmission of genes via high genetic relatedness outweighed the costs, resulting in 'inclusive fitness' benefits (West *et al.*, 2007). This explanation required relatedness to be above zero, and thus altruism could only occur when kin selection or positive assortment for relatedness was occurring (Archetti *et al*., 2011). This type of behaviour appears to be common in the animal kingdom (Platt & Bever, 2009). However, cooperation is not confined to kin groups and there are many examples of cooperation between unrelated individuals (Platt & Bever, 2009). Hence, additional explanations for cooperation amongst non-kin are needed - these potential explanations include reciprocity, mutualism and manipulation (Clutton-Brock, 2009).

Reciprocity occurs when cooperative behaviour is favoured by the probability of future mutual interactions. Natural selection favours strategies leading to reciprocity when different individuals interact repeatedly in potentially cooperative situations (Boyd & Richerson, 1988). Mutualism is an interaction in which the inclusive fitness of each individual is increased by the action of its partner. A common example of this behaviour is found in certain species of ants (*Iridomyrmex spp*) and caterpillars

(*Jalmenus evagoras*). In this example, the ants protect the caterpillars from predation, in exchange for a nutrient-rich secretion (Connor, 1995). Both insects benefit from this exchange. Although not all cooperation is 'equal', there are examples where organisms are manipulated into cooperating, and while they may still benefit, their inclusive fitness may not increase as much as the manipulating individual. In a study on keas (*Nestor notabilis*), two birds had to work together to activate an apparatus which released food, however, only one of the birds would receive the food. Commonly, the social hierarchy of the birds determined who received the food. In the dyadic test situations, three of the 'dominant' keas manipulated their respective partners to open the apparatus. The dominance status of the keas enabled them to force cooperation (Tebbich *et al*., 1996).

Recent studies show that even if the conditions for cooperation among unrelated individuals provided by reciprocity, mutualism and manipulation as described above are not met - cooperative behaviour can still be maintained (Dobata & Tsuji, 2013; Frank, 2010). Such explanations are provided by the properties of so-called 'public goods', and thus the conditions which support the emergence of cooperative behaviours under such scenarios need to be investigated to augment our understanding of social evolution.

1.2 Public Goods

One potential route to cooperation among non-relatives, and a main focus of this thesis, is through the production of public goods. The concept of public goods first originated in the context of economics (Samuelson, 1954) where public goods were defined as a benefit that is 'non-excludable' and 'non-rivalrous'. Essentially, this means that no individual can be excluded from the public good and that the good's availability is not reduced when used. True public goods of this form are thought to be extremely uncommon. The closest examples of near-unlimited goods are proposed to be resources such as air (McInerney *et al*., 2011). Hence, many 'public' goods might be better described as 'common' or 'club goods' (Archetti *et al*., 2011). However, despite this, the same public good principles are generally thought to pertain to these phenomena in a biological context.

The concept of public goods has been applied to biological systems, especially in the study of evolutionary biology and social evolution. In this context, a public good is generally defined as a substance which is costly to produce for the producing individual, and that improves the fitness of other individuals in the population. Public goods have been frequently described in microorganisms. The secretion of enzymes by bacteria in biofilms is often viewed as a public good – the enzymes are costly to produce and other microbes in the colony gain from such enzymes within a biofilm (Drescher *et al.*, 2014). For example, *Pseudomonas aeruginosa* secretes proteases such as elastase, in response to a las quorum sensing system which allows bacterial cell-to-cell communication via extracellular signalling molecules (Mukherjee & Bassler, 2019). These enzymes digest proteins such as elastin and collagen (Diggle *et al*., 2007). The digested proteins can be utilised by nearby bacteria as a source of food (Smith & Schuster, 2019). Thus, these proteases act as a public good because they are costly to produce and other, potentially non-protease-producing bacteria in the environment can ingest pre-digested substrate, which provides them with a fitness benefit. This scenario sets up the potential for the existence of cheats, who can gain fitness benefits from the presence of the digestive enzymes produced by others, but not pay the cost of making them (Frank, 2010). Therefore, the existence of public goods in this type of scenario creates a potential evolutionary dilemma that cannot result in an evolutionarily stable strategy, unless cheats who benefit but do not cooperate can be punished.

The research in this thesis is focused on identifying and evaluating a potential example of public goods in the form of substances secreted by mothers onto the surface of the eggs they lay, in the fruit fly *Drosophila melanogaster.* The conditions for the production and potential benefits of such public goods appear to exist. In fruit flies, it has been observed that unrelated females aggregating in groups on food patches significantly increase their egg-laying (Bailly *et al*., 2021; Sarin & Dukas, 2009; Wertheim *et al*., 2002). This suggests the potential for transfer of any public goods present on eggs increases when females occur in groups of non-relatives. Furthermore, within a food patch, female fruit flies make precise egg placement decisions in relation to any other eggs already present in the environment. For example, females can lay their eggs in a dispersed manner so that each egg is

separated from any other, or they can lay near to or in direct contact with existing eggs within a patch, i.e. within an egg ‘cluster’ (2 or more eggs directly in contact with one another on an egg-laying substrate). Throughout this thesis, I use the term “egg cluster” to refer to this situation in which one egg is in direct physical contact with at least one other egg. From the perspective of public goods theory, what is interesting is that these egg clusters are often made up of eggs with mixed maternity (around 79% being typical for eggs laid by females maintain in groups of 4, Fowler, Friend and Chapman, unpublished data). Thus, females living in groups of non-relatives lay more eggs than isolated females, and many of those eggs are laid within clusters of mixed maternity. Thus, if there are public goods with the potential to be shared across eggs, then females could gain benefits by placing their eggs with those of others. One possibility is that eggs could be better protected in a cluster if concentrations of defence compounds on egg surfaces are increased by the close proximity of eggs. Consistent with this idea, female *D. melanogaster* secrete anti-cannibalism pheromones which cloak the surface of the eggs they lay (Narasimha *et al.* 2019). There is also evidence from other species of Diptera that eggs can be provisioned with anti-microbial peptides (AMPs). For example, females of the Mediterranean fruit fly (*Ceratitis capitata*) coat their eggs with ceratotoxin peptides secreted from the female reproductive tract accessory glands (Marchini *et al*., 1997). These ceratotoxins are broad-spectrum, powerful anti-microbials that diffuse out onto the egg-laying substrate from even individual eggs (Marchini *et al*., 1997), which fits with the potential for them to represent a public good. The provisioning of eggs with AMPs has not been reported in *D. melanogaster*, but it is known that *D. melanogaster* females do express several AMP genes in their reproductive epithelium, the products of which have the potential to be secreted into the reproductive tract and onto eggs.

Anti-cannibalism or anti-microbial defence compounds associated with eggs could potentially act as public goods and may aid egg survival, hatching or larval performance - and thus increase fitness. These benefits would be accrued due to lowered infection and reduced cannibalism rates. However, this scenario would represent a public goods dilemma (Frank, 2010). In mixed maternity clusters, some flies could exploit the potential benefits of diffusible protective compounds made and secreted by other ‘producer’ females and withhold the production of their own. This

represents the ‘free-rider’ problem of potential destabilisation of the system via the invasion of non-producing cheats.

In other examples of cooperative behaviours that have been studied, it is usually found that there is a mechanism by which free riders are punished, or that there are mechanisms to ensure tight reciprocity between cooperators. For example, in vampire bats (*Desmodus rotundus*), a reciprocal cooperative behaviour is maintained within the context of sharing of blood meals between non-relatives. Cheating bats that receive blood meals from others but do not give them, are remembered by their social group and denied blood meals from others in the future. Thus, their fitness is lower than the bats which do share blood meals in a reciprocal fashion (Schweinfurth & Call, 2019). This example clearly shows how this cooperative behaviour can be stabilised and maintained in the long term. Other ways in which cooperation involving public goods could be maintained are via kin-directed recognition or through positive assortment leading to interactions between kin or cooperative individuals.

For the scenario I am exploring in this thesis, of the potential for protective egg surface substances acting as public goods in fruit flies, it is not clear what might be a mechanism for punishing any free riders. It is feasible perhaps that females could detect the presence of non-producers and reduce the proportion of egg clustering accordingly (to remove public good benefits from cheaters). For example, the anti-cannibalism substance secreted onto eggs as mentioned above (Narasimha *et al.* 2019) also functions as a sex pheromone, which could potentially be detected as a ‘cluster / not cluster’ signal by producing females. However, this is only a hypothesis and further work is required to establish this. Another puzzling feature of egg clustering that needs to be considered is that laying eggs together in clusters is predicted to increase the level of competition between the larvae hatching from clustered eggs (Hoffmeister & Rohlfs, 2001). This could set up the potential for an increased risk of exhaustion of food resources for offspring emerging from eggs laid in clusters. These two aspects of considering egg clustering as a mechanism for the sharing of public goods are problematic in terms of how such cooperation might be maintained. This is explored below where I also introduce the possibility that a new

branch of non-linear public goods theory could help explain cooperation in this system.

The topic of the problem of cooperation in public good systems was first described in 'The Tragedy of the Commons' (Hardin, 1968). This introduced the idea of collective action and free riders. In this scenario, it was assumed positive assortment or enforcement was required for public goods to evolve (Archetti & Scheuring, 2012). However, this conclusion was reached because it was assumed that public goods benefits would accrue linearly. A more recent theory of how cooperation via public goods can evolve or be maintained without positive assortment and enforcement removes this assumption. Building from the observation that, across biological systems, non-linear relationships of biological traits with fitness are potentially very common (Kimmel *et al.*, 2019), Archetti *et al*., 2020, have proposed 'non-linear public goods theory' (Figure 1.1) (Archetti *et al*., 2020).

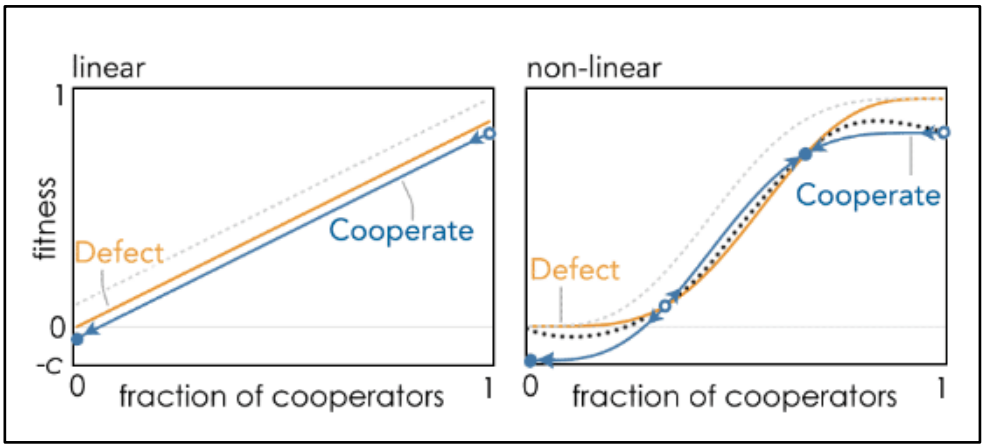

Figure 1.1: Relationship of fitness against the fraction of co-operators in linear and non-linear cooperative systems*. In the linear system (left), there is no point along the line where cooperation can be maintained, as cheats (defectors) have always have higher fitness than cooperators. However, in the non-linear system (right) there are areas where defectors or cooperators have higher fitness, depending upon the fraction of cooperators in the population. This scenario can promote the maintenance of cooperation. Figure reproduced with permission of the authors (Archetti et al., 2020).*

This body of theory shows that if there is indeed a non-linear relationship between fitness and the frequency of cooperators, then cooperation, and in turn public goods, can stably exist even in the presence of some defectors (Figure 1.1, right-hand panel). Applying this theory to the example of *D. melanogaster* females provisioning their eggs with protective compounds, such as AMPs which act as public goods,

seems particularly fruitful. It could explain the phenomenon of protective egg provisioning with public good benefits, without the need for sophisticated punishment mechanisms. It also has the potential to create a model system which allows theories of non-linear public goods to be tested.

1.3 Conspecific cannibalism in *D. melanogaster*

Under nutritional stress, *D. melanogaster* larvae have been found to consume conspecific eggs and larger, later stage conspecific larvae. Females appear to protect their eggs against this phenomenon by coating their eggs with the cuticular hydrocarbon (CHC) 7,11-HD, which has been shown to have anti-cannibalism properties and is deposited in the wax layer of the egg (Narasimha *et al*., 2019). Larvae depend on chemical cues from their environment to forage for food (Billeter & Wolfner, 2018). The 7,11-HD maternally deposited pheromone 'leak-proofs' the eggs, and essentially conceals them from other larvae. Details of the production of 7,11-HD are known. For example, one enzyme that has been identified in the production of the CHC 7,11-HD in the fly oenocyte, and on which I focus here, is a desaturase encoded by the gene *Fad2* (also known as *desatF*) (Shirangi *et al*., 2009).

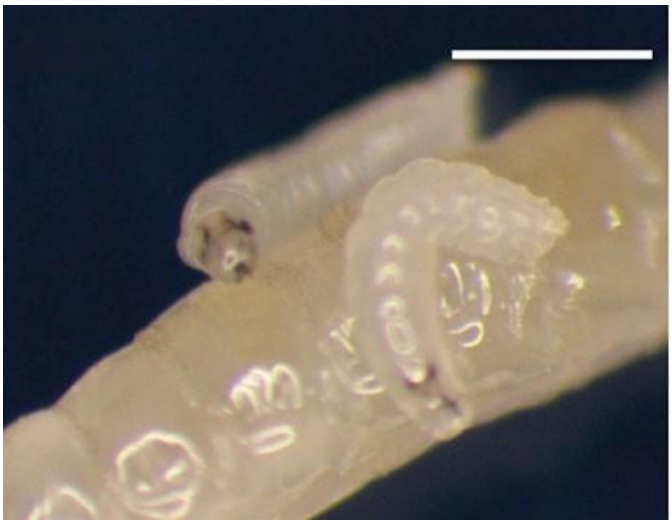

Figure 1.2: Two first instar larvae of D. melanogaster attacking and eating a conspecific third instar wandering larva *(Vijendravarma, R., Narasimha, S. & Kawecki, T., 2013).*

Nutritional stress appears to increase the probability of cannibalism among *D. melanogaster* larvae. Therefore, eggs laid on poor quality egg substrates would be expected to suffer from elevated cannibalism. However, the exact amount of nutritional stress required to promote cannibalism to a high level is uncertain. Vijendravarma *et al.* 2013, show that cannibalism behaviour can rapidly evolve under prolonged exposure to a low-nutrient diet over multiple generations. If eggs or larvae experiencing poor nutritional quality substrates do experience an increase in cannibalism, we would expect to see an increase in the gene expression of anti-cannibalism genes such as *Fad2* in response, in females laying eggs on such substrates. This was one idea I tested in this thesis by assessing the gene expression of *Fad2* and other related anti-cannibalism genes involved in the production of the 7,11-HD sex pheromone (Table 1.1).

By investigating the gene expression of 'anti-cannibalism genes', I wanted to evaluate the potential evidence for 7,11-HD as acting as a public good in egg clusters. For example, *fatp1* is a gene which aids the synthesis of CHCs (Wicker-Thomas *et al*., 2015) such as 7,11-HD. Thus, an increase in the expression of *fatp1* could indicate an increase in the production of 7,11-HD. By exposing flies to different social environments and egg-laying substrates, and then analysing the differences in expression of anti-cannibalism genes, I aimed to infer the extent to which 7,11-HD acts as a public good. However, many aspects of this scenario remain unknown. For example, it is uncertain that this kind of pheromone could mask surrounding eggs in addition to 'focal' individual eggs, as 7,11-HD appears to be found mostly in the sub-surface wax layer of eggs rather than directly on the egg surface. If 7,11-HD is non-diffusible, it might be acting as a 'private' rather than public good. It would also be useful to know whether eggs low in 7,11-HD that are laid in clusters of eggs containing higher 7,11-HD are at a decreased or increased risk of cannibalism. My gene expression studies along with such data will allow a deeper evaluation of whether 7,11-HD can act as a public good.

***Table 1.1:* Summary of the tested genes involved in the production of the anti-cannibalism sex pheromone 7,11-HD.** *Shown are the gene names, the function of these genes and the rationale for their inclusion in the study. Also included are the parts of the fly's body where the genes are most expressed to identify genes of interest in the abdomen and preferably the fat body or reproductive systems, as this would indicate the possibility of the gene product being provisioned on the egg. Body part expression is displayed as enrichment which is a measure of the abundance of a gene in a particular tissue relative to that in the whole fly. Expression level data and IDs were obtained from Fly Atlas and Flybase, respectively (Leader et al., 2018; Thurmond et al., 2019).*

Gene name	**Flybase ID**	**Body part (Enrichment value)**	**Function**	**Rationale for inclusion in study**	**References**
desat1	FBgn0086687	Mostly enriched in heart (6.6) and Fat body (5.9).	Iron ion binding activity and stearoyl-CoA 9-desaturase activity. Involved in several processes, including cuticle hydrocarbon biosynthetic process, mating behaviour and pheromone biosynthesis.	*Desat1,* part of the desaturase pathway, contributes to the synthesis of CHCs, potentially including 7,11-HD. Therefore, its expression may correlate with 7,11-HD levels.	(Narasimha *et al.*, 2019), (Chertemps *et al.*, 2006), (Leader *et al.*, 2018) (FlyAtlas). (Thurmond *et al.,* 2019) (Flybase)
ppk23	FBgn0030844	No enrichment and highest FPKM (Fragments Per Kilobase of transcript per Million mapped reads) in the head of females.	Contributes to pheromone perception and male courtship behaviour.	Required for the detection of 7,11-HD pheromone. So, expression may correlate with 7,11-HD production. However, mostly expressed in the head, rather than the abdomen.	(Lu *et al.*, 2012), (Narasimha *et al.*, 2019), (Leader *et al*., 2018)(FlyAtlas) Thurmond *et al*., 2019)(Flybase)

Fad2	FBgn0029172	Highly expressed in the female fat body (8.7) and heart (20).	Encodes LCFAs to synthesize di-unsaturated LCFAs. It is involved in the synthesis of the female pheromones.	Biosynthesis of 7,11-HD requires the enzymatic action of *desatF* (*Fad2*) in the *oe*s. Thus, expression of this gene should be directly correlated with the production of 7,11-HD.	(Leader *et al*., 2018)(FlyAtlas)(Narasimha *et al.*, 2019), Thurmond *et al.,* 2019)(Flybase) (Chertemps *et al*., 2006)
Fatp1	FBgn0267828	Mostly expressed in the female Eye, hindgut and Heart (8.1, 8.8, 7.1). However, it is also expressed in the fat body (3.4).	Required for CHC synthesis in adult oenocytes.	Contributes to the synthesis of CHCs, including 7,11-HD. Therefore, expression may correlate with 7,11-HD levels.	(Wicker-Thomas *et al.*, 2015), (Leader *et al.*, 2018)(FlyAtlas)

1.4 Anti-microbial peptides in *D. melanogaster*

The second type of potential public goods I investigated were anti-microbial peptides that might be deposited on egg surfaces. In *D. melanogaster,* the humoral and cellular immune response involves the production of anti-microbial peptides (AMPs) which are released in response to bacterial and fungal pathogens. Two main signalling pathways, Toll and Imd, are involved in the immune response and a key role in activating both occurs via peptidoglycan recognition proteins (Figure 1.3; Hultmark, 2003).

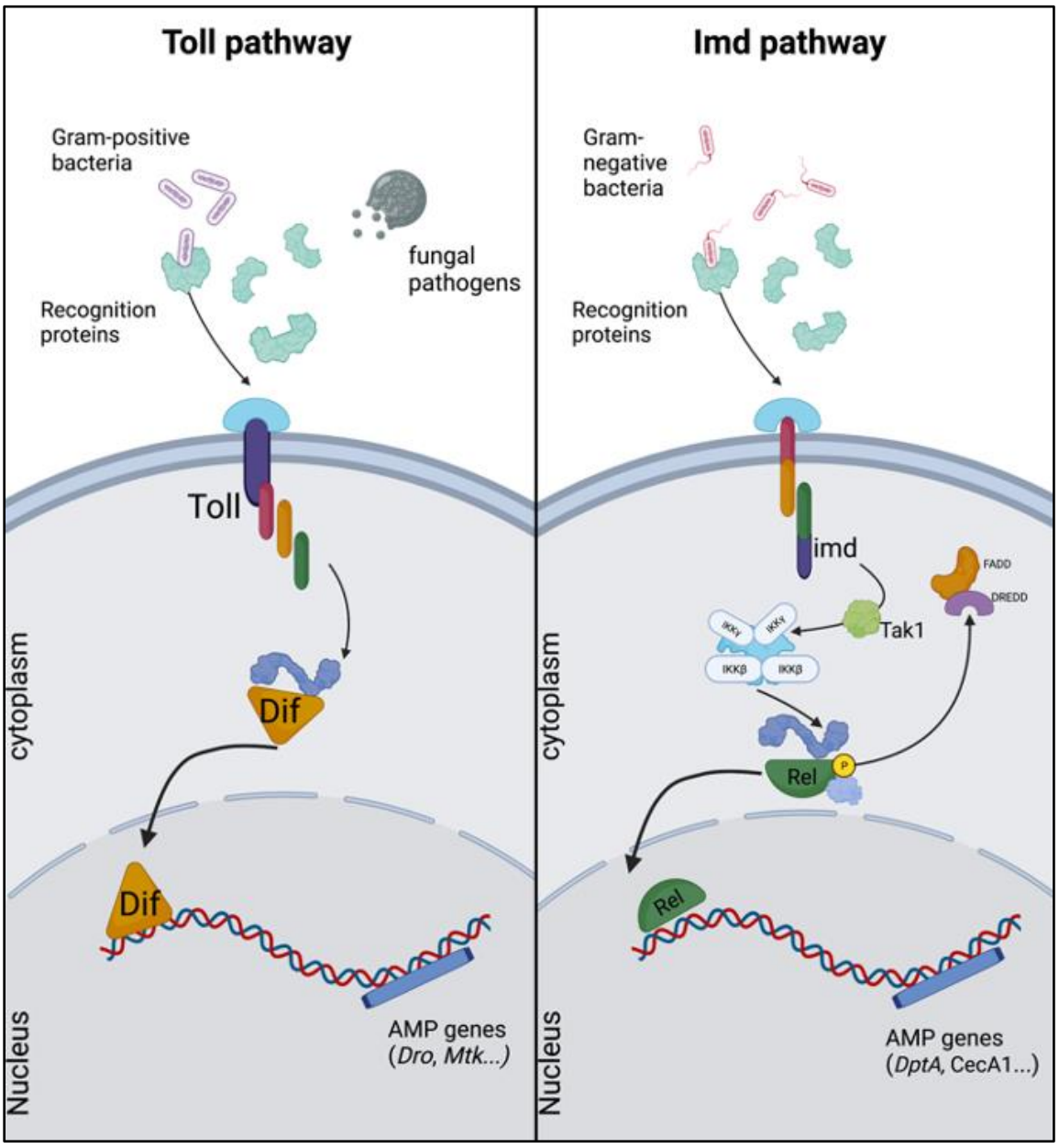

Figure 1.3: Diagram showing the Toll and Imd immune pathways In D. melanogaster. *The transcription factors Dif and Rel (on which I focused in this thesis) are shown in their respective pathways (Toll and Imd). (Created with BioRender.com).*

The Toll pathway is primarily activated in response to the presence of Gram-positive bacteria and upon fungal infections (Hoffmann, 2003). The *D. melanogaster* genome encodes three proteins in the Rel family: Dorsal (*dl*), Dif (*Dif*) and Relish (*Rel*) (Table 1.2). Mutations in these proteins all reduce the effectiveness of the anti-fungal response (Hoffmann & Reichhart, 2002). An important part of the activation of the Toll pathway is mediated by a cystine-knot cytokine–growth factor-like polypeptide, called Spaetzle (Table 1.2; Hoffmann & Reichhart, 2002). After the detection of Gram-positive bacteria or fungal pathogens via extracellular recognition factors, Spaetzle is cleaved to its active form as the result of a proteolytic cascade, leading to the activation of the Toll pathway (Valanne *et al*., 2011). The NF-kB Protein Dif is in a complex with an inhibitor protein called Cactus. The activation of the Toll pathway results in the dissociation of Cactus from Dif (Hoffmann, 2003; Hoffmann & Reichhart, 2002). The protein Dif is a transcription factor which promotes the transcription of many genes that appear to function in the immune response (De Gregorio *et al.*, 2001).

The Imd pathway is activated by Gram-negative bacteria and the presence of fungi. It is regulated by the activity of the NF-kB protein Relish (Park *et al.*, 2004). Receptors on the cell membrane (PGRP-LC, PGRP-LE) bind to peptidoglycans found on Gram-negative bacteria cell walls, this sends an intracellular signal to imd (Kleino & Silverman, 2014). This causes imd to bind with FADD and DREDD (Figure 1.3), with DREDD cleaving imd. Then via K63-ubiquitination, TAK1 is recruited which then goes on to activate the IκB kinase (IKK) complex (Myllymäki *et al*., 2014) (JNK pathway is also activated by TAK1 at this point, Park *et al*., 2004). IKK then phosphorylates the inhibitory protein IκB present on Relish, while DREDD cleaves the inhibitory C-terminal (Kleino & Silverman, 2014; Myllymäki *et al*., 2014). After this, the transcription factor Relish can move to the nucleus where it promotes the transcription of various AMPs (Hoffmann & Reichhart, 2002). This pathway controls the expression of most of the *D. melanogaster* AMPs (Myllymäki *et al.*, 2014). Therefore, I predicted it would be useful to analyse Imd as well as Toll pathway genes in my study (Table 1.2 and Figure 1.3), as the expression of Imd genes should correlate with the number of AMPs being produced. Specifically, for my study, *Dif* was selected to gain insight into the production of AMPs related to the toll pathway, *Rel* was selected as the member of the Imd pathway, and *Mtk* to investigate the

production of the AMP Metchnikowin. I reasoned that if the production of these molecules increased after mating when egg production is elevated, it could suggest that these AMPs are involved in the egg provisioning process and potentially act as public goods.

In the medfly *C. capitata*, the female reproductive accessory glands produce a secretion with strong antibacterial properties (Marchini *et al*., 1997) which is coated onto the surface of the eggs, potentially making them more resistant to bacterial infection. The family of AMPs which are secreted in the medfly are known as ceratotoxins (*Ctx*). This gene family that encodes the Ctx peptides has been identified in *C. capitata and Ceratitis rosa* (Rosetto *et al*., 2003). However, there is as yet no known *Ctx* homologue in *D. melanogaster*, and we also lack information on AMPs on the *D. melanogaster* egg surface. However, consistent with the potential for egg surface public goods, some AMPs have been found to be expressed in the female reproductive tract of *D. melanogaster*, for example, drosocin and drosomycin (Ferrandon *et al.*, 2007). These could potentially coat egg surfaces in a similar way to the Ctx peptides in *C. capitata*. The Toll and Imd pathways result in the production of drosocin and drosomycin AMPs in the fat body (Hetru *et al*., 2003). Hence genes in these pathways are good candidates to investigate, especially those expressed in the fat body or ovaries. These features governed the choice of candidate genes for investigation in this study (Table 1.2).

Table 1.2: Review of the genes related to immunity pathways (Toll and Imd) and the production of AMPs in D. melanogaster. *Shown are the gene names, the function of these genes and the rationale for their inclusion in the study. Also included are the parts of the fly's body where the genes are most expressed to identify genes of interest in the abdomen and preferably the fat body or reproductive systems, as this would indicate the possibility of the gene product being provisioned on the egg. Body part expression is displayed as enrichment which is a measure of the abundance of a gene in a particular tissue relative to that in the whole fly. Expression level data and IDs were obtained from Fly Atlas and Flybase, respectively (Leader et al., 2018; Thurmond et al., 2019).*

Gene name	**Flybase ID**	**Body part (Enrichment value)**	**Function**	**Rationale for inclusion in study**	**References**
Dif	FBgn0011274	Most enrichment: Crop (5.8), heart (6.2), hindgut (4.2), fat body (3.7). Lower in ovary (0.2), fairly enriched in spermatheca (Mated:3.8, Virgin:2.7)	Dorsal-related immunity factor encodes a transcription factor that contributes to zygotic function of the Toll pathway, notably the regulation of anti-microbial peptides (AMPs).	Important transcription factor that promotes the expression of many immunity related genes, and AMPs resulting from the Toll pathway.	(Valanne *et al.*, 2011), (Tapadia & Verma, 2012), (Thurmond *et al.,* 2019)(Flybase), (Leader *et al*., 2018)(FlyAtlas)
Rel	FBgn0014018	Highest in hindgut (4.3) Ovary (0.7) Fat body (2.0) Malpighian Tubules (3.0).	Relish (Rel) encodes a transcription factor and the downstream component of the immune deficiency pathway, which regulates the antibacterial response and other less characterized cellular processes.	Important part of the Imd pathway. This helps produce AMPs which may potentially be found on the egg.	(Hultmark, 2003) (Myllymäki *et al*., 2014) Thurmond *et al.,* 2019) (Flybase), (Leader *et al*., 2018) (FlyAtlas)

Mtk	FBgn0014865	Enriched in: Head (9.3) Fat body (0.5).	*Metchnikowin (Mtk)* expression is strongly stimulated in the first 6 hr after mating. Sex-peptide (SP), a male seminal peptide transferred during copulation, is the major agent eliciting transcription of *Mtk* and of other AMP genes.	Associated with an AMP produced in the fat body. It increases in expression after mating. Part of the Toll pathway.	(Peng *et al*., 2005), (Hetru *et al.*, 2003) Thurmond *et al.,* 2019) (Flybase), (Leader *et al.*, 2018) (FlyAtlas)
spätzle	FBgn0003495	Enriched in the ovary (1.5), crop (1.5), and rectal pad (2.3).	It contributes to function of the Toll signalling cascade, which plays a key role in various developmental and immune processes, notably embryonic dorso-ventral patterning and regulation of anti-microbial peptides.	Important part of the toll pathway. Thus, it is associated with the production of AMPs and it is enriched in the ovary.	(Valanne *et al*., 2011) Thurmond *et al.,* 2019) (Flybase), (Leader *et al.*, 2018) (FlyAtlas)
Imd	FBgn0013983	Enriched in the ovary (1.2).	Encodes a component of the immune deficiency pathway, which regulates the antibacterial response and other less characterized cellular processes.	Important part of the Imd pathway. It helps regulate the expression of Relish which is integral for the function of the Imd pathway.	(Myllymäki *et al.*, 2014) Thurmond *et al*., 2019) (Flybase), (Leader *et al.*, 2018) (FlyAtlas)

1.5 Aims and hypotheses

My overall research question for the thesis was: how do changes in the egg-laying substrate and social environment affect the expression of anti-cannibalism and anti-microbial potential public goods genes in *D. melanogaster?*

I aimed to determine how anti-cannibalism and immune gene expression was affected by the egg-laying substrate and the female's social environment (co-housing of females versus social isolation). Accordingly, I tested how these manipulations affected the propensity for fruit files to oviposit eggs in clusters (to test for the conditions that might promote the transfer of diffusible public goods, Chapter 2) before examining the associated changes in gene expression in females (Chapter 3).

I set up my main experiment with 4 treatments across 3 different time points. The time points consisted of 3 different groups which were sampled before mating (virgin), 3 hours after mating (3h) and flies sampled 6 hours after mating (6h). This made it possible to see how the expression of the target genes changed across the post mating timeframe. By investigating virgin flies, how these genes are expressed without being exposed to males (and mating) could be determined. There was uncertainty surrounding when flies would invest in potential public goods during the oviposition process and so having multiple time points was useful to determine at which point the expression of public goods-related genes were highest. With regard to the treatments, female *D. melanogaster* were either kept in groups of 4 or socially isolated and allowed to lay eggs on either a standard diet or a low-nutrient diet ('diet' and 'egg-laying substrate' are used interchangeably in this context). Females kept together with others were expected to perceive the potential for increased public goods benefits from laying eggs, potentially in clusters, with others. The use of the low nutrient egg-laying substrate on top of this social manipulation was to increase the probability of cannibalism. Studies have shown that under nutritional stress *D. melanogaster* larvae may consume conspecifics larvae and eggs (Narasimha *et al*., 2019; Vijendravarma *et al*., 2013). Here I used a diet containing 25% of the yeast and sugar found in the standard diet to increase the perceived risk of cannibalism among females laying eggs on that substrate and thus induce potential effects on

egg clustering (Chapter 2) as well as on the expression of anti-cannibalism 7,11-HD genes (Chapter 3). The specific hypotheses I tested are listed in the Table below:

Table 1.3: The hypotheses tested in this thesis, *including the rationale for each hypothesis and the relevant supporting references. The first two hypotheses were tested in Chapter 2, while the remaining four in Chapter 3.*

Hypotheses	Rationale	*References*
CHAPTER 2		
Grouped females will be more likely to cluster their eggs than isolated females.	This pattern would occur assuming there are benefits to be gained from potential public goods produced by non-kin flies.	Marchini *et al*., 1997; Platt & Bever, 2009
Females laying eggs on the low-nutrient substrate will be more likely to cluster their eggs compared to females laying on the standard diet.	This outcome is predicted due to potential increased fitness if benefits from public goods are present. The low-nutrient egg-laying substrate is expected to promote cannibalism.	Archetti *et al*., 2020
CHAPTER 3		
The relative expression of 'anti-cannibalism related genes' will be higher in females laying on the low-nutrient diet compared to the standard diet.	On the basis that there is a higher risk of cannibalism in females egg-laying on the low-nutrient diet, and that the levels of 7,11-HD will increase to counter this.	(Narasimha *et al*., 2019; Vijendravarma *et al*., 2013)
The relative expression of 'anti-cannibalism related genes' will be higher in females maintained in groups in comparison to solitary flies.	Would occur if there is a greater risk of egg cannibalism from larvae, when more non-kin eggs are laid in clusters.	(Khodaei & Long, 2020; Vijendravarma *et al*., 2013)
The relative expression of 'Anti-microbial related genes' will be higher in solitary females compared to those kept in a group.	This assumes that females in a group may be more likely to rely on AMPs produced by other flies, whereas solitary flies must produce AMPs independently.	(Archetti *et al*., 2011; Marchini *et al*., 1997)

Chapter 2: The effect of egg-laying substrates and the social environment on female fecundity and egg placement decisions

2.1 Abstract

Where to lay eggs, how many eggs to lay and in what configuration are key choices that can determine the fitness of organisms such as fruit flies. These decisions are likely to be impacted by the type of egg-laying substrate available and the social environment, particularly the number of additional conspecifics laying upon the same egg-laying substrate at the same time. These effects are of particular interest in the context of the potential for public good benefits accrued by protective, diffusible molecules placed by females on the surface of their eggs. I investigated here the specific effect of the egg-laying substrate and the social environment on a female's oviposition behaviour. I tested the hypothesis that females would lay more eggs in clusters together under conditions where putative public good benefits would be expected to be higher – i.e. on poor quality egg-laying substrates where the probability of cannibalism is higher, and when laying eggs together with other females due to the possibility to gain benefits of eggs laid in mixed maternity clusters. The results were not consistent with the existence of potential public good benefits because there was significantly less, rather than more, clustering of eggs on the poor-quality substrate, and the social environment also had no significant effect on egg clustering. Therefore, the fitness benefits of clustering eggs remain unclear, and if egg surfaces contain diffusible protective substances, their spread to other eggs does not appear to be mediated by the mechanism of egg clustering.

2.2 Introduction

Investment in eggs (how many to lay, and where to lay them) is a vitally important contributor to an individual's overall fitness. This can select for the evolution of specific reproductive strategies in organisms that lay eggs on or in external substrates. This can include mechanisms to vary the overall number of eggs laid (Flatt, 2020). In addition, the location and timing of oviposition are also important fitness-related traits (Churchill *et al*., 2021; Manjunatha *et al*., 2008). Placing eggs in unfavourable locations that carry a risk of predation, infection or insufficient nutrients to support offspring development, for example, can ultimately decrease the chances of offspring survival and thus decrease fitness (Sato *et al*., 2021). Conversely, egg placement in environments that are rich in nutrients, or safe from predators, could be extremely beneficial (Miller *et al*., 2011). Oviposition decisions may be especially important in species which do not exhibit parental care, such as in *Drosophila melanogaster* fruit flies (Lihoreau *et al*., 2016; Trumbo, 2018).

Many adaptations to enable the selection of suitable oviposition sites appear to exist and females of many species can detect and adjust their oviposition decisions according to biotic and abiotic factors. For example, *D. melanogaster* females are able to detect the nutritional quality and texture of a potential oviposition site, the local humidity and the presence of microbes (Sato *et al*., 2021; Vesterberg *et al*., 2021). Once an ideal nutritional substrate is found, females can lay many eggs on the same patch of food. However, if the offspring are restricted to utilising that same food patch, then ovipositing females must also ensure that the food patch does not become over-depleted. In *D. melanogaster,* eggs are often laid on rotting fruit (Lihoreau *et al*., 2016). *D. melanogaster* egg-laying behaviour is complex and requires considerable reproductive investment. Up to 100 eggs a day can be laid during peak fecundity periods (Flatt, 2020). Eggs are laid one at a time by females, with a search period occurring between the oviposition of each egg (Yang *et al*., 2008). However, the mechanisms used for determining these oviposition conditions, and the underlying pathways involved, and the benefits of laying in specific configurations, are not yet well known. The potentially large effects of egg placement on fitness suggest that oviposition rate and egg placement decisions should be

subject to strong selection. Thus, it is important to understand such oviposition decisions and the factors which cause them to vary, such as variation in substrate conditions, quality and the prevailing competitive or social environment in which eggs are produced and laid. A particular puzzle remains about the potential benefits of laying eggs in specific patterns in different environmental conditions. The idea I test here is that there are potential public goods benefits.

Both oviposition behaviour and the rate of egg-laying can be affected by the social environment, the egg-laying substrate and by the nutritional environment to which mothers are exposed. For example, diet has significant effects on fecundity (Mirth *et al*., 2019), with high fat (Liao *et al*., 2021) and low protein (Krittika & Yadav, 2020) diets both reported to reduce fecundity. Dietary restriction (DR), a term used to describe a reduction in nutrients in the diet, typically by 20-30% (to a lower level but without starvation), has also been shown to generally reduce fecundity. Typically, a reduction in protein in the diet reduces egg production in *D. melanogaster* (Chapman & Partridge 1996). However, fecundity is also highly plastic and decreases while DR is applied, then rapidly increases once standard diets are resumed (Chapman *et al*., 1994; Sultanova *et al*., 2021; Trevitt *et al*., 1988).

A reduction in fecundity in response to lower levels of protein may occur because fewer resources are allocated to reproduction over survival in comparison to the situation for standard diets (Maklakov & Chapman, 2019). In addition, the oviposition selection site decisions may vary according to the external environment. As mentioned above, the nutrients available in a potential egg-laying patch are critical for the successful development of offspring (Miller *et al*., 2011). Therefore, a female may plastically reduce the number of eggs laid in a patch based on what that patch can potentially support, even if she has the capacity to lay additional eggs. Essentially, the number of eggs being laid may be determined by a female's dietary status, by a female's decision based on the quality of the oviposition patch or level of potential competition at that patch (Fowler *et al*., 2022; Miller *et al*., 2011; Mirth *et al*., 2019).

Along with dietary variation, the social environment also has significant effects on fecundity in *D. melanogaster* fruit flies (Bailly *et al*., 2021; Churchill *et al*., 2021;

Fowler *et al*., 2022). For example, females maintained in social isolation before mating are reported to lay significantly more eggs in the 24 hours after mating, relative to females previously kept in social groups or housed with eggs or “deposits” of other females (Fowler *et al*., 2022). This suggests that females can remember the social environments in which they have been maintained and that they detect the presence of other females via the deposits they leave behind. This potentially allows them to adjust their fecundity according to the perception of competition for egg-laying sites, overall resource competition or potential benefits from public goods. Churchill *et al*. (2021) also reported an effect of pre-mating social grouping on female fecundity. The fecundity plasticity exhibited by females is also highly sophisticated, as females show additional and contrasting responses to their proximate immediate social environment - increasing egg-laying as group size increases (Bailly *et al*., 2021). However, the fitness benefits of these striking and plastic effects on fecundity in response to the social environment are not known.

The oviposition behaviour of *D. melanogaster* fruit flies has been shown to change depending on the patchiness of the substrate, with females laying more eggs on aggregated egg-laying substrates in comparison to single dispersed substrates (Churchill *et al*., 2021). In addition, when patches of food are dispersed, the females are more likely to lay their eggs together on one patch with those of other females, instead of using all the patches, which is what occurs when patches are in closer proximity. Females also show egg-laying preferences for different patches of food based on acidity, humidity and temperature (Chen & Amrein, 2017; Enjin *et al.*, 2016; Winkler *et al.*, 2020). Much less known about the patterns of micro-placement behaviours that occur within a patch, such as egg clustering, in which two or more eggs are physically touching. That these egg clustering patterns are also potentially important for fitness is supported by the observations outlined below. The experiment conducted in this Chapter aimed to investigate egg clustering behaviour, specifically, to test the effects of egg-laying substrates and social environment on egg clustering and whether the pattern of egg-laying was consistent with the predictions of public goods benefits.

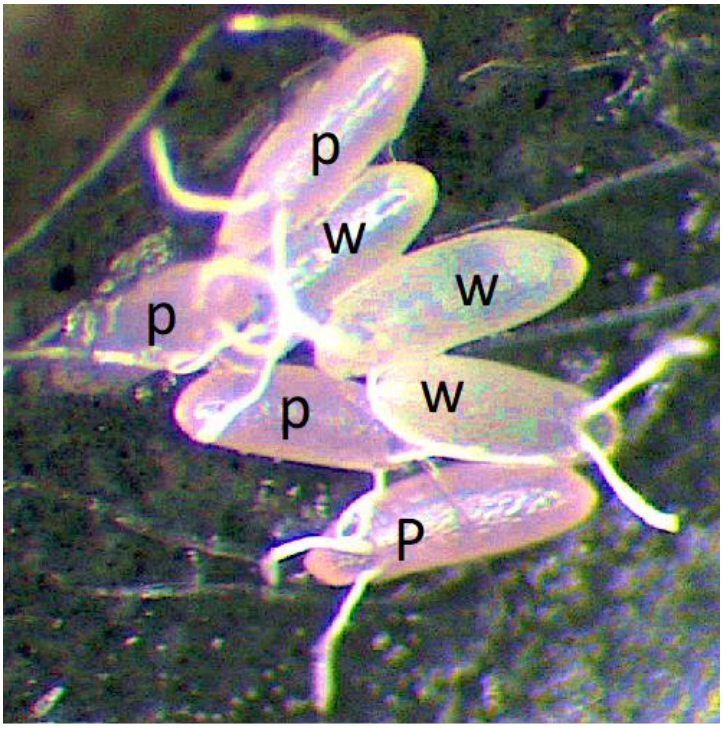

Figure 2.1: Example of a mixed maternity egg cluster. *The pink eggs (p) are from a wild-type female dyed with Sudan Red and the white eggs (w) are from a standard female). Emily Fowler, Lucy Friend, Tracey Chapman, unpublished data.*

Under laboratory conditions fruit flies often place their eggs in clusters within vials. These clusters can also be mixed maternity, meaning different individuals lay eggs adjoining each other (Figure 2.1). In the unpublished data (Emily Fowler, Lucy Friend, Tracey Chapman), it was found that the majority of the focal eggs were not clustered (73.9 ± 5.4%). However, when the eggs were in clusters most were of mixed maternity, containing focal and non-focal eggs (mixed maternity: 20.5 ± 3.9%, focal-only clusters: 5.5 ± 1.8%). From these data, it is expected that, in general, most egg clusters (79%) in this wild-type population will be of mixed maternity. The phenomenon of mixed maternity clusters is an evolutionary puzzle, as the clustering of eggs should increase competition for resources or increase the risk of larval cannibalism due to the larger number of kin and non-kin larvae (Khodaei & Long, 2020; Vijendravarma *et al*., 2013). For this behaviour to be selected for and maintained, there should be a demonstrable fitness benefit. One possible explanation for mixed maternity clustering could be if ‘public goods’ are present on these eggs. This could be in the form of defensive compounds which provide an advantage not only to the egg secreting them but also to the other eggs in the

cluster. For example, medflies (*Ceratitis capitata)* lay their eggs in clusters (Thomas *et al*., 2001) and the eggs are coated with broad-acting anti-microbial peptides (AMPs) (Marchini *et al*., 1997). These AMPs inhibit microbial growth in the areas surrounding the eggs. Thus, it is possible that these AMPs could act as a public good in egg clusters, as any eggs (from unrelated individuals) laid within the zone of any microbial inhibition they cause could also benefit. There are many unknowns regarding the production of AMPs in medflies, including whether medfly females can adjust the amount of anti-microbial provisioning and whether provisioning would be dependent on the size of the cluster, or the presence of eggs from other females. However, if these AMPs can act as public goods, in the absence of mechanisms for punishing potential free riders, this opens up the potential for "cheats" (Morsky *et al*., 2020). Females that cheat by not provisioning their eggs with anti-microbials, avoid the costs of producing AMPs. Instead, they can position their eggs next to eggs that have been provisioned with AMPs and gain the fitness benefits. Whether *D. melanogaster* eggs are similarly provisioned with anti-microbials is not yet known and is investigated in Chapter 3.

A second type of potential public good that could be found on *D. melanogaster* eggs is anti-cannibalism molecules. The female-specific sex pheromone 7,11 heptacosadiene (7,11-HD) can also act as an 'anti-cannibalism' pheromone (Narasimha *et al*., 2019). Eggs provisioned with this pheromone are protected from cannibalism by conspecific larvae. Although the exact mechanism by which 7,11-HD protects eggs is unknown, it has been hypothesised that the pheromone masks the eggs, making them undetectable by larvae (Narasimha *et al* 2019). If this pheromone also protects other adjacent eggs in an egg cluster, then it could also potentially act as a public good. In this context, egg investment and placement are important because they would impact upon the potential benefits of diffusible protective molecules, and both are expected to be responsive to the oviposition and social environment. These factors are also expected to affect the expression of genes whose products potentially act as public goods, which is investigated and reported in Chapter 3.

Here I set up an experiment to test for the effects of diet and social environment on fecundity and egg placement. I tested whether egg placement occurred in a manner

that might reflect the existence of public goods. In this Chapter, I report the experiment and effects of the dietary and social environment. In Chapter 3, I report the effects of those manipulations on the expression of potential 'public goods' genes. I set up four different treatments (Figure 2.3). Female *D. melanogaster* were kept in groups of 4 or socially isolated and allowed to lay eggs on a standard or a low-nutrient diet. The low-nutrient food substrate was chosen to promote the opportunity for cannibalism, as studies have shown that under nutritional stress *D. melanogaster* larvae may consume conspecifics larvae and eggs (Narasimha *et al*., 2019; Vijendravarma *et al*., 2013). I selected a diet containing 25% of the yeast and sugar found in the standard diet (for the recipe see below) and this was expected to result in an increase in the risk of cannibalism and thus potential effects on egg clustering and on anti-cannibalism gene expression (Chapter 3).

Following the public goods hypothesis, I expected to see more egg clustering in the grouped flies compared to the flies kept alone. This was based on the assumption that single females could potentially have less to gain from laying eggs in clusters because they would not be able to gain public goods benefits from eggs laid by other females. In addition, this expectation does assume that females have a mechanism for determining their own versus other females' eggs, or for assessing the maternity of eggs in clusters. If not, then there may be no differences in clustering according to the social environment. In addition, fecundity was expected to be higher in the grouped female treatments (Bailly *et al*., 2021). Therefore, in the grouped treatment, there may be more opportunities to place eggs in clusters. Oviposition site quality is also expected to affect egg placement. In the low-nutrient substrate, flies may react to reduced resource availability by laying fewer eggs overall (Mirth *et al*., 2019). Alternatively, females could also adjust the placement of eggs they oviposit as a response to the quality of egg-laying substrate, such as favouring dispersed eggs as opposed to clustered eggs. Laying eggs in clusters more frequently and pooling together costly public goods could result in increased offspring survival. However, the benefits of any such strategy could also be balanced by the potential for cannibalism of eggs, which is expected to be higher in the low-nutrient environment (Vijendravarma *et al*., 2013).

Table 2.1: The main hypothesises discussed in Chapter 2, *the rationale behind each of them along with supporting references.*

Hypothesises	Rationale	*References*
CHAPTER 2 *Grouped females will be more likely to cluster their eggs than isolated females.*	Assumes there are benefits to be gained from potential public goods produced by non-kin flies.	Marchini *et al*., 1997; Platt & Bever, 2009
Females laying eggs on the low-nutrient substrate will be more likely to cluster their eggs compared to females laying on the standard diet.	This pattern would occur due to potential increased fitness if benefits from public goods are present. The low-nutrient egg-laying substrate is expected to promote cannibalism.	Archetti *et al*., 2020

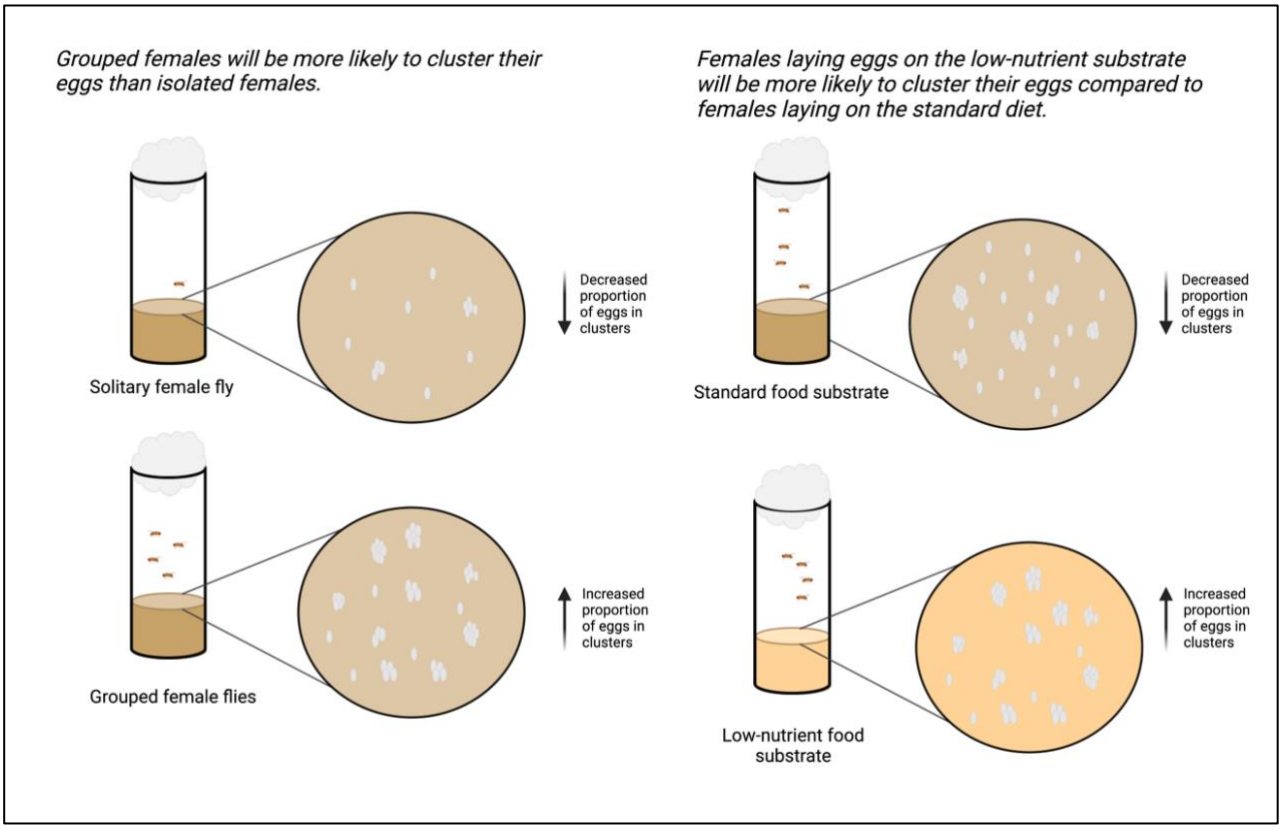

Figure 2.2: Chapter 2 experimental design. *This shows the rationale behind the two main hypotheses from Chapter 2, listed in Table 2.1. Including the effect of social environment and quality of substrate on egg clustering. Left: Flies were kept solitary or in groups of 4 (half on low-nutrient substrate, half on standard substrate); the proportion of eggs laid in clusters was predicted to increase in the grouped flies, relative to the solitary flies (Table 2.1). Right: Flies were kept on a low-nutrient egg-laying substrate and a standard substrate (for both grouped and solitary flies); the proportion of eggs laid in clusters was expected to be greater on the low-nutrient substrate, compared to the standard food substrate (Table 2.1)*

2.3 Methods

Testing for Social and Egg-laying Substrate Effects on Fecundity and Egg Placement

The *D. melanogaster* Dahomey strain used in these experiments was collected in the 1970s in Benin, Africa and has since been maintained since then in large outbred stocks with overlapping generations at 25°C, 50% relative humidity and a 12:12h light: dark cycle. Stocks were maintained in cages with glass bottles containing standard sugar-yeast-agar (SYA) food (100 g brewer's yeast, 50 g sucrose, 15 g agar, 30 ml Nipagin (10% w/v solution in 95% ethanol), 3 ml propionic acid, 1 l dH_2O).

Eggs were collected from across 3 Dahomey stock cages using 6 grape juice agar plates (275 ml dH_2O, 150 ml concentrated red grape juice, 12.5 g agar, 10.5 ml Nipagin (10% w/v solution in 95% ethanol)) with a small amount of fresh yeast paste on the surface. Once the eggs hatched, 3000 first instar larvae were picked and placed in 30 vials of standard SYA food (100 larvae per vial) to develop to adulthood under standardised conditions. Adult flies were collected as virgins using ice anaesthesia within 8h of eclosion and maintained in same-sex groups of 10 in standard SYA vials until reproductively mature (at 4-5 days old). Females were then allocated at random to social and egg-laying substrate treatments in a fully factorial design and transferred under CO_2 anaesthesia to new vials according to these treatments. These treatments along with the different time-points are visualised in Figure 2.3 below. For the social treatment, females were housed alone or in same-sex groups of 4. For the substrate treatment, females were kept on either standard SYA food (**S**) or 'low-nutrient' SYA food (**L**). The standard food was as described above and the low-nutrient SYA food contained 25% of the yeast and 25% of the sugar of the standard food amounts (i.e. 25 g and 12.5 g per litre, respectively).

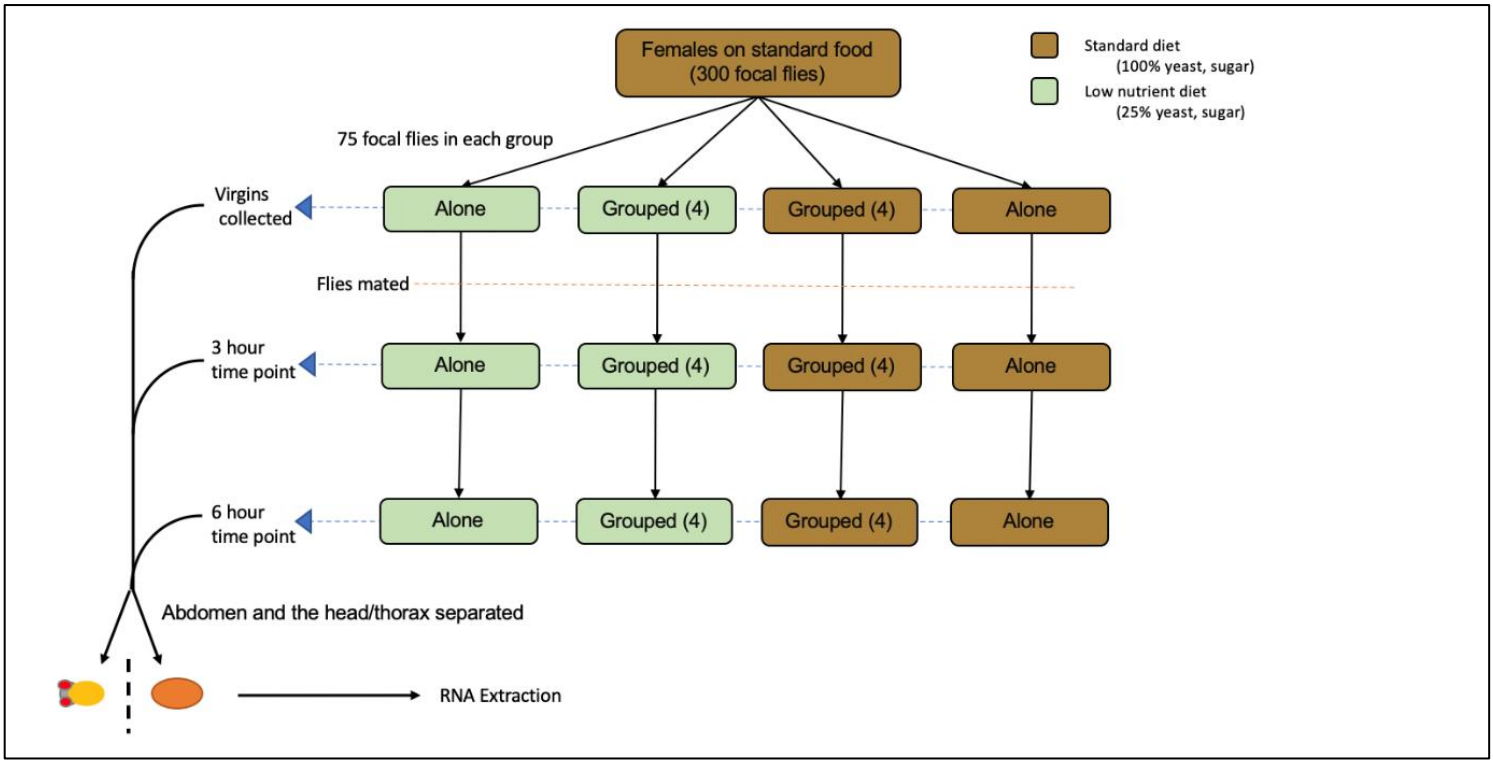

Figure 2.3: Experimental design for testing for effects of social environment and egg-laying substrate *on female fecundity and egg placement (this chapter) and gene expression (Chapter 3). Females were kept alone or in groups of 4 on standard (brown) or low (green) protein diets.*

Table 2.2: The number of focal flies under each treatment, includes all three time points (25 flies in each)

Social treatment	**Egg-laying substrate treatment**	
	Low quality	Standard quality
Alone	75	75
Grouped (1 focal, 3 non focal females)	75	75

Table 2.2 shows the number of flies in each treatment. These were divided into 3 time-points, so in a given time point there were 25 flies per treatment. 25 virgin females from each treatment were frozen in liquid nitrogen 2 days after the set-up of the social treatments. These virgins made up the first time point (V) for the gene expression analysis (Chapter 3). All remaining females were mated by introducing 2 or 6 males (for the alone vs grouped treatments, respectively) to each vial under CO_2 anaesthesia. Flies were then given 2h to mate. Mated females were moved, using CO_2 anaesthesia to fresh S or L vials for oviposition, with the same social environment as before, and the males were discarded. Three hours after mating, 25 females from each treatment were collected and frozen using liquid nitrogen to

comprise the 3h after mating time point for the work in Chapter 3. 6h after mating, the remaining 25 flies from each treatment were also sampled and frozen in the same way. The egg placement decisions of females from the 4 treatments were examined at both 3 and 6h after the mating had taken place and the flies were removed. These samples of flies were then used to investigate gene expression data in Chapter 3.

Effect of Egg-laying Substrate and Social Exposure on Fecundity and Egg Placement 3 and 6h after mating

After the females were mated, they were moved into fresh vials, under CO_2, keeping the diet and social treatment the same, while the males were discarded. Egg placement data were then recorded from those vials after 3h and 6h (once the flies had been removed and frozen). Using a data collection sheet (Supplementary information; Figure S.5) the number and placement of eggs in each vial was recorded. Two or more eggs touching each other was classified as a 'cluster'. The following measurements were recorded: the number of single eggs (eggs not in contact with other eggs) and the number of eggs making up each individual cluster. From this, the total number of eggs and total number and size of clusters along with the proportion of eggs in clusters was calculated.

The clusters were then sorted into size order and ranked, starting with the largest cluster to the smallest. Figures were created in (4.0.2)(R Core Team, 2020) (see supplementary information for code: Figure S.2). In addition, box plots containing fecundity data were also created in R using ggplot2 (Wickham, 2016) and the packages 'ggpubr' (Kassambara, 2020) and 'dplyr' (Wickham *et al*., 2021).

Statistical analysis of laying substrate and social effects on fecundity and egg placement 3 and 6h after mating

R (4.0.2) (R Core Team, 2020) was used for all Figures and statistical analyses (code provided in supplementary information). To analyse the effect of social environment and diet on egg clustering proportion, the number of eggs in clusters and the number of single eggs per vial were bound together using cbind() to create

the response variable in a generalized linear model (GLM) with quasibinomial error structure (used to account for over-dispersion) and a logit link function (vials with no clusters present were excluded), using the base 'stats' package included in R (R Core Team, 2020). The 3h and 6h time point data were analysed in two separate models. Social environment and diet were included as independent variables. The interaction between the independent variables was initially included in each model but was found to have no significant effect on egg clustering proportion in either case, so was dropped from the final models (Supplementary information: Figure S.3). Statistical significance values were derived from the analysis of deviance tests of the final models using the anova() function with an *F*-distribution. To test for differences in fecundity between the low-nutrient and standard diet, the total number of eggs was used as the dependent variable in a GLM with a quasipoisson error structure to account for overdispersion, and a log link function. The 3h and 6h time points and two different social groups were analysed separately in four different models (Supplementary information: Figure S.4). Statistical significance values were derived from the analysis of deviance tests of the final models using the anova() function with an *F*-distribution. This analysis was then repeated using the number of clusters as the response variable, using a 3h and 6h, alone and grouped treatment model for each.

2.4 Results

The effect of egg-laying substrate and social environment on fecundity 3 and 6h after mating.

I tested the effect of egg-laying substrate treatment on the total number of eggs present in the vials. These analyses showed that the substrate treatment had no statistically significant effect on the total number of eggs laid in either of the time points or social group treatments (Table 2.2). There was also no clear effect of the egg-laying substrate (Figure 2.4).

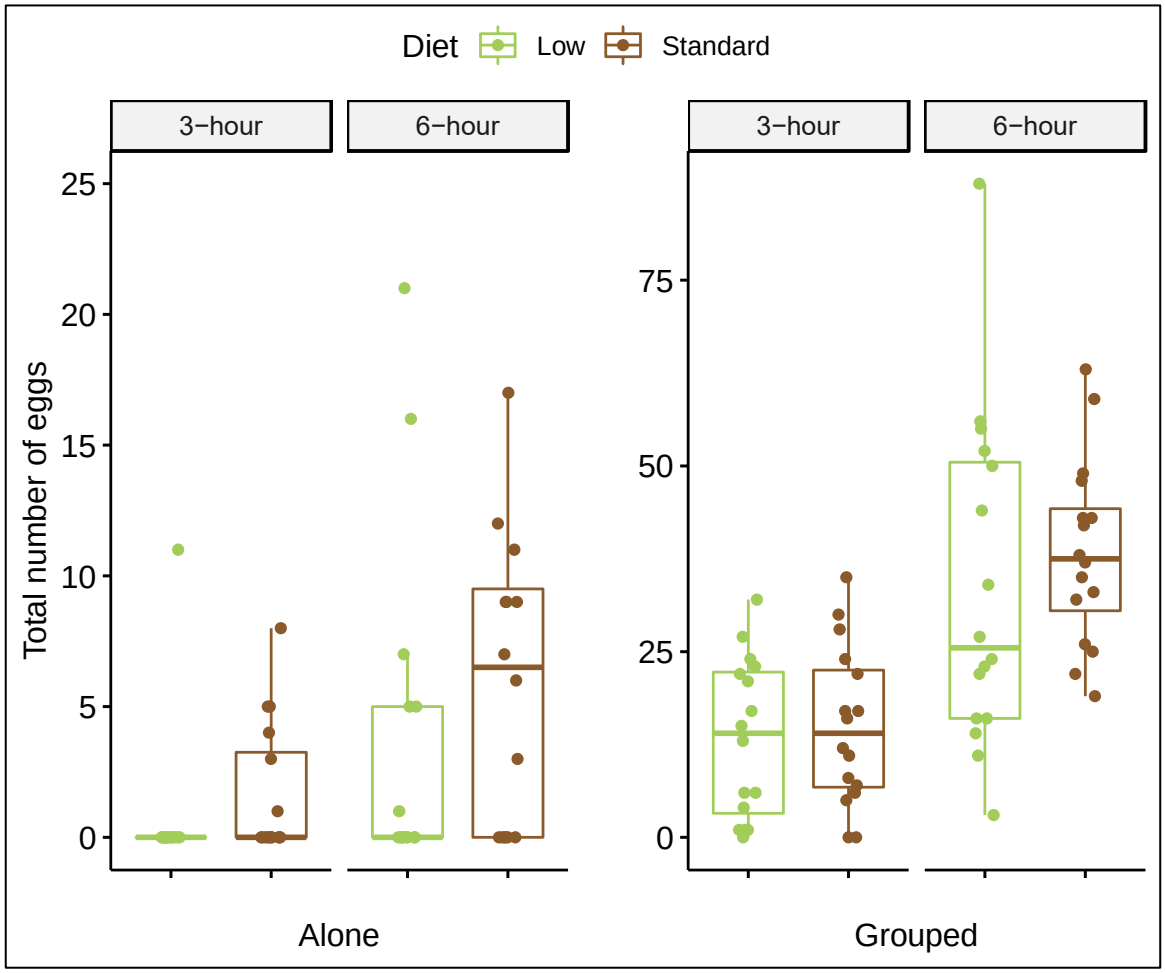

Figure 2.4: The effect of egg-laying substrate and social treatment on the number of eggs per vial 3h and 6h after mating. *Box plots showing the total number of eggs per vial across the 3h and 6h time points and in both social treatments (a 49-egg vial outlier in the 6h Alone Standard treatment was excluded). The left panel shows the number of eggs laid by the solitary flies while the right shows the grouped female's eggs 3h and 6h post-mating. The quality of the egg-laying substrate is shown in green (low nutrient) and brown (standard). Each data point represents an individual vial (sample size n=16 vials for all treatments in each time point).*

Table 2.3: Analysis of the effect of egg-laying substrate and social treatment on the total number of eggs laid. *Below are the results from the ANOVA conducted on four separate GLMs (3h and 6h/alone and grouped) in R, using the quasi-Poisson model and 'F' test to correct for overdispersion. This analysis tested for a significant difference in the total number of eggs, due to the effect of dietary treatment.*

Social treatment	Time point	F	Degrees of freedom	Residual Degrees of freedom	Deviance	Residual Deviance	P-value
Solitary	3h	0.83	1	30	6.30	118.9	0.368
	6h	2.21	1	30	32.7	347.2	0.148
Grouped	3h	0.68	1	30	1.37	290.1	0.683
	6h	0.57	1	30	5.44	282.2	0.455

Effect of social environment and egg-laying substrate on the proportion of eggs laid in clusters 3 and 6h after mating

To investigate the effect of social environment and egg-laying substrate on egg clustering, the proportion of eggs found in clusters per vial was calculated (Figure 2.5). These box plots appeared to show a trend for egg clustering to be greater on the standard diet in comparison to the low-nutrient diet across both time points and social environments.

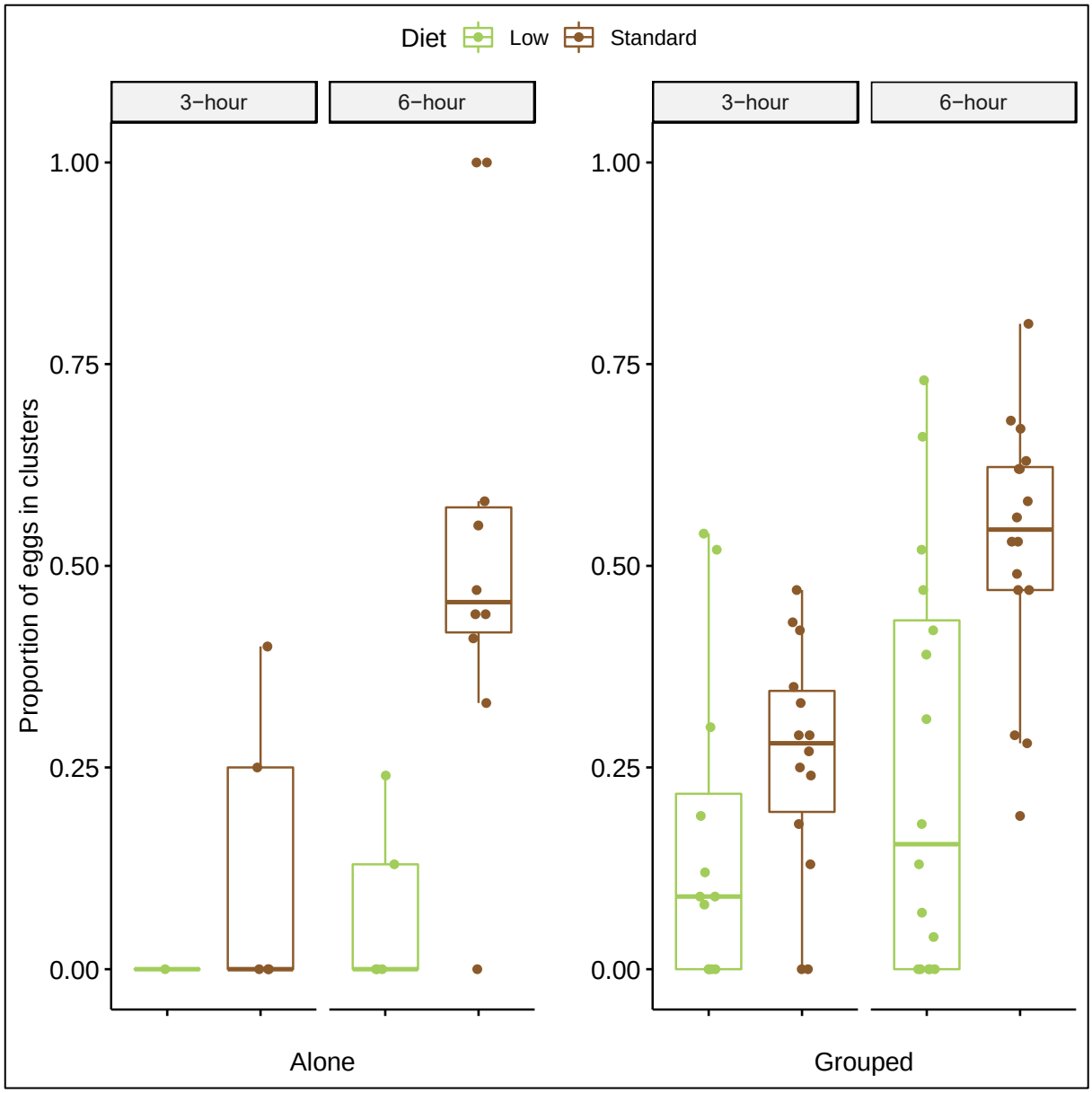

Figure 2.5: The effect of social grouping and egg-laying substrate on the proportion of eggs laid in clusters 3h and 6h after mating. *The box plots show the proportion of eggs which were in a cluster of eggs (physical contact with each other, with vials containing fewer than 2 eggs excluded as they cannot cluster). The left panel shows the proportion of the solitary females' eggs in clusters 3h and 6h post-mating. The right shows the proportion of the grouped female's eggs in clusters 3h and 6h post-mating. The quality of the egg-laying substrate is shown in green (low nutrient) and brown (standard). Each data point represents an individual vial (sample size left to right: n=1, n=5, n=5, n=10, n=12, n=14, n=16, n=16).*

These patterns were explored in the statistical analysis, which showed that in the 3h and 6h time points, females maintained on the low-nutrient treatment laid a significantly lower proportion of their eggs in clusters compared to those maintained on the standard substrate, though the effect at the 3h time point was marginally non-

significant (3h: $F_{(1,34)}$= 3.87, P = 0.057; 6h: $F_{(1,46)}$= 10.84, P = 0.002; Table 2.3, Figure 2.5). The social group treatment had no significant effect on the proportion of eggs laid in clusters, at either time point (3h: $F_{(1,33)}$ = 2.81, P = 0.103; 6h: $F_{(1,45)}$ = 1.34, P = 0.253; Table 2.3, Figure 2.5) and there was no significant interaction between social and diet effect on the proportion of eggs laid in clusters (Supplementary information: Figure S.11).

Table 2.4: Analysis of the effect of social and egg-laying substrate treatments on the proportion of eggs laid in clusters 3 and 6h after mating. *Shown are the results from the ANOVA completed on two separate GLMs (3h and 6h) in R, using the quasi-binomial model and 'F' test to correct for overdispersion in the proportion data. This analysis tested for a significant difference between the proportion of eggs found in clusters, due to the effect of dietary/social treatment Significant p values indicated in* ***bold****).*

Effect	Time point	F	Degrees of freedom	Residual Degrees of freedom	Deviance	Residual Deviance	P-value
Diet	3h	3.87	1	34	7.92	78.1	0.058
	6h	10.76	1	46	57.6	291.5	**0.002**
Social	3h	2.81	1	33	5.75	72.3	0.103
	6h	1.34	1	45	7.17	284.3	0.253

<u>The effect of egg-laying substrate and social treatment on the size and abundance of egg clusters produced 3 and 6h after mating</u>

I calculated the number of clusters and their respective sizes (number of eggs) across all vials separated by treatment. The rank-size distribution (Figure 2.6) shows all clusters across all vials ranked and separated by treatment. The largest cluster was ranked as 1, then in descending size order, the remaining clusters were ranked, the larger the rank the smaller the cluster. This allowed a visual comparison of how

the clusters were affected by the social environment and egg-laying substrate treatment. A qualitative comparison of this metric at the 3h time point (Figure 2.6) suggested that the grouped standard (SG) and grouped low-nutrient diet (LG) treatments had larger egg clusters than the alone females (SA), and that egg clusters tended to be larger on the standard diet (SG) treatment. No egg clusters were observed in the low nutrient-alone (LA) treatment. Note though that the alone treatments had 1 female per vial and the grouped treatments 4, which could potentially influence the clustering effect.

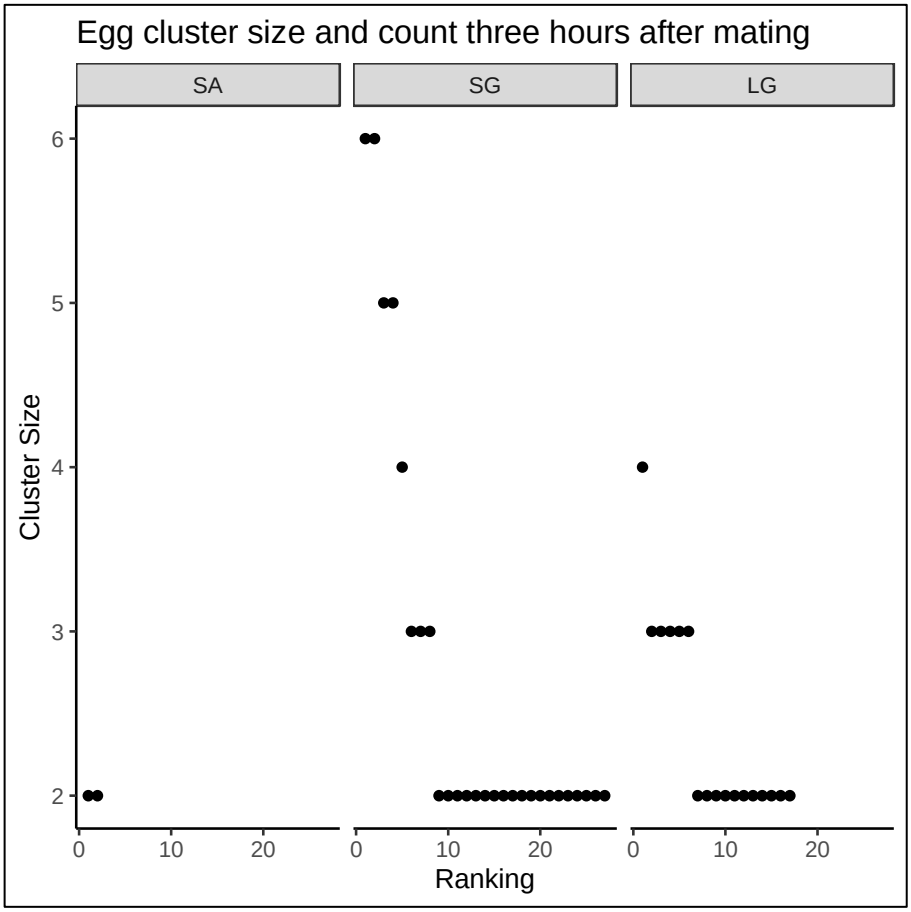

Figure 2.6: Effect of social environment and egg-laying substrate treatment on egg cluster size and count 3h after mating. *The graph shows the frequency of egg clusters of different sizes for females laying eggs on Standard (S) or Low (L) diets and maintained Alone (A) or in Groups (G). These clusters are from vials in which the flies were removed 3h after mating. The x-axis shows the ranking of egg clusters of different sizes (y-axis), in descending size order (6 = largest cluster size, 2 = smallest cluster size). No clusters were present in the LA (Low-nutrient diet and Alone) treatment for this time point.*

The same comparison at the 6h time point shows a similar pattern (Figure 2.7) with larger egg clusters being observed in groups kept on the standard diet (LG, SG), and with grouped treatments having larger clusters than alone.

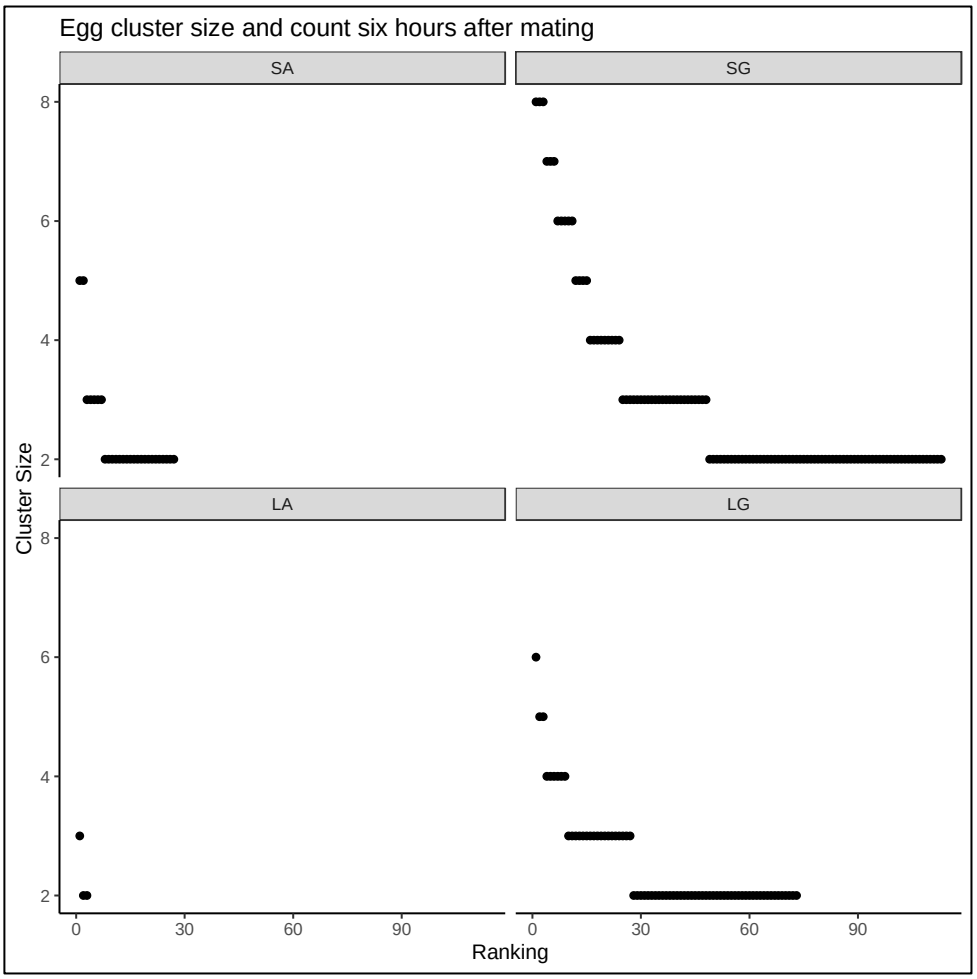

Figure 2.7: Effect of social environment and egg-laying substrate on egg cluster size and number 6h after mating. *The graph shows the frequency of egg clusters of different sizes for the female egg-laying on Standard (S) or Low (L) food substrates and maintained Alone (A) or in Groups (G). These clusters are from vials in which the flies were removed 6h after mating. The x-axis shows the ranking of egg clusters of different sizes (y-axis), in ascending order (8 = largest cluster size, 2 = smallest cluster size).*

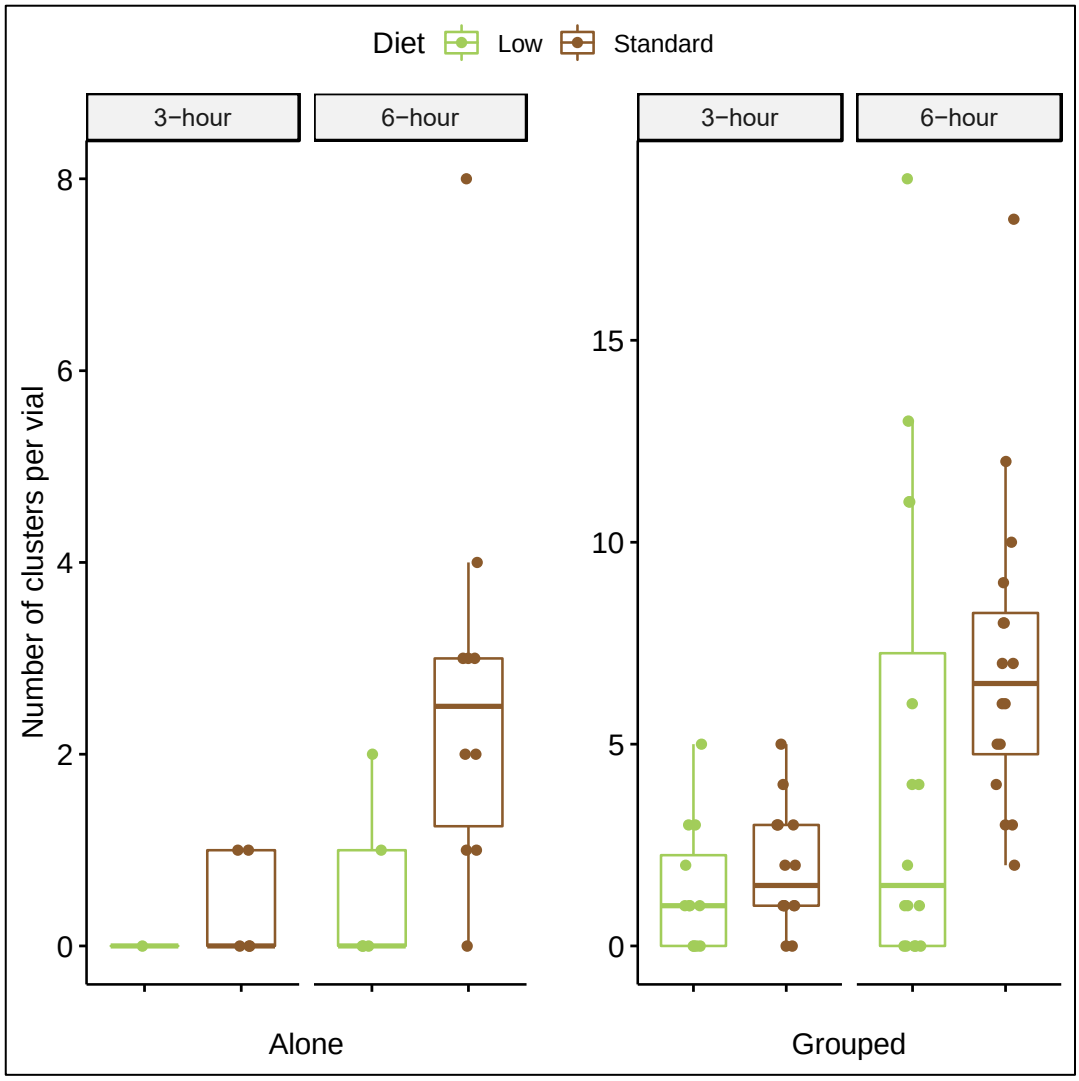

Figure 2.8: The effect of diet on the number of egg clusters per vial 3h and 6h after mating. *Box plots showing the number of clusters per vial across the 3h and 6h time points and in both social treatments (vials with less than 2 eggs were excluded). The left panel shows the number of clusters produced by the solitary flies while the right graph shows the grouped female's eggs clusters 3h and 6h post-mating. The quality of diet is shown in green (low nutrient) and brown (standard). Each data point represents an individual vial (sample size left to right: n=1, n=5, n=5, n=10, n=12, n=14, n=16, n=16).*

Table 2.5: The number of vials and mean number of egg clusters per vials *separated by time point and treatment (only includes vials with ≥ 2 eggs(L= Low-nutrient, S= Standard, A=Alone, G=Grouped).*

Time point	Treatment	Number of vials	Mean number of clusters per vials
3h	LA	1	0
	SA	5	0.2
	LG	12	1.13
	SG	14	1.8
6h	LA	5	0.25
	SA	10	1.8
	LG	16	4.56
	SG	16	7.06

I analysed statistically the effect of dietary treatment on the number of clusters per vial. Separate models were run for 3h and 6h and for solitary/grouped flies. It was found that flies on the standard diet had a significantly larger number of clusters compared to the low-nutrient diet flies, which can be seen in Figure 2.8). This effect on the number of clusters was only found in the solitary flies in both 3h and 6h time points, not in the grouped flies (there is only a small number of vials in the 3h time point) (In solitary flies: 3h: $F_{(1,30)}$ = 5.94, P = 0.021; 6h: $F_{(1,30)}$= 10.0, P = 0.004). In addition. Due to the fact that the grouped fly vials contained 4 times as many flies as the solitary fly vials, no analysis on the effect of social environment was completed.

Table 2.6: The effect of social and egg-laying substrate treatments on the number of clusters per vial. *Shown are the results from the ANOVA completed on four GLMs (3h and 6h/alone and grouped) in R, using the quasi-Poisson model and 'F' test to correct for overdispersion. This analysis tested for a significant difference in the total number of egg clusters, due to the effect of dietary/social treatment (Significant p values indicated in* ***bold****.).*

Social treatment	Time point	F	Degrees of freedom	Residual Degrees of freedom	Deviance	Residual Deviance	P-value
Solitary	3h	5.94	1	30	2.77	8.32	**0.021**
	6h	10.0	1	30	22.1	54.2	**0.004**
Grouped	3h	1.32	1	30	2.29	54.2	0.259
	6h	1.78	1	30	8.67	139.7	0.192

2.5 Discussion

Overall, the results showed no support for the public goods hypotheses tested. The first hypothesis was that grouped females would be more likely to cluster their eggs than isolated females. This prediction was not supported and provided no evidence for potential benefits to be gained from “public goods” in egg clusters of mixed maternity. The second hypothesis was that females maintained on the low-nutrient diet would lay more of their eggs in clusters. The findings also went against this idea and instead showed that low-nutrient ovipositing females laid a significantly lower proportion of their eggs in clusters compared to those laying on the standard diet. This does not support the idea that egg clustering is more common when females lay eggs under low-nutrient food conditions, which might increase the probability of cannibalism, and thus public good benefits that protect against this. The results are explored in detail further below.

Grouped females were not more likely to cluster their eggs compared to isolated females

The results showed no statistically significant difference in the proportion of eggs in clusters between the solitary and the grouped female treatments. This was contrary to the prediction, which was derived from the assumption that if females were producing public goods, flies which laid their eggs in these clusters would be able to gain from the public goods produced by eggs from other females. There are several potential explanations for the lack of support for the hypothesis. It is possible that females do not recognise their own eggs versus those laid by other females. Evidence for recognition of kin versus non-kin eggs has been shown, although not in adult flies. For example, larvae have been reported to cannibalise unrelated eggs more frequently than kin eggs (Khodaei & Long, 2020). Furthermore, clusters of larvae are reported to be more numerous when more closely related kin were present in the social environment (Khodaei & Long, 2019). This suggests that some level of egg recognition may be possible, although the kin recognition in this example was by larvae and not adult flies. Furthermore, fruit flies might rarely find themselves in a situation in the wild where they are completely solitary during oviposition opportunities. This could lead to negligible selection for fitness benefits gained by

altering oviposition preferences based on the frequency of close kin in the environment.

It is also possible that public goods effects do not benefit eggs laid in close proximity, or that egg clustering occurred for a different reason. Solitary flies might also benefit from laying eggs in clusters for a different reason than do females in groups. The hypothesis tested here assumed that the benefit of public goods is present as the cooperation between non-kin females might allow some flies to produce less of their own costly public goods and nevertheless still benefit. However, it could also be the case that laying eggs in clusters is an efficient way of distributing protective compounds across a female's own eggs even when no other flies are present. For example, if a minimum threshold of 7,11-HD pheromone is required to protect eggs from cannibalism, then it could be more efficient for individual females to produce enough 7,11-HD sufficient to protect a cluster of their eggs rather than individual eggs laid separately.

Females laying eggs on a low-nutrient diet are less likely to cluster their eggs

The second hypothesis tested was that the females laying eggs on the low-nutrient substrate (in which the risk of cannibalism was predicted to be higher and thus public good benefits from egg clustering potentially higher) would be more likely to cluster their eggs compared to standard diet females. The results provided no support for this hypothesis. Females laying eggs on the standard diet laid a significantly higher, not lower, proportion of eggs in clusters relative to those on the low-nutrient diet. There was also a significantly greater number of egg clusters per vial on the standard relative to the low-nutrient diet. The results show that, even though we observed significant plasticity in egg clustering, we do not yet understand the full range of fitness benefits involved.

Egg clustering is a behaviour found not only in *Drosophila* but other arthropods. For example, mites (*Iphiseius degenerans*), ticks (*Rhipicephalus sanguineus*), and ladybirds (*Aphidecta obliterata* and *Adalia bipunctata*) (Faraji *et al*., 2002; Ramos *et al*., 2013; Timms & Leather, 2007) all exhibit egg clustering. Potential benefits of egg clustering have been identified in some species. For example, in ticks, it has been

found that clusters with larger numbers of eggs increased the larval maximum survival period (Ramos *et al*., 2013). In ladybirds, the ideal size of an egg cluster was reported to vary across different host trees (Timms & Leather, 2007). Egg clustering in mites may be an adaptation to counter-attacking prey, rather than for protection against cannibalism (Faraji *et al*., 2002). It has also been found that in *Chlosyne lacinia*, a species of butterfly, egg clustering protects against desiccation in very dry conditions (Clark & Faeth, 1998). Additional studies of the effects of varying substrate qualities on the frequency of egg-laying patterns are needed to understand the potential benefits and factors that cause egg clustering to vary. In species that show cannibalism, such as *D. melanogaster* (Khodaei & Long, 2020; Vijendravarma *et al*., 2013), the potential costs and benefits are potentially more complex. Either the benefits of clustering (reduced predation) outweigh the costs (cannibalism), or there is a method of limiting cannibalism such as suggested by the presence of the 7,11-HD pheromone (Narasimha *et al*., 2019).

The prediction was that laying eggs in clusters more frequently might lead to public goods benefits that could result in increased offspring survival. This effect would be expected to be heightened in the low-nutrient laying substrate vials. In the standard diet, there were expected to be sufficient resources for females to produce protective molecules such as AMPs and pheromones for their own eggs, thus clusters would carry no benefit. This prediction was not supported. There are several possible explanations. For example, while laying eggs in clusters could lead to higher localized concentrations of 7,11-HD and thus protect more eggs in the cluster from cannibalism, the clustering itself may lead to increased levels of cannibalism because of the proximity of newly hatched larvae to their conspecific eggs within a cluster. However, the idea that *D. melanogaster* eggs would be more susceptible to cannibalism from hatchlings of nearby eggs has not been investigated. The relationship between egg-egg distance and the likelihood of cannibalism would be interesting to establish, since it could be a potential explanation for females reducing egg clustering in environments such as the low-nutrient diet in which cannibalism could be more likely (Vijendravarma *et al*., 2013).

I hypothesised that eggs would be found in clusters more frequently on the low-nutrient food, partly due to increased reliance on public goods. However, this also

assumed that public goods are produced at the same amount regardless of the quality of the egg-laying substrate's nutritional quality. Theorised public goods such as the AMPs and sex pheromones that were the focus of this project, are likely to be costly to produce. Under actual or perceived nutritional stress there is the potential for the synthesis of these compounds to be reduced. Thus, assuming 7,11-HD is affected in this way, egg clustering could become even riskier. The results of the gene expression analysis of 7,11-HD related genes in Chapter 3 explore this topic. Cannibalism risk offers an explanation for the decrease in egg clustering on low-nutrient egg-laying substrates, although there could be other reasons. It has been shown that fly immunity (including the expression of key AMP related genes) is improved when flies have the perception of, or access to, more dietary carbohydrates such as glucose (Galenza *et al*., 2016; Ponton *et al*., 2020). If public goods are more challenging to produce under the perception of nutritional stress via the detection of poor egg-laying substrates, then females may be less likely to lay eggs in clusters as the eggs they oviposit beside may carry reduced public goods. This could potentially make any reliance on other eggs for public goods benefits a riskier strategy. Conversely in the standard food, the presence of public goods on eggs may be more certain due to the perception of an adequate nutritional environment.

There was no significant difference between the number of eggs laid between the two diets in the grouped treatments. A change to fecundity wasn't tested as a hypothesis, but it was still possible that the quality of the substrate could have had an effect on the fecundity of females. Diet has been shown to affect egg production in various ways, for example, diet has a plastic effect on fecundity (Chapman *et al*., 1994; Sultanova *et al*., 2021; Trevitt *et al*., 1988), and the production of eggs has been shown to depend on multiple factors such as the specific nutrients found in food and activation of nutrient-sensing pathways (Krittika & Yadav, 2019; Mirth *et al*., 2019). Despite this, no effect was found in this study, while the quality of substrate did have an effect on the proportion of eggs found in clusters. It may be that using a diet 25% of the standard amount of yeast provided the fruit flies with the perception of sufficient food to produce eggs at the same rate as the standard, while the substrates were still different enough for the amount of egg clustering to be affected. If the flies lay the same number of eggs across the treatments, then any differences

in clustering are really due to the placement decisions of the flies, rather than due to fewer clusters being present due to fewer eggs being laid overall.

Another potential explanation for the differences in the number of eggs in clusters may be due to variations in the textures of the egg-laying substrates. It has been found that some species of fly, e.g. *Drosophila biarmipes and D. melanogaster*, have preferences for substrates based on food hardness (preferring softer substrates for egg-laying; Silva-Soares *et al*., 2017; Zhang *et al*., 2020). It may be that differences between the hardness of the low- versus standard egg-laying substrates could explain the differences in egg clustering. The effect of substrate texture as well as content would be interesting to test further.

Conclusion

In summary, there was no evidence for eggs being laid in response to the potential for public goods benefits arising from variation in the social environment or quality of egg-laying substrates. Indeed, it was found that reducing the nutritional quality of the diet resulted in flies being less, rather than more likely to cluster their eggs. This suggests that the fitness benefit of clustering eggs is reduced when flies are maintained and lay eggs on a low-nutrient diet, compared to the standard nutritional diet. The implications of this in terms of gene expression are explored further in Chapter 3.

Chapter 3: Expression of anti-cannibalism and anti-microbial genes in females in response to the egg-laying substrate and social environment

3.1 Abstract

Public goods are a potentially important component of cooperative behaviour. Goods are 'public' if they are costly to produce and benefit not only the producer, but other unrelated individuals (including non-producers). In this thesis, I am investigating whether there is any evidence for public goods type benefits in the placement or composition of eggs on substrates in *Drosophila melanogaster*. In Chapter 2, I found no evidence that the egg-laying substrate or social environment affected egg placement in a manner that would support public goods benefits via adaptive egg clustering. Hence egg clustering may occur for alternative reasons. The results also suggested that the mechanism for mediating any potential public goods benefits that arise from protective egg surface substances is not egg clustering *per se*. In this Chapter, I tested directly for changes in the expression of six candidate public goods genes that have the potential to produce protective molecules found on the surface of eggs. These included three genes related to the production of the 7,11-HD sex pheromone, which is reported to have anti-cannibalism properties, and three genes related to the production of anti-microbial peptides (AMPs). The results showed that the nutritional quality of the egg-laying substrate and the social environment both had significant effects on the expression of these potential 'public goods' related genes. However, these patterns of expression were only partially in line with the expectations of the public good hypothesis. Females laying on a low-nutrient diet showed an increased level of expression of anti-microbial genes such as *Dif* and *Mtk*, compared to females laying on the standard diet. Maintaining females together with other females in a grouped social environment also increased the expression of all anti-cannibalism-related genes tested (*Fad2, fatp1, desat1*) compared to females maintained in social isolation. Under the public goods hypothesis, I predicted that there would be more variation in the expression of the candidate genes in the grouped relative to the solitary flies, potentially reflecting the presence of non-expressing free riders and co-operators. Two genes tested, *Dif* and to a lesser extent

desat1, showed this pattern, but the rest did not. Overall, this study shows that the expression of potential public goods related genes is significantly influenced by variation in the quality of egg-laying substrates and social environments. Further work is now needed to investigate the significance of these changes and whether any of the candidates fit within the public goods framework.

3.2 Introduction

Previous studies have revealed that there are strong effects of the social environment on traits such as reproductive investment (Bailly *et al*., 2021; Fowler *et al*., 2022). These effects can be observed in individuals of both sexes (Fowler *et al*., 2022). For example, in *Drosophila melanogaster* fruit flies, females kept in same-sex groupings adjust their overall reproductive investment into fecundity, potentially to guard against over-exploitation of resources. In addition, *D. melanogaster* males that have been exposed to rival males mate for significantly longer (Bretman *et al*., 2009) and in some contexts significantly faster (Fowler *et al*., 2022) than do males held in isolation. These plastic responses of males are associated with increased ejaculate transfer (Wigby *et al.*, 2009) and lead to increased reproductive success (Bretman *et al*., 2009), consistent with the idea that they represent adaptive responses to the threat of sperm competition. Manipulations of the social environment and the arrangement of egg-laying substrates can also affect the placement of eggs by females (Bailly *et al*., 2021; Churchill *et al*., 2021). The significance of such changes to egg placements is not yet known. One possibility, which I tested here, is that the number, composition or placement of eggs varies according to the social environment in order to gain public good benefits. Specifically, I tested the hypothesis that females laying their eggs in an environment in which other unrelated females are also doing so, could gain benefits from the presence of diffusible molecules on egg surfaces, that provide protection across eggs of different females due to their anti-microbial or anti-cannibalistic effects (Marchini *et al*., 1997; Narasimha *et al*., 2019).

Public goods in the context of evolutionary biology refers to a substance which is costly to produce for the individual, but which improves the fitness of others in the

population. In Chapter 1.2 I introduced the example of public goods in bacterial biofilms. However, examples have also been found in insects. For example, in the ant *Pristomyrmex punctatus*, all workers are involved in both their own asexual reproduction and cooperative tasks. This creates a potential dilemma as it is advantageous to an individual ant's fitness to reproduce more and cooperate less. Studies have shown that the 'cheaters' survived and reproduced more than co-existing workers. However, groups containing only cheaters failed to produce any offspring (Dobata & Tsuji, 2013), acting as a brake on high frequencies of cheaters and stabilising cooperation.

In the current study conducted in this Chapter, I tested whether anti-microbial peptides (AMPs) and the female sex pheromone 7,11-HD genes are expressed in a manner consistent with the idea that they represent public goods. The rationale is that 7,11-HD has been identified as an anti-cannibalism pheromone, which is present on eggs and can protect eggs from cannibalism (Narasimha *et al*., 2019). It is also possible that AMPs could be present on the surface of *D. melanogaster* eggs, as has been shown for the medfly *Ceratitis capitata* (Marchini *et al*., 1997).

If producers of public goods, and cheaters, exist in a population we might expect to see this reflected in the expression of genes involved in the production of the public good. For example, cheaters would be expected to have a lower expression of public goods-related genes than producers. Furthermore, if cheating is a plastic trait, then females would only "cheat" if there were other females in the environment producing the public good. Under this plasticity assumption, we would expect to see more variation in public goods related gene expression when females were in a group than when they were isolated (since isolated females cannot cheat and benefit from the public goods of others). It is this theory I set out to test in this chapter. To do this, I first identified genes involved in the production of potential "egg surface" public goods, as described below.

Candidate public goods genes

Anti-cannibalism gene candidates - fad2, *fatp1 and desat1*

The cuticular hydrocarbon (CHC) 7,11-HD is a sex pheromone that also masks eggs from conspecific larvae, and eggs that lack this pheromone are vulnerable to

cannibalism (Narasimha *et al*., 2019). Therefore, the presence of potential public goods benefits from anti-cannibalism effects could in principle be detected by the activity of genes involved in sex pheromone production such as *Fad2*, *fatp1 and desat1. Fad2* is expressed in female oenocytes and encodes a desaturase (desatF). DesatF catalyses the reaction to introduce the second double bond into fatty acid precursors (Sato & Yamamoto, 2020). Desaturase F is essential for the normal production of 7,11-HD and other CHCs produced by female *D. melanogaster* (Chertemps *et al*., 2006). *fatp1* and *desat1* are also both associated with the production of CHCs such as 7,11-HD (Chertemps *et al*., 2006). The biogenesis pathway of CHCs generally consists of long-chain fatty acid (LCFA) synthesis, desaturations, elongation to very long-chain fatty acids (VLCFAs), and removal of the carboxyl group (Sato & Yamamoto, 2020; Wicker-Thomas *et al*., 2015). Fatty acid transport protein 1 (*fatp1*) encodes a transmembrane protein and is involved in the cellular uptake of long-chain fatty acids with a role in the catabolism of fatty acids as well as the synthesis of triglycerides. It has been shown that the expression of *fatp1* is essential to the function of larval and adult oenocytes (Wicker-Thomas *et al*., 2015). *Desat1* and *Fad2* both produce desaturases (desaturase1 and desaturaseF, respectively). Desat1 acts on saturated fatty acids, creating precursors to monoene pheromones in males, and diene pheromones in females. DesatF action is required to transform these precursors into dienes (Chertemps *et al*., 2006), this step only occurs in females and produces diene pheromones such as 7,11-HD.

An increase in the expression of *Fad2, fatp1 and desat1* is expected to result in more 7,11-HD being produced and potentially deposited in the wax layer of eggs. Thus, we expect the expression of these genes to covary with egg production, mating and/or the social environment. Given that cannibalism is expressed more strongly under conditions of starvation (Vijendravarma *et al*., 2013), then it is predicted that the expression of anti-cannibalism genes should also be sensitive to the nutritional environment on which eggs are placed and in which larvae will then develop.

Anti-microbial gene candidates – Dif, *Rel* and *Mtk*

Following a similar rationale to the above, it is expected that anti-microbial peptides such as drosocin and drosomycin could also provide eggs with anti-microbial protection. Therefore, the production of such AMPs and their potential as public

goods could be revealed by analysing the expression of genes from the Toll and Imd innate immunity pathways.

As a proof of principle, it has been shown in the medfly (*Ceratitis capitata*), that anti-microbial peptides (AMPs) are produced in the reproductive tissues of adult females and diffusible AMPs are secreted onto egg surfaces during oviposition (Marchini *et al*., 1997). These AMPs are broad-acting and potent. In anti-microbial assays, a single medfly egg causes a visible zone of inhibition when assayed in a bacterial clearing assay against gram-positive, gram-negative and eukaryotic microbes (Marchini *et al*., 1997). The diameter of the growth inhibition zone in such clearance assays increases with the number of eggs tested, suggesting an additive effect of an increased concentration of AMPs. The number of such AMPs in the oviposition environment may be socially responsive, as a study found that a greater proportion of females initiated oviposition into kumquat fruits if another medfly was present (Prokopy & Duan, 1998) though this effect may be inconsistent (Dukas *et al*., 2001). The genes encoding egg-surface AMPs in *C. capitata* have been identified as Ceratotoxins (*Ctx*), which are a family of genes seemingly restricted to a few tephritid fruit fly species and are structurally similar to another family of AMPs in *D. melanogaster*, cecropins (Rosetto *et al*., 1993, 1996, 2003). Cecropins and ceratotoxins have been shown to comprise a superfamily of peptides (Tamang & Saier, 2006). Two of the genes in the *D. melanogaster* cecropins family are known as *CecA1* and *CecA2* (Ramos-Onsins & Aguadé, 1998). These genes encode AMPs with activity against Gram-negative bacteria and are mainly expressed in the fat body (Hoffmann *et al*., 1996; Ramos-Onsins & Aguadé, 1998). These genes are mostly regulated by the imd pathway, and potentially act on this pathway by influencing Relish via the suppression of the Dredd protein (Hedengren-Olcott *et al*., 2004; Ryu *et al*., 2006; Tingvall *et al*., 2001) (Figure 1.3).

It is possible that *D. melanogaster* may deposit AMPs on the surface of their eggs in a similar manner to the medfly described above. *D. melanogaster* females lay eggs in a decomposing environment containing abundant microbial species, some of which may be pathogenic. Egg surface AMPs, such as anti-cannibalism pheromones, could potentially act as public goods if they diffuse from the egg into the surrounding area. Therefore, all of the eggs in a given cluster (inside the zone of

inhibition) could stand to benefit from the presence of AMPs on a neighbouring egg. No specific egg-surface AMPs have yet been described in *D. melanogaster*. Therefore, candidate genes were selected on the basis of whether they had appropriate expression patterns and also on selecting genes to represent both major immune pathways. *D. melanogaster* has a large number of AMPs, testing all of these without knowing what will be on the egg surface was out of the scope of this study. Instead of this, two upstream genes involved in the two main pathways that lead to the production of AMPs were chosen (*Dif* and *Rel*). This would potentially capture any increases in AMP expression more generally. *Mtk* was also chosen because there is evidence that this specific AMP is regulated in relation to mating (Peng *et al*., 2005), so it was a good candidate for an egg surface AMP.

Gram-positive bacterial and fungal pathogens are detected via recognition proteins resulting in the activation of proteolytic cascades and the cleavage of spaetzle (Hoffmann, 2003). This results in the activation of the Toll pathway, and eventually leads to the increased expression of the transcription factor *Dif*, which then in turn increases the expression of Toll-related AMPs such as drosomycin (Valanne *et al*., 2011). *Rel* is another transcription factor which acts in a related manner. However, for *Rel* to activate and amplify the expression of AMP genes, the Imd immunity pathway must first be activated by Gram-negative bacteria (Myllymäki *et al*., 2014). *Rel* then upregulates AMP producing genes resulting in the production of AMPs such as diptericin (Hoffmann & Reichhart, 2002). Increases in the expression of *Dif* or *Rel* in response to the perception of egg-laying substrates or to the social environment would be expected to lead to elevated AMP production. In addition to these key transcription factors, I also selected *Mtk* which directly encodes the AMP Metchnikowin. This AMP is an anti-fungal peptide and is activated by the Toll signalling pathway (Moghaddam *et al*., 2017). A previous study had shown a significant increase in the expression of *Mtk* 6 hours after mating in *D. melanogaster* (Peng *et al*., 2005). Therefore, *Mtk* appeared as a promising candidate for a socially responsive AMP.

In general, if a gene encodes a product that acts as a public good, then we'd expect its relative expression level to be correlated with the number of eggs being produced. This could be evident as a higher expression after mating when egg production

generally increases. However, the level of variation in the gene expression could also be indicative of public goods and specifically the cooccurrence of co-operators and cheats (Platt & Bever, 2009). If public goods are present on eggs, and candidate genes such as *Fad2* and *Mtk* correlate with their production, then cooperative 'producers' would show high levels of gene expression and 'cheats' little to no expression. This would result in a larger coefficient of variation among groups containing both types of individuals (the socially grouped treatments).

In this Chapter, I conducted an experiment designed to investigate the effects of egg-laying substrate and social environment on the expression of anti-cannibalism and anti-microbial genes. I derived samples from the experiment described in Chapter 2 (Figure 2.3). Females were either kept in groups of 4 or in social isolation and subjected to two dietary manipulations of their egg-laying substrates: a standard (100%) diet or a low-nutrient (25%) diet. Only the abdomens of the females were analysed for gene expression as this body part is expected to contain genes whose products could coat the surface of eggs ovulated from the ovaries (table 1.1 and 1.2). For example, AMPs are produced in the fat body (Leclerc & Reichhart, 2004) while 7,11-HD is produced in the oenocytes (Wicker-Thomas *et al*., 2015), both of which are expressed in the abdomen (Krupp & Levine, 2010). Gene expression for each candidate gene of interest was measured at three different time points. Once before mating, three hours after mating and six hours after mating. This allowed an assessment of how much each candidate gene was activated by mating and by social exposure, as well as how much the expression changed throughout the period of egg production and oviposition.

I predicted that the anti-cannibalism genes would increase in expression when females were maintained on the low-nutrient egg-laying diet, since 7,11-HD protects eggs from cannibalism (Narasimha *et al*., 2019) and the low-nutrient egg-laying substrate is expected to increase the risk of cannibalism (Vijendravarma *et al*., 2013). Similarly, group exposure was also expected to increase the risk of cannibalism due to more non-kin larvae being present in the vials compared to the solitary flies.

The expectations for the expression of the AMP genes were derived from public good theory but could also be shaped by findings that dietary manipulations can affect the quality of the immune response (Unckless *et al*., 2015). For example, the immune response and production of AMPs can be impaired or weakened when flies experience low levels of dietary carbohydrates such as glucose (Galenza *et al*., 2016). High sucrose (Yu *et al*., 2018) and low-protein: high-carbohydrate diets can upregulate the expression of AMPs (Ponton *et al*., 2020). Therefore, having an egg-laying substrate lower in carbohydrates and proteins (low-nutrient diet) could negatively impact the immune response, leading to lower AMP gene expression. I predicted anti-microbial gene expression would be higher per female, on average, in the solitary compared to the grouped females. This was based on potential for public goods benefits, and that solitary females would all need to produce AMPs and thus would not benefit from the AMPs on the eggs of other females. However, the expression of the AMPs should also covary with the number of eggs produced, which could counteract that effect and produce higher AMP expression in the grouped environments. The specific hypotheses tested are shown below:

Table 3.1: Hypotheses tested in Chapter 3, *including the rationale behind these hypotheses and any accompanying references.*

Hypothesis	Rationale	*References*
Anti-cannibalism candidate genes:		
(i) The relative expression of 'anti-cannibalism related genes' will be higher in females laying on the low-nutrient diet compared to the standard diet.	On the basis that there is a higher risk of cannibalism in females egg-laying on the low-nutrient diet, and that the levels of 7,11-HD will increase to counter this.	(Narasimha *et al*., 2019; Vijendravarma *et al*., 2013)
(ii) The relative expression of 'anti-cannibalism related genes' will be higher in females maintained in groups in comparison to solitary flies.	Would occur if there is a greater risk of egg cannibalism from larvae, when more non-kin eggs are laid in clusters.	(Khodaei & Long, 2020; Vijendravarma *et al*., 2013)
Anti-microbial candidate genes:		
(iii) The relative expression of 'Anti-microbial related genes' will be higher in solitary females compared to those kept in a group.	This assumes that females in a group may be more likely to rely on AMPs produced by other flies, whereas solitary flies must produce AMPs independently.	(Archetti *et al*., 2011; Marchini *et al*., 1997)
Both candidate gene categories		
(iv) Females kept in groups will have higher variation in relative expression levels of anti-microbial and anti-cannibalism related genes compared to socially isolated females.	This assumes that cheats and co-operators are present, more variation in the expression levels between individual females would be expected.	(Archetti *et al*., 2011; Platt & Bever, 2009)

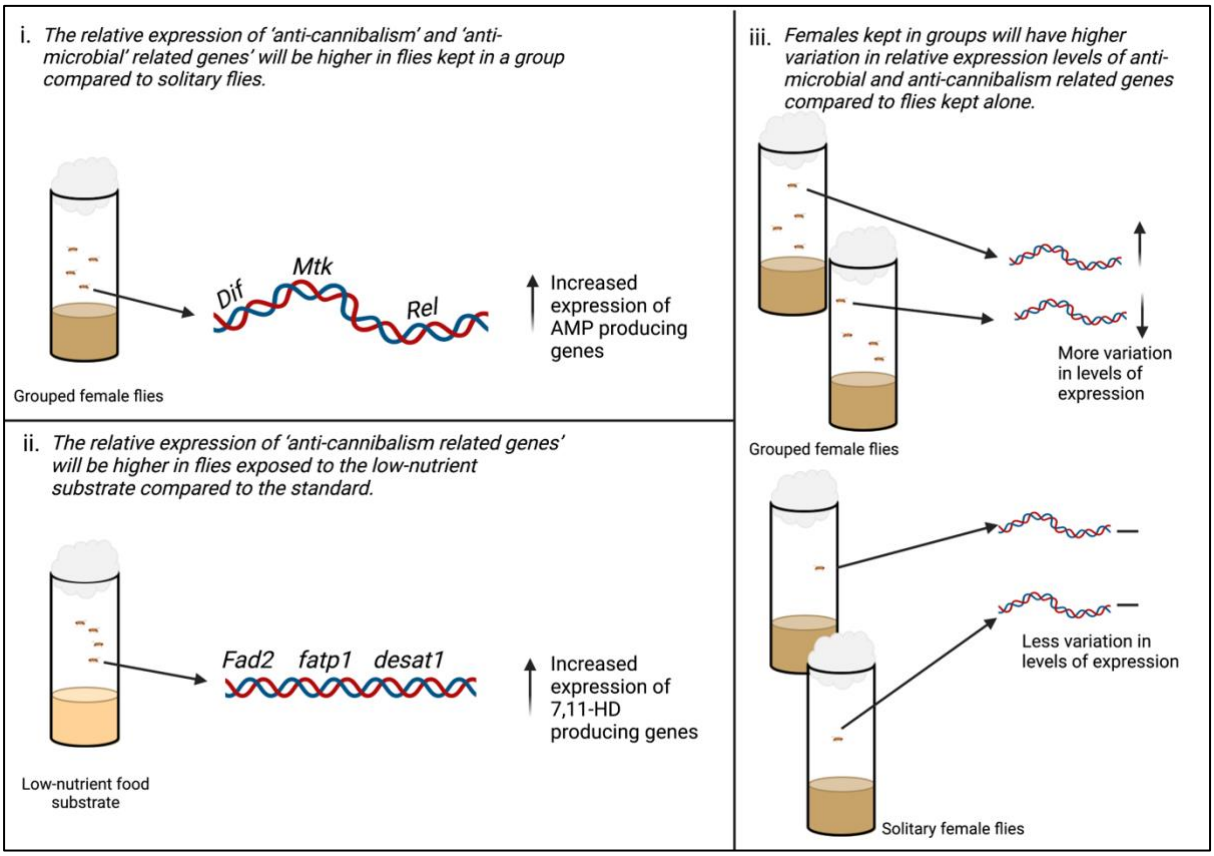

Figure 3.1: Chapter 3 Graphical abstract*. This visualises the expected outcomes of the three tested hypotheses from Chapter 3. This includes how the social environment and quality of substrate affect the expression of anti-cannibalism and anti-microbial related genes, as well as changes to variation due to social environment. (i): This shows the flies which were kept in groups of 4, having an increased relative expression of both the anti-cannibalism and the anti-microbial genes (although, Dif, Mtk, and Rel are all anti-microbial genes. ii: This figure shows flies kept on the low-nutrient food substrate having a higher relative expression of the anti-cannibalism genes, compared to flies kept on the standard substrate. (iii): The final figure shows the flies kept in groups as having more variation in the relative expression levels across all 'public goods' genes, relative to the solitary flies which have lower levels of expression in these genes.*

3.3 Methods

Experimental design

The flies analysed in this chapter were the same flies that were used in Chapter 2. Therefore, up to this point, the experimental design follows the previous method. The full description of the experimental set-up is described in Chapter 2, section 2.2. In brief, wild-type first instar larvae were placed in vials of standard food to develop to adulthood under standardised conditions. Adult flies were then collected as virgins using ice anaesthesia within 8h of eclosion and maintained in same-sex groups. These were kept on standard food until they were reproductively mature (at 4-5 days old). Females were then allocated at random to the social and egg substrate diet treatments, this is visualised in Figure 2.3. For the social environment treatments, females were housed either alone or in same-sex groups of 4. For the egg-laying substrate treatments, females were kept on either standard SYA food (**S**) or 'low-nutrient' SYA food (**L**). The standard food was as described in Chapter 2.2. The low-nutrient food contained 25% of the yeast and 25% of the sugar of the standard food. The flies were then maintained on these treatments for two days until mating. Following this, the flies were removed from the vials as described below and data regarding egg placement were recorded (chapter 2).

The four treatments were as follows:

Table 3.2: The number of focal flies under each treatment, includes all three time points (25 flies in each)

Social treatment	**Egg-laying substrate treatment**	
	Low quality	Standard quality
Alone	75	75
Grouped (1 focal, 3 non focal females)	75	75

Mating and Sampling flies for RNA extraction

Prior to the mating, 25 virgin females from each treatment group were frozen in liquid nitrogen. Flies were extracted from the vials using an electronic pooter and placed in a 2ml Eppendorf tube (with a small hole in the lid) and snap-frozen in liquid nitrogen.

For the flies kept in groups, one female from a group of 4 was chosen at random to be the focal fly (deciding which fly to pick before extracting it from the social group, to avoid selecting flies that were the easiest to capture). The remaining flies were then discarded. These virgins made up the first time point (V) for the gene expression analysis.

All remaining females were mated by introducing 2 or 6 males (for the alone vs grouped treatments, respectively) to each vial under CO_2 anaesthesia. Flies were then given 2h to mate. Mated females were moved, using CO_2 anaesthesia to fresh S or L vials for oviposition and all males were discarded. Three hours after mating, 25 females from each treatment were collected and frozen using liquid nitrogen to comprise the 3h after mating time point for the work in Chapter 3. 6h after mating the remaining 25 flies from each treatment were also sampled and frozen. The 3h and 6h time point flies were both frozen in liquid nitrogen in the same method as was used for the virgins. The flies were then kept at -80°C.

Preparation of abdomen samples for RNA extraction

The tubes containing the flies were removed from the -80°C freezer and immediately placed on dry ice. Then an upside-down petri dish was placed on dry ice under a microscope. Each fly was removed from the original tube and placed on this petri dish, then the abdomen was separated from the head and thorax. The abdomen was then directly placed into a new tube, which was also kept on dry ice, while the head and thorax were discarded. This was completed for all of the sampled flies. After this, abdomen samples were placed back into the -80°C freezer until RNA extraction.

RNA extraction, DNase and Reverse transcription

RNA was extracted from individual female abdomens (1 female abdomen per extraction) for 7 females per treatment and time point (total = 84 individuals). Thus, there were 7 biological replicates for each treatment. To minimise bias in RNA concentration due to batching effects, extractions from the four different treatments were conducted in parallel during each extraction session. RNA was extracted

according to miRvana miRNA isolation kit *(Invitrogen*, ThermoFisher – Cat# AM1561) instructions, and quantified using a Nanodrop 8000. The samples were then diluted to 200 ng/µL and any genomic DNA contamination was removed using the Invitrogen Turbo DNA-*free* kit (ThermoFisher, cat# AM1907). The amount of RNA was re-quantified and normalised across samples before being reverse transcribed to cDNA according to kit instructions (QuantiTect reverse transcription kit, *Qiagen* #205311). Assuming 100% RNA to cDNA conversion, the final cDNA concentration of all samples was 12.26 ng/µL in 30 µL. The resulting cDNA was then stored at -20°C until use.

Primer design and optimisation

Table 3.3: Primer sequences and efficiencies. *All of the primers were used at a concentration of 5pmol/µL. αTub84B and eIF1A were the reference genes used in this experiment.*

Gene name	FBgn number	Sequence of forward primer	Sequence of reverse primer	% efficiency
Fad2	FBgn0029172	ACCGGAGTGCTTTACGAATCC	GTGGTCTTCCGATTGCTTAGC	105.41%
fatp1	FBgn0267828	TCTAACAGCTCTCTGGGCCTA	GTCCGCCACCGTGTAGTTG	97.90%
desat1	FBgn0086687	AAGTGGCAGACGTGCATCTTA	GATGACCAGAATCACTCGCAG	102.77%
Dif	FBgn0011274	GGAGCCGACAAGCAATATAATCC	GTAGTTGCACACTTCGATGGT	104.76%
Rel	FBgn0014018	GGTGATAGTGCCCTGCATGT	CCATACCCAGCAAAGGTCGT	96.54%
Mtk	FBgn0014865	GCTACATCAGTGCTGGCAGA	TTAGGATTGAAGGGCGACGG	105.83%
αTub84B	FBgn0003884	CACACCACCCTGGAGCATTC	CCAATCAGACGGTTCAGGTTG	100.53%
eIF1A	FBgn0026250	TATCCGGCATCCACAAAGCG	GCGGCCATAAATCAAACCGT	98.11%

Primer sequences were obtained from FlyPrimerBank (Hu *et al*., 2013) for the genes of interest, and synthesised by Eurofins Genomics (Germany). Two pairs of primers were selected for each gene. The amplification efficiency of each primer set at a concentration of 5pmol/µl was tested in a qRT-PCR assay (according to the conditions below) using a five-fold serial dilution of female cDNA, with a starting cDNA concentration of 10ng/µl. Ct values were plotted against log ng of total cDNA to generate a standard curve. The slope of the standard curve was used in the following equation to calculate the primer efficiency.

$$E = 10^{\frac{-1}{m}}$$

All primers had an efficiency of 90-110% at a primer concentration of 5pmol/µl.

Experimental qRT-PCR

The cDNA from all female samples was diluted to 2 ng/µl using molecular grade dH_2O. A mastermix was made using the primers (at 5 pmol/µl) and iTaq universal SYBR green supermix (Bio-rad #1725121) and water, in the following volumes:

Per reaction:

1 µl forward primer

1 µl reverse primer

10 µl iTaq universal SYBR green supermix

3 µl H_2O

Table 3.4: A representation of the qPCR plate sample layout. *Each cell is 3 wells on the PCR plate.*

T1, alone S	**T2, grouped S**	**T3, alone L**	**T4, grouped L**
3hrA17	3hrA67	3hrA117	3hrA167
3hrA18	3hrA68	3hrA118	3hrA168
3hrB19	3hrB69	3hrB119	3hrB169
3hrB20	3hrB70	3hrB120	3hrB170
3hrC21	3hrC71	3hrC121	3hrC171
3hrC22	3hrC72	3hrC122	3hrC172
3hrD23	3hrD73	3hrD123	3hrD173
NTC	IPC	----	----

First, 15 µL of the mastermix was added to each active well. Then, 5 µL of the experimental cDNA was added according to the example above. A “no-template” negative control which contained dH_2O instead of cDNA, and an IPC (interplate

calibrator) which contained a standard sample of female cDNA and the *fatp1* primers, were included on every plate. For the IPC, cDNA and the primers were identical across every plate. This allowed the standardisation of all the results so different plates could be compared (Hellemans *et al*., 2008).

The qRT-PCR was then run on a CFX Connect machine (*Bio-rad*) according to the cycling protocol in Table 3.4.

Table 3.5: PCR thermal cycle protocol used for the experimental qRT-PCR.

Thermal cycling protocol			
	Polymerase activation and DNA denaturation	95°C for 30s	
Amplification	Denaturation	95°C for 5s	x 40 cycles
	Annealing/extension and plate read	60°C for 30s	
	Melt curve analysis	65-95°C in 0.5°C increments for 5 sec/step	

Statistical analysis

In order to analyse all three time points together, an interplate calibration was used. The IPC results from across all the PCR plates were used to normalise all the expression data in Excel. This was done by dividing the Cq value of the target/reference gene by the Cq value of the IPC that was run on its corresponding plate, then the resulting value was multiplied by the mean of the two IPC values (Collins *et al*., 2021). After this, any samples with a difference of more than 0.5 ct value in their technical repeats had their outliers removed or were completely removed if no value was within 0.5 ct of any other technical repeat. Following this, a mean ct value of the remaining technical replicates was taken for each sample. Ct

values of targets were converted into expression values, relative to the mean Ct value of two reference genes (*αTub84B* and *eIF1A*) using the following formula.

$$Relative\ expression = 2^{-(mean\ target\ ct - mean\ reference\ ct)}$$

To analyse the effect of social environment and egg-laying substrate on the relative expression of the various candidate genes, the relative expression was used as the response variable in a linear model. A linear model was created for each gene at each time point including diet and social environment as independent variables. The interaction between these two variables was also analysed. This interaction was non-significant for every model, so was removed from the model (ANOVA outputs given in the Supplementary information; Figures S.14 – S.19). Statistical significance values were derived from the analysis of deviance tests of the final models using the anova() function .

To analyse the differences between the coefficient of variation values, the R package cvequality (Version 0.1.3; Marwick and Krishnamoorthy., 2019) was used. For each gene, at each time point, the MSLRT (modified-signed-likelihood test) tested for significant differences between the coefficients of variation of relative expression between two treatments (diet and social environment were tested separately). This shows if there is a statistically significant change in the amount of variation in the relative expression across flies in one treatment compared to another. For example, significantly more varied relative expression in the low-nutrient diet compared to the standard diet in a certain gene.

R (4.0.2) (R Core Team, 2020) was used for all Figures with the packages: ggplot2 (Wickham, 2016), 'ggpubr' (Kassambara, 2020), 'dplyr' (Wickham *et al*., 2021). Statistical analyses (code provided in supplementary information) were completed using the base 'stats' package included in R (R Core Team, 2020).

3.4 Results

3.4.1 Effect of egg-laying substrates

(i) ***Virgins***: low-nutrient egg-laying diet significantly increased the relative expression of one tested anti-microbial gene (*Mtk*) in virgin flies.

In general, there was no significant effect of either dietary substrate or social group on the expression of the target genes in virgin females (Figure 3.2). The exception was for *Mtk*, which showed significantly reduced expression on the standard diet for both social treatments ($F_{(1,24)}$ = 10.087, P = 0.004).

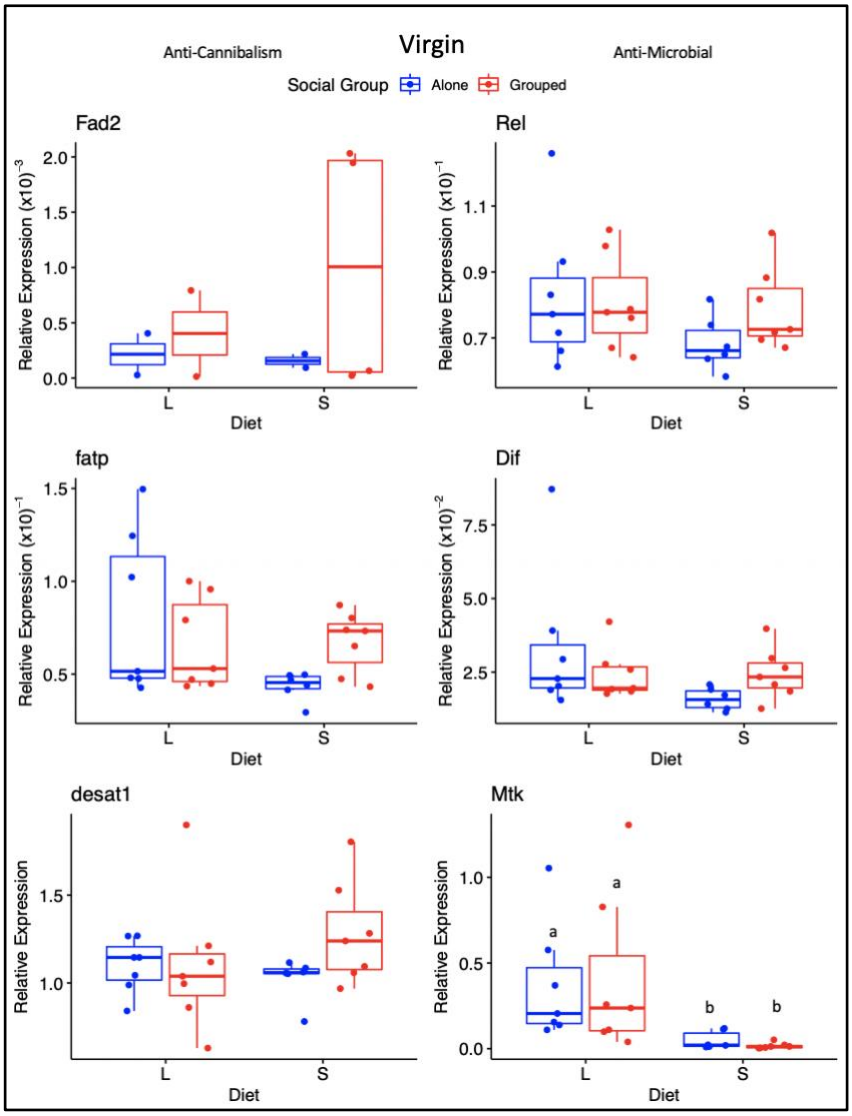

Figure 3.2: The effect of social group and egg-laying substrate manipulation on the expression of anti-cannibalism and anti-microbial genes in virgin female abdomens. *Box plots showing the relative expression of the six different target genes of flies before mating (left: Anti-cannibalism, right: anti-microbial. The different social groups are shown in blue (Alone) and red (Grouped); the different egg-laying substrates are shown on the x-axis (Low and Standard). Significant differences in gene expression were found only for Mtk - letters not in common indicate a significant difference at p< 0.05.*

The results of the statistical analysis for the virgin female gene expression data are shown below in Table 3.5. It was expected the low-nutrient egg-laying substrate would result in a reduced expression of AMP related genes, thus *Mtk*'s expression in the virgin flies ran counter to the prediction.

Table 3.6: The effect of social group and egg-laying substrate manipulation on the expression of anti-cannibalism and anti-microbial genes in virgin female abdomens. *The table shows the statistical outputs of the ANOVAs run on the linear models for each gene, with the social environment and egg-laying substrate (diet) as the fixed effects. Significant p-values indicated in* ***bold****.*

Effect of:	Gene	Degrees of freedom	Residual Degrees of freedom	Sum of Squares	Sum of squares residual	Mean of squares residuals	F	P-value
Diet	*Fad2*	1	7	$4.3x10^{-10}$	$4.4x10^{-9}$	$6.3x10^{-10}$	0.669	0.440
	fatp1	1	24	0.002	0.02	0.0008	2.464	0.130
	desat1	1	24	0.024	1.79	0.07	0.324	0.575
	Dif	1	24	0.0005	0.005	0.0002	2.159	0.155
	Rel	1	24	0.0004	0.006	0.0002	1.628	0.214
	Mtk	1	24	0.88	2.08	0.09	10.087	**0.004**
Social	*Fad2*	1	7	$7.6x10^{-10}$	$4.4x10^{-9}$	$6.3x10^{-10}$	1.208	0.308

	fatp1	1	24	$9x10^{-4}$	0.02	0.0008	0.115	0.737
	desat1	1	24	0.0005	1.79	0.07	1.466	0.238
	Dif	1	24	$1.8x10^{-6}$	0.005	0.0002	0.008	0.929
	Rel	1	24	0.0001	0.006	0.0002	0.469	0.500
	Mtk	1	24	0.0001	2.08	0.09	0.001	0.971

(ii) ***Females 3h after mating***: Social environment and egg-laying substrate had no significant effect on gene expression in female abdomens 3 hours post-mating

There was no significant difference in relative gene expression for any of the 6 genes across the different social treatments or egg-laying dietary substrates 3h after mating (Figure 3.3, Table 3.6).

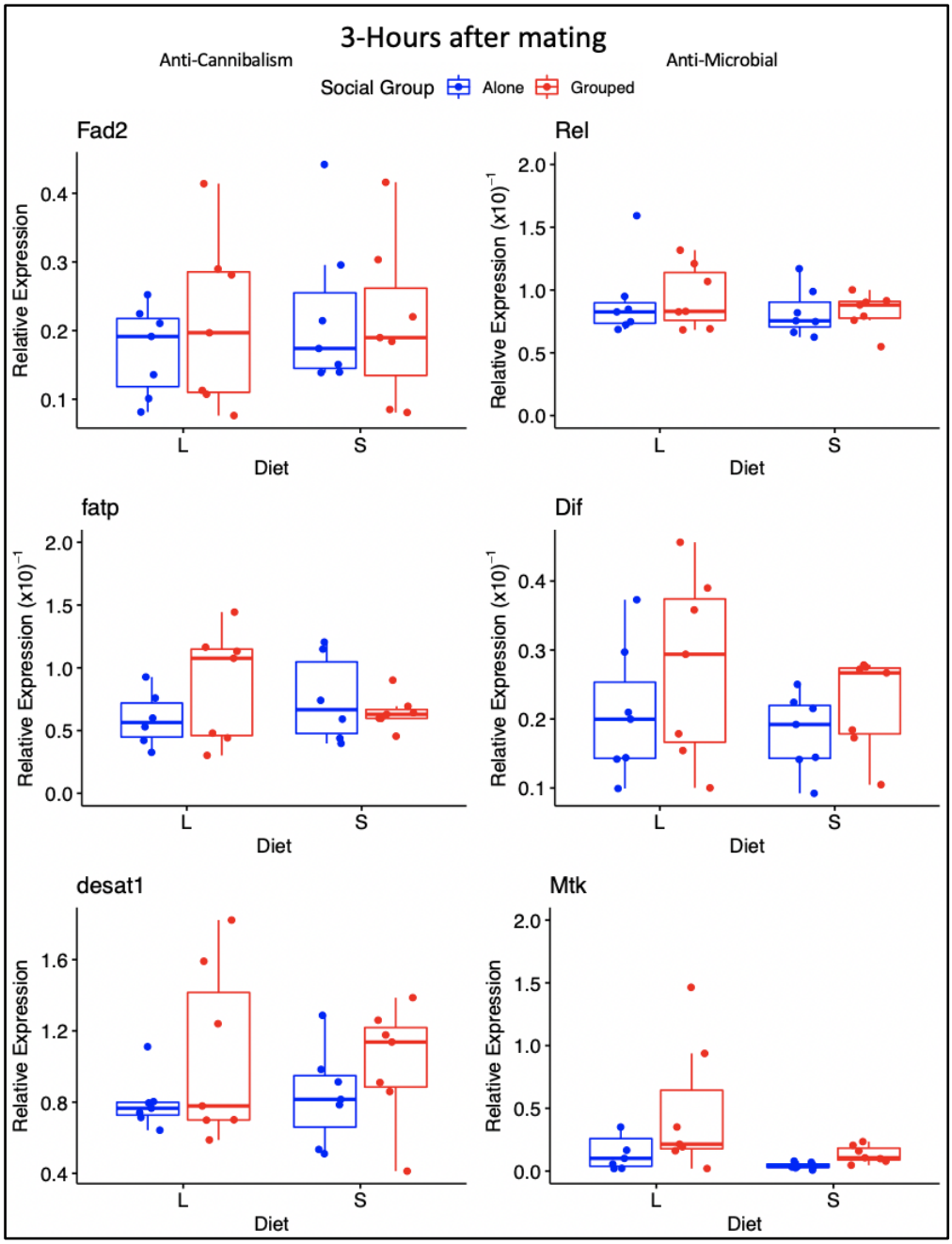

Figure 3.3: The effect of social group and egg-laying diet manipulation on the expression of anti-cannibalism and anti-microbial genes in female abdomens 3h after mating. *Box plots showing the relative expression of the six different target genes of flies which were frozen three hours after mating (left: Anti-cannibalism, right: anti-microbial. The different social groups are shown in blue (Alone) and red (Grouped); the different egg-laying substrates are shown on the x-axis (Low and Standard).*

Table 3.7: The effect of social group and egg-laying diet manipulation on the expression of anti-cannibalism and anti-microbial genes in female abdomens 3h after mating. *The table shows the statistical outputs of the ANOVAs run on the linear models for each gene, with the social environment and egg-laying diet as the effects.*

Effect of:	Gene	Degrees of freedom	Residual Degrees of freedom	Sum of Squares	Sum of squares residual	Mean of squares residuals	F	P-value
Diet	*Fad2*	1	25	0.005	0.281	0.011	0.409	0.528
	fatp1	1	23	0.0001	0.024	0.001	0.118	0.734
	desat1	1	25	0.00001	2.669	0.107	0.0001	0.992
	Dif	1	25	0.0001	0.002	$8.4x10^{-6}$	1.429	0.243
	Rel	1	25	0.0007	0.013	0.0005	1.372	0.253
	Mtk	1	25	3.704	47.64	1.906	1.944	0.176
Social	*Fad2*	1	25	0.002	0.281	0.011	0.135	0.717
	fatp1	1	23	0.0004	0.024	0.001	0.402	0.533
	desat1	1	25	0.357	2.669	0.107	0.080	3.344
	Dif	1	25	0.0002	0.002	$8.4x10^{-6}$	2.452	0.130
	Rel	1	25	0.00002	0.013	0.0005	0.055	0.816
	Mtk	1	25	0.598	47.64	1.906	0.314	0.580

At 3h post-mating, there was no evidence that the expression of the candidate genes tested conformed to the patterns predicted by the existence of public goods.

(iii) ***Females 6h after mating:*** Females kept in groups showed a significant increase in expression of 7,11-HD synthesis genes, and females kept on a low-nutrient diet for egg-laying had significantly increased expression of anti-microbial genes (excluding *Rel*), six hours after mating.

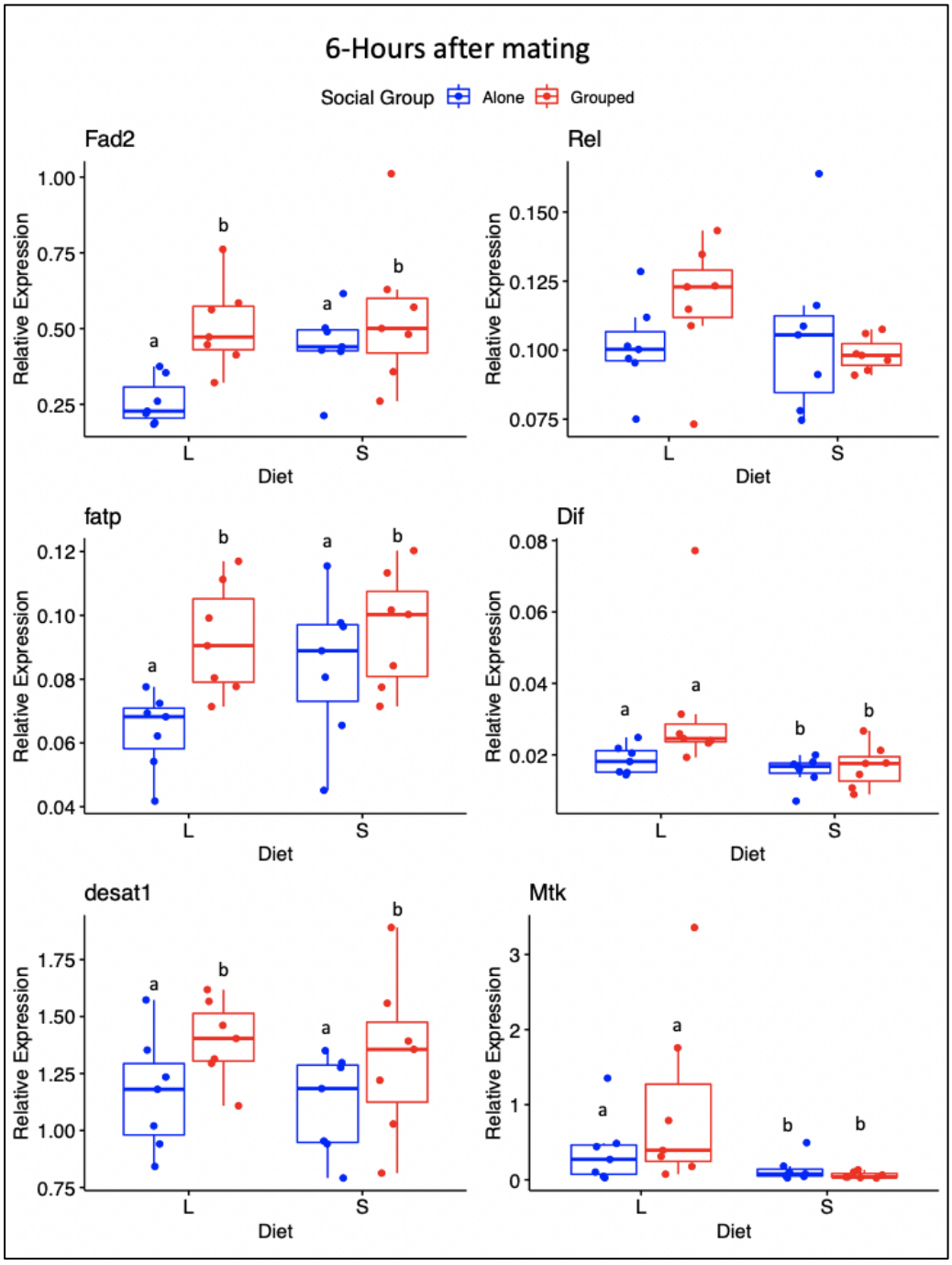

Figure 3.4: The effect of social group and egg-laying substrate manipulation on the expression of anti-cannibalism and anti-microbial genes in female abdomens 6h after mating. *Box plots showing the relative expression of the six different target genes of flies which were frozen six hours after mating (left: Anti-cannibalism, right: anti-microbial. The different social groups are shown in blue (Alone) and red (Grouped); the different egg-laying diets are shown on the x-axis (Low and Standard). Letters not in common indicate significant differences between the groups ($p < 0.05$).*

At 6h post-mating, there was a pattern for the expression of anti-cannibalism genes to show a significant response to the social environment and for the anti-microbial genes to the egg-laying diet (Figure 3.4).

In the 6h time point, the social and egg-laying substrate treatments had significant effects on the expression of several of the candidate genes. For example, all three genes associated with the production of 7,11-HD had significantly higher levels of relative expression in the grouped relative to the solitary females (*Fad2:* $F_{(1,25)}$ = 8.465, P = 0.007*; fatp1:* $F_{(1,25)}$ = 8.284, P = 0.008*; desat1:* $F_{(1,25)}$ = 5.362, P = 0.029). In contrast, the egg-laying dietary treatment had no statistically significant effect on the expression of any of the anti-cannibalism genes. This was not expected as it was hypothesised that the expression of the 'anti-cannibalism' genes would be more nutritionally responsive. However, the fact that grouped flies showed a higher level of expression of the 7,11-HD genes was predicted.

With regard to the immunity-related genes, both *Dif* and *Mtk* had significantly greater levels of expression in the low-nutrient egg-laying substrate relative to the standard (*Dif:* $F_{(1,25)}$ = 4.76, P = 0.039 *; Mtk:* $F_{(1,25)}$ = 5.528, P = 0.027). However, egg-laying diet showed no significant effect in *Rel* ($F_{(1,25)}$ = 0.826, P = 0.372). This was counter to predictions, as it was hypothesised that the expression of immunity-related genes would be lower under low-nutrient conditions. In addition, none of the anti-microbial genes were significantly affected by the social treatment (Table 3.7).

Table 3.8: The effect of social group and egg-laying substrate manipulation on the expression of anti-cannibalism and anti-microbial genes in female abdomens, 6h after mating. *The table shows the statistical outputs of the ANOVAs run on the linear models for each gene, with the social environment and egg-laying diet as the effects. Significant p values indicated in* ***bold*** *(Raw ANOVA outputs in Supplementary information: Figures S.14 – S.19).*

Effect of:	Gene	Degrees of freedom	Residual Degrees of freedom	Sum of Squares	Sum of squares residual	Mean of squares residuals	F	P-value
Diet	*Fad2*	1	25	0.086	0.633	0.025	3.387	0.078
	fatp1	1	25	0.001	0.008	0.0003	2.879	0.102
	desat1	1	25	0.026	1.587	0.063	0.414	0.526
	Dif	1	25	0.0006	0.003	0.001	4.762	**0.039**
	Rel	1	25	0.0004	0.011	0.0004	0.826	0.372
	Mtk	1	25	2.389	10.80	0.432	5.528	**0.027**
Social	*Fad2*	1	25	0.214	0.633	0.025	8.465	**0.007**
	fatp1	1	25	0.002	0.008	0.0003	8.284	**0.008**
	desat1	1	25	0.340	1.587	0.063	5.362	**0.029**
	Dif	1	25	0.0004	0.003	0.001	3.093	0.091
	Rel	1	25	0.0001	0.011	0.0004	0.576	0.321
	Mtk	1	25	0.459	10.80	0.432	1.062	0.313

3.4.2 Effect of social environments

(iv) ***Virgin females and females 3h and 6h after mating***: Effect of social environment and egg-laying diet on gene expression in female abdomens pre-(v) and post-mating (3h and 6h)

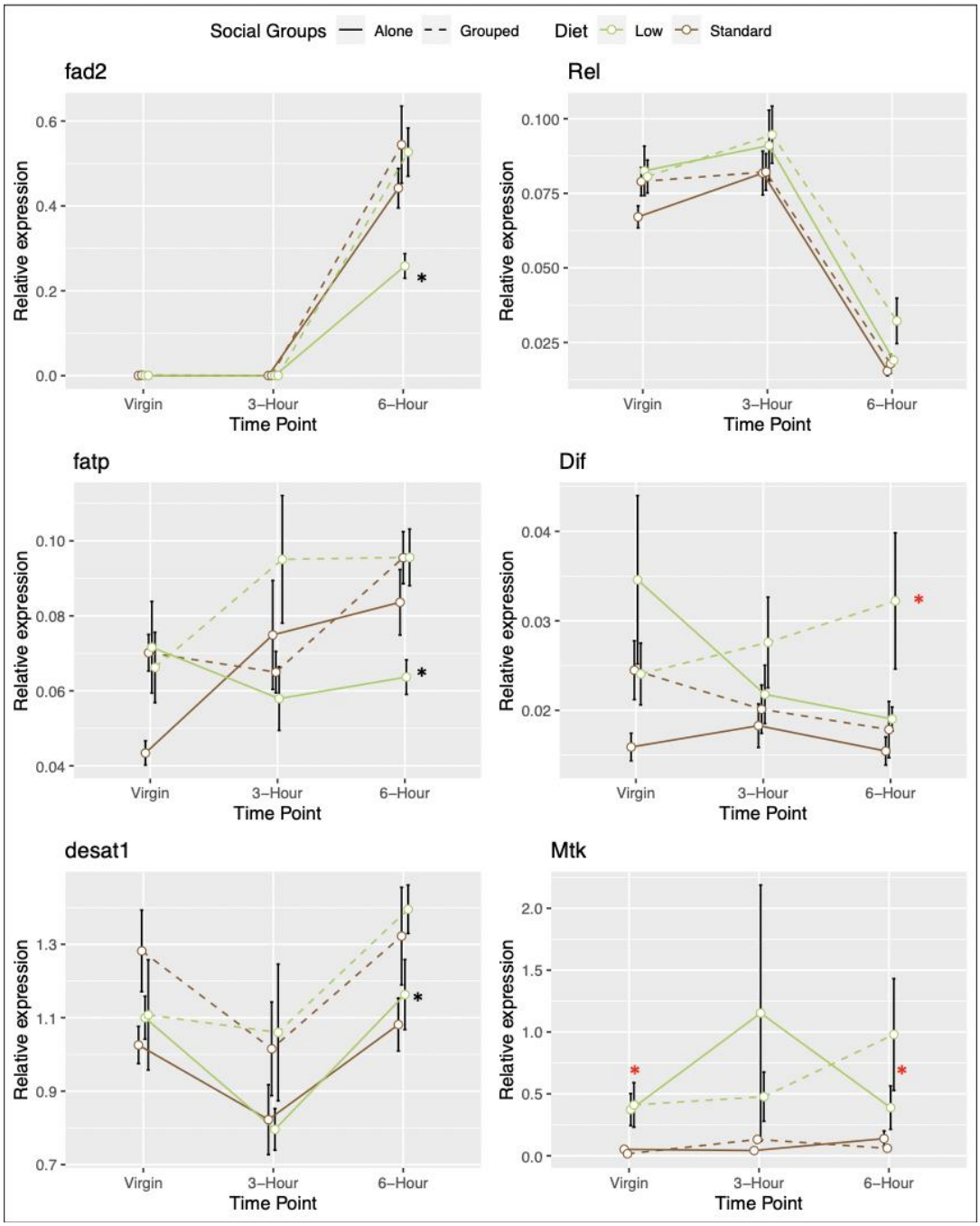

Figure 3.5: Effect of social and egg-laying substrate diet treatments on gene expression in female abdomens across different time points. *Line graphs showing the mean relative levels of gene expression± SE (left; anti-cannibalism related genes, right; anti-microbial related genes), at three different time points. 'Virgin' files were sampled before mating took place and females were also sampled 3 and 6h after mating. The two different egg-laying diets are differentiated by colour (L = Green; S = Brown); The social groups are denoted by a solid line (alone) and a dashed line (grouped). Statistical significance is represented by red asterisks (significant difference due to diet) and black asterisks (significant difference due to social group), ($p < 0.05$).*

Some genes had different levels of expression across different time points (Figure 3.5). For example, *Fad2* and *Rel* both changed significantly at the 6-hour time point. *Fad2* increased in expression in each treatment while *Rel* decreased. In addition, there was a significant effect of diet on the expression of *fad2*, but not *Rel*, at 6 hours. Overall, the results show that it was only 6h after mating that differences in the expression of the candidate genes were seen.

3.4.3 Variation in gene expression in anti-cannibalism and AMP candidate genes

(v) Egg-laying diet and social environment had a limited effect on the extent of gene expression variation .

Table 3.9: Coefficient of variation of relative expression across social and egg-laying substrate treatments for all time points. *Coefficient of variation for each treatment in every gene tested – for the Virgin females (V) and the females sampled three hours (3hr) and six hours (6hr) after mating.*

V	Fad2	fatp	Dif	Rel	Desat1	Mtk
T1 (alone, S)	0.563	0.174	0.236	0.122	0.119	1.054
T2 (grouped, S)	1.106	0.244	0.356	0.159	0.229	1.036
T3 (alone, L)	1.236	0.543	0.749	0.265	0.14	0.919
T4 (grouped, L)	1.365	0.374	0.357	0.181	0.359	1.156
3hr	Fad2	fatp	Dif	Rel	Desat1	Mtk
T1 (alone, S)	0.503	0.464	0.31	0.234	0.322	0.589
T2 (grouped, S)	0.562	0.209	0.308	0.178	0.319	0.518
T3 (alone, L)	0.382	0.372	0.46	0.344	0.188	2.373
T4 (grouped, L)	0.584	0.515	0.486	0.268	0.465	1.1
6hr	Fad2	fatp	Dif	Rel	Desat1	Mtk
T1 (alone, S)	0.274	0.275	0.27	0.286	0.194	1.175
T2 (grouped, S)	0.442	0.192	0.363	0.063	0.266	0.69
T3 (alone, L)	0.298	0.192	0.214	0.161	0.217	1.19
T4 (grouped, L)	0.28	0.188	0.625	0.193	0.125	1.219

Mtk showed the largest variation in expression between individual flies in the majority of treatments, usually followed by *Fad2* (Figure 3.6, Table 3.8).

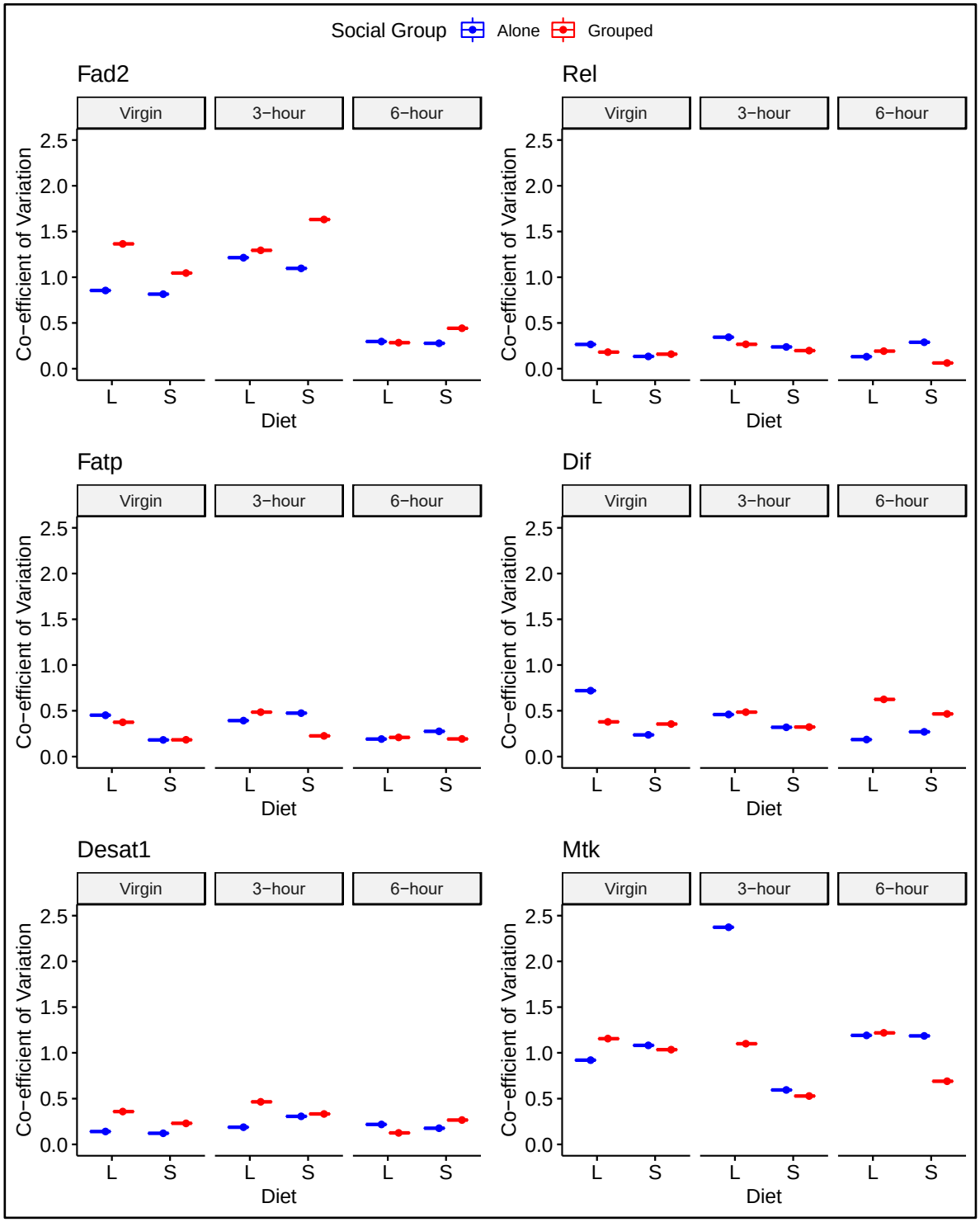

Figure 3.9: The effect of social and egg-laying substrate treatments on the coefficient of variation of relative expression. *Shown is the coefficient of variation for each gene at each time point according to the type of egg-laying diet on the x-axis (Low and Standard) and social grouping (Blue: alone, Red: grouped). The three time points are shown at the top of each graph. The larger the coefficient of variation, the more variation in the relative expression across the samples for each treatment.*

An MSLRT test was completed to determine if there was a significant difference in the amount of variation between the social groups in these genes. Most genes did not vary significantly in expression variation across the different treatments (Figure 3.6). However, by 6h post-mating, there was significantly more variation in *Dif* expression between individual females when they were grouped, compared to isolated females ($t_{(27)}$ = 8.178, P = 0.004). This pattern was also found in virgin flies in *Desat1* ($t_{(26)}$ = 6.529, P = 0.011) and *Dif* ($t_{(26)}$ = 5.360, P = 0.021). The coefficient of variation did not generally vary across the treatments maintained on the different egg-laying substrates. The two significant results (*Dif* 6hr and *desat1* virgins) did not vary markedly between the lowest and highest relative expression values (Supplementary information; Tables S.2 and S.3). Therefore, only two of the 6 genes showed evidence for greater variation in gene expression in the grouped flies.

3.5 Discussion

The results showed that the expression of candidate public goods genes in female abdomens was responsive to variation in egg-laying substrates and the social environment. There was some support for the public goods hypotheses among the patterns of expression observed in anti-cannibalism but not anti-microbial genes.

At 6 hours after mating, the relative expression of all anti-cannibalism genes was significantly higher in the abdomens of the grouped females relative to the solitary flies. This finding supported the predictions as it was assumed that due to non-kin larvae being present in the grouped treatments, there would be a greater risk of cannibalism, and so increased production of 7,11-HD could be beneficial. The hypothesis that low nutrient conditions in the egg-laying substrates would also lead to increased expression in 7,11-HD related genes was not supported at any time point.

For the anti-microbial genes, the relative expression levels of both *Mtk* and *Dif* were significantly higher in the low-nutrient egg-laying substrate compared to the standard diet at 6h post-mating, as well as in virgin females for *Mtk*. This was counter to predictions. It was also hypothesised that solitary flies would have higher levels of expression of anti-microbial genes compared to the grouped flies and this was also not supported, as there were no significant differences in expression according to social environment at any time point. The analysis of the coefficient of variation showed that *Dif* (6h mated females) and *desat1* (virgin females) exhibited significantly more variation between individual females when they were in groups compared to when they were isolated. This was in line with the hypothesis that individual females kept in groups would show greater variation in gene expression compared to the solitary flies (mixtures of putative producers and cheaters). These results are explained in terms of each of the hypotheses tested below'.

Hypothesis 1: No effect on the expression of anti-cannibalism genes across differing egg-laying substrates

In contrast to the prediction, there was no statistically significant difference in the relative expression of the anti-cannibalism genes between the low-nutrient and standard egg-laying substrate. It was assumed that there would be a greater risk of cannibalism in flies maintained on the low-nutrient substrate (Vijendravarma *et al*., 2013). Therefore, it was proposed that in response to this increased risk, production of the anti-cannibalism pheromone, 7,11-HD (Narasimha *et al*., 2019), would have increased. This increase would be reflected in an increase in gene expression for genes such as *Fad2, fatp1 and desat1*.

There are several possible reasons for the lack of support for the hypothesis. The first is that anti-cannibalism genes are not supported as candidates for the production of public goods. However, in the absence of cost and benefit information, and any knowledge of variation in gene expression patterns across a range of differing social contexts, it may be premature to rule out the involvement of public goods. It is also possible that the low-nutrient diet used did not have as much of an effect on the rate of cannibalism as expected. However, Vijendravarma (2013) showed that the same type of low-nutrient diet as deployed here (25% yeast and 25% sugar in comparison to the standard diet) did increase cannibalism. Therefore, the egg-laying substrate manipulations I used should have been sufficient to increase cannibalism risk. Despite this, there was no significant effect on anti-cannibalism gene expression, which is interesting, especially given I observed that females had the capacity to regulate the expression of anti-cannibalism genes in response to the social environment (see below).

It is also possible that longer exposures of females to the poor and good egg-laying substrates, or exposure to even lower quality substrates might increase the possibility of detecting gene expression changes. It might also be interesting to investigate gene expression when flies are kept on low-nutrient diets for multiple generations. Another possibility is that the production of 7,11-HD could carry a fitness benefit under poor nutrient conditions, but that its production becomes too costly under those conditions. Variation in sex pheromone production has been

observed to correlate with dietary protein intake in male *Bactrocera dorsalis* fruit flies (Gui *et al*., 2023) and it would be interesting to compare the fitness costs of expression for anti-cannibalism versus AMP expression across a range of different dietary conditions.

Hypothesis 2: Grouped flies had increased expression of anti-cannibalism genes

I found that grouped females showed significantly higher levels of expression of the anti-cannibalism genes: *Fad2, fatp1,* and *desat1*, compared to the solitary flies, consistent with hypothesis 2 (Table 3.1). Greater expression of these genes is expected to lead to higher production of 7,11-HD, which could be beneficial, though this relationship needs to be confirmed. To fit the public good idea, *D. melanogaster* females should be able to display some mechanism of kin-recognition (Khodaei & Long, 2020). Khodaei and Long (2020) found that larvae interacted with non-kin eggs for longer than related eggs, and they were more likely to cannibalise non-kin eggs. This shows there are potential mechanisms mitigating kin cannibalism. Thus, the presence of 7,11-HD in the solitary fly eggs (where all egg and larvae are related) would be less important than it would be in the socially grouped females that contain both kin and non-kin larvae. The results of the gene expression analysis in this Chapter provided evidence that the production of 7,11-HD (measured through the proxy of the expression of genes in its biosynthetic pathway) showed plasticity and responded to the social environment, which fits with the results of the Khodaei & Long (2020) study and supports the public goods hypothesis.

Despite the finding that cannibalism is more likely to occur among non-kin (Khodaei & Long, 2020), I found no significant difference in the proportion of eggs in clusters between the grouped and solitary flies in Chapter 2. Thus, egg clustering may not be the relevant mechanism for mediating any potential public goods effects. Another possibility is that the increased expression of 7,11-HD producing genes in the grouped flies affected the probability of egg clustering itself minimising and clustering differences. The potential risk of cannibalism caused by an increased prevalence of clustering could be countered by the increase in 7,11-HD, allowing *D. melanogaster* to oviposit in clusters at the same rate and gain from any fitness benefits which

clustering brings. Furthermore, while my results in this chapter do not directly supply any evidence of 7,11-HD acting as a public good, they could suggest that females continue to cluster their eggs even when exposed to increased cannibalism risk, and to protect them opt instead to increase expression and production of a costly pheromone (7,11-HD) (Narasimha *et al*., 2019).

Hypothesis 3: Social grouping had no effect on the expression of anti-microbial genes

Social grouping had no statistically significant effect on the relative expression of anti-microbial genes, counter to hypothesis 3. The original prediction feeding into this hypothesis assumed the existence of public goods and that the females in a group would be more likely to rely on AMPs produced by other flies and deposited in clusters, whereas the solitary flies would be required to produce all necessary AMPs individually. An underlying assumption was that egg clustering would occur more frequently in the grouped flies than the solitary. However, the results of Chapter 2 did not find such an effect. The results suggest that anti-microbials are produced at a similar level regardless of the social environment.

One explanation could be that AMPs and the genes that encode them do not function as public goods, yet more information is likely needed before this can be ruled out. Alternatively, AMP production may be so critical to fitness that regardless of whether it is possible to save energy (Hanson *et al*., 2019) by relying on other flies to potentially produce AMPs as public goods, it could be too risky for eggs to be laid without AMP protection. This doesn't contradict my finding that AMP-related genes increase in expression due to low-nutrient exposure, as in that scenario, more AMP production could be required to offset the disadvantages to offspring health due to the diet (Unckless *et al*., 2015). Following this idea, rather than egg clusters being a useful strategy to reduce the investment in AMPs for the same returns in offspring survivability, egg clusters might increase offspring survivability regardless of a change in AMP production. Thus, producing AMPs at a certain rate provides a fitness benefit, and the egg clustering may give an additional benefit (regardless of mixed or single maternity). This proposed increase in survivability could still be due to more AMPs potentially acting as public goods in one cluster. However, so far

these results do not provide direct evidence of public goods or suggest any forms of punishment for potential cheats.

Another potential reason for the lack of a significant effect of social environment on anti-microbial gene expression could be due to the fact that not all AMPs were tested here and only a selected few AMPs are produced in response to upregulation of the candidate genes *Dif*, *Rel* and *Mtk* that were tested (Kim & Kim, 2005; Moghaddam *et al*., 2017; Park *et al*., 2004). Hence additional AMPs could act as public goods in eggs. Ait would be interesting to investigate additional AMP-producing genes in the future.

Hypothesis 4: Social group and egg-laying substrate quality affected the variation in *Dif* and *desat1* expression

By analysing the coefficients of variation of relative gene expression levels, I found that, though most genes did not vary in the degree of expression variation across social groups and egg-laying substrates, two genes did, namely *Dif*, and *desat1*. In the virgins and at the 6h post-mating time point, grouped females showed greater variation in *Dif* expression compared to the solitary females. The hypothesis assumed that females kept in groups would have higher variation in relative expression levels of anti-microbial and anti-cannibalism related genes compared to flies kept alone. This was based on the idea that in the grouped flies, public good co-operators and free riders would be present, increasing expression level variation. Hence some flies could conceivably cooperate and produce more public goods (in this case, AMPs and potentially 7,11-HD) and have a higher relative expression level. Meanwhile, some would produce fewer (cheats/free riders), indicated by a lower relative expression. Socially isolated females were expected to show low coefficients of variation as there is no opportunity to cheat.

If the identified difference in the pattern of variation is indeed a sign of co-operators and cheats, this would suggest that the AMPs produced by *Dif*, such as drosomycin (Hoffmann & Reichhart, 2002), are potentially acting as public goods (Archetti *et al*., 2011). However, as discussed in the introduction to this chapter, *Dif* is a transcription factor affecting other genes, not only AMP production (Govind, 1999; Le Bourg,

2011). Therefore, there could be other unrelated processes which are causing the social environment to have an effect on the variation in gene expression, either in addition to, or instead of, drosomycin production. The significant difference in *Dif* expression variation between the grouped and the solitary flies in the virgin females and 6-hour post-mating time point (although not at 3h), and there was also a marginally significant difference in response to the egg-laying diet at 6h post mating. Whether all this expression level variation is linked to public goods and/or other roles of *Dif* remains to be tested. In order to further investigate this, direct investigations of the presence of AMPs like drosomycin on the surface of eggs would be useful.

Low-nutrient diet increased the relative expression of *Dif* and *Mtk*

Though it did not form one of the hypotheses tested, I also observed that two of the selected anti-microbial related genes, *Dif* and *Mtk,* had significantly higher levels of relative expression in the low-nutrient diet substrate compared to the standard diet, at 6 hours after mating. The relative expression of anti-microbial related genes might be expected to be higher in flies exposed to the standard diet compared to the low-nutrient diet on the basis that on the low-nutrient diet, the immune response of the flies would be weaker due to a lower level of dietary protein and carbohydrates (Galenza *et al*., 2016; Ponton *et al*., 2020). Interestingly the results showed the opposite pattern. One possibility for the unexpected effect might be pleiotropy or a lack of sampling of the whole range of different AMPs. For example, in comparison to the anti-cannibalism genes which were all involved in the production of 7,11-HD (Narasimha *et al*., 2019; Wicker-Thomas *et al*., 2015), the selected anti-microbial genes may have had additional general functions. A wider range of AMPs would be interesting to test, but the scale of investigations was necessarily limited by feasibility in the tests I describe here.

Three AMP genes were chosen for analysis, one which directly produced an AMP (*Mtk*) and two, *Dif* and *Rel*, which related more generally to the immunity pathways in *D. melanogaster*, Toll and Imd respectively (Hoffmann & Reichhart, 2002; Hultmark, 2003). *Dif* and *Rel* are transcription factors (Tanji *et al*., 2010) and may have additional effects related to development which could also be influenced by diet and social environment (Govind, 1999; Le Bourg, 2011). Despite this, they still remain a promising indicator of immune function through their primary roles in AMP

production. Therefore, one reason that *Dif* showed an effect based on dietary changes while *Rel* did not, could suggest that the Toll pathway, of which *Dif* is a major part (Hultmark, 2003)(Figure 1.3), could be more relevant to AMP production post-mating and during the oviposition process. Drosocin and drosomycin are AMPs, produced in the Toll pathway, which have been found to be expressed in the female reproductive tract of the fruit fly (Ferrandon *et al.*, 2007). Thus, these AMPs could potentially coat egg surfaces. The Toll pathway, regulated by *Dif* expression, results in the production of these AMPs in the fat body (Hetru *et al*., 2003).

There are a few potential reasons why the low-nutrient diet increased the relative expression of *Dif* and *Mtk*. For example, the low-nutrient diet might have limited the immune response. However, the increase in gene expression did not occur until 6h post-mating suggesting that any increase in the immune response mostly occurs late into the process of egg production and oviposition. So, it is possible that even though AMP production usually suffers while nutrient levels are low, in the scenario where flies must produce eggs while under low-nutrient substrate conditions, they must produce more AMPs if their offspring are less likely to survive under the low-nutrient conditions (Markow *et al*., 2009). Nutrient levels in the egg-laying substrates might also be giving the females a cue about the likelihood of their offspring encountering pathogens, and further work is needed to determine how females assess pathogen risk at oviposition sites and adjust AMP production accordingly.

Another potential answer relates to the findings in Chapter 2, where flies kept on the low-nutrient quality substrate were found to be less likely to oviposit eggs in clusters compared to those kept on the standard diet. Assuming AMPs are acting as public goods, flies may not need to produce as many AMPs in the standard diet as clustering is more common. Thus, a reduced number of AMPs could still protect multiple eggs. Whereas, in the low-nutrient diet where flies were less likely to cluster their eggs, AMP production needs to increase in order to ensure each egg is protected to the same degree.

Conclusion

Overall, the nutritional quality of egg-laying substrates and the social environment were found to have significant effects on the expression of potential 'public goods' related genes. A low-nutrient egg-laying substrate increased the relative expression of anti-microbial genes such as *Dif* and *Mtk* (and resulted in females being less likely to cluster their eggs, Chapter 2). Maintaining flies in a grouped environment increased the expression of all the tested 7,11-HD producing genes (*Fad2, fatp1, desat1*) when compared to solitary females (However, the social environment had no significant effect on egg clustering in Chapter 2). These results provide evidence for the fitness benefits of egg clustering, 7,11-HD, and anti-microbial peptides, as well as how these behaviours and genes may interact. Further analysis into the presence of AMPs and 7,11-HD on the surface of eggs could provide more direct evidence of these compounds acting as 'public goods'.

Chapter 4: General Discussion

In this thesis research I investigated whether changes in the egg-laying and social environment affected oviposition and the expression of putative 'public goods' related genes in *D. melanogaster*. Diet, egg-laying substrates and the social environment influence a wide range of phenotypes in *D. melanogaster*, including fecundity (Bailly *et al*., 2021; Fowler *et al*., 2022; Mirth *et al*., 2019), oviposition (Churchill *et al*., 2021), immunity (Unckless *et al*., 2015) and cannibalism (Vijendravarma *et al*., 2013). Diet and the social environment can also affect gene expression in a variety of ways (Baenas & Wagner, 2019; Schneider *et al*., 2017). I first investigated the effect of diet, egg-laying substrate and the social environment on oviposition behaviour (number and placement of eggs) (Chapter 2). In Chapter 3, I then investigated whether these diet and social environment manipulations altered the relative expression levels of six different potential 'public goods' anti-cannibalism and anti-microbial genes in the females laying those eggs.

I found that flies maintained and laying eggs on a low-nutrient substrate were significantly less likely to oviposit eggs in clusters, relative to those maintained on a standard substrate. In contrast, the social environment had no significant effect on the proportion of eggs laid in clusters. In terms of potential public goods effects, I found that the expression of all the genes related to the production of the potential anti-cannibalism gene 7,11-HD was lower in the solitary in comparison to the grouped females. In addition, the immunity-related genes *Dif* and *Mtk* showed lower levels of relative expression in females maintained and laying on the standard compared to the low-nutrient food substrate. Overall, the results showed that the social and nutritional environment can significantly affect the number and pattern of egg-laying by females. However, there was only limited evidence that the social and nutritional environment affected the expression of anti-cannibalism and anti-microbial genes in a way predicted by public goods genes theory. Thus, there was little evidence that the production of diffusible public goods on the surface of eggs explained why eggs were laid more frequently in communal clusters when females were held in groups. The wider context of these results and some future research directions are outlined below.

4.1 Oviposition behaviour is dictated by the nutritional and social environment

Few studies have so far investigated the microecology of oviposition behaviour in *D. melanogaster*. However, there is growing interest in this topic, given recent discoveries of striking plasticity in egg-laying and placement (Fowler *et al*., 2022) and the finding that, when females lay eggs together in groups, the majority of eggs in clusters are of mixed maternity (Fowler, Friend, Chapman, unpublished data). Females have also been found to change their oviposition behaviour depending on the patchiness of the substrate, with more eggs being laid on aggregated substrates in comparison to single-patch substrates that are spread out from one another (Churchill *et al*., 2021). When patches of food were dispersed, the females were also more likely to oviposit eggs together on one patch with other females, rather than using all the patches equally. When the patches were placed in closer proximity to each other, females distributed their eggs more evenly. The distribution of resources appeared to have a greater effect on oviposition behaviour than did the social environment and sexual competition. Less is known about the patterns of micro-placement behaviours that occur within a patch, such as egg clustering, in which two or more eggs are physically touching. There are some data on the preference of females to oviposit in the presence of others from the Tephritid fruit flies. A 1998 study conducted on the medfly (*Ceratitis capitata*) found that a greater proportion of females initiated oviposition into kumquat fruits if another medfly was present (Prokopy & Duan, 1998). However, a later related study did not find any evidence for this effect (Dukas *et al*., 2001). Thus, additional studies of social effects on egg-laying rate and placement in the natural context, are needed.

D. melanogaster females show selectivity towards egg-laying sites and use various olfactory and gustatory cues to choose a preferred oviposition site (Yang *et al*., 2008). Oviposition site choice in females is plastic and non-random, and fitness benefits of the plastic decisions seem likely, though have not yet been identified. As shown by Churchill *et al*., 2021, when food patches were placed further apart, flies were more likely to lay eggs in the same patches, whereas when the patches were together the eggs were more evenly distributed (Churchill *et al*., 2021). This

suggests there is a benefit of females placing eggs in closer proximity to those of other females. Females held in groups with other females are also reported to exhibit a stronger preference for laying eggs on substrates that are novel to them compared to when females experience the novel food substrate alone (Sarin & Dukas, 2009). These findings suggest that there could be benefits to placing eggs in closer proximity to those of others. It is possible that the presence of eggs on a substrate communicates to other females that the food is of good quality or that egg placement behaviours protect eggs from disease. Other benefits to egg placement choices may come in the form of public goods, which was the idea tested in this thesis. Overall my results did not provide consistent support for the public goods idea, though identified new features of egg placement and of the plastic expression of anti-cannibalism and anti-microbial genes. However, the ultimate benefits and potential costs of egg placement behaviour remain to be discovered.

4.2 Expression of anti-microbial peptide genes is modified by the nutritional but not social environment

Innate immunity in fruit flies is mediated by the Toll and Imd pathways, which regulate the production of AMPs (Tanji *et al*., 2010). When these pathways are activated by the detection of bacterial and fungal pathogens, the expression of the transcription factors *Dif* and *Rel* increases, resulting in the production of various anti-microbial peptides (AMPs) (Hoffmann, 2003). Studies have shown that innate immunity capacity and activation in fruit flies can be influenced by dietary manipulations. For example, high dietary glucose can lead to increased pathogen loads (Unckless *et al*., 2015), a low-protein: high-carbohydrate diet can initiate upregulation in the expression of anti-microbial peptides (Ponton *et al*., 2020) and a high-sugar diet can activate both JNK (c-Jun NH 2-terminal kinase) and Toll pathways (Yu *et al*., 2018). My analysis from Chapter 3 found that *Dif* and *Mtk* expression increased when females were placed in adulthood on a low-nutrient diet and laid eggs on that same dietary substrate. This suggests that under low-nutrient conditions the Toll pathway is activated more than it is for females maintained as adults on the standard diet. This effect was not found for *Rel* and thus the Imd pathway. Together, my results show how specific ratios of macronutrients can affect

immune function and the expression of key immune genes. Previous studies have shown that an individual's immune status is responsive to the level of carbohydrates in the diet. Out of a selection of different diets with varying macronutrients, it was determined that, when subjected to a chronic enteric infection, flies on a diet with added glucose had increased survival (Galenza *et al*., 2016). On the other hand, when diets contain high levels of dietary glucose, flies are more susceptible to infection (Unckless *et al*., 2015). So, dietary glucose in particular can have important effects on immune function, though other macronutrients are also important (Ponton *et al*., 2020). The influence of diet on the expression of immunity genes is interesting because it suggests that an individual's nutritional and immune status are linked. The reasons for the increase in AMP production (specifically Toll pathway genes) that I found on the low-nutrient diet are uncertain. A high sucrose diet is reported to activate the Toll pathway (Yu *et al*., 2018). Whereas in my study, the diet which was low in sucrose (and protein) resulted in the same activation of the Toll pathway (increased expression of *Dif* and *Mtk*) as did the standard diet. Since AMPs are costly to produce (Hanson *et al*., 2019), an increase in the production of AMPs while resources in the environment are low suggests that the potential increase in AMPs under these conditions accrues a fitness benefit. As suggested in Chapter 3.4, the fact this increase in expression was only found 6 hours after mating, could suggest that immune function and the production of AMPs is particularly important during the process of egg production and oviposition.

In comparison to the clear effects of diet, the effect of the social environment on immunity and the expression of immune genes was less clear. Research regarding the effect of social grouping on immunity is scant. One exception is the study of Leech *et al*. (2019), which suggested that social contact in fruit flies does not have a predictable impact on immune responses (Leech *et al*., 2019). The findings from my research in Chapter 3 showed that the social environment had no consistent or significant effect on the expression of the immune genes *Dif*, *Rel,* or *Mtk*. Thus, there was no evidence to suggest that social grouping affected the expression of immunity-related genes or the innate immune response as a whole. Additional studies including a wider selection of genes encodings AMPs and immune genes from many different pathways would be useful.

4.3 Expression of anti-cannibalism pheromone genes is modified by the social but not nutritional oviposition environment

The sex pheromone 7,11-HD has been shown to have anti-cannibalism properties (Narasimha *et al*., 2019) – it is a cuticular hydrocarbon (CHC) and multiple genes are responsible for its production, including *desat1* (Dallerac *et al*., 2000), *Fad2* (also known as *desatF*) (Chertemps *et al*., 2006) and *fatp1* (Wicker-Thomas *et al*., 2015). All of these genes aid in the production of CHCs and the 7,11-dienes, including 7,11-HD, which show sex-specific expression in females. These CHCs are produced by abdomen cells known as oenocytes (Wicker-Thomas *et al*., 2015) and they influence a range of different phenotypes. For example, oenocyte-specific reduction of desaturase activity has been shown to affect CHC production, but also fecundity, mating behaviour and lifespan (Joseph *et al*., 2018). There are few investigations so far on the anti-cannibalistic effects of sex pheromones such as 7,11-HD (Narasimha *et al*., 2019). My experiment adds new findings on 7,11 HD's anti-cannibalism and other potential phenotypic effects, through my investigations of the expression of its pre-cursor genes and the effect that social environment has on their expression. The expression of 7,11-HD precursor genes has previously been reported to vary across different species of *D. melanogaster* (Billeter & Wolfner, 2018; Dallerac *et al*., 2000) and it would be very interesting to probe whether this variation is also associated with differing social networks in those species.

It is likely that the risk of cannibalism is elevated among non-kin (Khodaei & Long, 2020). Hence my findings that 7,11-HD precursor gene expression responds to the social environment supports the idea that anti-cannibalism pheromone production increased in an environment with a heightened risk of cannibalism (grouped versus alone treatments). However, whether this pheromone also acts as a public good is not yet proven and so further experimentation is required (see below).

It is also possible that 7,11-HD's social responsiveness is not related to cannibalism at all and may be related to an alternative function of 7,11-HD. For example, 7,11-HD also acts as an aphrodisiac pheromone and is detected by males through the activity of *ppk23* (Liu *et al*., 2020; K. Sato & Yamamoto, 2020; Toda *et al*., 2012).

Females kept in groups might produce more of this pheromone in response to the presence of other females because such groups indicate the potential for higher sexual competition and the need to attract a mate. However, the elevated expression of anti-cannibalism precursor genes in grouped females was found only at the 6h time point, which could mean that the primary association is with the increased production of eggs by that point, supporting the initial cannibalism hypothesis. The precise reason for the social responsiveness of the anti-cannibalism genes isn't yet certain, However, based on my results, I propose that 7,11-HD functions not as a public good, but is elevated under conditions when there is a perceived risk of cannibalism in the environment.

4.4 Future research directions

In my experiments in Chapter 3, I found that the expression of the anti-cannibalism genes was socially responsive. However, the ultimate significance of this effect is not yet known. I designed, but did not have time to complete, some follow-up experiments to address this. These plans and the research rationale underpinning them are described below.

Effect of egg surface Anti-Microbial Peptides (AMPs) and anti-cannibalism molecules on egg hatching and larval survival

I designed two additional experiments to further investigate the effects of AMPs and 7, 11-HD on egg survival, in order to test for evidence that these compounds have a fitness benefit. In the first, the aim was to determine the survival rate of 'washed' eggs compared to unwashed eggs. The rationale was that, if they exist as in the medfly *Ceratitis capitata* (Marchini *et al*., 1997), washing eggs in a saline solution would wash off any such egg surface AMPs. In doing this, it would be possible to test whether washed eggs could gain benefits from neighbouring non washed eggs that had retained their egg surface AMPs. Since 7, 11-HD is found in the wax layer of the egg rather than the surface (Narasimha *et al*., 2019), it was thought unlikely that washing eggs would also remove 7,11-HD (see separate experiment designed to tackle anti-cannibalism, below). As well as the washing treatments, I also needed to be able to identify the eggs of focal versus non focal females. This was to be

achieved by using food dyes. I had proposed to use a technique developed in previous research (Fowler, Friend, Chapman, unpublished data) in which it was shown that it is possible to label mothers by raising them and feeding them on diets containing dyes such as Sudan-red and Sudan-black. Such females lay eggs that are labelled with a subtle pink and grey colour, respectively.

I proposed to test the effect of washing on egg hatching and larval survival in solitary and grouped eggs. One variation of this experiment I proposed was one in which survival and hatching rates of washed and unwashed egg clusters would be scored, with: unwashed eggs in a cluster, washed eggs in a cluster, and a cluster containing both washed and unwashed eggs (Figure 4.1).

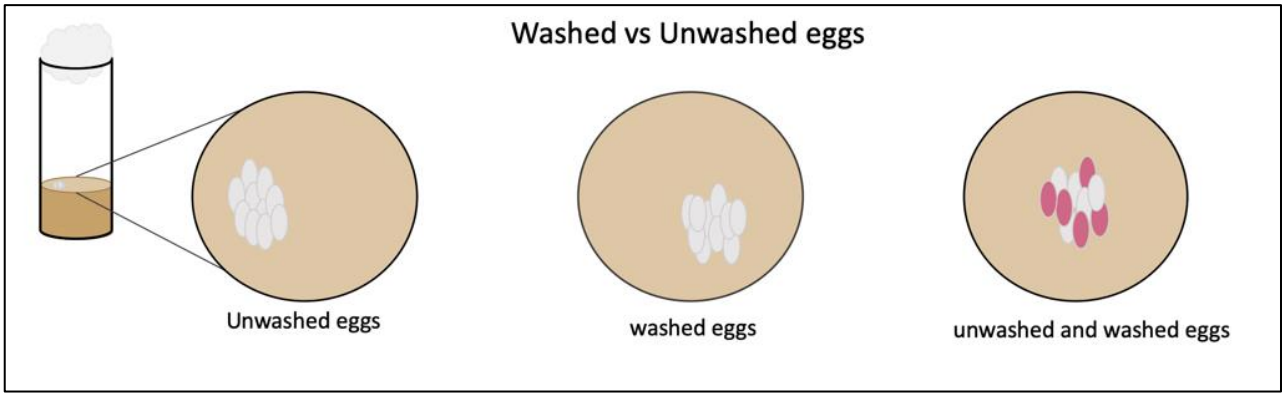

Figure 4.1: The experimental outline for testing the effect of egg surface AMPs on egg hatching. *Shown are diagrams of vials containing a food substrate and three egg treatments. Each of the three vials has a different treatment group made up of unwashed eggs, washed eggs, and a mixture of the prior two.*

I determined that comparing the survival rates of the eggs in these treatments would provide the data to test three predictions:

1. Solitary washed eggs have a lower chance of survival compared to solitary unwashed eggs.
2. Washed eggs in groups have a higher chance of survival than washed eggs alone.
3. Washed eggs mixed with unwashed eggs have a higher rate of survival compared to washed eggs.

I predicted that washed eggs would have a lower survival rate in comparison to unwashed eggs, due to the presence of hypothesised AMPs (and potentially other beneficial egg surface substances). If the potential AMPs act as public goods, then we would expect to see no significant difference in survival rates between the mixed clusters (washed and unwashed eggs) and the clusters only containing unwashed eggs. This would provide evidence that compounds on the egg surface can act as 'public goods'. However, in order to determine that these compounds are specifically AMPs, further analysis into the specific compounds found on the egg surface would also be required.

Survivability of oenocyte-less fly eggs

I also planned experiments to investigate the effect of the potential anti-cannibalism molecule 7,11-HD on egg survival and hatching. The 7,11-HD pheromone is produced in the oenocyte cells that line the inside of the upper abdomen wall (Wicker-Thomas *et al*., 2015). Thus, flies which lack oenocytes would not be able to synthesise 7,11-HD and potentially provision their eggs with it. This study would consist of comparing the survivability of eggs laid by oenocyte-less versus control wild-type females. There are multiple ways this experiment could be done, including by following the rationale of the unwashed/washed egg method outlined in Figure 4.1. Eggs laid by oenocyte-less females could be used to test how the presence of 7, 11-HD affects survivability and egg hatching. In addition, by creating mixed egg clusters comprising both egg types, it should be possible to determine if 7,11-HD is acting as a public good in an egg cluster. Evidence for this idea would be if there was no significant difference in survivability and egg hatching between the mixed egg and the wild-type egg clusters. Further information regarding the likelihood of cannibalism between oenocyte-less fly eggs and the wild-type eggs could also be investigated, although this would require more in-depth observation of the larvae following egg hatching. In addition, the outlined experiments could be extended further by analysing egg survivability in different dietary treatments such as the standard and low-nutrient diets (Chapter 2), or under dietary manipulations of yeast-to-sugar ratios (Ponton *et al*., 2020).

4.5 More genes

In addition to the experiments described above, the experiments described in this thesis could be built upon by analysing the expression pattern of additional putative public goods genes. In Chapter 3, it was feasible to study the expression of 6 different genes at the scale required. Three of these were related to AMP production. *Mtk* directly produces the AMP Metchnikowin (Moghaddam *et al*., 2017), while *Dif* and *Rel* are transcription factors which affect the expression and phenotypic effects of many other genes involved in the *D. melanogaster* innate immune system (Govind, 1999; Kim & Kim, 2005). However, there are many more genes specifically encoding AMPs which could be investigated to inform the specific predictions, e.g. *Drs* (drosomycin) and *CecA1* (Cecropin) (Hanson *et al*., 2019), and it would also be useful to explore the role of AMPs involved in immunity at the specific time during which egg production and oviposition are maximised. This would provide an opportunity to study whether these AMPs could potentially function as public goods, or whether they are nutritionally, but not socially responsive, as I found in Chapter 3. While it is useful to look at specific genes, a more comprehensive approach could also be taken through whole genome transcriptomic sequencing. The 6 genes used in my experiment were chosen due to their known association with 7,11-HD and AMP production. However, there are likely more genes which are not known, or less studied, which also contribute to the production of 7,11-HD and AMPs. By completing an unbiased gene expression analysis on the full transcriptome of the fly, patterns of gene expression could be identified, along with transcription factors influencing the expression of genes of interest. This could make it possible to find related genes and perhaps new genes of interest which could be studied in future experiments.

4.6 Conclusions

Overall, oviposition behaviour in fruit flies is an important area of research which warrants further study. Egg placement decisions are crucial behaviours contributing to fitness and represent a model for study into how behaviour is shaped by evolution. Specifically, the behaviour of egg clustering which I have investigated in this thesis raises many questions concerning the ultimate significance of competition and cooperation in the fruit fly. In this study, I found that the behaviour of egg clustering was nutritionally responsive. In addition, some of the genes hypothesised to potentially act as public goods were found to be socially responsive (7,11-HD) and others nutritionally sensitive (Toll-related AMPs). These results provide an insight into the nature of egg clustering and provide potential insights into the fitness consequences.

References:

Archetti, M., & Scheuring, I. (2012). Review: Game theory of public goods in one-shot social dilemmas without assortment. *Journal of Theoretical Biology*, *299*, 9–20. https://doi.org/10.1016/j.jtbi.2011.06.018

Archetti, M., Scheuring, I., Hoffman, M., Frederickson, M. E., Pierce, N. E., & Yu, D. W. (2011). Economic game theory for mutualism and cooperation. *Ecology Letters*, *14*(12), 1300–1312. https://doi.org/10.1111/j.1461-0248.2011.01697.x

Archetti, M., Scheuring, I., & Yu, D. (2020). *The Non-Tragedy of the Non-Linear Commons*. https://doi.org/10.20944/PREPRINTS202004.0226.V1

Axelrod, R., & Hamilton, W. D. (1981). The evolution of cooperation. *Science*, *212*(4489), 1390–1396. https://doi.org/10.1126/SCIENCE.7466396

Baenas, N., & Wagner, A. E. (2019). *Drosophila melanogaster* as an alternative model organism in nutrigenomics. *Genes & Nutrition*, *14*(1). https://doi.org/10.1186/S12263-019-0641-Y

Bailly, T. P. M., Kohlmeier, P., Etienne, R. S., Wertheim, B., & Billeter, J.-C. (2021). Social modulation of oogenesis and egg-laying in *Drosophila melanogaster*. *BioRxiv*, 2021.09.13.460109. https://doi.org/10.1101/2021.09.13.460109

Billeter, J. C., & Wolfner, M. F. (2018). Chemical Cues that Guide Female Reproduction in *Drosophila melanogaster*. *Journal of Chemical Ecology 2018. 44*(9), 750–769. https://doi.org/10.1007/S10886-018-0947-Z

Bourke, A. F. G. (2013). An expanded view of social evolution. *Principles of Social Evolution*, 1–27. https://doi.org/10.1093/ACPROF:OSO/9780199231157.003.0001

Boyd, R., & Richerson, P. J. (1988). The evolution of reciprocity in sizable groups.

Journal of Theoretical Biology, *132*(3), 337–356. https://doi.org/10.1016/S0022-5193(88)80219-4

Bretman, A., Fricke, C., & Chapman, T. (2009). Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1662), 1705–1711. https://doi.org/10.1098/RSPB.2008.1878

Chapman, T., Trevitt, S., & Partridge, L. (1994). Remating and male-derived nutrients in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, *7*(1), 51–69. https://doi.org/10.1046/J.1420-9101.1994.7010051.X

Chen, Y., & Amrein, H. (2017). Ionotropic Receptors Mediate *Drosophila* Oviposition Preference through Sour Gustatory Receptor Neurons. *Current Biology*, *27*(18), 2741-2750.e4. https://doi.org/10.1016/J.CUB.2017.08.003

Chertemps, T., Duportets, L., Labeur, C., Ueyama, M., & Wicker-Thomas, C. (2006). A female-specific desaturase gene responsible for diene hydrocarbon biosynthesis and courtship behaviour in *Drosophila melanogaster. Insect Molecular Biology*, *15*(4), 465–473. https://doi.org/10.1111/j.1365-2583.2006.00658.x

Churchill, E. R., Dytham, C., Bridle, J. R., & Thom, M. D. F. (2021). Social and physical environment independently affect oviposition decisions in *Drosophila. Behavioral Ecology*, *32*(6), 1391–1399. https://doi.org/10.1093/BEHECO/ARAB105

Clark, B. R., & Faeth, S. H. (1998). The evolution of egg clustering in butterflies: A test of the egg desiccation hypothesis. *Evolutionary Ecology*, *12*(5), 543–552. https://doi.org/10.1023/A:1006504725592/METRICS

Clutton-Brock, T. (2009). Cooperation between non-kin in animal societies. *Nature 2009, 462*(7269), 51–57. https://doi.org/10.1038/nature08366

Collins, D. H., Wirén, A., Labédan, M., Smith, M., Prince, D. C., Mohorianu, I., Dalmay, T., & Bourke, A. F. G. (2021). Gene expression during larval caste determination and differentiation in intermediately eusocial bumblebees, and a comparative analysis with advanced eusocial honeybees. *Molecular Ecology*, *30*(3), 718–735. https://doi.org/10.1111/MEC.15752

Connor, R. C. (1995). The Benefits of Mutualism: A Conceptual Framework. *Biological Reviews*, *70*(3), 427–457. https://doi.org/10.1111/J.1469-185X.1995.TB01196.X

Dallerac, R., Labeur, C., Jallon, J. M., Knipple, D. C., Roelofs, W. L., & Wicker-Thomas, C. (2000). A Δ9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *97*(17), 9449–9454. https://doi.org/10.1073/PNAS.150243997

De Gregorio, E., Spellman, P. T., Rubin, G. M., & Lemaitre, B. (2001). Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proceedings of the National Academy of Sciences*, *98*(22), 12590–12595. https://doi.org/10.1073/PNAS.221458698

Diggle, S. P., Griffin, A. S., Campbell, G. S., & West, S. A. (2007). Cooperation and conflict in quorum-sensing bacterial populations. *Nature 2007*, *450*(7168), 411–414. https://doi.org/10.1038/nature06279

Dobata, S., & Tsuji, K. (2013). Public goods dilemma in asexual ant societies. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(40), 16056–16060. https://doi.org/10.1073/PNAS.1309010110

Drescher, K., Nadell, C. D., Stone, H. A., Wingreen, N. S., & Bassler, B. L. (2014). Solutions to the public goods dilemma in bacterial biofilms. *Current Biology : CB*, *24*(1), 50. https://doi.org/10.1016/J.CUB.2013.10.030

Dukas, R., Prokopy, R. J., Papaj, D. R., & Duan, J. J. (2001). Egg-laying Behavior of

Mediterranean Fruit Flies (Diptera: Tephritidae): Is Social Facilitation Important? *The Florida Entomologist*, *84*(4), 665–671.

Enjin, A., Zaharieva, E. E., Frank, D. D., Mansourian, S., Suh, G. S. B., Gallio, M., & Stensmyr, M. C. (2016). Humidity Sensing in *Drosophila*. *Current Biology*, *26*(10), 1352–1358. https://doi.org/10.1016/J.CUB.2016.03.049

Faraji, F., Janssen, A., & Sabelis, M. W. (2002). The benefits of clustering eggs: The role of egg predation and larval cannibalism in a predatory mite. *Oecologia*, *131*(1), 20–26. https://doi.org/10.1007/S00442-001-0846-8/METRICS

Ferrandon, D., Imler, J. L., Hetru, C., & Hoffmann, J. A. (2007). The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nature Reviews. Immunology*, *7*(11), 862–874. https://doi.org/10.1038/NRI2194

Flatt, T. (2020). Life-History Evolution and the Genetics of Fitness Components in *Drosophila melanogaster. Genetics*, *214*(1), 3. https://doi.org/10.1534/GENETICS.119.300160

Fowler, E. K., Leigh, S., Bretman, A., & Chapman, T. (2022). Plastic responses of males and females interact to determine mating behavior. *Evolution; International Journal of Organic Evolution*, *76*(9), 2116. https://doi.org/10.1111/EVO.14568

Fowler, E. K., Leigh, S., Rostant, W. G., Thomas, A., Bretman, A., & Chapman, T. (2022). Memory of social experience affects female fecundity via perception of fly deposits. *BMC Biology*, *20*(1), 1–13.

Frank, S. A. (2010). A general model of the public goods dilemma. *Journal of Evolutionary Biology*, *23*(6), 1245–1250. https://doi.org/10.1111/J.1420-9101.2010.01986.X

Galenza, A., Hutchinson, J., Campbell, S. D., Hazes, B., & Foley, E. (2016). Glucose

modulates *Drosophila* longevity and immunity independent of the microbiota. *Biology Open*, *5*(2), 165–173. https://doi.org/10.1242/BIO.015016

Govind, S. (1999). Control of development and immunity by Rel transcription factors in *Drosophila*. *Oncogene 1999 18:49*, *18*(49), 6875–6887. https://doi.org/10.1038/sj.onc.1203223

Gui, S., Yuval, B., Engl, T., Lu, Y., & Cheng, D. (2023). Protein feeding mediates sex pheromone biosynthesis in an insect. *ELife*, *12 :e83469* https://doi.org/10.7554/ELIFE.83469

Hamilton, W. D. (1964). The genetical evolution of social behaviour. II. *Journal of Theoretical Biology*, *7*(1), 17–52. https://doi.org/10.1016/0022-5193(64)90039-6

Hanson, M. A., Lemaitre, B., & Unckless, R. L. (2019). Dynamic Evolution of Antimicrobial Peptides Underscores Trade-Offs Between Immunity and Ecological Fitness. *Frontiers in Immunology*, *10*, 2620. https://doi.org/10.3389/FIMMU.2019.02620/BIBTEX

Hanson, M. A, Dostálová, A., Ceroni, C., Poidevin, M., Kondo, S., & Lemaitre, B. (2019). Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *ELife*, *8*. https://doi.org/10.7554/ELIFE.44341

Hardin, G. (1968). The Tragedy of the Commons. *Source: Science, New Series*, *162*(3859), 1243–1248.

Hedengren-Olcott, M., Olcott, M. C., Mooney, D. T., Ekengren, S., Geller, B. L., & Taylor, B. J. (2004). Differential activation of the NF-κB-like factors relish and Dif in *Drosophila melanogaster* by fungi and gram-positive bacteria. *Journal of Biological Chemistry*, *279*(20), 21121–21127. https://doi.org/10.1074/jbc.M313856200

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J. (2008).

qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*(2), 1–14. https://doi.org/10.1186/GB-2007-8-2-R19/COMMENTS

Hetru, C., Troxler, L., & Hoffmann, J. A. (2003). *Drosophila melanogaster* Antimicrobial Defense. *The Journal of Infectious Diseases*, *187*, 327–334. https://doi.org/10.1086/374758

Hoffmann, J. A. (2003). The immune response of *Drosophila*. *Nature 2003*, *426*(6962), 33–38. https://doi.org/10.1038/nature02021

Hoffmann, J. A., & Reichhart, J. M. (2002). *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology 2002, 3*(2), 121–126. https://doi.org/10.1038/ni0202-121

Hoffmann, J. A., Reichhart, J. M., & Hetru, C. (1996). Innate immunity in higher insects. *Current Opinion in Immunology*, *8*(1), 8–13. https://doi.org/10.1016/S0952-7915(96)80098-7

Hoffmeister, T. S., & Rohlfs, M. (2001). Aggregative egg distributions may promote species co-existence-but why do they exist? *Evolutionary Ecology Research*, *3*, 37–50.

Hu, Y., Sopko, R., Foos, M., Kelley, C., Flockhart, I., Ammeux, N., Wang, X., Perkins, L., Perrimon, N., & Mohr, S. E. (2013). FlyPrimerBank: an online database for *Drosophila melanogaster* gene expression analysis and knockdown evaluation of RNAi reagents. *G3 (Bethesda, Md.)*, *3*(9), 1607–1616. https://doi.org/10.1534/G3.113.007021

Hultmark, D. (2003). *Drosophila* immunity: paths and patterns. *Current Opinion in Immunology*, *15*(1), 12–19. https://doi.org/10.1016/S0952-7915(02)00005-5

Joseph, N. M., Elphick, N. Y., Mohammad, S., & Bauer, J. H. (2018). Altered pheromone biosynthesis is associated with sex-specific changes in life span and

behavior in *Drosophila melanogaster*. *Mechanisms of Ageing and Development*, *176*, 1–8. https://doi.org/10.1016/J.MAD.2018.10.002

Kassambara, A. (2020). *ggpubr: "ggplot2" Based Publication Ready Plots*.

Khodaei, L., & Long, T. A. F. (2019). Kin recognition and co-operative foraging in *Drosophila melanogaster* larvae. *Journal of Evolutionary Biology*, *32*(12), 1352–1361. https://doi.org/10.1111/JEB.13531

Khodaei, L., & Long, T. A. F. (2020). Kin Recognition and Egg Cannibalism by *Drosophila melanogaster* Larvae. *Journal of Insect Behavior*, *33*(1), 20–29. https://doi.org/10.1007/s10905-020-09742-0

Kim, T., & Kim, Y. J. (2005). Overview of innate immunity in *Drosophila*. *Journal of Biochemistry and Molecular Biology*, *38*(2), 121–127. https://doi.org/10.5483/BMBREP.2005.38.2.121

Kimmel, G. J., Gerlee, P., & Altrock, P. M. (2019). Time scales and wave formation in non-linear spatial public goods games. *PLOS Computational Biology*, *15*(9), e1007361. https://doi.org/10.1371/JOURNAL.PCBI.1007361

Kleino, A., & Silverman, N. (2014). The *Drosophila* IMD pathway in the activation of the humoral immune response. *Developmental & Comparative Immunology*, *42*(1), 25–35. https://doi.org/10.1016/J.DCI.2013.05.014

Krittika, S., & Yadav, P. (2019). An overview of two decades of diet restriction studies using *Drosophila*. *Biogerontology*, *20*(6), 723–740. https://doi.org/10.1007/S10522-019-09827-0/TABLES/2

Krittika, S., & Yadav, P. (2020). Dietary protein restriction deciphers new relationships between lifespan, fecundity and activity levels in fruit flies *Drosophila melanogaster*. *Scientific Reports 2020, 10*(1), 1–8. https://doi.org/10.1038/s41598-020-66372-4

Krupp, J. J., & Levine, J. D. (2010). Dissection of Oenocytes from Adult *Drosophila melanogaster. JoVE (Journal of Visualized Experiments)*, *41*, e2242. https://doi.org/10.3791/2242

Le Bourg, É. (2011). The NF-kB like factor DIF has weaker effects on *Drosophila melanogaster* immune defenses than previously thought. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, *181*(6), 741–750. https://doi.org/10.1007/S00360-011-0567-1/FIGURES/7

Leader, D. P., Krause, S. A., Pandit, A., Davies, S. A., & Dow, J. A. T. (2018). FlyAtlas 2: a new version of the *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Research*, *46*(D1), D809–D815. https://doi.org/10.1093/NAR/GKX976

Leclerc, V., & Reichhart, J. M. (2004). The immune response of *Drosophila melanogaster*. *Immunological Reviews*, *198*(1), 59–71. https://doi.org/10.1111/J.0105-2896.2004.0130.X

Leech, T., Evison, S. E. F., Armitage, S. A. O., Sait, S. M., & Bretman, A. (2019). Interactive effects of social environment, age and sex on immune responses in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, *32*(10), 1082–1092. https://doi.org/10.1111/JEB.13509

Liao, S., Amcoff, M., & Nässel, D. R. (2021). Impact of high-fat diet on lifespan, metabolism, fecundity and behavioral senescence in *Drosophila*. *Insect Biochemistry and Molecular Biology*, *133*, 103495. https://doi.org/10.1016/J.IBMB.2020.103495

Lihoreau, M., Poissonnier, L. A., Isabel, G., & Dussutour, A. (2016). *Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods. *The Journal of Experimental Biology*, *219*(16), 2514–2524. https://doi.org/10.1242/JEB.142257

Liu, T., Wang, Y., Tian, Y., Zhang, J., Zhao, J., & Guo, A. (2020). The receptor channel formed by ppk25, ppk29 and ppk23 can sense the *Drosophila* female pheromone 7,11-heptacosadiene. *Genes, Brain, and Behavior*, *19*(2). https://doi.org/10.1111/GBB.12529

Lu, B., LaMora, A., Sun, Y., Welsh, M. J., & Ben-Shahar, Y. (2012). ppk23-Dependent Chemosensory Functions Contribute to Courtship Behavior in *Drosophila melanogaster*. *PLOS Genetics*, *8*(3), e1002587. https://doi.org/10.1371/JOURNAL.PGEN.1002587

Maklakov, A. A., & Chapman, T. (2019). Evolution of ageing as a tangle of trade-offs: energy versus function. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1911), 20191604. https://doi.org/10.1098/rspb.2019.1604

Manjunatha, T., Hari Dass, S., & Sharma, V. K. (2008). Egg-laying rhythm in *Drosophila melanogaster. Journal of Genetics*, *87*(5), 495–504. https://doi.org/10.1007/S12041-008-0072-9/METRICS

Marchini, D., Marri, L., Rosetto, M., Manetti, A. G. O., & Dallai, R. (1997). Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata*. *Biochemical and Biophysical Research Communications*, *240*(3), 657–663. https://doi.org/10.1006/BBRC.1997.7694

Markow, T. A., Beall, S., & Matzkin, L. M. (2009). Egg size, embryonic development time and ovoviviparity in *Drosophila* species. *Journal of Evolutionary Biology*, *22*(2), 430–434. https://doi.org/10.1111/J.1420-9101.2008.01649.X

Marwick, B. and K. Krishnamoorthy 2019 cvequality: Tests for the Equality of Coefficients of Variation from Multiple Groups. R software package version 0.1.3. Retrieved from https://github.com/benmarwick/cvequality, on 05/01/2019

McInerney, J. O., Pisani, D., Bapteste, E., & O'Connell, M. J. (2011). The public goods hypothesis for the evolution of life on Earth. *Biology Direct*, *6*(1), 1–17. https://doi.org/10.1186/1745-6150-6-41/FIGURES/1

Miller, P. M., Saltz, J. B., Cochrane, V. A., Marcinkowski, C. M., Mobin, R., & Turner, T. L. (2011). Natural variation in decision-making behavior in *Drosophila melanogaster*. *PloS One*, *6*(1). https://doi.org/10.1371/JOURNAL.PONE.0016436

Mirth, C. K., Nogueira Alves, A., & Piper, M. D. (2019). Turning food into eggs: insights from nutritional biology and developmental physiology of *Drosophila*. *Current Opinion in Insect Science*, *31*, 49–57. https://doi.org/10.1016/J.COIS.2018.08.006

Moghaddam, M. R. B., Gross, T., Becker, A., Vilcinskas, A., & Rahnamaeian, M. (2017). The selective antifungal activity of *Drosophila melanogaster* metchnikowin reflects the species-dependent inhibition of succinate-coenzyme Q reductase. *Scientific Reports*, *7*(1). https://doi.org/10.1038/S41598-017-08407-X

Morsky, B., Smolla, M., & Akçay, E. (2020). Evolution of contribution timing in public goods games. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1927). https://doi.org/10.1098/RSPB.2020.0735

Mukherjee, S., & Bassler, B. L. (2019). Bacterial quorum sensing in complex and dynamically changing environments. *Nature Reviews Microbiology 2019*, *17*(6), 371–382. https://doi.org/10.1038/s41579-019-0186-5

Myllymäki, H., Valanne, S., & Rämet, M. (2014). The *Drosophila* Imd Signaling Pathway. *The Journal of Immunology*, *192*(8), 3455–3462. https://doi.org/10.4049/JIMMUNOL.1303309

Narasimha, S., Nagornov, K. O., Menin, L., Mucciolo, A., Rohwedder, A., Humbel, B. M., Stevens, M., Thum, A. S., Tsybin, Y. O., & Vijendravarma, R. K. (2019). *Drosophila melanogaster* cloak their eggs with pheromones, which prevents cannibalism. *PLOS Biology*, *17*(1), e2006012. https://doi.org/10.1371/JOURNAL.PBIO.2006012

Nowak, M. A. (2006). Five rules for the evolution of cooperation. *Science*, *314*(5805), 1560–1563. https://doi.org/10.1126/SCIENCE.1133755/SUPPL_FILE/NOWAK.SOM.PDF

Park, J. M., Brady, H., Ruocco, M. G., Sun, H., Williams, D. A., Lee, S. J., Kato, T., Richards, N., Chan, K., Mercurio, F., Karin, M., & Wasserman, S. A. (2004). Targeting of TAK1 by the NF-κB protein Relish regulates the JNK-mediated immune response in *Drosophila*. *Genes & Development*, *18*(5), 584. https://doi.org/10.1101/GAD.1168104

Peng, J., Zipperlen, P., & Kubli, E. (2005). *Drosophila* Sex-Peptide Stimulates Female Innate Immune System after Mating via the Toll and Imd Pathways. *Current Biology*, *15*(18), 1690–1694. https://doi.org/10.1016/j.cub.2005.08.048

Platt, T. G., & Bever, J. D. (2009). Kin competition and the evolution of cooperation. *Trends in Ecology & Evolution*, *24*(7), 370–377. https://doi.org/10.1016/J.TREE.2009.02.009

Ponton, F., Morimoto, J., Robinson, K., Kumar, S. S., Cotter, S. C., Wilson, K., & Simpson, S. J. (2020). Macronutrients modulate survival to infection and immunity in *Drosophila*. *Journal of Animal Ecology*, *89*(2), 460–470. https://doi.org/10.1111/1365-2656.13126

Prokopy, R. J., & Duan, J. J. (1998). Socially Facilitated Egglaying Behavior in Mediterranean Fruit Flies. *Behavioral Ecology and Sociobiology*, *42*(2), 117–122. http://www.jstor.org/stable/4601428

Ramos-Onsins, S., & Aguadé, M. (1998). Molecular Evolution of the Cecropin Multigene Family in *Drosophila*: Functional Genes vs. Pseudogenes. *Genetics*, *150*(1), 157–171. https://doi.org/10.1093/GENETICS/150.1.157

Ramos, R. A. N., Giannelli, A., Dantas-Torres, F., & Otranto, D. (2013). Effect of egg clustering on the fitness of Rhipicephalus sanguineus larvae. *Parasitology*

Research, *112*(4), 1795–1797. https://doi.org/10.1007/S00436-012-3229-7/FIGURES/1

Rosetto, M., Marchini, D., De Filippis, T., Ciolfi, S., Frati, F., Quilici, S., & Dallai, R. (2003). The ceratotoxin gene family in the medfly *Ceratitis capitata* and the Natal fruit fly *Ceratitis rosa* (Diptera: Tephritidae). *Heredity 2003*, *90*(5), 382–389. https://doi.org/10.1038/sj.hdy.6800258

Rosetto, Marco, Manetti, A. G. O., Giordano, P. C., Marri, L., Amons, R., Baldari, C. T., Marchini, D., & Dallai, R. (1996). Molecular Characterization of Ceratotoxin C, a Novel Antibacterial Female-Specific Peptide of the Ceratotoxin Family from the Medfly *Ceratitis capitata*. *European Journal of Biochemistry*, *241*(2), 330–337. https://doi.org/10.1111/J.1432-1033.1996.00330.X

Rosetto, Marco, Manetti, A. G. O., Marchini, D., Dallai, R., Telford, J. L., & Baldari, C. T. (1993). Sequences of two cDNA clones from the medfly *Ceratitis capitata* encoding antibacterial peptides of the cecropin family. *Gene*, *134*(2), 241–243. https://doi.org/10.1016/0378-1119(93)90100-H

Ryu, J. H., Ha, E. M., Oh, C. T., Seol, J. H., Brey, P. T., Jin, I., Lee, D. G., Kim, J., Lee, D., & Lee, W. J. (2006). An essential complementary role of NF-κB pathway to microbicidal oxidants in *Drosophila* gut immunity. *The EMBO Journal*, *25*(15), 3693–3701. https://doi.org/10.1038/SJ.EMBOJ.7601233

Samuelson, P. A. (1954). The Pure Theory of Public Expenditure. *The Review of Economics and Statistics*, *36*(4), 387. https://doi.org/10.2307/1925895

Sarin, S., & Dukas, R. (2009). Social learning about egg-laying substrates in fruitflies. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1677), 4323. https://doi.org/10.1098/RSPB.2009.1294

Sato, A., Tanaka, K. M., Yew, J. Y., & Takahashi, A. (2021). *Drosophila suzukii* avoidance of microbes in oviposition choice. *Royal Society Open Science*, *8*(1). https://doi.org/10.1098/RSOS.201601

Sato, K., & Yamamoto, D. (2020). Contact-Chemosensory Evolution Underlying Reproductive Isolation in *Drosophila* Species. *Frontiers in Behavioral Neuroscience*, *14*. https://doi.org/10.3389/FNBEH.2020.597428

Schneider, J., Atallah, J., & Levine, J. D. (2017). Social structure and indirect genetic effects: genetics of social behaviour. *Biological Reviews of the Cambridge Philosophical Society*, *92*(2), 1027–1038. https://doi.org/10.1111/BRV.12267

Schweinfurth, M. K., & Call, J. (2019). Reciprocity: Different behavioural strategies, cognitive mechanisms and psychological processes. *Learning & Behavior*, *47*(4), 284. https://doi.org/10.3758/S13420-019-00394-5

Shirangi, T. R., Dufour, H. D., Williams, T. M., & Carroll, S. B. (2009). Rapid Evolution of Sex Pheromone-Producing Enzyme Expression in *Drosophila*. *PLOS Biology*, *7*(8), e1000168. https://doi.org/10.1371/JOURNAL.PBIO.1000168

Silva-Soares, N. F., Nogueira-Alves, A., Beldade, P., & Mirth, C. K. (2017). Adaptation to new nutritional environments: larval performance, foraging decisions, and adult oviposition choices in *Drosophila suzukii*. *BMC Ecology*, *17*(1), 21. https://doi.org/10.1186/S12898-017-0131-2

Smith, P., & Schuster, M. (2019). Public goods and cheating in microbes. *Current Biology*, *29*(11), R442–R447. https://doi.org/10.1016/J.CUB.2019.03.001

Sultanova, Z., Ivimey-Cook, E. R., Chapman, T., & Maklakov, A. A. (2021). Fitness benefits of dietary restriction. *Proceedings of the Royal Society B*, *288*(1963). https://doi.org/10.1098/RSPB.2021.1787

Tamang, D. G., & Saier, M. H. (2006). The Cecropin Superfamily of Toxic Peptides. *Journal of Molecular Microbiology and Biotechnology*, *11*(1–2), 94–103. https://doi.org/10.1159/000092821

Tanji, T., Yun, E. Y., & Ip, Y. T. (2010). Heterodimers of NF-κB transcription factors DIF and Relish regulate antimicrobial peptide genes in *Drosophila*. *Proceedings of the National Academy of Sciences*, *107*(33), 14715–14720. https://doi.org/10.1073/PNAS.1009473107

Tapadia, M. G., & Verma, P. (2012). Immune response and anti-microbial peptides expression in Malpighian tubules of *Drosophila melanogaster* is under developmental regulation. *PloS One*, *7*(7). https://doi.org/10.1371/JOURNAL.PONE.0040714

R Coding Team (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing.

Tebbich, S., Taborsky, M., & Winkler, H. (1996). Social manipulation causes cooperation in keas. *Animal Behaviour*, *52*(1), 1–10. https://doi.org/10.1006/ANBE.1996.0147

Thomas, M. C., Eugene Woodruff, R., Steck, G. J., Thomas, M. C., Heppner, J. B., Woodruff, R. E., Weems, H. V, Steck, G. J., & Fasulo, T. R. (2001). Mediterranean Fruit Fly, *Ceratitis capitata* (Wiedemann) (Insecta: Diptera: Tephritidae)1 Phyllophaga of the Caribbean View project Mediterranean Fruit Fly, *Ceratitis capitata* (Wiedemann) (Insecta: Diptera: Tephritidae) : EENY-214/IN371, Rev. 9/2001. EDIS 2004 (8).https://doi.org/10.32473/edis-in371-2001

Thurmond, J., Goodman, J. L., Strelets, V. B., Attrill, H., Gramates, L. S., Marygold, S. J., Matthews, B. B., Millburn, G., Antonazzo, G., Trovisco, V., Kaufman, T. C., Calvi, B. R., Perrimon, N., Gelbart, S. R., Agapite, J., Broll, K., Crosby, L., Dos Santos, G., Emmert, D., … Baker, P. (2019). FlyBase 2.0: the next generation. *Nucleic Acids Research*, *47*(D1), D759–D765. https://doi.org/10.1093/NAR/GKY1003

Timms, J. E. L., & Leather, S. R. (2007). Ladybird egg cluster size: relationships between species, oviposition substrate and cannibalism. *Bulletin of*

Entomological Research, *97*(6), 613–618. https://doi.org/10.1017/S0007485307005354

Tingvall, T. Ö., Roos, E., & Engström, Y. (2001). The imd gene is required for local Cecropin expression in *Drosophila* barrier epithelia. *EMBO Reports*, *2*(3), 239–243. https://doi.org/10.1093/EMBO-REPORTS/KVE048

Toda, H., Zhao, X., & Dickson, B. J. (2012). The *Drosophila* Female Aphrodisiac Pheromone Activates ppk23+ Sensory Neurons to Elicit Male Courtship Behavior. *Cell Reports*, *1*(6), 599–607. https://doi.org/10.1016/J.CELREP.2012.05.007

Trevitt, S., Fowler, K., & Partridge, L. (1988). An effect of egg-deposition on the subsequent fertility and remating frequency of female *Drosophila melanogaster*. *Journal of Insect Physiology*, *34*(8), 821–828. https://doi.org/10.1016/0022-1910(88)90157-6

Trumbo, S. T. (2018). Juvenile hormone and parental care in subsocial insects: implications for the role of juvenile hormone in the evolution of sociality. *Current Opinion in Insect Science*, *28*, 13–18. https://doi.org/10.1016/J.COIS.2018.04.001

Unckless, R. L., Rottschaefer, S. M., & Lazzaro, B. P. (2015). The Complex Contributions of Genetics and Nutrition to Immunity in *Drosophila melanogaster*. *PLoS Genetics*, *11*(3). https://doi.org/10.1371/JOURNAL.PGEN.1005030

Valanne, S., Wang, J.-H., & Rämet, M. (2011). The *Drosophila* Toll signaling pathway. *Journal of Immunology*, *186*(2), 649–656. https://doi.org/10.4049/JIMMUNOL.1002302

Vesterberg, A., Rizkalla, R., & Fitzpatrick, M. J. (2021). Environmental influences on for-mediated oviposition decisions in *Drosophila melanogaster*. *Journal of Neurogenetics*, *35*(3), 262–273. https://doi.org/10.1080/01677063.2021.1950713

Vijendravarma, R. K., Narasimha, S., & Kawecki, T. J. (2013). Predatory cannibalism in *Drosophila melanogaster* larvae. *Nature Communications 2013*, *4*(1), 1–8. https://doi.org/10.1038/ncomms2744

Wertheim, B., Dicke, M., & Vet, L. E. M. (2002). Behavioural plasticity in support of a benefit for aggregation pheromone use in *Drosophila melanogaster*. *Entomologia Experimentalis et Applicata*, *103*(1), 61–71. https://doi.org/10.1046/J.1570-7458.2002.00954.X

West, S. A., Griffin, A. S., & Gardner, A. (2007). Evolutionary Explanations for Cooperation. *Current Biology*, *17*(16), 661–672. https://doi.org/10.1016/J.CUB.2007.06.004

Wicker-Thomas, C., Garrido, D., Bontonou, G., Napal, L., Mazuras, N., Denis, B., Rubin, T., Parvy, J. P., & Montagne, J. (2015). Flexible origin of hydrocarbon/pheromone precursors in *Drosophila melanogaster*. *Journal of Lipid Research*, *56*(11), 2094–2101. https://doi.org/10.1194/JLR.M060368/ATTACHMENT/22AF8CDF-8297-4EB0-9ECE-6C7194BEC473/MMC1.ZIP

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Wickham, H., François, R., Müller, K., & Henry, L. (2021). *dplyr: A Grammar of Data Manipulation*.

Wigby, S., Sirot, L. K., Linklater, J. R., Buehner, N., Calboli, F. C. F., Bretman, A., Wolfner, M. F., & Chapman, T. (2009). Seminal Fluid Protein Allocation and Male Reproductive Success. *Current Biology*, *19*(9), 751–757. https://doi.org/10.1016/J.CUB.2009.03.036

Winkler, A., Jung, J., Kleinhenz, B., & Racca, P. (2020). A review on temperature and humidity effects on *Drosophila suzukii* population dynamics. *Agricultural and*

Forest Entomology, *22*(3), 179–192. https://doi.org/10.1111/AFE.12381

Yang, C. H., Belawat, P., Hafen, E., Jan, L. Y., & Jan, Y. N. (2008). *Drosophila* egg-laying site selection as a system to study simple decision-making processes. *Science*, *319*(5870), 1679. https://doi.org/10.1126/SCIENCE.1151842

Yu, S., Zhang, G., & Jin, L. H. (2018). A high-sugar diet affects cellular and humoral immune responses in *Drosophila*. *Experimental Cell Research*, *368*(2), 215–224. https://doi.org/10.1016/j.yexcr.2018.04.032

Zhang, L., Yu, J., Guo, X., Wei, J., Liu, T., & Zhang, W. (2020). Parallel Mechanosensory Pathways Direct Oviposition Decision-Making in *Drosophila*. *Current Biology*, *30*(16), 3075-3088.e4. https://doi.org/10.1016/j.cub.2020.05.076

Supplementary information

Raw data link:

https://www.dropbox.com/scl/fo/lj5jmaxmdpjv96siewy1k/h?rlkey=0rfpdv1lzp6t50g0o2tfdts2q&dl=0

```
setwd("~/Desktop/MScR/Data/code/eggcount")
library(ggpubr)
library(dplyr)
ec_alone <- read.csv("ec_alone.csv")
ec_alone <- subset(ec_alone, total_eggs >"1")
time_point.labs <- c("3-hour", "6-hour")
names(time_point.labs) <- c("3","6")
ec_alone$trt <-factor(ec_alone$trt,
                      levels = c('3','1'), ordered = TRUE)
ec_prop_alone <- ggboxplot(ec_alone, "trt", "prop", color = "diet", ylim=c(0,1), add =
"jitter", bxp.errorbar = FALSE, bxp.errorbar.width = 0.4, notch = FALSE) + labs(x = "Alone", y
= "Proportion of eggs in clusters") +
   scale_color_manual(values=c("darkolivegreen3","tan4"), name="Diet", labels=c("Low",
"Standard")) +
   scale_x_discrete(breaks = c(3, 1), labels = c("", "")) +
   facet_wrap(~time_point, labeller = labeller(time_point = time_point.labs))
ec_prop_alone

ec_grouped <- read.csv("ec_grouped.csv")
ec_grouped <- subset(ec_grouped, total_eggs >"1")
time_point.labs <- c("3-hour", "6-hour")
names(time_point.labs) <- c("3","6")
ec_grouped$trt <-factor(ec_grouped$trt,
                        levels = c('4','2'), ordered = TRUE)
ec_prop_grouped <- ggboxplot(ec_grouped, "trt", "prop", color = "diet", ylim=c(0,1), add =
"jitter", bxp.errorbar = FALSE, bxp.errorbar.width = 0.4, notch = FALSE) + labs(x = "Grouped",
y = "") +
   scale_color_manual(values=c("darkolivegreen3","tan4"), name="Diet", labels=c("Low",
"Standard")) +
   scale_x_discrete(breaks = c(4, 2), labels = c("", "")) +
   facet_wrap(~time_point, labeller = labeller(time_point = time_point.labs))
ec_prop_grouped

library(ggpubr)
ec_graphs <- ggarrange(ec_prop_alone, ec_prop_grouped, ncol = 2, nrow  = 1,
common.legend = TRUE)
ec_graphs
```

Figure S.1: The effect of diet, social environment and time-point on the proportion of eggs found in clusters*. This code produces Figure 5, it shows box plots representing the differences between the different dietary and social treatments and the two time-points.*

```
library(ggplot2)
setwd("~/Desktop/MScR/Data/code/eggcount")

long_data <- data %>% gather(cluster_id, cluster_size, C1:C19)

eggcount_6hr <- read.csv("eggcount_rank_6hr.csv")
library(tidyr)
eggcount_6hr <- eggcount_6hr %>% drop_na(Ranking)
trt.labs <- c("SA", "SG", "LA", "LG")
names(trt.labs) <- c("1","2","3","4")
ggplot(data = eggcount_6hr, aes(x = Ranking, y = cluster_size)) +
  geom_point() +
  ggtitle("Egg cluster size and count six hours after mating") +
  ylab("Cluster Size") +
  theme_bw() +
  theme(panel.border = element_blank(), panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), axis.line = element_line(colour = "black")) +
  facet_wrap(~trt, labeller = labeller(trt = trt.labs))

library(ggplot2)
setwd("~/Desktop/MScR/Data/code/eggcount")
eggcount_3hr <- read.csv("eggcount_rank_3hr.csv")
library(tidyr)
eggcount_3hr <- eggcount_3hr %>% drop_na(Ranking)
trt.labs <- c("SA", "SG", "LA", "LG")
names(trt.labs) <- c("1","2","3","4")
ggplot(data = eggcount_3hr, aes(x = Ranking, y = cluster_size)) +
  geom_point() +
  ggtitle("Egg cluster size and count six hours after mating") +
  ylab("Cluster Size") +
  theme_bw() +
  theme(panel.border = element_blank(), panel.grid.major = element_blank(),
panel.grid.minor = element_blank(), axis.line = element_line(colour = "black")) +
  facet_wrap(~trt, labeller = labeller(trt = trt.labs))
```

Figure S.2: The size and ranking of egg clusters. *This code produces Figure 6 and Figure 7, which present the variation in the sizes of number of clusters found across each treatment (code in italics show the line used to convert the egg count data into a useable input, by making each cluster an individual row).*

```
setwd("~/Desktop/MScR/Data/code/eggcount")
library(ggpubr)
library(dplyr)
ec <- read.csv("ec.csv")
ec_na <- subset(ec, total_eggs >"1")
ec_tp3 <- subset(ec, time_point == "3")
ec_tp6 <- subset(ec, time_point == "6")
attach(ec_tp3)
y <- cbind(egg_cluster, single_eggs)
ec_tp3_model<-glm(y~diet + social, data = ec_tp3, family = quasibinomial)
anova(ec_tp3_model, test = "F")

attach(ec_tp6)
y <- cbind(egg_cluster, single_eggs)
ec_tp6_model<-glm(y~diet + social, data = ec_tp6, family = quasibinomial)
anova(ec_tp6_model, test = "F")
```

Figure S.3: The effect of diet and social environment on the proportion of eggs laid in clusters. *This Figure shows the code used to create the Generalised Linear Models (GLM) and run the subsequent ANOVAs which tested for the proportion of eggs laid in clusters for a social and/or dietary effect. Initially the analysis interactions ('*' instead of '+' in the model) were also measured but were removed once no interactions were found.*

```
setwd("~/Desktop/MScR/Data/code/eggcount")
library(ggpubr)
library(dplyr)
ec_grouped <- read.csv("ec_grouped.csv")
ec_alone <- read.csv("ec_alone.csv")
alone_tp3 <- subset(ec_alone, time_point == "3")
grouped_tp3 <- subset(ec_grouped, time_point == "3")
grouped_tp3_model <-glm(total_eggs~diet, data = grouped_tp3, family = quasipoisson)
anova(grouped_tp3_model, test = "F")
alone_tp3_model <-glm(total_eggs~diet, data = alone_tp3, family = quasipoisson)
anova(alone_tp3_model, test = "F")
alone_tp6 <- subset(ec_alone, time_point == "6")
grouped_tp6 <- subset(ec_grouped, time_point == "6")
grouped_tp6_model <-glm(total_eggs~diet, data = grouped_tp6, family = quasipoisson)
anova(grouped_tp6_model, test = "F")
alone_tp6_model <-glm(total_eggs~diet, data = alone_tp6, family = quasipoisson)
anova(alone_tp6_model, test = "F")
```

Figure S.4: The effect of diet and social environment on the total number of eggs. *Shows the code used to create the Generalised Linear Models (GLM) and run the subsequent ANOVAs which tested for the differences in the total number of eggs as a result of dietary or social environment. The same code is used for the analysis of the number of single eggs and the number of clusters. However, 'total_eggs' was replaced with 'single_eggs' and 'number_of_clusters' respectively.*

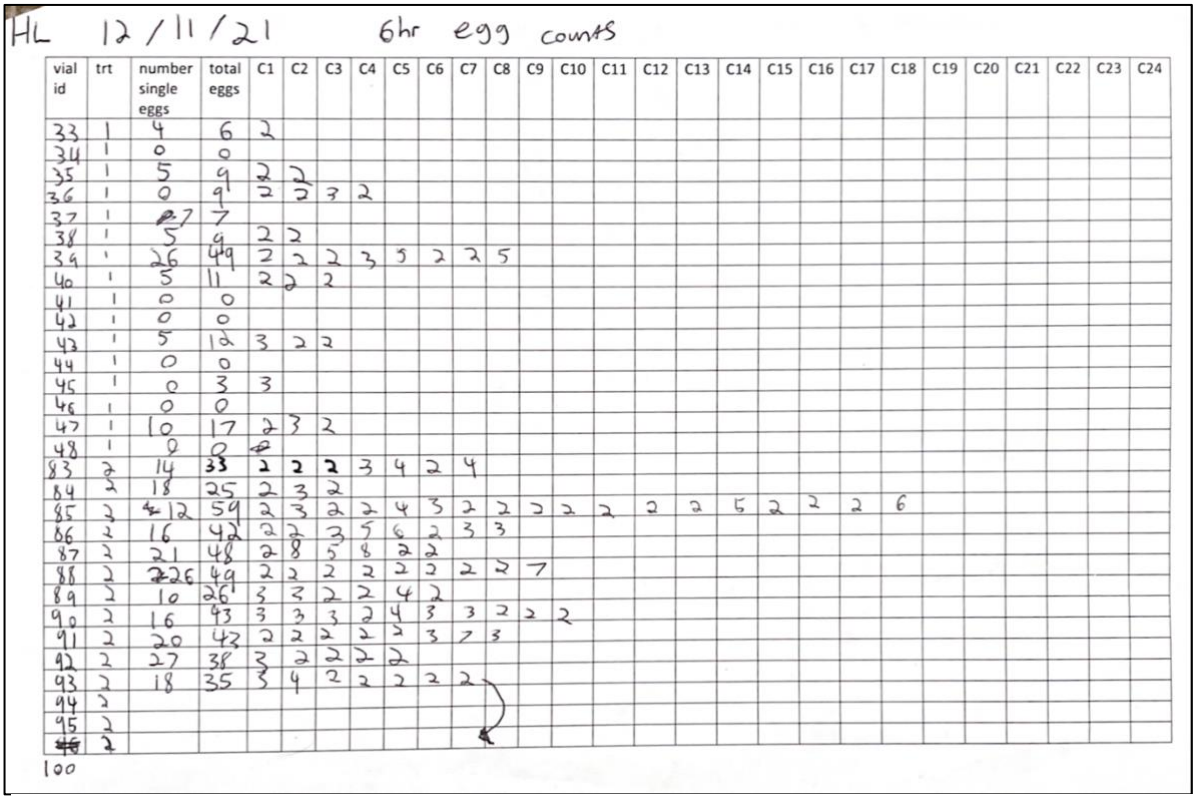

Figure S.5: Example of the egg count data collection sheet *used to record the information on the number of eggs, single eggs, and clusters. This specific sheet shows the data for the 6h time point.*

```
setwd("~/Desktop/MScR/Data/code/Normalised_data")
library(ggplot2)
library(plyr)
nm_fad2 <- read.csv("nm_fad2.csv")
snm_fad2<- ddply(nm_fad2, c("diet_social", "time_point", "diet", "social"), summarise,
N    = length(Relative_expression),
mean = mean(Relative_expression),
sd   = sd(Relative_expression),
se   = sd / sqrt(N)
)
pd <- position_dodge(0.1)
fad2_tp <- ggplot(data = snm_fad2, aes(x = factor(time_point), y = mean, group =
diet_social, colour = diet, linetype = social)) +
geom_errorbar(aes(ymin=mean-se, ymax=mean+se), linetype=1, width=.1, position=pd,
colour="black") +
geom_line(position=pd) +
geom_point(position=pd, size=2, shape=21, fill="white") +
scale_color_manual(values=c("darkolivegreen3","tan4"), name="Diet", labels=c("Low",
"Standard")) +
scale_linetype_manual(values=c(1, 2), name="Social Groups", labels=c("Alone",
"Grouped")) +
scale_x_discrete(breaks = c(0, 3, 6), labels = c("Virgin", "3-Hour", "6-Hour")) +
xlab("Time Point") +
ylab("Relative expression") +
ggtitle("fad2")
fad2_tp

library(ggpubr)
time_point_graphs <- ggarrange(fad2_tp, Rel_tp, fatp_tp, Dif_tp, desat1_tp, Mtk_tp,
ncol = 2, nrow  = 3, common.legend = TRUE)
time_point_graphs
```

Figure S.6: Code to create the line graphs in Figure 11. *This code uses fad2 as the main example.*

```
setwd("~/Desktop/MScR/Data/code/")
library(ggpubr)
library(dplyr)

#Anti-microbial

Mtk_6hr <- read.csv("6hr_Mtk.csv")
Mtk_6hr$Relative_expression <- Mtk_6hr$Relative_expression
Mtk_6hr$Diet <-factor(Mtk_6hr$Diet, levels = c('L','S'), ordered = TRUE)
Mtk_6hr_bp<- ggboxplot(Mtk_6hr, "Diet", "Relative_expression", color = "Social_group",
ylim=c(0, 2), add = "jitter", bxp.errorbar = FALSE, bxp.errorbar.width = 0.4, notch = FALSE,
title = "Mtk") + labs(x = "Diet", y = expression("Relative Expression")) +
scale_color_manual(values=c("Blue","Red"), name="Social Group", labels=c("Alone",
"Grouped"))
Mtk_6hr_bp

#Anti-cannibalism

Fad2_6hr <- read.csv("6hr_Fad2.csv")
Fad2_6hr$Relative_expression <- Fad2_6hr$Relative_expression
Fad2_6hr$Diet <-factor(Fad2_6hr$Diet, levels = c('L','S'), ordered = TRUE)
Fad2_6hr_bp<- ggboxplot(Fad2_6hr, "Diet", "Relative_expression", color = "Social_group",
ylim=c(0, 0.8), add = "jitter", bxp.errorbar = FALSE, bxp.errorbar.width = 0.4, notch = FALSE,
title = "Fad2") + labs(x = "Diet", y = expression("Relative Expression (x10)")) +
scale_color_manual(values=c("Blue","Red"), name="Social Group", labels=c("Alone",
"Grouped"))
Fad2_6hr_bp
desat1_6hr_bp

bp_trans_6hr <- ggarrange(Fad2_6hr_bp, Rel_6hr_bp, fatp_6hr_bp, Dif_6hr_bp,
desat1_6hr_bp, Mtk_6hr_bp, ncol = 2, nrow  = 3, common.legend = TRUE)
bp_trans_6hr
```

Figure S.7: This shows the code used to create the box plots showing the effect of diet and social treatment on relative expression *(Figure 8, 9, and 10). This example only shows the code for Fad2 and Mtk at the 6h time point. The same code was used for each gene at each time point and then arranged into three Figures using the ggarrange line of code at the bottom of the Figure.*

```
setwd("~/Desktop/MScR/Data/code/cov/")
library(ggpubr)
library(dplyr)
fad2_cov <- read.csv("fad2_cov.csv")
fad2_cov
time_point.labs <- c("Virgin", "3-hour", "6-hour")
names(time_point.labs) <- c("0", "3","6")
fad2_cov$diet <-factor(fad2_cov$diet,
levels = c('L','S'), ordered = TRUE)
fad2_bp<- ggboxplot(fad2_cov, "diet", "cov", color = "social.group", ylim=c(0, 2.5), add =
"jitter", bxp.errorbar = FALSE, bxp.errorbar.width = 0.4, notch = FALSE, title = "Fad2") +
labs(x = "Diet", y = "Co-efficient of Variation") +
facet_wrap(~time_point, labeller = labeller(time_point = time_point.labs)) +
scale_color_manual(values=c("Blue","Red"), name="Social Group", labels=c("Alone",
"Grouped"))
fad2_bp

library(ggpubr)
time_point_graphs <- ggarrange(fad2_bp, Rel_bp, fatp_bp, Dif_bp, desat1_bp, Mtk_bp, ncol
= 2, nrow  = 3, common.legend = TRUE)
time_point_graphs
```

Figure S.8: This shows the code used to create the boxplots for the coefficient of variation *(Figure 12).*

```
setwd("~/Desktop/MScR/Data/code/")
fad2_V <- read.csv("V_fad2.csv")
fad2_V
fad2_V_anova <- lm(formula = Relative_expression ~ Diet + Social_group, data = fad2_V)
anova(fad2_V_anova)
```

Figure S.9: The line of code used to analyse the effect of social and dietary treatments on relative expression, *using a linear model and an ANOVA.*

```
#cov analysis

library(cvequality)
Mtk_6hr_cv_test<-
 with(Mtk_6hr,
    asymptotic_test(Relative_expression,
                Diet))
Mtk_6hr_cv_test

Mtk_6hr_cv_test2 <-
with(Mtk_6hr,
mslr_test(nr = 1e4,
Relative_expression,
Diet))
Mtk_6hr_cv_test2
```

Figure S.10: Example code used to test for the effect of diet and social environment on the coefficient of variation.

Table S.1: The effect of dietary (blue) and social environment (green), on the coefficient of variation of the relative gene expression *in flies for each gene of interest at each time point (significant p-values in* ***bold)***

Effect	Target gene	Time point	Test statistic	P-value
Diet	*Fad2*	V	0.014	0.907
		3h	- 0.005	1
		6h	0.192	0.661
	fatp1	V	1.634	0.201
		3h	0.917	0.338
		6h	0.198	0.657
	desat1	V	0.311	0.577

		3h	0.451	0.5018
		6h	0.841	0.359
	Dif	V	2.035	0.154
		3h	1.709	0.191
		6h	3.935	**0.047**
	Rel	V	1.169	0.28
		3h	1.692	0.193
		6h	0.091	0.763
	Mtk	V	0.151	0.698
		3h	3.328	0.068
		6h	0.03	0.862
Social	*Fad2*	V	0.128	0.721
		3h	0.216	0.642
		6h	0.082	0.774
	fatp1	V	3.714	0.054
		3h	- 0.002	1
		6h	1.956	0.162
	desat1	V	6.529	**0.011**
		3h	1.751	0.186
		6h	0.019	0.889
	Dif	V	5.360	**0.021**

		3h	0.042	0.838
		6h	8.178	**0.004**
	Rel	V	1.465	0.226
		3h	0.530	0.467
		6h	0.825	0.364
	Mtk	V	0.217	0.642
		3h	1.031	0.310
		6h	0.219	0.640

Table S.2: The highest and lowest level relative expression values in each treatment for the solitary and grouped flies *for each target gene, shown at the three different time points.*

		Fad2		fatp		Dif		Rel		Desat1		Mtk	
Time point	Social group	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
V	Alone	6.03E-05	0.0004	0.0287	0.1245	0.0113	0.0871	0.058	0.126	0.7773	1.2683	0.0094	1.0545
	Grouped	1.42E-05	0.002	0.0436	0.1001	0.0126	0.0421	0.0641	0.1028	0.629	1.9002	0.0034	1.306
3hr	Alone	8.03E-06	0.0006	0.0326	0.1205	0.0082	0.0373	0.0617	0.1592	0.5105	1.23	0.0079	7.3529
	Grouped	1.6E-05	0.0013	0.0338	0.1443	0.0096	0.0456	0.0501	0.1317	0.3766	1.8219	0.0209	1.4637
6hr	Alone	0.1844	0.6168	0.0418	0.1155	0.0071	0.0249	0.0748	0.1642	0.7929	1.5728	0.0249	1.3528
	Grouped	0.2604	1.011	0.0714	0.1303	0.0089	0.0771	0.0731	0.1432	0.8128	1.8903	0.0235	3.3589

***Table S.3: The highest and lowest level relative expression values for the low-nutrient diet and standard diet for** each target gene shown at the three different time points.*

		Fad2		fatp		Dif		Rel		Desat1		Mtk	
Time point	Diet	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
V	S	6.03E-05	0.002	0.0287	0.0872	0.0113	0.0398	0.058	0.1018	0.7773	1.804	0.0034	0.13
	L	1.42E-05	0.0008	0.0427	0.1245	0.0156	0.0871	0.0608	0.126	0.629	1.9002	0.0397	1.306
3hr	S	1.6E-05	0.0013	0.037	0.1205	0.0082	0.0278	0.0501	0.117	0.3766	1.3863	0.0079	0.2351
	L	8.02E-06	0.0011	0.0326	0.1443	0.0099	0.0456	0.0683	0.1592	0.5876	1.8219	0.0205	7.3529
6hr	S	0.2128	1.011	0.0452	0.1203	0.0071	0.034	0.0748	0.1642	0.7929	1.8903	0.0235	0.4953
	L	0.1844	0.7613	0.0418	0.1303	0.0153	0.0771	0.0731	0.1432	0.8416	1.6183	0.0268	3.3589

Proportion of eggs in clusters analysis

Excluding interactions:

3h

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			35	85.994		
diet	1	7.9210	34	78.073	3.8662	0.05772 .
social	1	5.7495	33	72.323	2.8063	0.10334

6h

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			47	349.07		
diet	1	57.576	46	291.49	10.7611	0.002006 **
social	1	7.172	45	284.32	1.3404	0.253067

Including interactions:

3h

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			35	85.994		
diet	1	7.9210	34	78.073	3.8995	0.05698 .
social	1	5.7495	33	72.323	2.8304	0.10223
diet:social	1	1.7876	32	70.536	0.8800	0.35523

6h

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			47	349.07		
diet	1	57.576	46	291.49	10.8430	0.001962 **
social	1	7.172	45	284.32	1.3506	0.251433
diet:social	1	6.679	44	277.64	1.2578	0.268155

Figure S.11: The effect of diet and social environment on the proportion of eggs found in clusters. *ANOVA outputs of the proportion of eggs analysis. This Figure shows 4 tables of the ANOVA outputs created in R (4.0.2). This includes the interactions between the independent variables which were removed due to no significance*

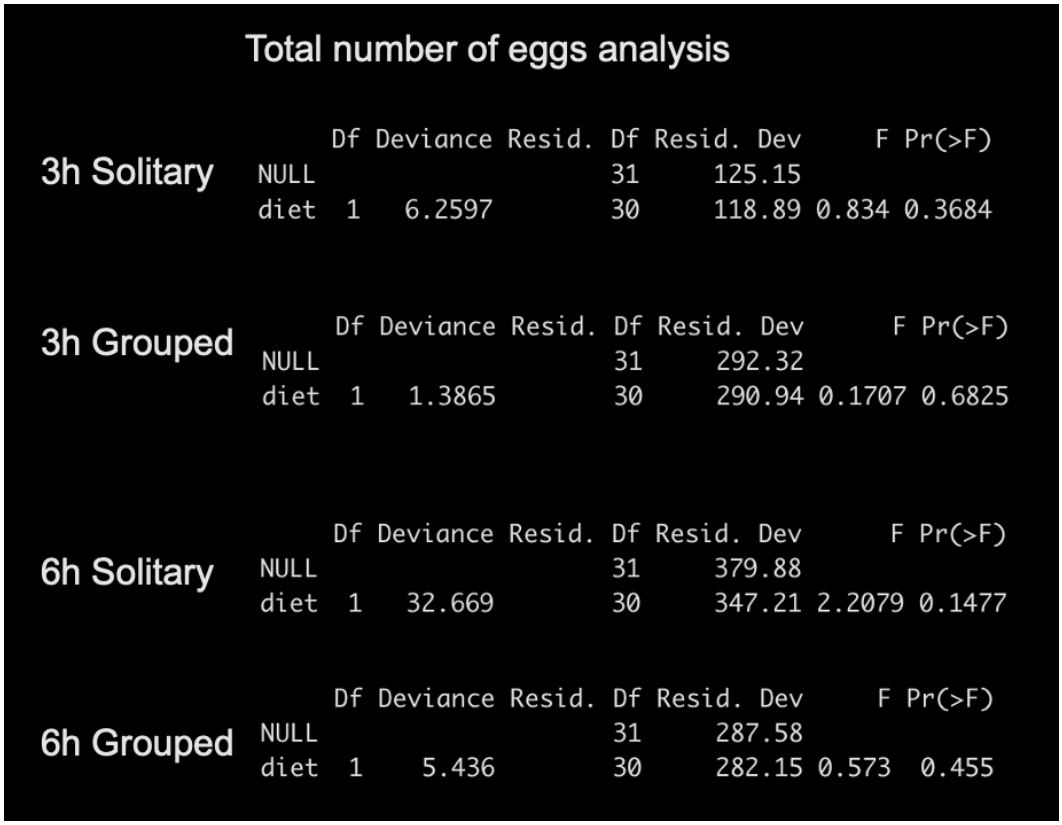

Figure S.12: the effect of diet on the total number of eggs. *ANOVA outputs of the total number of eggs analysis. This Figure shows 4 tables of the ANOVA outputs created in R (4.0.2).*

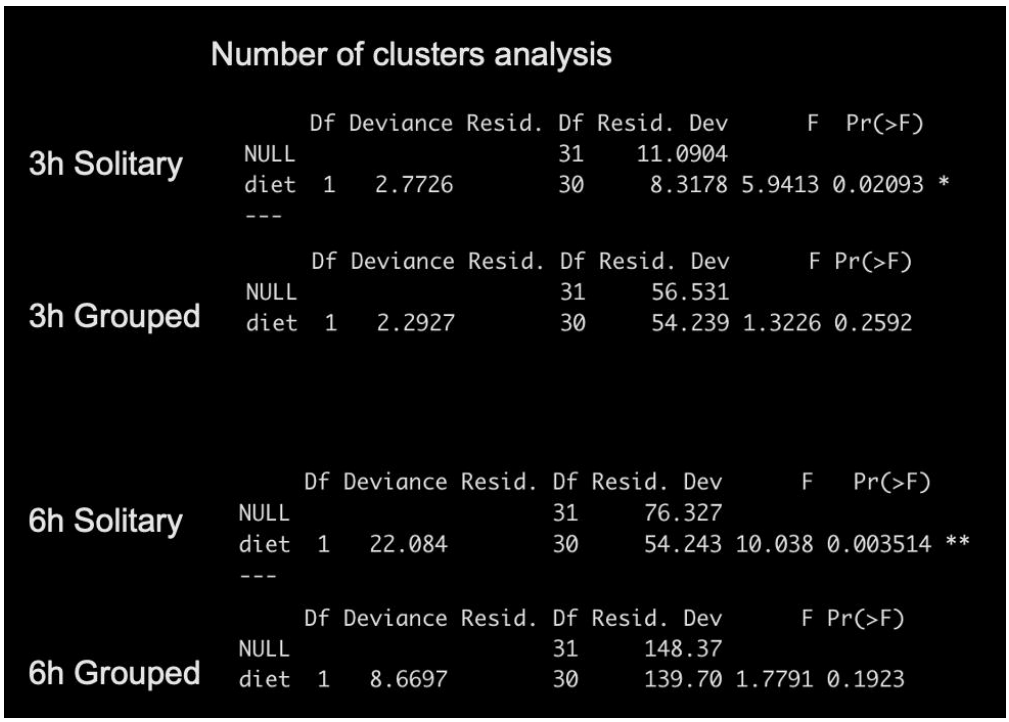

Figure S.13: The effect of diet on the number of clusters per vial. *ANOVA outputs of the number of clusters analysis. This Figure shows 4 tables of the ANOVA outputs created in R (4.0.2).*

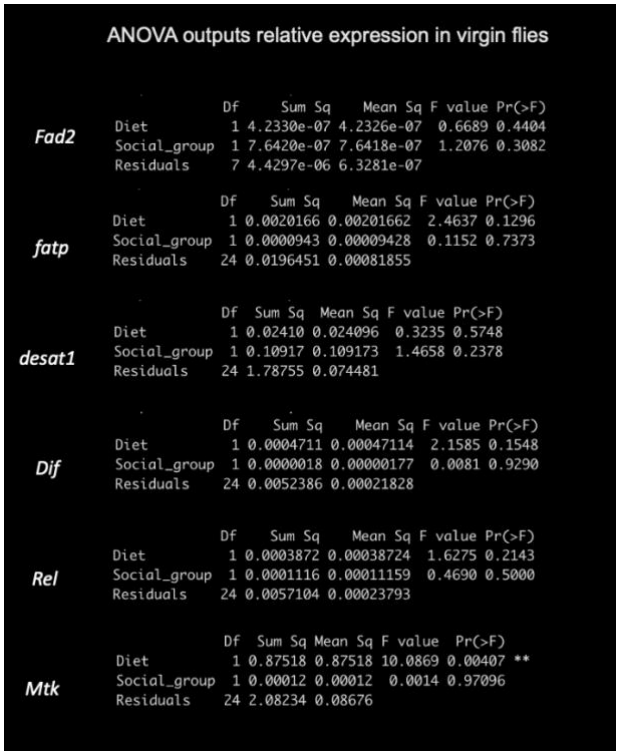

Figure S.14: The effect of Social group and diet on the relative expression levels in virgin flies. *ANOVA outputs of the number of clusters analysis. This Figure shows 6 tables of the ANOVA outputs created in R (4.0.2) from linear models.*

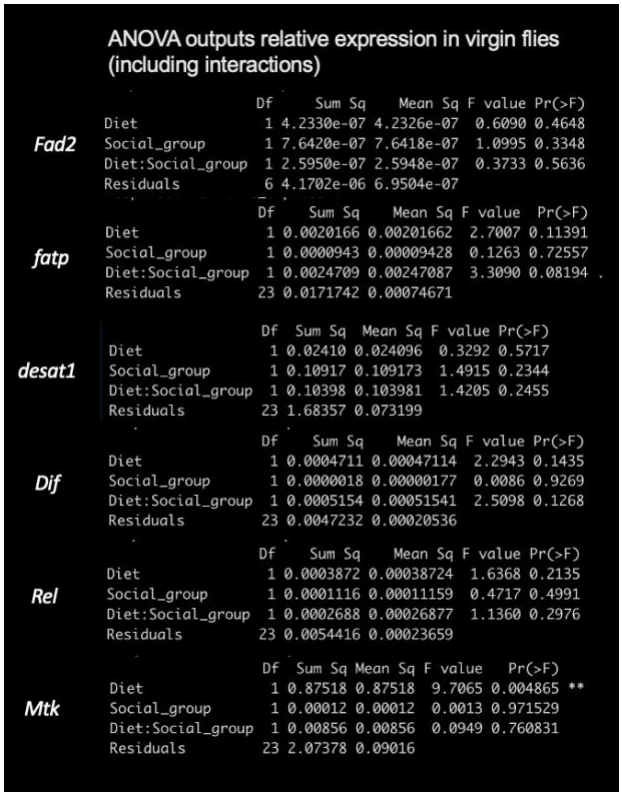

Figure S.15: The effect of Social group and diet on the relative expression levels in virgin flies (including interactions). *ANOVA outputs of the number of clusters analysis. This Figure shows 6 tables of the ANOVA outputs created in R (4.0.2) from linear models.*

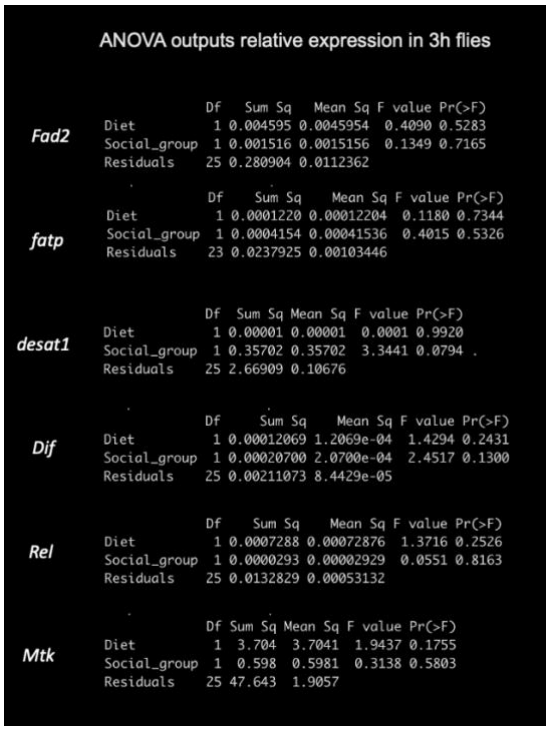

Figure S.16: The effect of Social group and diet on the relative expression levels in 3h flies. *ANOVA outputs of the number of clusters analysis. This Figure shows 6 tables of the ANOVA outputs created in R (4.0.2) from linear models.*

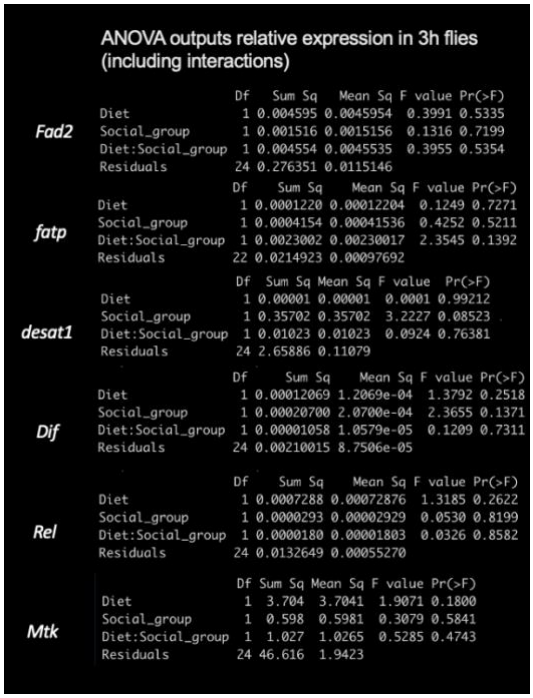

Figure S.17: The effect of Social group and diet on the relative expression levels in 3h flies (including interactions). *ANOVA outputs of the number of clusters analysis. This Figure shows 6 tables of the ANOVA outputs created in R (4.0.2) from linear models.*

Figure S.18: The effect of Social group and diet on the relative expression levels in 6h flies. *ANOVA outputs of the number of clusters analysis. This Figure shows 6 tables of the ANOVA outputs created in R (4.0.2) from linear models.*

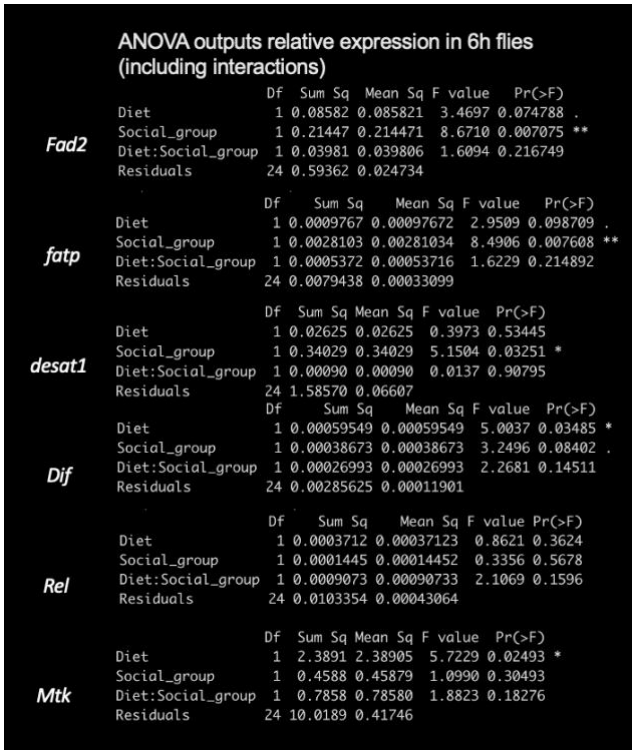

Figure S.19: The effect of Social group and diet on the relative expression levels in 6h flies (including interactions). *ANOVA outputs of the number of clusters analysis. This Figure shows 6 tables of the ANOVA outputs created in R (4.0.2) from linear models.*