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Happy or Hangry Families:

Does kinship mediate cooperation and cannibalism in *Drosophila melanogaster* larvae?

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Abstract:

The fruit fly *Drosophila melanogaster* has a long history as a research model for studies on behaviour and group dynamics. When individuals are grouped in an environment and resources decrease, then they may behave co-operatively with one another or antagonistically compete. Hamilton's Law states that if the benefit of helping a related individual out-weighs the cost to its direct fitness, then the individual's indirect fitness will increase when they help their kin compared to helping an unrelated stranger. Yet it is unknown whether kinship mediates larval behaviour to encourage co-operation and/or lessen antagonistic behaviours between consanguineous individuals. In this thesis I set out to examine and understand how kinship might be important to the evolution of sociality and anti-social behaviours in *Drosophila melanogaster* larvae. First, I set out to determine if kinship mediates the co-operative feeding cluster behaviour between 3rd instar larvae and whether there are evolutionary benefits to co-operating with kin over unrelated conspecifics in three separate assays. I changed the average degree of relatedness between individuals in the social environment and measured the characteristics of the feeding cluster (size, frequency and number of larvae) in the first assay. In the second assay I measured the weight and survivorship of the matured adult flies, and in the third assay I measured the proportion of related individuals in the clusters. From these assays, I was able to determine that the higher the degree of relatedness between individuals resulted: in increasing clusters with relatives, in increasing cooperation between related individuals in cluster formation, and evidence of benefits to co-operating with kin than strangers (with increased survivorship and weight in females as a predictor of fecundity). Secondly, I also examined how kinship might affect the expression of cannibalistic behaviour towards related individuals compared to unrelated conspecifics. I conducted assays with 2nd instar larvae, giving them the choice to cannibalize related eggs or unrelated eggs. I was able to determine that there is some evidence of cannibalism occurring with larvae more often eating unrelated eggs, however there were also some unexpected results which occurred. For example, there was no difference in the median time to the first incidence of cannibalism nor differences in the duration of the cannibalistic interactions. As such, more experiments need to be conducted to support this hypothesis and to better understand any underlining mechanisms that might be in play. Kinship selection might be the key to unlocking the underlining factors of why individuals behave pleasantly towards one another and antagonistically with rivals; and eventually understanding the genes behind those behaviours might also help us understand our own evolutionary origins of sociality.

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Chapter 1

Evolution of sociality and how kinship selection might mediate social and anti-social behaviour in *Drosophila melanogaster* larvae.

Abstract:

The evolution of sociality and social behaviours have been long standing concepts which researchers have been interested in understanding. When resources start to become depleted and the environment becomes harsher, groups of individuals will often either cooperate to access or compete with each other to acquire resources; in these conditions behaviours might be plastic responses depending on whom the individual is interacting with (either relatives or strangers). Hamilton's states that if the benefit to co-operating with kin members out-weighs the cost to the individual's direct fitness then the individual's indirect fitness will increase when they help their kin compared to helping an unrelated stranger. Currently *Drosophila melanogaster* has been used in many different studies to understand the evolution of sociality, social and anti-social behaviours; yet it is still unknown whether kinship might mediate larval behaviour. *Drosophila melanogaster* are known to exhibit both co-operative behaviours in the form of cluster feeding and antagonistic behaviours in the form of cannibalism; yet there might be differences in how individuals behave between consanguineal conspecifics compared to that of strangers. Understanding how kinship might encourage cooperation and lessen cannibalistic behaviours towards kin, helps to understand the underlining mechanism of social behaviours and perhaps how sociality might have evolved in groups and species.

Introduction:

"Let us try to teach generosity and altruism, because we are born selfish. Let us understand what our own selfish genes are up to, because we may then at least have the chance to upset their designs, something that no other species has ever aspired to do" (Dawkins 1976).

Like the genes in Richard Dawkin's quote, behaviour is a complicated subject and not fully understood, and often is dependent on the scientific area of study (Levitis, Lidicker, and Freund 2009; Dugatkin 2019). Levitis *et al.* (2009) define behaviours as "internal coordinated responses of whole living organisms to stimuli, excluding developmental response changes" Behaviours variation have some genetic basis, and are often adaptive (Dugatkin 2019), and increase the fitness of an individual. It is often the case that individuals will behave selfishly to maximize their fitness by both acquiring as many resources as possible and by passing on as many genes to the next generation (Dawkins 1976). Individuals can achieve this by either behaving agreeably with others (co-operating) or antagonistically (competing) (Dawkins 1976; Dugatkin 2019). Individuals often aggregate in groups to either mate, find resources, or decrease the chance of predation (Allee 1930). However, when resources start to become limited in the environment, individuals often become drawn into competition for the remaining resources, and this is the case with many insect species, such as *Drosophila melanogaster,* which are known to cannibalize each other when resources become limited (Richardson *et al.* 2010; Vijendravarma *et al.* 2013). However, it has also been observed in *Drosophila melanogaster,* that larvae form groups or feeding clusters to access resources that they are unable to acquire on their own (Dombrovski *et al.* 2017). However, in many species, kinship, the degree of relatedness between individuals (Hamilton 1964; Breed 2014), might decrease cannibalistic behaviour towards relatives (Bekoff 1992; Bilde and Lubin 2001; Bolívar-Silva *et al*. 2017) and kinship might promote co-operative behaviour. Therefore, in my thesis, I will focus on how kinship mediates co-operative and cannibalistic behaviour in *Drosophila melanogaster* larvae.

Evolution of Sociality and Behaviour:

Behaviour, and more importantly group behaviour, is one of many plastic phenotypes that have been used to understand the interaction of genes with environmental factors and the evolutionary history of species in general. One area which has been greatly benefited from the understanding of this interaction is the evolution of sociality, or the degree to which individuals associate and help with one another in conspecific groups (Lin and Michener 1972; Garcia and De Monte 2012; Procter *et al*. 2016). Many species have complex social systems, for example in ants and bees, colonies are often directed towards altruistic behaviours which benefit the entire colony as opposed to a single individual. These colonies often develop specialized castes such as workers to collect food and maintain the colony, or the larger warrior caste which defend the colony; these castes are needed in order for the colony to survive (Danforth 2002; Procter *et al* 2016). However, there is a cost of the individual's direct reproductive fitness as these specialized castes are often sterile or sacrifice their reproduction so that the queen can reproduce (Danforth 2002; Procter *et al* 2016). Yet, not all species have altruistic social systems; in many species the degree of social (co-operative) and anti-social (antagonistic) behaviours is dependent on the degree of relatedness between individuals in the population or group (Hamilton 1964; Pamilo 1984). In the case of marmots (*Marmota jlaviventris*), the social system differs depending on the condition of the environment, to either exploit resources that may become available or to avoid predation (Armitage 1999; Armitage 2012; Garcia and De Monte 2012). There is often a trade-off with some decrease in reproductive potential when individual females allocate resources to their own survival/ growth over egg production, or they trade off their development and survival in exchange for increased reproductive success via more eggs (Armitage 1998). To combat this problem, marmots behave more sympathetically by forming “kin groups” with multiple females co-operatively breeding and caring for the offspring together, which in turn actually increases the reproductive potential through direct fitness gains and allows for females to acquire the necessary energy to survive (Armitage 1999; Armitage 2012). In other social systems and species there are more mutualistic interactions and behaviours that occur which are in turn more selfish than altruistic. For example, in primates, many species evolved from harem troops of one male with many females to a social system of bond-pairs (Shultz, Opie, and Atkinson 2011). In this social system, individuals will group and be social (co-operative) but only if there is a benefit to doing so, such as predator avoidance or acquisition of resources (Shultz, Opie, and Atkinson 2011), and thus increase their survivability or reproductive fitness. Otherwise, individuals will not associate with other bond-pairs. Although social systems promote protagonistic behaviours, the individuals which aggregate into groups actually do so to gain selfish fitness benefits.

Group formation itself has advantages, however, there maybe some disadvantages to individuals form living in close proximity to other conspecifics. Possible disadvantages to aggregating include sharing of resources, conflict between conspecifics, or possibly attracting predators with more ease. However, as long as there is a genetic basis for these social behaviours to occur, such as specific genes which code for these behaviours to be beneficial and to promote some advantage to an individual’s fitness (Wilson 1975; Garcia and De Monte 2012), then these behaviours will occur as long as there is an increase fitness. Even in altruistic social systems these altruistic behaviours are thought to arise from selfish desires, as individuals will try to maximize their fitness both directly and indirectly (Hamilton 1964) rather than to simply help each other, such as in the case of directed or empathy-based altruism (De Waal 2008). One solution to the apparent disadvantages mentioned earlier is kinship selection, or kinship theory, which can be used to describe why individuals might behave selflessly or co-operatively towards other individuals despite it being beneficial to behave more selfishly, or in some cases aggressively, towards conspecifics. Hamilton (1964) proposed a set of ideas on kin selection and defined the conditions in which behaving selflessly and increasing another individual's fitness is beneficial. Hamilton proposed in 1964, that helping individuals would be beneficial if there is the increase in the individual's own inclusive fitness which out-weighs the direct fitness loss of helping that individual (Hamilton 1964; Breed 2014). Thus, helping kin would be more beneficial than helping a stranger as more similar genes will be passed on. However, for kin selection to be beneficial, it frequently requires individuals to be able to distinguish relatives from non-relatives since they would only gain indirect benefits from helping the former, rather than the latter (Hamilton 1964; Pamilo 1984; Breed 2014). It is important to note that kin recognition is not always needed for cooperation between individuals if there is a high certainty that individuals in their local environment are relatives. For example, in bird species where offspring are laid in nests of copulating pairs, siblings may be certain that they are related to each other based on being raised and laid in the same nest, assuming no egg-dumping was present (Hamilton 1964; Beecher 1988). Similarly, in bees and ant species, female queens lay their eggs in the colonies where they are heavily guarded and cared for (Pamilo 1984; Crosland 1989; Danforth 2002) which means that individuals are certain that they are co-operating with kin and therefore gain benefits. However, in species similar to *Drosophila melanogaster*, where either polyandry occurs readily (Childress and Hartl 1972; Arnqvist and Nilsson 2000) and/or multiple females lay their eggs in the same location, there may be a high level of uncertainty of who is a relative and who is a non-relative (Beecher 1988; Sokolowski 2001; Tinette, Zhang and Robichon 2004).

In *D.melanogaster*, where individuals do not know relatives from non-relatives just by where they were laid, kin recognition might be a necessary mechanism to determine who is a relative and this is fundamental in understanding how kinship might mediate both the social (co-operative) and anti-social (cannibalistic) behaviours individuals display to each other, based on how closely related individuals are in the population.

Kinship and Cooperation Behaviour:

There are several different theories why individuals will cooperate in groups that do not require kinship (Trivers 1971; Dugatkin 1997; Dugatkin 2019). ‘By-product mutualism’ is one such strategy in which a group will cooperate to perform a task that one individual could not do alone (Brown, 1983), but it does not require individuals to be related. In birds, such as blue jays and house sparrows, kinship recognition might not be necessary. Blue jays and sparrows call in order to signal where food can be found and recruit other individuals to feed (Summers-Smith 1963). This can be a form of ‘By-product mutualism’ because the more birds that are present in the feeding area results in a lesser chance that the founder bird will be killed by any nearby predators (Elgar 1986, Dugatkin 2002). In these scenarios, individuals benefit by finding resources and attracting one another, as the founder bird gains benefits in the reduction of the chance to be killed by predators as the finder bird attracts more conspecifics to itself, and the recruited conspecifics gain increased chances of finding resources and thus increased survivability. ‘Reciprocity’ is another system of cooperation which does not necessarily require kinship selection. Reciprocity requires several factors to be beneficial which include: 1. individuals forming coalitions or alliances, 2. the exchange of gifts or favours, and 3. the ability to remember individuals who have helped from those who have not (Dugatkin 2002). For example, in vampire bats (*Desmodus rotundas*), individuals would share food with starving members, and these starving individuals remember which bats helped them and later would assist those individuals if they require food (Wilkinson 1984, Wilkinson 1985, Wilkinson 1990). These individuals do not necessarily need to be related to benefit from co-operating with one another. Similarly, in primate species like baboons (*Papio anubis*), individuals would form coalitions with each other to combat rivals in mating and social encounters (Packer 1977; Croft *et al.* 2015) which greatly increases the chances of successful mating and acquiring resources by utilizing both group cooperation in addition to the reciprocal exchange of favours or gifts. This system is ideal in species that have long term needs to form groups and to develop these social relationships, however, in species with ephemeral social environments this system would not be as effective.

In *Drosophila melanogaster*, adult females lay their eggs on rotting fruits. The larvae which hatch from those eggs feed in this environment, and when resources start to become scarce, will sometimes aggregate to form co-operative feeding clusters (Dombrovski *et al.* 2017). These larvae cooperate in clusters in order to reach more nutritious food deeper in the media while also allowing for air to circulate by a tunnel (Dombrovski *et al.* 2017); this is done once they reach the 3rd instar (approximately 48-72 hours post hatching). At first glance ‘By-product mutualism’ or ‘reciprocity’ could be responsible for cooperation behaviour between *Drosophila* larvae, except that the clustering behaviour only lasts for a short time, about 18 hours, where clusters are constantly shifting (Flagg 1971; Dombrovski *et al.* 2017). Since *Drosophila* memory is primarily olfactory based, lasting no more than a few hours (Davis 2011), it is unlikely larvae are using reciprocity as a cooperation system, as there are far too many individuals in these shifting ephemeral environments for larvae to remember every possible favour and which to return at a later point in time. ‘By-product mutualism’ would suggest that the degree of relatedness (i.e. kinship) might not have an effect on the degree of individuals co-operating in clusters. This means that if the average degree of relatedness changed in the cluster, then there should be no difference between a population of individuals who are highly related compared to a population where individuals are unrelated or have a low degree of relatedness. Nevertheless, for ‘By-product mutualism’ to be a successful strategy there must be a degree of exploitation between individuals to maximize their fitness, while at the same time some insurance that individuals would not defect if given the chance (Dugatkin 2002). ‘By-product mutualism’ also creates an environment that may be more competitive than co-operative, which in turn might cause clusters to break apart more easily as individuals would either scramble to gain as many resources as possible, or by interfering with other individuals’ access to these resources (Bakker 1959, 1961; De Jong 1976). Thus, ‘By-product mutualism’ as a co-operative strategy alone might not accurately describe the co-operative clustering behaviour, but in combination with kin selection it might be an effective strategy which *D.melanogaster* larvae could employ to gain both direct and indirect benefits.

The ability to recognize kin may be crucial to understanding how individuals interact with one another in populations or groups where many unrelated females lay eggs in close proximity with each other, and why kin selection might be used as an effective strategy to gain any indirect benefits from co-operating with relatives. As mentioned before, *Drosophila* *melanogaster* are a species where many unrelated females lay eggs in close proximity to one another and thus both relatives and strangers might be present in clusters. If this is the case, then there needs to be some mechanism for individuals to recognize kin if there is going to be any kinship selection. In previous studies on *D. melanogaster* it has been suggested that larvae are capable of using both taste and smell to recognize individuals (Scherer, *et al.* 2003; Khurana and Siddiqi 2013; Apostolopoulou *et al*. 2015). Adult flies have been observed to behave differently to individuals based on the scent or taste of their gut microbiome and aromatic hydrocarbons, which is inherited from their mothers (Lizé *et al.* 2013) and in larvae there are the *ppk* genes that can detect maternal hydrocarbon cues on eggs, which can similarly effect larval behaviour depending on the scent (Liu *et al.* 2018). However more recently, vision, in combination with mechanosensory cues, such as body contractions, may also be a way for larvae to recognize each other (Dombrovski *et al.* 2017). Dombrovski *et al.* in their 2017 study, used visually impaired mutant larvae and in addition to genetically altered the mechanosensory genes in other larvae (that were also raised in isolation) after which the larvae were transferred to a new cluster. They found that the visually impaired mutant larvae and mechanosensory altered larvae both had different motor profiles (contraction lengths and rates) from control larvae, which meant that these larvae were incapable of co-ordinating and co-operating in clusters (Dombrovski *et al.* 2017). Dombrovski *et al.* (2017) also theorized that motor profile matching was the key for co-operative behaviour to be successful (Dombrovski *et al.* 2017), and this could be the mechanism for kin selection to occur. Any combination of these sensory systems might be used to recognize kin; thus, kinship selection in combination with ‘By-product mutualism’ might be the system used in clustering behaviour to gain both direct benefits and indirect benefits from co-operating with kin. In addition, since there is a genetic basis to many behaviours, including sensory behaviours and foraging behaviours (Sokolowski 2001), these behaviours might encourage relatives to cooperate with one another more often than with unrelated individuals because they will be more compatible. Kinship selection and 'By-product mutualism' might be the reasons for *Drosophila* larvae to form feeding clusters and cooperate with each other when resources become more toxic or scarcer, but this might also instead lead to conflict between individuals.

Kinship and Anti-social (Cannibalism) Behaviour:

While scarcity of resources in the environment can lead to individuals co-operating (as described above), more often it leads to individuals to compete and for conflict to occur. Conflict over resources is ubiquitous, but the degree of conflict and where it is directed, varies from species to species. In ant colonies *(Rhytidoponera confusa*), there is a difference in the level of aggression between related individuals and strangers (Crosland 1989). In Crosland's study (1989), the degree of aggression varied between different ant colonies and the number of attacks was different for related and unrelated ants. More importantly, Crosland (1989) stated that there might be a genetic component to this aggressive behaviour, which means that if kin discrimination (or kin selection) was present then related individuals might behave very similarly towards each other in comparison to strangers. Food deprivation is one such factor that might cause conflicts and cannibalism in other species, such as in the larvae of the granary weevil, *Sitophilus granaries*, and the maize weevil, *Sitophilus zeamais* (Bolívar-Silva *et al.* 2017). In these weevil species, many unrelated females often lay their eggs in close proximity to each other on the grain kernels, and once the eggs hatch, they will engage in contest competition for resources (Bolívar-Silva *et al.* 2017). Bolívar-Silva *et al.* (2017) tested whether density and different species of weevils leads to cannibalism and found evidence that higher levels of aggression and cannibalism occurred between larvae at higher larval and egg densities. High population densities can cause the limited resources in an area, such as grain kernels, to deplete more quickly, which in turn could cause the weevils to resort to trying to acquire resources from other means as their environment becomes harsher.

The migratory desert locust, *Schistocerca gregaria,* is a great example of how harsh environmental conditions coupled with high population densities could result in cannibalistic behaviours. Every couple of years during the gregarious phase, vast amounts of these pests converge to form highly dense feeding groups (Bazazi *et al.* 2008; Richardson *et al.* 2010). These groups or travel long distances to search for food (Ashall and Ellis 1962; Van Huis *et al.* 2008). However, due to the harsh conditions of the deserts, (i.e. droughts or the loss of their limited food supplies) causes the need for nymphs and adults to find resources in other ways, namely each other. The locusts cannibalize each other to acquire necessary resources such as salts, nitrogen, and proteins (Simpson *et al.* 2006; Bazazi *et al.* 2008; Richardson *et al.* 2010). Cannibalism can be beneficial as it can be a fast way to gain resources that would be otherwise not available, also it can serve to decrease the number of conspecifics and future competitors from a population (Richardson *et al.* 2010). However, cannibalism can also be detrimental, as even in *S. gregaria,* the nymphs run the risk of injuring themselves when they cannibalize conspecifics and from being cannibalized themselves by other larger individuals (Richardson *et al.* 2010; Vijendravarma *et al*. 2013). One other issue which might arise from the cannibalism of conspecifics would be consuming kin members. Consuming kin members would be problematic as any potential indirectly gained benefits of shared traits would be lost (Hamilton 1964; Richardson *et al* 2010; Breed 2014). To prevent this issue of losing indirect fitness benefits, kin selection coupled with kin recognition can be used to selectively cannibalize unrelated conspecifics. Although, whether or not kinship does mediate cannibalism in species like *Drosophila melanogaster* larvae is still not known.

In recent years *Drosophila melanogaster* has been used as a model species to study behaviour and the evolution of sociality between individuals (Wu *et al.* 2003; Sokolowski 2010; Camiletti and Thompson 2016; Durisko *et al*. 2014) but fruit fly larvae have also been shown to exhibit cannibalistic behaviours (Vijendravarma *et al.* 2013; Ahmad *et al.* 2015; Yang 2018). Larvae in their 2nd instar phase (approximately 32-48h post hatching) have been recorded feeding on adult carcasses, on 3rd instar larvae (Vijendravarma *et al.* 2013; Yang 2018), and on eggs (Ahmad *et al.* 2015; *but see* Narasimha *et al.* 2019). Since females aggregate to lay their eggs in rotting fruits, once their offspring hatch and start feeding they will consume the locally available resources and cause the environment to become depleted and deteriorated. These conditions can lead to the expression of cannibalistic behaviours. Although there has been much research on kin mediated cannibalism in other species (Bilde and Lubin 2001; Bayoumy and Michaud 2015; Barkae, Scharf and Ovadia 2016; De Nardin *et al.* 2016; Saitoh and Choh 2018), it is currently unknown if kin selection mediates the cannibalistic interactions to reduce or prevent cannibalism of related conspecifics compared to unrelated ones in *Drosophila melanogaster* larvae. In addition, there is little known about kin selection in *D. melanogaster larvae.*

Thesis Objectives and Predictions:

In studying the effects of kinship on both co-operative clustering behaviour and antagonistic cannibalistic behaviour, a better understanding of the evolution of sociality and anti-sociality can be attained. I chose to focus on *Drosophila melanogaster* larvae as there is evidence of both cooperation, in the form of feeding clusters, (Dombrovski *et al.* 2017) and cannibalism in larvae (Vijendravarma *et al.* 2013; Yang 2018; Narasimha *et al.* 2019). In addition, there is a body of evidence to support that many behaviours, including the *foraging/dg2* genes responsible for foraging, have a genetic component (Sokolowski 2001). This signifies that behaviours could be inherited and can be cross generational. Since the fruit fly genome has been sequenced, we can determine which genes are associated with their subsequent behaviours (Camiletti and Thompson 2016). *Drosophila melanogaster* populations can be manipulated by genetic cues, social cues, and/or environmental cues, which means we can study the interactional effects of these cues on behaviour. Other potential factors which could be understood from studying the effects of kinship on behavioural expression, is how they might be affected by both biotic predators, such as other conspecifics in the social environment or predatory wasps (Sokolowski 2001; Vijendravarma *et al.* 2013) and the risk of starvation due to resource loss. To date, there have been very few studies on the affects kinship has on the behavioural spectrum of *Drosophila melanogaster* larvae and the underlining mechanism that mediates cannibalistic or co-operative interactions. The focus of my master's study research will be to determine how social group dynamics change with the presence of relatives, and whether or not kinship will lead to more co-operative behaviours and less cannibalism towards related individuals in *Drosophila melanogaster.*

The objective of my master's research thesis will be to test if kinship mediates cannibalism and co-operative behaviours in *Drosophila melanogaster* larvae. Specifically, kinship promotes co-operative feeding behaviours, and individuals gain more evolutionary fitness benefits from co-operating with relatives more than with unrelated individuals in *Drosophila melanogaster* 3rd instar larvae. Thus, I predict that if kinship does mediate cooperation among relatives then there should be more cooperation and benefits between relatives compared to unrelated individuals. If kinship does not mediate co-operative behaviours, then there should be no difference between co-operating with relatives compared to unrelated individuals. In addition, I will test if kinship mediates cannibalistic behaviour in *Drosophila melanogaster* 2^{nd} instar larvae. I predict that if kinship does mediate cannibalism, then 2^{nd} instar larvae will behave differently towards related eggs then compared to unrelated ones; in which there will be less cannibalism of related eggs and that there should be a difference in the amount and time of interactions between the two types of eggs.

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Chapter 2:

Kin recognition and co-operative foraging in *Drosophila melanogaster* larvae

Abstract:

A long-standing goal for biologists and social scientists is to understand the factors that lead to the evolution and maintenance of co-operative behaviour between conspecifics. To that end, the fruit fly, *Drosophila melanogaster*, is becoming an increasingly popular model species to study sociality, however, most of the research to date has focused on adult behaviours. In this study, we set out to examine group feeding behaviour by larvae, and to determine whether the degree of relatedness between individuals mediates the expression of this co-operative behaviour. In a series of assays, we manipulated the average degree of relatedness in groups of third instar larvae that were faced with resource scarcity, and measured the size, frequency and composition of feeding clusters, as well as the fitness benefits associated with co-operation. We found evidence that suggests *D. melanogaster* larvae are capable of kin recognition (something that has not been previously described in this species), as clusters were more numerous, larger, and involved more larvae, when more closely related kin were present in the social environment. These findings are discussed in the context of the correlated fitness-associated benefits of co-operation, the potential mechanisms by which individuals may recognize kin, and how that kinship may play an important role in facilitating the manifestation of this co-operative behaviour.

Introduction:

As resources become limited in an individual's environment, it is faced with the increasing prospect of conflict with local conspecifics. While engaging in such competition (be it directly or indirectly) is a common response to scarcity, it is not an inevitable outcome. Indeed, in some species, rather than act antagonistically towards others, some individuals will engage in co-operative behaviours in order to acquire more resources (Dugatkin 1997). Such behaviours come at a cost to the interacting individuals but can yield a net benefit to the participants. Understanding the underlying factors that lead to the emergence and evolution of co-operation in certain species has been a long-standing goal of both biological and social scientists. Of the four major (but not necessarily mutually-exclusive) pathways that have been developed to explain co-operation: reciprocity, 'By-product mutualism', group selection and kinship (Dugatkin 2002), the latter – kinship theory (Hamilton 1964) – has yielded the greatest insights into cases of apparent altruism in many different species and contexts (West-Eberhard 1975; Smith 2004; Foster *et. al* 2006; Marshall 2016). In this model, individuals gain both direct fitness benefits from the joint prosocial activity, as well as accruing indirect fitness benefits via the positive impact of co-operation on the survival and reproduction of relatives, with greater degrees of co-operation favoured as the relatedness between individuals within groups increases (Hamilton 1964; West-Eberhard 1975). Thus, by examining the expression of co-operative behaviour across social groups differing in their degree of consanguinity, we can better understand how social behaviours evolve.

The fruit fly, *Drosophila melanogaster*, has a long and distinguished history as a model organism in scientific studies of genetics, development and behaviour (Weiner 2000; Brookes 2001). In recent years, it has also been championed as an excellent species in which to study *social* behaviour, due to their wide range of behavioural phenotypes, the plastic expression of these behaviours, and the availability of numerous tools to examine the genetic and neurobiological underpinnings of these behaviours (Wu *et al.* 2003; Sokolowski 2010; Durisko and Dukas 2013; Durisko *et al*. 2014; Camiletti and Thompson 2016; Lihoreau *et al.* 2016). While most studies of social behaviour have focused on adult flies (*e.g.* Tinette *et al.* 2004; Simon *et al.* 2011; Billeter *et al.* 2012; Martin and Long 2015), there is also great potential insight into the evolution of co-operation which can be gained from examining behaviour at the larval stages (*e.g.* Wu *et al.* 2003; Durisko *et al.* 2014). In the wild, *D. melanogaster* seek out decaying fruits, vegetables, and other plant matter as a site for feeding, mating and/or ovipositing of eggs (*see* Reaume and Sokolowski 2006; Markow and O'Grady 2008; Markow 2015; Soto-Yéber *et al*. 2018). Once the eggs have hatched, larvae have limited mobility, and typically are restricted to foraging in the area where their mothers oviposited them (Sokolowski 1985). As time passes, the cumulative feeding of the growing larvae, the accumulation of their excreted waste products, the effects of secreted digestive enzymes, and the continued microbiotic activity in the environment, leads to a progressive loss of suitable forageable resources near the surface, a pattern that is also seen in lab-raised populations (Gordon and Sang 1941; Sang 1949; Gregg *et al.* 1990; Ashburner *et al.* 2005; Louis and de Polavieja 2017). Additionally, these processes contribute to the increasing liquification of the upper layers of the environment, which prevents individuals from easily accessing any nutrients buried deeper down, due to the instability of air passages (Dombrovski *et al.* 2017). Faced with the risk of starvation, some larvae will engage in cannibalism, consuming conspecific larvae (Vijendravarma *et al*. 2013) and/or eggs (Ahmad *et al*. 2015; *but see* Narasimha *et al.* 2019). However, an alternate strategy adopted by some third-instar *D. melanogaster* larvae is to aggregate into foraging groups, wherein individuals, using both visual and mechanosensory cues, synchronize their digging behaviours (Wu *et al.* 2003; Justice *et al*. 2012; Dombrovski *et al.* 2017). Together, the cluster of larvae is collectively able to dig a well (*sensu* Louis and de Polavieja 2017) that allows access to the deeper, more nutritious, regions of their environment, while still allowing respiration (through their posterior breathing spiracles) via a common air cavity (Dombrovski *et al.* 2017). A recent study by Dombrovski *et al.* (2017) demonstrated that motion in clusters is highly co-ordinated and is necessary for the formation of large, stable clusters. In their assays individuals that were blind or socially isolated (during a critical period in which they acquire visual recognition of the movements of other larvae), did not integrate effectively into cluster groups, likely due to different motor profiles that impeded their ability to move in unison. Based on their findings, the authors suggested that membership into clusters may be selective and could potentially be limited to "…those larvae who were together during a distinct developmental period … or even sibs" (Dombrovski *et al.* 2017).

Female fruit flies are capable of laying many eggs in a short period of time (*e.g.* Long *et al.* 2006), so there is great potential that individual larvae *may* have the opportunity to interact with kin (of varying degrees of relatedness). While kin recognition is not, strictly speaking, required for co-operation between relatives (as long as individuals are more likely to interact with kin than non-kin; Pizzari *et al*. 2014), due to *D. melanogaster* females' tendency towards polyandry (Markow 2002), and their aggregation at ovipositing sites (Hoffmann and Nielsen 1985; Markow and O'Grady 2008; Schwartz *et al.* 2012), there is likely considerable variation in the familial composition of the social environment, both in the lab and in the field (Chippindale *et al.* 2015). Thus, the presence of a plastic, kin-based, co-operative response to conspecifics when faced with a nutritional challenge in this species likely requires some form of mechanism(s) for kin recognition. Adult *D. melanogaster* appear to have the capacity to recognize kin, which may be based on cues associated with the specific gut microbiome community arising from the local environment (*e.g.* Lizé *et al*. 2014; Carazo *et al*. 2015; Le Page *et al*. 2017; *but see* Chippindale *et al.* 2015; Martin and Long 2015). While larvae are able to recognize each other as conspecifics using vision, smell, mechanosensation, and gustation (*reviewed in* Dombrovski *et al.* 2017), to the best of our knowledge there has been no published evidence that larvae have the ability to distinguish kin from non-kin. In this study, we set out to examine whether the expression of *D. melanogaster* larvae's co-operative foraging behaviours was mediated by the degree of kinship present in their environment. We did so by testing whether larval co-operative feeding behaviour, the size and/or membership of feeding clusters varied in response to experimental changes in the degree of consanguinity within a local social environment. Furthermore, we quantified the benefits associated with co-operative foraging, so as to better understand how kin selection may have shaped the evolution of this behaviour.

Materials and Methods:

Population History and Maintenance

The focal *Drosophila melanogaster* individuals used in our assays were obtained from the outbred, *wild-type*, population *Ives* (hereafter 'IV'), which originated from a sample of 200 mated females collected in Amherst MA, USA in 1975 (Rose 1984). In addition, we also used competitors from the IV-*bw* population, which was created through the introgression of the recessive, brown-eyed allele, *bw* into the IV genetic background via repeated back- crossing. Both populations are maintained, in non-overlapping generations, at large size (~3500 adults per generation) under standardized conditions (incubated at 25ºC, 60% RH on a 12hr-L:12hr-D diurnal light cycle) (Martin and Long 2015). Flies are housed in standard narrow *Drosophila* vials (95mm H x 28.5mm OD; VWR International, 75813-160) containing 10mL of a banana, killed-yeast and agar media, set at an initial density of approximately of 100 eggs/vial and are cultured *en masse* every 14 days (Rose 1984, Martin and Long 2015). Egg-to-adult survival rates for IV and IV-*bw* flies are the same, and larvae in both populations exhibit very similar group feeding behaviours (Khodaei *et al.* 2019)

Establishment of kinship treatment groups

In order to determine whether the behaviour and/or development of larval *D. melanogaster* were influenced by the degree of within-group relatedness present in their social environment, we experimentally manipulated the average kinship of target IV individuals within in vial. We did so by creating families by mating virgin IV females (collected within ~8h of their eclosion from pupae) individually to a single IV male, and then left them to oviposit in a vial for about 48h before being discarded. Starting 8-9 days later, we collected males and females as they eclosed as virgins, from each of the family vials, and housed them in same-sex groups. Using these flies, as well as similarly-aged virgin IV females, collected from the stock population, we established three experimental treatment groups of differing in their overall degree of relatedness: high, intermediate, and low (for our estimates of means and variances of the coefficients of relatedness present in each treatment group, please refer to *Supplemental File 1*). To create individuals for the high relatedness treatment, we combined 5 virgin sisters (females from the same family) with 5 brothers (males obtained from a different family). The resulting offspring have relatedness coefficients ranging from a maximum of 0.5 (for full siblings) to a minimum of 0.25 (for double-first cousins). To create individuals for the intermediate relatedness treatment, we combined 5 brothers from the same family with 5 unrelated females from culture populations, which yielded offspring with relatedness coefficients varying from a maximum of 0.5 (for full siblings) to a minimum of 0.125 (for first cousins). To create individuals in the low relatedness treatment, 5 unrelated IV females were mated with 5 unrelated IV males which yielded offspring with relatedness coefficients of a maximum of 0.5 (for full siblings) to a minimum of 0 (for completely unrelated individuals). In all treatments, males and females were combined in a well-yeasted vial and given a minimum of 18-24h to mate (and potentially remate) before being used to generate offspring used in our assays.

Experiment 1: Characterization of feeding clusters

In this first experiment, we set out to describe the characteristics and frequencies of feeding clusters manifested in vials in which individuals differed in their mean degree of relatedness to each other. We did so by first establishing our experimental vials (32 replicate vials for each of the high and intermediate treatments, and 40 vials for the unrelated treatment) by transferring (without anesthesia) the adult egg-laying flies into fresh vials containing 10ml of media for 18h. At that time flies were discarded, and the number of eggs was trimmed by hand to a density of 200 eggs/vial. This is twice the typical larval density of the IV population and was done to expedite the depletion of the nutritional resources near the media surface. Starting 24h after vial establishment, we observed the clustering behaviour of larvae during three sessions, spaced three hours apart, on each of the three consecutive days. Measurements were taken from every vial in each session (within a 30 minute window of each other) by 4-5 well-trained observers who were blind to the nature of the treatment groups. Observers were haphazardly assigned vials to measure in each of the sessions, to ensure that there were no systematic biases in which observer measured which vials. During each session, we counted the number of feeding clusters present in the vial, which was defined as a grouping of 3 or more larvae which were all feeding in a downward direction creating a depression in the food media, which were visible along the side of the vial. Clusters were defined as having ended when the larvae were no longer feeding in a co- ordinated manner and the depression had dissipated/collapsed upon itself. Feeding clusters were defined as being separate from one another if there was evidence that larvae in the two groups were not working together (*i.e.* oriented in opposite directions) and separated by at least 0.5mm between the two groups. For those clusters that formed along the side of the vial, we measured, from the outside of the vial, the width (the distance from one side of the foraging group to the other side at the food surface level) and the depth (the distance from the food surface level to the lowest point in the foraging group) of each the clusters using a caliper to the nearest 0.5 mm. We also counted the number of larvae along the contour of the feeding cluster that were visible through the transparent wall of the vial as an index of how many larvae were co-operating.

Experiment 2: Assessing consequences of larval co-operative behaviour on survivorship and development

In this second experiment, we set out to quantify how co-operative feeding between larvae may impact the growth and survival of *D. melanogaster* larvae. We did so by establishing experimental crosses, as described above with 30 replicate vials for each of the high, the intermediate, and the low treatments by transferring (without anesthesia) the adult egg-laying flies for ~18h overnight into oviposition chambers. These chambers were constructed from inverted half-pint polypropylene bottles (Genesee Scientific; 32-130) which covered petri dishes (35mm diameter; Nunc, 12-565-90) containing grape juice/ agar medium (Sullivan *et al.* 2000) for ovipositing. The next morning, all adult flies were discarded, and we transferred 50 eggs from each replicate dish into a new vial (containing 10ml of media) along with 150 similarly- aged eggs obtained from the IV-*bw* stock into each of the vials. These vials were then placed in an incubator for 13 days. The number of adult *wild-type* (red-eyed) flies present in the vial was then counted to measure egg-to-adult survivorship, and 3 males and females from a subset of these vials (30/treatment) were collected and immediately frozen for later weighing. Prior to weighing, flies were dried overnight in a 70°C oven. We weighed flies on a Sartorius M5 microbalance (Sartorious, Gottinger, Germany) to the nearest 0.001mg, and calculated the mean weights of males and females for each of the vials.

Experiment 3: Measuring feeding cluster membership as it relates to kinship

To further determine if larvae are more willing to feed co-operatively with relatives than with unrelated individuals, we conducted a third assay, by establishing experimental vials in the exact same manner as described for experiment 2 above. This experiment consisted of 60 replicate vials in the high treatment, 59 vials in the intermediate treatment, and 47 vials in the low treatment. Starting approximately 30 hours after the vials were established (when larval age is about 48 hours) and feeding clusters start to form, we used a spatula to carefully scoop out the larvae present in one randomly chosen cluster per vial and placed them into a new vial containing 10ml of media. Vials were returned to the incubator for 13 days, at which time all eclosed adults were removed. The number and sex of (target) *wild-type* (red-eyed) flies were counted, as well as the number of (competitor) brown-eyed flies.

Statistical Analyses

All statistical analyses were performed using R version 3.3.2 (R Core team 2016). In all analyses we used vials as the unit of replication. For our first experiment, we compared the frequency of cluster formation using a generalized linear model (GLM), with quasipoisson error distribution, where the independent variable was the relatedness treatment and the dependent variable was the number of clusters in each vial across all sessions. We determined whether the treatment means were different from each other using a likelihood-ratio Chi-square test using the *Anova* function in the *car* package (Fox and Weisberg 2011), followed by a Tukey HSD test implemented by the *glht* function in the *multcomp* package (Hothorn *et al*. 2008) to determine the specific location of differences between treatments. We estimated the size of each of the cluster's depression observed as the area of a triangle using our width and depth measurements, and then calculated the mean cluster depression areas measured across all sessions. As not all the data from the treatment groups was normally-distributed (determined using a Shapiro-Wilks test), so we decided to compare the position of their medians using a Kruskal-Wallis rank-sum test, followed by a Dunn's post-hoc comparison using the *kruskalmc* function in the *pgirmess* package (Giraudoux 2016) to identify specific differences between treatments. As the mean number of larvae present in clusters met parametric assumptions, we used Analysis of Variance (ANOVA), followed by a Tukey HSD test to compare the means of the three relatedness treatments.

For the second experiment, the survivorship was analyzed using a GLM with a quasibionomial error distribution, where the response variable was the number of larvae (out of the initial 50) that survived to adulthood, and the independent variable was the relatedness treatment. The above statistics were followed up with a likelihood-ratio Chi-square test and

Tukey HSD post-hoc tests to determine if the means were similar across treatments. Before analyzing the weight data, we first calculated the means for each of the males and females collected from each vial to avoid pseudo-replication. Mean weights of males and females were compared between relatedness treatments using ANOVA and Tukey HSD post-hoc tests.

For the third experiment, we compared the composition of the sampled clusters between treatments using a GLM with quasibionomial error distribution, where the response variable (fraction of all eclosed offspring that had red eyes), and the independent variable was the relatedness treatment. We also analyzed the sex-ratio of the wild-type (IV) adults present in each vial using a GLM with quasibionomial error distribution. For both these models, the significance of differences in treatment means were assessed using likelihood-ratio Chi-square tests followed by Tukey HSD post-hoc tests (if necessary).

Results:

In our first experiment, we set out to determine if differences in overall group relatedness was associated with the difference in the frequency, size and/or membership of feeding clusters. The mean total number of clusters differed between treatments GLM: LR χ^2=59.5, df=2. p=1.212 x10^{-13}), with more clusters observed in the high and medium treatments compared to the low relatedness treatment (Figure 1a). The median size of the cluster depressions also varied between the relatedness treatments (Kruskal-Wallis test: χ^2= 11.0, df = 2, p-value = 0.004), with larger clusters in vials where there was higher overall relatedness between larvae (Figure 1b). The mean number of larvae present in the clusters also differed between treatments (ANOVA: Treatment, $F_{2,101}$=16.45, p=6.54x10^{-7}) with progressively more larvae observed in clusters in vials of increasing average relatedness (Figure 1c).

In our second experiment, we observed that the mean survivorship differed between treatments (GLM: LR χ^2 =10.8, df= 2, p=0.005) with more individuals successfully reaching adulthood in the high relatedness treatments compared to the other two treatments (Figure 2a). The mean weight of both females and males differed significantly across treatments (ANOVA: ♀: $F_{2,87}$= 19.18, p=1.26x10^{-7}; ♂: $F_{2,87}$= 6.44, p=0.002). Females developing in vials with a greater overall degree of relatedness were heavier (Figure 2b), while for males, those from the intermediate treatment weighed more, on average, than those from the low treatment, while the average weight of males from the high treatment did not differ significantly from either of the other two groups (Figure 2c).

In our third experiment, we examined whether varying the mean degree of relatedness of target (*wild- type*) flies influenced the probability that these flies would participate in a feeding cluster. Overall, the number of larvae that were collected from our sampled clusters did not differ between treatments (GLM: LR χ^2 =3.3, df= 2, p=0.19), however the mean proportion of larvae in each sample that were *wild-type* did exhibit significant differences between treatments (GLM: LR χ^2 =11.4, df= 2, p=0.003). Specifically, post-hoc testing revealed that as the average relatedness of wild-type offspring increased in a vial, so did their representation in the feeding cluster (Figure 3). No significant differences in sex ratios were observed between treatments (GLM: LR χ^2 =2.72, df=2, p=0.256).

Discussion:

In this study, we set out to determine whether the phenomenon of larval group foraging behaviour in *D. melanogaster* was mediated by the degree of relatedness between individuals in their social environment. We found that the magnitude of expression of this co-operative behaviour differed between larval groups in which the extent of kinship had been experimentally manipulated. In our first assay we saw an overall pattern that in treatments with higher overall degrees of relatedness, that clusters were more frequently observed, and were larger in overall size and membership. This pattern was also seen in our third assay where we found that more closely related larvae were more likely to be found in an early-establishing feeding cluster than groups of focal individuals of lower degrees of kinship (by counting the number of adult flies which had red wild-type IV eyes) Together, these results indicate that *D. melanogaster* larvae are clearly able to distinguish who is closely related to themselves, and to adjust their co-operative behaviour accordingly. The differences in the expressed behaviours observed between treatments suggests the presence of a kin-recognition mechanism in this species, rather than a generic co-operative response. In both the wild and in the lab, the combination of female polyandry and aggregation at oviposition sites (Hoffmann and Nielsen 1985; Markow 2002; Markow and O'Grady 2008; Schwartz *et al.* 2012) has the consequence that individual larvae are likely to encounter others whose degree of consanguinity can vary tremendously. Our experiments show that they are able to respond plastically to this variation in order to access resources in their environment that would otherwise be inaccessible.

Participation in a feeding group can be very beneficial for larvae, as we saw in our second assay. Vials in which individuals were more closely related had higher rates of survival, and female weights (a strong predictor of lifetime fecundity, *e.g.* Lefranc and Bundgaard 2004) that were greater than those from vials were the overall degree of relatedness was lower. Male weight did not differ substantially between treatments, which may be potentially associated with male larvae being more starvation resistant (Huey *et al.* 2004), and requiring fewer resources to survive than females. All else being equal, the superior performance of larvae in the high relatedness treatment is somewhat surprising, as in many cases such groups (of relatively low genetic diversity) fare worse than groups of higher genetic diversity, if the latter groups are better able to exploit a wider range of resources, and consequently experience reduced intra-specific competition (*reviewed in* Hughes *et al.* 2008). In our study system, there is likely limited opportunity for this type of niche partitioning, as *D. melanogaster* is a generalist forager (Soliman and van Herrewege 1988), and it is unlikely that such dietary polymorphisms exists in our populations, which have been maintained on the same type of media for hundreds of generations (Long *et al.* 2013). Instead, the significant fitness-associated differences between treatments are likely mediated by the abundance of kin in a vial, as close relatives were more likely to cooperate than others, as seen in our first and third assays. This co-operation permits greater access to otherwise unattainable resources (the nutritionally- rich lower layers of media) (Dombrovski *et al.* 2017), and in turn, benefits those that are participating in these resource-poor environments if they experience less competition. Increased group foraging at lower depths may also benefit those that are not participants in these groups, as there are fewer competitors present near the surface. As we did not measure the size or survival of the competitor IV-*bw* flies we do not know whether this occurred in our assay. However, in a study by Saltz *et al.* (2012), it was observed that at high densities, inbred groups of *D. melanogaster* larvae (carrying a phenotypic marker) that developed in the presence of groups of *wild-type* conspecifics which varied in their genetic diversity, had higher survivorship when the *wild-type* groups were genetically uniform than when they were genetically diverse. If genetically diverse groups (of low relatedness) are less likely to form feeding groups, all larvae near the habitat surface may experience greater competition for resources. Our findings suggest that the expression of social foraging may have widespread and complex ecological and evolutionary consequences.

Situations in which individuals will collectively work together to accomplish a task that cannot be done individually does not necessarily require kin selection (Trivers 1971; Dugatkin 1997; Dugatkin 2019), our results are most consistent with this model. If this was a case of larvae exclusively engaging in 'By-product mutualism' (Brown 1983) and kinship was an irrelevant element, then we would have expected that the frequencies and magnitudes of this co-operative behaviour to have been roughly the same across all vials, as they all contained the same amount of media and same number of larvae. Instead, we saw differences between means/median behaviour of larvae in the three relatedness treatments. These differences strongly suggest that there is some cost (and/or risk) of forming feeding alliances, and that these costs are more likely to be overcome, and co-operation more likely to be exhibited if surrounded with kin. From this we infer the presence of some type of kin selection at work. The differences between treatments that we observed is not likely to reflect 'reciprocity' (Trivers 1971), as the life history and ecology of this species restricts the opportunity for co-operative feeding interactions to the early third instar larval stage. This, along with the environment's crowded conditions, and the ephemeral nature of these feeding alliances, means there is low probability of future interaction with past partners making it implausible that a larva would be able to remember its past interactions with conspecifics. Overall, our observed pattern of cooperation is best understood through an inclusive fitness framework, wherein this behaviour has been selected for in *D. melanogaster*, presumably because the indirect fitness benefits, moderated by the degree of shared genes, outweighs the direct fitness costs of cluster feeding (Hamilton 1964).

Given the clear benefits of group feeding seen in our assays, why are *D. melanogaster* larvae not *more* co-operative? We hypothesize that the answer to this question is inherently linked to the issue of kin selection and kin recognition. While a, large, established feeding cluster is able to acquire otherwise inaccessible resources, getting to that point can be risky, especially to the initial pioneering larvae. First, those individuals who found a feeding cluster must remain relatively stationary compared to other larvae, which makes them relatively more vulnerable to being parasitized by predatory wasps, (Wertheim *et al.* 2005), or to being cannibalized by younger, hungry, larvae (Ahmad *et al.* 2015). Additionally, before they can reach the lower, nutritious resources, the pioneers must expend energy digging a hole that may collapse (and suffocate them), with the likelihood of failure increasing if others in the group abandon the cluster prematurely. Finally, before they can be consumed, the unexploited resources may first need to be socially digested via the production and excretion of digestive enzymes of the pioneering larvae (Gregg *et al.* 1990; Sakaguchi and Suzuki 2013). Thus, there are few immediate benefits and considerable uncertainty, investments, and risks involved in initiating a feeding cluster. Kinship selection may help mediate these costs as relatives might be less likely to abandon relatives as their efforts benefit both themselves directly by being able to access the untapped resources, as well as indirectly through the reproductive success of their relatives. Additionally, larvae also may be less likely to abandon a proto- cluster that contains kin if in doing so it increases the kin's risk of mortality (and ultimately results in an indirect fitness loss). Pioneering larvae might also be at risk of desiccation from being exposed to the air for longer periods of time (Cole 1995; Xie and Zhang 2007; Johnson and Carder 2012) which could result from abandonment in earlier stages of clustering. Successfully recruiting and maintaining enough pioneering larvae to be able to dig a sufficiently deep well may be a challenge, which may be facilitated when kin are present. The sharing of costs/benefits among relatives may in turn result in the appearance of a positive relationship between individual fitness and the number/density of conspecifics (*i.e.* an 'Allee effect', *sensu* Stephens *et al.* 1999). The potential hazards and costs associated with initiating a feeding cluster in the absence of indirect fitness benefits may potentially explain why group feeding is not more common among all larvae, and studies of the dynamics of early cluster forming among kin and non-kin is a potentially valuable area for future studies of social behaviour.

While our experimental results are consistent with the presence of a kin-recognition system among *D. melanogaster* larvae, they do not reveal what mechanism(s) are used. There are several strong possibilities, based on previous research in this species, which we shall consider. First, since it is known that there are many substances that can elicit specific olfactory and gustatory responses in larvae (*reviewed in* Scherer, *et al.* 2003; Khurana and Siddiqi 2013; Apostolopoulou *et al*. 2015), they may also be using taste or smell to recognize kin, or kin-associated cues. In adults, flies behave differently towards conspecifics based on the phenotypes associated with the gut microbiomes that develop in different environments (Lize *et al*. 2013), which may also play a role in kin recognition (*e.g.* Carazo *et al.* 2015; Le Page *et al.* 2017, *but see* Martin and Long 2015). However, in all our assays all larvae developed in the same type of environment, so they are likely to exhibit very similar microbiotic cues. A second possibility to consider is that the larvae are using visual cues. While this may seem initially unlikely due to the obscuring nature of the liquefied environments through which larvae burrow, it may be that sight, in combination with mechanosensory sensations may be useful in working with kin. In Dombrovski *et al*'s (2017) study of group-feeding, they observed that visually- impaired transplanted larvae had trouble co- ordinating their movements with the other larvae within a cluster. They concluded that sight played an important role in co- ordinating contractile cycles and speculated that individuals of similar motor profiles would have an easier task of synchronizing their digging and feeding behaviours. Since some of the individual variation in larval feeding behaviour has a genetic basis (*e.g.* Bauer and Sokolowski 1984; Sokolowski *et al*. 1984; Wu *et al.* 2003; Sewell *et al.* 2009), it may be more likely that two close relatives (especially if they are of similar developmental age) will phenotypically match than two unrelated individuals, and consequently be more efficient in their co-operative foraging. Furthermore, the specific pattern of contractions established by the larval pioneers may create a positive feedback loop, wherein joining the cluster is easier for those individuals who exhibit a compatible motor profile, which in turn leads to increasing the familial representation in the behaviourally co- ordinated feeding cluster (if kin are more likely to be behaviourally similar than non-kin). This may also explain the presence of larvae from both the focal (IV) and competitor (IV-*bw*) populations in feeding clusters sampled in our third assay, if these are cases where, by coincidence, kin and non-kin exhibited similar and compatible motor profile phenotypes. Studies of the behavioural phenotypes and interactions between individual larvae within clusters differing in the degree of relatedness may prove useful in confirming the mechanism(s) by which larvae appear to recognize their relatives.

In our third assay we observed no significant difference between treatments in the sex ratios of target flies sampled from feeding clusters. This was somewhat a surprising outcome, as we had previously seen in our second assay that females appeared to benefit more from group foraging (manifested as greater adult weight) than males, and previous studies have found that the sexes differ in their larval competitive abilities (Nunney 1983), which had led us to speculate that we might see sex biases in cluster membership. The absence of differences may reflect that no differences in behaviours exist between larvae, that the magnitude of the differences are too small for us to detect, or that our experimental design may have missed any behavioural dimorphisms in cluster feeding. On the latter point, it should be noted that our sampling of clusters was done in the earliest portion of the third instar larval stage, when clusters were relatively small. Follow-up studies may want to examine the membership of clusters sampled at later time points to see if any differences in composition are present.

This study was motivated by *Drosophila melanogaster*'s increasingly use as a model species to understand the evolution of social behaviour. Our results indicate that even at the larval level, that kinship may play an important role in mediating the expression of co-operative behaviour, potentially revealing the presence of a previously undescribed kin recognition system present in larvae. This inferred ability also shows a certain degree of granularity, as seen in the differences between vials from the high and intermediate relatedness treatments, suggesting that this species is sensitive to the composition of its social environment. We hope that these findings contribute to the better understanding of the factors that may lead to the evolution of sociality and altruism.

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Figures:

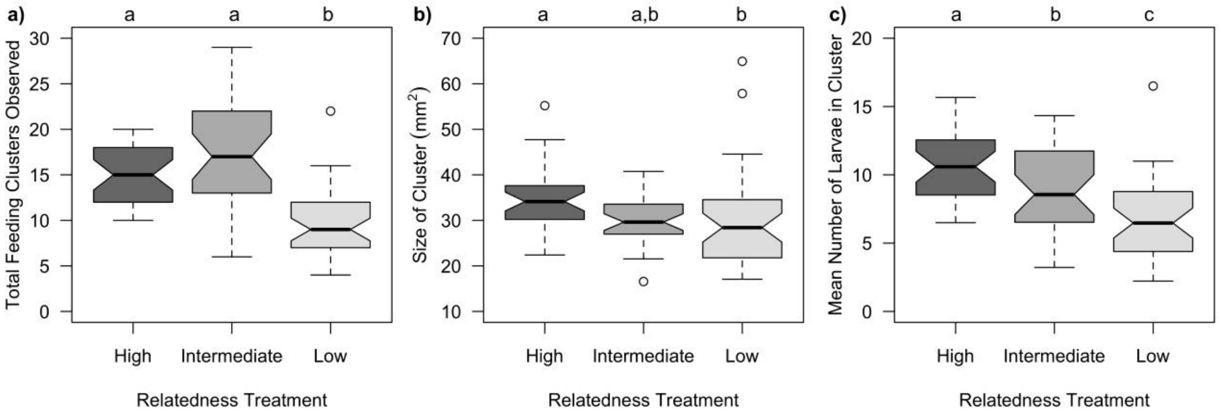


Figure 1: Boxplots illustrating a) the total number of feeding clusters observed b) the mean size of the cluster depression and c) the mean number of larvae in feeding clusters in vials of *Drosophila melanogaster* where the degree of relatedness between individuals had been experimentally manipulated. Each box encloses the middle 50% of data (Inter-Quartile Range, IQR), with the location of the median represented by a horizontal line with the notch depicting the 95% confidence interval around the median (*i.e* ±1.57 x IQR/√sample size). Values >±1.5 x the IQR outside the box are considered outliers and depicted as open circles. Whiskers extend to the largest and smallest values that are not outliers. Boxplots that are not sharing a letter have significantly different means (Figure 1a and 1c) or medians (Figure 1b).

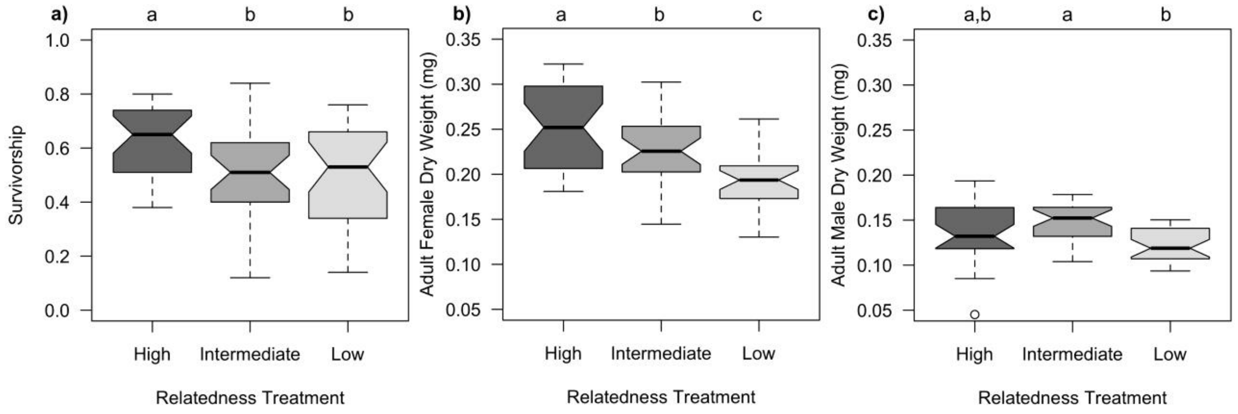


Figure 2: Boxplots illustrating a) the egg-to-adult survivorship of target (*wild-type*) *Drosophila melanogaster*, b) the mean dry weight of target adult females, and c) the mean dry weight of target adult males, in vials of where the degree of relatedness between individuals had been experimentally manipulated. Boxplot components as in Figure 1. Boxplots that are not sharing a letter have significantly different means.

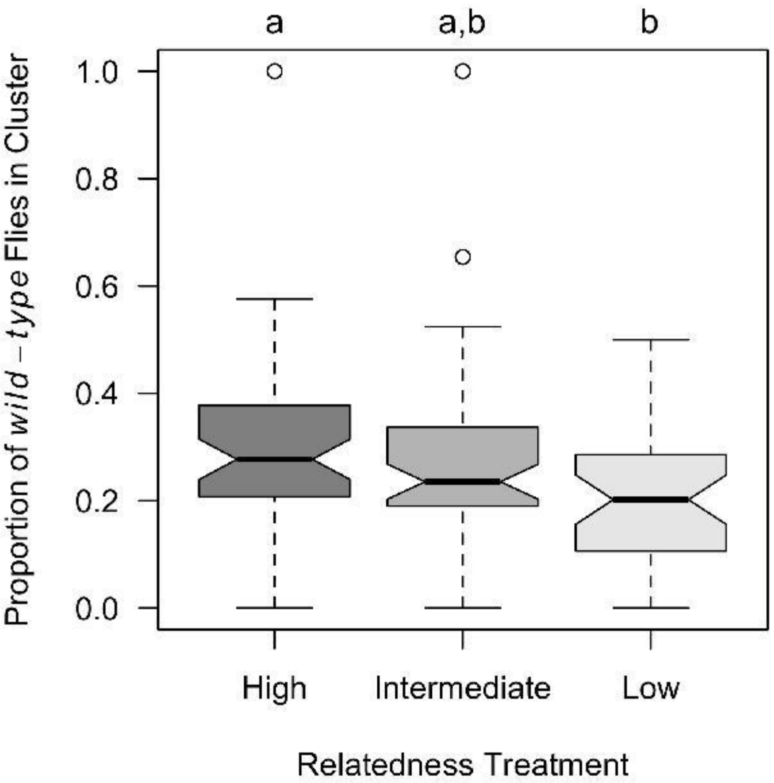


Figure 3: Boxplots illustrating proportion of target (*wild- type*) *Drosophila melanogaster,* developing from a sampled feeding cluster, from vials of where the degree of relatedness between individuals had been experimentally manipulated. Boxplot components as in Figure 1. Boxplots that are not sharing a letter have significantly different means.

Supplementary Information, Simulations, and Figures:

Simulations of degree of relatedness across the three experimental treatments.

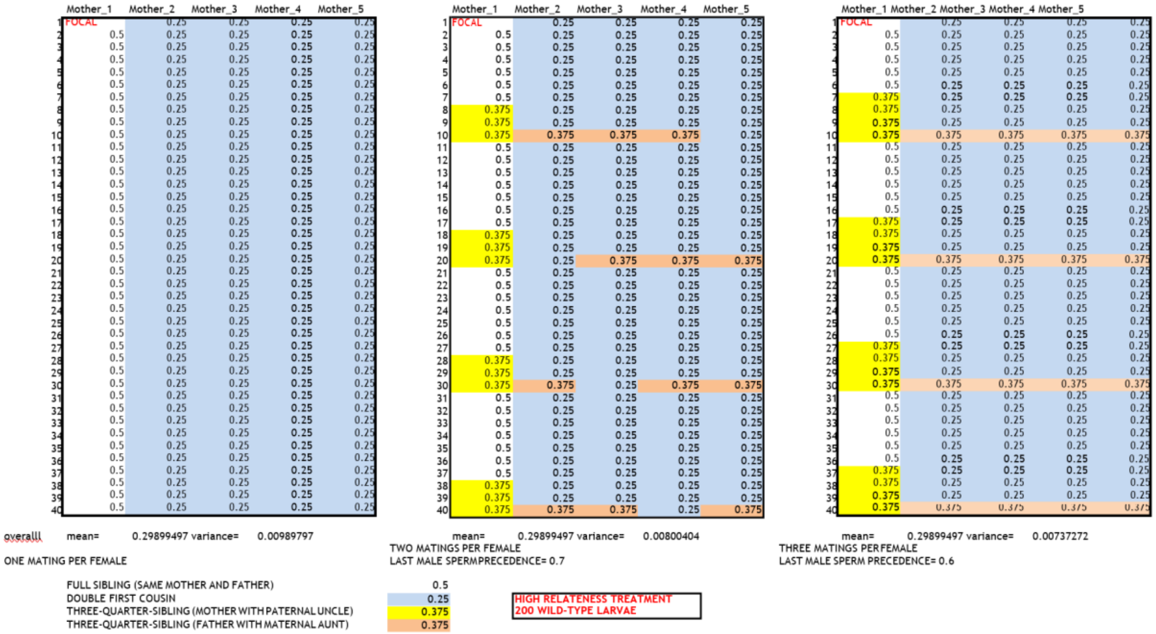
(see SUPPLEMENTAL FILE “SIMULATIONS.xlsx”; pictures of which are included below)

In our experiments, we collected adult male and female flies as virgins from a number of different families, and used these sets of full-siblings to establish three experimental groups (“high”, “intermediate” and “low”) to generate the target larvae. To create individual larvae in the “high relatedness” treatment, we combined 5 virgin sisters (females from the same family) with 5 brothers (males obtained from a different family). The resulting offspring have relatedness coefficients ranging from a maximum of 0.5 (for full siblings) to a minimum of 0.25 (for double-first cousins). To create individuals in the “intermediate relatedness” treatment, we combined 5 brothers from the same family with 5 unrelated females from culture populations, which yielded offspring with relatedness coefficients varying from a maximum of 0.5 (for full siblings) to a minimum of 0.125 (for first cousins). To create individuals in the “low relatedness” treatment, 5 unrelated IV females were mated with 5 unrelated IV males, which yielded offspring with relatedness coefficients of a maximum of 0.5 (for full siblings) to a minimum of 0 (for completely unrelated individuals).

While these different combinations of sires and dams in each treatment will yield offspring groups of different mean degrees of relatedness, to better estimate these specific values, we also simulated the coefficients of relatedness within each treatment group from the perspective of a given focal individual, and calculated the mean coefficient of relatedness across all target larvae. The results of these thought experiments are printed below, and the relevant worksheets “SIMULATIONS.xlxs” is available as a supplemental file (of which pictures can be found below). In these simulations, we assumed that each of the five females produced the same number of offspring. We also assumed that each of the five males has an equal reproductive success (that is they each mated with the same number of females). We examined scenarios where females only mated once, where females mated twice (with last male sperm precedence =0.7), and where females mated thrice (with last male sperm precedence =0.6, and each of the other two males having equal mating success)*. While the variances in relatedness coefficients differed slightly between these three scenarios, the overall mean coefficients of relatedness in each group remained the same.

Based on our simulations of vials containing 200 wild-type larvae (as in Experiment 1), the overall mean coefficient of relatedness in the "high relatedness" vials is ~0.299, in the "intermediate relatedness" vials is ~0.198 and in the "low relatedness" vials is ~0.098. Based on our simulations of vials containing 50 wild-type larvae (Experiments 2 & 3), the overall mean coefficient of relatedness between these focal larvae in the "high relatedness" vials is ~0.296, in the "intermediate relatedness" vials is ~0.194 and in the "low relatedness" vials is ~0.092.

* These two-mating and three-mating sperm precedence values are in line with those reported in Laturney M, van Eijk R, Billeter JC (2018) Last male sperm precedence is modulated by female remating rate in *Drosophila melanogaster*. Evolution Letters, 2(3):180-189.



	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0.25	0.25	0.25	0.25
2	0.5	0.25	0.25	0.25	0.25
3	0.5	0.25	0.25	0.25	0.25
4	0.5	0.25	0.25	0.25	0.25
5	0.5	0.25	0.25	0.25	0.25
6	0.5	0.25	0.25	0.25	0.25
7	0.5	0.25	0.25	0.25	0.25
8	0.5	0.25	0.25	0.25	0.25
9	0.5	0.25	0.25	0.25	0.25
10	0.5	0.25	0.25	0.25	0.25
11	0.5	0.25	0.25	0.25	0.25
12	0.5	0.25	0.25	0.25	0.25
13	0.5	0.25	0.25	0.25	0.25
14	0.5	0.25	0.25	0.25	0.25
15	0.5	0.25	0.25	0.25	0.25
16	0.5	0.25	0.25	0.25	0.25
17	0.5	0.25	0.25	0.25	0.25
18	0.5	0.25	0.25	0.25	0.25
19	0.5	0.25	0.25	0.25	0.25
20	0.5	0.25	0.25	0.25	0.25
21	0.5	0.25	0.25	0.25	0.25
22	0.5	0.25	0.25	0.25	0.25
23	0.5	0.25	0.25	0.25	0.25
24	0.5	0.25	0.25	0.25	0.25
25	0.5	0.25	0.25	0.25	0.25
26	0.5	0.25	0.25	0.25	0.25
27	0.5	0.25	0.25	0.25	0.25
28	0.5	0.25	0.25	0.25	0.25
29	0.5	0.25	0.25	0.25	0.25
30	0.5	0.25	0.25	0.25	0.25
31	0.5	0.25	0.25	0.25	0.25
32	0.5	0.25	0.25	0.25	0.25
33	0.5	0.25	0.25	0.25	0.25
34	0.5	0.25	0.25	0.25	0.25
35	0.5	0.25	0.25	0.25	0.25
36	0.5	0.25	0.25	0.25	0.25
37	0.5	0.25	0.25	0.25	0.25
38	0.5	0.25	0.25	0.25	0.25
39	0.5	0.25	0.25	0.25	0.25
40	0.5	0.25	0.25	0.25	0.25

overalll mean= 0.29899497 variance= 0.00989797

ONE MATING PER FEMALE

	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0.25	0.25	0.25	0.25
2	0.5	0.25	0.25	0.25	0.25
3	0.5	0.25	0.25	0.25	0.25
4	0.5	0.25	0.25	0.25	0.25
5	0.5	0.25	0.25	0.25	0.25
6	0.5	0.25	0.25	0.25	0.25
7	0.5	0.25	0.25	0.25	0.25
8	0.375	0.25	0.25	0.25	0.25
9	0.375	0.25	0.25	0.25	0.25
10	0.375	0.375	0.375	0.375	0.25
11	0.5	0.25	0.25	0.25	0.25
12	0.5	0.25	0.25	0.25	0.25
13	0.5	0.25	0.25	0.25	0.25
14	0.5	0.25	0.25	0.25	0.25
15	0.5	0.25	0.25	0.25	0.25
16	0.5	0.25	0.25	0.25	0.25
17	0.5	0.25	0.25	0.25	0.25
18	0.375	0.25	0.25	0.25	0.25
19	0.375	0.25	0.25	0.25	0.25
20	0.375	0.25	0.375	0.375	0.375
21	0.5	0.25	0.25	0.25	0.25
22	0.5	0.25	0.25	0.25	0.25
23	0.5	0.25	0.25	0.25	0.25
24	0.5	0.25	0.25	0.25	0.25
25	0.5	0.25	0.25	0.25	0.25
26	0.5	0.25	0.25	0.25	0.25
27	0.5	0.25	0.25	0.25	0.25
28	0.375	0.25	0.25	0.25	0.25
29	0.375	0.25	0.25	0.25	0.25
30	0.375	0.375	0.25	0.375	0.375
31	0.5	0.25	0.25	0.25	0.25
32	0.5	0.25	0.25	0.25	0.25
33	0.5	0.25	0.25	0.25	0.25
34	0.5	0.25	0.25	0.25	0.25
35	0.5	0.25	0.25	0.25	0.25
36	0.5	0.25	0.25	0.25	0.25
37	0.5	0.25	0.25	0.25	0.25
38	0.375	0.25	0.25	0.25	0.25
39	0.375	0.25	0.25	0.25	0.25
40	0.375	0.375	0.375	0.25	0.375

mean= 0.29899497 variance= 0.00800404

TWO MATINGS PER FEMALE

LAST MALE SPERMPRECEDENCE= 0.7

	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0.25	0.25	0.25	0.25
2	0.5	0.25	0.25	0.25	0.25
3	0.5	0.25	0.25	0.25	0.25
4	0.5	0.25	0.25	0.25	0.25
5	0.5	0.25	0.25	0.25	0.25
6	0.5	0.25	0.25	0.25	0.25
7	0.375	0.25	0.25	0.25	0.25
8	0.375	0.25	0.25	0.25	0.25
9	0.375	0.25	0.25	0.25	0.25
10	0.375	0.375	0.375	0.375	0.375
11	0.5	0.25	0.25	0.25	0.25
12	0.5	0.25	0.25	0.25	0.25
13	0.5	0.25	0.25	0.25	0.25
14	0.5	0.25	0.25	0.25	0.25
15	0.5	0.25	0.25	0.25	0.25
16	0.5	0.25	0.25	0.25	0.25
17	0.375	0.25	0.25	0.25	0.25
18	0.375	0.25	0.25	0.25	0.25
19	0.375	0.25	0.25	0.25	0.25
20	0.375	0.375	0.375	0.375	0.375
21	0.5	0.25	0.25	0.25	0.25
22	0.5	0.25	0.25	0.25	0.25
23	0.5	0.25	0.25	0.25	0.25
24	0.5	0.25	0.25	0.25	0.25
25	0.5	0.25	0.25	0.25	0.25
26	0.5	0.25	0.25	0.25	0.25
27	0.375	0.25	0.25	0.25	0.25
28	0.375	0.25	0.25	0.25	0.25
29	0.375	0.25	0.25	0.25	0.25
30	0.375	0.375	0.375	0.375	0.375
31	0.5	0.25	0.25	0.25	0.25
32	0.5	0.25	0.25	0.25	0.25
33	0.5	0.25	0.25	0.25	0.25
34	0.5	0.25	0.25	0.25	0.25
35	0.5	0.25	0.25	0.25	0.25
36	0.5	0.25	0.25	0.25	0.25
37	0.375	0.25	0.25	0.25	0.25
38	0.375	0.25	0.25	0.25	0.25
39	0.375	0.25	0.25	0.25	0.25
40	0.375	0.375	0.375	0.375	0.375

mean= 0.29899497 variance= 0.00737272

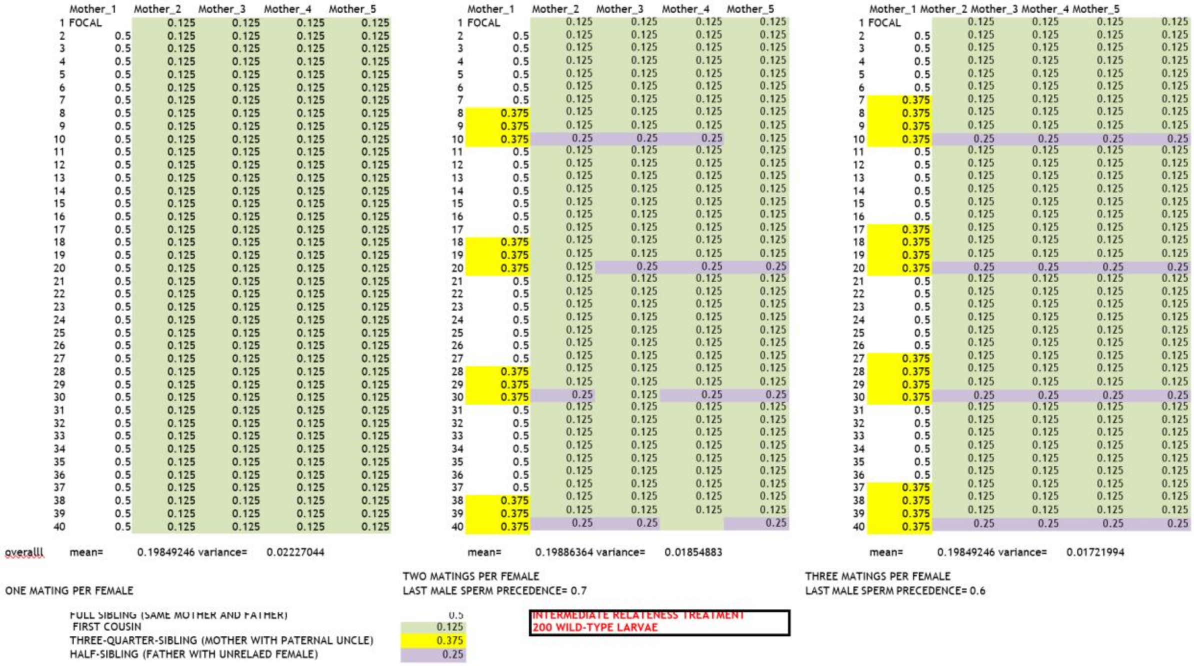
THREE MATINGS PER FEMALE

LAST MALE SPERM PRECEDENCE= 0.6

FULL SIBLING (SAME MOTHER AND FATHER)	0.5
DOUBLE FIRST COUSIN	0.25
THREE-QUARTER-SIBLING (MOTHER WITH PATERNAL UNCLE)	0.375
THREE-QUARTER-SIBLING (FATHER WITH MATERNAL AUNT)	0.375

HIGH RELATENESS TREATMENT
200 WILD-TYPE LARVAE

Supplementary Figure P1: Picture of Simulation 1 with High relatedness treatment larvae average relatedness at females mating with 1 male, females mating with 2 males, and females mating with 3 males. Subsequent results can be found in Supplementary Figure S1 below.



	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0.125	0.125	0.125	0.125
2	0.5	0.125	0.125	0.125	0.125
3	0.5	0.125	0.125	0.125	0.125
4	0.5	0.125	0.125	0.125	0.125
5	0.5	0.125	0.125	0.125	0.125
6	0.5	0.125	0.125	0.125	0.125
7	0.5	0.125	0.125	0.125	0.125
8	0.5	0.125	0.125	0.125	0.125
9	0.5	0.125	0.125	0.125	0.125
10	0.5	0.125	0.125	0.125	0.125
11	0.5	0.125	0.125	0.125	0.125
12	0.5	0.125	0.125	0.125	0.125
13	0.5	0.125	0.125	0.125	0.125
14	0.5	0.125	0.125	0.125	0.125
15	0.5	0.125	0.125	0.125	0.125
16	0.5	0.125	0.125	0.125	0.125
17	0.5	0.125	0.125	0.125	0.125
18	0.5	0.125	0.125	0.125	0.125
19	0.5	0.125	0.125	0.125	0.125
20	0.5	0.125	0.125	0.125	0.125
21	0.5	0.125	0.125	0.125	0.125
22	0.5	0.125	0.125	0.125	0.125
23	0.5	0.125	0.125	0.125	0.125
24	0.5	0.125	0.125	0.125	0.125
25	0.5	0.125	0.125	0.125	0.125
26	0.5	0.125	0.125	0.125	0.125
27	0.5	0.125	0.125	0.125	0.125
28	0.5	0.125	0.125	0.125	0.125
29	0.5	0.125	0.125	0.125	0.125
30	0.5	0.125	0.125	0.125	0.125
31	0.5	0.125	0.125	0.125	0.125
32	0.5	0.125	0.125	0.125	0.125
33	0.5	0.125	0.125	0.125	0.125
34	0.5	0.125	0.125	0.125	0.125
35	0.5	0.125	0.125	0.125	0.125
36	0.5	0.125	0.125	0.125	0.125
37	0.5	0.125	0.125	0.125	0.125
38	0.5	0.125	0.125	0.125	0.125
39	0.5	0.125	0.125	0.125	0.125
40	0.5	0.125	0.125	0.125	0.125

overalll mean= 0.19849246 variance= 0.02227044

ONE MATING PER FEMALE

	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0.125	0.125	0.125	0.125
2	0.5	0.125	0.125	0.125	0.125
3	0.5	0.125	0.125	0.125	0.125
4	0.5	0.125	0.125	0.125	0.125
5	0.5	0.125	0.125	0.125	0.125
6	0.5	0.125	0.125	0.125	0.125
7	0.5	0.125	0.125	0.125	0.125
8	0.375	0.125	0.125	0.125	0.125
9	0.375	0.125	0.125	0.125	0.125
10	0.375	0.25	0.25	0.25	0.125
11	0.5	0.125	0.125	0.125	0.125
12	0.5	0.125	0.125	0.125	0.125
13	0.5	0.125	0.125	0.125	0.125
14	0.5	0.125	0.125	0.125	0.125
15	0.5	0.125	0.125	0.125	0.125
16	0.5	0.125	0.125	0.125	0.125
17	0.5	0.125	0.125	0.125	0.125
18	0.375	0.125	0.125	0.125	0.125
19	0.375	0.125	0.125	0.125	0.125
20	0.375	0.125	0.25	0.25	0.25
21	0.5	0.125	0.125	0.125	0.125
22	0.5	0.125	0.125	0.125	0.125
23	0.5	0.125	0.125	0.125	0.125
24	0.5	0.125	0.125	0.125	0.125
25	0.5	0.125	0.125	0.125	0.125
26	0.5	0.125	0.125	0.125	0.125
27	0.5	0.125	0.125	0.125	0.125
28	0.375	0.125	0.125	0.125	0.125
29	0.375	0.125	0.125	0.125	0.125
30	0.375	0.25	0.125	0.25	0.25
31	0.5	0.125	0.125	0.125	0.125
32	0.5	0.125	0.125	0.125	0.125
33	0.5	0.125	0.125	0.125	0.125
34	0.5	0.125	0.125	0.125	0.125
35	0.5	0.125	0.125	0.125	0.125
36	0.5	0.125	0.125	0.125	0.125
37	0.5	0.125	0.125	0.125	0.125
38	0.375	0.125	0.125	0.125	0.125
39	0.375	0.125	0.125	0.125	0.125
40	0.375	0.25	0.25		0.25

mean= 0.19886364 variance= 0.01854883

TWO MATINGS PER FEMALE
LAST MALE SPERM PRECEDENCE= 0.7

	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0.125	0.125	0.125	0.125
2	0.5	0.125	0.125	0.125	0.125
3	0.5	0.125	0.125	0.125	0.125
4	0.5	0.125	0.125	0.125	0.125
5	0.5	0.125	0.125	0.125	0.125
6	0.5	0.125	0.125	0.125	0.125
7	0.375	0.125	0.125	0.125	0.125
8	0.375	0.125	0.125	0.125	0.125
9	0.375	0.125	0.125	0.125	0.125
10	0.375	0.25	0.25	0.25	0.25
11	0.5	0.125	0.125	0.125	0.125
12	0.5	0.125	0.125	0.125	0.125
13	0.5	0.125	0.125	0.125	0.125
14	0.5	0.125	0.125	0.125	0.125
15	0.5	0.125	0.125	0.125	0.125
16	0.5	0.125	0.125	0.125	0.125
17	0.375	0.125	0.125	0.125	0.125
18	0.375	0.125	0.125	0.125	0.125
19	0.375	0.125	0.125	0.125	0.125
20	0.375	0.25	0.25	0.25	0.25
21	0.5	0.125	0.125	0.125	0.125
22	0.5	0.125	0.125	0.125	0.125
23	0.5	0.125	0.125	0.125	0.125
24	0.5	0.125	0.125	0.125	0.125
25	0.5	0.125	0.125	0.125	0.125
26	0.5	0.125	0.125	0.125	0.125
27	0.375	0.125	0.125	0.125	0.125
28	0.375	0.125	0.125	0.125	0.125
29	0.375	0.125	0.125	0.125	0.125
30	0.375	0.25	0.25	0.25	0.25
31	0.5	0.125	0.125	0.125	0.125
32	0.5	0.125	0.125	0.125	0.125
33	0.5	0.125	0.125	0.125	0.125
34	0.5	0.125	0.125	0.125	0.125
35	0.5	0.125	0.125	0.125	0.125
36	0.5	0.125	0.125	0.125	0.125
37	0.375	0.125	0.125	0.125	0.125
38	0.375	0.125	0.125	0.125	0.125
39	0.375	0.125	0.125	0.125	0.125
40	0.375	0.25	0.25	0.25	0.25

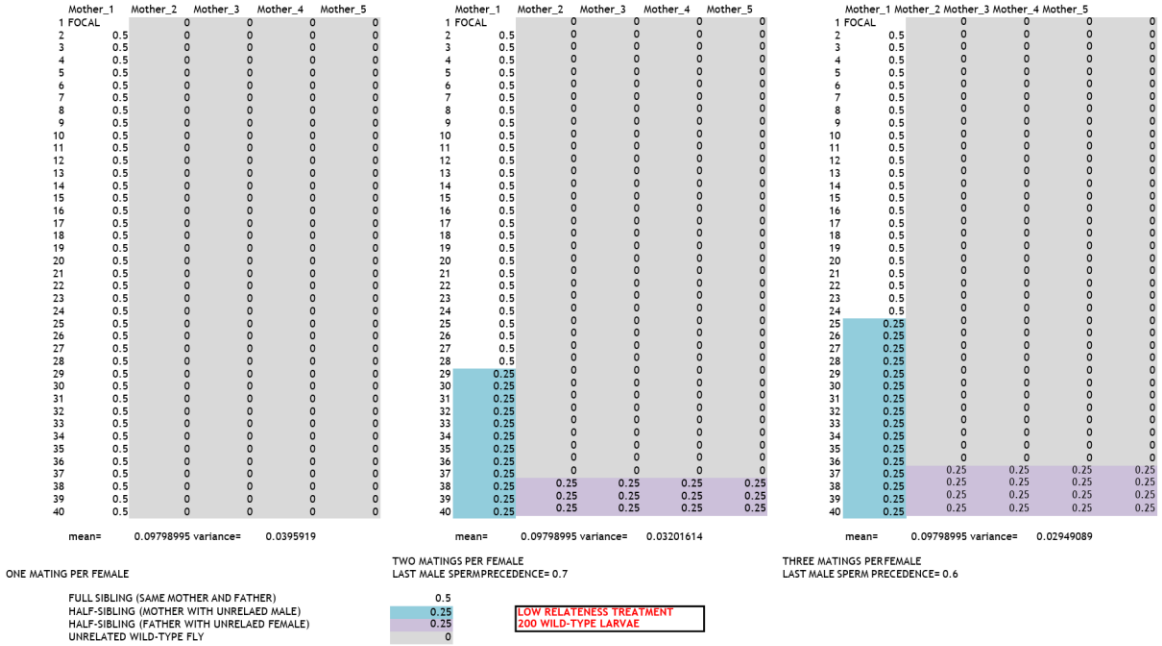
mean= 0.19849246 variance= 0.01721994

THREE MATINGS PER FEMALE
LAST MALE SPERM PRECEDENCE= 0.6

FULL SIBLING (SAME MOTHER AND FATHER)	0.5
FIRST COUSIN	0.125
THREE-QUARTER-SIBLING (MOTHER WITH PATERNAL UNCLE)	0.375
HALF-SIBLING (FATHER WITH UNRELAED FEMALE)	0.25

INTERMEDIATE RELATENESS TREATMENT
200 WILD-TYPE LARVAE

Supplementary Figure P2: Picture of Simulation 2 with Intermediate relatedness treatment larvae average relatedness at females mating with 1 male, females mating with 2 males, and females mating with 3 males. Subsequent results can be found in Supplementary Figure S2 below.



	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0	0	0	0
2	0.5	0	0	0	0
3	0.5	0	0	0	0
4	0.5	0	0	0	0
5	0.5	0	0	0	0
6	0.5	0	0	0	0
7	0.5	0	0	0	0
8	0.5	0	0	0	0
9	0.5	0	0	0	0
10	0.5	0	0	0	0
11	0.5	0	0	0	0
12	0.5	0	0	0	0
13	0.5	0	0	0	0
14	0.5	0	0	0	0
15	0.5	0	0	0	0
16	0.5	0	0	0	0
17	0.5	0	0	0	0
18	0.5	0	0	0	0
19	0.5	0	0	0	0
20	0.5	0	0	0	0
21	0.5	0	0	0	0
22	0.5	0	0	0	0
23	0.5	0	0	0	0
24	0.5	0	0	0	0
25	0.5	0	0	0	0
26	0.5	0	0	0	0
27	0.5	0	0	0	0
28	0.5	0	0	0	0
29	0.5	0	0	0	0
30	0.5	0	0	0	0
31	0.5	0	0	0	0
32	0.5	0	0	0	0
33	0.5	0	0	0	0
34	0.5	0	0	0	0
35	0.5	0	0	0	0
36	0.5	0	0	0	0
37	0.5	0	0	0	0
38	0.5	0	0	0	0
39	0.5	0	0	0	0
40	0.5	0	0	0	0

mean= 0.09798995 variance= 0.0395919

ONE MATING PER FEMALE

	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0	0	0	0
2	0.5	0	0	0	0
3	0.5	0	0	0	0
4	0.5	0	0	0	0
5	0.5	0	0	0	0
6	0.5	0	0	0	0
7	0.5	0	0	0	0
8	0.5	0	0	0	0
9	0.5	0	0	0	0
10	0.5	0	0	0	0
11	0.5	0	0	0	0
12	0.5	0	0	0	0
13	0.5	0	0	0	0
14	0.5	0	0	0	0
15	0.5	0	0	0	0
16	0.5	0	0	0	0
17	0.5	0	0	0	0
18	0.5	0	0	0	0
19	0.5	0	0	0	0
20	0.5	0	0	0	0
21	0.5	0	0	0	0
22	0.5	0	0	0	0
23	0.5	0	0	0	0
24	0.5	0	0	0	0
25	0.5	0	0	0	0
26	0.5	0	0	0	0
27	0.5	0	0	0	0
28	0.5	0	0	0	0
29	0.25	0	0	0	0
30	0.25	0	0	0	0
31	0.25	0	0	0	0
32	0.25	0	0	0	0
33	0.25	0	0	0	0
34	0.25	0	0	0	0
35	0.25	0	0	0	0
36	0.25	0	0	0	0
37	0.25	0	0	0	0
38	0.25	0.25	0.25	0.25	0.25
39	0.25	0.25	0.25	0.25	0.25
40	0.25	0.25	0.25	0.25	0.25

mean= 0.09798995 variance= 0.03201614

TWO MATINGS PER FEMALE
LAST MALE SPERMPRECEDENCE= 0.7

	Mother_1	Mother_2	Mother_3	Mother_4	Mother_5
1	FOCAL	0	0	0	0
2	0.5	0	0	0	0
3	0.5	0	0	0	0
4	0.5	0	0	0	0
5	0.5	0	0	0	0
6	0.5	0	0	0	0
7	0.5	0	0	0	0
8	0.5	0	0	0	0
9	0.5	0	0	0	0
10	0.5	0	0	0	0
11	0.5	0	0	0	0
12	0.5	0	0	0	0
13	0.5	0	0	0	0
14	0.5	0	0	0	0
15	0.5	0	0	0	0
16	0.5	0	0	0	0
17	0.5	0	0	0	0
18	0.5	0	0	0	0
19	0.5	0	0	0	0
20	0.5	0	0	0	0
21	0.5	0	0	0	0
22	0.5	0	0	0	0
23	0.5	0	0	0	0
24	0.5	0	0	0	0
25	0.25	0	0	0	0
26	0.25	0	0	0	0
27	0.25	0	0	0	0
28	0.25	0	0	0	0
29	0.25	0	0	0	0
30	0.25	0	0	0	0
31	0.25	0	0	0	0
32	0.25	0	0	0	0
33	0.25	0	0	0	0
34	0.25	0	0	0	0
35	0.25	0	0	0	0
36	0.25	0	0	0	0
37	0.25	0.25	0.25	0.25	0.25
38	0.25	0.25	0.25	0.25	0.25
39	0.25	0.25	0.25	0.25	0.25
40	0.25	0.25	0.25	0.25	0.25

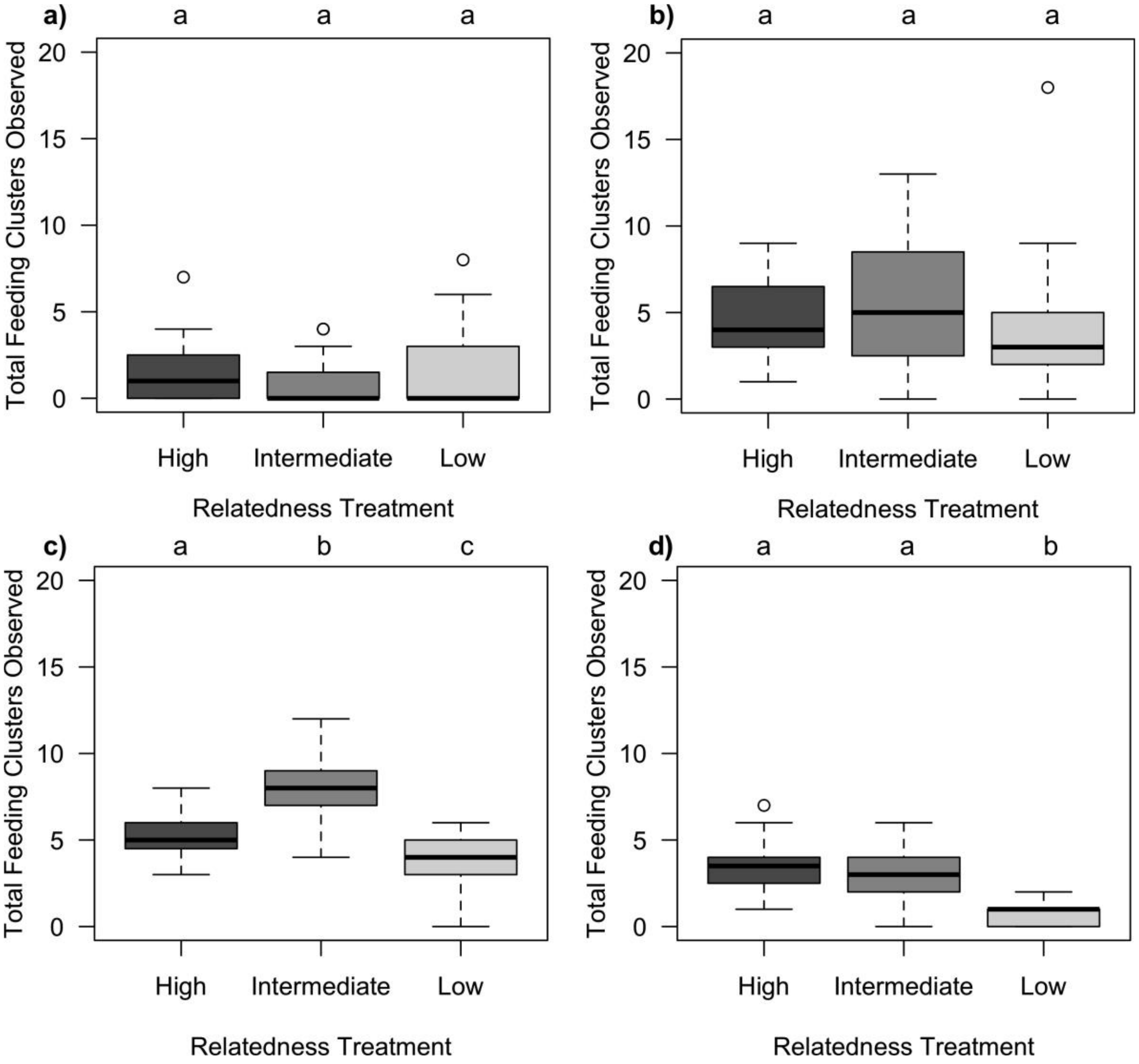
mean= 0.09798995 variance= 0.02949089

THREE MATINGS PERFEMALE
LAST MALE SPERM PRECEDENCE= 0.6

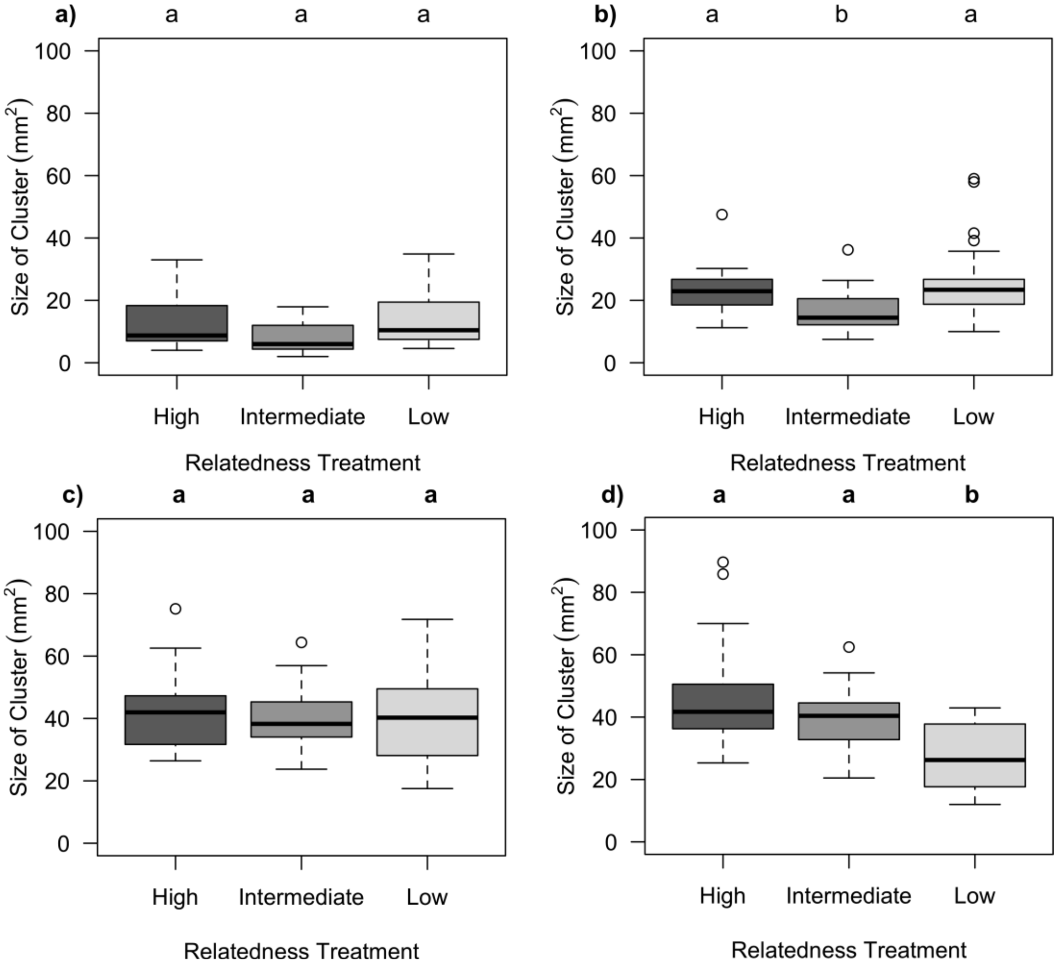
FULL SIBLING (SAME MOTHER AND FATHER)	0.5
HALF-SIBLING (MOTHER WITH UNRELAED MALE)	0.25
HALF-SIBLING (FATHER WITH UNRELAED FEMALE)	0.25
UNRELATED WILD-TYPE FLY	0

LOW RELATENESS TREATMENT
200 WILD-TYPE LARVAE

Supplementary Figure P3: Picture of Simulation 3 with Low relatedness treatment larvae average relatedness at females mating with 1 male, females mating with 2 males, and females mating with 3 males. Subsequent results can be found in Supplementary Figure S3 below.

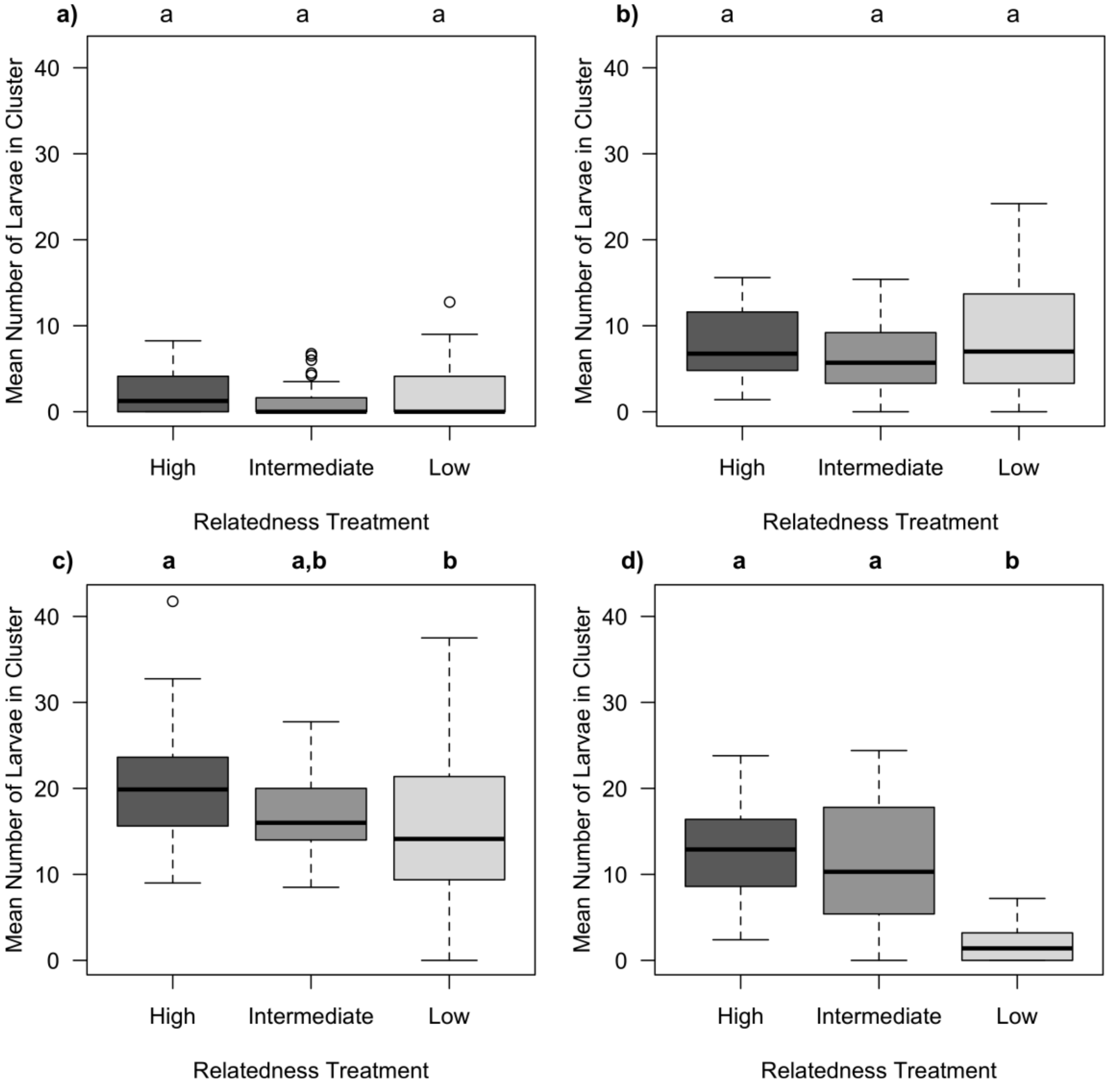


Supplementary Figure S1: Boxplots illustrating the total number of feeding clusters observed on a) the first, b) the second, c) the third and d) the fourth day of observation in vials of *Drosophila melanogaster* where the degree of relatedness between individuals had been experimentally manipulated. Each box encloses the middle 50% of data (Inter- Quartile Range, IQR), with the location of the median represented by a horizontal line. Values >±1.5 x the IQR outside the box are considered outliers and depicted as open circles. Whiskers extend to the largest and smallest values that are not outliers. Boxplots that are not sharing a letter have significantly different means according to a Tukey's HSD test.



Supplementary Figure S2: Boxplots illustrating the mean size of the cluster depressions observed on a) the first, b) the second, c) the third and d) the fourth day of observation in vials of *Drosophila melanogaster* where the degree of relatedness between individuals had been experimentally manipulated. Each box encloses the middle 50% of data (Inter- Quartile Range, IQR), with the location of the median represented by a horizontal line.

Values >±1.5 x the IQR outside the box are considered outliers and depicted as open circles. Whiskers extend to the largest and smallest values that are not outliers. Boxplots that are not sharing a letter have significantly different medians using a Multiple comparison test after Kruskal-Wallis.



Supplementary Figure S3: Boxplots illustrating the mean number of larvae in feeding clusters observed on a) the first, b) the second, c) the third and d) the fourth day of observation in vials of *Drosophila melanogaster* where the degree of relatedness between individuals had been experimentally manipulated. Each box encloses the middle 50% of data (Inter-Quartile Range, IQR), with the location of the median represented by a horizontal line. Values >±1.5 x the IQR outside the box are considered outliers and depicted as open circles. Whiskers extend to the largest and smallest values that are not outliers. Boxplots that are not sharing a letter have significantly different means according to a Tukey's HSD test.

Chapter 3:

Kin recognition and egg cannibalism by *Drosophila melanogaster* larvae

Abstract:

Cannibalism is a widespread behavioural phenomenon that is often thought to be an adaptive plastic response to limited environmental resources. However, cannibalism can potentially come at a fitness cost to an individual if one consumes relatives, due to the potential loss of indirect fitness benefits. One way in which this cost could be avoided is by the selective avoidance of cannibalizing kin in favour of consuming non-kin conspecifics. Here, we examined whether fruit fly (*Drosophila melanogaster*) 2nd instar larvae differ in their interactions with groups of related and unrelated eggs, and whether this is associated with differential rates of cannibalism. Our experiment revealed that, at this developmental stage larvae appear to be able to distinguish between full-sibling eggs and non-kin eggs, as they behaved differently towards these two groups. Larvae approached groups of unrelated eggs more frequently and spent more time overall associating with them than they did with groups of related eggs. Furthermore, larvae cannibalized unrelated eggs significantly and more frequently than kin eggs. These results are consistent with a kin-selection behavioural strategy that maximizes both direct and indirect fitness benefits. We discuss these findings in the context of this species' natural history, and the potential mechanisms of kin recognition. This study contributes to the growing number of studies examining the evolution of social behaviours using this model species.

Introduction:

When resources start to become scarce in an environment, this frequently puts individuals into conflict with each other through competition for those remaining resources. This conflict is sometimes characterized by antagonistic interactions, with one of the most extreme examples being cannibalism, in which individuals consume other conspecifics (Pfennig 1997; Richardson *et al.* 2010). For example, in the nymph stage of the migratory desert locust, *Schistocerca gregaria*, individuals exhibit cannibalistic behaviours when at high densities, as resources become limited (Pfennig 1997; Bazazi *et al.* 2008). Under these conditions, individuals engage in cannibalism to acquire essential salts, nitrogen and proteins (Simpson et al. 2006; Bazazi et al. 2008; Richardson *et al.* 2010). Cannibalism in this context is thought of as an adaptive plastic response as it allows one to immediately aquire the nutrients needed to grow and survive, while also potentially reducing the intensity of future competition through the reduction in conspecifics (Pfennig 1997; Richardson *et al.* 2010; Scharf 2016). However, there are also potential disadvantages associated with cannibalism that might lead to selection acting against it. First, is the risk of increased exposure to pathogens. The consumption of conspecifics may facilitate the spread of parasites or pathogens and may lead to epidemics in the population (Lockwood 1989; Pfennig *et al.* 1991; Pfennig *et al.* 1998). A second potential issue with cannibalism is that individuals may injure themselves as they attempt to cannibalize living conspecifics (Pfennig 1997; Richardson *et al.* 2010). Depending on the severity of the injury, individuals might either contract a pathogen or be at risk of being cannibalized themselves. Finally, cannibalism may be disadvantageous if an individual consumes their relatives, and in doing so loses out on the indirect fitness benefits they would have gained from the survival of their kin (Hamilton 1964; Breed 2014; Marshall 2016). Depending on the relative magnitude of this loss to one's inclusive fitness, selection may potentially act against the evolution of cannibalism. Given its complex nature and the costs and benefits associated with it, understanding the factors that contribute to the expression of cannibalism will help understand under what conditions may lead to cannibalism spreading via adaptive evolution.

One potential solution to the problem associated with cannibalizing one's relatives would be the presence of a kinship recognition mechanism, whereby cannibals have the ability to distinguish between related and unrelated individuals in their local environment and adjust their behaviours accordingly to avoid the former in favour of the latter (Hamilton 1964; Waldman 1988; Walls and Roudebush 1991). Kinship recognition, and its effect on the expression of aggressive behaviours has been reported in many species (Dewsbury 1988; Crosland 1990; Pravosudova *et al.* 2001; Viblanc *et al.* 2016). For instance, in Columbian ground squirrels, *Urocitellus columbianus*, where aggressive behaviours during the short breeding period often leads to infanticide of conspecifics, individuals act more aggressively towards non-kin than towards recognized relatives (Viblanc *et al.* 2016), a strategy that should result in enhanced indirect fitness gains (Hamilton 1964, Breed 2014). Whether kinship mediates the expression of cannibalism between individuals has also been the subject of much inquiry (*e.g.* Bry and Gillet 1980; Duelli 1981; Schausberger and Croft 2001; Anthony 2003; Michaud and Grant 2004; Pervez *et al.* 2005; Guedes *et al.* 2007). In wolf spiders, *Pardosa milvina*, females carry their young in eggs sacs, and are known to cannibalize juvenile spiderlings (Anthony 2003). However, while virgin females are indiscriminate in what spiderlings they cannibalize, mated females are less likely to cannibalize spiderlings, and are also able to determine which spiderlings are related to them and which were not (Anthony 2003). This behaviour is consistent with a strategy to maximize fitness through both the direct fitness gains from cannibalism and avoidance of indirect fitness losses that would arise by consuming one's offspring.

Here, we examined whether or not the expression of egg cannibalism by *Drosophila melanogaster* larvae is mediated by kinship. In addition to its long history as a model species in genetic studies (Weiner 2000; Brookes 2001), fruit flies are also increasingly being used to study social behaviours (*see* Wu *et al.* 2003; Sokolowski 2001, Sokolowski 2010; Durisko and Dukas 2013; Durisko *et al.* 2014; Camiletti and Thompson 2016). In this species, females aggregate around fermenting vegetation, where they lay their eggs, and upon hatching, their offspring remain and develop until pupation (Spieth 1974; Reaume and Sokolowski 2006). During the ~72 hours of their juvenile phase *D. melanogaster* larvae grow rapidly (Kaznowski *et al.* 1985), and consume 3-5 times their body weight in yeasts and decaying matter each day (Chiang and Hodson 1950). Larvae typically engage in scramble-based exploitative competition, first on the surface, and later by burrowing (Ashburner and Thompson 1978) wherein individuals attempt to acquire resources before they can be obtained by conspecifics (De Jong 1976; Joshi *et al.* 1996). However, as resources become depleted in these ephemermal environments, larvae will also engage in interference competition (De Jong 1976; Joshi *et al*. 1996), of which cannibalism is an extreme form. In *D. melanogaster,* larval cannibalism is expressed in times of nutritional stress and can involve individuals consuming other larvae (Vijendravarma *et al.* 2013), adult carcasses (Yang 2018) and/or eggs (Ahmad *et al.* 2015; *but see* Narasimha *et al*. 2019). Due to the high fecundity and rapid egg production (*reviewed in* Ashburner and Thompson 1978) of ovipositing *D. melanogaster* females, there is great potential larvae will encounter close relatives, and due to communal egg-laying (Spieth 1974; Reaume and Sokolowski 2006) that they will also interact with non-kin. To the best of our knowledge, there has been no published study of *D.*

melanogaster which has examined whether prospective cannibalistic larvae differentiate between these two groups, or even have the capacity to distinguish between them. Recently, Khodaei and Long (2019) reported that the expression of group-feeding behaviour in 3rd instar *D. melanogaster* larvae differed with the mean degree of consanguinity in their social group. The plasticity in this behaviour indicates that this species is capable of kin recognition at this later developmental stage. As such, we set out to see whether the cannibalistic behaviour of 2nd instar *D. melanogaster* larvae was also mediated by the presence of kin. We chose to focus on egg cannibalism as there is evidence from other insect species of larvae selectively cannibalizing unrelated eggs over related eggs (Bilde and Lubin 2001; Bayoumy and Michaud 2015; Barkae *et al.* 2016; De Nardin *et al.* 2016; Saitoh and Choh 2018). If kinship does influence cannibalism, we predict that larvae will act differently around related eggs than unrelated eggs and will be more likely to cannibalize the latter group, but, if kinship is not a factor than there should be no difference between the incidence of cannibalism of unrelated and related eggs.

Materials and Methods:

Population History and Maintenance

All ‘related’ eggs and larvae used in this study were obtained from a large, outbred population of *Drosophila melanogaster*. This population, *Ives*, (hereafter, ‘IV’) originated from a sample of 200 mated females collected in Amerherst MA, USA in 1975 (Rose 1984). We culture these flies in vials containing 10ml of a banana, killed-yeast and agar media and incubated them at 25ºC, 60% RH on a 12hr-L:12hr-D diurnal light cycle (Martin and Long 2015). The populations size is ~3500 adults at the start of per generation and is cultured *en masse* every 14 days. At this time, flies are pooled between vials, using light CO_2 anesthesia, and transferred to new vials for 2-5 hours at which time adults are discarded, and the density of eggs is trimmed by hand to ~100/vial. To obtain unrelated eggs, we used flies from the IV-*bw* population, which was created through introgressing the recessive, brown-eyed allele, *bw* into the IV genetic background via repeated backcrossing, but is otherwise maintained in the same manner and under the same conditions as the IV population.

Establishment of familial and non-familial groups

We set out to examine the potential effect of kinship in mediating egg cannibalism by *D. melanogaster* larvae. To do this, we began by collecting virgin IV males and females (collected within ~8h of eclosing from pupae), which we kept in same-sex groups in vials containing media and ample live yeast. We used these flies to set up ‘families’, by placing a single male and female into an un-yeasted vial containing 10ml of media. These ‘older sibling’ vials were left in the incubator for 48 hours. At that time, we transferred the pair of flies from each family, using light CO_2 anesthesia, into a smaller ‘younger sibling’ egg-laying chamber (Kartell Polyethylene 7ml vials 226245-10), whose cap contained non-nutritive media. This non-nutritive media consisted of 25g agar dissolved in 1L of water, mixed with 0.5g of methylparaben dissolved in 20ml of anhydrous alcohol, with food-grade green food dye added to provide visual contrast, and the surface of the media lightly scored to encourage oviposition by the females. At the same time, we transferred sets of ~100 mated females from the IV-*bw* population into half-pint laying chambers outfitted with 35mm ∅ petri dish lids (Nunclon) that also contained non-nutritive media for ovipositing. The older sibling vials and the two types of chambers were returned to the incubator for ~18 h.

Observation Setup and Data Collection Protocol

Larval behaviours were monitored in observation arenas which consisted of 20ml of non-nutritive media in a 35mm ∅ petri dish whose inner walls has been treated with Vaseline (Buckley 1940) and to prevent the larvae from escaping. Into these dishes we first transferred 10 eggs from one of the IV families' 'younger sibling' vials, and a second set of 10 (unrelated) eggs from the IV-*bw* petri dish. These two clusters of eggs were placed on opposite sides of the observation area, approximately 15mm apart. Into this arena we immediately added a single 2nd instar larvae which was obtained from 'older sibling' vial corresponding the same family from which the 'younger sibling' eggs had been collected. We filmed arenas from above (at a distance of ~23cm) using a JVC Everio GZ-HM440U video camera on a time-lapse setting (1 frame s^{-10}) in a humidified chamber in a quiet room for the next ~6-8 hours.

On each video, prior to analysis, we defined a circular zone of interest 7.8mm in diameter centered around each of the two patches of eggs. The position of each larvae was then tracked manually during a frame-by-frame video playback, and when the larvae entered/exited into either of these circles the time of these events were recorded, as was the incidence of any cannibalism during these interactions (by visually observing when larvae climbed over eggs and consumed them leaving behind the damaged egg casing). Data were collected from each video until either the focal larvae escaped the arena, any of the eggs hatched, or the video ended. From this data we were able to calculate the latency to first larval-egg interaction, the number of interactions with related and unrelated eggs, and the duration of each of these interactions. In total, we analyzed videotapes made of the behaviour of 130 larvae, of which 108 interacted with at least one of the egg patches.

Statistical Analyses

All statistical analyses were performed using R version 3.3.2 (R Core Team 2016). In all analyses the arena/larvae were used as the unit of replication, unless stated otherwise. For each individual larva we calculated the total number of interactions with the related and unrelated egg patches, as well as the mean time spent in proximity with each of the egg patches.

Our first set of analyses focused on the first and second interactions of the focal larvae with eggs. We compared the frequency of the first interaction with a related egg patch to the frequency of the first interaction with an unrelated egg patch using a binomial test (using the *binom.test* function, with H_o p=0.5), and compared the latency to first interaction and the duration of the first interactions using Mann-Whitney tests (as the data were non-normally distributed). We examined whether there were differences in the frequencies of switching patch type for those larvae that had first encountered a related egg patch with those that had first encountered an unrelated egg patch using a McNemar test (using the *mcnemar.test* function). We compared the median durations of the second interactions for all four possible combinations of first-second interaction groups using a Kruskall-Wallis test. We also used a Kruskall-Wallis test to compare the median net difference in duration between the first and second interactions for each larva for these four groups.

There were 96 videos in which larvae visited both the related and the unrelated egg patches at least once each video. From each of these videos we counted the total number of interactions with each type of egg patch, as well as the mean duration of interactions with each type of egg patch and compared the medians of the two groups using paired Mann-Whitney tests.

To understand if larval cannibalistic behaviour differed towards related compared to unrelated eggs, we focused on the first instance of cannibalism observed, and compared the frequencies of related and unrelated prey using a binomial test (with H_o p=0.5). We also examined whether there was any difference in the median latency to interaction, the median number of egg patch interactions prior to the first cannibalism, and the median duration of the egg patch interaction, between those larvae who first cannibalized a related egg and those who have first cannibalized an unrelated egg using Mann-Whitney tests, and calculated the Cliff's effect sizes using function in the *effsize* package (Torchiano 2016).

We also tested whether, overall, larvae were more likely to interact with related or unrelated egg patches by comparing the total number of interactions observed across all videos with each type of egg patch using a binomial test (with H_o p=0.5), and used a Chi-square test to determine whether rates of cannibalism of eggs differed between egg patch type.

Results:

In our assay, larvae were equally likely to initially interact with a related or an unrelated patch of eggs (number of first interactions with related eggs = 54, number of first interactions with unrelated eggs = 54; binomial test, H_o=0.5: p= 1, 95 %CI : 0.40- 0.60), and there was no significant difference in how long it took for that first interaction to occur ($\tilde{x}_{related}$=470s, $\tilde{x}_{unrelated}$=775s: Mann-Whitney test: W = 1629.5, p= 0.29). Similarly, there was no significant difference in the larvaes' frequency of encountering a related or unrelated egg patch in their second interaction (number of second interactions with related eggs = 50, number of first interactions with unrelated eggs = 56; binomial test, H_o=0.5 :,p= 0.63, 95 %CI : 0.44-0.63). The likelihood that a larva would visit a related or an unrelated egg patch on its second interaction was not significantly associated with the type of egg patch encountered on its first interaction (McNemar's χ^2 = 0.138, df = 1, p = 0.710, Table 1). There was no difference in the duration between a larvae's first and second interaction with an egg patch associated with the identity of either patch type (Kruskal-Wallis χ^2= 3.49, df = 3, p = 0.32), however larvae spend slightly less time interacting with their second patch if they had also visited it during their first interaction compared to if they switched patch types ($\tilde{x}_{\text{same patch}}$=30s, $\tilde{x}_{\text{switch patch}}$=40s: Mann-Whitney test: W = 830, p = 0.04).

When examining the behaviour of the 96 larvae that visited both patch types at least once each video, we found that larvae interacted more with unrelated egg patches than they did with related egg patches ($\tilde{x}_{\text{related}}$=4, $\tilde{x}_{\text{unrelated}}$=6; Paired Mann-Whitney test: V = 2381, p = 0.002; Figure 1). While there was no difference in the median time spent in association with either egg patch type ($\tilde{x}_{\text{related}}$=34.38s, $\tilde{x}_{\text{unrelated}}$=39.17; Paired Mann-Whitney test: V = 2381, p = 0.002), larvae spent more time overall in proximity to unrelated eggs than they did in proximity to related eggs ($\tilde{x}_{\text{related}}$=115, $\tilde{x}_{\text{unrelated}}$=185s; Paired Mann-Whitney test: V = 3050.5, p = 0.002).

Of the 130 larvae that interacted with at least one egg patch, we observed 26 individuals that cannibalized at least one egg. Of these first acts of cannibalism, 22 involved an unrelated egg, and only 4 involved a related egg (binomial test, (H_o=0.5): p= 0.0005, 95 %CI: 0.65 - 0.97). There was no statistically significant difference in the median time to cannibalism for those that first consumed a relative and a non-relative ($\tilde{x}_{\text{related}}$= 1715s, $\tilde{x}_{\text{unrelated}}$=3115s; Mann-Whitney test: W = 56, p = 0.429, Cliff's delta=0.27, 95% CI: -0.24 - 0.67), or for the duration of the interaction ($\tilde{x}_{\text{related}}$= 145s, $\tilde{x}_{\text{unrelated}}$=130s; Mann-Whitney test: W = 32, p = 0.41, Cliff's delta=-0.27, 95% CI: -0.69 – 0.28). Interestingly, larvae that first cannibalized a non-related egg, visited more than twice as many patches beforehand than those that first cannibalized a related egg, but the difference in medians was not statistically significant ($\tilde{x}_{\text{related}}$= 3 interactions, $\tilde{x}_{\text{unrelated}}$=7.5 interactions; Mann-Whitney test: W = 68, p-value = 0.09, Figure 2).

When all 1526 observed interactions of larvae with eggs were considered, 858 involved the unrelated egg patch, which is more than would be expected by chance (binomial test, H_o=0.5, p= 1.271×10^{-6}). Of the 61 observed instances of egg cannibalism, significantly more involved an unrelated egg than would be expected based on the numbers of interactions alone (Pearson's Chi-square test: $\chi^2 = 5.26$, df = 1, p = 0.022, Table 2).

Discussion:

In this study, we set out to determine whether kinship mediates the expression of cannibalistic behaviour in *D. melanogaster* larvae by examining how they interact with groups of related and unrelated eggs. In our experiments we saw evidence that larvae cannibalize eggs, that they have significantly more interactions with groups of unrelated eggs than with groups of related eggs, and most interestingly, appear to cannibalize the former group more than the latter.

In our study we chose to focus on cannibalism in *D. melanogaster,* as this species is becoming an increasingly popular model to study the expression and evolution of social behaviour, as well as its underlying genetic and neurobiological foundations (Wu et al. 2003; Sokolowski 2010; Durisko and Dukas 2013; Durisko *et al.* 2014; Camiletti and Thompson 2016). When conceiving our experiments, we were inspired by the work of Ahmad *et al.* (2015) who first reported that larval *D. melanogaster* cannibalized eggs. However, a recent study by Narasimha *et al.* (2019) (which was published after the completion of our assays) has challenged the existence of egg cannibalism in this species. Narasimha *et al.*'s research found that female *D. melanogaster* females attach hydrocarbons to the waxy layer of the egg-shell to 'mask' their offspring's scent from prospective cannibalistic larvae. They argued that these maternally provisioned chemicals were sufficient to prevent cannibalism of viable eggs, and suggested that the only reason Ahmad *et al.* (2015) observed egg cannibalism in their study was because their egg-handling protocol likely damaged the egg's wax layer, undermining the protective effects of the mother's hydrocarbons. While our observation of egg cannibalism by larvae would appear to be in direct conflict with Narasimha *et al*.'s (2019) conclusions, it should be noted that in their assay of egg cannibalism their eggs were only 1-2h old, and their cannibalism assays lasted a total of 12 hours. In contrast, we obtained eggs from chambers where females had up to ~18h to lay, and our assays ran for an additional 6-8h. Thus, it is possible that in our assays the protective scent in the egg's shell may have lost its potency or was otherwise disrupted at this later point in the embryo's development. The presence of this olfactory camouflage may explain the relatively low rate of cannibalism observed in our assays (~4% of all egg-larvae interactions, and ~24% of all larvae that interacted with egg patches), while the greater potential range of egg ages in our assays may explain some of the between and within-patch variation in behaviour, and are interesting topics for future studies.

Ovipositing females transfer a diverse bacterial community onto the surface of the eggs they lay (Wong *et al.* 2011), which is later consumed by the hatched larvae (along with the egg's chorion) and becomes established in the larvae's gut (Bakula 1969), which might provide scent/taste cues that could be used by larvae to distinguish between kin and non-kin. A fly's environment can influence the composition of its microbiome (Blum *et al.* 2013), and as such larvae may be able to perceive differences in the cues associated with related eggs and unrelated eggs. Although we do not know the mechanism by which 2nd instar larvae distinguish between the two types of eggs, our observation that larval interactions differed between the unrelated and related egg patches does suggest there is *some* mechanism present at this developmental stage that permits individuals to recognize closely related kin. Overall, larvae had more (and cumulatively longer) interactions with unrelated egg patches than with related egg patches, and eggs that were kin were less likely to be cannibalized (both initially and overall) than those that were not kin. In this species, females may lay many eggs at one site, and larvae have a limited capacity for pre-pupal dispersal (Spieth 1974; Reaume and Sokolowski 2006). Such population viscosity increases the likelihood of interacting with close relatives, which favours the evolution of social behaviours that result in indirect fitness benefits (Hamilton 1964). In *D. melanogaster* 3rd instar larvae, Khodaei and Long (2019) found evidence that co-operative feeding behaviour was more likely to arise if there was a greater degree of relatedness within a social group. Our results extend our understanding of how selective cannibalism by larvae can increase direct fitness by the acquisition of resources and eliminating competitive rivals, while simultaneously avoiding indirect fitness losses via the death of relatives. In *D. melanogaster* conspecific cannibalism can happen at many stages (Vijendravarma *et al.* 2013; Ahmed *et al.* 2015; Yang 2018) and is likely an important source of natural selection (*e.g.* it led to the evolution the anti-cannibalistic deceptive chemical traits described by Narasimha *et al.* 2019), and it would be interesting to explore the relationship between cannibalism and kin-recognition in other contexts. For instance, as variation at the *for* locus is associated with differences in larval locomotory behavioural phenotypes (Osbourne *et al.* 1997; Sokolowski 2001) different genotypes may differ in in their likelihood of encountering close relatives, and might conceivably also express conspecific cannibalism avoidance differently. Furthermore, Vijendravarma *et al.* (2013) found that larvae from populations that had been experimentlally evolved on poor-quality food were more likely to engage in cannibalism than larvae from control populations, and it would be interesting whether there were also any corresponding changes in kin avoidance behaviours.

Such studies would further our understanding about how the expression of cannibalism behaviour is influenced by both direct and indirect selection.

It is worth noting not all of our analyses detected differences in the behaviour of larvae towards related and unrelated eggs that were consistent with our *a priori* predictions about kinship's mediating effects. For instance, when examining what patch larvae visited during their second interaction, we had expected that we would see differences between those larvae that had first encountered a patch of related eggs, and those that contained unrelated eggs. We had predicted that larvae who had first encountered related eggs would be less likely to revisit that patch, and more likely to seek out prey that were not kin; while those that had first encountered unrelated eggs would be less likely to move to a different patch. Instead, the rates of patch revisiting and patch switching were similar between groups. Similarly, we had expected that, in addition to visiting kin egg patches less frequently than patches containing unrelated eggs, that larvae would exhibit shorter interactions with the former, compared to the latter groups, consistent with avoidance of relatives. Instead, we saw no statistically significant difference in their time in proximity with either type of egg. These results suggest that despite the advantages of avoiding kin, it might be difficult to achieve, potentially due to environmental factors effecting kin recognition and/or neurological constraints. The putative cues to kinship may be subtle and may be masked by anti-cannibalistic hydrocarbons (*see* Liu *et al.* 2018; Narasimha *et al.* 2019), making it more difficult to determine whether eggs are related or not. In our assay all mothers were housed under similar environmental conditions, which might further limit the opportunity for family-specific microbiome-associated cues to arise. Larval *Drosophila* can learn and form memories from scent cues, which can last for several hours (Scherer *et al.* 2003; Davis 2011) which might allow them to remember the location of related eggs in their environment. If larvae revisit patches later on, these memories should make the assessment of whether or not to cannibalize those eggs more efficient. Interestingly, we saw that those larvae that switched egg patch types between their first and second interaction spent more time associating with the new egg patches compared to those that revisited patches. However, if the olfactory differences between the egg patches are subtle, cognitive constraints in larvae may require similarly long interaction times for related and unrelated eggs alike. The limitations listed above might also explain why in our assays some larvae cannibalized related eggs despite the close physical proximity of unrelated eggs. Those larvae whose first act of cannibalism involved related eggs had interacted with fewer patches and tended to engage in cannibalism faster than those larvae who first cannibalized unrelated eggs. This could indicate an uninformed decision on part of these kin-consuming larvae, or simply less selectivity due to greater nutritional stress in these individuals. In our assay, we did not starve larvae prior to the start of our assay (*unlike* Vijendravarma *et al.* 2013 *or* Yang 2018) but there might still have been inter-larval variation in hunger. Identifying the limits to the expression of kin cannibalism avoidance will help better understand the conditions under which it can evolve.

Overall, our study indicates that while *D. melanogaster* larvae in times of nutritional stress may engage in cannibalism, rather than preying indiscriminately on eggs, they appear to avoid relatives in favour of unrelated eggs, a pattern that is consistent with predictions of inclusive fitness theory. While kin selection theory is usually applied to understand the establishment of social behaviour, it can also be used to understand anti-social behaviour (Pfennig 1997; Richardson *et al.* 2010). It is our hope that our findings stimulate further research on the proximate and ultimate causes of this interesting behavioural phenotype.

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Figures and Table:

Table 1: Identity of egg patches encountered during first two interactions by focal *Drosophila melanogaster* larvae.

	Second interaction with related egg patch	Second interaction with unrelated egg patch
First interaction with related egg patch	37	16
First interaction with unrelated egg patch	13	40

Table 2: Number of cannibalistic and non-cannibalistic interactions of focal *Drosophila melanogaster* larvae with patches of related and unrelated eggs.

	Interactions that involved cannibalism	Interactions that did not involve cannibalism
Related egg patch	17	651
Unrelated egg patch	44	844

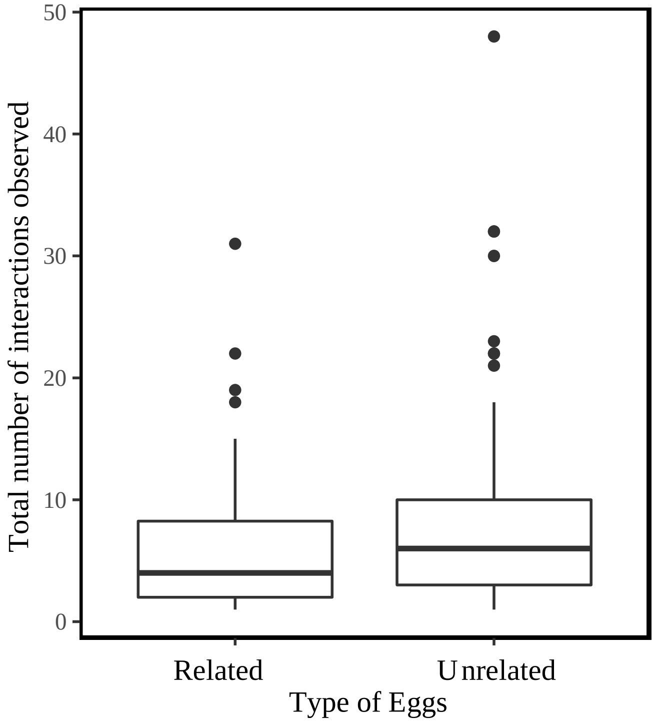

Figure 1: Boxplots illustrating the total number of interactions with unrelated and related eggs patches for the 96 *Drosophila melanogaster* larvae that visited each type of patch ≥ 1 times. Each box encloses the middle 50% of data (Inter-Quartile Range, IQR), with the location of the median represented by a horizontal line with the depicting the median. Values >±1.5 x the IQR outside the box are considered outliers and depicted as solid circles. Whiskers extend to the largest and smallest values that are not outliers.

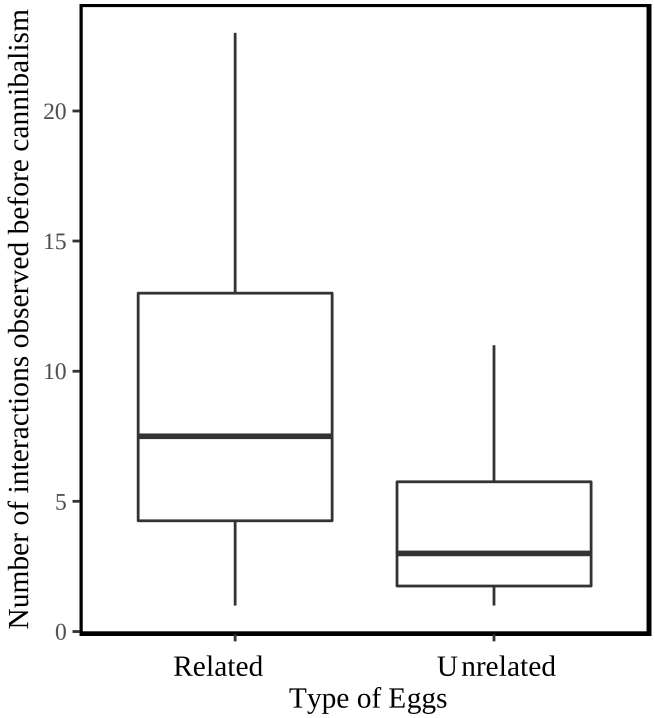

Figure 2: Boxplot illustrating the total number of interactions with unrelated and related eggs patches prior to first observed act of cannibalism for the 26 *Drosophila melanogaster* larvae that cannibalized ≥ 1 eggs. Each box encloses the middle 50% of data (Inter-Quartile Range, IQR), with the location of the median represented by a horizontal line with the depicting the median. Whiskers extend to the largest and smallest values that are not outliers.

Chapter 4

The role of kinship as a mediator of cooperation and cannibalism in *Drosophila larvae.*

Summary of Thesis:

Chapter 1:

All environments have a finite amount of resources and as these resources become increasingly scarce, individuals will either cooperate with each other to acquire new resources or competition between them can lead to antagonistic conflicts. The fruit fly *Drosophila melanogaster* has a long history as a research model for studies on behaviour. Recently it has been shown that *Drosophila* larvae can group together to form co-operative feeding clusters in order to access more nutritious food sources (Dombrovski *et al.* 2017). However, there is also some evidence that suggests larvae can be cannibalistic towards conspecifics if resources become too low (Vijendravarma *et al.* 2013). Kinship might be a factor which mediates these two behaviours at different ends of the social behaviour spectrum and may shed new light in understanding how sociality develops.

The goal of this thesis was to first examine whether kinship is a mediating factor that promotes cooperation between kin and provides more benefits compared to strangers, and additionally whether kinship is a factor that decreases cannibalism towards related individuals compared to unrelated conspecifics. In chapter 1, I discussed the factors which lead to the expression of social and anti-social behaviours in many different species. I discussed why cooperation would be beneficial and why individuals would cooperate more with kin to gain both direct and indirect fitness benefits. Primarily, individuals cooperate because they are selfish and want to pass on their genes by both direct and indirect fitness gains as described by Hamilton’s Law (Hamilton 1964; Breed 2014). In addition, I discussed the benefits, and consequences of cannibalizing conspecifics and why kinship selection and recognition might cause individuals to selectively cannibalize unrelated conspecifics over kin. For my experimental assays I wanted to observe if kinship mediates the co-operative feeding clusters between 3rd instar larvae and whether there are evolutionary benefits to co-operating with kin over unrelated conspecifics. In addition, I set out to examine how kinship might decrease cannibalistic behaviours towards related individuals compared to unrelated conspecifics in 2nd instar larvae.

Chapter 2:

In the first half of my thesis research, I wanted to see if changing the average degree of relatedness between 3rd instar larvae in the social environment would affect the level of cooperation between individuals. In the first assay, I measured characteristics of the feeding clusters (size, frequency and number of larvae), and I was able to determine the effects of having more related individuals in groups. It was observed that the more related the individuals were in a group: the more often they clustered together, the larger the clusters were, and the more individuals joined the clusters, compared to unrelated individuals who did not cluster as much. This allowed for related individuals to access more resources and potentially gain more benefits to survive. To see if kinship selection helps related individuals gain more evolutionary benefits, I measured the weight and survivorship once the larvae matured into adult flies. I observed that, on average, groups of more larvae with higher overall degrees of relatedness, had greater egg-to-adult survivorship rates, and adult females in these groups were much larger (weight being a strong indicator of fertility, see Lefranc and Bundgaard 2004) compared to unrelated conspecifics. Overall, this suggests that there are benefits to co-operating with relatives as individuals would gain direct benefits from co-operating and indirect benefits in increased fitness of related females passing on more shared genes. For my third assay I wanted to see if relatives aggregated into feeding clusters more than if they grouped with unrelated individuals when the average degree of relatedness changed. I measured the proportion of related individuals, focal IV (red-eyed) flies, to unrelated competitor IV-*bw* (brown-eyed) flies and found that more focal larvae clustered together if they were more closely related. Together, the results of these assays showed that relatives are more likely to group together, the clusters are larger and have more potential to gain resources, and there are evolutionary fitness benefits to co-operating with relatives compared to unrelated conspecifics.

The mechanism behind these larvae co-operating could be due to 'By-product mutualism' (Brown, 1983), however if the cooperation was only due to this strategy then there would be no differences between the larval treatment groups. What is more likely is that the larvae are using kin selection coupled with 'By-product mutualism' and using kin recognition in the form of sight and matching profiles to cluster (Dombrovski *et al.* 2017) either by being related or by sharing similar genotypes but not being related (Sokolowski 2001, Wu *et al.* 2003). Understanding why *Drosophila melanogaster* larvae cooperate and the underlining mechanism may help shed light on how sociality developed and why it might be valuable to cooperate with kin over strangers. However, when resources become scarce individuals will more likely behave antagonistically rather than cooperate and in extreme cases cannibalize conspecifics.

Chapter 3:

In my second thesis research project, I wanted to investigate if cannibalism could be mediated by kinship in 2nd instar *Drosophila* larvae by decreasing the rates of cannibalism of related eggs compared to unrelated eggs. When resources start to become scarce in the environment, for example, when population densities are high (Bazazi *et al.* 2008; Richardson *et al.* 2010), this frequently leads individuals into conflict with each other through competition and sometimes cannibalism, in which individuals consume other conspecifics (Richardson *et al.* 2010). Cannibalism might be adaptive, as the cannibal gains the needed nutritional resources, and by eliminating the conspecifics, they might also potentially increase their access to more resources in the future (Richardson *et al.* 2010; Scharf 2016). However, if the cannibal consumes a kin member then they will lose any indirect fitness gains they would have reaped if that conspecific had survived to reproduce and pass on shared alleles between them (Hamilton 1964; Breed 2014; Marshall 2016). Thus, examining the factors that contribute to the expression of cannibalism, such as kinship, will help understand evolutionary consequences that might arise from the expression of this behaviour and why cannibalism would be an adaptive trait.

To determine if kinship mediates the expression of cannibalism, I took 2nd instar larvae, and placed them in an arena with the choice to cannibalize related eggs or unrelated eggs. I filmed and then measured: the number of interactions, the time of each interaction, and the type of each interaction (cannibalistic or not). To test if kinship is associated with the expression of cannibalism towards eggs, I compared the rates of larval interactions with eggs and the types of interactions between related and unrelated eggs. When comparing the rates of cannibalism, I found that larvae on average did not cannibalize eggs much, however there was some evidence that when they do cannibalize eggs, they were almost 4 times more likely to cannibalize unrelated eggs than related ones. I found that although larvae visited unrelated eggs more often than related eggs, there was no difference in the mean time spent per interaction between related and unrelated eggs. However, the total time spent with unrelated egg patches was longer than with related ones. Interestingly, I found that larvae tended to return to the same location they visited before and take longer to explore unknown new eggs than familiar ones. This indicates there is kin recognition, and that this information shapes the behavioural decisions involving cannibalism. Cannibalism can be an effective way of gaining resources, such as in the desert locust *Schistocerca gregaria* (Bazazi *et al.* 2008; Richardson *et al.* 2010). Nonetheless, overall it was not that prevalent in the cannibalism assay. The reason why cannibalism may not be as prevalent, may be due to adult female flies masking the eggs scent upon laying them (Narasimha *et al.* 2019), thus rendering them invisible to larvae. However, when this defense mechanism fails, larvae are more likely to cannibalize unrelated eggs over related ones. In addition, since female flies mask their eggs using the “maternal sex pheromone 7,11-HD which deters egg cannibalism” and larvae use the pickpocket regulator genes to detect these odors (see Liu *et al.* 2018; Narasimha *et al.* 2019), this could then act as a mechanism to detect eggs which have the same scent as the larvae. Detecting the scent would allow for larvae to eliminate unrelated conspecifics to avoid future competition while increasing the expression of shared genes in the future. More research is needed to determine if kinship does mediate antagonistic interactions like cannibalism in *Drosophila melanogaster* larvae.

Future Directions:

This research can be used in several future avenues which involve behaviour and its implications on the origins of sociality in groups. In chapter 2 we were able to measure clustering behaviour and quantify the benefits of co-operating with relatives in the form of increased female weight and survivorship. In the third assay we were able to determine that individuals on average grouped with relatives compared to non-kin. However, in this assay I did not know the exact degree of relatedness between each of the individuals within a cluster, nor was I aware of whether the larvae left or stayed in the same feeding clusters. One potential future study would be to track and measure how much time individuals spend with relatives compared to unrelated individuals, and to get an accurate degree of relatedness of individuals within each cluster. This might be achieved by marking focal larvae with a protein or indicator and tracking the movement of the marked larvae and whether the focal individuals aggregate into clusters together more often than with unrelated individuals. Since the *Drosophila* genome has been sequenced and we are aware of several genes that contribute to behaviours, including *forage* which influence foraging behaviour (Sokolowski 2001), then we might be able to look for other genes associated with behaviours. Furthermore, we could also conduct DNA finger printing , for example through gene candidates and knock-outs or mitochondrial comparisons or using recombinant inbreed lines, to both get an accurate degree of how closely individuals are related to each other, but we can also measure the genetic motor profiles to determine if individuals with similar genotypes are also more likely to cooperate than those with dissimilar genetics. This would help get a better insight as to whether 'By-product mutualism' is the sole mechanism by which larvae cluster or if it is some combination involving kin selection.

A potential experiment which could shed light on this argument of whether kinship is the dominant factor which mediates cooperative clustering in 3rd instars would be to change the populations to use the *rover* and *sitter* phenotypes. In Fitzpatrick *et al*'s study (2007) they argued that in poorer food media which phenotype had greater fitness was dependent on the frequency of the phenotype (were the rarer phenotype had greater fitness). Similarly in future studies, instead of using IV and IV-*bw* we use rover and sitters and vary the degree of relatedness (similar to what was done in our studies) to test and see if it is sharing phenotypic shared forage gene phenotypes or overall relatedness, as well as testing to see if negative or positive frequency-dependent selection describes this behaviour.

The experiment in chapter 3 revealed that cannibalism by larvae, involved unrelated eggs more frequently than related eggs. However, overall cannibalistic behaviour might be deterred due to female adult flies 'cloaking' their eggs' scent and making them 'invisible' to the larvae (Narasimha *et al.* 2019); more studies are needed to determine whether this cloaking lasts until the eggs hatch or if it fades later on in development and might lead to cannibalism. A future study could entail the elimination of the heptacosadiene (7,11-HD) pheromone and/or knock-out the pickpocket 23 (*ppk23*) gene, mentioned in Narasimha *et al.*'s 2019 study, in the cannibalistic larvae to see if the larvae are able to recognize related eggs and whether this behaviour changes as a result. Alternatively, we could assess if the larvae change their behaviour when they are introduced to other conspecifics which are wounded and could be cannibalized. There is evidence to suggest that larvae will cannibalize adult and older 3rd instar larvae (Vijendravarma *et al.* 2013; Yang 2018), but it is unknown whether kin selection might be a deterring factor to prevent or lessen the rates of cannibalism on consanguineal individuals. I believe more research is needed to better understand if larvae are able to distinguish between kin and non-kin when they are able to detect them, and whether these behaviours could be a cross generational phenomenon or found in other *Drosophila* species.

Concluding remarks:

Understanding behavioural variation and its relation to group dynamics and evolutionary theory requires an integrative biology approach. This thesis is meant to bridge the gap from individual/group behaviours to population and evolutionary genetics. Behaviours are difficult to analyze and the mechanism behind why those behaviours are beneficial or detrimental is not always easy to measure or to determine. However, by experimentally manipulating the social environment, we can see whether behaviours change and how group dynamics change in a population or environment. In chapter 2 I found that an individual's behaviour changes to be more co-operative towards relatives and that there are potential fitness benefits to co-operating with relatives (primarily increase fecundity in females and survivorship overall). Since many behaviours are known to have a genetic basis, (Ayyub *et al.* 1990; Sokolowski 2001; Durisko *et al.* 2014; Camiletti and Thompson 2016) then the traits that would increase fecundity and fitness will propagate those genes and other genes in those individuals. Subsequently, this might change the genetics of the population if more of those genes are propagated to the next generation, which might cause evolution to occur, similar to what might happen with kinship promoting cooperation in fruit fly larvae. Antagonistic behaviours can also be affected by kinship and in turn cause differences in the social interactions between kin and strangers. In chapter 3, I found that *Drosophila* larvae engage in the cannibalism of conspecific eggs when resources in the environment are depleted and that kin are consumed less frequently compared to unrelated eggs. This selective preference for consuming non-kin can cause the overall degree of relatedness to increase in a population, thus the gene of this behaviour will also propagate in the next generation and change the genetic frequency of the future population. The environment effects the way individuals will behave towards each other and kinship might further influence the differences in these behaviours. As Dawkins's put it, "you can make some inferences about a man's character if you know something about the conditions in which he has survived and prospered." (Dawkins 1976). Thus, we can better understand how behaviours have shaped our experience and the evolution of sociality by understanding differences between social interactions of individuals.

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