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Disentangling the roles of social and physical environment in driving reproductive plasticity

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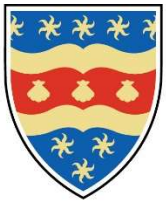

UNIVERSITY OF
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DISENTANGLING THE ROLES OF SOCIAL AND PHYSICAL ENVIRONMENT IN DRIVING REPRODUCTIVE PLASTICITY

by

EMILY ROSE CHURCHILL

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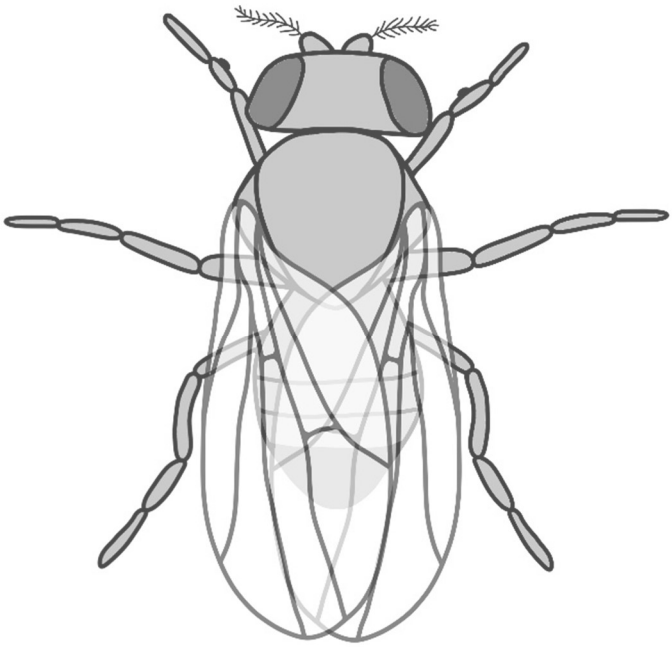
Disentangling the roles of social and physical environment in driving reproductive plasticity



by

Emily Rose Churchill

“I have to monitor how fruit flies do the nasty”

“Gross”

Gilmore Girls

Amy Sherman-Palladino & Daniel Palladino

For Mike

A fiercely intelligent Scientist. A caring and compassionate supervisor. A much-loved friend.

I miss you

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Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

The following list contains the citation of all works published during my studies at the University of Plymouth.

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Emily Rose Churchill

Disentangling the roles of social and physical environment in driving reproductive plasticity

Abstract

Resource patchiness results in spatially fluctuating population densities and thus variable levels of competition. If different traits are favoured in different population densities, individuals should adjust their physiology and behaviour in response, to maximise their fitness. In this thesis I manipulate resource distributions to investigate the implications this has on the social environment, and the sex differences in subsequent reproductive responses.

Under high levels of perceived sperm competition, male *Drosophila melanogaster* will lengthen copulation durations. Despite this being a well-established responses, little is known about the biological mechanism that facilitates this behaviour, as most of the studies that have demonstrated it have done so by comparing solitary males to paired (or grouped) males – social environments unlikely to be experienced by wild-living males. Given that resource patchiness affects encounter rates, I predicted that this ecological variability might be one of the natural stimuli affecting plasticity in mating-related traits in the wild. Here I demonstrate that these sperm competition-linked responses respond to spatial heterogeneity: males on clustered food resources

were found in closer proximity to rivals, and consequently had longer copulation durations (Chapter 3).

I then expand on this work by matching individual movement patterns in a variably patchy environment with individual-level mating behaviour and fitness (Chapter 4). I show that clustered food resources not only result in increased encounter rates, but also reduced home range size. Surprisingly, however, I find no evidence of fitness impacts associated with these responses.

Alongside increased copulation duration, males also increase sperm transfer, improve sperm quality and alter seminal fluid composition after exposure to rivals – and intriguingly have longer lifespans, although it is not understood how or why. To attempt to better understand this unexpected result, I compare survival impacts in males exposed to direct rival interactions (exposed to likely injurious aggression) and males only exposed to rivals via a barrier (Chapter 2). It appears that indirect exposure is beneficial for starvation resistance, but direct interactions result in longer lifespans. In addition, I discover that these survival benefits are only activated by male and not female presence.

Comparatively little is known about female responses to environmental variability and conspecific presence. This is surprising given that females have greater control over progeny investment due to their role in oviposition. I address this knowledge gap by exploring the impact of resource patchiness and competition on oviposition decisions. I demonstrate that females show a preference for clustered resources (with a higher number of females choosing to lay on clustered patches, and also then laying on a higher number of patches), likely due to the benefit of cooperative foraging in larvae. Interestingly, the impacts of competition differed when exposure to rivals was experienced prior to, rather than during, oviposition. After prior exposure to competition, females laid fewer

eggs (Chapter 5) whereas this effect disappeared when females were laying in the presence of another female (Chapter 6). This suggests that those females were copying conspecific oviposition decisions, minimising exploration costs and enabling them to lay a higher number of eggs.

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Chapter 1: General introduction

What do you call a fly with no wings?

A walk

1.1 Social interactions and individual responses

Social dynamics in changing population densities

The level of competition is determined by population density and the quality, quantity, and distribution of available resources. There are many different types of competition that individuals might experience: two important ones in the context of resource distributions are exploitative and interference competition. The first of these, exploitative competition, occurs when individuals are competing for consumption of shared resources (such as food, or rearing or mating sites) and interact indirectly (Rodrigues et al., 2016, Jensen, 1987). This type of competition will result in a fitness cost for the loser because the resource will be depleted before they gain access. By contrast, interference competition is a direct interaction (often via attack) between individuals where the winner will obstruct access to the resource (Rodrigues et al., 2016, Jensen, 1987) – for example, gaining access to susceptible mates, or monopolising a given food resource. Given these resources cannot be shared, and losers in these interactions will have to instead search for an alternative, there is a greater fitness cost to the losers in these competitive interactions.

When population density is high, exploitative competition is high, given resources are comparatively scarce. As a result, individuals receive a smaller proportion of the available resources, and experience crowding stress. In plant crops for example, very high-density results in a decrease in nutrient uptake, and shading causing a reduction in the rate of photosynthesis, resulting in a negative impact on yield (Chen et al., 2021, Boomsma et al., 2009, Hashemi et al., 2005). In addition, overcrowding can result in stress-induced premature

flowering; a sign that the individual cannot effectively respond to the stress and instead invests in reproduction (Boomsma et al., 2009, Takeno, 2016).

Increasing density has physiological and morphological consequences for animals too. The energetic equivalence rule is that there is an inverse relationship between population density and body size (Nee et al., 1991, Damuth, 1981) and this has been supported by a number of mammal studies (Finn et al., 2018, Zedrosser et al., 2006, Fa and Purvis, 1997, Robinson and Redford, 1986). This will be likely to have fitness consequences for the smaller individuals, as smaller body sizes have been linked to reduced offspring production (Honěk, 1993, Sand, 1996, Rideout and Morgan, 2010, Darwin, 1871) and variation in offspring sex ratio (Kölliker et al., 1999, Booksmythe et al., 2017, Calsbeek and Sinervo, 2004). Although in contradiction to this, there is evidence that density can lead to more attractive individuals: in zebra finches, group housing leads to enhanced plumage and song development, plus increased frequency of courtship and aggression (Bölting and von Engelhardt, 2017).

Furthermore, the social environment can directly impact offspring sex ratio; e.g., von Engelhardt et al. (2009) observed a more female biased sex ratio in guinea pigs, under unstable maternal social conditions (i.e., variable group dynamics). Biased sex ratios lead to an increased local density of one sex, and thus result in an increase in intrasexual competition and often aggression.

Aggression in competitive interactions

Aggressive interactions in contests are costly for both participants. Individuals must therefore consider whether the benefit of gaining the fought-over resource is worth these costs (Lane

and Briffa, 2017). Often individuals will settle conflict via visual displays of strength/size (e.g., push-ups in lizards (Brandt, 2003), and head-bobs and stomps in mandrills (Laidre, 2005)), so that injurious fights do not occur, and costs are not encountered. For the same reason, threat displays used as a "bluff" also often take place (Andersson, 1980). Whether or not these displays escalate, and a fight takes place is determined by the resource value and chance of winning for both combatants (Parker, 1974, Arnott and Elwood, 2008).

Escalation regularly occurs in territorial species. This is likely to be because territories are highly valuable, providing the holder with access to multiple resources – e.g., food, shelter and mates. Therefore, individuals attempting to establish territories have more to gain (or lose) from any competitive encounters. Good territories often come with long-term hierarchical benefits too, as seen in lobster cockroaches (Ewing, 1972). Male lobster cockroaches engage in a number of defensive behaviours to defend such territories; including chasing, fencing, charging and head-butting (Ewing, 1967, Kramer, 1964).

Aggressive behaviours observed in male and female *Drosophila* are similar to those observed in lobster cockroaches (Dow and Schilcher, 1975, Jacobs, 1960, Sturtevant, 1915, Ueda and Kidokoro, 2002, Bath et al., 2017). Not only do females compete for food, shelter and mates like their male conspecifics, but they must also compete for quality oviposition sites. Contests in females are often driven by access to mates or oviposition/nesting sites, as individuals compete to increase their lifetime reproductive success.

Sperm competition – an evolutionary perspective

Competition occurs not only between individuals, but within individuals too. Sperm competition occurs in polygamous species: females mate multiply, and therefore rival male sperm typically compete in their reproductive tract to fertilise their eggs (Parker, 1970). Males have evolved in response to this, developing traits to increase an individual's chance of successful fertilisation. These traits will be selected for over many generations, via sexual selection, resulting in those traits becoming further exaggerated (Andersson, 1994).

A commonly observed example of this is increased testes size in males associated with increased levels of polyandry, which is observed in a range of invertebrates (Hosken and Ward, 2001, Gage, 1994, Simmons and García-González, 2008), amphibians (Jennions and Passmore, 1993, Kusano et al., 1991), reptiles (Kahrl et al., 2019), fish (Rowley et al., 2019, Stockley et al., 1997), birds (Pitcher et al., 2005, Coker et al., 2002, Brown and Brown, 2003), and mammals (Harcourt et al., 1995, Breed and Taylor, 2000, Preston et al., 2003). This increase in testes size is linked to an increase in sperm size or number (Schulte-Hostedde and Millar, 2004, Simmons and García-González, 2008, Gomendio and Roldan, 1991, Møller, 1989). There is also evidence of increased sperm motility in multi-male breeding systems (Møller, 1988), and variation in other reproductive organs (Ramm et al., 2005).

In addition to this, penis morphology is altered in response to increased competition for mates in many taxa. For example, penis growth rate is quicker when waterfowl are exposed to more forced extra-pair copulations (Brennan et al., 2017). And length of the mammalian baculum (the bone located inside the penis) is also longer under male competition (Dixson and Anderson, 2004). Penis morphology has significant consequences for reproductive

success (Stockley et al., 2013, House and Simmons, 2003, Arnqvist and Danielsson, 1999, Hosken and Stockley, 2004), and has functionality beyond sperm transfer (Gallup et al., 2003).

Behavioural strategies have evolved to cope with the increased competition for mates. Nuptial gifts are given to potential mates to persuade them to succumb to copulation, and can evolve under high perceived sperm competition (Wedell, 1991). These gifts usually provide additional nutritional benefits to the recipient, and their consumption can result in increased offspring production (Steele, 1986, Gwynne, 1988).

Sperm competition – an individual's perspective

Individuals' plastic behavioural responses to varying levels of sperm competition also have fitness consequences. When competition for mates is high, individuals must invest more in energetically expensive processes to compete with their conspecifics. For males, attracting a mate is more difficult when the females can afford to be more choosy (i.e., when in good condition with large energy reserves), so under high competition they must increase their courtship efforts (Santori et al., 2020, Churchill, 2016, Tauber and Eberl, 2002) or increase forced extra-pair copulation attempts (Wysocki and Halupka, 2004).

Once a female has accepted copulation, males will lengthen the duration of mating when predicting high levels of sperm competition (Bretman et al., 2009, Lane et al., 2015, Klemme and Firman, 2013, Flay et al., 2009, García-González and Gomendio, 2004). Increased copulation durations have been linked to an increase in sperm transfer (Engqvist and Sauer, 2003, Simmons and Parker, 1992); but longer copulations could also be a form of mate-guarding by males (Vitta and Lorenzo, 2009).

To improve their chances of siring success when facing increased competition, males increase both the number and quality of sperm they produce (Gage and Barnard, 1996, Moatt et al., 2014, Giannakara et al., 2016, Ramm and Stockley, 2009) as well as the number of sperm that they transfer to the female during copulation (Price et al., 2012, Candolin and Reynolds, 2002). Males are also able to alter the composition of their ejaculate (Fedorka et al., 2011, Ramm et al., 2015, Bartlett et al., 2017). Ejaculate contains multiple seminal fluid proteins which males transfer alongside their sperm, to better enable their sperm to outcompete others (Wolfner, 2002, Wigby et al., 2009). Many seminal fluid proteins alter the receiving females' behaviour in a way that is beneficial to the male's fitness; for example, inhibition of female re-mating (Scott, 1986) and stimulation of egg-laying (Herndon and Wolfner, 1995). They are also involved in displacement of rival sperm (Clark et al., 1995) and enable more efficient sperm storage (Neubaum and Wolfner, 1999). Some seminal fluid proteins provide longevity benefits for the female too (Lung et al., 2001), although many are also inadvertently detrimental to female longevity due to toxicity that has evolved to kill rival sperm cells (Lung et al., 2002, Mueller et al., 2007).

Another behavioural response that increases with the presence of rival males is mate-guarding: with increasing rival presence, males guard females for longer (Jarrige et al., 2016, Rondeau and Sainte-Marie, 2001). An alternative strategy for preventing female re-mating, is the transfer of mating plugs which are also impacted by perceived sperm competition risk – for example, in nematodes copulatory plug size increases in higher male density environments (Canales-Lazcano et al., 2019).

Preventing re-mating is particularly important in species where “sneaker” males exist; e.g., in cuttlefish. Males can adopt one of two strategies: “guarders” will guard their mated females, whereas “sneakers” will alter their colouration to mimic females, and then sneak past the guarding males to fertilise their females (Hanlon et al., 2005). Males can change strategy throughout their lifetime; a plastic response that is likely to have evolved due to the highly male-biased sex ratio (up to 11 males to one female) and hence high competition for female mates (Hanlon et al., 2005).

1.2 Spatial heterogeneity in the physical environment

A history of spatial distribution research

The social environment dictates the level of intraspecific competition that an individual is exposed to, but the physical environment can impact variation in the social environment. For example, where resources are clustered local population density is increased, causing increased encounter rates among individuals, and therefore increased aggressive interactions (Emlen and Oring, 1977, Lim et al., 2014).

More dispersed resources (and smaller- and low-quality resources) lead to an increased foraging time, because individuals must travel further as they search (Spaethe et al., 2001, Monaghan et al., 1994, Lester et al., 2010). In some species, more complex environments further increase foraging time, as individuals must traverse more barriers during their search (e.g., freshwater fish searching in turbid waters (Murray et al., 2016)). However in others, complex environments decrease foraging time as they provide shelter enabling individuals to take a more direct, and thus more efficient, route (e.g., in harvester ants (Fewell, 1988)).

Wherever possible, individuals take the most direct route to the resource to minimise searching costs – as observed in slime moulds (Nakagaki et al., 2000)

Plant roots must respond to highly dispersed nutrients within the soil by elongating and thickening their roots, and developing more branches (Goss et al., 1993), although this is an energetically expensive strategy (Caldwell, 1979). Increased foraging time leads to a number of fitness costs for individuals of any species: increased energy expenditure and increased consumption to overcome this energetic cost (MacArthur and Pianka, 1966, Wiersma et al., 2005), plus, reduced time for alternative tasks such as predator vigilance and reproduction (Fortin et al., 2004, Godin and Smith, 1988).

Territoriality and lekking (the aggregation of males engaging in courtship displays) can occur when resources can be defended and female choice is high (Kirkpatrick and Ryan, 1991). Although this will reduce costs acquired from increased foraging, it does heighten competition, and therefore results in increased costs (and benefits) associated with this (Fiske et al., 1998, Clutton-brock et al., 1992, Whitham, 1986, Hart, 1987). However, these costs can be counteracted by knowledge of the local area. For example, bees that return to favourable resource patches have a higher fitness, because they return to a guaranteed high-quality resource (Carter et al., 2012), and slime moulds also show a preference for previously explored areas (Patino-Ramirez et al., 2019).

Due to the increased costs of searching and the reduced encounter rates when resources are dispersed, individuals tend to reside in well-connected habitats. Therefore, abundance and diversity are higher in more clustered and connected habitats, providing additional fitness

benefits to residing individuals (Gripenberg and Roslin, 2005, Aguilar et al., 2019, Theodorou et al., 2020, Rotenberry and Wiens, 1980). This will have evolutionary impacts for populations, as individuals can more easily adapt in diverse environments, and hence ecosystem stability is often higher in more diverse habitats (Yachi and Loreau, 1999, Tilman and Downing, 1994, Harding, 1999).

Resource patchiness on a smaller spatial scale

Most studies investigating the impacts of resource patchiness were undertaken at large spatial scales, to understand the implications at an ecological level. Fewer studies have been completed at a local scale in controlled environments, but these studies provide an important insight into individual (rather than population) responses. Despland and Simpson (2000) showed that even at a fine scale, locusts travelled further when foraging on dilute food sources, and aggregated near optimal resources. The same was true in woodlice when the dispersed food was of a low quality, however the opposite was true when they were given higher quality food patches (Hassall et al., 2002).

There is evidence that complex environments have similar impacts to that of low-density environments: reduced encounter rates, reduced courtship, increased mating latencies and reduced re-mating rates (in fruit flies: Malek and Long (2019); and in two-spotted gobies: Myhre et al. (2012)). Although surprisingly, Malek and Long (2019) found offspring production was increased in complex environments. This is likely to be due to these environments enabling harm avoidance in females from multiple matings. However, this seems to vary across species, given that in the three-spined stickleback, courtship intensity was increased under more complex environments (Candolin et al., 2007, Dzieweczynski and Rowland, 2004).

Another important aspect of fitness that is impacted by resource patchiness is oviposition. This is particularly challenging for metamorphosing insects as at each stage of development (egg, larval instars, pupae and adults), individuals will likely face different nutritional and environmental stresses due to the variation in their potential mobility. Therefore, females must consider the changing needs of their offspring in oviposition decisions. For example, in fruit flies females consider larval travel in their oviposition location decisions: with site preference for sucrose disappearing when larvae are capable of migrating to the sucrose source once hatched (Schwartz et al., 2012). Competitive and cooperative interactions between offspring are both found in different circumstances and understanding how parental fitness benefits from either positive or negative interactions between larvae can be complex. For example, cowpea beetle females reduce larval competition by avoiding sites with existing larvae – using vibration cues from their movements when choosing where to oviposit (Guedes and Yack, 2016). However in many species, offspring benefit from cooperative foraging (caterpillars: Fitzgerald and Peterson (1988); Clark and Faeth (1997); flatworms: Cash et al. (1993); sawflies: Ghent (1960); and treehoppers: Cocroft (2005)), and females will preferentially lay in the presence of conspecific larvae. Optimal oviposition strategy will likely vary in changing environments, and thus spatial heterogeneity will impact plastic responses at all life stages.

1.3 The importance of plasticity

Surviving in a changing climate

Future climate change scenarios include elevated mean temperatures, increased variability in temperatures and severe alterations to weather systems (Petit et al., 1999, Mirza, 2003, van

Aalst, 2006, Smith et al., 2012). These changes to weather patterns are in turn constraining geographic boundaries, and impacting the spatial and temporal distribution of resources and populations (Guisan and Zimmermann, 2000, Perry et al., 2005, Hijmans and Graham, 2006). Parmesan and Yohe (2003) demonstrated that climate change is causing significant range shifts and advanced Spring events for over 250 species across the globe. In addition to this, it is also reducing the total abundance of a number of species (Thomas et al., 2004, Bellard et al., 2012, Urban, 2015), yet increasing the abundance of others (Liao et al., 2017, Høye et al., 2021).

Many organisms exist in complex habitats, with fluctuating environments. As these environments change, so does resource value and competition. When resources are scarce, competition is heightened and the value of resources (whether food, mates or nesting or oviposition sites) increases. Resources that are physically isolated can be more easily defended than those that are highly aggregated. Therefore, levels of aggression experienced will be likely to be altered by both the abundance of local resources, and the density of the population (number of rivals) (Lim et al., 2014, Kilgour et al., 2020).

The role of phenotypic plasticity in adaptation

Climate change will have a huge impact on the physical and social environment experienced for all flora and fauna (Hyvönen et al., 2007, Mitchell et al., 1993, Karlsson and Wiklund, 2005, Smith, 1958). Being able to adapt to changing scenarios with quick and often reversible responses will enable individuals to better cope with stresses caused by environmental variability. The ability of individual genotypes to respond to variation in surroundings by producing different phenotypes, is known as phenotypic plasticity (West-Eberhard, 1989). At

a population level plasticity increases ability to cope with short-term variation, increasing the persistence of populations, as it gives them more time to evolve in response to long-term irreversible changes in climate. However, the impact of phenotypic plasticity on evolution is unclear. There is a long-standing debate over whether phenotypic plasticity acts as a constraint for natural selection, limiting the potential for variation, or whether it could actually be a fundamental component of evolution (Thompson, 1991, Ducatez et al., 2020, West-Eberhard, 2005, Wright, 1931, Schmalhausen, 1949). Whether or not plasticity is essential in facilitating adaptation is yet to be resolved, but there is now considerable evidence emerging of the heritability of plastic traits (Nussey et al., 2005, Pelletier et al., 2007, Scott et al., 2018, Bergstrom et al., 2012, Araya-Ajoy and Dingemanse, 2017, Scheiner and Lyman, 1989) which appears to be linked to the predictability of the current environment (Oostra et al., 2018, Ashander et al., 2016, Reed et al., 2010, Snell-Rood, 2013).

Phenotypically plastic responses have been observed in a wide range of plants (Wahl et al., 2001, MacKenzie and Kundariya, 2020, Gratani et al., 2006, Labra et al., 2017), animals (Houslay et al., 2020, Mathur and Schmidt, 2017, Menz et al., 2017, Kim, 2016, Brumm et al., 2009, Carter et al., 2012) and fungi (Slepecky and Starmer, 2009, Muggia et al., 2014, Soll, 2002, Promislow, 2005), and they have important consequences for fitness. Plastic traits can be physiological; for example, body colour variation in response to light in aphids (Tougeron et al., 2021) and in response to seasonal variation in lizards (Pellitteri-Rosa et al., 2020). They can also be morphological, such as the increase of stomatal density in response to elevated precipitation in the Canadian sugar maple (Zhu et al., 2020), or the increase in shell roundedness observed in freshwater snails in the presence of certain predators (Brönmark et al., 2011). Alternatively, plastic response can be behavioural; for example, female clownfish

increase parental care when in high nutrition environments (Barbasch and Buston, 2018) and blackbirds sing at a higher frequency in response to anthropogenic noise (Slabbekoorn and Peet, 2003).

Reproduction versus survival: A trade-off

Plasticity enables genotypes to alter their phenotype, depending on their environment and current condition. If a phenotype is in a good condition and in expectation of a favourable environment for offspring survival, they will be able to afford the energetic expense of reproduction and therefore will invest in offspring reproduction. Alternatively, when an individual is stressed and close to death, they may invest in terminal investment – investing all their remaining energy into reproducing for a final time, prioritising passing on their genes rather than conserving energy to survive (Clutton-Brock, 1984).

There are many behaviours that are crucial for successful reproduction, but all come with some energetic cost: contests for mates (Lane and Briffa, 2017, Marler and Moore, 1988), courtship (Cordts and Partridge, 1996, Bennett and Houck, 1983), copulation (Jordan and Brooks, 2010, Wigby and Chapman, 2005, Rolff and Siva-Jothy, 2002), spermatogenesis (Olsson et al., 1997, Pitnick, 1996) and egg production (Visser and Lessells, 2001, Rosenheim, 1999). For these behaviours to be economical, the benefit of initiating the behaviours must outweigh the costs. Individuals must trade-off costs versus benefits of completing these behaviours in order to maximise their lifetime reproductive success. In this thesis, I will investigate the impacts of variable social and physical environments on reproduction and survival, and therefore potential lifetime reproductive success.

1.4 In this thesis…

Drosophila – a behavioural model system to understanding reproduction and fitness

I will be using the model species *Drosophila melanogaster* in all studies presented in this thesis. In the wild, *Drosophila* feed and breed on fallen fermenting fruit, colonised by yeast (Begon, 1982). This resource is patchy and ephemeral, with individual fruits naturally varying in size, quality, and proximity. As a result, local population density and sex ratio can vary considerably (Markow, 1988, Soto-Yéber et al., 2018). This variation will impact the frequency at which individuals encounter conspecifics, and thus will alter an individual's perception of existing and future levels of competition.

Drosophila are a polygamous species (both males and females mate multiply) and form social groups on fallen fruits (Dukas, 2020). This tendency to aggregate means that competition for mates is often high (Dobzhansky and Pavlovsky, 1967, Imhof et al., 1998). Individuals have physiological and behavioural plastic responses to make them better competitors and increase their chances of acquiring resources and successfully siring offspring. For example, they are able to respond to changing nutrient availability (Churchill, 2016, Churchill et al., 2019, Davies et al., 2019, Morimoto and Wigby, 2016, Terashima and Bownes, 2004), temperature (Clemson et al., 2016, Sambucetti et al., 2015, Comeault and Matute, 2021, Zerulla et al., 2017), and population density and sex ratio (Sultanova and Carazo, 2019, Mazzi et al., 2009, Fanara and Werenkraut, 2017). Individuals also alter their behaviours as they, and their mating partners, age (Churchill, 2016, Churchill et al., 2019, Lüpold et al., 2010, Mossman et al., 2019, Dhole, 2014).

Under high competition, males must often fight off rival males to gain access to females. Once a male identifies a competitor, he will orientate towards them and raise and twist his wings, before charging forwards (Dow and Schilcher, 1975, Jacobs, 1960). The recipient male will then choose to either flee or reciprocate, often then engaging in tussling and head-butting (Jacobs, 1960, Dow and Schilcher, 1975, Sturtevant, 1915). This antagonistic behaviour will continue until a winner gains access to the female (or other resource).

Another form of pre-copulatory competitive investment found in males is investment in elaborate courtship behaviours: chasing, wing extensions (raising one or both wings to ~90° in the direction of the female), abdomen taps, genital licks and mounting attempts (Spieth, 1952, Manning, 1959). These behaviours are associated with the female accepting copulation. Females must either accept the male's courtship efforts, or respond with rejection behaviours: fleeing, kicking, fluttering of their wings, or the extrusion of their ovipositor (Spieth, 1974, Spieth, 1952). Female receptiveness determines the length of courtship displays and dictates if and when copulation takes place (Bastock and Manning, 1955).

Male responses to competition continue during copulation. Under increased perceived sperm competition, males extend mating durations (Bretman et al., 2009, Bretman et al., 2010, Moatt et al., 2013), likely to increase sperm transfer (Price et al., 2012) and possibly as a form of mate-guarding too (as has been observed in *Triatoma*: Vitta and Lorenzo (2009)). Males also alter the composition of their ejaculate after exposure to consexual competition: increasing sperm quality (Moatt et al., 2014), and altering the seminal fluid proteins transferred to increase sperm competitive ability (Fedorka et al., 2011, Wigby et al., 2009, Wolfner, 2002).

After successful copulation, females must compete for the best oviposition sites. The behaviours they initiate are similar to those observed in male aggression: approaching, lunging, head-butting, shoving and wing raises (Ueda and Kidokoro, 2002, Chapman and Wolfner, 2017, Bath et al., 2017). Females also use oviposition decisions to maximise their fitness. They can respond to variation in resource composition (Sumethasorn and Turner, 2016), variation in resource texture (Silva-Soares et al., 2017), changes to the surrounding environment (Abbott and Dukas, 2016), and can even respond to decisions made by conspecifics (Lihoreau et al., 2016, Malek and Long, 2020, Tait et al., 2020). Despite their greater influence on progeny fitness, female reproductive responses have received less research attention than their male conspecifics, and little is known about oviposition responses. In two chapters of this thesis, I focus specifically on how female interactions affect this critical component of fitness.

Research aims

Most investigations into the impact of spatial heterogeneity have been landscape-scale studies using vertebrates (e.g., Holmes and Schultz, 1988, Koenig et al., 1998, Pruetz and Isbell, 2000, Ryer and Olla, 1995). Here however, I test these principles at a relatively small scale so that individual responses can be explored. To my knowledge, this is the first attempt to explore laboratory-based principles of plastic responses to competition and present them in a more ecologically relevant context, where resources are patchily distributed; and I investigate these responses in both males and females.

The impacts of the presence of rivals, and the perceived likelihood of sperm competition on reproductive behaviour have been demonstrated by empirical studies in a wide range of

organisms: including in insects (Gage, 1995, McCullough et al., 2018), platyhelminths (Giannakara et al., 2016), rodents (Firman et al., 2018, Ramm and Stockley, 2009), frogs (Buzatto et al., 2015), and lizards (Kustra et al., 2019). The behavioural and physiological impacts in *Drosophila* are especially well-known and include: increased mating duration (Bretman et al., 2009); increased proportion of live sperm (Moatt et al., 2014); increased sperm transfer (Price et al., 2012); and altered seminal fluid protein production (Fedorka et al., 2011). However, less is known about the long-term impacts for the longevity of each individual. There is some evidence that survival is increased in virgins after exposure to rivals (Moatt et al., 2013, Bretman et al., 2013b), yet aggressive interactions in many species can be injurious and have damage costs regardless of outcome (Lane and Briffa, 2017). In Chapter 2, I aim to disentangle the impacts of exposure to rivals from the impacts of aggressive encounters on individual survival and reproductive responses.

Like many studies on competition and perceived sperm competition risk, in Chapter 2 I manipulated exposure to rivals using solitary and paired males (Moatt et al., 2013, Lizé et al., 2012, Price et al., 2012, Bretman et al., 2013a, Bretman et al., 2009). Friberg (2006) was the first to show an increased mating duration in *D. melanogaster* in response to rival male cuticular hydrocarbons. Bretman et al. (2009) later demonstrated this response by manipulating direct exposure to rival males (comparing males in groups of one, two and four), and since then their work has been cited over 200 times. Although this method is still widely used, the conditions created in this artificial laboratory environment are not obviously translatable to conditions experienced in the wild. It seems improbable that any individual would experience either high density in a small volume with no chance of escape, or a completely solitary existence in wild-living populations, where local population density is

more variable. Because most of this work has been undertaken in laboratory conditions, it is not yet known what mechanisms could be driving these responses in natural populations of *Drosophila.* In the wild, *Drosophila* feed on fallen fermenting fruit, a resource that is naturally patchy and ephemeral, and varies in size and proximity (Begon, 1982). This would lead to variation in local population density in time and space, with increased encounter rates found where resources are clumped (Emlen and Oring, 1977). In Chapter 3 of this thesis, I use small-scale variations in the physical environment (uniform, clustered or dispersed resources) to understand how this variation in resource distribution affects the social environment and thus perceived sperm competition risk – establishing whether resource patchiness could be a potential driver of the observed male plastic responses in terms of reproductive investment.

In Chapter 4, I then test this principle in more detail using marked males to track individual responses to variable resource distributions and associated conspecific density. *Drosophila* are territorial (Lim et al., 2014), and using marked individuals, I investigate home range size and distance travelled, and link this to subsequent copulation durations and fitness responses. Such territoriality may also help to explain the variation observed in behavioural responses by individuals from the same enclosure, given it is possible that this is due to differences in male hierarchical position (Goodman, 1979).

Females have a greater degree of control over investment into progeny than males in most species, as they often have control over paternity (Birkhead and Møller, 1993, Rodriguez-Enriquez et al., 2013, Ward, 1993), number and ratio of offspring via ejaculate manipulation (Elgar et al., 2000, Passera et al., 2001, Sato and Karino, 2010), and generally choose the nesting or oviposition site location. Given this greater degree of control, it is surprising that

the responses of females to resource availability, local population density and competition has received less attention than male responses. In Chapter 5, I begin to address this knowledge gap by exploring the impact of prior exposure to rivals and resource patchiness (using the same clustered and dispersed resource distributions as used in the previous two chapters) on oviposition decisions and subsequent fitness.

In males, responses in reproductive behaviour to prior exposure to rivals differs from current exposure to rivals. For example, mating duration in *D. melanogaster* increases after prior exposure to rivals (Bretman et al., 2009, Moatt et al., 2013, Price et al., 2012), but decreases when rival exposure occurs during copulation (Bretman et al., 2009) possibly due to harassment from rivals. In females, the costs and benefits of consexual presence are more complex. In addition to the costs of competing (e.g., costs of aggressive interactions (Bath et al., 2017, Ueda and Kidokoro, 2002) and potential cannibalism in future larvae (Vijendravarma et al., 2013)), there are also benefits of cooperation (e.g., oviposition copying behaviours reducing sampling costs (Malek and Long, 2020) and cooperative feeding aggregations in larvae (Khodaei and Long, 2020)). In my final chapter, I explore the differences in oviposition decisions when females are exposed to mated and virgin consexuals whilst ovipositing, and the subsequent consequences for individual fitness.

Chapter 2: Contrasting impacts of competition on survival

What do you call a fly with no wings or no legs?

A roll

Abstract

In polygamous species, each male's sperm must compete against that of rivals to fertilise a female's egg. In these species, males are expected to plastically adjust their physiology and behaviour in response to increasing perceived sperm competition risk, to maximise their potential fitness. It has been reliably established in *Drosophila* that after exposure to potential rivals, males will increase the duration of subsequent copulations. However, how they respond to the presence of female conspecifics is not yet known. In this study I show investment in copulation duration is only activated in the presence of potential rivals, and response to males in the presence of females is similar to that of males in solitude. Intriguingly, it is also well-established that this exposure to rivals in males increases their longevity. This is surprising, and as yet has no clear biological explanation. I compare longevity impacts in males exposed to direct interactions with males, and those exposed only to indirect interactions (and thus should not experience the negative impacts of aggression) to better understand this unexpected phenomenon and reveal that indirect exposure appears to be beneficial for starvation resistance, but direct interactions result in longer lifespans.

2.1 Introduction

Consequences of competition

Individuals must engage in aggressive interactions to gain access to resources needed for survival or reproduction. However, aggression is costly: fights could result in a severe injury or death for the loser, and it is likely that even the winning party will undergo energetic costs in completing the aggressive actions (Lane and Briffa, 2017). In addition, there are opportunity costs (time spent fighting reduces time available for foraging and mate-searching), and increased risk of predation due to decreased vigilance (Hess et al., 2016, Dunn et al., 2004).

Individuals must continuously reassess whether the potential gains from a contest are worth the risk of injury (Payne, 1998). Winning the fight results in that individual securing access to the fought-over resource (often food, a mate, or a good oviposition or nesting site) that is vital for improving their lifetime reproductive success. Plus, in many species, winning aggressive encounters ensures a higher position in the hierarchy (Meese and Ewbank, 1973, Sbragaglia et al., 2017), which will lead to additional future benefits. Individuals must trade-off the (often) short-term benefits of fighting and reproduction versus the long-term benefit of a longer lifespan.

Sperm competition and other trade-offs

Reproduction-longevity trade-offs are often observed in males at high population densities, due to an increased perceived sperm-competition risk. Males of a range of species have been shown to increase sperm production and transfer, and copulation duration (Bretman et al., 2009, Candolin and Reynolds, 2002, Price et al., 2012, García-González and Gomendio, 2004,

Gomendio and Roldan, 1991, Kiss et al., 2019, Klemme and Firman, 2013) when exposed to rival males prior to mating, despite the increased longevity costs associated with these plastic behaviours (Chapman, 1992, Creighton et al., 2009, Dewsbury, 1982, Boulton and Shuker, 2015, Olsson et al., 1997). However, paradoxically it has also been found that exposure to a rival male has longevity benefits: Moatt et al. (2013) and (Bretman et al., 2013b) found that an increased perceived sperm competition risk increases survival in virgin males.

A wide range of studies have investigated the effects of male rival presence on reproductive behaviours and longevity in *Drosophila melanogaster* (Price et al., 2012, Moatt et al., 2014, Bretman et al., 2017, Bretman et al., 2010, Lizé et al., 2012). But comparatively few have attempted to understand the effects caused by female presence and varying population sex ratios (although note: Sultanova and Carazo (2019), Mazzi et al. (2009), Chechi et al. (2017)), depsite instability of sex ratios often occurring in natural populations (Pitnick, 1993).

Aggressive interactions in Drosophila melanogaster

D. melanogaster population densities in the wild greatly fluctuate, as their habitats are inherently patchy. They inhabit patches of rotting fruit in sites such as orchards and compost heaps (Begon, 1982). As these patches are a source of food and oviposition sites, and thus potential mates, motivation to fight for access to patches is high in both males and females. When engaging in aggression, male *Drosophila* exhibit a range of fighting behaviours, including wing raises, charging, lunging, tussling, and head-butts (Dow and Schilcher, 1975, Jacobs, 1960, Sturtevant, 1915), and aggressive behaviours initiated by females are characteristically similar (Bath et al., 2017, Ueda and Kidokoro, 2002, Chapman and Wolfner, 2017).

It is not yet known whether fruit flies form hierarchal systems, but some individuals do establish distinct territories (Hoffmann, 1987), and there is evidence that territorial males have better mating success (Hoffmann and Cacoyianni, 1989). It is therefore likely that winners will continue to be rewarded for their efforts even after the fight has concluded – likely improving their lifetime reproductive success.

Assessing survival: Lifespan versus starvation resistance

There are several ways in which survival has been influenced and quantified in *D. melanogaster*: including by desiccation resistance (Hoang, 2001), starvation resistance (Moatt et al., 2013), temperature resistance (Hangartner et al., 2017, Manenti et al., 2014), via immune responses (Helenius et al., 2009) or by observing lifespan (Galin et al., 2019, Partridge et al., 1987b, Lee, 2008). This study investigates the effects of exposure to competition on lifespan and starvation resistance.

In laboratory populations, *Drosophila* can survive for over 50 days (Linford et al., 2013), although it is thought that lifespan is much shorter in wild populations due to predation and other stressors (Rosewell and Shorrocks, 1987, Behrman et al., 2015). Effects on lifespan observed in the laboratory are therefore often only observed in late-life (likely later than an individual would be expected to survive in the wild) and are potentially just artefacts of the *ad libitum* resources provided under controlled conditions. However, the opposite is true of starvation resistance: any effect observed under resistance to starvation would likely be accelerated compared to effects observed in natural populations as lifespan under starvation can be as short as three days (Churchill, 2016, Moatt et al., 2013). In this study, I use both approaches to determine whether they reveal similar outcomes.

Aims of this investigation

Although the effects of rival male presence have been widely studied, nothing is known about the effects of exposure to females on male *D. melanogaster* reproductive behaviours and survival. As sex ratios and density vary in wild-living populations, exposure to females will vary, and thus it is important that we understand how this might impact male fitness. In this chapter I address these omissions with two distinct experiments. The first of these experiments investigates direct (full contact) and indirect (exposed to visual, olfactory, and auditory cues only) interactions between males in the presence of females, and how this impacts copulatory behaviours, fitness, activity levels and starvation resistance. The second compares direct and indirect male interactions separately to female interactions, and the impact this has on the same copulatory behaviours, fitness and longevity.

Testing both direct and indirect male interactions enables further clarification on the differences between the impacts of increased perceived sperm competition risk alone (indirect), versus the combined impacts of increased perceived sperm competition risk and aggression. Despite the vast existing knowledge of the effect of perceived sperm competition risk on reproductive behaviours and survival in *Drosophila* (Moatt et al., 2013, Bretman et al., 2013a, Garbaczewska et al., 2013), it is not yet known how aggressive interactions may interfere with these effects. Furthermore, in this study I also measure whether the presence of females has similar or differing impacts on male responses.

2.2 Experiment One: Methods

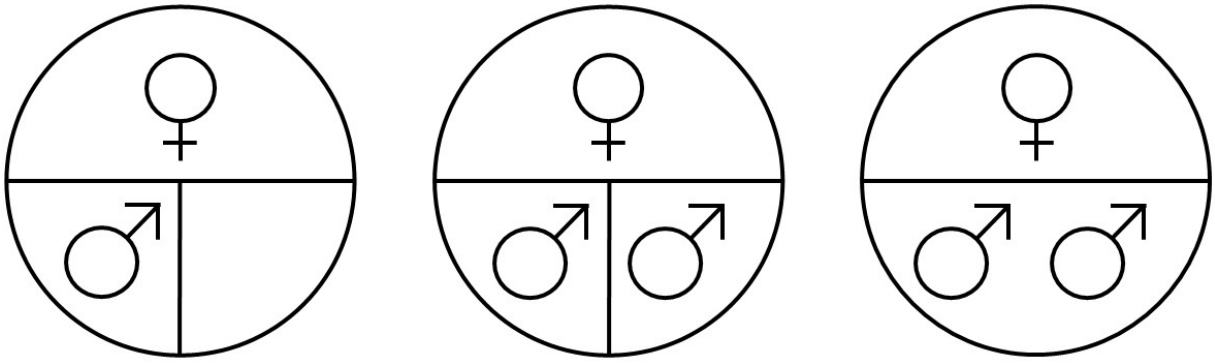
All *D. melanogaster* used in this study were housed and tested in standard laboratory conditions: a 12hour light:dark cycle (08:00 – 20:00h GMT), at 25°C. Flies originated from a laboratory population (Canton-S), and populations were housed in 40ml vials containing 7ml of a standard agar-based medium of 40g of yeast per litre (Appendix 1. i). Between 20 and 30 *Drosophila* were housed in each vial, and all vials were pooled and randomly redistributed into new vials to limit the impacts of drift, inbreeding and selective sweeps.

I collected test flies from parent vials with a standardised maximum density: six males and six females were allowed to breed for 70-98 hours before being removed. I did this to ensure that density does not exceed a maximum crowdedness level, and therefore food availability does not limit larval development. Individuals were removed from these vials within six hours of eclosion to ensure virginity, and immediately aspirated into treatment conditions under light ice anaesthesia.

Manipulating age and exposure to rival competitors

There were six treatments in this investigation: solitary males (control), paired males (referred to as direct interactions) and paired males separated by a mesh divider (referred to as indirect interactions); all at either age one day old or 12 days. All were housed in the presence of a single stimulus female to encourage aggression, also separated by a mesh divider. The setup of these vials is illustrated in Fig. 1.

The dividers for the treatment vials were made from transparent acetate, cut to the diameter of the vials, with six pin-sized holes along each edge. This allowed individuals to sense each other through visual, olfactory and auditory cues, but prevented them from fully interacting – and in the case of the female, prevented them from mating.



a. b. c.

Figure 1. Experiment one: male exposure treatments

a. Solitary male treatment: one male confined to one compartment

b. Paired males separated treatment: two males separated by a perforated acetate divider

c. Paired males treatment: two males in a double-spaced compartment, able to fully interact with one another

Sixty-six males were tested in mating assays (22 per treatment) and investigations were completed across 56 days. Final sample sizes for the longevity investigation ranged from 22 – 25. One day old flies were not included in this reproductive behaviour analysis because, despite being sexually mature, they showed very low copulatory success rate. Of the 22 males given the opportunity to mate, only four (18.2%) successfully mated (no treatment effect: $\chi^2 = 0.283$, d.f. = 2, $p = 0.868$).

Mating behavioural assays

Twelve-day old flies were transferred to individual chambers of a mating apparatus comprising an array of 16 holes (chambers) drilled into a Perspex block (Fig. 2) – each one 650mm^3 in volume and containing 0.01g of active yeast granules added to encourage copulation.

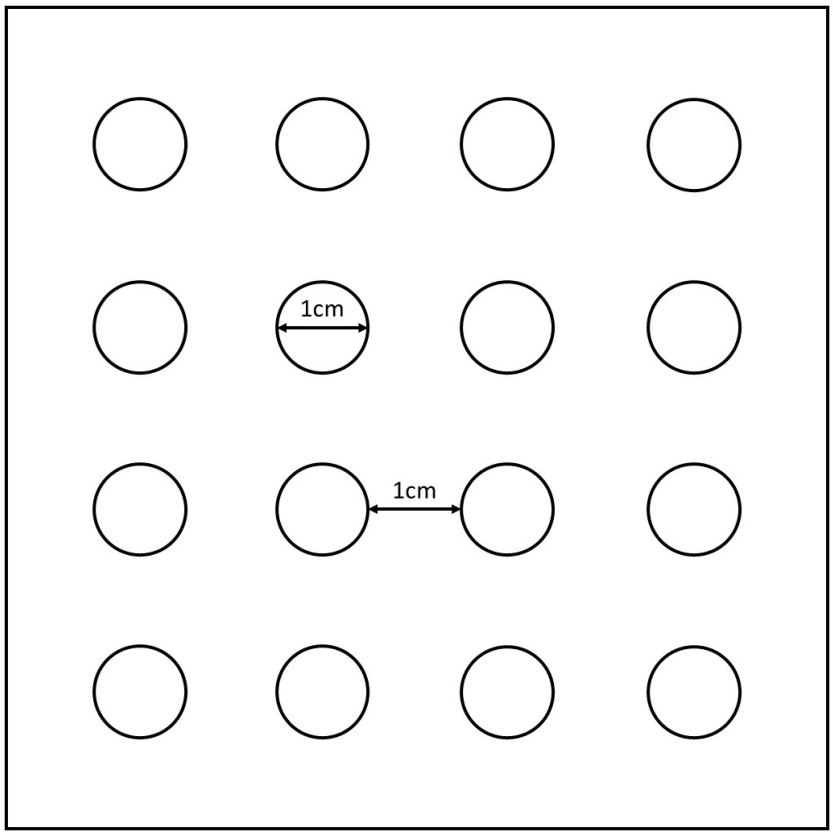


Figure 2. The platform – containing 16 mating chambers

Each male was paired with a standard seven-day old female in the chamber (Churchill et al., 2019). Males were transferred to the mating chamber first, followed by females. The pair remained separated by a horizontally placed acetate sheet for five minutes prior to introduction to enable them to acclimatise. Once the separating sheet had been removed and

the pair had been introduced, all mating behaviours were recorded using a Basler camera and Media Recorder software (Noldus, 2018).

Video footage was recorded for two hours and reproductive behaviours subsequently quantified. Courtship latency was calculated in seconds, from the time in which the pair were first introduced, to the time until the male initiated the first wing extension. Latency to copulate is equal to courtship duration: it begins with the first wing extension and ends when the male successfully mounts the female to begin copulation. As male mounting is also seen as a courtship attempt, this was only recorded as the start of copulation when the pairs' genitals met. Finally, copulation duration starts as courtship ends – when the male successfully mounts the female. Copulation was recorded as ended when the pair had fully separated. In cases where the pair took more than two minutes to separate (when both individuals were kicking the other and attempting to walk away), mating duration was not included in the analysis.

Courtship and copulation did not always take place, so observation windows were set. Observation windows were 30 minutes for courtship latency, and 75 minutes for copulation latency.

Once the pair had finished mating, the female was transferred from the mating chamber to a standard vial. She was left to freely oviposit for seven days, before being removed. Fourteen days after her removal, the total number of eclosed adults were counted to give an indication of fitness.

As not all mating chambers were used, the number of mating pairs in the surrounding chambers (henceforth referred to as 'neighbours') varied. The number of neighbours for each pair in each assay batch was monitored, to determine whether this impacted behaviour of the focal male.

Quantifying activity and survival

Eighty-eight one day old flies and 74 12-day old flies were used in an investigation into the effects of male-male interactions on activity and starvation resistance.

D. melanogaster were aspirated into a 5mm by 65mm glass tube, containing 0.5ml of starvation agar (Appendix 1. iii). These were then placed into the *Drosophila* Activity Monitor (DAM) (DAM, Trikinetics, Waltham, MA, USA); pictured in Fig. 3. This measures the activity levels of each fly, by counting the number of times the infrared beam that passes through the glass tube is broken by movement. A time of death can also be calculated from this data – the time at which the infrared beam is no longer being broken by movement. Resistance to starvation was used as a proxy for longevity; a technique that has been used many times since Rose et al. (1992) first established a link between the two factors (Moatt et al., 2013, Graves et al., 1992, Wit et al., 2013).

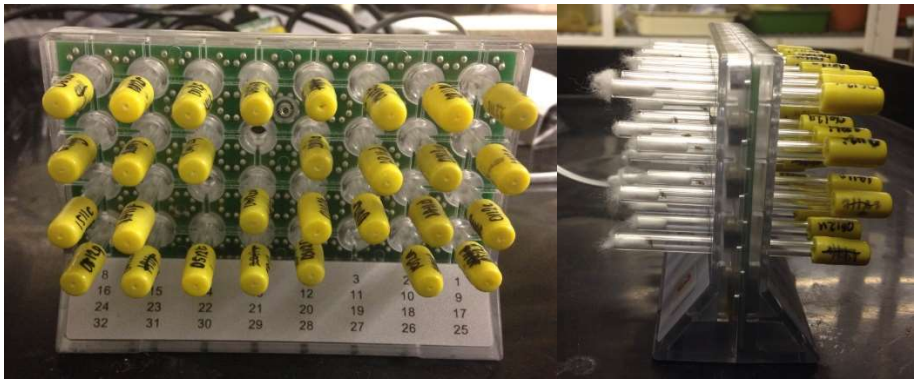


Figure 3. *D. melanogaster* in the *Drosophila* Activity Monitor

The output from the DAM system was extracted and time of death was calculated using R version 4. 0. 3 (R Core Team, 2019); this code can be found in Appendix 2.

Body size measurements

Once testing had been completed, males and females were frozen to -18°C and their left wings were removed. Wing lengths of each individual were measured to the nearest 0.005mm under a binocular microscope (Meiji PKL-2 equipped with a graticule) at 40x magnification.

Measurements were taken from the intersection of the anterior cross vein and the longitudinal vein, to the intersection of L3 with the distal wing margin, as described by Partridge et al. (1987c); shown in Fig. 4.

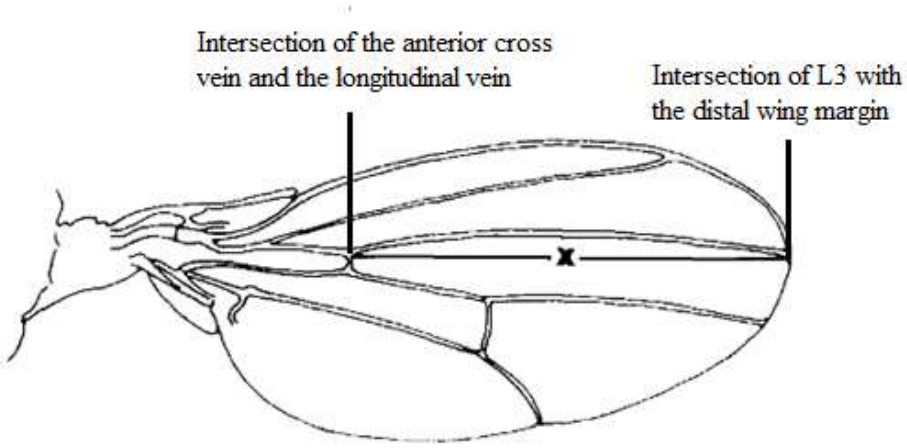


Figure 4. Method used to measure individual wing sizes

'X' highlights the two landmarks used – diagram adapted from Partridge et al. (1987c)

Previously collected data has shown that the size of the left wing and the right wing of *D. melanogaster* are not significantly different (Churchill, unpublished; males: left wing: $\bar{x}$ = 1.24, SD = 0.069, right wing: $\bar{x}$ = 1.24, SD = 0.060, t(11) = 0.642, p = 0.524; females: left wing: $\bar{x}$ = 1.39, SD = 0.09, right wing: $\bar{x}$ = 1.40, SD = 0.08, t(11) = -0.699, p = 0.489), so where the left wing was absent or damaged the right wing was used.

When flies were observed in pairs for mating behavioural assays, the size difference between the mating pair was also calculated by subtracting the male's wing length from the female's wing length.

Statistical analysis

Twenty-two males were tested in each of the three mating behaviour treatments (66 total), and between 19 – 23 individuals were assessed in each treatment in the investigation into

starvation resistance (142 individuals across the three competition treatments and two age classes). All statistical analyses were conducted in R version 4. 0. 3. (R Core Team, 2019).

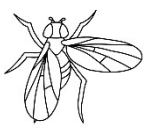
I tested normality assumptions using Shapiro-Wilk tests; linear models were used for the data that met these assumptions. Count data were analysed using GLMs with quasiPoisson error distribution to account for overdispersion. Finally, Cox proportional hazards models and ANCOVAs were used to assess the effect of treatment on starvation resistance and relative activity.

2.3 Experiment Two: Methods

In this second experiment I explored how the presence (via direct and indirect interactions) of rival males and also females impact male reproductive behaviours, fitness and longevity (as opposed to starvation resistance). *D. melanogaster* used in this second experiment originated from the same stock and were maintained in the same conditions described for the previous experiment.

Manipulating access to rival male competitors and females

There were four treatments in this study: solitary males (control; Fig. 5a), paired males (referred to as indirect interactions; Fig. 5b), paired males separated by a perforated divider (referred to as direct interactions; Fig. 5c), and finally solitary males exposed to a female (via a perforated divider; Fig. 5d). The dividers used were the same as those used in the previous experiment.



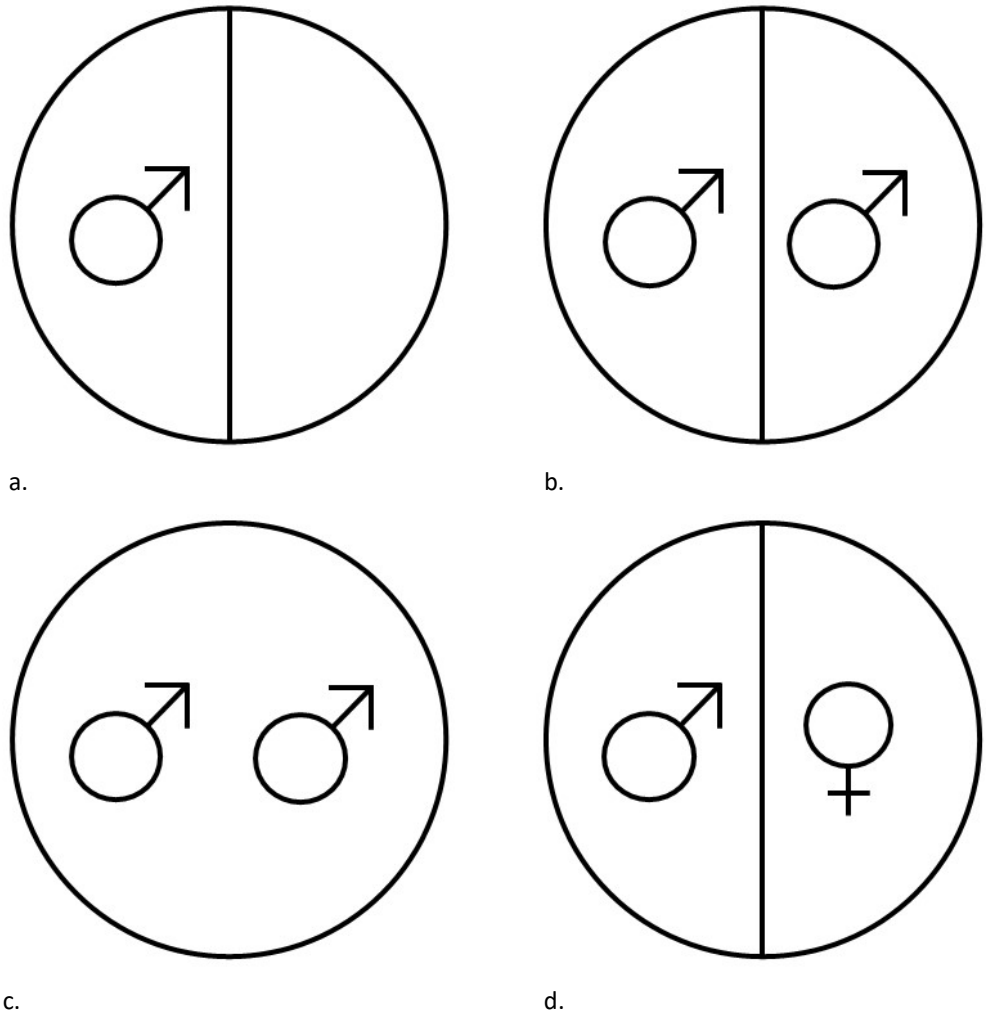


Figure 5. Experiment two: Male exposure treatments

a. Solitary male: one male confined to one compartment

b. Indirect interactions: two males separated by a perforated acetate divider

c. Direct interactions: two males in a double-spaced compartment, able to fully interact with one another

d. Female exposure: one male and one female separated by a perforated acetate divider

Test flies remained in these treatments for seven days, before being transferred to either mating behavioural assays or longevity testing.

Mating behavioural assays

To prevent the presence of neighbouring mating pairs from influencing the focal male's behaviour, a different mating assay technique was used in this second experiment. Half of the seven-day old flies were tested: pairs were aspirated into standard food vial. Then the sponge bung was lowered to reduce the flies' available space to $7cm^3$ – to increase their encounter rate and increase the chance of successful courting and copulation. Additional yeast granules were also provided here, for the same reasons.

It was not possible to film the mating pair using this technique, so all behaviour data was collected live. Because of this, it was not possible to place the vials inside an incubator, so assays were completed on a warming plate set to 27°C to maintain a constant internal temperature.

Quantifying longevity

The same treatments were used in the longevity investigation, but males remained in the treatment vials: 13 solitary males, 18 males indirectly exposed to male rivals, 13 males directly exposed to male rivals, and 17 males indirectly exposed to females. I checked these vials daily, and counted the number of individuals that remained alive, to monitor the effect of interactions on longevity. Rivals (wing-clipped for identification) and females were replaced every seven days, and flies were aspirated to fresh agar-based medium every 14 days. In cases where rivals or females died before focal flies, the rival was removed and replaced within 24 hours of death.

Body size measurements

Individuals were removed from the DAM or mating environment and their wing size was measured (as described above, and in Partridge et al. (1987c)).

Statistical analysis

One hundred and forty-four individuals were tested in mating assays (24 in solitary and female presence treatments, and 48 in paired and separated male treatments as both males were used) – although final sample sizes differed throughout the investigation due to individual variance (e.g., failure to successfully copulate). Final sample sizes for the longevity investigation ranged from 10 – 12 across the four treatments.

All statistical analyses were conducted in R version 4. 0. 3 (R Core Team, 2019). Effects of treatment on mating related behaviours were analysed using linear mixed effects models, with replicate included as a random effect to account for when both males from paired or separated treatments were tested. The R package lmerTest (Kuznetsova et al., 2017) was used to generate p values using Satterthwaite approximation for degrees of freedom.

Binomial variables were assessed using generalised linear mixed models (GLMs) with a binomial error distribution, and count data were analysed using GLMs with a quasiPoisson error distribution – in all tests, replicate vial was nested within treatment to account for potential vial effects. Finally, Cox proportional hazards models were used to assess the effect of treatment on starvation resistance.

2.4 Experiment One: Results

Effect of prior exposure to competition on mating behaviours

All males courted females but not all successfully mated (95.5%); however, there was no significant effect of housing condition on likelihood to copulate ($\chi^2 = 21.5$, d.f. = 63, $p = 0.238$).

Neither courtship latency ($\log_{10}$: $F_{2,63} = 0.0253$, $p = 0.975$) nor copulation latency (i.e., duration of courtship, when taking into account number of neighbours in the surrounding chambers: $\log_{10}$: $F_{2,58} = 1.08$, $p = 0.347$) were significantly affected by a male's exposure to rival males prior to mating.

Because the mating apparatus could contain between 1 – 16 mating pairs at a time, I tested the effect of the number of pairs in the apparatus to look for possible neighbour effects. Copulation latency decreased with increasing number of immediately adjacent neighbours in the mating apparatus ($\log_{10}$: $F_{2,58} = 3.17$, $p = 0.0494$; Fig. 6). Males with zero neighbours took, on average, 18 minutes 53 seconds to begin copulation; compared to those with one and two neighbours who took 12 minutes 11 seconds and 11 minutes 4 seconds respectively.

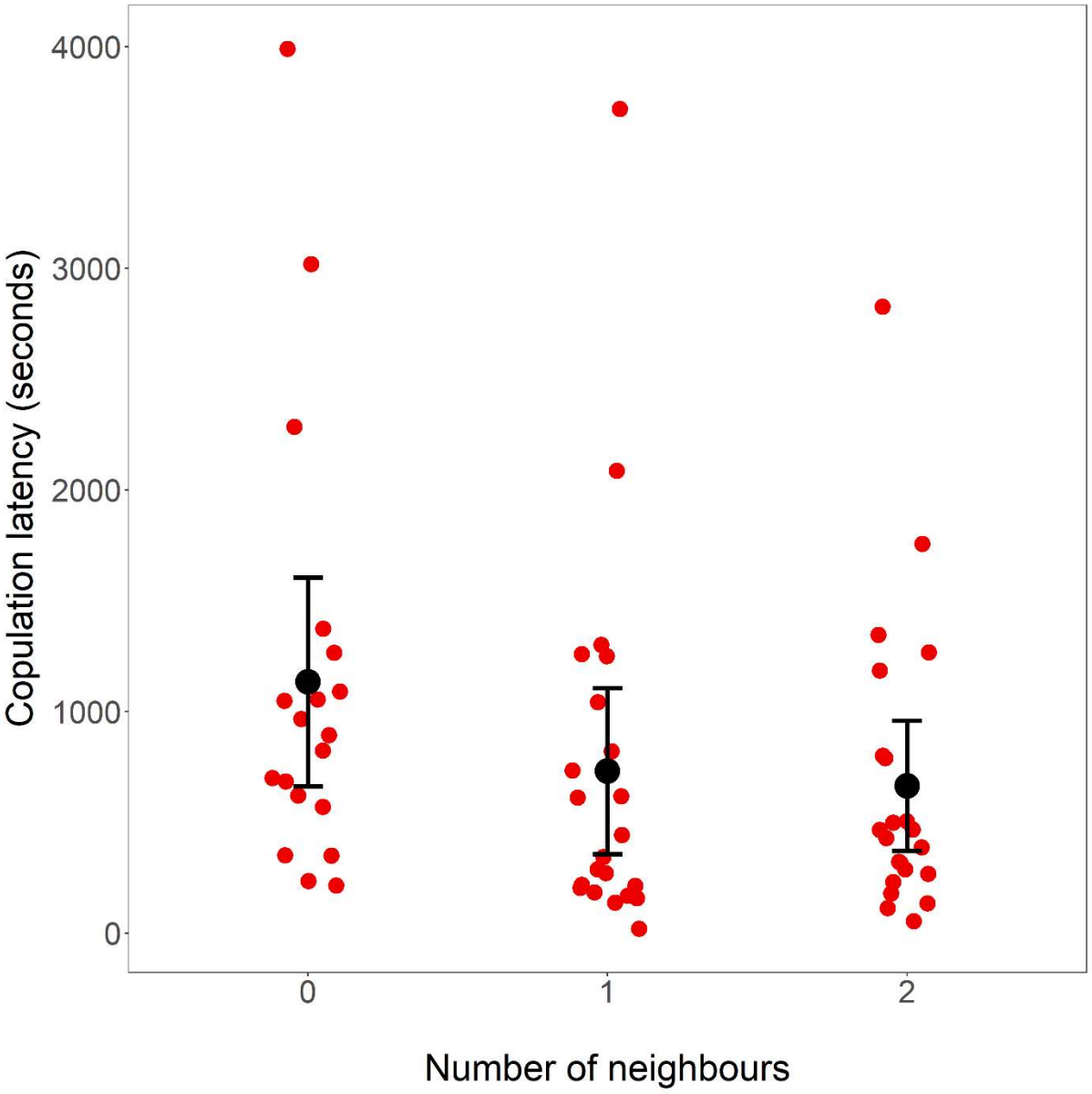


Figure 6. The effect of neighbours in the mating apparatus on latency to copulate

Means (black dot) and 95% confidence intervals of copulation latency are shown in seconds

Exposure to rival male presence prior to mating did not affect the duration of copulation ($F_{1,52} = 0.945$, $p = 0.395$; Fig. 7). Male size was included in this model, as it impacts copulation duration (Lefranc and Bungaard, 2000). As expected, increasing male size lead to increased copulation durations across all treatments ($F_{1,52} = 4.13$, $p = 0.0473$; Fig. 8).

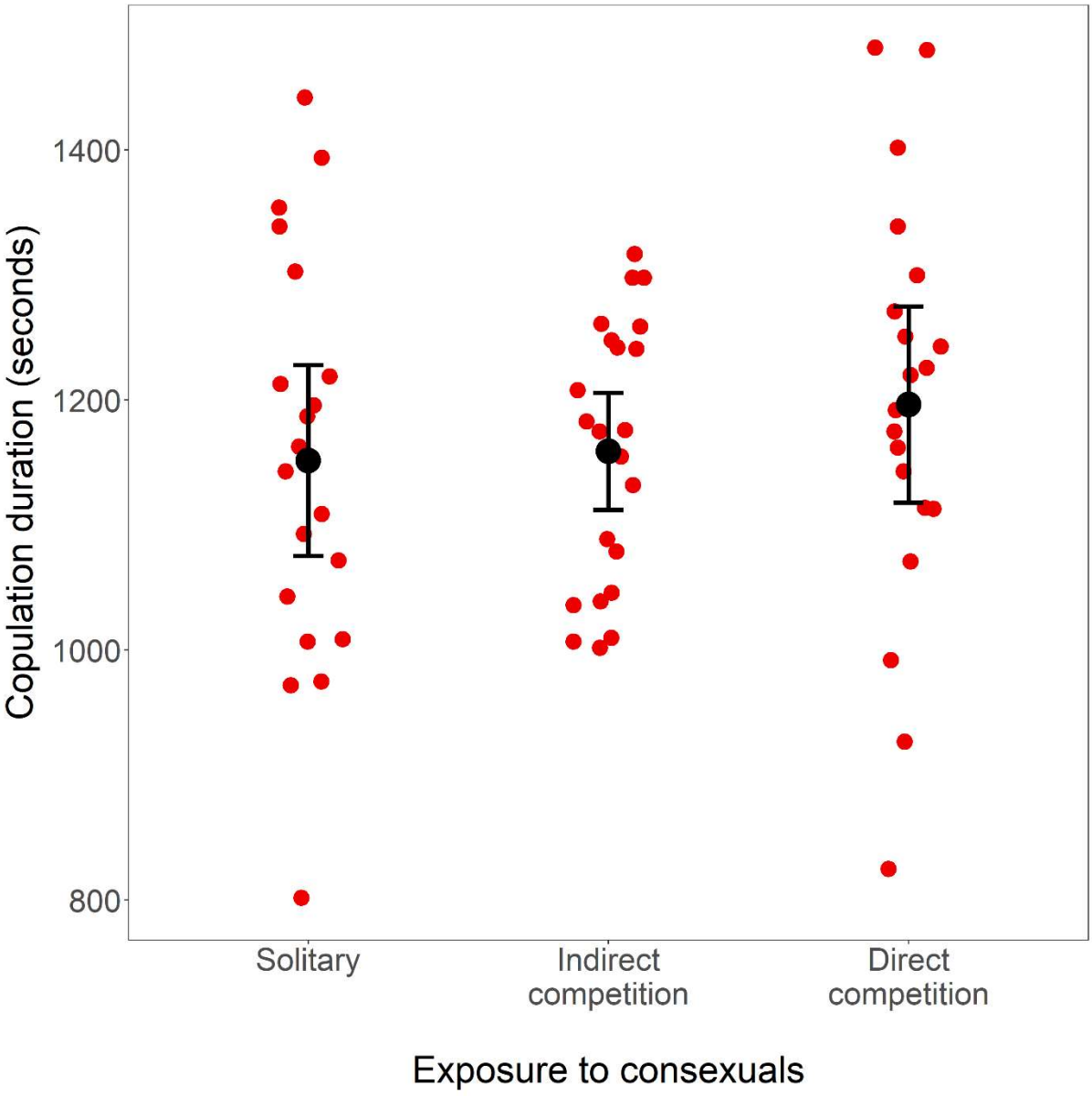


Figure 7. The effect of competition treatment on copulation duration

Means (black dot) and 95% confidence intervals of copulation duration are shown in seconds

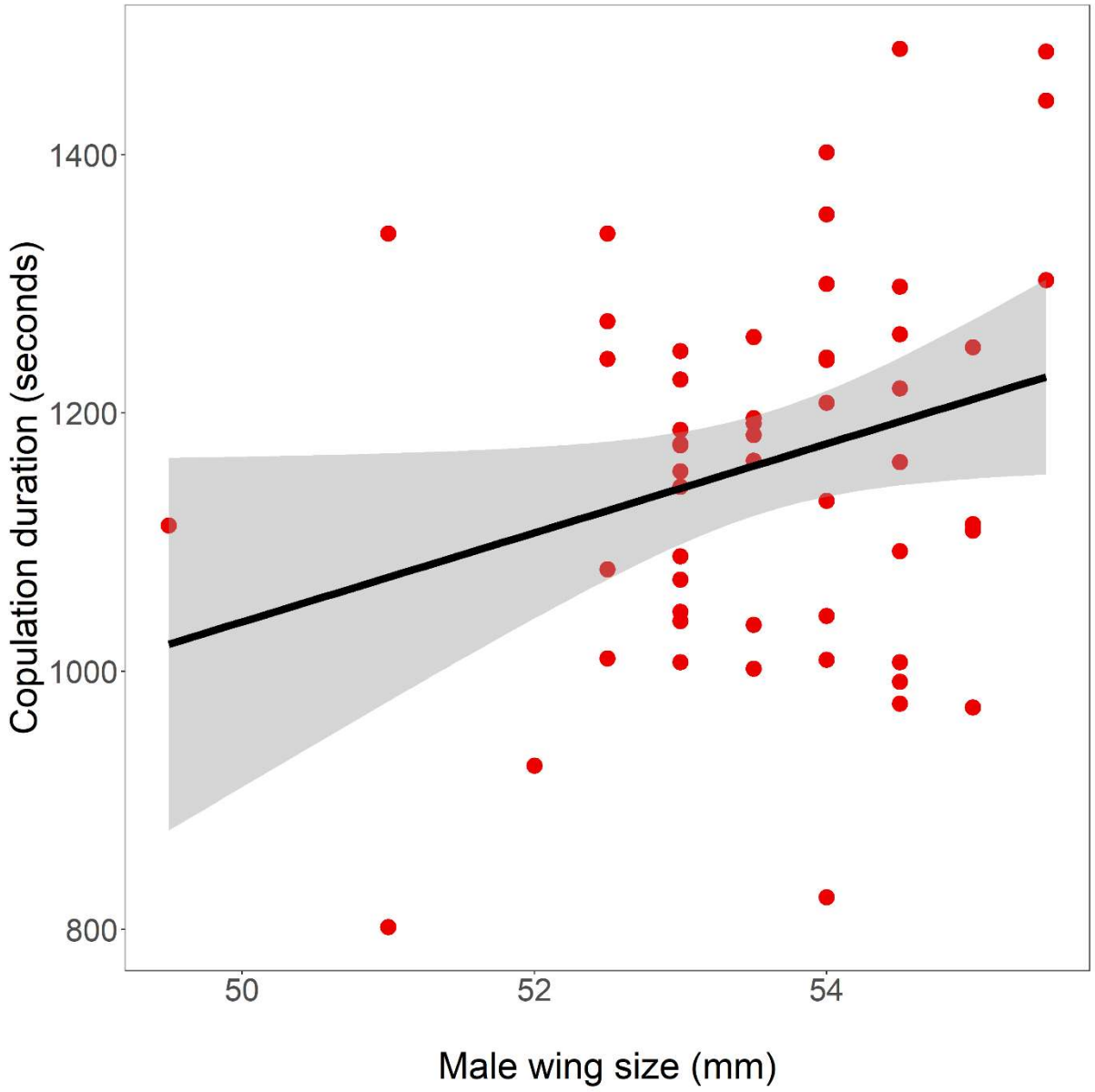


Figure 8. The correlation between male size (measured using left wing lengths) and duration of copulation Black line shows the line of best fit, and grey shadowing shows the accompanying standard error

Effect of prior exposure to competition on fitness

Whether the male was housed in solitude, or in direct or indirect contact with rival males, did not alter individual fitness (number of offspring sired: $\chi^2 = 827$, d.f. = 52, p = 0.831; Fig. 9).

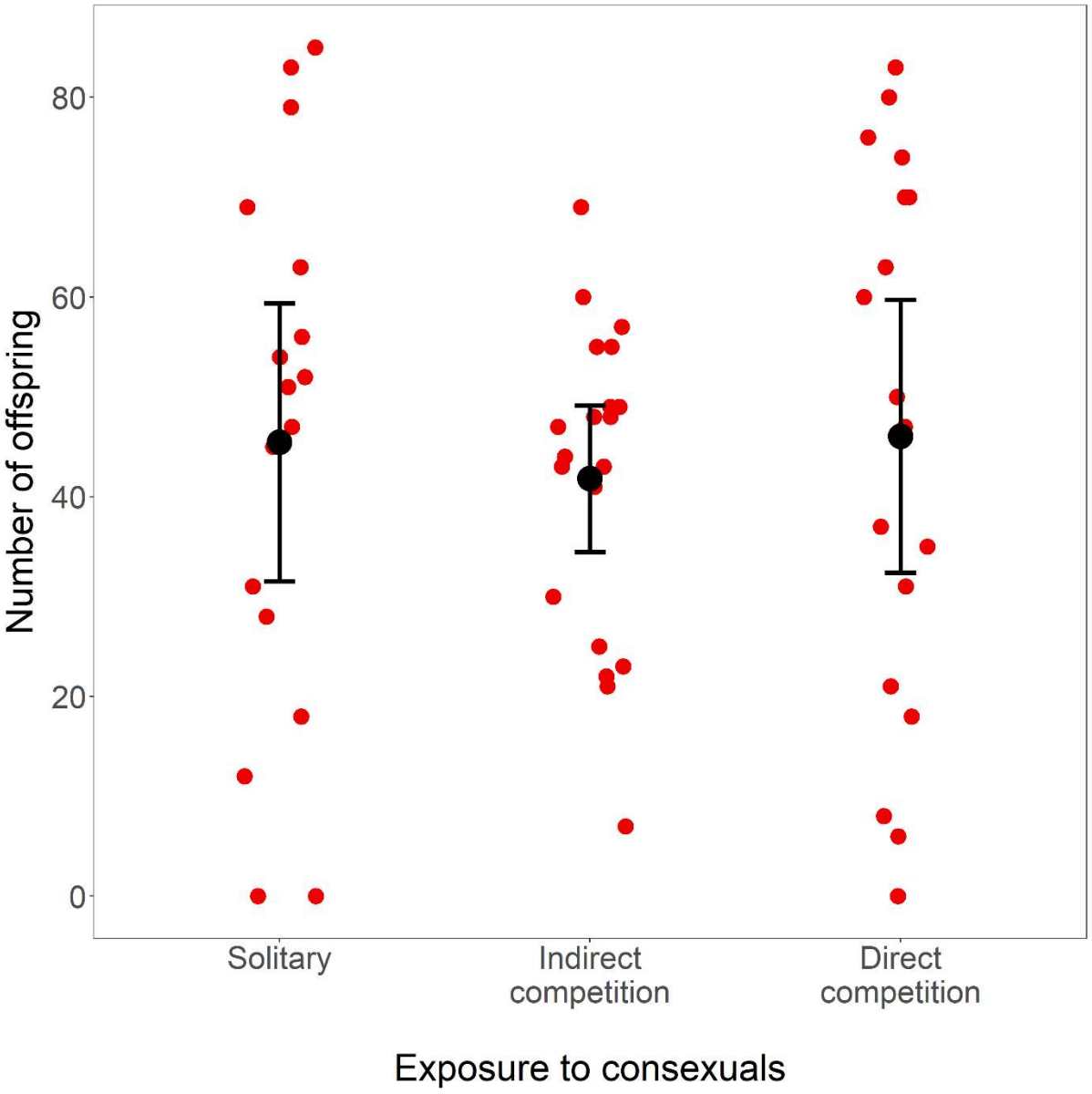


Figure 9. The effect of competition treatment on male fitness (number of offspring that successfully reached the adult life stage)

Means (black dot) and 95% confidence intervals are shown

Effect of age and prior competition on starvation resistance

Age and prior exposure to competition had complex impacts on starvation resistance. Total starvation resistance was longer in older males ($F_{1,114}$ = 9.83, p = 0.00218) but housing condition had no impact ($F_{2,114}$ = 1.88, p = 0.157); whereas survivorship was affected more by

housing condition (although this was not significant: $\chi^2 = 5.71$, d.f. = 2, p = 0.0575) than age ($\chi^2 = 0.0271$, d.f. = 1, p = 0.869); see Fig. 10 and Fig. 11.

In twelve-day olds, housing condition significantly impacted total resistance to starvation ($F_{2,56} = 6.46$, p = 0.00300). Post-hoc pairwise comparisons showed that males in indirect competition (130.2 hours) lived an average of 40.4 hours longer than males in direct competition (89.8 hours; $F_{1,39} = 17.4$, $p << 0.001$). Housing condition also impacted survivorship ($\chi^2 = 8.96$, d.f. = 2, p = 0.0114): males in direct competition had a significantly steeper decline to solitary males ($\chi^2 = 4.15$, d.f. = 1, p = 0.0416) and those exposed to indirect competition ($\chi^2 = 8.76$, d.f. = 1, p = 0.00308). But in one day old males, there was no impact of housing condition on resistance to starvation (linear model: $F_{2,56} = 0.462$, p = 0.632; Cox proportional hazards model: $\chi^2 = 2.36$, d.f. = 2, p = 0.308).

In males that were exposed to indirect competition, older males (130.2 hours) had a longer starvation resistance (by 57.4 hours) than younger males (72.8 hours; $F_{1,36} = 16.3$, $p << 0.001$). However, there was no effect of age on survivorship ($\chi^2 = 0.559$, d.f. = 1, p = 0.455). No effect of age was found when comparing solitary males (linear model: $F_{1,37} = 1.17$, p = 0.286; Cox proportional hazards model: $\chi^2 = 0.128$, d.f. = 1, p = 0.720) or males exposed to direct competition (linear model: $F_{1,39} = 0.980$, p = 0.328; Cox proportional hazards model: $\chi^2 = 0.435$, d.f. = 1, p = 0.509).

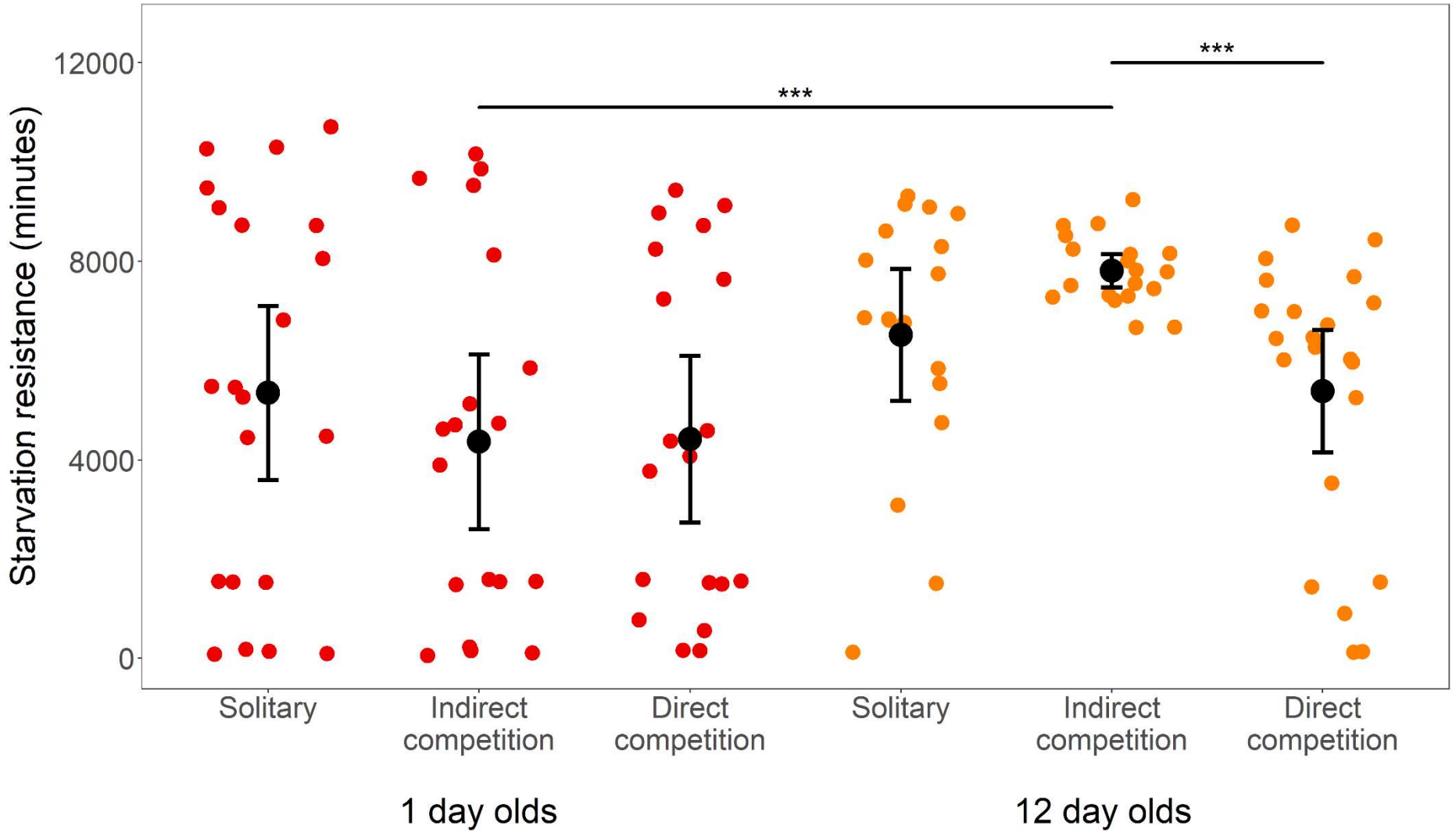


Figure 10. The effect of age and competition treatments on overall starvation resistance

Means (black dot) and 95% confidence intervals are shown in minutes

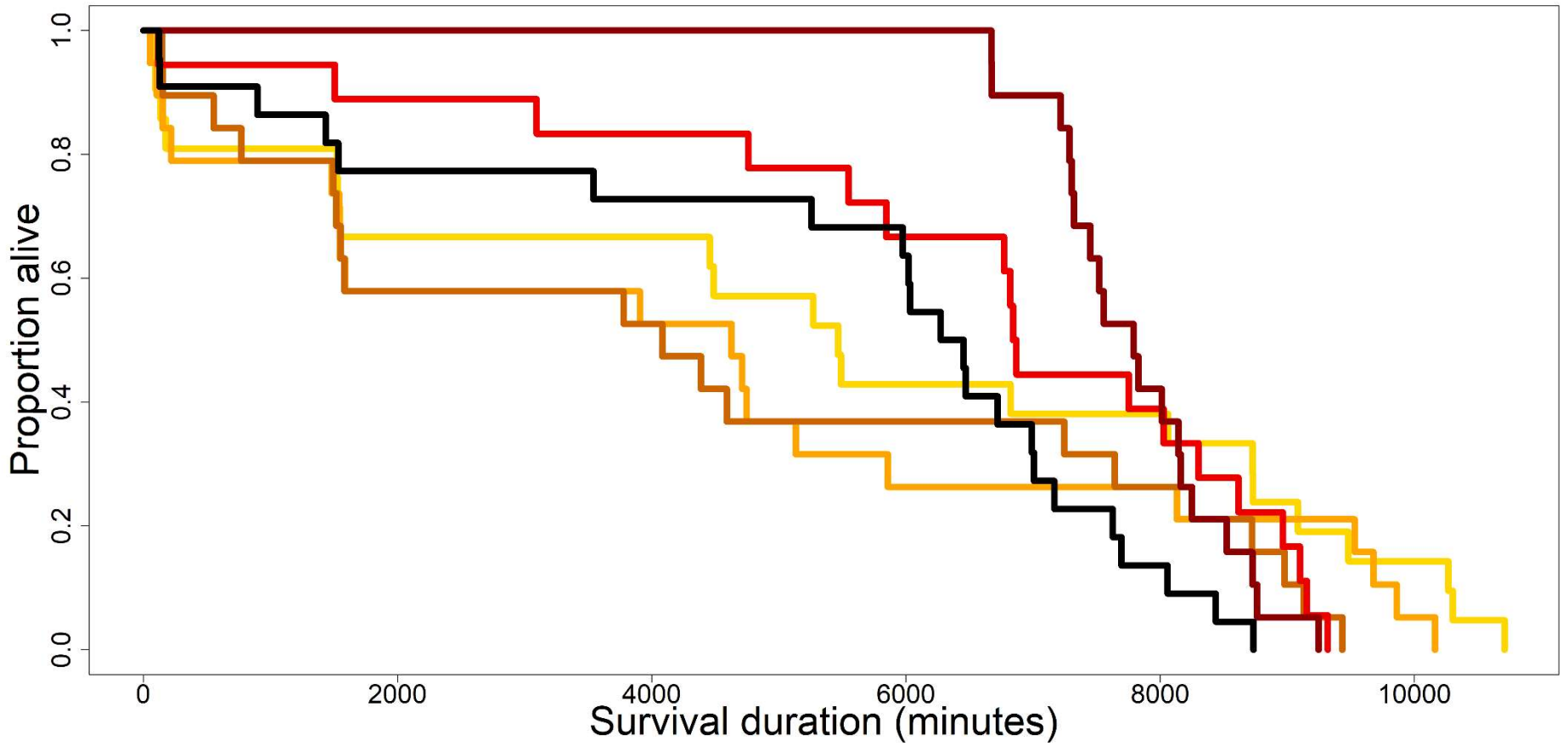


Figure 11. Proportional survivorship for both 1 and 12 day old flies in solitary, separated and paired competition conditions

One day old individuals: yellow – solitary; light orange – indirect competition; dark orange – direct competition

Twelve day old individuals: light red – solitary; dark red – indirect competition; black – direct competition

Effect of age and competition on activity levels

Young flies had a higher relative activity than older flies (log_{10}: $F_{1,114} = 7.86$, $p = 0.00594$; Fig. 12). But housing condition did not affect relative activity (log_{10}: $F_{2,114} = 0.199$, $p = 0.820$).

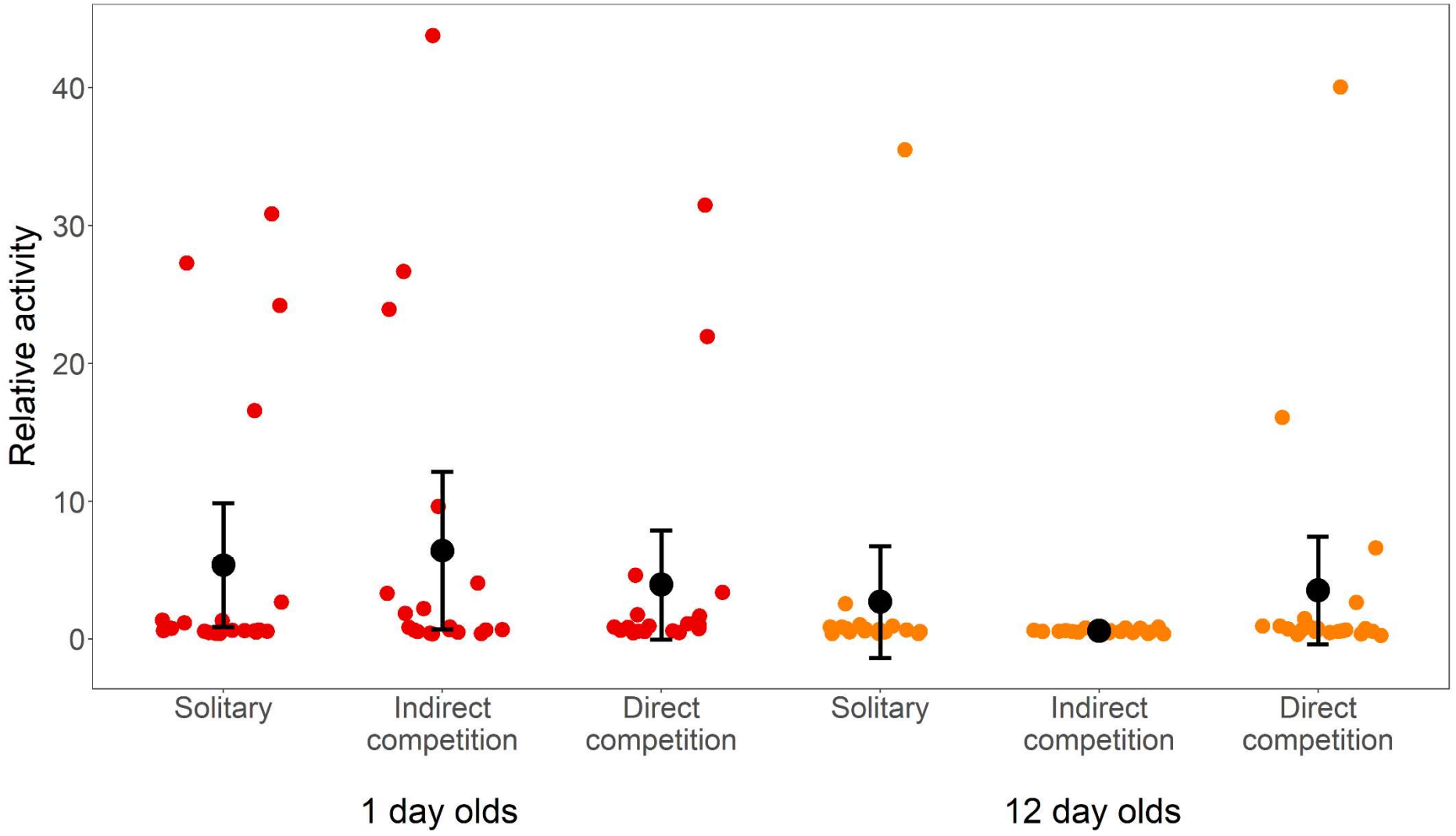


Figure 12. The effect of age and housing condition on relative activity

Means (black dot) and 95% confidence intervals are shown

2.5 Experiment Two: Results

Effect of prior exposure to competition and female presence on mating behaviours

Not all males courted (99.3%), and not all were successful in mating (89.6%), but in both cases there was no significant impact of housing condition on event likelihood (courtship: $\chi^2 = 8.31$, d.f. = 94, p = 0.236; copulation: $\chi^2 = 54.0$, d.f. = 94, p = 0.636).

There was no significant effect of a male's prior exposure to rival males or female on courtship latency (log_{10}: F = 1.14, p = 0.499) or copulation latency (log_{10}: $F_{3,123} = 0.246$, p = 0.864).

Previous presence of potential rival males or potential mates significantly affected subsequent mating duration (F = 4.07, p = 0.00854; Fig. 13). Post-hoc pairwise comparisons showed paired males mated for an average of 22 minutes 17 seconds – 110 seconds longer than solitary males (20 minutes 27 seconds; F = 6.81, p = 0.0114); and 129 seconds longer than males exposed to a female (20 minutes 8 seconds; F = 8.80, p = 0.00428).

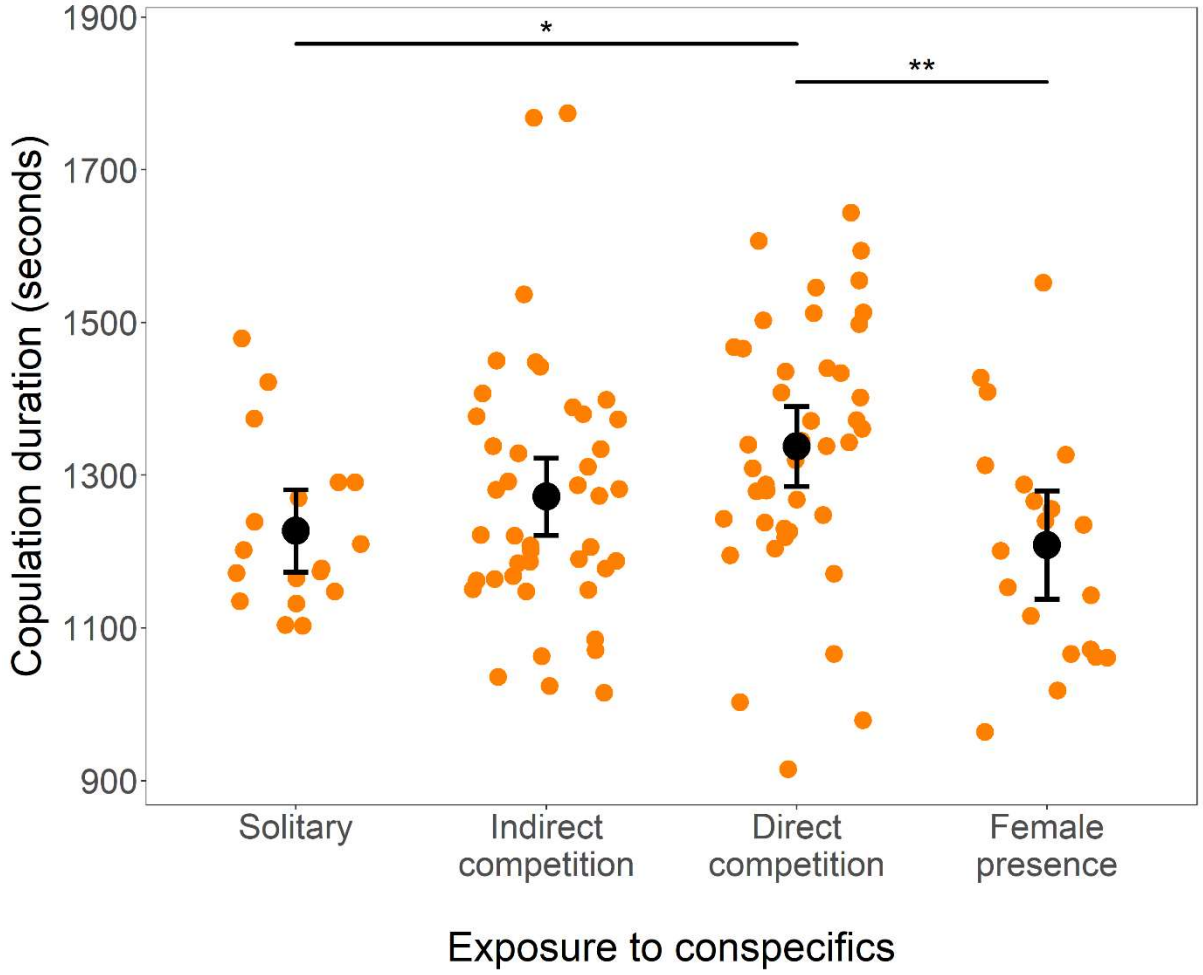


Figure 13. The effect of prior exposure to rival males and females on copulation duration
Means (black dot) and 95% confidence intervals are shown in seconds

Effect of prior exposure to competition and female presence on fitness

Fitness (number of offspring to successfully reach adult life stage) was not affected by treatment, though the treatment effect was approaching significance ($\chi^2 = 1631$, d.f. = 76, p = 0.0566; Fig. 14).

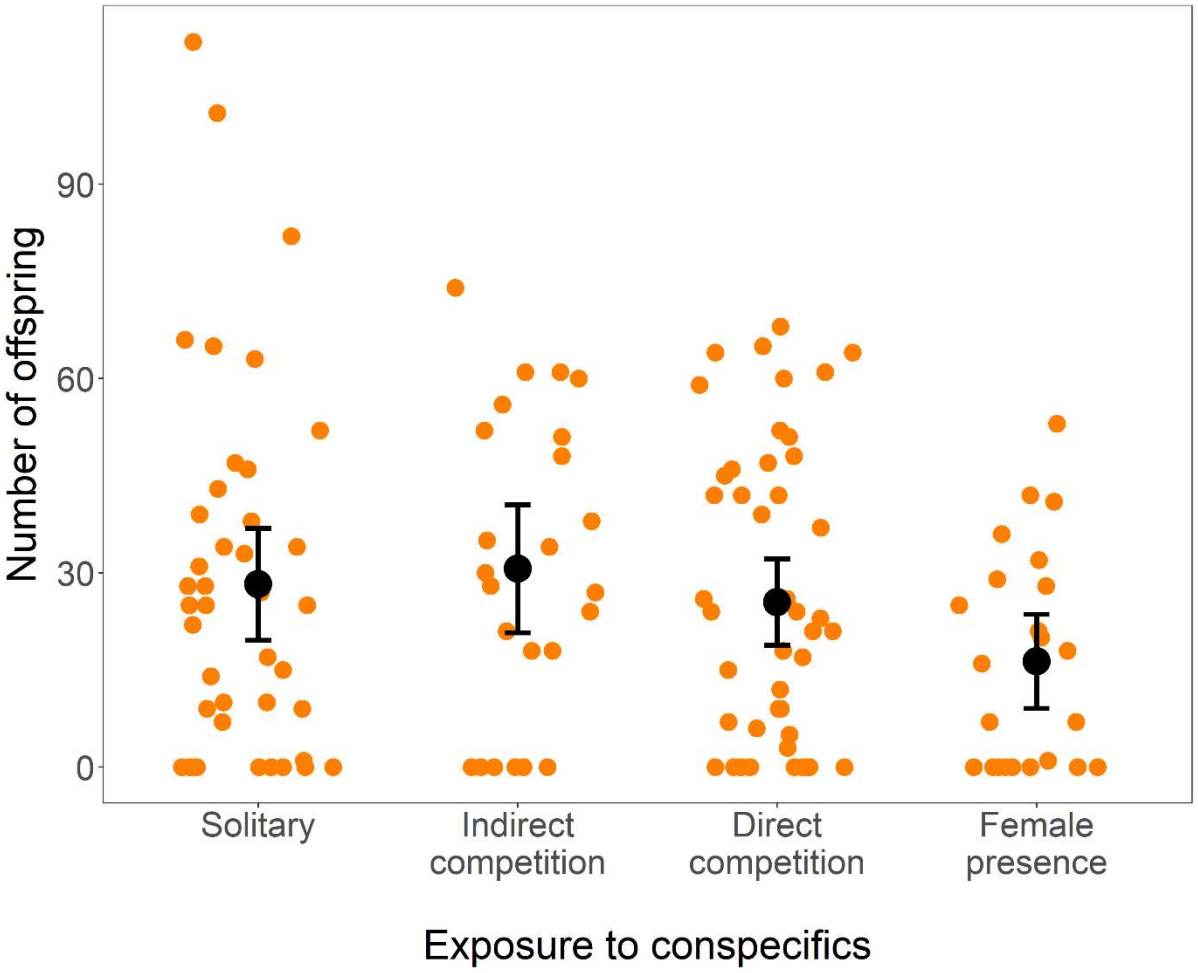


Figure 14. The effect of prior exposure to rival males and females on number of offspring that successfully reached adult life stage

Means (black dot) and 95% confidence intervals are shown

Effect of prior exposure to competition and female presence on longevity

Prior exposure to potential male rivals and mates significantly impacted total overall longevity (squared: $F_{3,40} = 5.37$, p = 0.00335; Fig. 15) and survivorship ($\chi^2 = 15.8$, d.f. = 3, p = 0.00128; Fig. 16).

Males that experienced direct exposure to rivals (74.8 days) lived for longer than solitary males (62.2 days; squared: $F_{1,21} = 7.45$, p = 0.0126), males that experienced indirect exposure

(58.4 days; squared: $F_{1,19} = 9.79$, $p = 0.00552$) and males that were exposed to the presence of females (56.5 days; squared: $F_{1,20} = 15.6$, $p << 0.001$).

The pattern of survivorship was also significantly slower in directly paired males, compared to males in all other housing conditions (solitary: $\chi^2 = 10.0$, d.f. = 1, $p = 0.00154$; indirect competition: $\chi^2 = 8.49$, d.f. = 1, $p = 0.00356$; female presence: $\chi^2 = 9.05$, d.f. = 1, $p = 0.00262$).

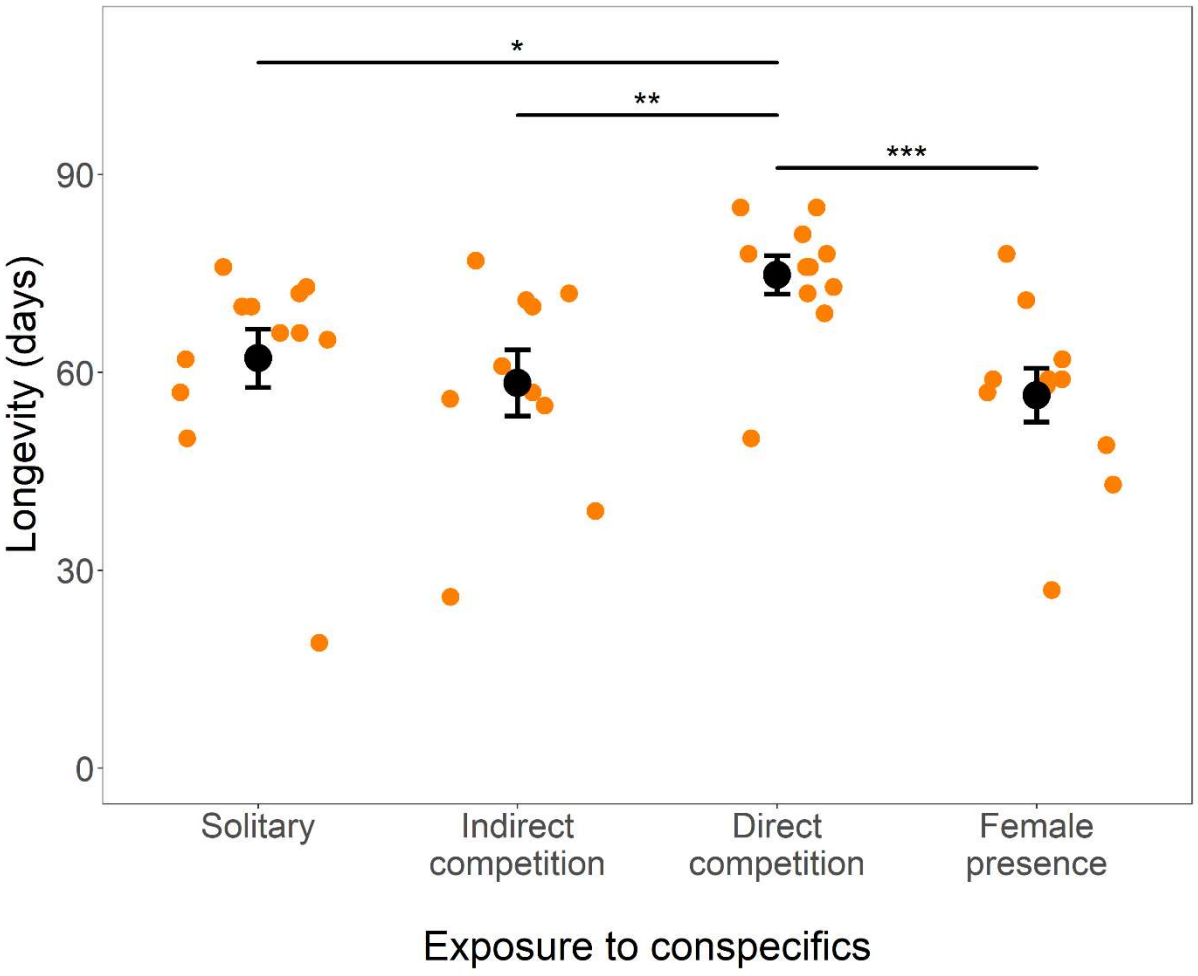


Figure 15. The effect of prior exposure to rival males and females on longevity
Means (black dot) and 95% confidence intervals are shown in days

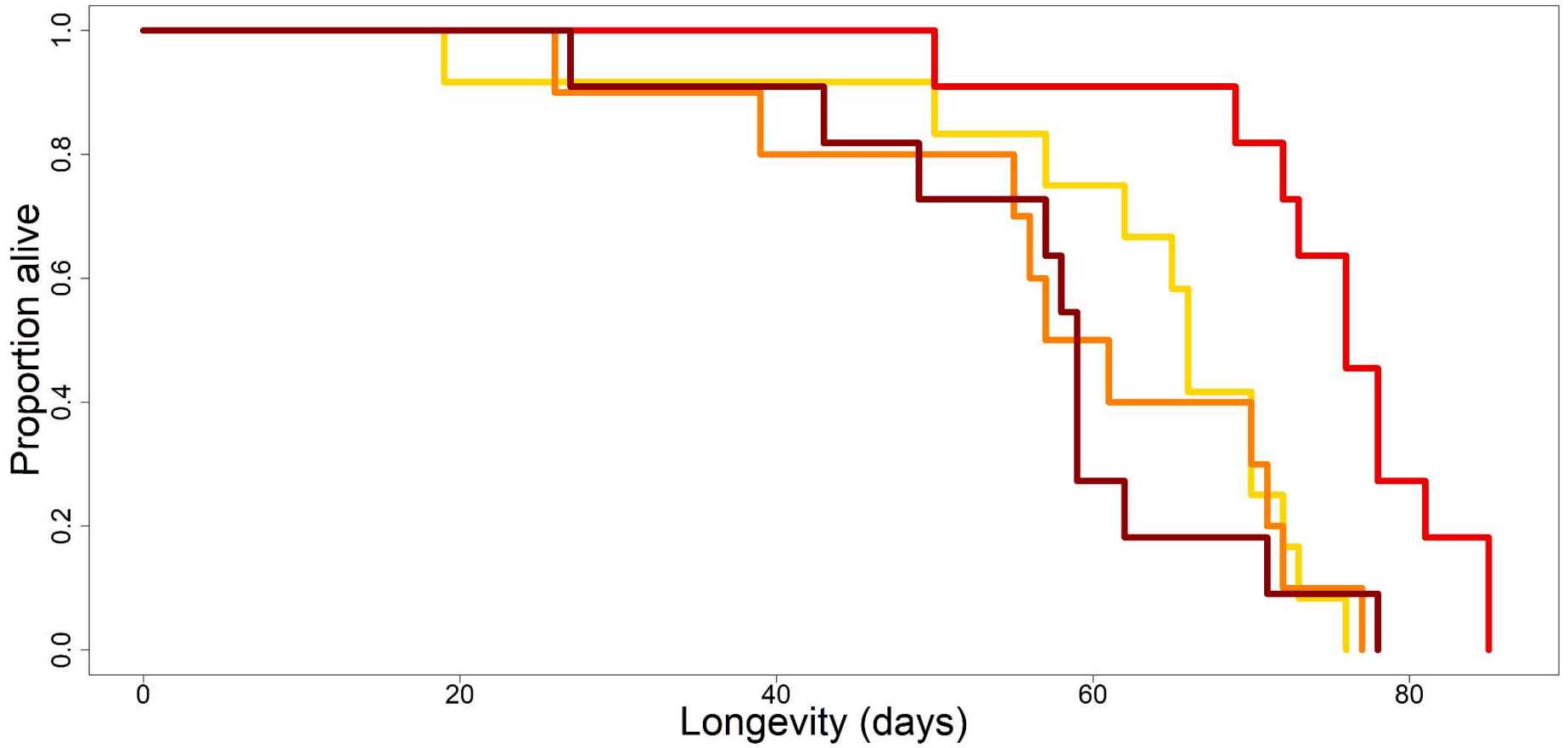


Figure 16. Proportional survivorship for males in solitary, separated and paired competition conditions and males exposed to females

Yellow – solitary males

Orange – separated males

Red – Paired males

Dark red – males in the presence of females

2.6 Discussion

Behaviour and fitness responses to conspecific exposure

Many previous studies have shown that males respond to prior exposure to rival males, by increasing their mating duration (Bretman et al., 2009, Bretman et al., 2013a, Moatt et al., 2013, García-González and Gomendio, 2004, Flay et al., 2009). This is thought to be a response to a higher perceived sperm competition, and is associated with an increase in sperm production and transfer (Moatt et al., 2014, García-González and Gomendio, 2004, Garbaczewska et al., 2013, Price et al., 2012, Fedorka et al., 2011). But in this study, I found conflicting evidence for this response. As expected, this response was found in males that experienced direct exposure to rivals, but unexpectedly it was not found in those that were unable to fully interact. This is surprising as these males were exposed to three cues (olfactory, auditory and visual cues), and responses to perceived sperm competition have previously been shown with exposure to only two of these cues (Bretman et al., 2017) – plus this effect has previously been found in paired males separated by a divider (Moatt et al., 2013).

When males were simultaneously exposed to the presence of females, the increased mating duration effect was not found in any of the housing conditions. There is limited evidence for the effect of varying sex ratios: most studies have focussed on the presence of rival males in the absence of females. One study by Mazzi et al. (2009) did investigate response to changing sex ratios, and they found that the response to increasing perceived sperm competition risk was present even with the presence of females. It is surprising that the males in my study did not respond to the increase in perceived sperm competition risk. There was an effect of male

size on the duration of copulation, but it is unlikely that this interfered in any effects caused by rival male presence: the effect of male size on copulation duration is also well-established (Partridge et al., 1987a, Partridge et al., 1987c, Lefranc and Bungaard, 2000), but males were allocated randomly to treatments and there should not have been any consistent bias as a result.

Copulation durations in males are longer after exposure to rivals prior to being introduced to the female mate (at least 24 hours previously (Bretman et al., 2010)) but are shorter when rival presence is experienced at the time of copulation (Bretman et al., 2009). Surprisingly, I found that the presence of neighbouring mating pairs in the mating apparatus reduced latency to initiate copulation. This was an unexpected confounding variable but as males only need two cues to detect and respond to the presence of rivals (Bretman et al., 2017), it is possible that males were able to detect other mating pairs in the surrounding chambers through olfactory and auditory cues. This increased speed to achieve successful copulation could possibly be a form of mate-guarding to prevent rivals from wining their mating opportunity. However, if rival presence could be detected by males in neighbouring chambers, it is surprising that the previously observed decrease in copulation duration was not found. Although this result is likely due to male harassment and here rivals were not able to harass the successfully mating male. This unexpected variable might compromise mating behaviour results in this first experiment, but will have no impact on either survival data or mating behaviour data in the second experiment – due to the change in assay technique.

This was the first study to my knowledge to compare the effect of exposure to females versus male rivals on male mating behaviours. This study suggests that as male reproductive

responses to females were comparable to the responses observed in the solitary control male (and not those exposed to rival males), that the male responses observed are indeed due to an increase in perceived sperm competition and not merely the presence of any conspecific.

Surprisingly, none of the observed behavioural responses to social environment were associated with any changes in fitness (measured via adult offspring produced after first copulation). The number of adult offspring produced was not affected by any of the housing conditions, in either of the two studies presented here, despite increasing perceived sperm competition and copulation durations often being linked to an increase in offspring produced in previous work (Bretman et al., 2009, Bretman et al., 2012, Price et al., 2012). However, this evidence has not been unanimous among published studies, as others have not discovered this effect (Bretman et al., 2012, Dobler and Reinhardt, 2016). But copulation and spermatogenesis are energetically expensive for males (Fowler and Partridge, 1989, Pitnick et al., 1995, Dewsbury, 1982), and it is unclear why males would copulate for longer and increase sperm transfer if it did not result in any fitness benefits. It is possible that fitness effects might have been observed under competitive mating environments – or that transgenerational fitness effects have been missed, as number of grand-offspring produced was not measured. With any laboratory-based study, it is important to consider results that may be an artefact of the laboratory environment (e.g., *ad libitum* food, and lack of environmental stressors) which may also be hiding possible fitness effects that would otherwise be found in field studies.

Effects of exposure to conspecifics on activity and survival

Another factor contributing to lifetime reproductive success, is overall longevity. In this study I observed impacts to starvation resistance and longevity to try to disentangle both short- and long-term impacts of conspecific presence.

When in close proximity to potential rivals, male *Drosophila* exhibit a range of aggressive behaviours: including kicking and tussling (Dow and Schilcher, 1975, Jacobs, 1960). These behaviours are likely to be energetically expensive – as is the case in similar courtship behaviours (Cordts and Partridge, 1996). I predicted that two opposing pressures would alter longevity responses. The first is that the energetic cost of aggression, alongside any injuries sustained from fights, would reduce lifespan and activity rate in males that were exposed to direct interactions with rivals (when compared to those exposed to indirect interactions). However contradictory to this, when compared to the solitary control treatment, I also predicted an increase in lifespan for all males exposed to rivals (both directly and indirectly) – as exposure to rivals has previously been linked to an increased resistance to starvation in virgin males (Moatt et al., 2013, Bretman et al., 2013b).

The results from these two studies were complex: survival responses to conspecific exposure were different when measured over the full lifetime compared to among starved flies, and there were significant impacts of age. Older males were more starvation resistant than younger males, possibly as these older flies have been able to consume what resources they need to withstand starvation, unlike the younger flies which have only had ~24 hours. Linked to this, younger flies had a higher relative activity when exposed to starvation. As these males are less starvation resistant, they are likely nearer the end of their lives and thus this increased

activity is possibly due to a late-life increased foraging effort (similar to terminal investment (Clutton-Brock, 1984)).

In older males, those that were indirectly exposed to rivals also had a longer starvation resistance than those that engaged in full interactions with males. This is possibly due to the impact of the energetic cost of aggression (Lane and Briffa, 2017). However, it is intriguing that the opposite was found in male longevity: males with direct exposure to rivals lived for longer than solitary males, males with indirect exposure to rivals, and males indirectly exposed to females. This is a surprising result that makes little biological sense as it is not yet known why exposure to rivals has survival benefits for males, despite this not being the first time this effect was observed (Moatt et al., 2013, Bretman et al., 2013b), as behavioural responses to rivals are energetically expensive (Fowler and Partridge, 1989, Pitnick et al., 1995, Dewsbury, 1982). But this result suggests that this benefit serves males more in the long-term longevity response – and not in the short-term response to starvation. Whatever the reason for this difference, this study makes it clear that the results of longevity and starvation resistance cannot be directly compared.

Chapter 3: Spatially clustered resources increase male aggregation and mating duration

Where you can you find a fly with no wings and no legs?

Right where you left it

A version of this chapter has been published, and is available online as:
Churchill, E. R., Bridle, J. R. & Thom, M. D. F. (2020) 'Spatially clustered resources increase male aggregation and mating duration in *Drosophila melanogaster*'. *Animal Behaviour,* 169, pp. 45-50.

Abstract

In environments where females mate multiply, males should adjust their behaviour and physiology in response to the perceived level of sperm competition to maximize their fitness. Evidence of such plasticity has been found in several laboratory and field studies, but little is known about the cues stimulating these responses in natural populations. One way in which males appear to assess sperm competition risk is through encounter rates with conspecific males. Such encounter rates may be driven by the spatial distribution of resources required by males (i.e., food patches or potential mates), which in turn affects local density. However, explicit links between resource distribution, male encounter rates and shifts in behaviour related to sperm competition have not been demonstrated. We found that when group size of *D. melanogaster* males was held constant, a small decrease in the distance between patches of food resources had striking effects on male behaviour. Compared to those from dispersed resources, males on clustered resources had a significantly reduced intermale distance (and hence encounter rate) and subsequently a longer non-competitive copulation duration, previously shown to be a reliable indicator of male perception of sperm competition risk. The aggregation of resources, operating via increased encounter rate, can stimulate shifts in behaviour affecting male sperm competition performance. Given that the spatial distribution of resources is typically variable in natural populations (and often unpredictable), selection is likely to favour the evolution of plasticity in sexual behaviour where resource aggregation increases the probability of sperm competition.

3.1 Introduction

Spatial heterogeneity in biotic and abiotic resources

The biotic and abiotic resources that organisms rely on for survival are generally not uniformly distributed in space, but have patchy distributions. Spatial heterogeneity has significant consequences for the distribution of the organisms themselves, a phenomenon which has been studied extensively at the landscape scale in a range of organisms (passerine birds: Holmes and Schultz (1988); gray langurs: Koenig et al. (1998); vervets and patas monkeys: Pruetz and Isbell (2000); chum salmon: Ryer and Olla (1995)). However, habitat configuration can also have fine-scale structure, with knock-on effects on individual distribution, and ultimately individual fitness, at a much more local scale. For example, when nectar sites were more dispersed, bumble bees (*Bombus occidentalis*) revisited sites more frequently and also showed decreased directionality in their foraging (Cartar and Real, 1997). In particular, clumped food resources are likely to lead to increased competition at high-density sites, due to increased encounter rates with competitors as they gather to access resources (Emlen and Oring, 1977).

The impact of density and competition

The requirement of both sexes to feed, the need for females to find effective nesting or ovipositing sites, and the fact that males will be attracted to locations where females (and therefore potential mating opportunities) are abundant mean that many species are likely to be found at higher density where there are patches of suitable food. Variation in population density affects the rate at which individuals encounter conspecific competitors and potential

mates. Where encounter rate is high, investment in traits such as sperm production and transfer, courtship and mating duration should be upregulated to maximise reproductive success in the social environment (Kokko and Rankin, 2006). Several empirical studies have supported this prediction, including in crickets (Gage and Barnard, 1996), beetles (McCullough et al., 2018), bugs (García-González and Gomendio, 2004), platyhelminths (Giannakara et al., 2016), fish (Candolin and Reynolds, 2002), and rodents (Firman et al., 2018, Ramm and Stockley, 2009).

Demonstrating that male encounter rate can stimulate plasticity in sexual traits has generally been achieved by housing males at varying densities in the laboratory, with the most common treatment comparing a singly housed male and a male housed with one or more conspecifics (Candolin and Reynolds, 2002, Gage and Barnard, 1996, Firman et al., 2018, Lizé et al., 2012, Moatt et al., 2013). This extreme manipulation of the total number of potential rivals is not intended to mimic the effects that males experience in nature, but rather is to demonstrate that such adaptive responses exist. Evidence for how such responses link to more ecologically realistic stimuli is lacking, although effects of sperm competition have been observed in natural populations. For example in lizards, increasing density resulted in lower sperm counts (Kustra et al., 2019) and in frogs increasing density resulted in increased offspring production – in individuals with thinner arms, where there was a trade-off between cost of spermatogenesis and increasing arm thickness (Buzatto et al., 2015). Given that patchiness in food resources is common in nature, and that resource distribution affects the degree of male-male competition (Emlen and Oring, 1977), small-scale variation in resource distribution that leads to local variation in encounter rate should drive plastic variation in the allocation

of resources by males to sexual behaviour that improves their chances of successful reproduction.

Drosophila *as a model organism for sperm competition*

Laboratory studies have repeatedly demonstrated that *Drosophila melanogaster* males are highly sensitive to the presence of other males, and that they increase their investment in sperm quality and ejaculate size (Moatt et al., 2014, Garbaczewska et al., 2013, Hopkins et al., 2019), adjust ejaculate composition (Hopkins et al., 2019, Fedorka et al., 2011, Wigby et al., 2009) and lengthen copulation durations (Bretman et al., 2009) when they perceive an elevated risk of sperm competition. Because *D. melanogaster* feed and breed on fermenting fruit colonised by yeast (Begon, 1982), they rely on a fundamentally patchy resource with individual fruits naturally varying in size, quality, and proximity to each other. Sex ratio and local population density of natural populations can vary considerably as a result (Markow, 1988, Soto-Yéber et al., 2018). This variation would likely affect the frequency at which males encounter male conspecifics, and hence alter their perception of potential sperm competition risk. Thus, such patchiness in natural food resources seems an ideal candidate for the type of ecological variability that might stimulate adjustment in postcopulatory processes in the wild.

Aims of this study

I tested the hypothesis that food patchiness alters the social environment and subsequent mating behaviours in this study. The only study to my knowledge in which local density is manipulated via varying food resource distributions whilst maintaining the absolute number of potential rivals throughout all treatment conditions. It is thus distinct from existing

competition studies which directly manipulate number of potential rivals (Hopkins et al., 2019, Bretman et al., 2009, Fedorka et al., 2011, Garbaczewska et al., 2013, Moatt et al., 2014, Wigby et al., 2009).

I tested whether behaviours linked to sperm competition respond to resource patchiness by exposing male *D. melanogaster* to three different food distributions (clustered, dispersed, and a uniform coverage control). In this way, local density was manipulated in an ecologically realistic way, but without changing the number of rivals between treatments. Duration of copulation was used as a proxy for males' perception of sperm competition risk – an association that has been demonstrated repeatedly in the laboratory (Bretman et al., 2009, Bretman et al., 2010, Bretman et al., 2012, Bretman et al., 2013a, Mazzi et al., 2009, Moatt et al., 2013). I predicted that (a) after experimental manipulation of the distribution of food resources, males on clustered resources would have a closer mean proximity to rivals (i.e., a higher encounter rate on average) and (b) males on clustered resources would subsequently mate for longer on average, indicating an adaptive response based on perception of increased sperm competition risk.

3.2 Methods

Rearing conditions

All fly rearing and experiments were conducted in a 12hour light:dark cycle (08:00 – 20:00h GMT), at 25°C. *D. melanogaster* used were from a laboratory population (Canton-S), and populations were cultured on 7ml of a standard agar-based medium (Appendix 1. i) in 40ml vials. Between 20 and 30 *Drosophila* were housed in each vial. To minimise any effects of inbreeding, drift and selective sweeps, every seven days I pooled adults from all vials and randomly redistributed them among new vials to start the next generation.

I collected test flies (180 total, 60 per treatment) from parent vials, each established with six males and six females allowed to breed for 70 – 98 hours. I removed test flies from parent vials within six hours of eclosion to ensure virginity; prior to this, individuals are not sexually mature (Strömnæs and Kvelland, 1962). They were then immediately aspirated under light anaesthesia into treatments. I collected virgin female flies for mating assays from the same parent vials and aspirated them into new vials in groups of four. Females were used in mating assays when they were seven days (+ 6-8 hours) old (Churchill et al., 2019).

Manipulating resource distributions and patchiness

Each replicate for each treatment consisted of four virgin males maintained in a 90mm plastic Petri dish for three days. I arranged food in each of these 45 dishes into one of three treatments: clustered, dispersed or uniform (control). Clustered and dispersed treatments both contained four plugs (discs cut from a tube of agar; 420mm^3 per patch) of standard

medium (Appendix 1. ii). The size of these patches is within the range of patch sizes where territorial behaviours have been observed (Hoffmann and Cacoyianni, 1990).

In the dispersed treatment, I placed the food patches at four equidistant points around the circumference of the Petri dish (as shown in Fig. 17a). In the clustered treatment, the food patches were placed in the centre of the Petri dish, in a square arrangement with adjacent food discs in contact (Fig. 17b). The uniform treatment was an even layer of 45ml standard medium covering the bottom of the dish, to the same height as the four food discs in the other two treatments (Fig. 17c). Volume and surface area were both greater in the uniform than that two patchy treatments, but given the number of flies food was assumed to be *ad libitum* in all.

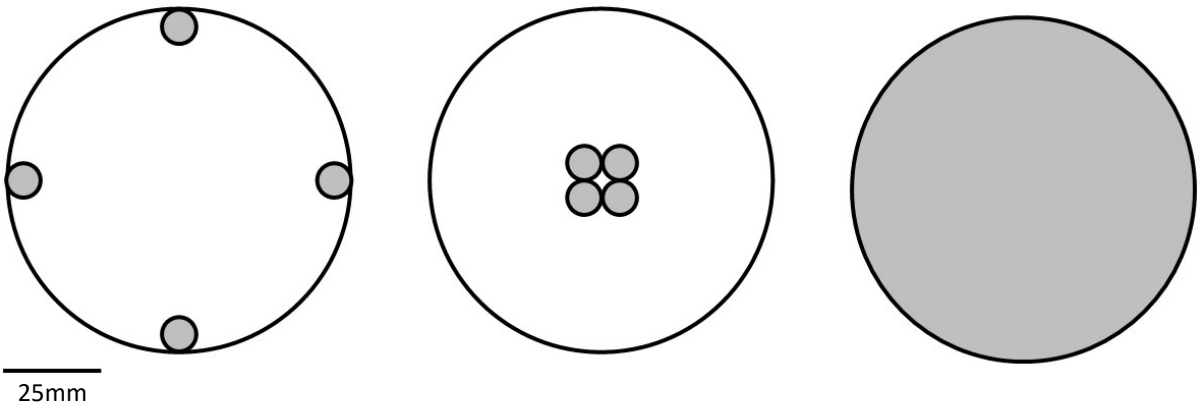


Figure 17. Petri dishes illustrating the three treatments

a. Dispersed treatment: food plugs 50mm apart along the edge of the square, 70mm apart on the diagonal

b. Clustered treatment: food plugs in direct contact with adjacent discs

c. Uniform treatment: food evenly distributed over the base of the dish

Quantifying male spacing behaviour

Treatment enclosures were placed in one of two incubators maintained under the same conditions as stock flies: 25°C on a 12:12 L:D cycle. I fitted each incubator with a Raspberry Pi (www.raspberrypi.org) connected to an 8MP Raspberry Pi Camera module (v2; www.thepihut.com). I photographed two to three enclosures in a balanced arrangement across all treatment combinations. I used frame capture software ('raspistill', Raspberry Pi) to collect one image every 15 minutes from 08:00-20:00 GMT (during the light part of the cycle). Pilot data suggested that individuals did not alter their position much when in these enclosures, and thus the interval was deemed sufficient. I then used ImageJ's multiple point selector tool (Schneider et al., 2012) to capture X and Y coordinates of each male, and then converted these into a set of six Euclidean pairwise distances between each of the four males (24,670 measurements across the three treatments and all time points). For 325 of the 4290 individual time-point photographs (7.6%) at least one male could not be accurately located. To minimise the effect of this missing data on the number of time points included per replicate, I used the mean (rather than the raw data) as the unit of analysis of the distances between each pair for each time point.

Reproductive behavioural assays

After 70 hours (± 1 hour) in a treatment, each male from each enclosure was allowed one opportunity to mate with a virgin female (N = 15; 60 individuals). I aspirated the pair into a standard food vial supplemented with ~0.03g active yeast granules. To reduce encounter latency, I limited the space in the vial to 7cm^3 by pushing down the vial bung.

Courtship latency was defined as the time from which the pair were first introduced until the male initiated his first wing extension. Latency to copulate (or courtship duration) started at the time of the first wing extension and ended with a male's successful mounting attempt. Copulation duration was recorded from successful mounting until the pair were fully separated (Chapter 2).

Not every male successfully courted (uniform: 81.8%; clustered: 86.4%; dispersed: 95.6%), and not all courting males mated (uniform: 75.0%; clustered: 86.8%; dispersed: 83.3%). I therefore set observation windows: a maximum of 90 minutes for copulation after the pair's initial introduction, and failures to court or mate within this time period were recorded.

Statistical analysis

Sample sizes were 15 replicates (N = 60 *Drosophila*) for each of the three treatments – of these, I photographed 11 from each treatment (33 in total) for data collection on inter-male distances.

All statistical analyses were conducted in R version 4. 0. 3. (R Core Team, 2019). I analysed the effect of food resource spatial distribution on total intermale distance using linear mixed effects models, with enclosure included as a random effect in all models to account for the nonindependence of the four males in a single treatment replicate. I modelled time point (numbered sequentially from first to last measurement and treated as continuous) as a fixed effect.

Using linear mixed effects models, I analysed treatment effects on mating-related traits, with replicate enclosure entered as a random effect to account for the fact that mating data was available for (up to) four males per enclosure. I initially entered time and point treatment as interacting predictor variables; if the interaction was non-significant I reran the model with both variables entered as main effects. I used the R package lmerTest (Kuznetsova et al., 2017) to generate p values using the Sattherthwaite approximation for degrees of freedom. To assess treatment effects on binomial variables (courtship and copulation success), I used generalised linear mixed models with a binomial error distribution, with replicate enclosure nested within treatment to account for possible enclosure effects. I used one-way ANOVAs to analyse the relationship between intermale distance and copulation durations, and then I used Levene's test to determine whether food resource spatial distributions effected the variance observed in mating behaviours.

3.3 Results

Effect of food resource distribution on intermale spacing

The spatial distribution of food resource patches significantly influenced the mean pairwise distance between the four males in the enclosure, and this interacted with the time course of exposure to treatment (treatment*time: $F_{2,4239} = 286$, $p << 0.0001$; Fig. 18, Table 1). On the final day of treatment, the time effect had stabilised (treatment*time: $F_{2,525} = 1.134$, $p = 0.322$), leaving a significant main effect of treatment on pairwise distance between males ($F_{2,30} = 32.268$, $p << 0.0001$; interaction removed; Table 1). Post-hoc testing confirmed that on this final day, pairwise distances between males in the dispersed treatment (44.02 ± 0.66mm SE) and the uniform treatment (39.35 ± 0.93mm SE) were both significantly greater than between males in the clustered food treatment (22.79 ± 0.86mm SE; dispersed vs. clustered: $F_{1,20} = 57.8$, $p << 0.0001$; uniform vs. clustered: $F_{1,20} = 27.9$, $p << 0.0001$; time remained in these models as a main effect). There was no significant difference in mean pairwise distance between males in the uniform and dispersed treatments ($F_{1,20} = 3.9$, $p = 0.061$).

Table 1. Details of statistical parameters from the linear mixed-model analyses

Parameter	Estimate	SE	*t*	*P*
Pairwise distance between males: full duration of treatment				
Clustered (intercept)	35.14	1.85	18.98	<0.0001
Uniform	-6.31	2.62	-2.41	0.0210
Dispersed	-3.93	2.62	-1.50	0.142
Time sequence	-0.13	0.01	-14.95	<0.0001
Uniform*time	0.21	0.01	17.23	<0.0001
Dispersed*time	0.28	0.01	23.03	<0.0001
Pairwise distance between males: final day of treatment[a]				
Clustered (intercept)	22.79	1.98	11.49	<0.0001
Uniform	16.56	2.78	5.96	<0.0001
Dispersed	21.22	2.78	7.64	<0.0001
Copulation duration				
Clustered (intercept)	1170.90	35.28	33.19	<0.0001
Uniform	-64.70	51.12	-1.27	0.212
Dispersed	-140.31	49.89	-2.81	0.008

Copulation duration; outliers removed[b]

Clustered (intercept)	1170.55	31.98	36.60	<0.0001
Uniform	-64.45	46.46	-1.39	0.173
Dispersed	-121.13	45.48	-2.66	0.011

Courtship latency

Clustered (intercept)	925.50	176.37	5.25	<0.0001
Uniform	-157.78	249.90	-0.63	0.531
Dispersed	92.17	245.20	-0.38	0.709

Copulation latency

Clustered (intercept)	954.33	183.00	5.22	<0.0001
Uniform	-254.07	262.09	-0.97	0.340
Dispersed	154.10	255.73	0.60	0.552

Model outputs are presented in the order they appear in the text. Response variables and data subsets are outlined in row headings, predictor variables in the 'Parameter' column

[a] Nonsignificant time*treatment interaction term removed

[b] Two outliers in the dispersed treatment with copulation duration values <600s removed

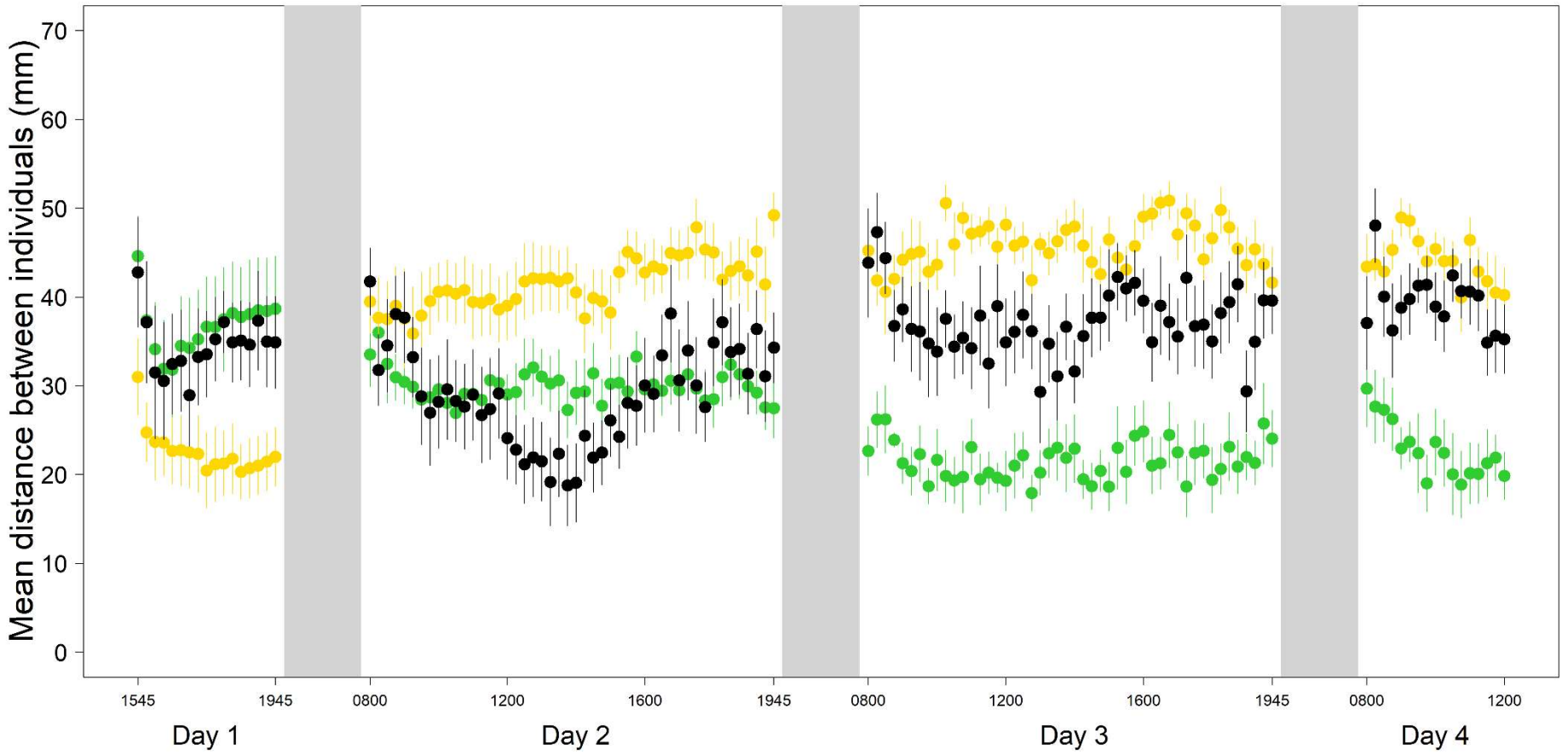


Figure 18. Mean intermale distance (mean of six pairwise distances between four focal flies per enclosure, averaged across 11 replicate enclosures) over time

Bars show standard errors of the mean for each time point. Grey blocks indicate period of dark (20:00 – 08:00h GMT), and are not to scale

Black = uniform treatment (evenly distributed food) Yellow = dispersed food patches

Green = clustered food patches

Not only were the inter-distances between males different between treatments, but the changes to the trajectory of mean average distances between individuals in the population were also significantly affected by food resource spatial distribution ($F_{2,3930}$ = 15.2, p << 0.0001; Fig. 18), showing that the temporal pattern of intermale distances is significantly different in the three resource distributions. All pairwise comparisons of trajectories were significantly different (clustered-dispersed: $F_{1,2480}$ = 617, p << 0.0001; clustered-uniform: $F_{1,2450}$ = 260, p << 0.0001; dispersed-uniform: $F_{1,2580}$ = 30.2, p << 0.0001).

Effect of food resource distribution on mating behaviour

Among those males that mated, copulation duration was significantly affected by food distribution previously experienced by males ($F_{2,42.5}$ = 3.96, p = 0.026; Fig. 19). Analysing the effect of treatment on the mean mating duration across all males in a replicate, a more conservative measure, confirmed a significant difference in mating durations between treatments ($F_{2,42}$ = 4.22, p = 0.021). Males from the clustered treatment mated for significantly longer (1170 ± 28s SE) than those from the dispersed treatment (1029 ± 28s SE), a difference of 2min 20s ($F_{1,28}$ = 6.59, p = 0.016). Copulation duration of males from the uniform treatment (1107 ± 23s SE) did not differ significantly from either of the other treatments (uniform vs. dispersed: $F_{1,28.5}$ = 2.22, p = 0.146; uniform vs. clustered $F_{1,28.5}$ = 1.96, p = 0.172). Copulation duration was also significantly longer with decreasing intermale distances irrespective of treatment ($F_{1,31}$ = 9.03, p = 0.00523; Fig. 20).

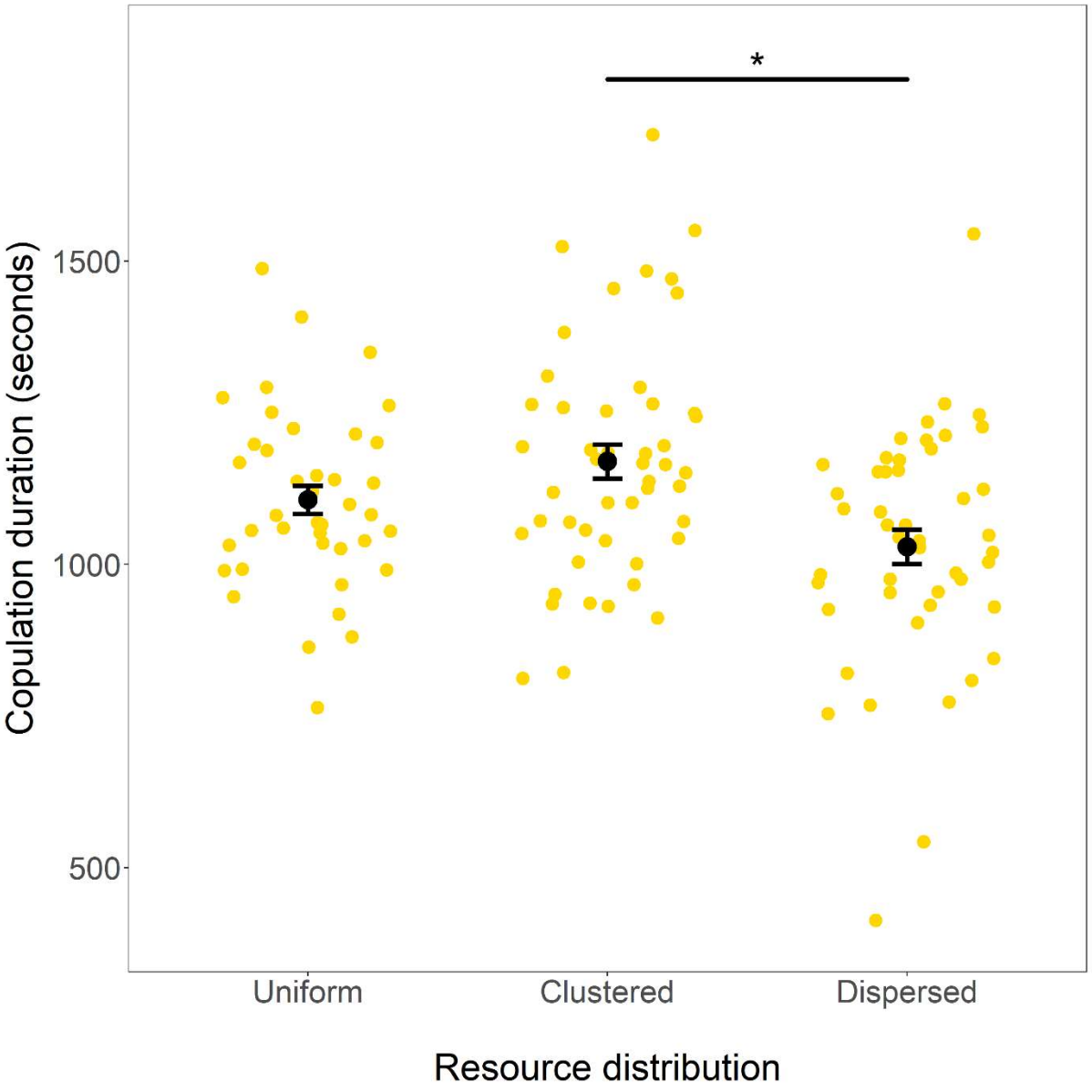


Figure 19. The effect of the spatial distribution of food on the duration of subsequent copulation

Mean copulation duration (in seconds) is shown as a black dot, with 95% confidence intervals

The treatment effect on copulation duration remained significant when the two mating duration values below 600s in the dispersed treatment were excluded from the analysis ($F_{2,40.9} = 3.55$, $p = 0.038$)

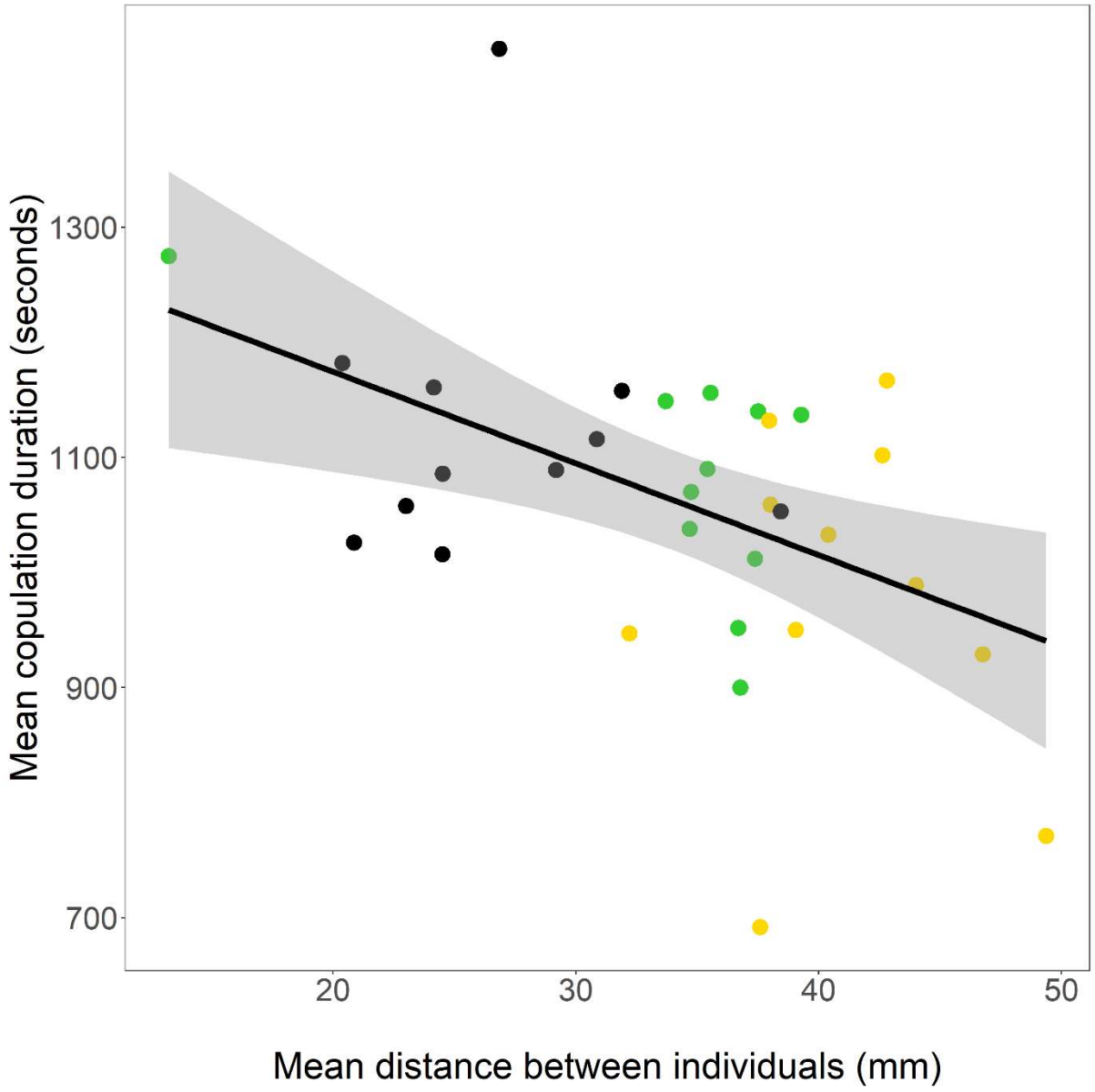


Figure 20. The effect of mean total intermale distance within treatments (mean of six pairwise distances between four focal males, averaged across replicate enclosures and across the four daylight testing periods) on mean duration of copulation

Black line shows the line of best fit, and grey shadowing shows the standard error of the line

Black = uniform treatment

Green = clustered food patches

Yellow = dispersed food patches

Food resource spatial distribution had no effect on the variance of mating behaviours observed (courtship latency: $F_{2,147} = 0.0793$, $p = 0.924$; copulation latency: $F_{2,127} = 1.856$, $p = 0.161$; copulation duration: $F_{2,132} = 1.070$, $p = 0.346$).

In total, 159 of 180 males (88.3%) courted the female. There was no significant effect of treatment on the proportion of males that courted (generalized linear model with binomial errors and enclosure nested within treatment; $\chi 2 = 118$, $p = 0.376$). Similarly, 144 (80%) males mated, and this was not influenced by treatment ($\chi 2 = 175$, $p = 0.286$). Neither the latency to start courting ($\log_{10}$: $F_{2,39.3} = 0.201$, $p = 0.818$; Fig. 21) nor the latency to start copulation ($\log_{10}$: $F_{2,30.4} = 1.257$, $p = 0.299$; Fig. 22) differed significantly between the three treatments.

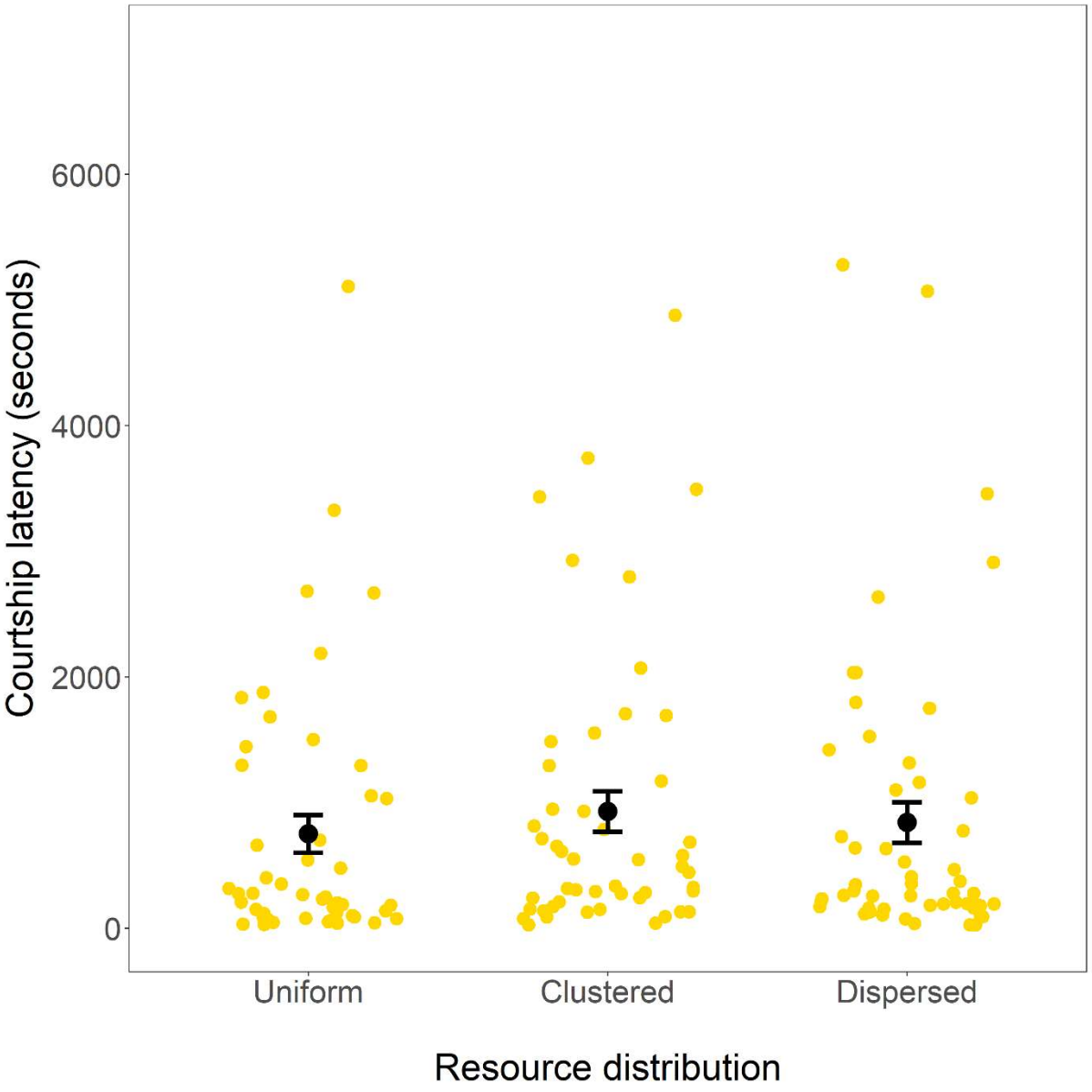


Figure 21. The effect of food resource spatial distribution on the latency of the male to begin courtship
Mean courtship latency (in seconds) is shown as a black dot, with accompanying 95% confidence intervals
Note that the analysis was performed on logged data, but untransformed values are presented here

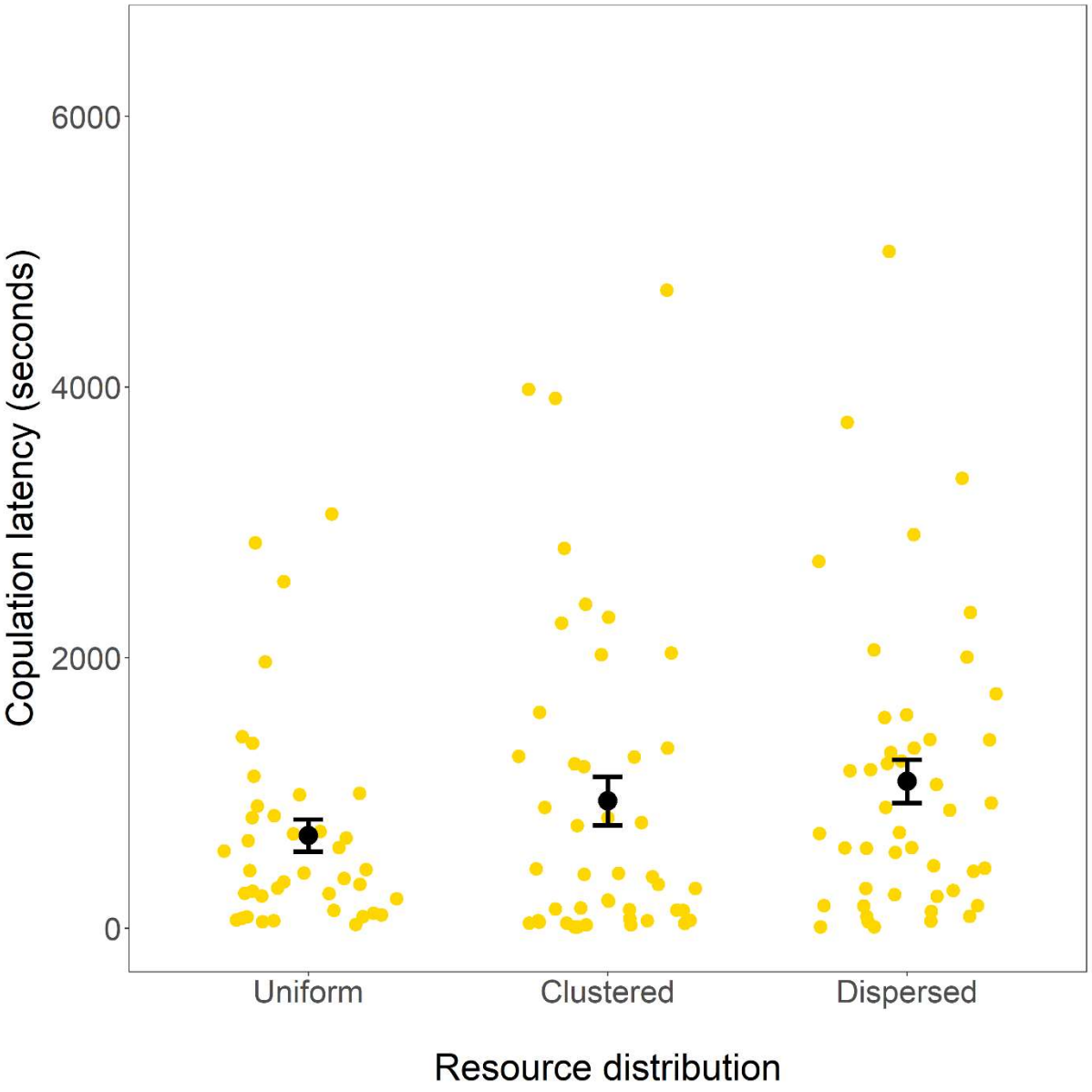


Figure 22. The effect of food resource spatial distribution on the latency of the pair to start successful copulation
Mean copulation latency (in seconds) is shown as a black dot, with accompanying 95% confidence intervals
Note that the analysis was performed on logged data, but untransformed values are presented here

3.4 Discussion

Plasticity in mating-related traits

The high degree of plasticity in mating-related traits in male *Drosophila* is well established (Churchill et al., 2019, Davies et al., 2019, Droney, 1998, Fricke et al., 2008, Jensen et al., 2015, Lefranc and Bungaard, 2000, Lüpold et al., 2010, Morimoto and Wigby, 2016, Ormerod et al., 2017, Schultzhaus et al., 2017). Variation in these traits is sensitive to conspecific male density in a manner which suggests that males adjust investment in anticipation of the intensity of sperm competition that they are likely to encounter during mating (Bretman et al., 2009). However how this level of plasticity relates to the variation in density and resource distributions observed in natural populations was previously unknown, as many laboratory studies tend to manipulate density in ways that seem unlikely to occur frequently in nature – e.g., singly housed males compared to a high density of males in a single vial.

I have shown that experimentally manipulating the patchiness of resources (while keeping group size constant) causes the same effect on a sperm competition-related trait as has been observed when directly manipulating local density. Despite the manipulations in my experiment occurring over very small spatial scales, these effects were similar in both direction and magnitude to those stimulated by keeping males in groups versus solitary housing; an approximately two-minute increase in mating duration in high-density males compared to low-density males (Bretman et al., 2009, Bretman et al., 2010, Bretman et al., 2013). Given that wild *D. melanogaster* encounter a patchy resource that is likely to alter male encounter rates at a similar scale to that demonstrated here (Markow, 1988, Soto-Yéber et al., 2018), this suggests that fine-scale variation in these environmental cues might influence

the resource allocation and continual adjustment of male traits associated with sperm competition, and thus mating success, in wild-living *Drosophila.*

Individual fitness effects

Although the effect of density on mating duration is a repeatable indicator of male perception of sperm competition risk, the benefits of this behaviour to males remains uncertain. In many species, increased mating duration has been linked to increased sperm transfer and offspring production (Edvardsson and Canal, 2006, Engqvist and Sauer, 2003, Sakaluk and Eggert, 1996). Yet in *Drosophila* the consequences of longer copulation durations are less clear, with some studies reporting an association with increased fitness (Bretman et al., 2009, Garbaczewska et al., 2013, Price et al., 2012), while others have not found such a link (Bretman et al., 2012, Dobler and Reinhardt, 2016). Whether males on the clustered food resource would have a higher fitness remains to be tested, but will almost certainly depend on mating order effects and the competing male's history of exposure to rivals (Bretman et al., 2012).

Latency to court and copulate

Varying spatial distribution of food resources had no impact on the latency of the male to begin courtship, or the time taken for the female to succumb to copulation. Courtship behaviours are energetically expensive (Cordts and Partridge, 1996), so poor quality males often take longer to begin courtship or court with less rigour – as has been observed in aging males (Churchill et al., 2019). As the males in this study were all tested at comparable ages and provided with equal nutrition, the only energetic expense that might reduce an

individual's strength is that of food resource searching (dispersed treatment) or aggressive interactions (clustered treatment).

There are costs and benefits associated with both clustered and dispersed resources in the wild. If dispersed, individuals must travel further in search of the resources: not only is this an energetic expense, but it also reduces potential mate-searching opportunities (Bonte et al., 2012). By comparison under a higher encounter rate, as experienced in clustered resource environments, mating opportunities will be rife. However, there will also be an increased chance of encountering rivals and therefore fights. *Drosophila* aggressive behaviours are likely energetically expensive (as courtship behaviours, with similar limb movements, have been shown to be (Cordts and Partridge, 1996)), and have an increased risk of harm. Behaviours include lunging, kicking, and wing raises (Sturtevant, 1915). It is therefore likely that varying the spatial distribution of food resources will impact an individuals' attractiveness and potential fitness. It is not yet clear whether these treatments did have any physiological effects on individuals, but if there were any effects, they were not detected in courtship responses.

The receptiveness of the female determines the length of time between when the male first initiates courtship and when copulation begins (Bastock and Manning, 1955). Females can either accept males' advances and succumb to copulation, or reject using a series of rejection responses including: fleeing, kicking, fluttering wings or ovipositor extrusion (Spieth, 1952, Spieth, 1974). It is therefore not wholly surprising that copulation latency did not vary, as it is largely under female control, and females were not directly manipulated in this experiment. It is however possible that females could respond to variations in male traits and overall

attractiveness, caused by environmental stressors or aggressive interactions with rivals. This effect has been reported in male response to female attractiveness in *Drosophila* (Lüpold et al., 2010), although no such effect was detected in this study.

Population dynamics and territoriality

Interestingly, the effect of food distribution on male distribution behaviour was observed in the absence of females. Females often follow social cues, and their grouping behaviour is promoted by aggregation pheromones (Bartelt et al., 1985, Duménil et al., 2016). By comparison, given their low feeding rate once adult (Wong et al., 2009), males are thought to aggregate near food resources primarily to seek mating opportunities. That these groups of males responded in their individual positioning to the distribution of food even in the absence of females is intriguing, and leaves open the question of the relative importance of females' social cues compared to the direct response of males to food resources. In general however, studies manipulating male density have tended to exclude females from the treatment phase (e.g., Bretman et al. (2009), Bretman et al. (2010), Lizé et al. (2012), Moatt et al. (2013), Price et al. (2012), Rouse and Bretman (2016)) meaning the effects of intersexual interactions on plastic responses to density is relatively unexplored.

There were also some intriguing dynamics operating in the intermale distances in the early stages of the treatment period in this study: in particular, males on the dispersed food patches initially experienced lower intermale distances than those on clustered food. This effect does not match behaviour expected in males attempting to defend food patches, and is the opposite to the pattern observed on the final days of treatment. Inspection of photographs from this treatment suggests that males on dispersed food patches initially cluster together

away from the food before sorting themselves into individual territories focused around each patch. Territorial behaviour in *D. melanogaster* has been observed under laboratory conditions, and appears to be driven by boundaries of food sources (Lim et al., 2014) so it is possible that multiple distinct territories could be established under these conditions – however, it remains unclear what is driving this initial clustering behaviour. Understanding this is the focus of my next chapter.

Future directions

This research adds to a small number of studies that demonstrate the effects of environmental heterogeneity on *Drosophila* behaviour. Yun et al. (2017) demonstrated that female fitness was higher in more spatially complex laboratory environments as a result of a reduction in sexual interactions and consequent mitigation of male harm. Similar effects were found when laboratory populations were presented with a refuge (Byrne et al., 2008). Such rapid shifts in behaviour, driven by ecological patchiness, have rarely been included in laboratory assays, but may have major effects on the demography and growth rate of populations exposed to spatial patchiness, through their effects on male reproductive skew and therefore effective population size. Such effects may have important evolutionary and ecological consequences in relatively patchy parts of a species' distribution, for example by increasing sexual conflict over shared resources (Pilakouta et al., 2016), or reducing maximum sustainable rates of evolution (Bridle et al., 2019, Bridle et al., 2009).

This study describes a potential mechanism that mediates the plastic sperm-competition linked responses in natural populations, via variation in local-scale resource spatial distributions. This mechanism could be tested in wild populations of *Drosophila*, as there have

been comparatively few field studies carried out using this species. It is not yet known how much variation exists in the mating duration of wild *D. melanogaster*, nor how the surrounding environment may impact this.

It would also be beneficial to further define this mechanism in the laboratory. Tracking individual males throughout the assay period would allow data to be collected on individual positions. As a result, each individual's specific distance from rivals could be more precisely matched to their copulation duration. Equally valuably, it would also enable an investigation into potential impacts of varying food resource spatial distribution on home range sizes and territoriality, and total distance travelled – and how these in turn impact copulation duration and ultimately fitness. Finally, my findings have raised an additional question: why on the first day of the testing period, do individuals raised on a dispersed patch distribution appear to have a shorter intermale distance than those housed on clustered patches? The following chapter attempts to answer these questions by repeating this study using individually marked flies.

Chapter 4: **Resource patchiness effects on male home range and social interactions**

Why did the fly fly?

Because the spider spied 'er

Abstract

The spatial distribution of essential resources influences the social environment that individuals encounter. I have previously shown that when resources are spatially aggregated, male *Drosophila* have higher encounter rates (closer mean proximity to potential rivals). But how this alters individual behaviour around food patches, and how individual behaviour impacts on subsequent fitness-related traits, has not yet been established. In this study I test the effect of variable spatial distributions of food resources on individual males' home range sizes and distances travelled. I show that in aggregated food resource distributions, males have smaller home range sizes compared to those on dispersed and uniform resources, and surprisingly, that distance travelled does not appear to be impacted by spatial heterogeneity. I then link these changes in males' social environment to fitness consequences: aggregated resource patches resulted in increased mating duration, but had no impact of production of offspring.

4.1 Introduction

Resource patchiness and the social environment

When resources are more aggregated, proximity to rivals and encounter rates increase (Emlen and Oring, 1977). This has important consequences for individual behavioural responses. For example, the number of rivals males are exposed to influences the level of aggression they exhibit – with males that experience intermediate levels of competition displaying more aggressive behaviours (Nandy et al., 2016). Compounding this, on aggregated resources increasing food availability also increases aggression – irrespective of encounter rate (Lim et al., 2014).

This will have important consequences for population dynamics and individual fitness due to ongoing winner-loser effects. When engaging in contests, individuals that have previously won are more aggressive and thus are more likely to continue winning and losers are more likely to continue losing (Hsu and Wolf, 2001, Hsu and Wolf, 1999, Drummond and Canales, 1998, Oliveira et al., 2011, Oliveira et al., 2009). One reason for this is that individuals that win, gain the benefits from acquiring the resource whereas the losers have expended energy into the aggressive encounter but have gained nothing. This winner-loser effect is observed over territoriality too: known as the 'residence effect', defending males are more likely to retain their territory than attacking males are to evict them (Fuxjager et al., 2009, Kemp and Wiklund, 2004, Krebs, 1982). Individual skill will also play a role in the winner-loser effect (Briffa and Lane, 2017), and will likely have long-term fitness consequences for the individuals.

Fitness impacts of spatial heterogeneity

In *D. melanogaster w*hen resources are clustered, males are in closer proximity to rivals, and mating duration is increased (Chapter 3; Churchill et al. (2020)). The study described in the previous chapter, demonstrated for the first time that this sperm competition-related behavioural response could be altered by experimentally manipulating the patchiness of resources while keeping group size constant. I thus demonstrated conclusively that resource patchiness could be a driver of plastic variation in the allocation of resources by males to sexual behaviour. But links between individual-level behaviour around resources and subsequent mating opportunities and fitness consequences remain to be explored. It is likely that under clustered resources offspring production would be higher, due to the previously established sperm competition-linked responses – such as increased sperm quality and altered seminal fluid composition (Fedorka et al., 2011, Wigby et al., 2009, Wolfner, 2002, Moatt et al., 2014).

Furthermore, it is likely that the variation in social environments generated by resource patchiness will also impact reproductive behaviours and fitness. For example, males that hold larger territories are more likely to successfully secure a mate (Hoffmann and Cacoyianni, 1989). Males will need to engage in aggressive behaviours to gain territories and fight off rivals for access to females, so one would think that increased aggression might result in greater mating success and/or fitness. However, as yet no evidence of this has been found (Nandy et al., 2016). This could potentially be due to the energetic costs of aggression and, for the loser especially, the cost of receiving injuries reducing expendable energy required for reproduction (Lane and Briffa, 2017). This is supported by the increase in aggression and

mating in the presence of increased food (Lim et al., 2014). Related to this, if males experience increased sampling costs (as experienced when resources are more dispersed (MacArthur and Pianka, 1966, Wiersma et al., 2005)) they would again have less expendable energy for copulation and production of offspring. Here I measure instantaneous fitness to better understand how it is impacted by variable physical and social environmental variability.

Aims of this study

In this chapter, I repeat the study described in Chapter 3 with two key differences: the males are marked so individuals can be identified, and I maintain the mated females to observe the number of adult offspring that are produced as an instantaneous measure of male fitness. These significant enhancements enable me to gain a better understanding of individual (rather than group) responses to resource patchiness, and hence answer the questions raised in the previous investigation.

I hypothesised that males would establish territories on the four patches, as territorial behaviour in *D. melanogaster* appears to be driven by the boundaries of food resources (Lim et al., 2014), and that when the resources are dispersed intermale distances and home range sizes would be larger due to the greater distance between patches. Linked to this, I predicted that males on dispersed food patches would have travelled further than those on clustered resources because they have a greater distance to cover to sample all available resources.

I finally predicted that these males with larger intermale distances and home ranges would subsequently copulate for less time than males in close proximity, caused by reduced

encounter rates with rivals and increased sampling costs. This would also likely be detectable in reduced offspring production.

4.2 Methods

Rearing conditions

All fly rearing and testing were conducted at 25°C in a 12hour light:dark cycle (08:00 – 20:00h GMT). *Drosophila melanogaster* used were from a laboratory population of LHm stock (originally collected by Larry Harshman from a Californian orchard) and were cultured on 7ml of a standard agar-based medium (Appendix 1. i), in 40ml vials. Between 20 and 30 individuals were housed in each vial, and all vials were pooled and randomly redistributed among new vials every seven days. This was done to minimise any impacts of inbreeding, drift, and selective sweeps.

I collected test flies (196 total) from parent vials, each established with six males and six females that were allowed to breed for 70-98 hours. Male test flies were removed within six hours to ensure virginity (Chapter 2). Virgin females to be used for mating assays were collected from the same vials, aspirated under light ice anaesthesia into new vials in groups of four, and aged to seven days old before mating (Chapter 3, Churchill et al. (2019)).

Marking individuals

I added Humbrol enamel paint (Humbrol, UK) to male thoraxes under light ice anaesthesia using a fine paintbrush. They were marked in one of four colours: red (Humbrol no. 60), yellow (no. 99), blue (no. 109) and grey (no. 196). Although I have found that this marking technique reduces individual copulation duration, this should not influence the results of this experiment as impact was the same for all colours (Appendix 4).

Manipulating resource distributions and patchiness

I placed four virgin males (one marked in each colour) into a 90mm Petri dish for three days. The food in each of these 49 dishes was arranged into one of three treatments as described in Chapter 3 and Churchill et al. (2020): clustered, dispersed and uniform. Clustered and dispersed treatments contained four food patches (discs of agar; 420mm^3 per patch) of standard medium (Appendix 1. iii). Further detail about the placement of patches can be found in Chapter 3 – Fig. 17.

Quantifying male spacing behaviour

I placed treatment enclosures in one of two incubators, maintained under the same conditions as stock flies: 25°C on a 12:12 L:D cycle. I fixed an Imaging Source camera (DFK 33UX226) overhead in each incubator, and enclosures were photographed in batches of three to four. I used IC Capture software (The Imaging Source, Germany) to collect one image every 15 minutes from 08:00-20:00 GMT (during the light part of the cycle). Pilot data and evidence from the previous experiment suggested that individuals did not frequently alter their position in the Petri dishes, and thus the interval was deemed sufficient.

I captured X and Y coordinates of each male using ImageJ's multiple point selector tool (Schneider et al., 2012), and then converted these into a set of six pairwise Euclidean distance between each of the four males, which was then used to calculate mean distance between males at each time point. A total of 16338 individual locations were taken from the 4257 images captured.

A male's isolation index was calculated using a similar method to the previously calculated pairwise distances. However, rather than averaging the pairwise distances across the four males in each enclosure (as for the unmarked flies in Chapter 3), the isolation index is the mean distance of each specifically-identified male from the other three males – giving four values per image.

Minimum distance travelled by each male was also calculated using Euclidean distances. The mean of the distances between an individual's consecutive locations across the four daylight periods was taken as the minimum distance that the male had travelled whilst in the treatment conditions.

I calculated male home range as 80% Minimum Convex Polygons, using the R packages adehabitatHR (Calenge, 2006). I assumed the 15-minute locations were independent, based on the observation that males could traverse the full enclosure many times during the 15-minute period. I excluded location points from the first period of daylight, to exclude the period of adjustment to and exploration of the arena, and to allow males time to acclimatise and establish home range patterns.

Reproductive behavioural assays

After 70 hours (± 2 hours) in the treatment enclosures, males were given one opportunity to mate with a virgin female; uniform: N = 63, clustered: N = 68, dispersed: N = 64. I aspirated the pair into a standard food vial supplemented with ~0.03g active yeast granules. To reduce encounter latency, I limited the space in the vial to 7cm^3 by pushing down the vial bung.

I measured the latency of the male to court, latency of the pair to copulate and copulation duration – as described in Chapter 3. Not every male successfully courted (uniform: 96.8%; clustered: 92.6%; dispersed: 90.6%), and not all courting males mated (uniform: 88.5%; clustered: 72.1%; dispersed: 79.3%).

Statistical analysis

Sample sizes were 16 replicates (N = 64 *Drosophila*) for the dispersed and uniform distribution treatments, and 17 replicates (N = 68 *Drosophila*) for the clustered treatment – of these, I photographed 11 from each treatment (33 in total) for data collection on intermale distances.

All statistical analyses were conducted in R version 4. 0. 3. (R Core Team, 2019). I analysed the effect of food resource spatial distribution on total intermale distance using linear mixed effects models, with enclosure included as a random effect in all models to account for the nonindependence of the four males in a single treatment replicate. I modelled time point (numbered sequentially from first to last measurement and treated as continuous) as a fixed effect.

Using linear mixed effects models, I analysed treatment effects on spacing behaviours and mating-related traits, with replicate enclosure again entered as a random effect. I initially entered time and point treatment as interacting predictor variables; if the interaction was non-significant I reran the model with both variables entered as main effects. I used the R package lmerTest (Kuznetsova et al., 2017) to generate p values using the Sattherthwaite approximation for degrees of freedom. To assess treatment effects on binomial variables (courtship and copulation success), I used generalised linear mixed models with a binomial

error distribution. I used one-way ANOVAs to analyse the relationship between spacing behaviours and mating behaviours, and then generalised linear models with a quasiPoisson error for effects on production of offspring. Finally, I used Levene's test to determine whether food resource spatial distributions effected the variance observed in behavioural responses.

Of the potential 17028 individual locations that could be collected, in 690 (4.1%) instances I was unable to accurately locate the individual. Of these 16338 individual location values, in 1638 (10.0%) the male's identity could not be detected. To minimise the effect of missing data on the number of time points included per replicate, the unit of analysis used was the mean (rather than the raw data) of the distances between each pair for each time point, the isolation index, the home range size and the minimum distance travelled (Chapter 3, Churchill et al. (2020)).

4.3 Results

Effect of food resource distribution on intermale spacing

The impact of the spatial distribution of food resource patches had a strikingly similar effect on the social environment to that I reported in the previous chapter. Food resource distribution significantly influenced the mean pairwise distance between the four males in the enclosures, and this interacted with the time course of exposure to treatment (treatment*time: $F_{2,3979} = 117$, $p << 0.0001$; Fig. 23, Table 2). As in Chapter 3, this significant interaction was present on the final day (although unlike in the previous chapter, the time effect on distance did not stabilise; treatment*time: $F_{2,339} = 22.4$, $p << 0.0001$; Table 2). Post-hoc testing confirmed that on this final day, pairwise distances between males in the dispersed treatment (44.78 ± 0.99mm SE) were significantly greater than those on the uniform treatment (35.74 ± 1.43mm SE) and clustered food treatment (24.51 ± 1.04mm SE; dispersed vs. clustered: $F_{2,30} = 17.9$, $p << 0.0001$; dispersed vs. uniform: $F_{1,20} = 8.43$, $p = 0.00875$; time remained in these models as a main effect). In addition, pairwise distances between males on the uniform treatment were significantly greater than those on clustered resources ($F_{1,20} = 8.21$, $p = 0.00956$).

In Chapter 3, I reported intriguing dynamics operating on the intermale distances on the first day of this treatment period: with males on dispersed resources having lower intermale distances than those on clustered food patches. However, the opposite pattern was true in this study, although the difference in spacing between these two treatments was not statistically significant (clustered vs. dispersed: $F_{2,30} = 0.790$, $p = 0.463$; Fig. 23, Table 2).

Variance of mean pairwise distances was equal between the three food resource distributions (Levene test of equal variance: $F_{2,30} = 2.07$, $p = 0.144$). Comparing replicates within treatments revealed significant variability in consistency of individual mean pairwise distances between individual enclosures in all three treatments (clustered: $F_{10,1333} = 26.1$, $p << 0.0001$; dispersed: $F_{10,1369} = 21.6$, $p << 0.0001$; uniform: $F_{10,1398} = 7.49$, $p << 0.0001$).

Table 2. Details of statistical parameters from the linear mixed-model analyses

Parameter	Estimate	SE	*t*	*P*
Pairwise distance between males: full duration of treatment				
Clustered (intercept)	37.09	2.16	17.19	<0.0001
Uniform	-3.11	2.99	-1.04	0.306
Dispersed	7.23	2.99	2.41	0.021
Time sequence	-0.14	0.01	-14.09	<0.0001
Uniform*time	0.14	0.01	11.97	<0.0001
Dispersed*time	0.17	0.01	14.32	<0.0001
Pairwise distance between males: final day of treatment				
Clustered (intercept)	146.07	36.56	4.00	<0.0001
Uniform	137.52	49.73	2.77	0.00600
Dispersed	-168.48	49.60	-3.40	0.000762
Time sequence	-0.98	0.30	-3.33	0.000967
Uniform*time	-1.03	0.40	-2.56	0.0109
Dispersed*time	1.53	0.40	3.81	0.000162

Pairwise distance between males: first day of treatment

Clustered (intercept)	42.48	4.07	10.43	<0.0001
Uniform	2.29	5.76	0.40	0.693
Dispersed	4.41	5.77	0.76	0.451
Time sequence	-0.24	0.07	-3.50	0.000505
Uniform*time	-0.26	0.10	-2.72	0.00665
Dispersed*time	0.12	0.10	1.22	0.223

Home range size

Clustered (intercept)	29.83	7.53	3.96	0.000426
Uniform	35.64	10.65	3.35	0.00222
Dispersed	41.15	10.65	3.86	0.000555

Home range size: clustered treatment

Day two	2.59	5.26	0.49	0.624
Day three	20.79	4.74	4.38	<0.0001
Day four (intercept)	11.01	3.93	2.80	0.00858

Home range size: uniform treatment

Day two	-5.91	3.98	-1.49	0.140
Day three	7.99	3.18	2.51	0.0136
Day four (intercept)	13.80	3.29	4.19	0.000537

Home range size: dispersed treatment

Day two	-2.12	6.28	-0.34	0.736
Day three	15.56	5.32	2.92	0.00427
Day four (intercept)	17.88	5.95	3.00	0.00848

Minimum distance travelled

Clustered (intercept)	179.22	17.35	10.33	<0.0001
Uniform	14.55	24.54	0.59	0.558
Dispersed	-2.94	24.54	-0.12	0.905

Copulation duration

Clustered (intercept)	1237.95	31.48	39.32	<0.0001
Uniform	-65.45	44.16	-1.48	0.145
Dispersed	-107.34	44.46	-2.41	0.0199

Courtship latency

Clustered (intercept)	2.72	0.10	27.94	<0.0001
Uniform	-0.15	0.14	-1.09	0.281
Dispersed	-0.07	0.14	-0.46	0.648

Copulation latency

Clustered (intercept)	2.45	0.13	19.40	<0.0001
Uniform	0.08	0.18	0.43	0.667
Dispersed	0.05	0.18	0.25	0.805

Number of offspring				
Clustered (intercept)	4.20	0.08	51.08	<0.0001
Uniform	0.06	0.11	0.52	0.60
Dispersed	0.15	0.11	1.35	0.179

Model outputs are presented in the order they appear in the text. Response variables and data subsets are outlined in row headings, predictor variables in the 'Parameter' column

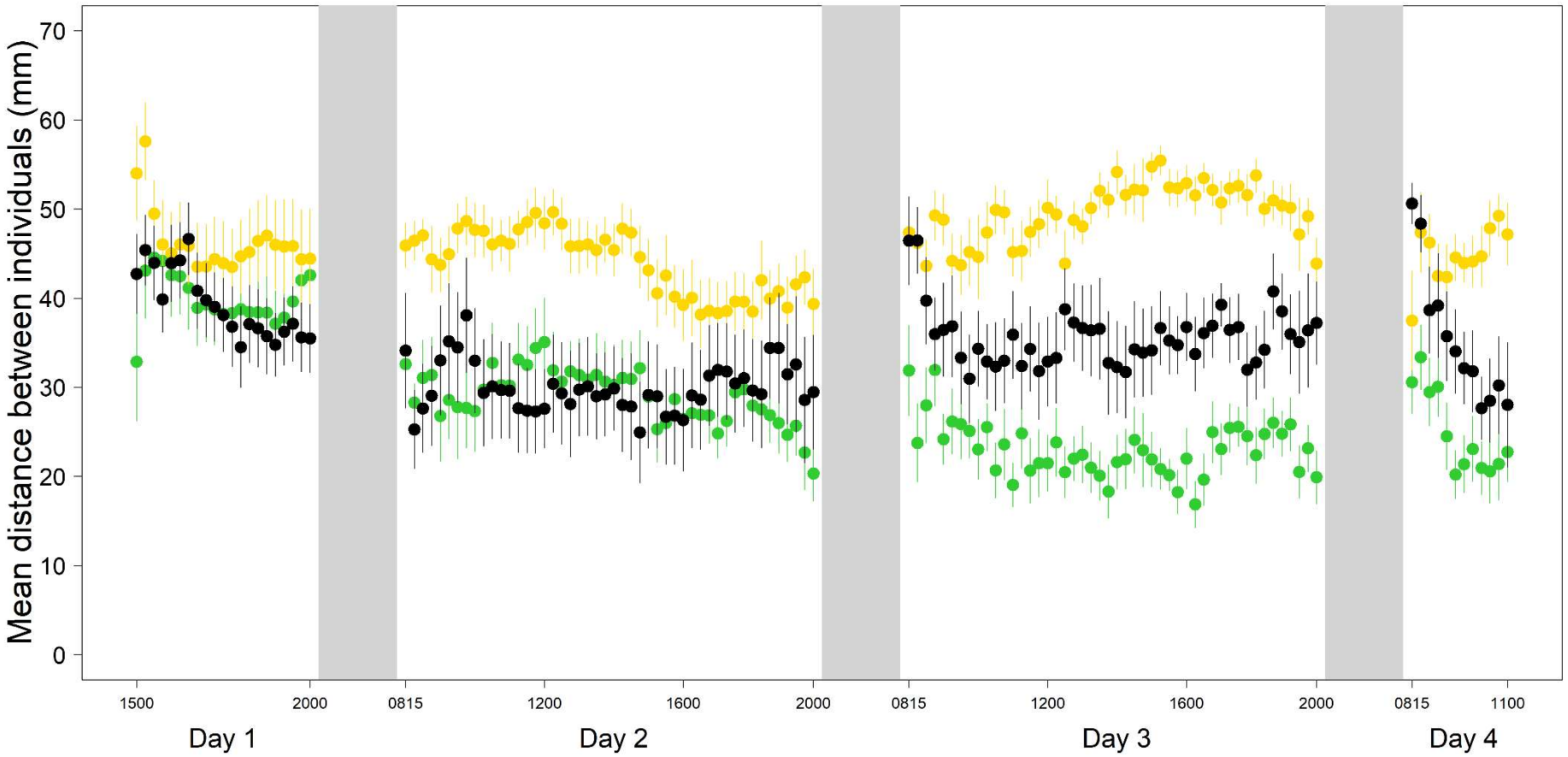


Figure 23. Mean intermale distance (mean of six pairwise distances between four focal flies per enclosure, averaged across 11 replicate enclosures) over time

Bars show standard errors of the mean for each time point. Grey blocks indicate period of dark (20:00 – 08:00h GMT), and are not to scale

Black = uniform treatment (evenly distributed food) Yellow = dispersed food patches

Green = clustered food patches

Effect of food resource distribution on home range size

Spatial distribution of food resources significantly affected the males' home ranges sizes ($F_{2,30}$ = 8.80, p << 0.001; Figs. 24, 25 and 26, Table 2). Those housed on clustered resources (979mm^2 ± 87mm^2 SE), had a substantially smaller mean home range size than those on dispersed (2329mm^2 ± 186mm^2 SE; $F_{1,20}$ = 23.2, p << 0.001) or uniformly distributed resources (2148mm^2 ± 203mm^2 SE; $F_{1,20}$ = 11.6, p = 0.00281). No significant difference of home range size was found between males on dispersed and those on uniform food resource distributions ($F_{1,20}$ = 0.193, p = 0.666).

Home ranges on the clustered resource were considerably more uniform in size than those on the remaining two treatments (Levene test of equal variances: $F_{2,129}$ = 13.1, p << 0.0001; note that this test ignores non-independence of home ranges within the same replicate). Comparing replicates within treatments revealed significant variability in consistency of home range sizes between individual enclosures in males house on uniform resources ($F_{10,33}$ = 3.76, p << 0.0001) but not in those housed on clustered ($F_{10,33}$ = 1.40, p = 0.224) or dispersed food ($F_{10,33}$ = 1.28, p = 0.280). In other words, in the uniform treatment the different replicates varied in how variable the four home range sizes were, whereas in the other two treatments there was greater consistency between replicates of the four constituent home range sizes per treatment.

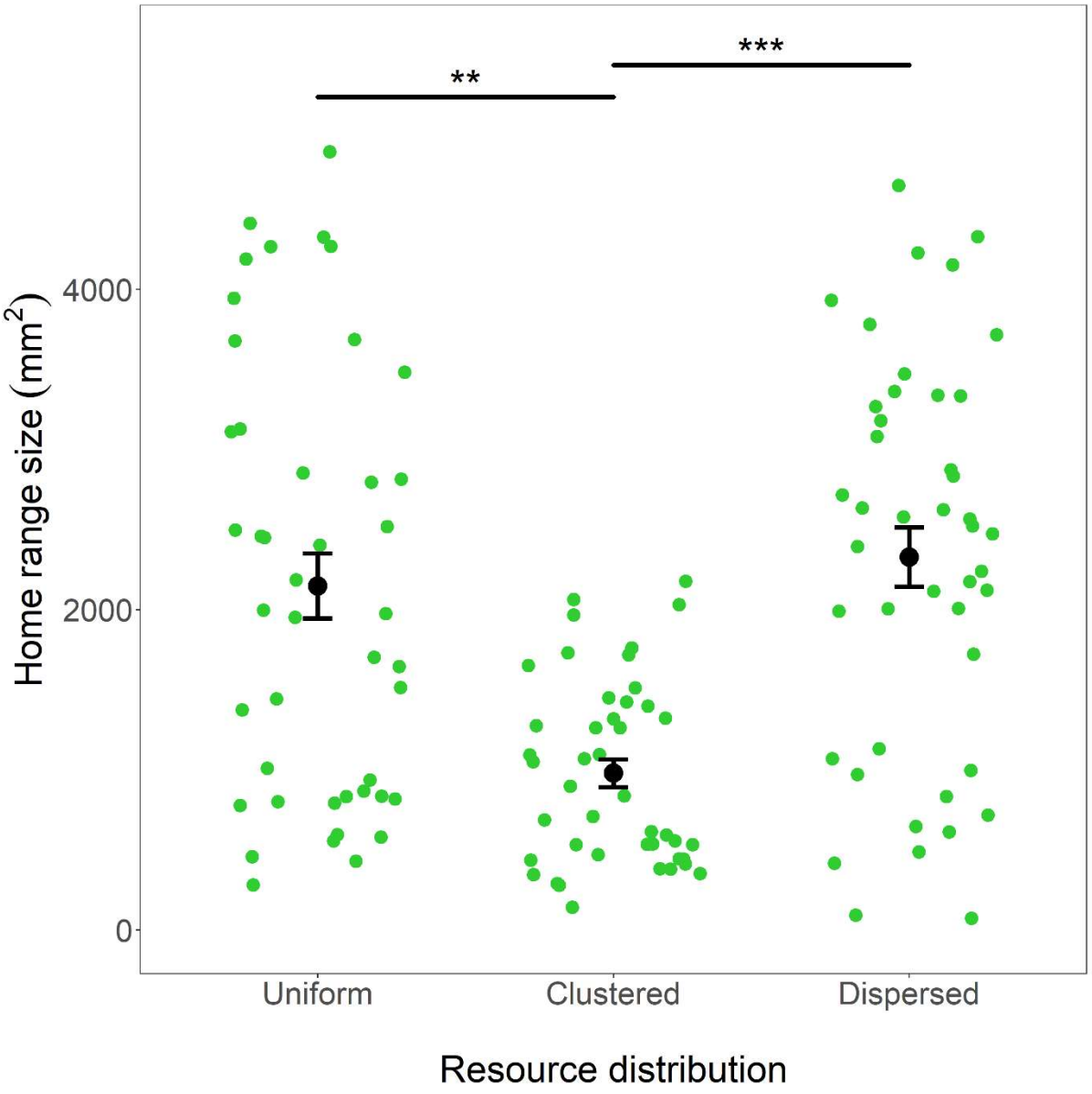
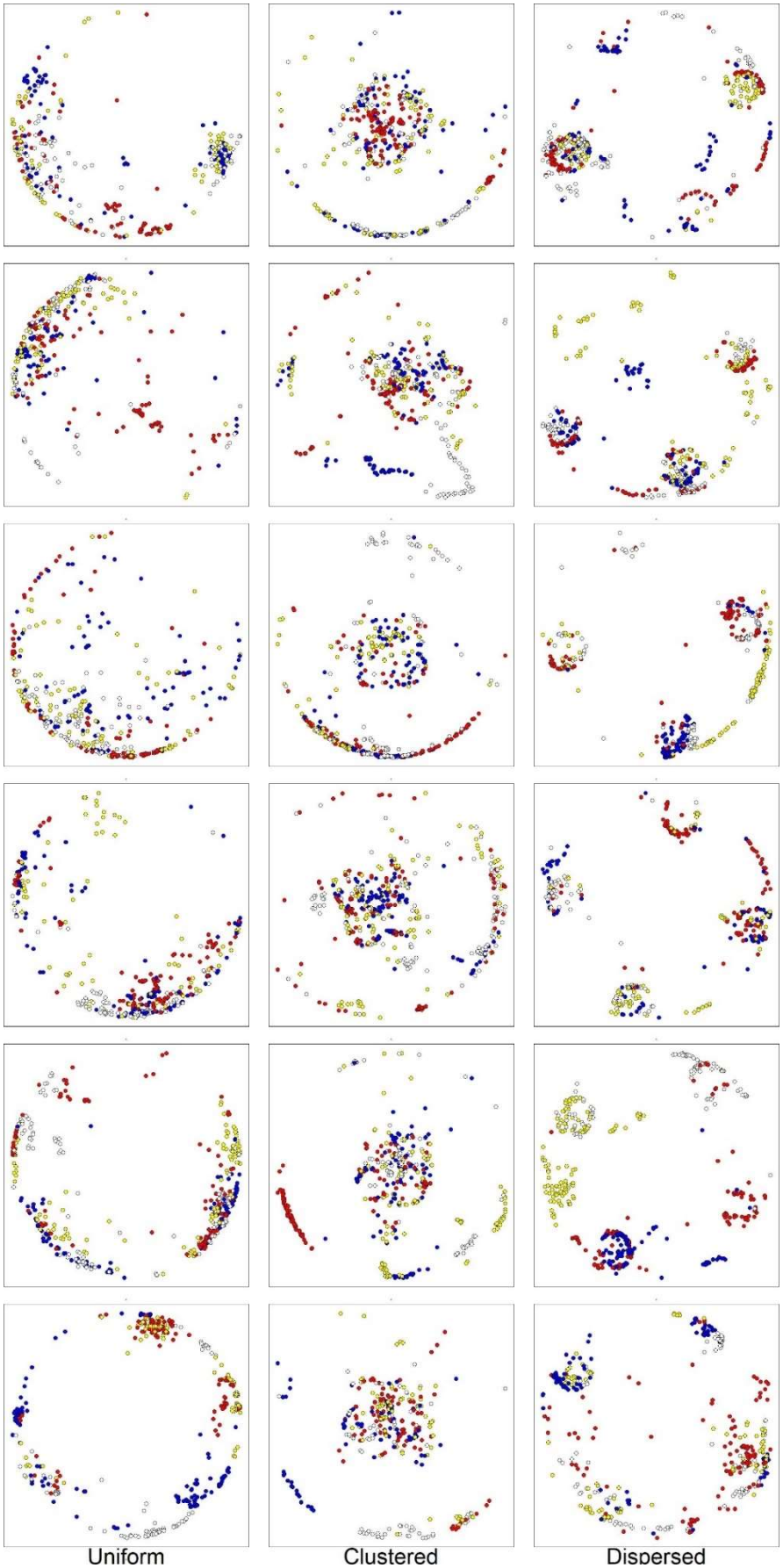


Figure 24. The effect of the spatial distribution of food on the male home range size

Calculated as Minimum Convex Polygons, excluding 20% of the most extreme location points and excluding the first day of treatment

Mean home range size in millimetres squared is shown as a black dot, with 95% confidence intervals


Uniform
Clustered
Dispersed

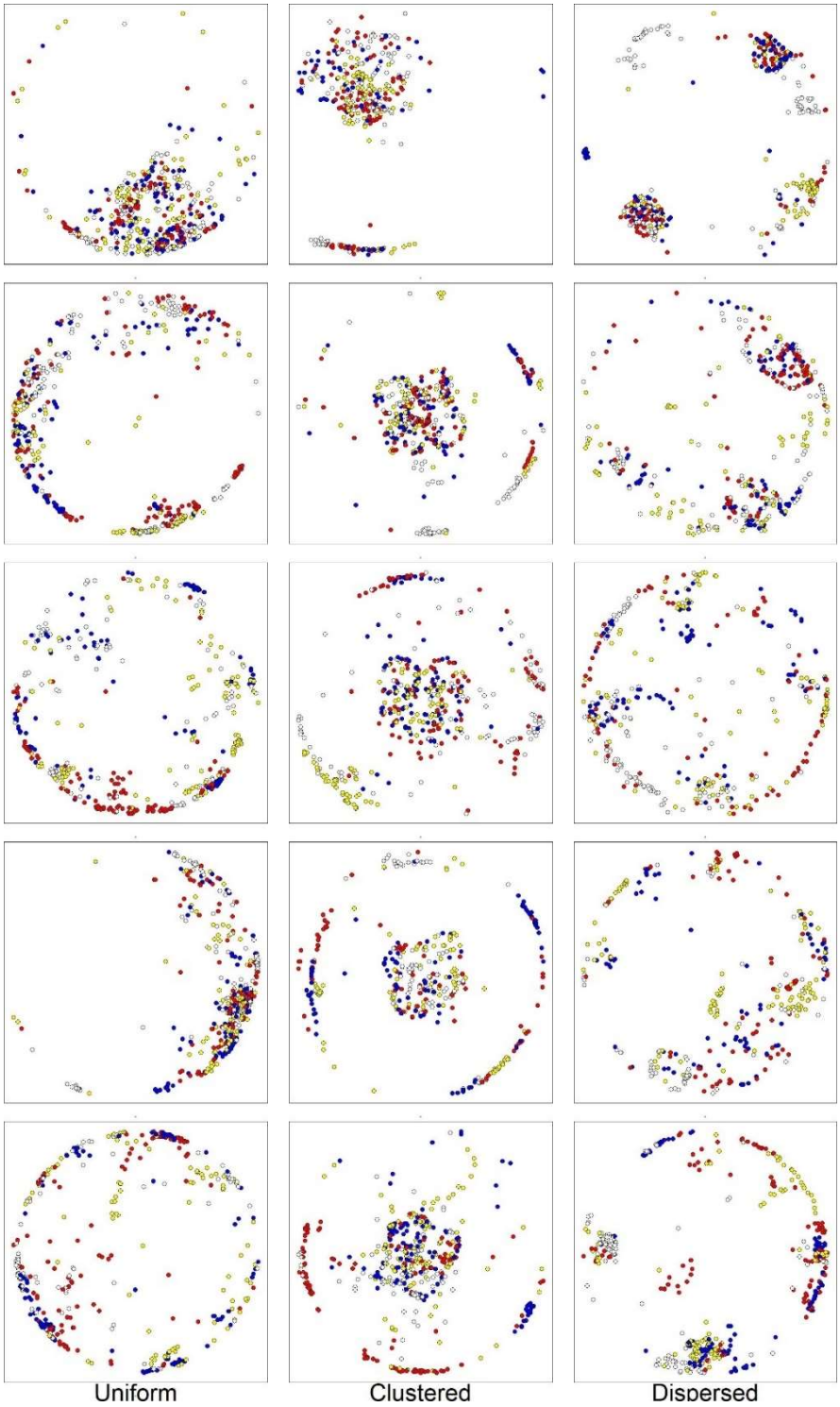
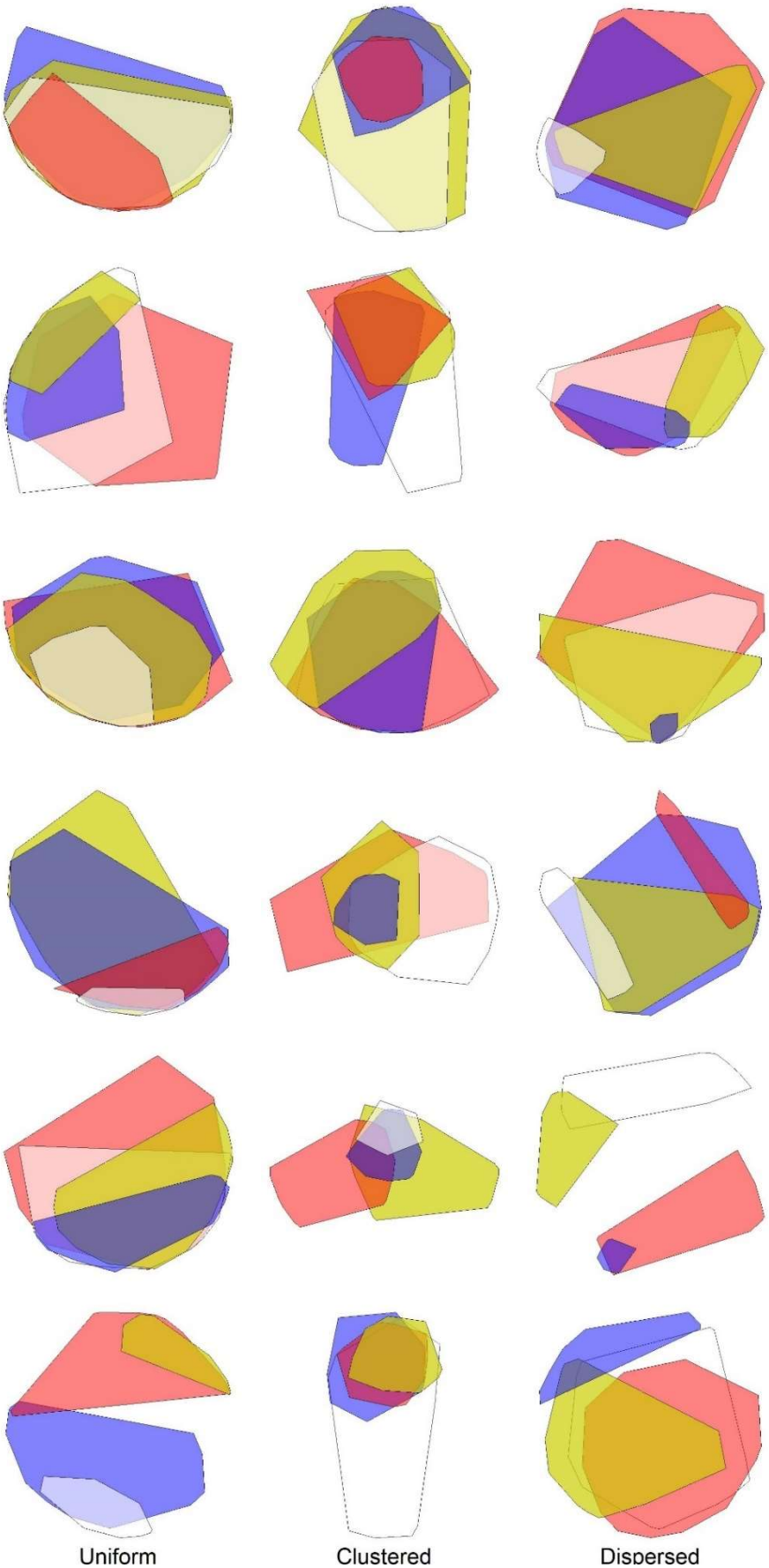


Figure 25. The observed locations of the four marked males (white, yellow, red and blue) for all 11 replicates in each of the three spatial distribution treatments


Uniform
Clustered
Dispersed

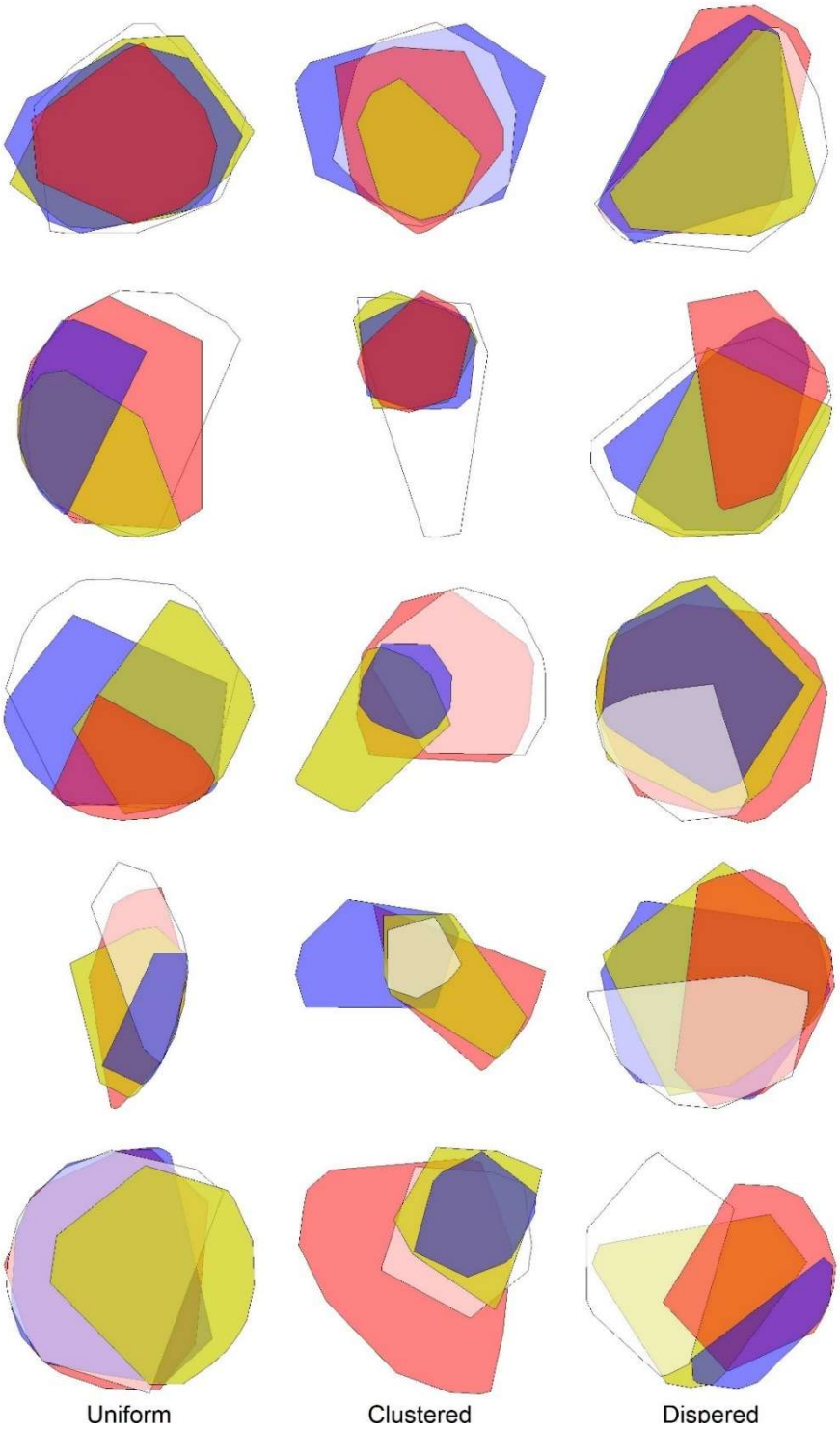


Figure 26. Home ranges (80% MCPs) of the four marked males (white, yellow, red and blue) for all 11 replicates in each of the three spatial distribution treatments

In all three treatments, home range size varied throughout the four periods of daylight testing; Fig. 27, Table 2. In all cases, home range size was larger on the third day compared to the second and fourth days (clustered: $F_{2,103} = 6.85$, $p = 0.00162$; dispersed: $F_{2,105} = 5.79$, $p = 0.00413$; uniform: $F_{2,111} = 11.0$, $p << 0.0001$).

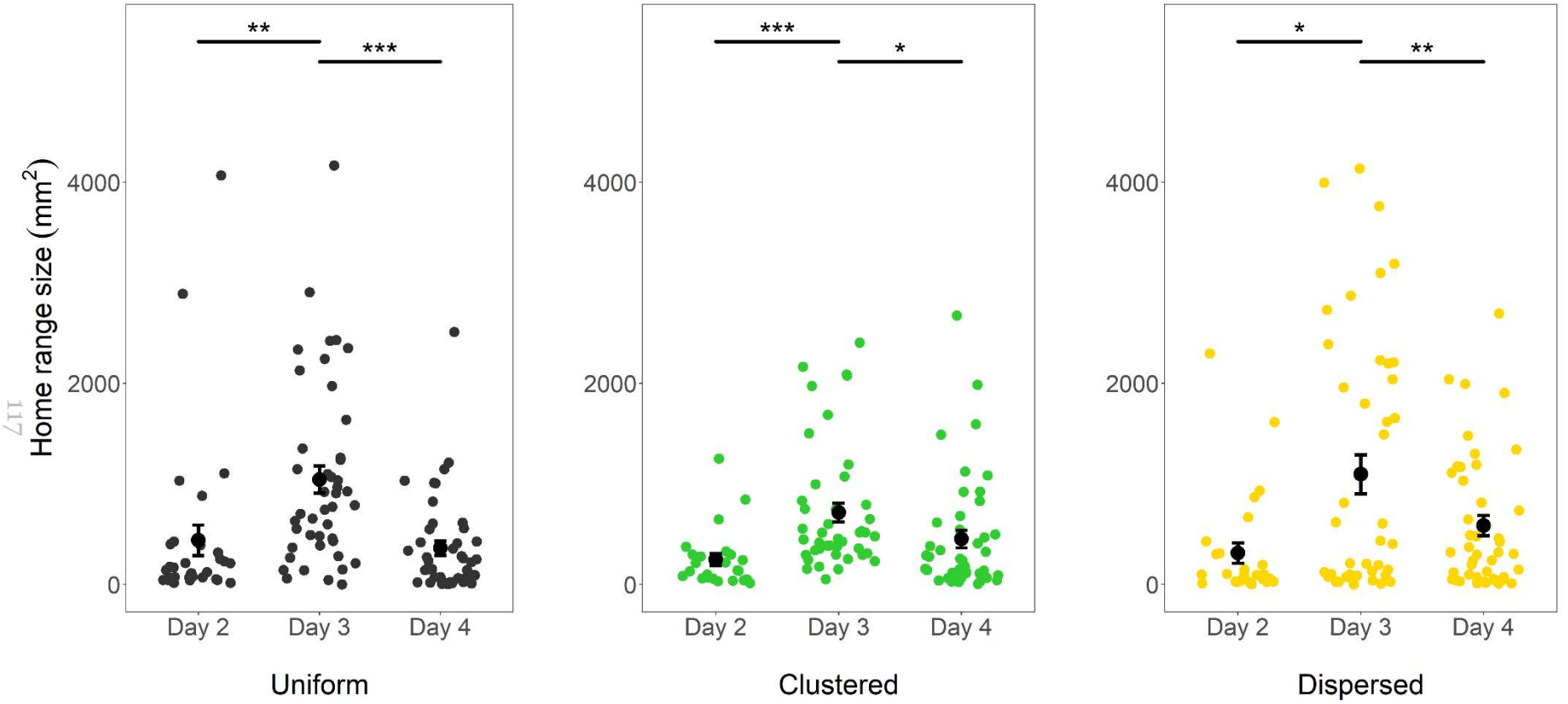


Figure 27. The effect of the spatial distribution of food on the male home range size

Calculated using Minimum Convex Polygons, excluding 20% of the most extreme location points

Mean home range size in millimetres squared is shown as a black dot, with 95% confidence intervals

The mean of the minimum distance travelled was not influenced by the spatial distribution of the food resources that males were housed on (uniform: 11.40 ± 0.53mm SE; clustered: 10.54 ± 0.45mm SE; dispersed: 10.37 ± 0.71mm SE; $F_{2,30} = 0.291$, $p = 0.749$; Fig. 28, Table 2), but minimum distance travelled was more variable in the dispersed and uniform treatments than in the clustered treatment (Levene test of equal variances: $F_{2,129} = 5.69$, $p = 0.00429$). There was however no variability in consistency of mean minimum distance travelled when comparing replicates within treatments in any of the three spatial distribution treatments (uniform: $F_{10,33} = 1.22$, $p = 0.312$; clustered: $F_{10,33} = 1.02$, $p = 0.448$; dispersed: $F_{10,33} = 1.93$, $p = 0.0758$).

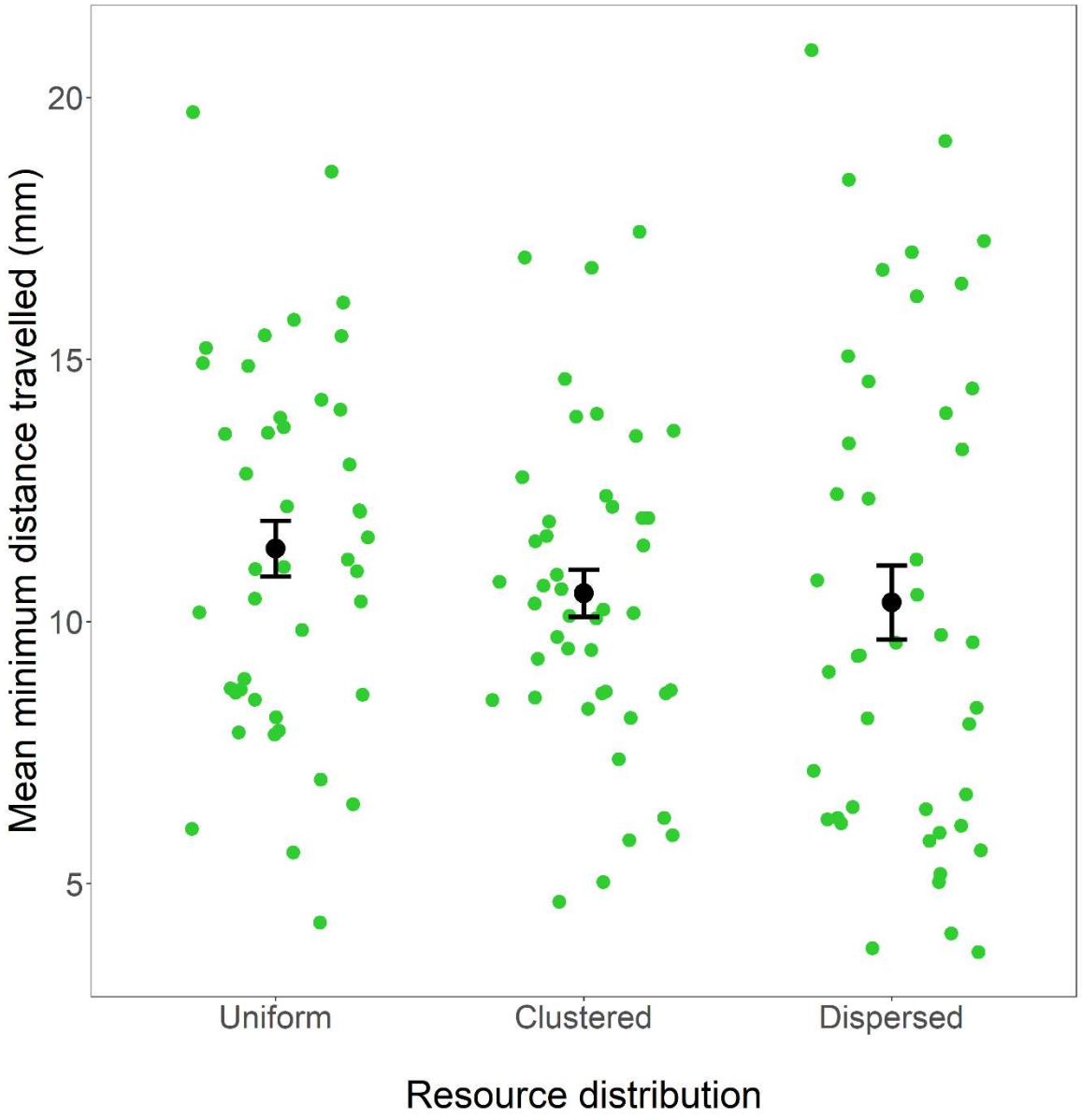


Figure 28. The effect of the spatial distribution of food on the mean minimum distance travelled between each 15-minute imaging period

Mean minimum distance travelled in millimetres cubed is shown as a black dot, with 95% confidence intervals

Effect of food resource distribution on mating behaviour

Males from the clustered treatment mated for significantly longer (1232 ± 26s SE) than those from the dispersed treatment (1133 ± 24s SE), a difference of 1min 39s ($F_{1,27}$ = 7.30, p = 0.0117; Fig. 29, Table 2). However, the overall effect of resource treatment on mating

duration was not significant ($F_{2,44}$ = 2.96, p = 0.0621); and copulation duration of males from the uniform treatment (1174 ± 25s SE) did not differ significantly from either of the other treatments (uniform versus dispersed: $F_{1,30}$ = 0.839, p = 0.367; uniform versus clustered: $F_{1,30}$ = 2.01, p = 0.167).

Mating duration variance was equal between the three distribution treatments (Levene test of equal variance: $F_{2,139}$ = 0.0448, p = 0.956; note that this test ignores non-independence of home ranges within the same replicate), and in between individual enclosures in all treatments (clustered: $F_{16,29}$ = 1.62, p = 0.127; dispersed: $F_{15,33}$ = 0.949, p = 0.525; uniform: $F_{15,33}$ = 1.17, p = 0.340).

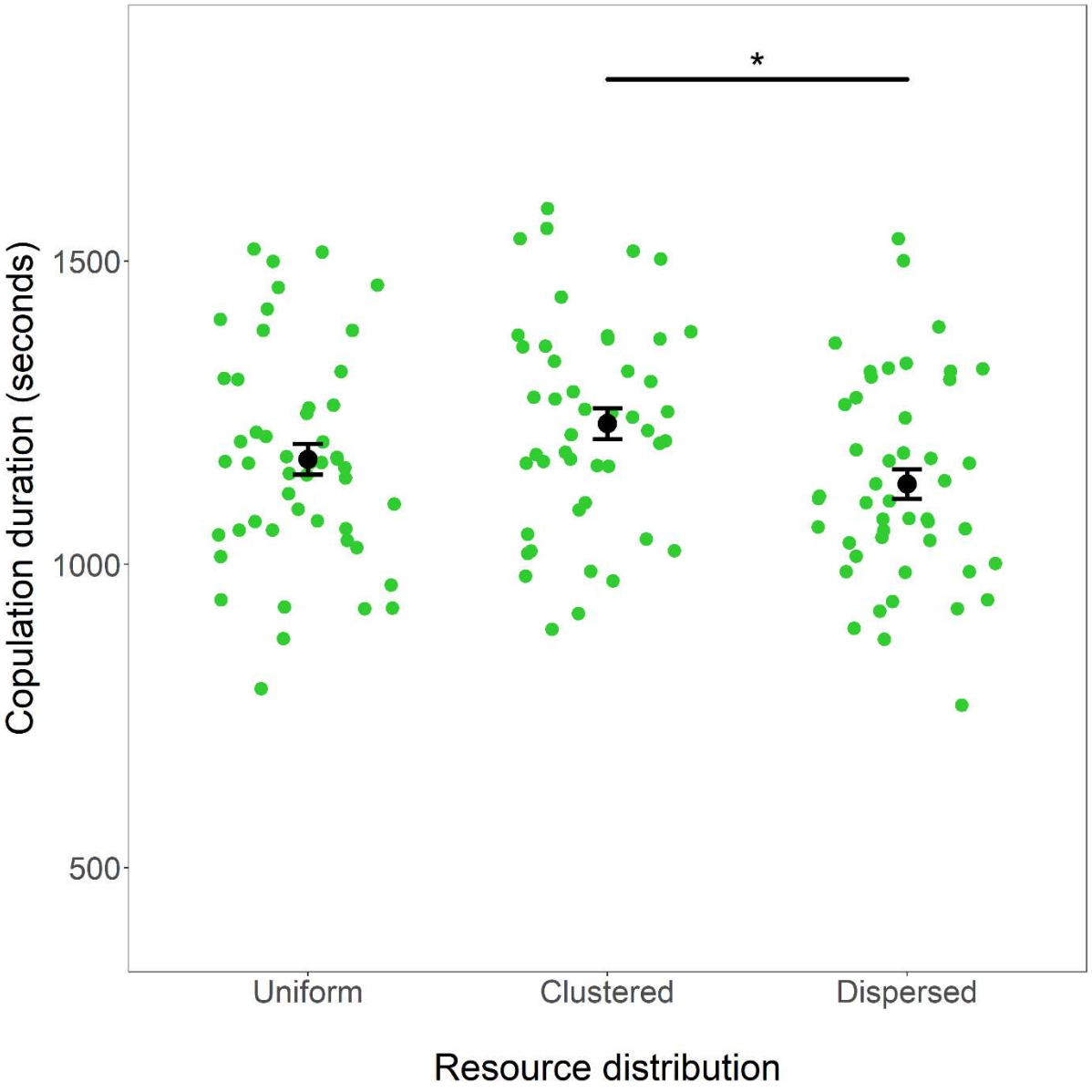


Figure 29. The effect of the spatial distribution of food on the duration of subsequent copulation
Mean copulation duration (in seconds) is shown as a black dot, with 95% confidence intervals

In total, 182 of 195 males (93.3%) courted the female. There was no significant effect of treatment on the proportion of males that courted (generalized linear model with binomial errors and enclosure plate nested within treatment; $\chi 2 = 192$, $p = 0.327$). Similarly, 157 (86.3%) of these males mated, and this was not influenced by treatment ($\chi 2 = 179$, $p = 0.123$).

Neither the latency to start courting (log_{10}: $F_{2,47} = 0.596$, $p = 0.555$; Fig. 30) nor the latency to start copulation (log_{10}: $F_{2,41} = 0.095$, $p = 0.910$; Fig. 31) differed significantly between the three treatments; see Table 2.

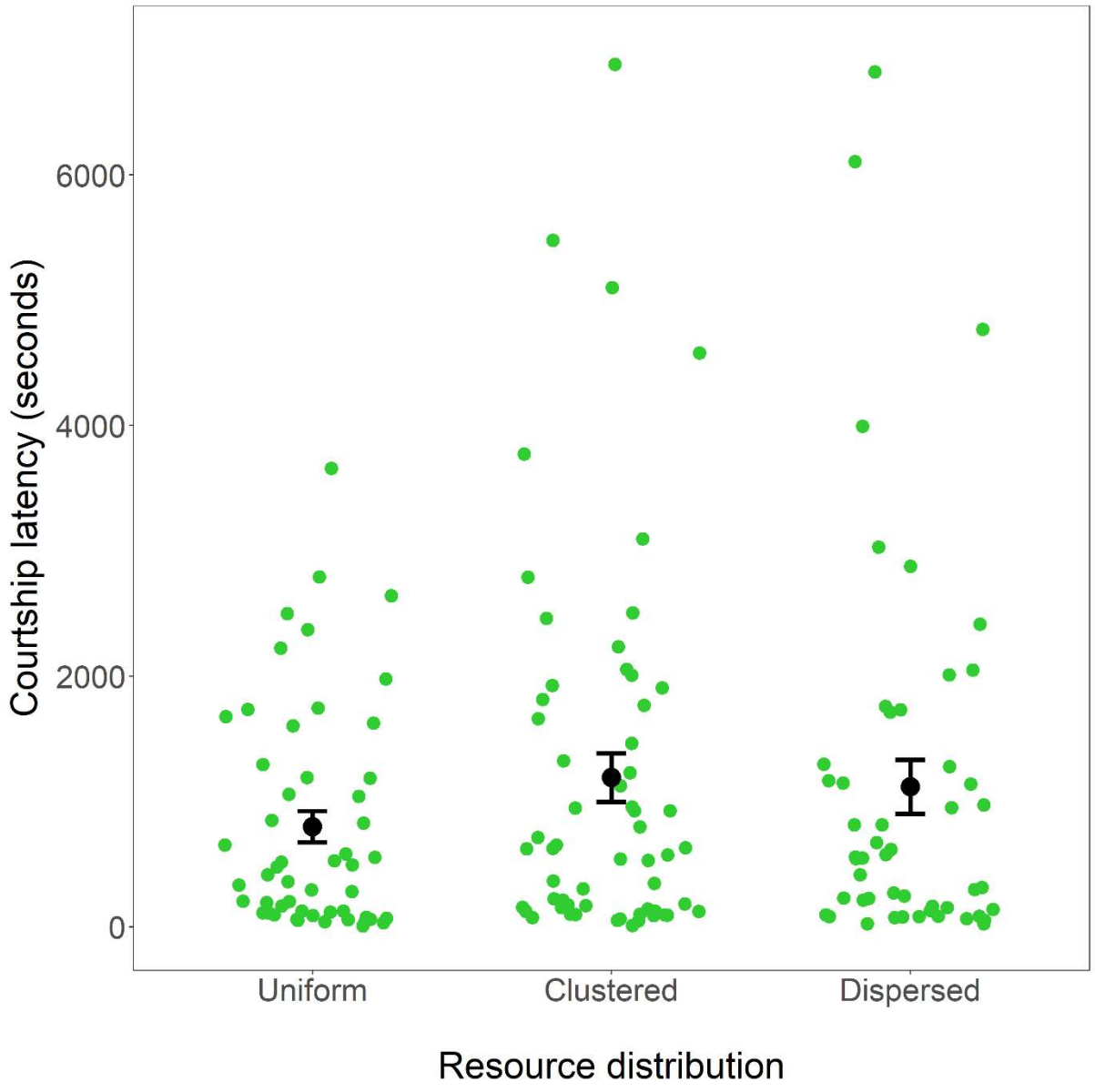


Figure 30. The effect of food resource spatial distribution on the latency of the male to begin courtship
Mean courtship latency (in seconds) is shown as a black dot, with accompanying 95% confidence intervals
Note that the analysis was performed on logged data, but untransformed values are presented here

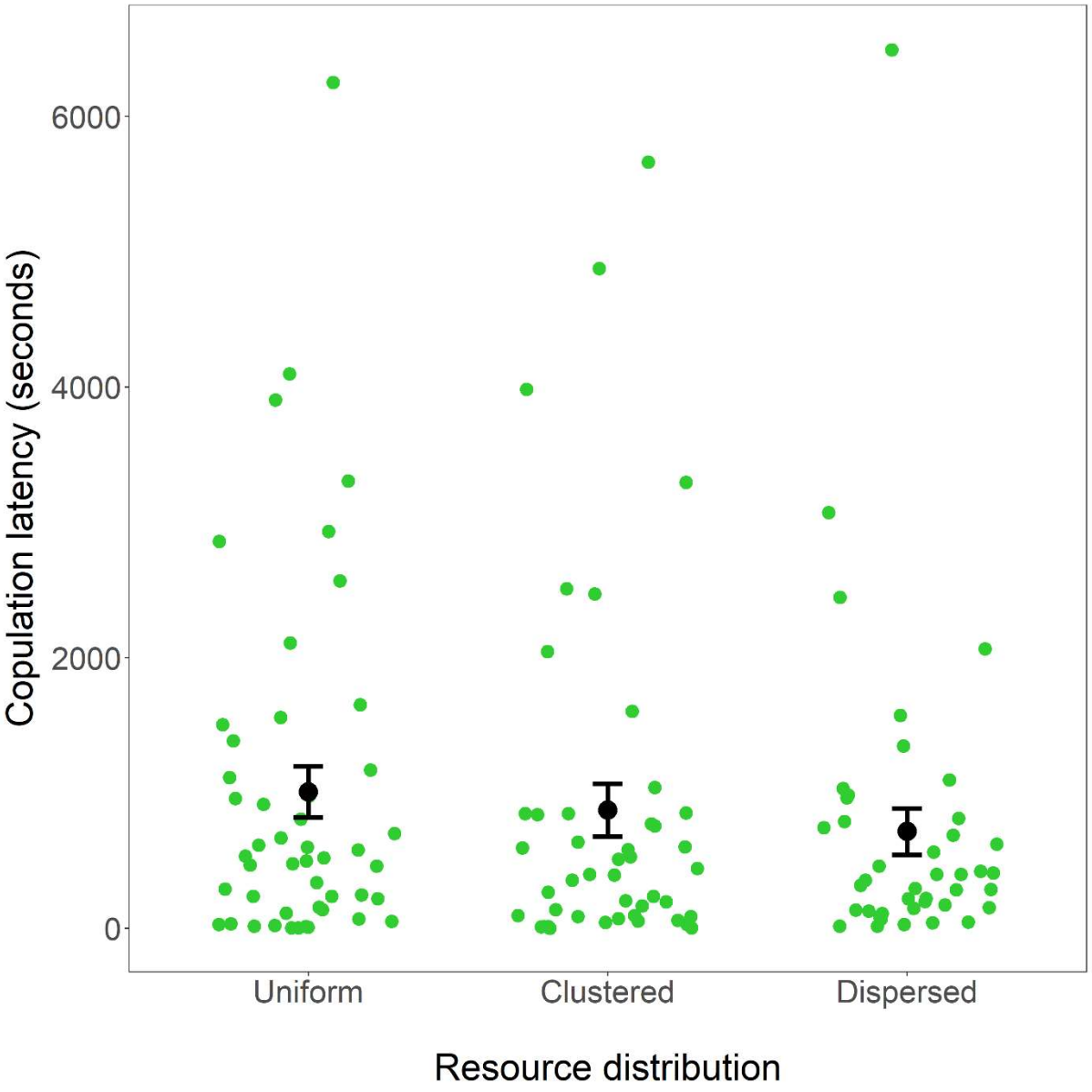


Figure 31. The effect of food resource spatial distribution on the latency of the pair to start successful copulation
Mean copulation latency (in seconds) is shown as a black dot, with accompanying 95% confidence intervals
Note that the analysis was performed on logged data, but untransformed values are presented here

Effect of food resource distribution on fitness

There was no significant effect of spatial distribution of food resources on the number of adult offspring produced ($\chi^2 = 40.3$, d.f. = 146, p = 0.391; Fig. 32, Table 2). Variation in the number of offspring produced (i.e., consistency across replicates) was equal over the three treatments

(Levene test of equal variance: $F_{2,146}$ = 1.54, p = 0.218; note that this test ignores non-independence of home ranges within the same replicate) and between individual enclosures in each of the three treatments (clustered: $F_{16,31}$ = 0.891, p = 0.585; dispersed: $F_{15,33}$ = 1.15, p = 0.355; uniform: $F_{15,36}$ = 0.894, p = 0.576).

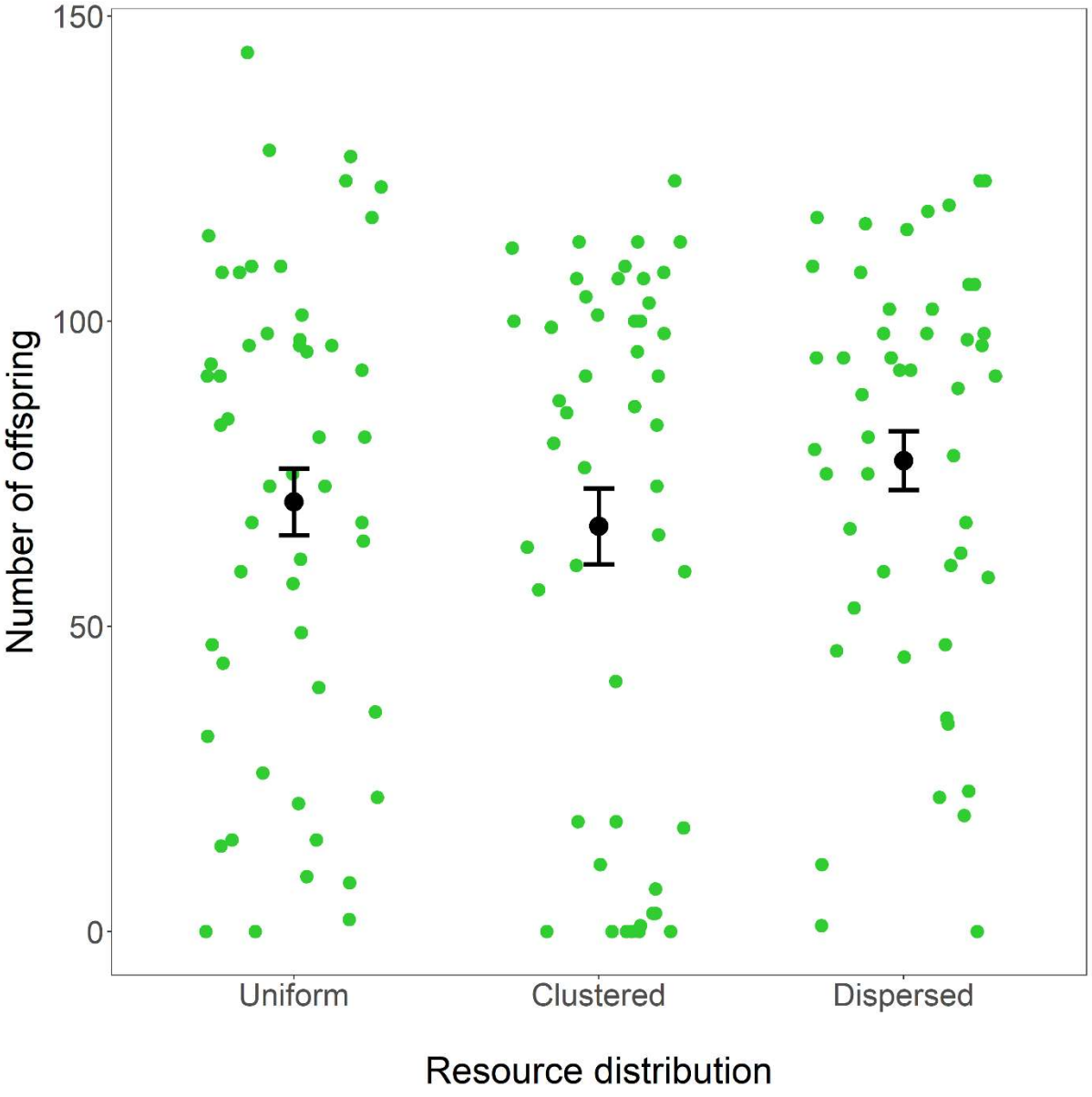
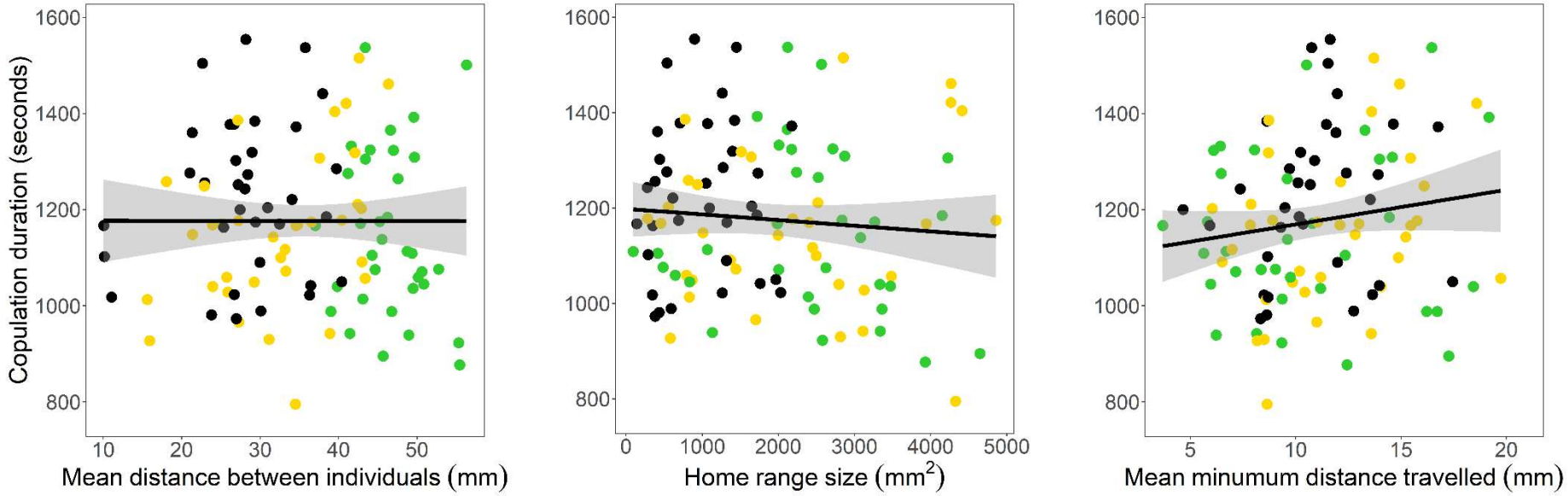


Figure 32. The effect of food resource spatial distribution on the number of successfully eclosed adult offspring Mean number of adult offspring is shown as a black dot, with accompanying 95% confidence intervals

Effect of intermale spacing and home range size on mating behaviour

Male isolation index (an individual male's mean distance from three potential rivals averaged across the four treatment days), did not have any significant effect on their subsequent copulation duration ($F_{1,44} = 0$, $p = 0.995$; Fig. 33a).

I found no evidence that male home range size ($F_{1,95} = 1.96$, $p = 0.165$; Fig. 33b) or mean minimum distance travelled ($F_{1,77} = 0.839$, $p = 0.362$; Fig. 33c) affected duration of copulation.



a. b. c.

Figure 33. The effect of social environment on male copulation duration

a. The effect of mean intermale distance within treatments (individual pairwise distances between four focal males, averaged across the four daylight testing periods)

b. The effect of male home range size, averaged across the four daylight testing periods

c. The effect of the minimum distance the male travelled, averaged across the four daylight testing periods

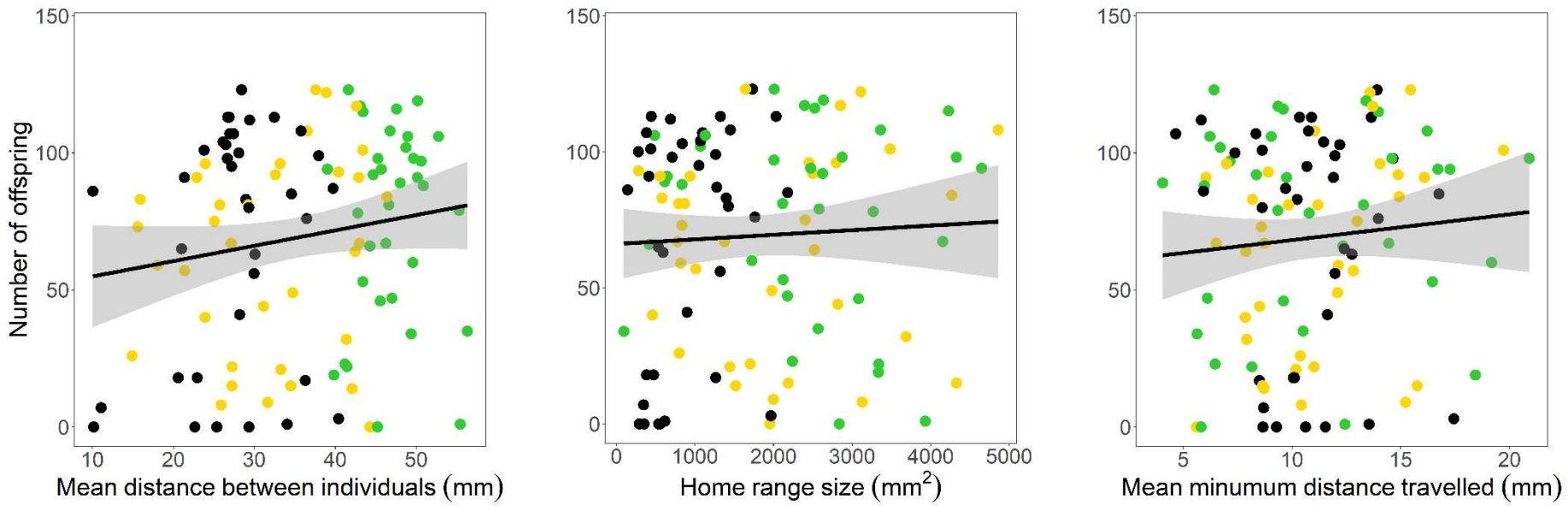
Black line shows the line of best fit, and grey shadowing shows the standard error of the line

Black = uniform treatment Green = clustered food patches Yellow = dispersed food patches

Effect of intermale spacing and home range size on fitness

Individual intermale spacing across the four treatment days, did not impact the number of adult offspring males produced ($\chi^2 = 57.2$, d.f. = 103, p = 0.101; Fig. 34a).

Home range size also did not impact the number of adult offspring produced by the male ($\chi^2 = 6.12$, d.f. = 103, p = 0.593; Fig. 34b); and neither did the mean minimum distance travelled ($\chi^2 = 17.0$, d.f. = 103, p = 0.374; Fig. 34c).



a. b. c.

Figure 34. The effect of social environment on the number of offspring produced

a. The effect of mean intermale distance within treatments (individual pairwise distances between four focal males, averaged across the four daylight testing periods)

b. The effect of male home range size, averaged across the four daylight testing periods

c. The effect of the minimum distance the male travelled, averaged across the four daylight testing periods

Black line shows the line of best fit, and grey shadowing shows the standard error of the line

Black = uniform treatment Green = clustered food patches Yellow = dispersed food patches

4.4 Discussion

Population dynamics and territoriality

Males housed on clustered resources were found in closer proximity than males housed on uniform and dispersed resources, plus males on dispersed resources were found to be further from their closest rival than those on uniform resources (Churchill et al., 2020). As expected, this result was true of the average across the four treatment days and on the final day – as was found in Chapter 3.

There were other impacts of the physical environment on the social environment. Spatial distribution of resources had a substantial impact on home range size: males on clustered resources held much smaller territories than males on dispersed or uniform treatments. In *Drosophila*, territorial behaviour seems to be driven by the boundaries of food resources (Lim et al., 2014), and because the potential food sources in this clustered treatment are in direct contact (Chapter 3, Churchill et al. (2020)), males did not need to travel far to sample all available resources – resulting in smaller home ranges. However, given there was a significant effect of home range size caused by resource patchiness, it is surprising that distance travelled was not also impacted by resource patchiness. As this experiment was conducted at a relatively small scale compared to the distance that *Drosophila* could inhabit in the wild (Soto-Yéber et al., 2018, Taylor and Powell, 1978), it is possible that enclosure size constrained individuals so that any impacts of resource distribution on population dispersal were not detected. Yet if this were true, it is intriguing that a difference in home range size was observed. There was significant amount of variance of both home range size and distance

travelled across the three treatments, suggesting that individual responses are extremely variable across populations – and individual responses may not be repeatable. It would be beneficial for future work to explore this further, in a range of enclosures varying in size.

Another unexpected result was that in all three resource distribution treatments, male home range sizes were larger on the third day than on the second or final day of treatment exposure. This contradicts the prediction that males would establish some form of territoriality and/or dominance hierarchy on the first day of exposure, and from then on would remain in their established home ranges. Instead, it appears as though males are attempting to broaden and improve their territory as time progresses, but with only three days to compare it is not possible to draw any accurate conclusions. It would be interesting to explore the impacts of time on territoriality by housing individuals in the enclosures over a longer period so an overall trend can be more accurately described.

I discovered significant variability in the intermale distances between enclosures in all three spatial distribution treatments. This means that individuals spacing responses (in their proximity from rivals) were not consistent across the enclosures suggesting that searching behaviours are therefore highly stochastic. I also found that variance of home range size and distance travelled was different between treatments – in both cases the responses were more consistent in males housed on clustered resources compared to those on dispersed or uniform resources. Males housed on clustered resources are all responding to the spatial distribution of food similarly, whereas responses observed on uniform and dispersed treatments are less consistent. This suggests that there are more potential spacing patterns that males could occupy in those latter two resource distributions. Furthermore, male

behaviour on the uniform treatment was highly variable between replicates, suggesting that males resolved the division of available space in different ways in the different replicates. The same was not true of the two patchy treatments, in which variance in home range size was consistent across treatments – suggesting that males found a consistent solution to sharing the available space. This could be an indication that in some enclosures there may have been one (or more) more dominant males that emerged. Without video data however, it is difficult to interpret how dominance may impact pairwise distances or territoriality. On the one hand, one might expect that more dominant males would have a larger territory and thus would be further from any potential competitors. On the other hand, if a male were to be holding a large territory, it is likely that rival males would be willing to engage in a fight for this – and if fights were occurring regularly, intermale pairwise distances would be short. Repeating this study with software-tracked individuals would provide clarification here.

Plasticity in mating-related traits

As was discovered in the previous chapter, experimentally manipulating resource patchiness without changing group size, caused the same impact on mating duration as has previously been observed after direct manipulation on local density (Bretman et al., 2009, Bretman et al., 2010, Moatt et al., 2013, Bretman et al., 2013). In this study the overall impact of resource distribution on mating duration was not significant, but males housed on clustered resources were still found to have a significantly longer copulation duration compared to those exposed to dispersed resources. It is possible that this difference is caused by impacts of the marking technique as was found in Appendix 4. However, results in Appendix 4 showed that marking

reduced copulation durations compared to control males, whereas in this study the males had slightly longer copulation durations than the unmarked males of Chapter 3.

As expected, effects on courtship and copulation success, as well courtship and copulation latencies, were also consistent with Chapter 3. Exposure to resource patchiness and variability in the social environment experienced by the males did not impact their willingness to court, or the willingness of their mates to succumb to copulation.

Individual fitness effects

The increased copulation duration in males housed on clustered resources did not result in an increase in fitness. No effect of resource distribution or isolation index was found on the number of offspring produced. Copulation is an energetically expensive activity for males (Pitnick et al., 1995, Fowler and Partridge, 1989, Dewsbury, 1982), so why they would increase their investment in this without gaining any fitness benefits is unclear. This is not the first study to find this result: Bretman et al. (2012) and Dobler and Reinhardt (2016) also found no evidence of fitness benefits in response to increased copulation duration under higher perceived sperm competition. However, others have discovered an increase in fitness (Bretman et al., 2009, Bretman et al., 2012, Price et al., 2012). I did not count the number of grand-offspring produced, but it is possible that fitness effects might have been discovered here. Alternatively, they may only be present after the first mating – i.e., would have been detected if males were given the opportunity to re-mate. Number of offspring produced is also influenced by the female condition too – either directly or via male mate choice (Churchill et al., 2019, Churchill, 2016, Nandy et al., 2012, Lüpold et al., 2010, Fricke et al., 2008). Therefore, I might have observed fitness effects if males were given the opportunity to choose

their potential mate. If multiple females were present in the enclosure alongside the males, not only could a mate choice trial be completed, but also offspring would develop in the same environment that sires were responding to, and this would be a more ecologically relevant test of potential fitness effects.

Furthermore, there was no evidence to suggest that a male's home range size impacted either mating success or subsequent offspring production. This was unexpected, as Hoffmann and Cacoyianni (1989) discovered territoriality increased likelihood of males securing a mate – although they also found no evidence of any fitness benefits besides this. Linked to this, I discovered no mating duration or fitness effects of the distance travelled. Foraging is energetically expensive (MacArthur and Pianka, 1966, Wiersma et al., 2005), so I expected to find that males that had travelled further in search of food, had less expendable energy resulting in shorter mating durations and fewer offspring produced, however this was not the case. I have no data on the aggressive interactions that males experienced whilst in the treatment enclosures, so it is possible that those that did not travel so far, instead opted for a different strategy and expended their energy on fighting others to maintain their territories – thus nullifying the benefits of avoiding foraging costs. It is also possible that as this study was completed a small-scale, that distances travelled were not great enough to have a negative impact on the males' energy reserves.

In contrast to Chapter 3

In Chapter 3, I observed that males on dispersed resources appeared to be initially aggregating on the first day of treatment, possibly to establish territories or hierarchies (although it is not yet known if *Drosophila* are hierarchical (Hoffmann, 1987)). Interestingly

however, on the first day of treatment in this study, males on dispersed resources were still found to be further from their closest rival than males on clustered resources. Exactly why this result differs in these two chapters is not yet certain, but it is likely that males are using this first day to acclimatise to their new surroundings and thus individual movements on his day are likely to be highly stochastic.

Future directions

This research builds upon the resource patchiness effects discovered in the previous chapter and has opened up some exciting questions for future investigation. I have demonstrated that resource patchiness impacts individual home ranges on a very small scale, but I also discovered an intriguing impact of time exposure on territoriality too. Future work should explore how home temporal patterns in home range size, by maintaining males in enclosures for longer periods. Research on home ranges in the wild suggests that home range size does not differ through time (Brown and Orians, 1970), however this is likely biased by the fact that in the wild territories have already been established and little is known about how territories may change in novel environments. Plus, any reported effects of temporal variability in wild-living populations are likely caused by variation in climate or seasonality as opposed to knowledge of the surrounding habitat, and so replicating these results in a controlled environment would be a valuable future study.

It would also be beneficial for this study to include enclosures in a range of different sizes to uncover the intriguing disconnect between the impacts of resource patchiness on home range size and distance travelled. Using video analysis software, and tracking the individuals in real-time, would provide more detailed data on home range sizes and the total distance

(rather than minimum distance discussed here). Equally valuably, it would enable an exploration into the impacts of territoriality on encounter rates and proximity to rivals.

Continuing to link these behaviours to subsequent impacts on reproductive behaviours and fitness is paramount, as it will have important consequences for population dynamics. Investigating fitness effects using a mate-choice experiment would be beneficial, particularly if the females were also located in-situ, as both individuals would be responding to manipulated environmental variability. However, currently little is known about how females might respond to resource patchiness. Although given that female oviposition decisions can be impacted by density and presence of conspecifics (Battesti et al., 2012, Sarin and Dukas, 2009, Malek and Long, 2020), and here I have shown that resource patchiness affects local density, it is likely that resource patchiness will also impact oviposition preferences. Before trying to understand the combined impact of males and females on patchy resources, we first need to understand how patchiness might alter female responses. By manipulating resources using the same methods as I used in these chapters, in my next chapter I will explore the impacts of resource patchiness and competition on oviposition decisions.

Chapter 5: **Social and physical environment independently affect oviposition decisions**

Why did the fly have a sore throat?

It was a hoarse fly

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Abstract

In response to environmental stimuli, including variation in the presence of conspecifics, genotypes show highly plastic responses in behavioural and physiological traits influencing reproduction. Although extensively documented in males, such female responses are rather less studied. We expect females to be highly responsive to environmental variation and to differentially allocate resources to increase offspring fitness, given the major contribution of mothers to offspring number, size, and developmental conditions. Using *Drosophila melanogaster*, I manipulate: (a) exposure to conspecific females, which mothers could use to anticipate the number of potential mates and larval density, and; (b) test how this interacts with the spatial distribution of potential oviposition sites, with females from higher densities expected to prefer clustered food resources that can support a larger number of larvae. I found that high density females were slower to start copulating and reduced their copulation duration, the opposite effect to that observed in previously group-housed males. There was a parallel, perhaps related, effect on egg production: females previously housed in groups laid fewer eggs than those housed in solitude. Resource patchiness also influenced oviposition behaviour: females preferred aggregated substrate, which attracted more females to lay eggs, and larger clutches. However, I found no interaction between prior housing conditions and resource patchiness, indicating that females did not perceive the value of different resource distributions differently when exposed to environments that could signal expected levels of larval competition. I show that, although exposure to consexual competition changes copulatory behaviours of males, the distribution of oviposition resources has a greater effect on oviposition decisions.

5.1 Introduction

Impacts of intra-sexual competition

Most individuals are likely to experience competition for resources (whether food, mates or nesting or oviposition sites) at some point in their lifetime. Population density and the distribution of resources determine the extent of the competition that individuals might face: more clustered resources result in increased encounter rates (Emlen and Oring, 1977) and therefore are associated with a greater degree of adult and juvenile competition. Because optimal responses often differ in high- and low-competition environments (Maynard Smith, 1979), animals which experience variation in local population density are expected to make physiological and behavioural plastic adjustments, in order to maximise their lifetime reproductive success. In more dense populations, competition will inevitably be heightened, and as a result lifetime reproductive success for some will be limited. Previous work has demonstrated that increased exposure to rivals can have substantial physiological and behavioural impacts in males of a vast range of species, when investing in reproduction (Droge-Young et al., 2016, Gage and Barnard, 1996, García-González and Gomendio, 2004, Gomendio and Roldan, 1991, Kustra et al., 2019, Kiss et al., 2019, Klemme and Firman, 2013, Rowley et al., 2019). *Drosophila* species have emerged as an important model organism for understanding these effects: males of these species are sensitive to the presence of potential competitors and adjust a range of behaviours and reproductive physiological responses accordingly, and in ways that vary among species (Bretman et al., 2009, Fedorka et al., 2011, Garbaczewska et al., 2013, Moatt et al., 2014, Lizé et al., 2012).

Surprisingly, given the greater contribution that variation in their behaviour could make to the fitness of offspring, less research attention has focused on equivalent plastic responses in females (but see Singh and Singh (2014) on density effects on female remating rates, and Battesti et al. (2012) and Sarin and Dukas (2009) on copying behaviours in ovipositing females). *Drosophila* feed on fermenting fallen fruit colonised by yeast (Begon, 1982), and females also use these fallen fruits for laying eggs too, as this is the primary source of nutrients for developing larvae. As a result, these fruit patches have a greater resource value to females, and therefore it is likely that their willingness to compete with rivals for access to these sites would be increased compared to male conspecifics. Here I start to address this knowledge gap, to understand how resource patchiness and exposure to potential competitors impact oviposition responses.

Aggression in females

Female *Drosophila* are aggressive towards other females, exhibiting a range of behaviours similar to those observed among fighting males (Bath et al., 2017, Chapman and Wolfner, 2017, Ueda and Kidokoro, 2002). The observation that aggression occurs between female conspecifics, suggests that females may be equally sensitive to the presence of same-sex rivals as males. However, the context in which this aggression manifests is likely to differ compared to males, given the activating neurones are sexually dimorphic (Schretter et al., 2020). Despite female aggression being common, prior to oviposition females can also show strong social attraction to conspecifics on food patches (Lihoreau et al., 2016), perhaps because of the benefits of shared feeding among larvae (Dombrovski et al., 2017). For this reason, larvae also seek social aggregations to enable them to more easily burrow into fruit

(Durisko and Dukas, 2013, Durisko et al., 2014). There is inevitably a trade-off between these benefits and increasing competition, which can even result in cannibalism (Vijendravarma et al., 2013). This tension between competition and cooperative feeding is to some degree mediated by relatedness, as closely related larvae are more likely to form cooperative feeding aggregations than unrelated larvae (Khodaei and Long, 2020). Nevertheless, under food restriction cannibalism is observed even within inbred laboratory strains with high mean relatedness (Vijendravarma et al., 2013).

Plasticity in oviposition choices

Females are able to respond to varying resource quality and spatial heterogeneity in oviposition sites (Sumethasorn and Turner, 2016, Abbott and Dukas, 2016, Lihoreau et al., 2016). Female *Drosophila* must alter their oviposition choices based on a trade-off between maximising their own fitness, and increasing individual offspring survival rate. Each stage of development (egg, larval instars, pupae and adult) will likely face different nutritional and environmental stresses due to the variation in their potential mobility and ovipositing females should alter their decisions accordingly.

During oviposition females can only assess the level of competition their larvae will face based on the number of existing eggs at a patch, or the number of pheromone markings by conspecifics (Malek and Long, 2020, Tait et al., 2020). They are able to make sophisticated oviposition choices, including assessing not only the nutritional quality of the resource but also the inter-patch substrate, considering potential energetic costs of larval travel (Schwartz et al., 2012). Rohlfs and Hoffmeister (2003) showed that an intermediate larval density was

optimal (here mortality rates were lowest), so to increase their fitness females should try to achieve this optimal larval density.

It seems likely that they may also be sensitive to intra-sexual encounter rate among adults as a proxy for likely future larval competition. However despite the strong evidence for density and encounter rate effects on male behaviour (Churchill et al., 2020, Bretman et al., 2009, Moatt et al., 2014, Fedorka et al., 2011, Price et al., 2012, Hopkins et al., 2019, Garbaczewska et al., 2013), and evidence that females are sensitive to the presence of conspecifics when making oviposition decisions (Malek and Long, 2020, Tait et al., 2020), studies on the effect of female encounter rate on subsequent oviposition behaviour are so far lacking (Gillmeister, 1999).

Aims of this study

Here I test whether females respond to the presence of other females during adulthood by subsequently plastically adjusting their oviposition behaviour according to expected level of competition their larvae might experience. As well as manipulating adult density, I tested to what extent socially induced plasticity interacts with the physical oviposition environment, using two different spatial orientations of oviposition resources: clustered and dispersed. Given that each female lays only one egg at a time (Yang et al., 2008), clustered eggs show evidence of repeated decisions to lay in the same site. I hypothesized that females would lay fewer eggs per patch on small, isolated patches, and more eggs per patch on clustered patches because this better facilitates larval travel between food sources. Furthermore, I expected that maternal oviposition decisions would be mediated by the female's experience of intra-sexual encounter rates prior to egg laying, with females from a high-density

environment investing more in offspring production in the expectation of high future competition among larvae – either by investing more resources per egg (and thus laying fewer eggs overall) or by ovipositing a higher number of less well-provisioned eggs.

5.2 Methods

All fly rearing and experiments were conducted at 25°C in a 12hour light:dark cycle (08:00 – 20:00h GMT), unless otherwise stated. Stock flies (originating from a Canton-S laboratory population) were housed in 40ml vials containing 7ml of a standard agar-based medium (Appendix 1. i). Approximately 25 *D. melanogaster* were held in each vial, and all vials were pooled and randomly redistributed every seven days to minimise any effects of inbreeding, drift, and selective sweeps.

Test flies were removed from parent vials within six hours of eclosion to ensure virginity, and were aspirated under ice anaesthesia. Parent vials were set up with a standardised maximum density to ensure food resources were not limiting – six males and six females were left to breed for 70-98 hours.

Prior experience of competition

Test females were housed in treatment vials for seven days in one of two treatments: singly housed (hereafter "solitary"), or in a group of six females ("grouped").

Copulation behaviours

Females were translocated to a new standard vial for copulation with a standard seven-day old male which had previously been housed alone since eclosion. Courtship and copulation behaviours were observed live, and latency to copulate (time from when pair were first introduced until the male successfully mounts the female) and copulation duration (until pair fully separates) were recorded in seconds.

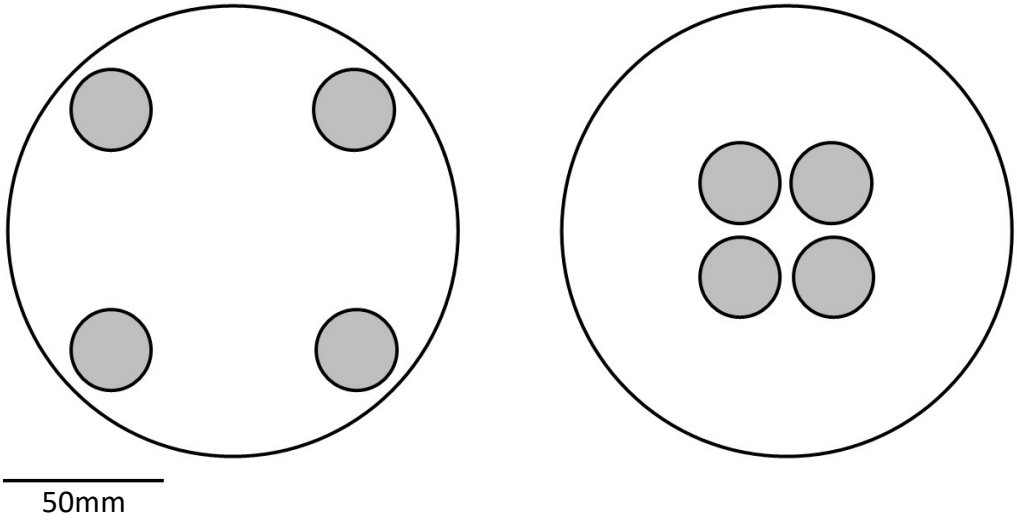
Oviposition distributions

Females that did not copulate within 90 minutes of being introduced to the male were excluded from subsequent (egg laying) stages of the experiment. Females which copulated were transferred to individual egg laying dishes, with oviposition substrate arranged in one of two spatial treatments: dispersed or clustered resources. Petri dishes were 140mm in diameter and contained four patches of agar-based medium (each 22mm in diameter, 7mm depth).

For the dispersed treatment, patches were located at four equidistant points around the circumference of the Petri dish, at an interpatch distance of 100mm (Fig. 35a). In the clustered treatment, patches were arranged in a square in the centre of the Petri dish (Fig. 35b). Given that Drosophila larvae travel at an average of 90µm per second (Heckscher et al., 2012), it would take those on dispersed patches ~18.5 minutes minimum to travel to a different resource patch, compared to less than one minute for those on clustered patches. Each patch was placed 3mm from the edge of the Petri dish, or from other patches, to keep total surface area available for oviposition constant between treatments. The base of each Petri dish was lined with filter paper, to which 10ml of distilled water was added to prevent food patches from drying out.

This arrangement resulted in four treatments: females from solitary and grouped treatments could lay in either dispersed or clustered resource plates in a fully factorial design. Sample sizes were: solitary/clustered resources: 22; solitary/dispersed resources: 22; grouped/clustered resources: 30; grouped/dispersed resources: 29. For the analysis of mating

behaviours only, I had an additional six solitary females for which there is no accompanying egg laying data due to incubator failure.



a. b.

Figure 35. Egg laying treatment enclosures

a. Dispersed resource distribution treatment: food discs located at the edge of the Petri dish, approximately 100mm apart

b. Clustered resource distribution treatment: food discs located in the centre of the Petri dish, with a 3mm gap adjacent discs

Females were left to oviposit eggs for 20±1 hours, before being relocated to a standard vial. Treatment enclosures were placed in three incubators, maintained at 25°C, under constant light for imaging. Each incubator held one Raspberry Pi (www.raspberrypi.org) connected to an 8MP Raspberry Pi Camera module (v2; www.thepihut.com). Frame capture software 'raspistill' was used to capture one image every 10 minutes. For each image, I recorded on which patch of the four food patches the female was found, or if she was not currently on a patch.

Once the female had been removed, egg-laying enclosures were photographed using a digital camera (Panasonic Lumix DMC-FT4) to allow counting of eggs laid per patch.

Fitness

The enclosures containing eggs were returned to the incubator, and after 21 days the emerged adults were counted and sexed. During the 21-day emergence period, the filter paper was replenished with 5ml of water every three to four days.

Immediately after females were removed from the enclosures (20±1 hours after introduction), they were given a further seven days to lay any remaining eggs in a standard vial in order to quantify total fitness, before being removed for wing size measurements to be taken. Number of male and female offspring were counted 14 days later.

Wing size measurements

After mating, males were sacrificed and their left wings were measured to enable an investigation into the impact of body size on their behaviours and overall fitness. This was repeated for females after the eight days allowed for oviposition. Wing measurements were taken from the intersection of the anterior cross vein and longitudinal vein, to the intersection of L3 with the distal wing margin – as shown in Chapter 2 and Partridge et al. (1987). The size difference between mating pairs was also calculated, by subtracting the male's wing length from the female's.

Statistical analysis

All statistical analyses were conducted in R 4. 0. 3 (R Core Team, 2019).

I tested the effects of treatment on response variables using mixed effects models with the appropriate error distribution (binomial error for egg presence/absence, negative binomial error distribution for the over-dispersed egg and offspring number, Gaussian for mating latency and duration) with the functions in packages lmerTest (Kuznetsova et al., 2017), and lme4 (Bates et al., 2015). This approach allowed me to fit vial identity as a random effect to account for shared housing of females in grouped treatment. However, in all models, the variance component for vial was estimated at non-significantly different from zero leading to a singular model fit, so I re-ran these using (generalized) linear models with the appropriate error distribution: binomial for the egg presence/absence data, quasiPoisson error for the egg and offspring number models to account for overdispersion, and Gaussian for mating latency and duration.

I analysed whether the number of patches that a female chose to lay on was influenced by either prior housing or oviposition substrate treatment using Chi-squared tests. Although this does not allow me to treat the effect of shared housing as a random effect, this was not a significantly confounding factor in any of the previous models.

5.3 Results

The impact of prior exposure to competition on female mating behaviours

All females were courted; and all group-housed females and all but 3 out of 60 single-housed females copulated, indicating that prior housing had no effect on whether females received courtship or subsequently copulated.

Group-housed females were >120 seconds slower to copulate than solitary females (solitary: 235 ± 47s; group housed: 373 ± 45s; linear model: $\log_{10}$: copulation latency: $F_{1,104} = 15.97$, $p < 0.001$; Fig. 2). The residuals from this model were non-normal (Shapiro-Wilk test $p = 0.010$) due to outliers in both treatments (Fig 36). Removing all values > 1000s (N = 4) normalized the residuals ($p = 0.820$) and the difference between treatments remained statistically significant ($F_{1,100} = 17.61$, $p < 0.0001$).

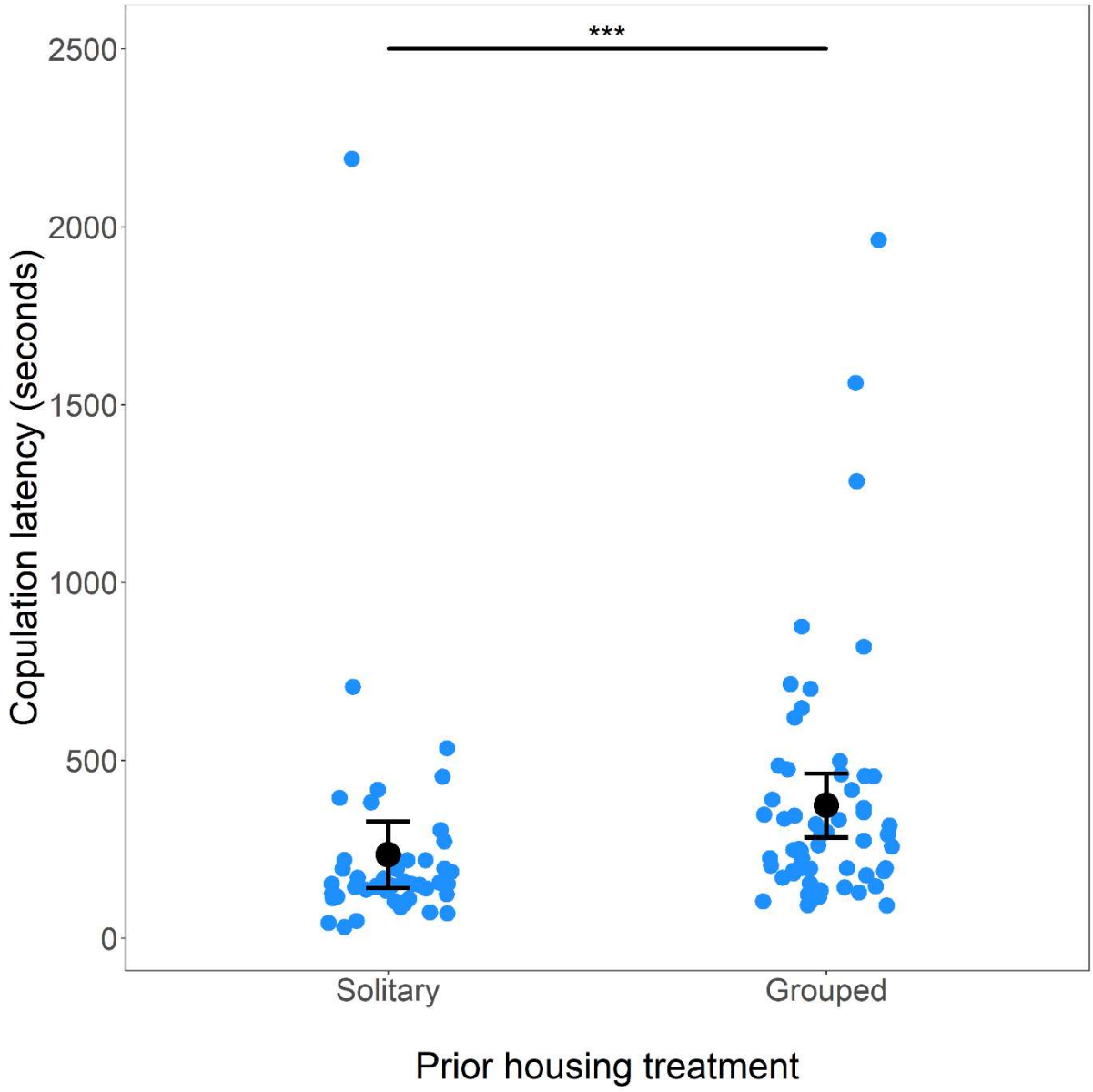


Figure 36. The effect of female prior housing density on latency to copulate

The difference in latency remained statistically significant after removal of the four points with values above 1000s

Note that the analysis was performed on logged data, but untransformed values are presented here

Means (black dot) and 95% confidence intervals of copulation latency are shown in seconds

As well as being slower to start copulating, co-housed females' mating duration was an average of 55 seconds less than that of solitary females (linear model: $\log_{10}$: mating duration:

$F_{1,102}$ = 4.97, p = 0.0255; Fig. 37). This difference remained significant after removal of an outlier in the group-housed group (linear model; $F_{1,101}$ = 4.10, p = 0.0418; Fig. 37).

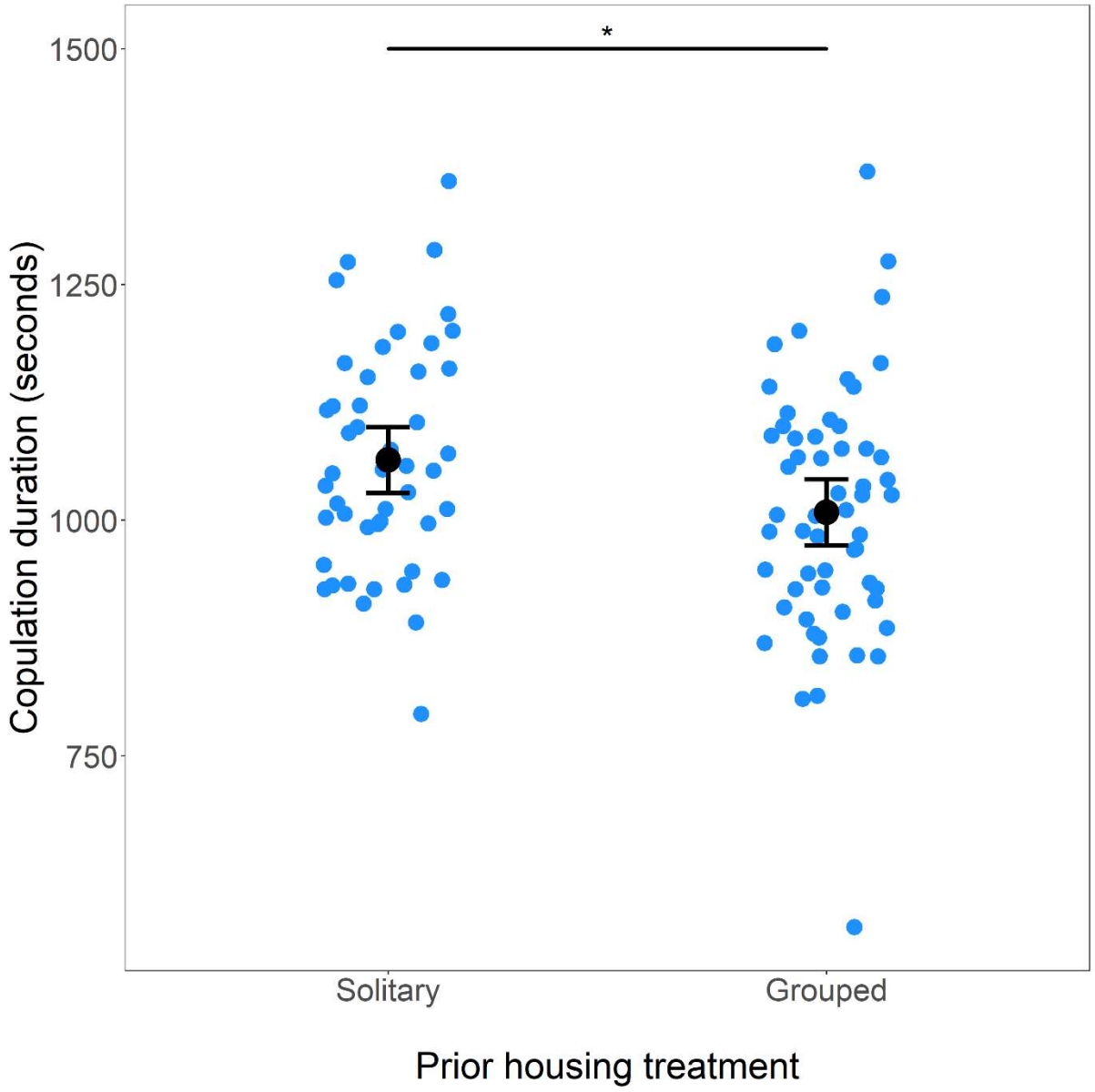


Figure 37. The effect of female prior exposure to competition on copulation duration

The difference in means remains significant after removal of the low outlier in the group-housed treatment

Note that the analysis was performed on logged data, but untransformed values are presented here

Means (black dot) and 95% confidence intervals of copulation duration are shown in seconds

Effects of prior housing density and resource spatial distribution on oviposition and fitness

Female oviposition behaviour could be influenced by two treatment conditions: prior housing (either group-housed or solitary) or current oviposition landscape (either clustered or dispersed food patches). Of these, clustered resources led to a significantly higher proportion of females laying in the 18 – 20 hours given, than did dispersed resources (general linear model with binomial errors; $X^2 = 4.88$, $p = 0.0272$; solitary odds ratio = 1.429, group-housed odds ratio = 1.269; Table 3), but no significant interaction between these variables influenced whether eggs were laid or not ($X^2 = 1.24$, $p = 0.265$), and although the percentage of females laying eggs was lower among group-housed females, prior housing treatment had no significant effect ($X^2 = 2.74$, $p = 0.098$; Table 3).

Table 3. The effect of competition and resource distribution on the percentage of females who laid at least one egg

		Prior housing treatment	
		Solitary	Group housed
Oviposition resource distribution	Clustered	90.9%	70.0%
	Dispersed	63.6%	55.5%

I found the opposite effect of these two treatments on number of eggs laid (excluding females that did not lay; Fig. 38). Prior housing significantly influenced clutch size, with group-housed females laying 22% fewer eggs than those from a solitary background (solitary: 18 ± 2 eggs; group housed: 14 ± 1 eggs; generalized linear model with quasiPoisson errors $F_{1,69} = 5.106$, $p = 0.027$), but in this instance no significant effect of food patchiness was found ($F_{1,68} = 0.073$, $p = 0.788$). Again, there was no interaction between treatments ($F_{1,67} = 0.076$, $p = 0.783$) in this model.

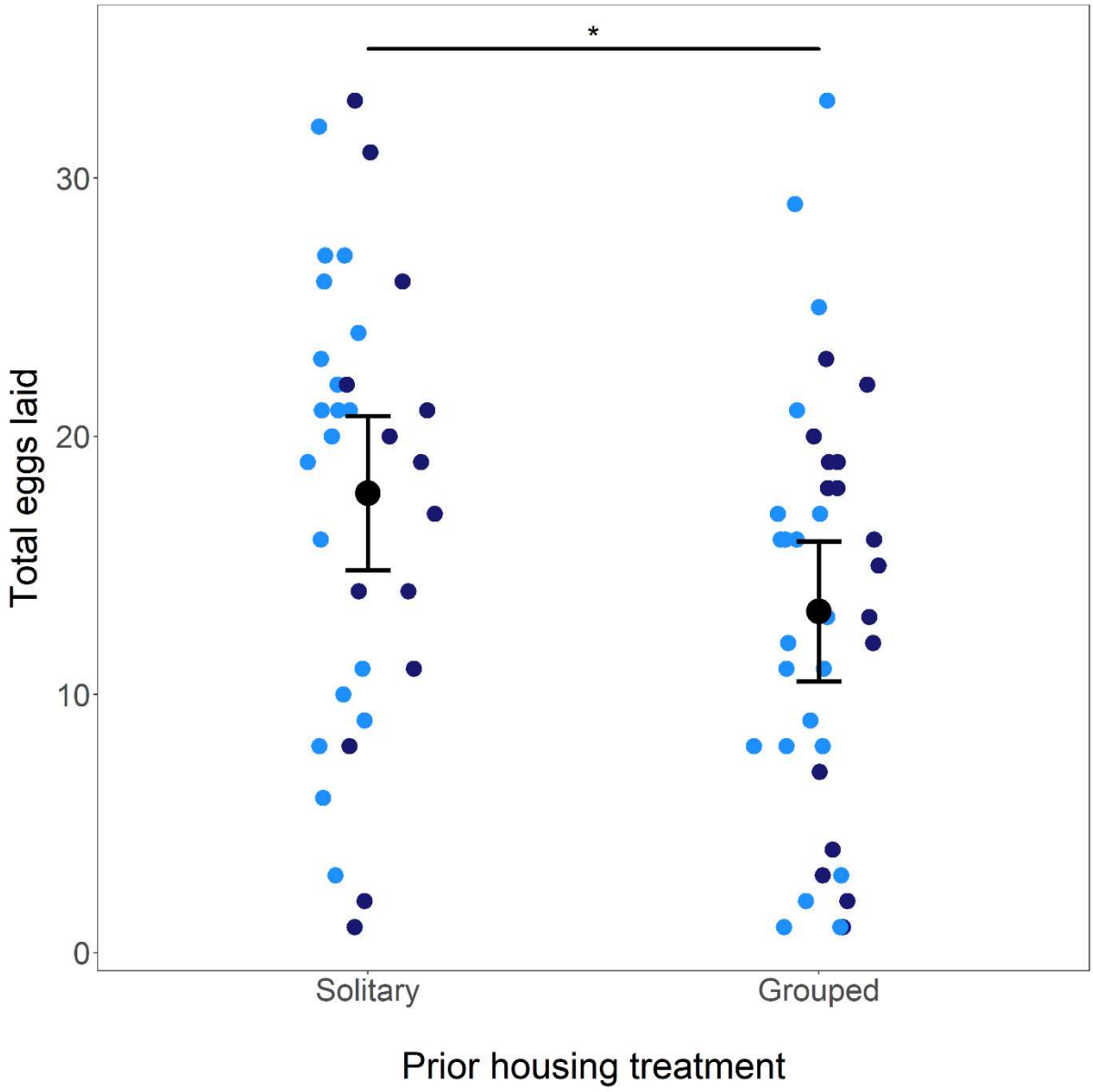


Figure 38. The effect of prior housing density on the total number of eggs laid on patches

Means (black dot) and 95% confidence intervals are shown

Light blue – clustered oviposition resources

Dark blue – dispersed oviposition resources

Surprisingly, these effects of prior housing and egg-laying resource distribution did not lead to any difference between treatments in the proportion of eggs that survived to adulthood (competition: $F_{1,34} = 0.115$, $p = 0.737$; oviposition substrate: $F_{1,63} = 0.0450$, $p = 0.833$;

treatment interaction: $F_{1,63}$ = 3.59, p = 0.0629). Neither treatment, nor the interaction, affected the sex ratio of offspring produced ($F_{1,85}$ < 2.145, p > 0.147).

Oviposition distribution and fitness amongst varying resource distributions

Females on dispersed resources spent less time on the food patches (34.2% of time on resources) than those on clustered resources (56.1%: raw number of records on food patches, linear model: $F_{1,99}$ = 18.64, p << 0.001; Fig. 39). Prior housing treatment had no equivalent effect ($F_{1,99}$ = 0.321, p = 0.572), and there was no interaction between the two treatments ($F_{1,99}$ = 0.155, p = 0.695). Females housed on dispersed oviposition resources spent less time exploring the resource patches (39 ± 4 images in which the female was on a resource patch) than those housed in enclosures where the resources were clustered (64 ± 4 observations on images).

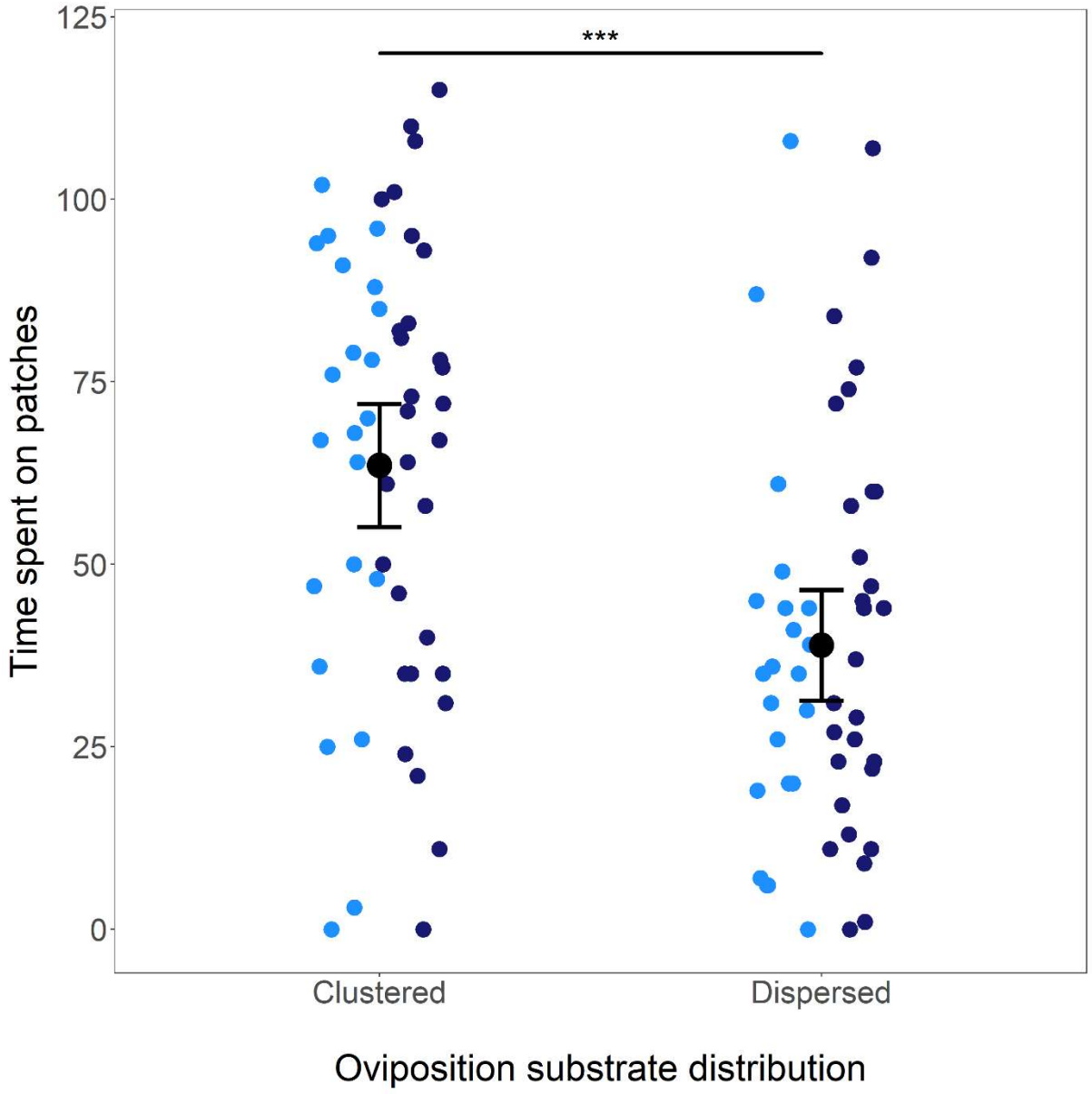


Figure 39. The effect of the spatial distribution of oviposition resources on the time females spent exploring those resources (measured as the number of images in which the female was observed on a food patch)
Means (black dot) and 95% confidence intervals are shown
Light blue – solitary females
Dark blue – grouped females

There was a significant positive correlation between the amount of time focal females spent on oviposition resource patches and the number of eggs that were laid on these oviposition patches (all $p << 0.001$; Fig. 40).

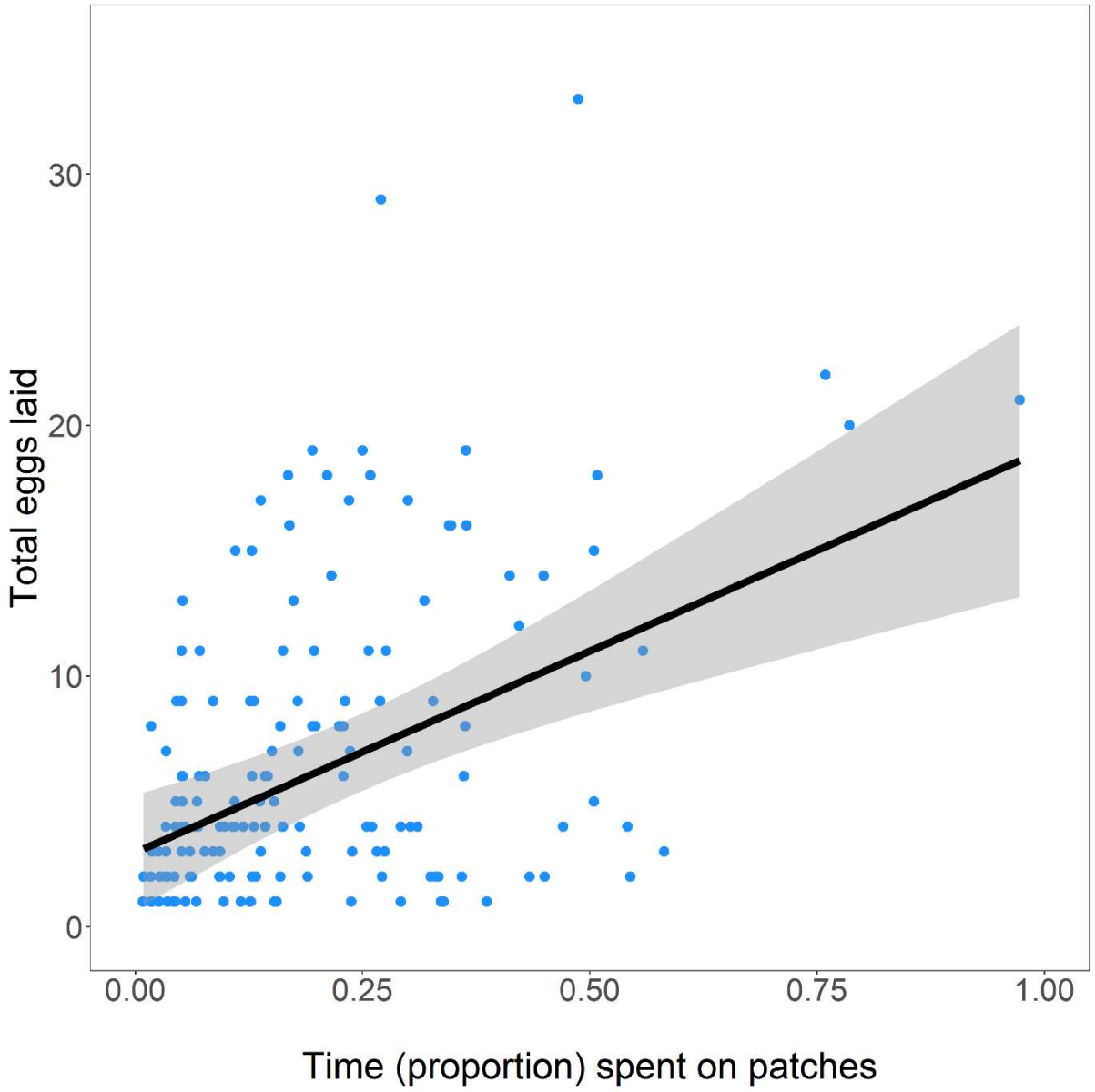


Figure 40. The correlation between the time females spent on oviposition patches and the number of eggs they oviposited on these patches

Line of best fit shown with accompanying standard error\

A similar pattern is observed in the effect on the number of patches oviposited on. Females laid eggs on more of the available patches when these patches were clustered than when they were dispersed ($\bar{x}$ = 0.92; mdn = 1.0; $\chi^2 = 32.06$, $p < 0.001$; Fig. 41). Again, there was no

effect of prior housing density on the number of patches on which eggs were observed ($\bar{x}$ = 2.31; mdn = 2.5; χ^2 = 5.44, p = 0.245).

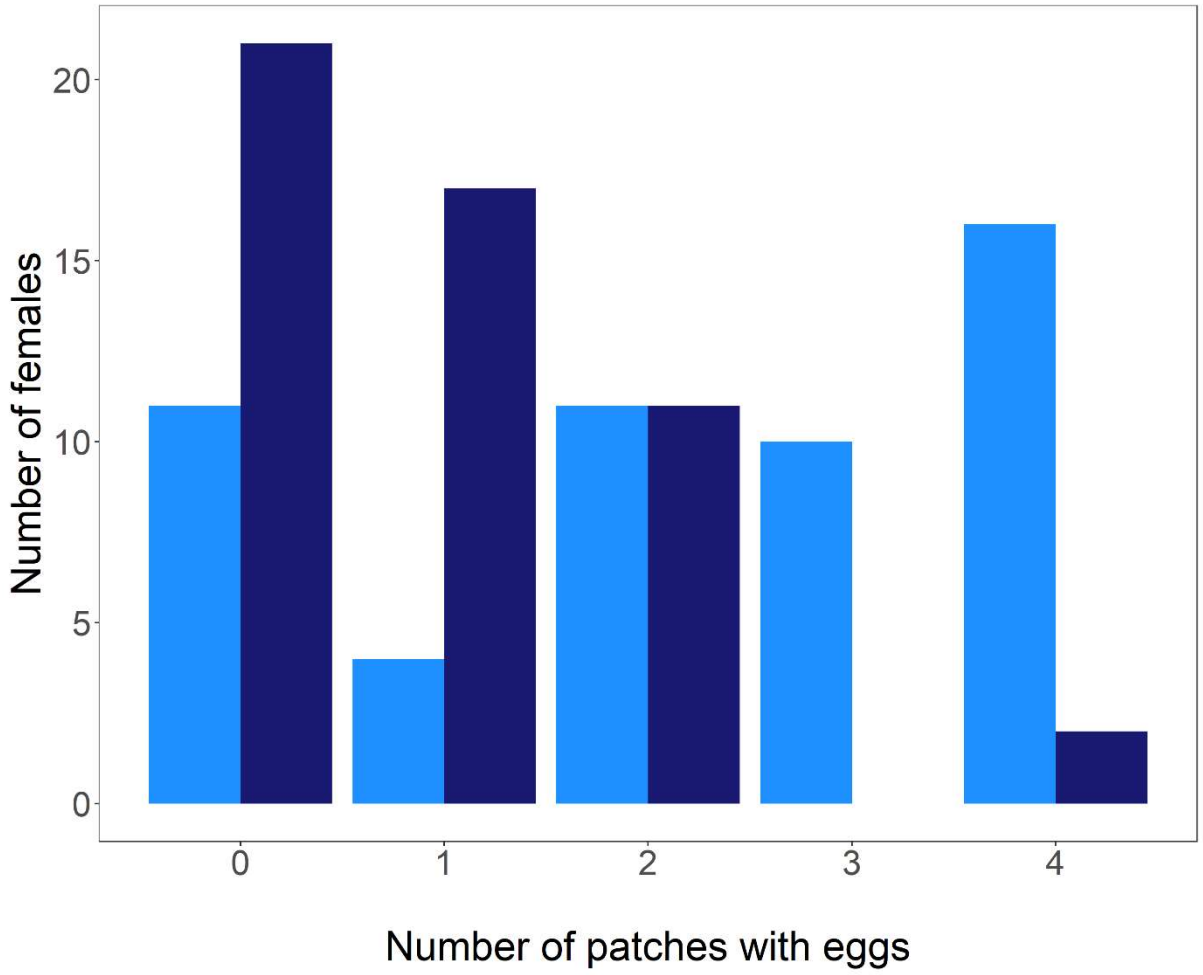


Figure 41. The number of females which laid on either zero, one, two, three or all four available oviposition patches in two different spatial arrangements

Light blue – clustered oviposition resources

Dark blue – dispersed oviposition resources

There was no effect of the number of patches that a female chose to lay on, on the number of offspring produced (χ^2 = 83.4, d.f. = 42, p = 0.910; Fig. 42).

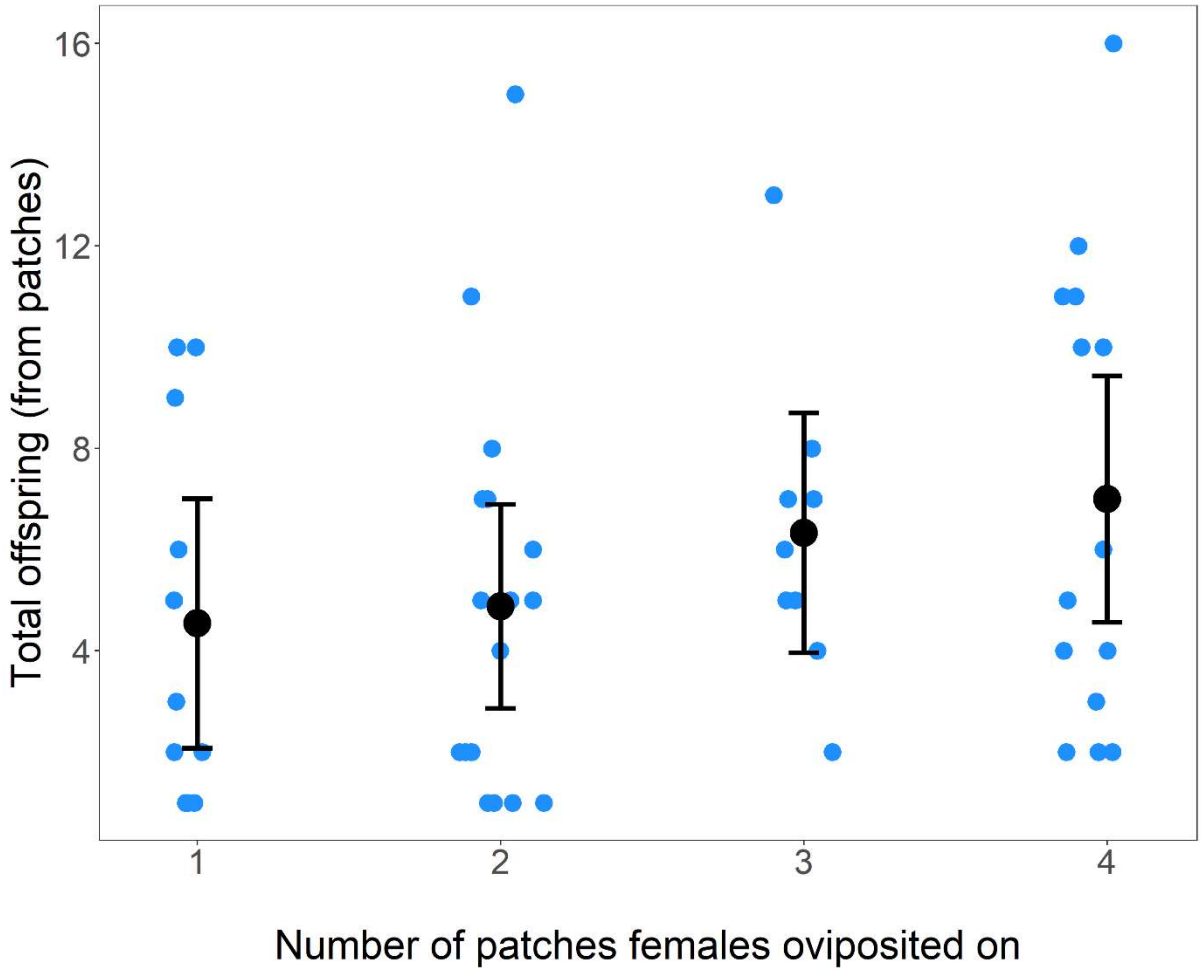


Figure 42. The number of patches oviposited on by females and the number of adult offspring that successfully emerged from those patches

Means (black dot) and 95% confidence intervals are shown

Oviposition distribution on individual resource patches

Across all treatments, females were more likely to lay on the edge of patches than on the top surface (sum of eggs on sides vs. top of 4 food patches; paired t-test, $t = -6.426$, $p < 0.001$; Fig. 43); and this pattern was consistent within each of the 4 treatment combinations (all $p < 0.013$).

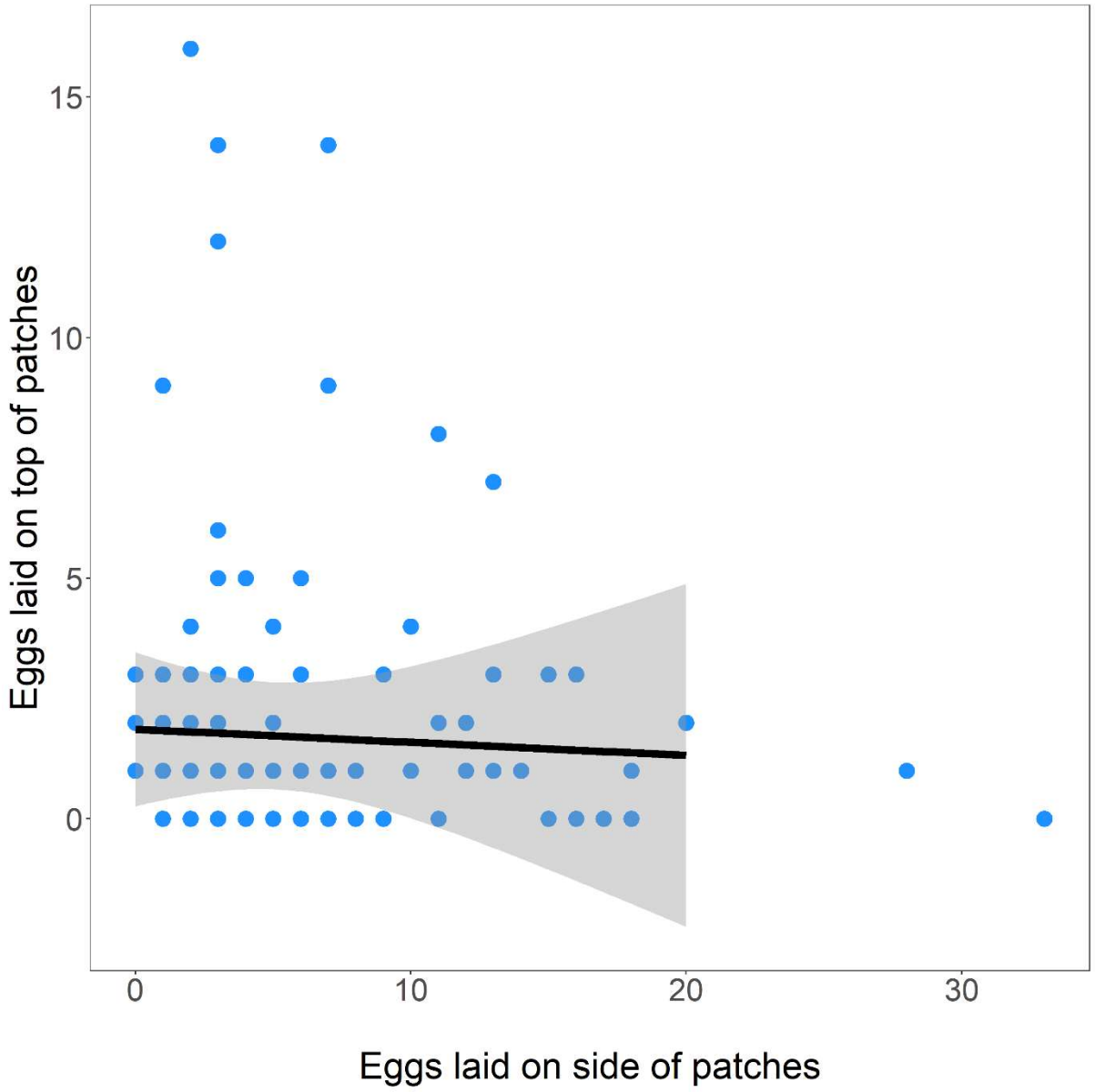


Figure 43. The relationship between the numbers of eggs oviposited on the tops and the sides and the patches Line of best fit shown with accompanying standard error

Post-treatment offspring production

To ensure that these results were not skewed due to females who have previously laid on patches ovipositing all of their fertilised eggs, I tested the effect of treatment on subsequent egg production. Of the 101 females given the opportunity to lay in both Petri dishes and vials,

70 laid in both, 10 laid in neither, 20 laid none in the Petri dishes but did lay in the vials, and only one laid in the Petri dishes but none in the vials. The probability of laying in vials was thus significantly positively influenced by whether or not eggs had been laid in the Petri dish (GLM with binomial errors: $\chi^2 = 20.8$, $p < 0.001$), but not by either treatment (both $p > 0.512$). The number of offspring produced across both Petri dish and vial was not significantly affected by either prior housing density ($\chi^2 = 14.50$, $p = 0.305$) nor resource distribution in the Petri dish ($\chi^2 = 14.22$, $p = 0.309$).

5.4 Discussion

The impact of prior exposure to density on mating behaviours

Females that had been housed in groups prior to mating took longer to start copulating and copulated for a shorter duration than females maintained in solitude since emergence. Assuming both copulation latency and duration have at least an element of female control (Spieth, 1974, Mazzi et al., 2009), these behaviours suggest a greater reluctance to mate among females from a group housed background. This matches findings from previous studies, which demonstrate that females are often choosier in higher-density mixed-sex populations (Lehmann, 2007, Atwell and Wagner, 2014, Willis et al., 2011, Scott et al., 2020), where the risks of remaining unmated are lower and there is less pressure to mate with the first available male. Here, I interpret the delayed start of copulation and the shorter mating duration as indicators of choosiness – females from a grouped background showed lower willingness to mate with the first available male, and mated with him for less time, in expectation of future mating opportunities. While females in the grouped treatment encountered only other females, they may use female encounter rate as evidence of generally higher population density, and hence a greater likelihood of encountering multiple males. However, the opposite expectation is also plausible – higher female encounter rate without encountering males may lead to an expectation of low male presence. The observed decrease in willingness to copulate at higher female densities could instead be due to a trade-off with previously increased energy expenditure in social behaviours experienced by group-housed females, or group-housed females could be slower to identify male conspecifics than those that have not previously been exposed to consexuals.

It is intriguing that these density effects on females are the opposite to those demonstrated for males exposed to consexual rivals prior to mating, which stimulates more extended copulation durations in *Drosophila melanogaster* and a number of other species (Bretman et al., 2009, Flay et al., 2009, García-González and Gomendio, 2004, Klemme and Firman, 2013). Male responses are interpreted as a reaction to a perceived increased risk of sperm competition, which males can best counteract by mating for longer – perhaps to increase the quantity of sperm transferred (Engqvist and Sauer, 2003, Simmons and Parker, 1992), but also possibly as a form of mate guarding (Vitta and Lorenzo, 2009). Because my measure of mating latency includes the time taken for males to initiate courtship, variation in mating latency might also be influenced by males' reduced willingness to court females from group housed backgrounds. Although there is evidence for females influencing copulation duration, it is clear that males can also affect this trait. So, an alternative explanation for the increased mating latency and reduced copulation duration observed is that this variability is due to male rather than female behaviour. Males paired with previously group-housed females may have detected apparent high density of other females via pheromones remaining on the focal female, given that males are known to be sensitive to the pheromones of other males carried on females (Friberg, 2006). This means that although the responses of males to female density have not yet been explored in this species, the lengthened latency to copulate and subsequent shortened copulation duration could be due to males anticipating additional mating opportunities, influencing how much they invest in the focal female.

Effects of prior housing density and resource spatial distribution on oviposition decisions and subsequent fitness effects

A higher proportion of females housed on clustered resources laid eggs, compared to those housed on dispersed oviposition resources. Those on clustered resources also laid on more of the available patches than those on dispersed resources. Clustered resources reduce the search time for females, meaning that they have more time available to lay eggs. This is supported by the data on the time spent on patches: females on the dispersed medium spent less time on patches than females housed on clustered resources. However, the observation that some females did not lay at all, particularly on the dispersed patches, suggests that dispersed patches may also be perceived as a less valuable resource than clustered patches. This may arise from the fact that females consider the degree to which larvae will need to travel when choosing oviposition sites (Schwartz et al., 2012), where clustered patches better facilitate social aggregation in larvae, and travel between patches, perhaps allowing for more efficient cooperative feeding (Dombrovski et al., 2017, Khodaei and Long, 2020, Rohlfs and Hoffmeister, 2003).

There may also be physical environmental explanations for the difference in laying success on clustered versus dispersed resources. Like others, I found that females laid more eggs on the edge of the resource patches than on the top surface (Chess and Ringo, 1985, Moore, 1952). Although I did not control for the fact that patch side area is greater than patch top area, the total area available to lay upon are the same under both resource distributions. The number of sheltered edges is increased in clustered resources, and as *Drosophila* preferentially lay on the edges of resources, this increase in sheltered edges could be driving the preference for

clustered egg-laying patches. Additionally, small patches of food are likely to dry out much more rapidly than larger patches, and clustering may help to mitigate this effect as well. A desiccated food resource will inevitably limit larval survival, providing another possible explanation for females preferring this arrangement of patches. Further work is necessary to discriminate between these explanations for female behaviour.

While the distribution of egg-laying patches influenced the probability of eggs being laid and the number of patches laid on, this physical environment had no effect on the number of eggs laid. By contrast, I found that prior housing treatment was important here: group housed females laid fewer eggs irrespective of egg-laying environment. Females engage in energetically expensive aggressive interactions with their consexual rivals in this species (Bath et al., 2017, Ueda and Kidokoro, 2002). These aggressive interactions could lead to a trade-off in which group-housed females have less energy available for oviposition. In addition, when females oviposit in the presence of rivals, they copy their oviposition behaviours to reduce sampling costs (Malek and Long, 2020). In the absence of such information, females may have been slower to choose where to deposit their clutch. Finally, females from the group housed treatment are likely to anticipate a high level of competition for their offspring. This may have caused them to reduce the size of the clutch – perhaps increasing the size of eggs, or the quality of provision, to improve their competitive advantage. However, although I did not measure egg size, I found no evidence to suggest there was a trade-off in the quality of eggs laid, or female investment per egg, as I found no treatment effects on the number of successfully eclosed adults from these eggs. This observed decrease in clutch size suggests the alternative prediction of an increased clutch size under high density was not true here. It is also possible that any fitness effects of egg investment were absent because of the benign

laboratory conditions (e.g., *ad libitum* food and constant temperature). Future work should test for fitness effects in a more stressful environment and over subsequent generations.

Fitness effects may also have not been detected because this experimental design required the removal of the females before they had completed oviposition of all fertilised eggs: this was necessary so that the location of oviposited eggs could be recorded, and larvae hatch after 22-24 hours at ~25°C (Fernández-Moreno et al., 2007, Markow et al., 2009). But if females had been given the opportunity to continue ovipositing on the patchy resources, it is quite plausible that their choice of egg location could have impacted overall fitness. Equally importantly, a benefit of clustering oviposition sites is likely to arise in the presence of non-related conspecific larvae, meaning that fitness benefits may have been observed had other females been given the opportunity to lay on the same resources.

In this study I have demonstrated that female density has significant effects on mating and egg-laying behaviour. Females from group-housed conditions are slower to accept mating and mate for less time, which I suggest is related to future opportunities to mate, and lay fewer eggs, which is likely to be due to anticipated competition for their larvae, which mothers infer from their own conspecific density as adults. Surprisingly however, although the physical arrangement of egg-laying patches also has significant effects on oviposition behaviour, with clustered resources being preferred over dispersed ones, there is no interaction between this physical stimulus and the social stimulus of perceived population density. While reproduction-related effects of density are already well known in males, equivalent study in females has so far been lacking. I have demonstrated that social environment has profound effects on females too. The way that the social environment affects both sexes simultaneously

should be of particular interest in the future, especially given the more pivotal role of females in the demography, persistence and evolvability of populations.

Chapter 6: **Interfemale competition versus cooperation during oviposition**

What do you call a fly that can dance?

A jitterbug

Abstract

Individuals show highly plastic physiological and behavioural responses, when faced with changing physical and social environments. These responses have been extensively documented in males, but responses in females have been comparatively less well-studied despite their greater impact on progeny and fitness, although new studies are now beginning to emerge. I have previously shown that females plastically adjust their oviposition behaviours in response to exposure to competition prior to laying and spatial heterogeneity in laying sites, but still little is known about how presence of consexuals whilst ovipositing may affect their oviposition decisions. Unlike in males, presence of conspecifics can be beneficial, due to the potential for cooperation. Larvae benefit from cooperative feeding as it is more efficient, however when resources become limiting, they can resort to cannibalism. Given that females have been shown to consider larval travel in their oviposition decisions, it is likely that they will choose sites with potential for this optimal larval foraging density for their future offspring. Here I manipulate resource patchiness and competition to uncover their impacts on females' oviposition decisions and subsequent fitness. I show that exposure to rival females (whether mated or not), decreases the distance between the sites where females opted to lay their eggs – as does dispersed compared to aggregated resources. These changes to oviposition decisions are likely to have long-term fitness effects for the females, and thus should be the focus of future investigations.

6.1 Introduction

Female response to consexuals

The spatial and temporal distribution of resources determines local population density and thus intraspecific encounter rates (Emlen and Oring, 1977) – and higher encounter rates results in increased potential competition. The optimal responses for individuals to increase their lifetime reproductive success will differ as the level of competition changes (Maynard Smith, 1979), so individuals must make plastic physiological and behavioural adjustments in response to this.

It has widely been established that density and competition alter reproductive responses and subsequent fitness in males (Friberg, 2006, Bretman et al., 2009, Firman et al., 2018, García-González and Gomendio, 2004, Flay et al., 2009). But given that females often have more control over investment into offspring, it is surprising that they have received little attention compared to males. For example, in many species the female has control over paternity (Birkhead and Møller, 1993, Rodriguez-Enriquez et al., 2013, Ward, 1993) and number and sex ratio of offspring via manipulation of ejaculates (Elgar et al., 2000, Passera et al., 2001, Sato and Karino, 2010). In addition, females usually choose the location of oviposition and nesting sites which can influence the offspring's potential nutritional environment, and risk of competition and predation.

Oviposition decisions

Female *Drosophila* exhibit aggressive behaviours towards consexuals (Bath et al., 2017, Chapman and Wolfner, 2017, Ueda and Kidokoro, 2002), and this likely has fitness

consequences for those competing females. When exposed to potential competition with consexuals, females lay fewer eggs (Churchill et al., 2021). However, females also have a social attraction to conspecifics on oviposition resources (Lihoreau et al., 2016). This could be due to the efficiency benefits of oviposition copying behaviours (Sarin and Dukas, 2009, Battesti et al., 2012), or perhaps due to consideration of the benefits of shared feeding among larvae (Dombrovski et al., 2017) although any such benefit must outweigh the effects of increased competition. There is trade-off between the consequences of aggression, and the benefits of cooperation, and this is likely impacted by varying spatial distribution of resources.

Female *Drosophila* in natural populations feed and lay upon fermenting fallen fruits, an inherently patchy and ephemeral environment (Begon, 1982). When resources are clustered, the potential for both competition and cooperation are heightened, due to an increased local population density and increased encounter rates (Emlen and Oring, 1977). Furthermore, resource searching costs are reduced as the distance between the resources decreases (Norberg, 1977), and so females are able to spend more time at egg-laying sites and thus lay more eggs (Chapter 5; Churchill et al., 2021).

In Chapter 5, I showed that prior exposure to rivals influenced the number of eggs that females oviposited, with females exposed to competition laying fewer eggs. This demonstrates the importance of understanding the impact of the social environment on female fitness, yet we still do not know how competition whilst ovipositing may impact laying location decision and subsequent fitness in *Drosophila*. This question has been explored a little in other invertebrates: e.g., number of eggs produced decreases with increasing competition in beetles (Halliday et al., 2019), and parasitoid wasps accepted potential hosts

more readily when searching in solitude compared to those searching in pairs (Visser, 1995). In this study I address this knowledge gap: I investigate how the presence of a rival during oviposition affects oviposition decisions, using mated and virgin females as potential competitors. Despite not being fertilised, virgin females still oviposit so could compete for oviposition sites; although they oviposit less frequently than their mated conspecifics (Menon et al., 2014) so motivation to compete is likely reduced. Regardless of mating status, female *Drosophila* can only lay one egg at a time (Yang et al., 2008), so where eggs are clustered this is evidence of repeated decision to lay at the same site, and thus is evidence of an oviposition preference.

As well as manipulating the social environment that ovipositing females were exposed to, I also tested how varying physical oviposition environments affects females' decisions, by comparing females allowed to lay on clustered and dispersed resources (repeating the approach used in Chapter 5). I hypothesised that females that were ovipositing in solitude would lay their eggs in closer proximity on fewer patches, to ensure their offspring benefit from shared feeding. This effect on solitary females would likely be exaggerated in those on dispersed resources (a pattern that was also found in (Chapter 5; Churchill et al., 2021)), to avoid the energetic costs of increased larval travel – something females have been shown to consider in their oviposition decisions (Schwartz et al., 2012).

6.2 Methods

All *D. melanogaster* husbandry and experiments were conducted at 25°C on a 12hour light:dark cycle (08:00 – 20:00h GMT), unless otherwise stated. Stock flies originated from a LHm laboratory stock population, and were housed in 40ml vials with 7ml of a standard agar-based medium (Appendix 1. i). Around 20-30 flies were held in each vial, and all vials were pooled and randomly redistributed weekly, to minimise effects on inbreeding, drift and selective sweeps within vials.

Parent generation vials with a standardised maximum density were set up to ensure food resources were not limiting – six males and six females were housed in each vial for three to four days. Test flies were collected from these vials within six hours of eclosion (to ensure virginity), and immediately transferred into treatment conditions under light ice anaesthesia.

Egg marking

Unmarked test females were housed in solitude in standard vials, for three days. Marked females were housed for three days in vials with 7ml of agar mixed with Rhodamine B fluorescent dye powder to 0.04M (Appendix 1. iv, Kamimura (2007)). Eggs laid by females that had ingested this agar fluoresced under a light wavelength of 556 nm allowing them to be differentiated from eggs laid by females housed on standard agar.

As others have shown (Marking, 1969, Alford et al., 2009), Rhodamine B had a negative impact on longevity: females fed continuously on Rhodamine B agar lived on average for 28 days less than standard-fed females (Rhodamine B: $\bar{x}$ = 49.8 days; standard: $\bar{x}$ = 78.2 days; linear model:

$F_{1,17} = 13.3$, $p = 0.00198$; Appendix 4). Although they were kept on Rhodamine B agar for only 3 days in this experiment, and all data was collected before females reached eight days old, I controlled for negative effects on health by balancing the Rhodamine B diet across all six treatments.

Competition and oviposition distributions

Three-day old females were paired with a standard seven-day old male, except for those being used as virgin competitors. Any that successfully mated within 120 minutes were transferred to one of the six following treatments: solitary, virgin competition, and mated competition, each housed on either clustered or dispersed oviposition resources. As noted above, half of the focal flies in each of the six treatments were fed Rhodamine B-stained agar diet, and half standard agar diet. Final sample sizes were 24 in each of the six treatments (12 per diet).

The six treatments were set up in 140mm diameter Petri dishes, each of which contained four patches of agar to allow for oviposition: each patch was 8.8mm in diameter and 7mm in depth. For the dispersed treatment, patches were located at four equidistant points around the circumference of the Petri dish, at an interpatch distance of 126mm (Fig. 44a). In the clustered treatment, patches were arranged in a square in the centre of the Petri dish (Fig. 44b). Each patch was placed 3mm from the edge of the Petri dish, or from other patches, to keep total surface area available for oviposition constant between treatments.

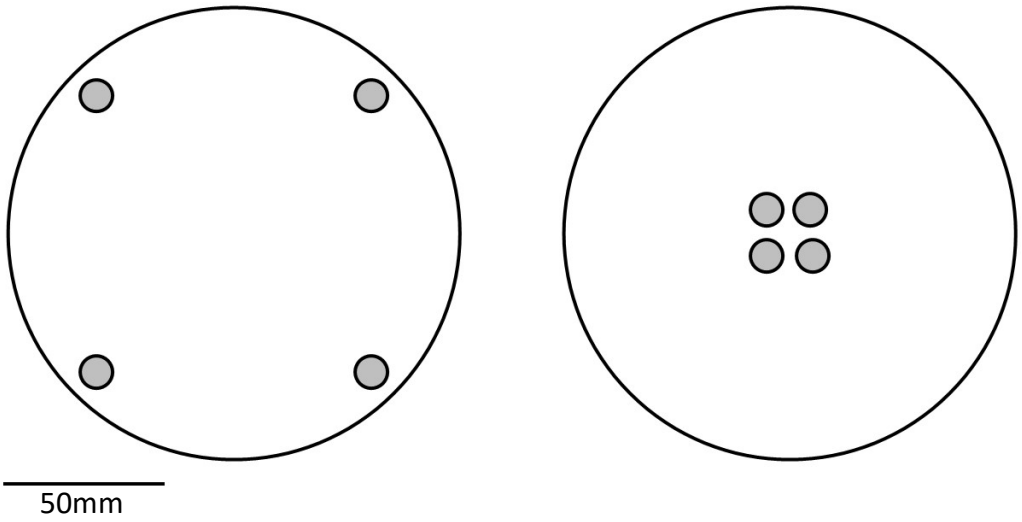


Figure 44. Egg laying treatment enclosures

a. Dispersed resource distribution treatment: food discs located at four equidistant points around the circumference of the Petri dish

b. Clustered resource distribution treatment: food discs located in a square arrangement in the centre of the Petri dish

Egg identification

Females were left to oviposit eggs for 18 – 20 hours in incubators at 25°C, under constant light for imaging. Enclosures containing two mated females were placed under an Imaging Source camera (DFK 33UX226) fixed overhead, and IC Capture software (The Imaging Source, Germany) was used to collect images every three minutes. For each image I recorded how many females were located on a patch, and if both were present on the same oviposition patch. Note that it was not possible to identify diet treatment from the photographs.

Once all females had been removed, patches were photographed using a digital camera (Panasonic Lumix DMC-FT4) to allow the number of eggs on each patch to be counted. Each egg was then viewed under a high-powered fluorescence microscope (Nikon with light

wavelength 480 – 600nm), and the female that laid each egg was identified via its fluorescence.

After testing, all individuals were frozen to -18°C, and their left wings sizes were measured – as described in Chapter 2 and Partridge et al. (1987).

Statistical analysis

All statistical analyses were performed using R 4. 0. 3 (R Core Team, 2019). I tested the effects of treatment on response variables using generalised linear models with the appropriate error distribution (binomial error for egg presence/absence, quasiPoisson error for the egg number models to account for overdispersion, and ANCOVAs for the proportion of eggs laid per patch).

I used one-sample and two-sample T tests to analyse the difference in time the two females spent simultaneously exploring the same patch versus different laying resource patches, and I analysed whether the number of patches that a female chose to lay on was influenced by either oviposition substrate treatment or competition type using Chi-squared tests.

6.3 Results

Effects of competition and resource spatial distribution on egg production

Not all females laid eggs (Table 4): likelihood to lay was not affected by spatial distribution of patches (general linear model with binomial errors; $X^2 = 0.139$, $p = 0.709$), or competition ($X^2 = 0.301$, $p = 0.860$). This contrasts with the result reported in Chapter 5, where a higher proportion of females laid when ovipositing on clustered resources compared to dispersed resources.

Table 4. The number of focal females that successfully oviposited

Competitor eggs were not included in this count

Numbers in bold show the overall percentage, additional numbers show unmarked (U) and Rhodamine B marked (R) percentages

		Competition		
		Solitary	Virgin	Mated
Oviposition resource distribution	Clustered	**83.3%**	**91.7%**	**79.2%**
		U: 100%	U: 83.3%	U: 91.7%
		R: 66.7%	R: 100%	R: 66.7%
	Dispersed	**83.3%**	**79.2%**	**83.3%**
		U: 100%	U: 83.3%	U: 83.3%
		R: 66.7%	R: 75.0%	R: 83.3%

In females that did lay, the number of eggs they laid was not affected by current level of competition (generalized linear model with quasiPoisson errors; $F_{2,117} = 479$, $p = 0.691$) or resource distribution ($F_{1,116} = 474$, $p = 0.199$; Fig. 45).

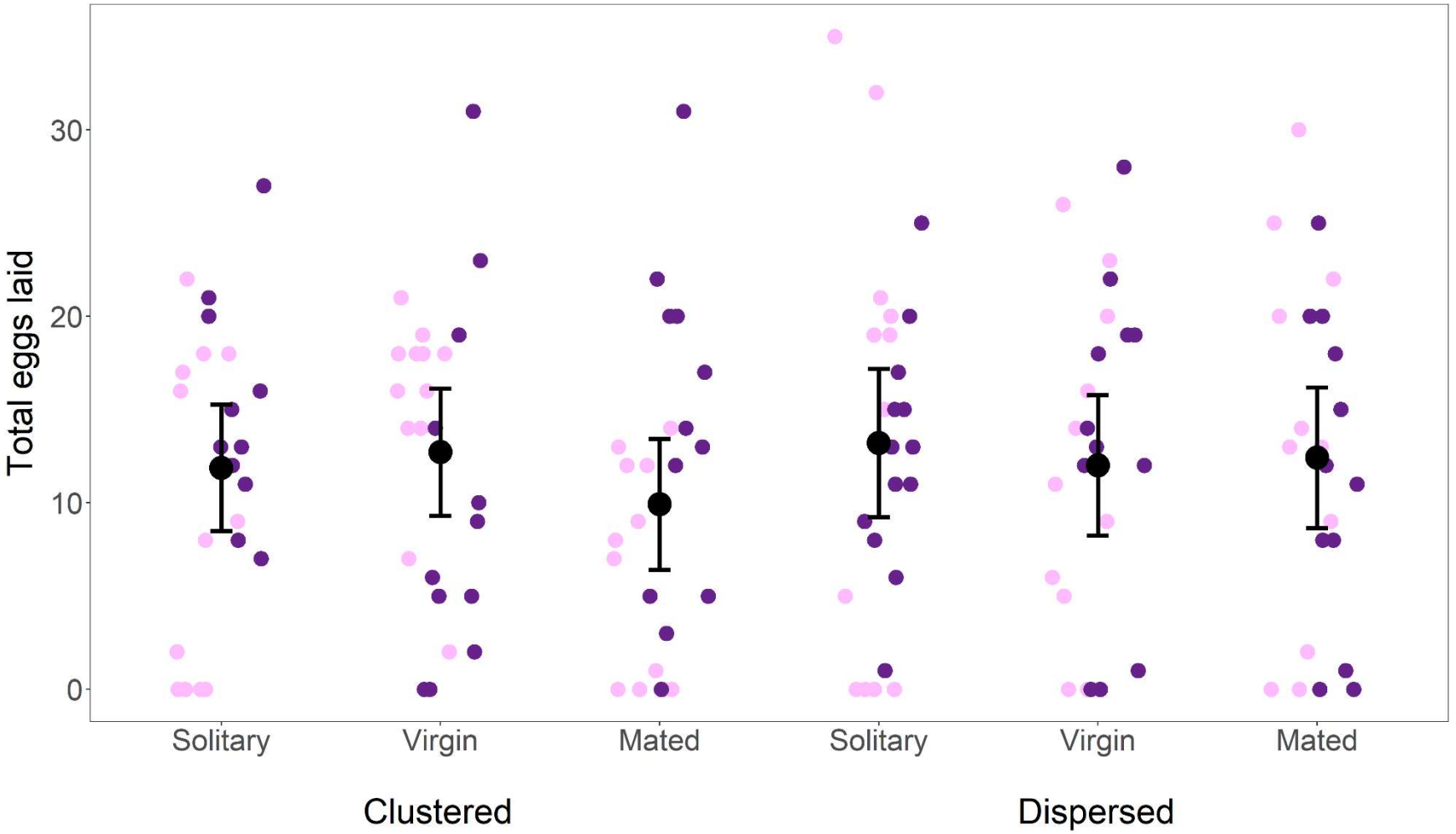


Figure 45. Total number of eggs laid in varying resource distributions and competition

Black dots show means, with accompanying 95% confidence intervals

Pink – Rhodamine B marked

Purple – Unmarked

The proportion of eggs laid on the patch with the highest number of eggs, in other words the degree to which females concentrated their egg laying on a single patch, was significantly affected by competition ($F_{2,116} = 4.77$, $p = 0.0102$; Fig. 46). Post-hoc tests showed that females exposed to competition laid a significantly higher proportion of their eggs on one patch than those that laid in solitude (solitude vs. virgin competitors: $F_{1,78} = 7.84$, $p = 0.00642$; solitude vs. mated competitors: $F_{1,76} = 6.37$, $p = 0.0137$).

The proportion of eggs laid on the patch with the highest number of eggs was also significantly higher when females were ovipositing on dispersed resources compared to clustered ($F_{1,116} = 71.1$, $p << 0.001$).

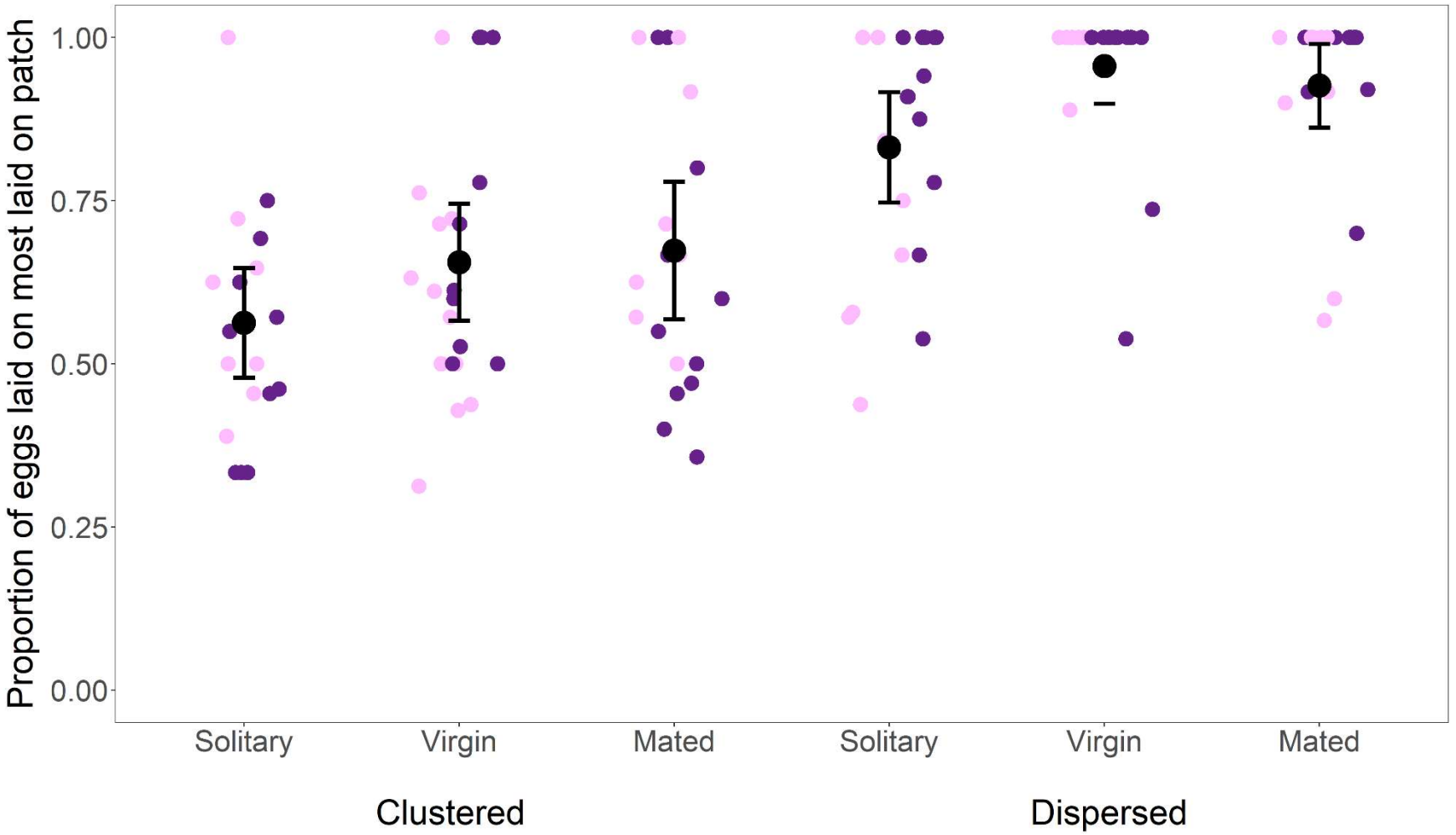


Figure 46. The effect of competition and resource distribution on the proportion of eggs laid on the highest laid-on patch

Black dots show means, with accompanying 95% confidence intervals

Pink – Rhodamine B marked

Purple – Unmarked

Oviposition decisions in response to competition

The number of eggs that competing females laid did not affect the number of eggs laid by the focal female (generalized linear model with quasiPoisson errors; $F_{1,94} = 744$, $p = 0.973$; Fig. 47).

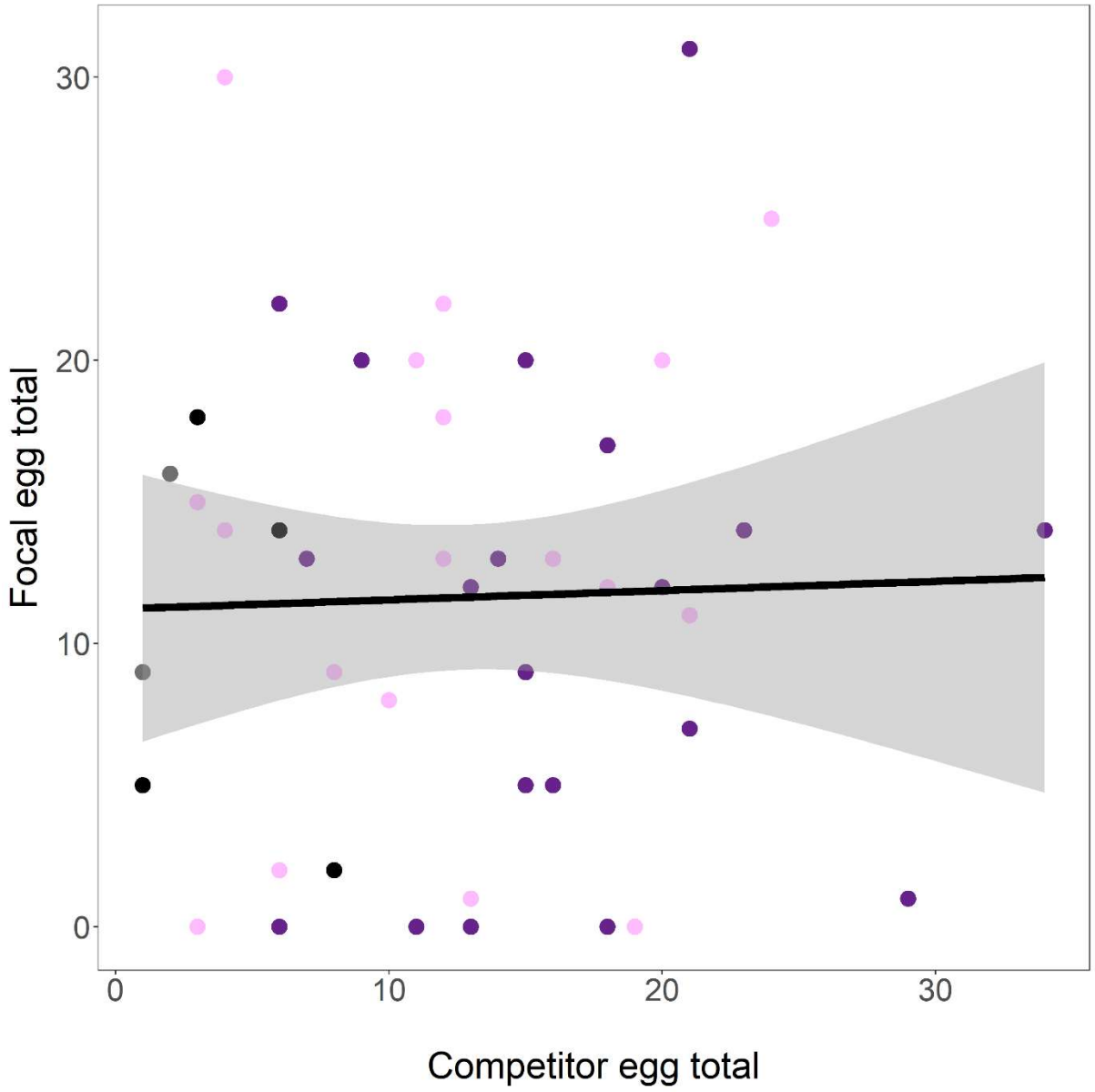


Figure 47. The total number of eggs laid by focal and competing pair females

Line of best fit shown with accompanying standard error

Pink – Mated competitor on dispersed resources

Purple – Mated competitor on clustered resources

Grey – Virgin competitor on dispersed resources

Black – Virgin competitor on clustered resources

Focal females that were housed on clustered resources laid on a greater number of patches than focal females that were housed on dispersed resources ($X^2 = 42.4$, $p << 0.001$; Fig. 48; as

found in Chapter 5). Exposure to competition had no impact on the number of patches that females chose to oviposit on (X^2 = 10.8, p = 0.216).

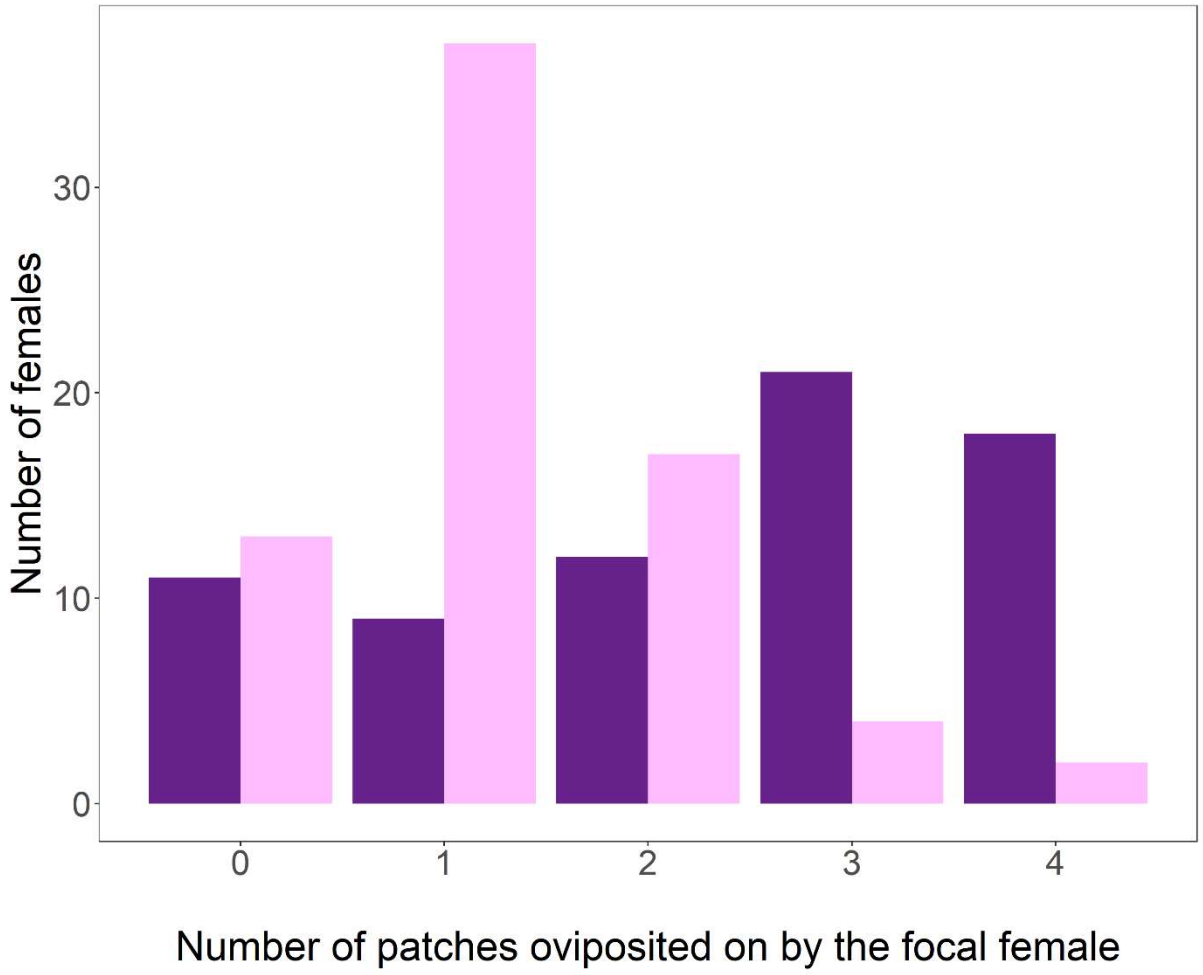


Figure 48. The number of patches that the focal females oviposited on

Pink – dispersed resources

Purple – clustered resources

When housed on clustered resources, more patches were laid on by both females than when females were housed on dispersed resources (clustered: $\bar{x}$ = 2.37; mdn = 3.0; dispersed: $\bar{x}$ = 1.25; mdn = 1.0; X^2 = 8.55, p = 0.0360; Fig. 49). As expected, fewer patches were laid on by both females when the competitor female was a virgin, compared to when both females

were mated (virgin competitor: $\bar{x}$ = 0.15; mdn = 0.0; mated competitor: $\bar{x}$ = 1.02; mdn = 1.0; $X^2 = 23.5$, $p << 0.001$; Fig. 49).

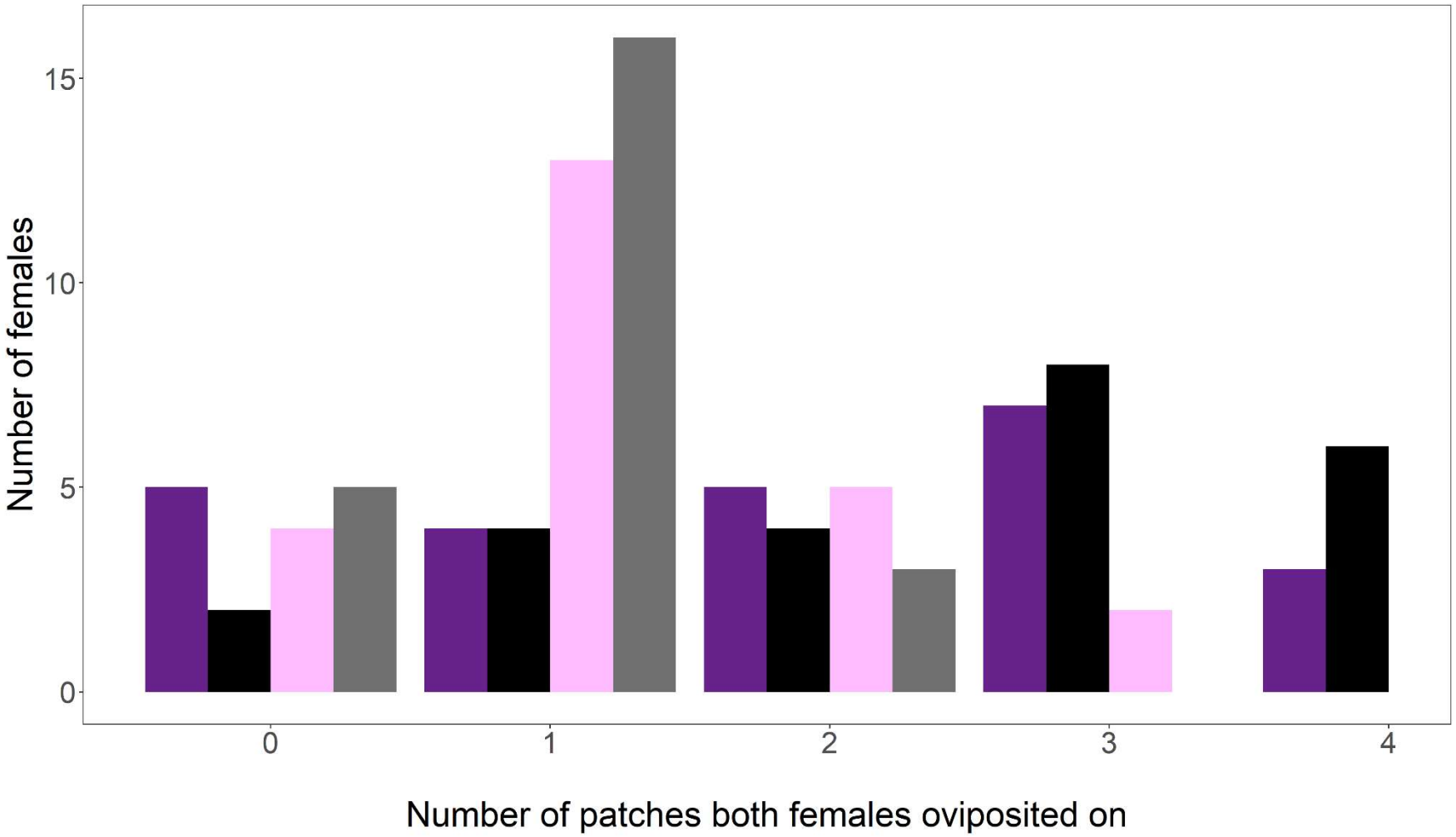


Figure 49. The number of patches that both competing females oviposited on

Pink – Mated competition on dispersed resources

Purple – Mated competition on clustered resources

Grey – Virgin competition on dispersed resources

Black – Virgin competition on clustered resources

Interactions between two mated females

In mated competition treatments, spatial distribution of oviposition resources did not affect the overall amount of time that both mated females spent exploring the four food patches ($F_{1,46}$ = 0.215, p = 0.645), unlike in Chapter 5.

Where both females were mated, those that oviposited on clustered resources spent more time on different patches (60.8%) than on the same patch (39.2%). This pattern was true of those that oviposited on dispersed resources too (different patches: 55.8%; same patches: 44.2%). However, in both cases, females spent more time on the same patch than expected at random (25%; clustered: t = 4.16, d.f. = 20, p << 0.001; dispersed: t = 2.49, d.f. = 23, p = 0.0207). There was no significant difference of time spent on the same patch between the two resource treatments (t = -0.587, d.f. = 31.5, p = 0.561).

6.4 Discussion

An overview of female responses

In the previous chapter, I considered how prior exposure to potential rivals in females affected their subsequent egg-laying decisions when they were allowed to oviposit without competition. In this chapter, I kept all females in isolated conditions until after mating, and then they were exposed to competition during oviposition. These chapters test different hypotheses – the expectation of future larval competition based on prior experience of adult density in Chapter 5, and on the presence of rivals during oviposition in Chapter 6. As a result, it is perhaps not surprising to find that some of the females' behavioural responses differed between these two chapters (highlighted in Table 5). Clearly, one explanation for these differences is that females experience or respond to prior density differently than how they experience the immediate presence of competitors. Male *Drosophila* also respond differently to prior versus current exposure to rivals: those exposed to rivals prior to mating lengthened their mating duration, but those exposed to rivals during mating shortened it (Bretman et al., 2009).

Alternatively, however, the differences between these chapters could be an effect of cooperation in the presence of a second female. Females have been observed to copy conspecific oviposition behaviours (Malek and Long, 2020, Duménil et al., 2016), so it is possible that the second females are influencing the focal females' oviposition choices. Furthermore, it is likely that the presence of a competitor female, or at least her eggs, alters the perceived value of the potential resources. There is existing evidence in *D. suzukii* that

presence of competitor eggs makes the resource less appealing for oviposition (Kidera and Takahashi, 2020), although it is not known if this applies to *D. melanogaster.*

Finally, it could be due to the size difference in patches between the two experiments. Although the size of the enclosure was kept the same, the size of patches was not. In this experiment, the patches needed to be smaller to limit space for potential larvae, and hence provide a source of potential competition. Whereas in Chapter 5, the patches needed to be larger, as I was interested in adult progeny produced, so it was important that the patches did not become desiccated for seven days (rather than in one day as needed in this study).

Table 5. An overview of the responses observed in Chapters 5 and 6, and the impact of competition and spatial distribution of oviposition resources

Response	Chapter 5: Prior housing density manipulated		Chapter 6: Competition during oviposition manipulated	
	Resource distribution	**Competition**	**Resource distribution**	**Competition**
Mating behaviours				
Mating latency	Not measured: exposure to resource distributions took place after mating	Group-housed females were slower to mate than solitary females	Not measured: exposure to resource distributions took place after mating	Not measured: exposure to competition took place after mating

Mating duration	Not measured: exposure to resource distributions took place after mating	Group-housed females had shorter mating durations	Not measured: exposure to resource distributions took place after mating	Not measured: exposure to competition took place after mating
Oviposition decisions				
Proportion oviposited	A higher proportion of females laid when laying on clustered resources	No effect observed	No effect observed	No effect observed
Number of eggs	No effect observed	Group-housed females laid fewer eggs	No effect observed	No effect observed

Number of patches laid on	Females laid on a higher number of patches when the patches were clustered	No effect observed	Females laid on a higher number of patches when oviposition patches were clustered	No effect observed
Proportion of eggs laid on highest laid-on patch	Not measured	Not measured	The proportion was lower when oviposition patches were clustered	The proportion was higher in females exposed to competitors (mated and virgin females)

Fitness responses

Number of adults (from patches)	No effect observed	No effect observed	Not measured: methods required manipulation of eggs	Not measured: methods required manipulation of eggs
Number of adults (from vials)	No effect observed	No effect observed	Not measured: methods required manipulation of eggs	Not measured: methods required manipulation of eggs

Time spent on patches				
Time spent on patches	Females on clustered resources spent more time on patches	No effect observed	No effect observed (only measured in females with a mated competitor)	Not measured: only measured in females with a mated competitor
Time spent on the same patch	Not measured: females did not have a competitor at time of oviposition	Not measured: females did not have a competitor at time of oviposition	Females that were given clustered oviposition resources spent more time on the same patch than on different patches	Not measured: only measured in females with a mated competitor

The impact of competition and spatial distribution on oviposition location decisions

Both focal and competitor females that oviposited on dispersed resources laid on fewer of the available patches than those ovipositing on clustered resources, likely to reduce the distance between eggs. This is supported by the result that those on dispersed resources laid a higher proportion of the eggs on the highest laid-on patch, and is concordant with the results from Chapter 5 (Churchill et al., 2021). This is likely to be because females consider the potential degree of larval travel in their oviposition decisions (Schwartz et al., 2012), and clustered patches better facilitate social aggregation in larvae, which in turn will enable more efficient cooperative feeding for the larvae (Dombrovski et al., 2017, Khodaei and Long, 2020).

The distribution of the eggs was further impacted by the competition that the focal females were exposed to whilst laying. Those that were ovipositing in the presence of mated and virgin competitors laid a higher proportion of eggs on the most laid-on patch. This was unexpected: I predicted that solitary females would deposit eggs in closer proximity to each other to obtain the optimal larval foraging density (Rohlfs and Hoffmeister, 2003), and that this would be a priority for them with no other females' decisions to consider. The contrasting result suggests that where there were two females in the treatment, they did not cooperate and lay in similar locations as expected (Malek and Long, 2020, Duménil et al., 2016). One possible explanation is differences in condition. As one female had been fed Rhodamine B-stained agar and the other standard agar, it is possible that the females did not perceive their conspecifics decisions to be reliable, and thus instead relied on private information. Reduced sampling costs is a benefit of copying behaviours, but acting on unreliable information is one of the downsides (Giraldeau et al., 2002, Laland, 2004). The finding that the number of competitor

eggs present on the patches did not impact the number of eggs laid by the focal females, corroborates this interpretation of the results. Previous studies investigating oviposition copying behaviours have not tested simultaneously ovipositing females – rather solitary females were given previously laid eggs, larvae or deposited pheromonal cues to explore (Malek and Long, 2020, Duménil et al., 2016, Golden and Dukas, 2014). It could be that this result was not replicated in my study, as females require the full clutch to be laid (or at least the presence of a more experienced/knowledgeable conspecific) to be able to make an accurate assessment of oviposition sites.

Interaction between two mated females

There was no impact of the spatial distribution of available oviposition resources on the amount of time that the focal female and the mated competitor spent exploring resource patches. This was not what was found in Chapter 5: in that experiment, females on dispersed resources spent less time on available patches (Churchill et al., 2021), likely due to increased sampling time. However, the females in Chapter 5 were laying in solitude, so the lack of resource distribution effect in this study could be due to oviposition copying behaviours (Malek and Long, 2020). It could also be due to the likely increased competition at clustered sites due to increased encounter rates: an increased number of aggressive encounters would reduce potential sampling time. It could be that females are trying to avoid competitors to avoid aggressive interactions, or increased larval competition (as it can lead to cannibalism (Vijendravarma et al., 2013)). If this explanation were correct, the fact that such a difference was not observed suggests that females on dispersed resources place greater value in copying

than those on clustered resources – likely due to the increased sampling cost (due to larger travelling distances).

In pairs laying on clustered oviposition patches, the two mated females spent more time on the same patch than predicted by the null hypothesis. This analysis only includes images where females were both observed to be present on an oviposition patch – i.e., when both females were potentially ovipositing as eggs could not be laid on the surrounding substrate. This suggests that females could be cooperating in their oviposition decisions as predicted.

The subsequent fitness effects of oviposition decisions

Although I discovered numerous effects of the physical and social environment on oviposition distribution, no overall fitness effects were observed. Unlike in Chapter 5, distribution of potential oviposition resources did not influence the likelihood of successful oviposition. This could suggest that the presence of a competitor female, or at least her eggs, alters the perceived value of the potential resources. There is existing evidence in *D. suzukii* that presence of competitor eggs makes the resource less appealing for oviposition (Kidera and Takahashi, 2020), although it is not known if this is true for *D. melanogaster* too.

In Chapter 5, I observed that females that had previously been exposed to rival females laid fewer eggs than those that had been housed in solitude (Churchill et al., 2021). I found no evidence of competition impacting the number of eggs oviposited here, although it is not surprising that current versus prior exposure to rivals impacts female behaviours differently. The same effect is observed in males, with those previously exposed to competition having a

longer mating duration, but those exposed to competition during mating having a shorter duration (Bretman et al., 2009).

It is possible that the reason there were no fitness effects was due to the contradicting benefits of cooperation but the detrimental impacts of competition taking place at the same time, when females were exposed to rivals – with the impacts effectively balancing out. If focal females copied competitor oviposition decisions, they will likely have begun ovipositing more quickly as resource sampling time was reduced (Malek and Long, 2020). However, females engage in aggressive interactions with potential rivals (Bath et al., 2017, Ueda and Kidokoro, 2002) – likely to establish dominance. These aggressive interactions would not only limit resource sampling time but would also come with an energetic expense and would thus limit the number of eggs the females were able to lay. Future work should investigate this further: filming the competing pair would enable one to establish what aggressive interactions, if any, were occurring, and how time spent on competition is traded off against time spent on egg laying or sampling the environment.

As was the case in Chapter 5, my experimental design required females to be removed from the enclosures after 20 hours, as the location of eggs needed to be recorded – and larvae hatch after 22 – 24 hours at ~25°C (Fernández-Moreno et al., 2007, Markow et al., 2009). This was before females had oviposited all their remaining fertilised eggs, and it is likely that had females been given the opportunity to continue ovipositing, that their oviposition decisions (number, location and proximity to rivals) will have impacted overall fitness.

My results here, compared to those presented in Chapter 5, demonstrate that the presence of rivals during oviposition has a different impact to when females are exposed to rivals prior to mating and oviposition. In this study it was not possible to disentangle the alternate effects of cooperation versus competition, and it is unlikely that both would be simultaneously impacting responses. It would be useful if future studies could attempt to disentangle these contradicting forces.

The impact of the physical arrangement of oviposition patches was less pronounced in this study, where the changes were experienced simultaneously with changes to the social environment – specifically the level of competition. Although fitness-related effects of competition have been widely studied in males, comparatively fewer studies have been completed in females. Considering the differing results presented here in this thesis, it would be beneficial for further investigation to be completed into the impact of the social and physical environment on oviposition responses – especially considering that females have a greater impact over progeny fitness, and thus overall population dynamics.

Chapter 7: **Final discussion**

What do you call a dead fly?

A flew

In this thesis I set out specifically to resolve a number of outstanding questions about how mating-related plasticity is mediated in a natural context, and how these responses are expressed in males and females. I did this by testing these responses in spatially variable environments that were designed to mimic the type of environmental variability that might be found in the natural habitat of *D. melanogaster*, but still could still be controlled under laboratory conditions enabling the manipulation of just one (or very few) variables. Here I have investigated the combined effects of competition and resource patchiness and demonstrated the differential impacts it can have in the two sexes: measuring short-term impacts on reproductive outputs, and long-term impacts on survival.

7.1 An overview

A mechanism for sperm competition-related responses

I show that manipulating resource patchiness whilst maintaining constant group size has the same effect on copulation duration (both in direction and magnitude) as does direct manipulation of local density. An increase in copulation duration in *D. melanogaster* is a widely-reported response to an increase in perceived sperm competition under laboratory conditions (Moatt et al., 2013, Bretman et al., 2009, Bretman et al., 2013a, Bretman et al., 2010). By manipulating local density in a more ecologically relevant context, here I report one possible mechanism by which the environment might alter reproductive investment in males in wild-living populations.

I then explored this mechanism further, using the same technique with individually marked males. I studied in more depth how these alterations to the physical environment impacted

the social environment. I found that clustered food resources lead to increased proximity to rivals and decreased home range size, in addition to increased copulation duration. Territoriality in *Drosophila* is driven by food resource boundaries (Lim et al., 2014), and has previously been shown lead to increased mating success (Hoffmann and Cacoyianni, 1989). However, despite the observed increase in copulation duration, I found no evidence of any fitness benefits of competition (via resource patchiness) or home range size.

Competition or cooperation?

The observed responses to competition differed between the two sexes. Whereas males increase investment in reproduction after exposure to rivals prior to copulation, females were found to depress reproductive investments. Females that were exposed to competition prior to copulation and oviposition, laid fewer eggs. However, conspecific presence does not necessarily result in competition for females, as they could instead cooperate. When ovipositing, females can copy choices made by other females (Malek and Long, 2020, Duménil et al., 2016). This is likely to be the reason for the shift in reproductive investment by females when presence of conspecifics is experienced during (rather than prior to) oviposition.

Resource patchiness also impacted reproductive investment in females. They showed a preference for clustered resources: a higher proportion laid when housed on clustered oviposition resources. Plus, in those that did lay, they laid on a higher number of patches compared to those ovipositing on dispersed resources. Clustered resources will better facilitate larval travel, a factor females take into consideration when ovipositing (Schwartz et al., 2012). For this reason, it is likely that females perceive clustered resources to be more valuable and hence show a preference for this spatial distribution.

Fight for survival

Intriguingly, exposure to increased perceived sperm competition increases survival in virgin males (Moatt et al., 2013, Bretman et al., 2013b), but it is not yet understood why. Instinctively, as exposure to rivals upregulates a number of energetically expensive processes (Moatt et al., 2013, Moatt et al., 2014, Fedorka et al., 2011, Garbaczewska et al., 2013, Pitnick, 1996, Dewsbury, 1982), it was first predicted that the opposite would be true. In this thesis I manipulated male intrasexual competition via two exposure treatments to investigate survival effects: direct exposure (exposure to likely injurious aggression) and indirect exposure (separated by a transparent divider – visual, olfactory and auditory cues only). Unfortunately, this provided little clarification on this conundrum. Males exposed to direct competition lived for longer than males housed in solitude, males exposed to indirect competition and males exposed to the presence of female conspecifics. However, when male survival was tested via starvation resistance rather than longevity, indirectly exposed males survived for longer than directly exposed males. This suggests that the exposure to direct competition has long-term but not short-term survival benefits for the males.

7.2 Future directions

Understanding survival effects

The survival response to competition was first discovered in *D. melanogaster* in 2013 (Bretman et al., 2013b, Moatt et al., 2013) yet we still do not understand why it occurs – this should be a focus of future studies. It is possible that production of sperm and other ejaculate

composites arise from a reduction in energy allocation elsewhere – such as other types of tissue production, immune responses or behavioural strategies.

How perceived competition affects longevity in females has not yet been discovered. This would also be a valuable avenue to explore given the surprising results found in males.

More on resource patchiness

Despite the strong impact on behavioural responses, no effect of resource patchiness on male fitness was discovered in Chapter 4. This could be because it was an instantaneous measure of fitness not a measure of an individual's lifetime reproductive success. Thus, fitness effects may be detected if offspring production were measured over multiple mating opportunities. Or it could be because the males were responding to environmental cues that their offspring were not exposed to – i.e., males were responding to resource patchiness in the enclosures, but offspring were raised in standard vials. It could also be because each male was provided with a standard female to mate with, and thus there was no element of mate choice. It would be valuable to explore whether any fitness effects would be detected if this experiment were repeated with females also present in the enclosure. In this scenario males must compete to gain access to mates, and any successful offspring would develop on the same patchy environments that the parent flies were responding to.

Another avenue that would be valuable to explore would be to include variation in food patch quality. This would likely have revealing consequences for female oviposition as we know they have a preference for a clustered distribution of resources (Chapter 5, Churchill et al. (2021)), and we know they have a preference for substrates containing sugar (Schwartz et al., 2012)

and ethanol (Sumethasorn and Turner, 2016), but how these preferences might interact is not known.

Modifying individual experience

Evidence of cooperation was found in ovipositing females: differences in responses to oviposition resource patchiness were observed when females were ovipositing in the presence of a consexual compared to when females were oviposition in solitude after prior exposure to competition. In males, it has been shown that relatedness and familiarity affect aggression (Carazo et al., 2015), mate choice (Tan et al., 2013), fitness (Carazo et al., 2015) and female harm (Carazo et al., 2014, Le Page et al., 2017). Hence it is likely that relatedness and familiarity would impact cooperation and oviposition copying – so future investigations should focus on understanding this.

As well as in the adult life stage, *Drosophila* larvae are also social (Anderson et al., 2016). They cooperate when feeding (Dombrovski et al., 2017), but can also be cannibalistic (Vijendravarma et al., 2013, Khodaei and Long, 2020). Using resource patchiness to understand how these two opposing strategies might persevere would be a significant next step for an experiment.

Field versus laboratory studies

Having established that biologically plausible mechanisms can stimulate sperm competition-linked traits in the laboratory, the next major step for this field of research is to uncover how these traits might be impacted by environmental conditions in wild-living

populations. It would be beneficial to repeat this experiment using wild caught flies *in situ* to gain a greater understanding of the biological relevance and to reveal how natural fluctuations in local population density impact reproductive responses.

7.3 Concluding remarks

Demonstrating the link between competition-related responses observed in the laboratory and small-scale patchiness of resources, places those widely-reported responses in a more biologically relevant context. Resource patchiness is an ecological factor likely to be influencing the social environment that wild-living individuals are exposed to, and thus should be an important focus for future research into uncovering how fitness-enhancing plasticity is mediated. The finding that female responses differ after manipulation of exposure to conspecifics prior to and during oviposition highlights the importance of investigating interacting environmental factors. Equally variably, the difference observed in male and female reproductive responses demonstrates that examining hypotheses in both sexes is crucial. Existing research has so far comparatively excluded female responses, despite the increased control they often have over investment in progeny. To understand variation in individual fitness, and ultimately population dynamics, we must consider the interaction between the two sexes with often competing reproductive goals in a dynamic social and physical environment.

Appendices

What's the difference between a fly and a bird?

A bird can fly, but a fly can't bird

Appendix 1: Recipes

1. i. Nutritious agar-based medium

Ingredients for 1000ml of agar:

- 40g yeast
- 40g sucrose
- 14g technical agar
- 1000ml of distilled water
- 40ml nipagin solution
    - 4g methyl-4-hydroxybenzoate
    - 40ml 100% ethanol

Method:

1. Combine the yeast, sucrose, technical agar and distilled water in a glass bottle and autoclave
2. Once cooled to 40-50°C, add the nipagin solution
3. Pour 7ml into each 40ml vial

1. ii. Distribution treatments agar-based medium

Ingredients for 1000ml of agar:

- 40g yeast
- 40g sucrose
- 14g technical agar
- 1000ml of distilled water
- 40ml nipagin solution
    - 4g methyl-4-hydroxybenzoate

- 40ml 100% ethanol

Method:

1. Combine the yeast, sucrose, technical agar and distilled water in a glass bottle and autoclave
2. Once cooled to 40-50°C, add the nipagin solution
3. Pour 35ml into each Petri dish
4. Cut the agar as required using an upturned vial and distribute in Petri dishes as described in chapters 4.2, 5.2, 6.2 and 7.2

1. iii. Starvation agar-based medium

Ingredients for 1000ml of agar:

- 14g technical agar
- 1000ml of distilled water
- 40ml nipagin solution
    - 4g methyl-4-hydroxybenzoate
    - 40ml 100% ethanol

Method:

1. Combine the technical agar and distilled water in a glass bottle and autoclave
2. Once cooled to 40-50°C, add the nipagin solution
3. Pour 30ml into each Petri dish
4. Cut and remove the agar using an empty DAM tube

1. iv. Fluorescence agar-based medium

Ingredients for 1000ml of agar:

- 40g yeast
- 40g sucrose
- 14g technical agar
- 1000ml of distilled water
- 40ml nipagin solution
    - 4g methyl-4-hydroxybenzoate
    - 40ml 100% ethanol
- 1.916g Rhodamine B – to make a 0.004M solution (Kamimura, 2007)

Method:

1. Combine the yeast, sucrose, technical agar and 950ml distilled water in a glass bottle and autoclave
2. Once cooled to 40-50°C, add the nipagin solution and Rhodamine B powder (rinsed in with remaining 50ml distilled water)
3. Pour 7ml into each 40ml vial

Appendix 2: Transforming DAM data to output files

2. i. R script to transform DAM data to an output file

```
layout<-read.csv("layout.csv",h=T)

layout$deathindex<-NA

#add column to collect index of final movement

layout$act<-NA

maxgap<-500

# = 6h gap (1 minute intervals)

sets<-14

for(set in 1:sets){

  dat<-read.table(paste("set",set,".txt",sep=""),sep="\t",h=T)

  # read in raw monitor file

  templayout<-layout[which(layout$fly %in% names(dat)),]

  # read in layout file, subset to flies that match monitor file

  flies<-nrow(templayout)

  # count how many flies are in this monitor file

  for(i in 1:flies){

    temp<-cbind(dat["index"],dat[which(names(dat) %in% templayout$fly)][i])

    # create temporary monitor file containing just the focal fly

    index<-templayout[templayout$fly==names(temp)[2],4]

    # find the start index of that fly

    maxindex<-templayout[templayout$fly==names(temp)[2],5]
```


```
    temp<-temp[temp$index>=index & temp$index<=maxindex,]

    # restrict temporary monitor file to match index

    movements<-temp[which(temp[,2]>0),]

    # find all non-zero instances

    if(sum(movements[,2])>0){

      movements$gap<-c(NA,movements[-1,1] - movements[-nrow(movements),1])

      # calculate gap

      if(max(movements$gap,na.rm=T)<maxgap) layout[which(layout$fly %in%
names(temp)),"deathindex"]<-max(movements$index,na.rm=T)

      else

        layout[which(layout$fly %in% names(temp)),"deathindex"]<-
movements[which(movements[,3]>maxgap)-1,1][1]

# find first index in which gap > maxgap

        layout[which(layout$fly %in% names(temp)),"act"]<-sum(movements[,2])

    }

  }

}

layout$survival<-layout$deathindex-layout$index

write.csv(layout,"DAMoutput.csv",row.names=FALSE)

# create final file
```

2. ii. Example of a 'layout' file

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	use	hole	date	damno	index	maxindex	set	fly	pvial	treatment	age	mtreatment	timein	timeout	timetreat	timediff	size
2	1	17	2303	2	10400	15900	1	f2203cb	0803c	3	1	3	19	18.75	23.75	0.25	53.5
3	1	28	2303	2	10400	15900	1	f2203da	0803d	2	1	2	19	18.75	23.75	0.25	55.5
4	1	24	2303	2	10400	15900	1	f2203bb	0903a	2	1	2	19	18.75	23.75	0.25	53
5	1	16	2303	2	10400	15900	1	f2203hb	1003a	3	1	3	19.5	19.75	24.25	-0.25	53.5
6	1	2	2303	2	10400	15900	1	f2203ib	1103a	3	1	3	19.5	19.75	24.25	-0.25	54

Figure 50. An example layout file

Appendix 3: An analysis of two reproductive behaviour assays

3. i. Introduction

A history of Drosophila

Drosophila are a widely studied model organism, used to provide answers to a vast array of questions in a wide range of fields – included within this are six Nobel Prize winning investigations. The first in 1933, led by Thomas Morgan Hunt, uncovered the role of chromosomes in heredity (NobelPrize.org). The most recent, discovered the molecular mechanisms required to control circadian rhythms (NobelPrize.org).

In addition to this, *Drosophila* have also been used in a number of behavioural studies. It is widely accepted that variable environments impact animal courtship (Zweifel, 1968, Greenfield and Rodriguez, 2004, Lampe et al., 2012) and mating behaviour (Elsey, 1982, Verner and Willson, 1966, Miskimen, 1966). Research on *Drosophila* species has further contributed to our understanding of the implications of variable environments. For example, Shenoi et al. (2016) discovered that *Drosophila melanogaster* evolve an increased rate of courtship, when exposed to larval crowding. Furthermore, another study by Best et al. (2012) found that female remating rate was affected by temperature both within and outside of courtship.

Mating durations are (often) increased under perceived sperm competition

Many studies have reported an increase in the length of mating durations when male *Drosophila* were exposed to a rival male prior to mating (Price et al., 2012, Moatt et al., 2013,

Bretman et al., 2009). As *Drosophila* are a polygamous species, both males and females mate with multiple mates (Dobzhansky and Pavlovsky, 1967). Sperm competition occurs when more than one males' sperm compete for the same female's eggs (Parker, 1970). It is the response to this perceived sperm competition that likely drives the increase in mating durations. Longer mating durations could be a form of mate-guarding (Alcock, 1994) as has been hypothesised in other taxa including drill flies (Rull et al., 2017), rice weevils (Flay et al., 2009) and golden egg bugs (García-González and Gomendio, 2004). It has also been shown that longer mating durations enable an increased number of viable sperm to be produced and transferred (Bretman et al., 2009, Moatt et al., 2014), which then leads to a greater chance of siring successful offspring.

However, there are some studies and further anecdotal evidence that contradict these findings. One such study is the one featured in Chapter 2: mating duration was not affected by exposure to potential sperm competition. A further study by Lizé et al. (2012) found no significant effect of previous exposure to rivals on mating duration. It is not yet clear why these contrasting results have been reported – it could be due to differences in methods, species, mating environments or individual differences. Table 6 provides further details on the range of findings surrounding this effect, and the methods used in each study.

Table 6. A review of the effects of increased perceived sperm competition risk (PSCR) on mating duration in a range of Drosophila species

Paper	Conclusions	PSCR level	Assay	Times recorded	Age	Cues exposed to	Yeast	*Drosophila* species
Bretman et al. (2009)	Prior exposure to PSCR increased mating duration, but current exposure decreased it. Latency was shorter when rivals were present in the arena. Paternity share is higher for males exposed to rivals.	1, 2, 4	Vial	Nearest minute	5 days	Full interactions	Bass plus live yeast	*D. melanogaster*
Mazzi et al. (2009)	2:1 had longest mating duration, 5:1 shortest.	1:1, 3:3, 2:1, 5:1	Vial	Start/end	19-23 days	Full interactions	Malt medium plus live yeast	*D. montana*

Bretman et al. (2010)	PSCR leads to longer mating duration – increased with exposure.	1, 2, 4	Vial	Start/end	0-19 days	Full interactions	Bass	*D. melanogaster*
Bretman et al. (2012)	PSCR increased mating duration. PSCR also increased number of offspring produced.	1, 4-8, 8-17	Vial	Start/end	4 days	Full interactions	Bass	*D. melanogaster*
Lizé et al. (2012)	No effect of prior exposure to PSCR. Mating duration increased with competition in space.	1, 2	Vial	Start/end	8 days	Full interactions	Corn-sugar-yeast agar	*D. bifasciata*

Price et al. (2012)	PSCR increased mating duration. PSCR increased number of offspring produced. PSCR resulted in more long sperm. Increase is found only with conspecifics.	1, 2	Vial	Start/end	5-6 days	Full interactions	Markow plus live yeast	*D. pseudoobscura*
Bretman et al. (2013)	PSCR increased mating duration and latency. No effect on likelihood to mate.	1, 2	Vial	Start/end	4 days	Full interactions	Bass	*D. melanogaster*
Moatt et al. (2013)	PSCR increased mating duration.	1, 2	Vial	Start/end	8-12 days	Divider	Sugar yeast 50g	*D. melanogaster*

Bretman et al. (2014)	PSCR increased mating duration. Mating duration showed heritable variation.	1, 2	Vial	Start/end	5-6 days	Full interactions	Markow plus live yeast	*D. pseudoobscura*
Rouse and Bretman (2016)	Responses to increased PSCR were observed for 12 hours. Responses were observed when rival has no wings or smell.	1, 2	Vial	Start/end	12-3 days	Full interactions	Bass	*D. melanogaster*

Bretman et al. (2017)	Males exposed to rivals mated for longer than single males (and with mirror). No difference in solitary and mirror males. Duration increased with heterospecific rivals, but not as much as conspecifics.	1, 1 (plus mirror), 2	5 days	Full interactions	*D. melanogaster, D. simulans, D. yakuba, D. pseudoobscura,* and D. *virilis*

Mating behavioural assay techniques

There are two widely used apparatus and techniques used when measuring mating duration in *Drosophila*. One is observing mating pairs in vials (Moatt et al., 2014, Bretman et al., 2010, Lizé et al., 2012), and another is observing mating pairs in smaller plastic wells – often referred to as arenas or chambers (Beaver and Giebultowicz, 2004, Hall, 1979, Nazari, 2011). However, to the best of my knowledge, there has not yet been a study comparing results found using both techniques.

This study aims to compare the impact of these two techniques using four treatments: mating pairs in a chamber versus those in a standard vial, with both solitary and paired males. Directly comparing the results found using these techniques will shed light on previous contradictory findings, and ensure that an effective method for measuring copulation durations is refined.

3. ii. Methods

Perceived sperm competition treatments

D. melanogaster (originating from the Canton-S strain) were maintained on a 12hour light:dark cycle (08:00 – 20:00h GMT) at a constant temperature of 25°C, in 40 ml vials containing 7ml of standard agar-based medium – of 40g of yeast per litre. Approximately 20-30 *Drosophila* were housed in each vial, and they were pooled and redistributed every seven days to minimise potential genetic effects.

Test flies were collected from standardised density vials: six males were placed in a vial with six females and allowed to breed for 70-98 hours to ensure food was not limiting for F1

offspring. They were then anaesthetised using ice and transferred to treatment conditions. Half of the test flies were maintained in solitude, and half in pairs. They were held in standard vials until aged to three days, before being transferred to the testing environment.

Reproductive behaviour assays

All focal flies were paired with a standard three-day old female, also collected from parent vials. Once in the mating set-up, pairs were observed for courtship latency, copulation latency and copulation duration (Chapter 2).

Once the pair had completed copulation, males were removed and sacrificed to measure the left-wing sizes (Partridge et al., 1987b). Females remained in the mating vials (or were transferred to a vial from a mating chamber) for seven days, to enable her to oviposit eggs. After this, females were removed to measure their left-wing sizes. Number of offspring to successfully reach the adult life stage were then counted 21 days after the mating event.

Mating chambers versus mating vials

To ascertain whether the type of mating environment was having a significant impact on the reproductive behaviours of fruit flies, two types of environment were tested.

Half of the focal flies from each treatment were aspirated into a mating chamber. The mating chambers were 4.2ml cylindrical wells and were situated in a platform that held 16 mating chambers, all spaced 1cm apart; see Fig. 51. Each well contained approximately 0.01g of brewer's yeast granules.

There were always four pairs of flies in the mating platform, in the central four chambers in a square formation (Fig. 51). As a result, each mating pair had two neighbouring mating pairs, balancing any effects caused by neighbours' pheromones – as discussed in Chapter 2.

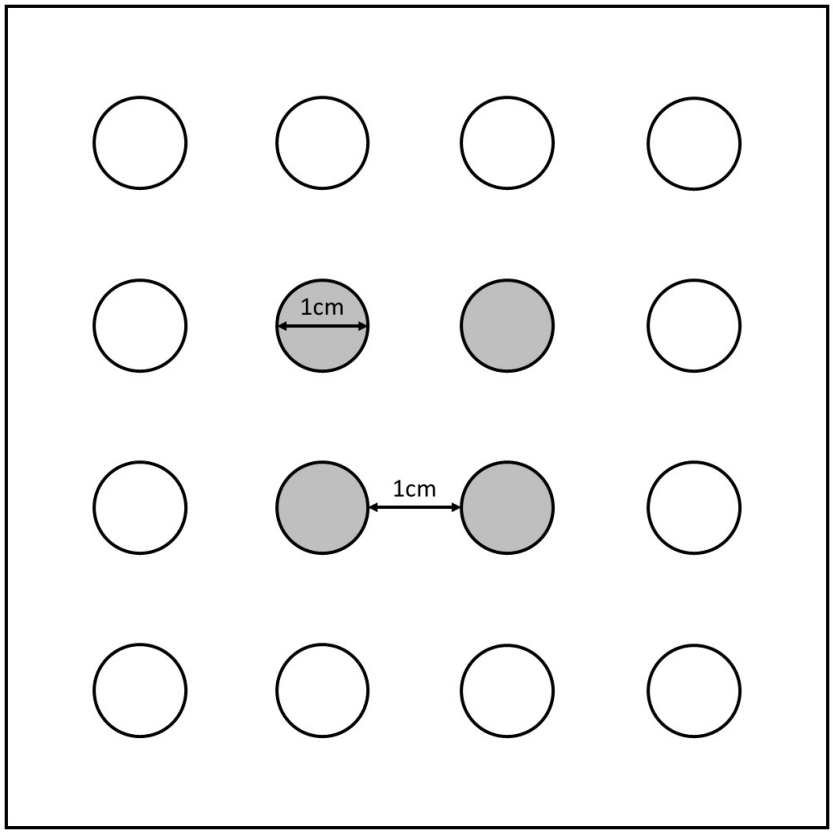


Figure 51. The platform containing 16 mating chambers – the chambers used in this study are shaded grey

The platform was placed in an incubator at 25°C, and reproductive behaviours were filmed using a Basler camera (Basler AG) and Media Recorder software (Noldus UK, 2018), and were watched back and analysed at a later date.

The remaining focal flies were aspirated into a mating vial: a standard agar-based medium vial (Appendix 1. i). Their space was restricted to $7cm^3$ using a sponge bung.

Assays were completed on the laboratory bench at room temperature and behaviours observed in real time. In order to control for the effects of varying temperatures, the temperature was recorded every minute and the mean average temperature for each assay block was calculated.

In both treatments, males were aspirated into the space first, and females second. Mating durations in this treatment were collected live. Mating durations in both methods were limited by identical time constraints: 30 minutes for courtship and 75 minutes for copulation (Nandy et al., 2012, Churchill et al., 2019).

Statistical analysis

All statistical analysis was completed using RStudio version 4. 0. 3 (R Core Team, 2019). All data was checked for normality using Shapiro-Wilk tests. If the data was not normally distributed, it was transformed using a natural log.

Binomial generalised linear models were completed to test whether exposure to perceived sperm competition or varying mating environments impacted the likelihood of the pair to successfully copulate.

Two-way ANOVAs and ANCOVAs were used to identify differences in all mating durations and number of successful offspring, between those pre-exposed to potential sperm competition

and those maintained in solitude in both mating environments (mating arenas and standard agar-based medium vials).

Additional variables were also included within the analysis to control for any effects they had on the results. These included: time, date, mating pair origin, parent origin, and temperature. Male and female size, and size difference between the mating pair, were also tested as size has been shown to effect mating duration (Turiegano et al., 2013, Partridge et al., 1987a, Partridge et al., 1987b, Partridge and Farquhar, 1983, Lefranc and Bungaard, 2000).

3. iii. Results

All males engaged in courtship, and all but one successfully copulated. There was no significant effect of exposure to potential sperm competition ($t = 0.003$, d.f. = 47, $p = 0.998$) or mating environment ($t = 0.003$, d.f. = 47, $p = 0.998$) on the likelihood of the pair to copulate.

Effect of rival exposure and mating environment on reproductive behaviours

Latency to court was significantly affected by the interaction between prior exposure to rival males, and the two different mating environments (log_{10}: $F_{1,44} = 5.12$, $p = 0.0286$). Solitary individuals had a longer latency in vials (solitary: 203 seconds; paired: 100 seconds), but paired individuals had a longer latency to court when mated in chambers (solitary: 60 seconds; paired: 126 seconds); Fig. 52.

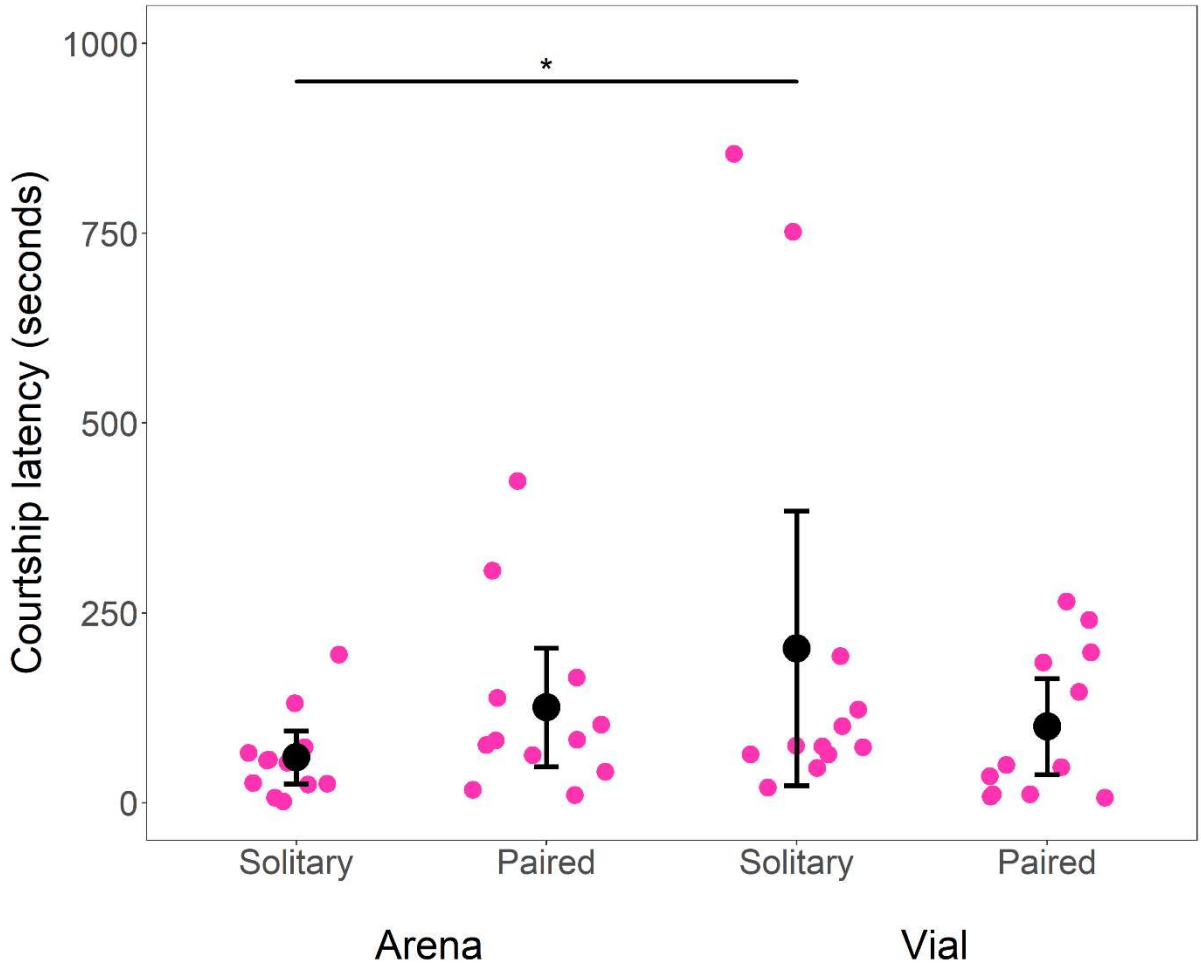


Figure 52. Mean latency to court in solitary and paired males, in both types of mating environment: arenas and vials

Black dots show means and 95% confidence intervals

Latency to copulate was significantly longer in males that were paired with a female in a mating arena (16 minutes) compared to those that were paired in a mating vial (six minutes) – when prior exposure to male rivals was controlled for (log_{10}: $F_{1,45} = 5.52$, $p = 0.0233$). But whether the individual was housed in solitary or paired conditions had no effect on copulation latency (log_{10}: $F_{1,45} = 1.14$, $p = 0.291$).

When males were exposed to a rival male prior to mating, the pair's copulation duration was significantly longer (after controlling for mating environment) (log_{10}: $F_{1,44} = 26.25$, $p << 0.01$; Fig. 53). Paired males mated in the arena mated for an average of 23 minutes – nearly five minutes longer than solitary males. Paired males mated in vials had a mean average duration of 21 minutes, compared to solitary males that only mated for an average of 18 minutes.

The mating environment had no significant effect on the duration of copulation (log_{10}: $F_{1,44} = 1.41$, $p = 0.241$).

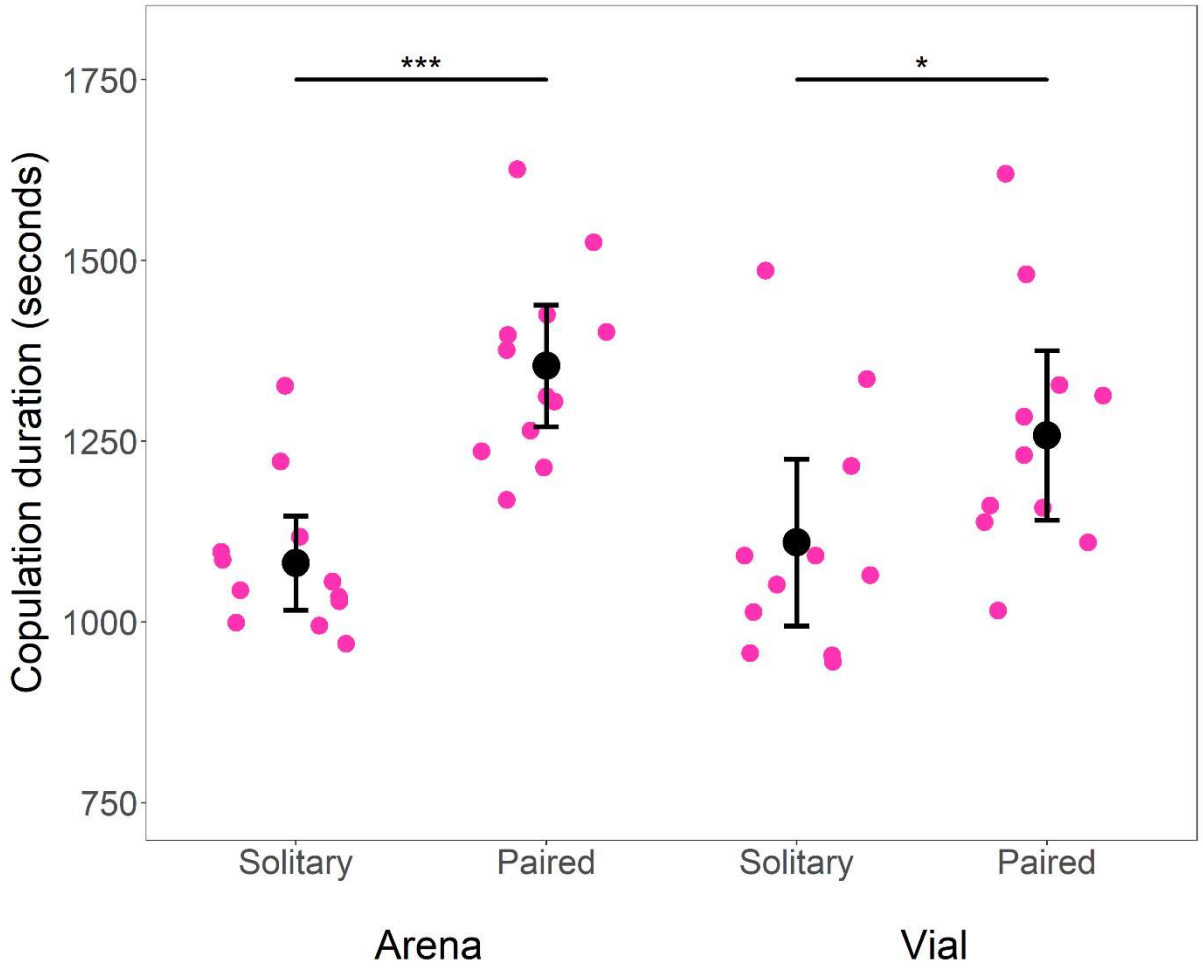


Figure 53. Copulation duration in solitary and paired males, in both types of mating environment: arenas and vials

Black dots show means and 95% confidence intervals

Effect of rival exposure and mating environment on fitness

The number of successful offspring the pair produced was affected by the interaction between the mating environment and prior exposure to rival males ($F_{1,42}$ = 7.05, p = 0.0111; Fig. 54). Solitary males mated in vials produced the fewest offspring, followed by paired males mated in an arena, then paired males mated in vials, and finally solitary males mated in an

arena produced the most successful offspring; a mean average of 55, 57, 70 and 75 respectively.

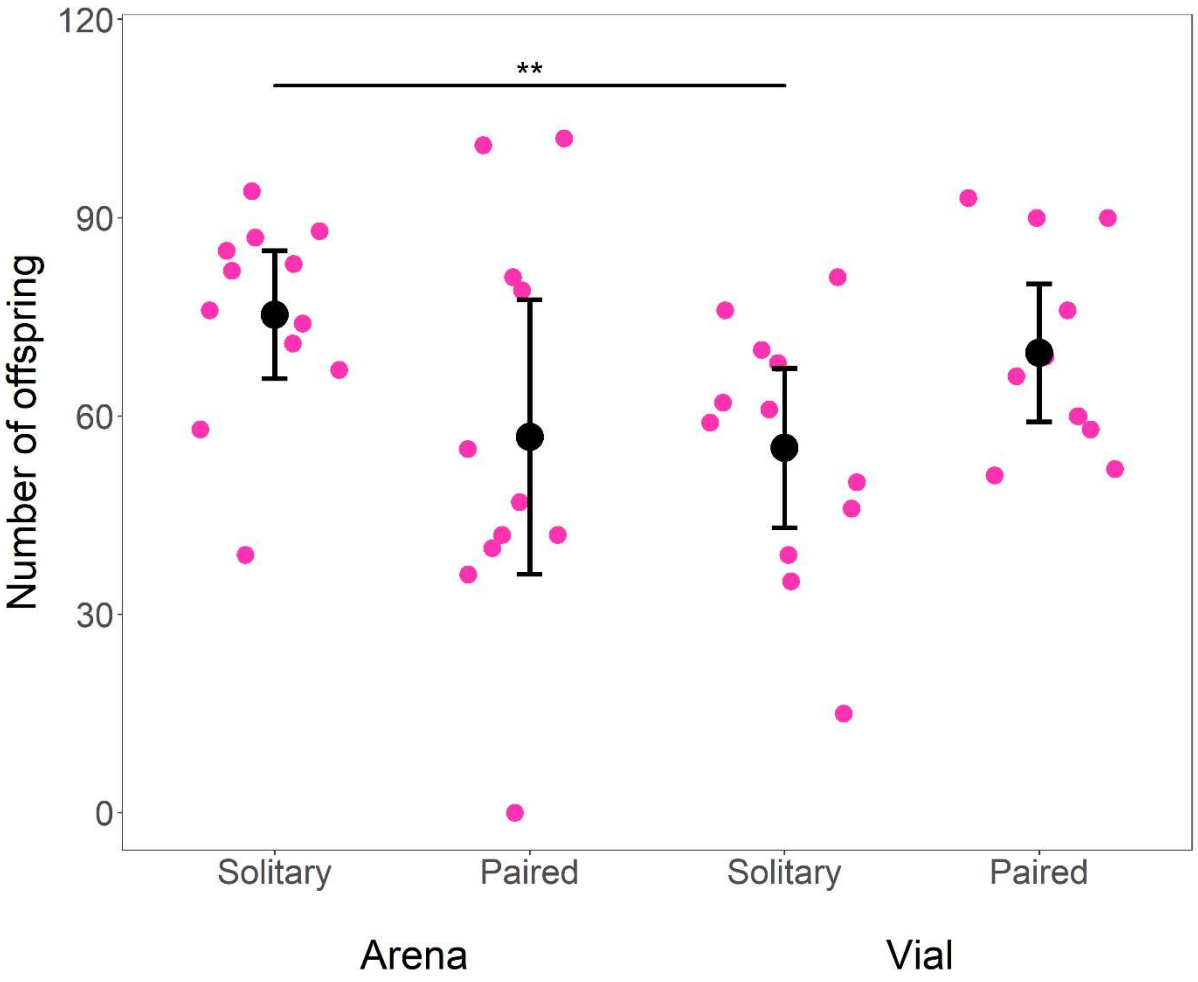


Figure 54. Number of successfully eclosed adults produced by solitary and paired males, in both types of mating environment: arenas and vials

Black dots show means and 95% confidence intervals

3. iv. Discussion

Perceived sperm competition increased copulation duration

Using both techniques, prior exposure to a rival male significantly lengthened mating duration. This result was as predicted, as similar results have previously been widely

published (Moatt et al., 2013, Price et al., 2012, Bretman et al., 2010, Bretman et al., 2009). An increased sperm competition will enable more sperm to be transferred (Moatt et al., 2014, Bretman et al., 2009), and will also act as a form of mate-guarding (Alcock, 1994).

However, this result contradicts that found in Chapter 2, which showed there was no effect to mating duration caused by perceived sperm competition. As this study matched the predicted result, I conclude that it is likely that the previous non-significant result was not due to a lack of variation or plasticity in our stock population of *D. melanogaster.* But there are several other factors that could have caused this difference in results.

The first of which, is that there were females present throughout the males' lifetimes in the experiment described in Chapter 2, unlike in this one. This will alter the sex ratio of the environment, and so will alter the number of potential mates that the male believes he has access to. As shown in Table 6, there are few studies that have investigated the effects of the presence of females on copulation duration, and these studies are not comparable to those done in the absence of female presence. Future research should aim to address this gap in knowledge.

The second key difference is that the number of neighbours present in the mating treatment was not kept constant in the Chapter 2 investigation. Further investigation highlighted that the number of pairs present in the mating platform was having a significant impact on the reproductive output of the mating pairs. To the best of my knowledge, no study has directly tested this effect. That being said, there a number of studies that have shown that rival male

presence at the time of mating does affect length of copulation (Lizé et al., 2012, Bretman et al., 2009).

Finally, it is possible that the effect is not consistent in populations. Anecdotal evidence suggests that other laboratories also find inconsistencies in the effects of perceived sperm competition on mating duration. However, this is not reflected in the published literature. Fanelli (2010) reported evidence of a positive publishing bias showing that there is an increased frequency of "positive" results in more competitive environments, and it could be due to this positive publishing bias that few of the non-significant results have been published.

Plasticity in other mating-related traits

Latency to court was affected by the interaction between prior housing conditions and the mating environment. Solitary males had a longer courtship latency in vials compared to arenas, whereas paired males had a longer courtship latency in arenas. It is not obvious why this result was found, but one hypothesis is that it could be because males housed in solitary conditions lack social experience, and so it took them longer to find their mate in the larger mating space.

Copulation latency was significantly longer when the pair mated in the mating chamber, compared to those mated in a vial. It is possible that this was due to the lack of availability of egg-laying sites: despite the pair being provided with yeast required for the potential offspring to feed upon, unlike in vials, females in the chamber were not provided with a suitable substrate upon which they could lay their eggs. It is likely that the female would be less willing

to mate (and so would therefore reject the males mating attempts more frequently, prolonging copulation latency) without somewhere to lay eggs after successful copulation.

It is also possible that the limited space of the mating chamber caused this effect. Males would have had less room to court the female, meaning their courtship behaviours may have been less effective, thus it took more time to persuade the female to succumb to copulation. It is worth mentioning however, that this would also be true of the females' rejection behaviours. Females would have had less room to flee from the advancing male and perform rejection behaviours, which would have the opposite effect.

When comparing males that mated in arenas, paired males produced fewer successful offspring than males housed in solitude. However, the reverse was true for males mated in vials. The latter result is consistent with that found by Bretman et al. (2009): both first and second mated males sired more offspring when exposed to potential sperm competition. It is not clear why this result was not found when pairs were mated in the mating chambers – further research will need to be completed to determine this.

A comparison of two techniques

Regardless of treatment, mating environment had no significant effect on the likelihood of the male to court the female, or the likelihood that the pair successfully copulated. Plus, the effects observed in copulation duration are similar in both the mating environments. I therefore conclude that future studies on reproductive durations, completed in both environments, will be comparable – if all other variables are kept constant.

However, the effects to individual fitness were not similar; it appears that mating environment has a significant effect on the number of successful offspring produced. It would therefore not be possible to directly compare results of offspring using the two difference mating techniques.

Equally, both latency to court and copulate were also affected by the mating environment. Effects on latencies are less widely published compared to copulation duration and individual fitness, but in any future studies, direct comparisons between studies could only be made if the same mating method were used.

Limitations to reproductive behaviour assays

Matings that take place in vials, are likely to be more similar to those that occur in the wild as their space is less confined, so these results are more applicable to natural populations. However, there are a number of limitations to using this method, so there are many valid reasons as to why you might opt to use the mating chamber technique instead.

One limitation is that it is not possible to film copulating pairs when they are in vials, which has many consequences. The behavioural observations must be completed at a set time and cannot be completed at a later date when better suits the investigator. This therefore reduces the number of pairs that can be mating at any one time, as it is not possible to watch a limitless number of pairs at once.

It is also not possible, unlike with video footage, to rewind time and double-check certain movements or behaviours to confirm individuals' intentions. This can result in missed

behaviours and incomplete datasets at best; or at worst, it could result in a full replicate being removed from the study.

Rewinding footage has an additional benefit. It also enables the observer to count and score multiple behaviours occurring simultaneously. For example, you could score all male courtship behaviours (chasing, wing extensions, tapping and licking) and female rejection responses (fleeing, kicking and extrusion of the ovipositor) for each mating pair.

A final advantage of video footage is that it is possible to remove human bias by requesting that a third party rename video files. Then the investigator will be blind to which treatment the individual has been exposed to, when scoring their behaviours.

On the other hand, there are also limitations to using the mating platform. In the mating chambers, pairs are not given access to standard agar-based medium as they are in vials. They are exposed to only live active yeast granules. This is a substance that females will not be able to lay eggs into, and it is therefore possible that it will affect her decision to mate or reject the courting male.

Plus, as mentioned previously, mating pairs have a much more confined space in a chamber. As a result, the female has less room to flee from the male and may therefore find it difficult to reject his advances. It is also possible that the pair perceive themselves to be in a denser environment than is true, because of their increased encounter rates with one another.

Selecting a technique

It is clear that both techniques have value, and it is important that all benefits and limitations to each technique are considered. If the aim of the study is to understand the impacts on a range of courtship behaviours and rejection responses, it is critical that the mating pair are filmed, and so assays must be completed using the mating platform.

If this is not the case, then either technique could be optimal depending on varying situations. Whichever technique is chosen, it will be possible to compare mating durations, although it would not be possible to directly compare offspring production and courtship and copulation latencies (as these were significantly affected by mating environment).

Appendix 4: An analysis of different marking techniques

4. i. Introduction

Dyes and other markers have been used in a range of invertebrate studies to uniquely identify individuals (Verspoor et al., 2015, Manier et al., 2010, Narisu et al., 1999, Brenner and Patterson, 1988, Cohen and Jackson, 1989, Sharon et al., 2010). Artificial markings are often not needed in larger species, as they have natural unique markings; however, in smaller species such as insects marking individuals enables us to study the impact of experimental manipulations in larger populations, whilst still being able to identify the individuals that had previously been manipulated.

Some studies have shown a range of survival effects of colour and application of dyes (Dickens and Brant, 2014, Naranjo, 1990, Valenca-Barbosa et al., 2016). There is also evidence to suggest that certain markers could alter individuals' behaviour and physiology, or even potentially the behaviour of others in their group (Walker et al., 2012, Pietz et al., 1993, Schwartzkopf-Genswein et al., 1997, Burley et al., 1982).

It is vital that effects of markers and dyes are established before they are used in studies, both to ensure that the marker has minimal impact on the study organism, and also that it works effectively for the chosen study. This investigation aims to understand the impact of wing and thorax markers on longevity and reproductive behaviours in *Drosophila melanogaster* – for use in future studies attempting to understand social and environmental impacts on individual responses.

4. ii. Methods

D. melanogaster used were collected from the LHm stock. All *D. melanogaster* were housed in 40ml vials containing 7ml of a standard agar-based medium (Appendix 1. i), in groups of approximately 20-30. They were exposed to a 12hour light:dark cycle (08:00 – 20:00h GMT), at 25°C. Test flies were collected from parent vials of a standard adult density – six females and six males were left to breed for three to four days.

Part one: The effect of wing markers on male longevity

The wings of male flies were marked using Corvina Grafix fine-tipped permanent marker pens (Corvina, Poland), under ice anaesthesia. Both wings were laid flat on a damp slide, and then the tip was dotted with ink (either coloured blue or red).

To test for handling effects, two control treatments were also set up: flies that had their wings 'marked' using distilled water alone, and flies without dyed wings.

A total of 112 male flies were used in this investigation – 28 per treatment. Individuals were maintained in groups of four, in standard vials. Each day, the number of flies alive were counted until all the flies had died.

Part two: The effect of thorax markers on male longevity and reproductive behaviour

The thoraxes of male flies were marked using a fine paintbrush with Humbrol enamel paint (Humbrol, UK) under light ice anaesthesia in one of four colours: red (Humbrol no. 60), yellow

(no. 99), blue (no. 109) and grey (no. 196). Two control treatments were again used to test for any handling effects: distilled water and non-dyed individuals.

Individuals were maintained in solitude in standard agar vials. Half of these vials were checked every two to three days, and any individuals that had died were noted.

The other half of all test flies were removed after seven days and paired with a standard seven day old female in a standard agar vial – supplemented with ~ 0.03g yeast. The pairs' mating behaviours were observed (latency of the male to begin courting, latency to copulate, and copulation duration; as described in Chapter 2) for an investigation into the behavioural impacts of marking.

A total of 120 male flies were used in this investigation – 10 per treatment in each of the two experiments.

Part three: The effect of thorax markers and Rhodamine B ingestion on female longevity

The thoraxes of female flies were marked using a fine paintbrush with Humbrol enamel paint (Humbrol, UK) under light ice anaesthesia in one of two colours: yellow (Humbrol no. 99) and grey (no. 196). Distilled water-marked and non-marked controls were used again, as described above.

Individuals in these four treatments were maintained in standard agar vials. An additional treatment was set up to assess the longevity effects of marking via ingestion of Rhodamine B. Rhodamine B can be seen in the abdomen by individuals that have ingested it, and it can also

be seen in the sperm and eggs produced (Kamimura, 2007). These females were housed in standard vials that contained agar-based medium mixed with Rhodamine B (Appendix 1. iv).

A total of 50 female flies were used in this investigation – 10 per treatment. Individuals were housed in solitude, and vials were checked for live flies every two to three days, until all individuals had died.

Statistical analysis

Statistical analysis was completed using RStudio version 4. 0. 3 (R Core Team, 2019). I used one-way ANOVAs and ANCOVAs to analyse the impact of marking techniques on longevity and reproductive behaviours. I used Shapiro-Wilk tests to check for normality, and longevity data were transformed via squaring when necessary and reproductive behaviour data were logged.

For a more detailed survival analysis, I used Cox proportional hazard models to analyse the effect of marking techniques on proportional survivorship.

All individuals left wing sizes were measured after death (as described in Chapter 2), and these data were included in models where the effect of size was significant.

4. iii. Results

Part one: The effect of wing markers on male longevity

There was no significant effect of any of the wing marking techniques on male longevity (double squared: $F_{3,108} = 0.138$, $p = 0.937$) or proportional survivorship (Cox proportional

hazards model: $X^2 = 0.580$, d.f. = 3, p = 0.901). Males survived for a mean average of between 43 – 46 days (control: $\overline{x}$ = 44.5 days; water: $\overline{x}$ = 43.5 days; red: $\overline{x}$ = 45.2 days; blue: $\overline{x}$ = 45.4 days).

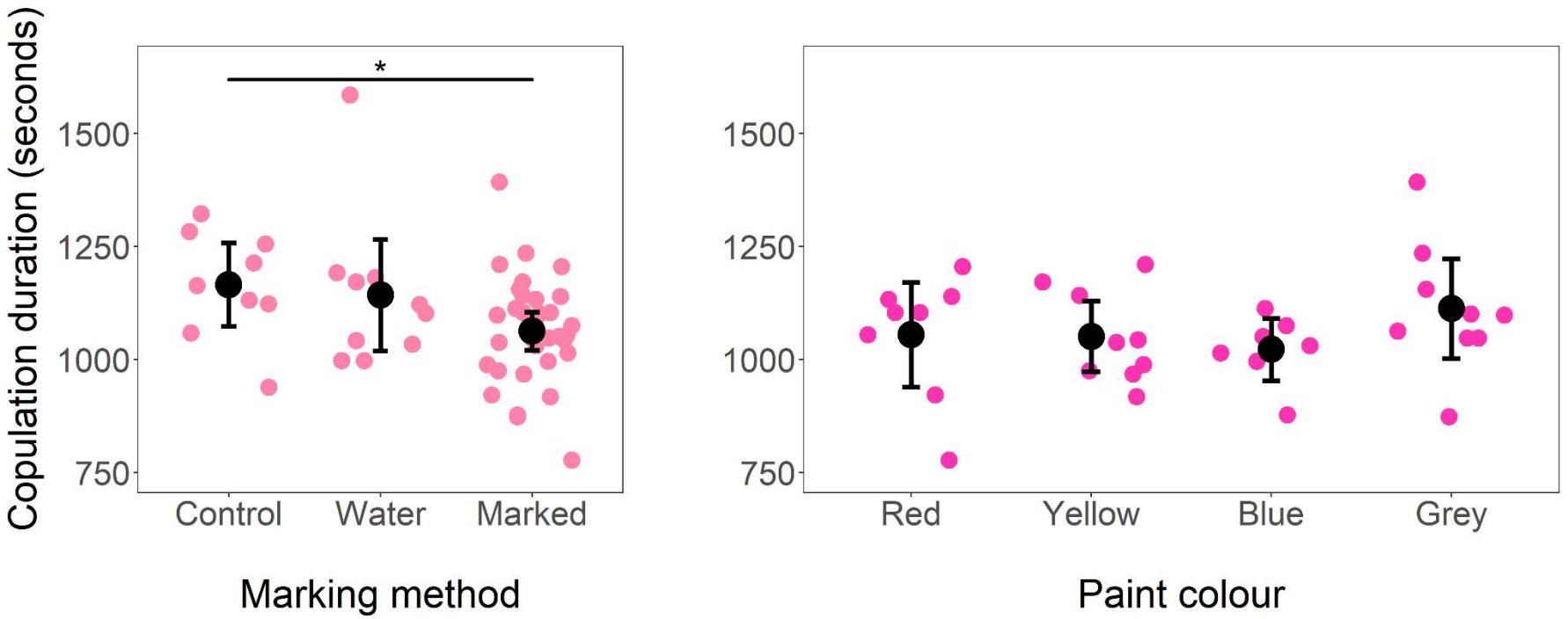
Part two: The effect of thorax markers on male longevity and reproductive behaviour

There was no significant effect of the enamel painting techniques on overall longevity (squared: $F_{2,54} = 0.508$, p = 0.604) or proportional survivorship (Cox proportional hazards model: $X^2 = 0.824$, d.f. = 2, p = 0.662). There was also no significant difference between the four chosen paint colours on longevity (squared: $F_{3,34} = 1.18$, p = 0.332) or proportional survivorship (Cox proportional hazards model: $X^2 = 1.16$, d.f. = 3, p = 0.762).

Males survived for a mean average of between 62 – 80 days (control: $\overline{x}$ = 77.2 days; water: $\overline{x}$ = 67.4 days; red: $\overline{x}$ = 67.7 days; yellow: $\overline{x}$ = 62.1 days; blue: $\overline{x}$ = 64.6 days; grey: $\overline{x}$ = 79.9 days).

All males courted their potential mates, but not all successfully mated. However, there was no significant effect of treatment on whether copulation occurred ($F_{5,54} = 38.2$, p = 0.415).

Marking did not impact courtship latency ($\log_{10}$ $\log_{10}$: $F_{2,55} = 0.275$, p = 0.761) or copulation latency ($\log_{10}$: $F_{2,48} = 0.958$, p = 0.391). However, marking did effect copulation duration ($\log_{10}$: $F_{2,47} = 3.54$, p = 0.0368; Fig. 55a), with control individuals mating for longer than those that were marked with enamel paint ($\log_{10}$: $F_{1,40} = 4.98$, p = 0.0314). But there was no significant difference between the four paints used ($\log_{10}$: $F_{3,29} = 0.716$, p = 0.550; Fig. 55b).



a.

b.

Figure 55. The effect of marking method on copulation duration

a. Marking method

b. Marking paint colour

Black dots show means and 95% confidence intervals in seconds

Part three: The effect of thorax markers and Rhodamine B ingestion on female longevity

The thorax paint-marking techniques significantly reduced overall female lifespan (squared: $F_{2,36} = 3.29$, $p = 0.0488$; Fig. 56). The unmarked females lived for a mean average of 78.2 days - ~23 days longer than water-marked females ($\overline{x} = 55.1$ days; squared: $F_{1,17} = 5.63$, $p = 0.0297$) and ~21 days longer than yellow-marked females ($\overline{x} = 57.4$ days; squared: $F_{1,17} = 6.62$, $p = 0.0198$). They also lived ~14 days longer than grey-marked males ($\overline{x} = 64.4$ days), but this difference was not statistically significant (squared: $F_{1,17} = 2.65$, $p = 0.122$). But there was no significant difference in the pattern of proportional survivorship (Cox proportional hazards model: $X^2 = 4.02$, d.f. = 2, $p = 0.134$; Fig. 57).

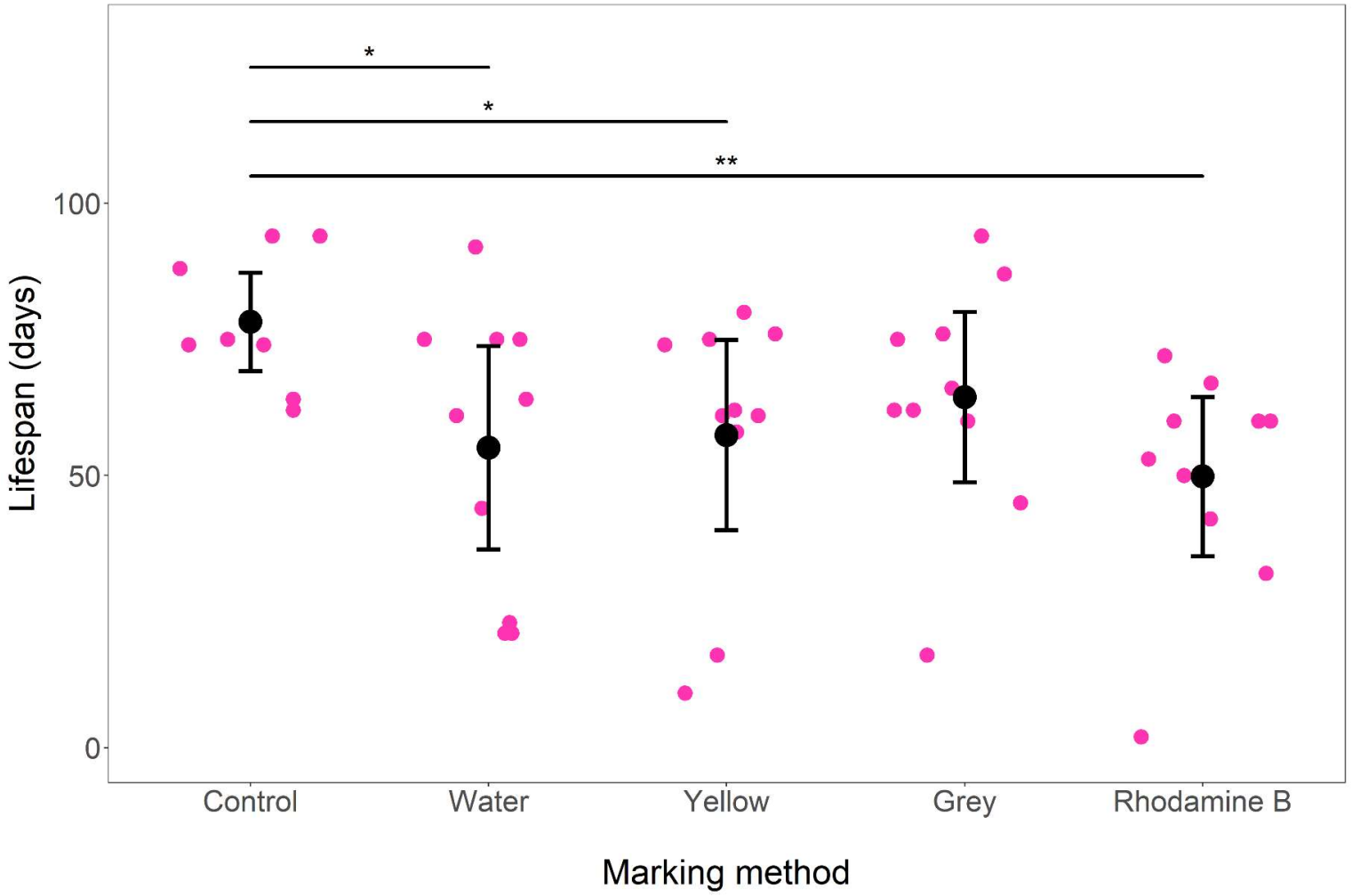


Figure 56. The effect of marking technique on female lifespan

Black dots show mean and 95% confidence intervals in days

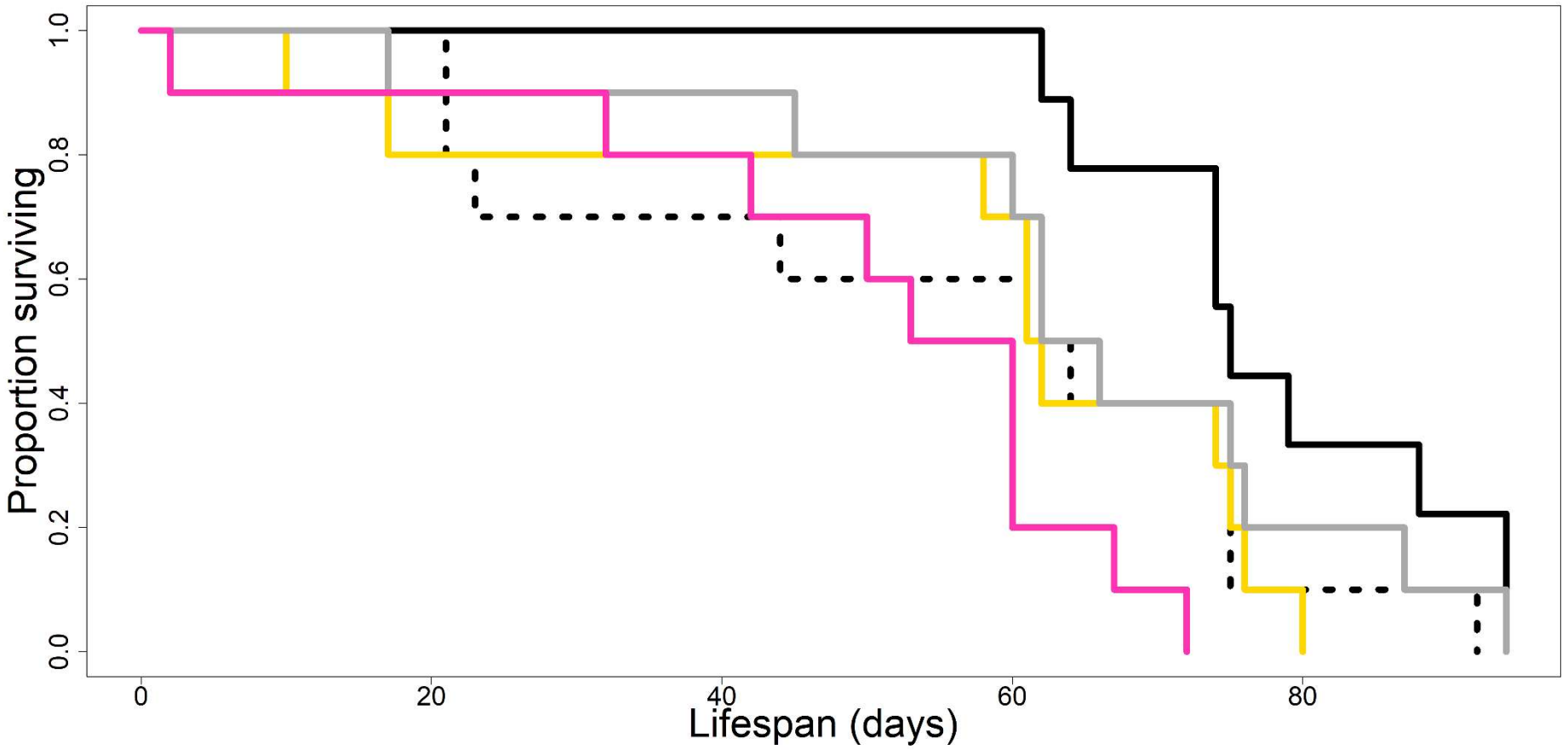


Figure 57. Proportional survivorship curves for all marking techniques

Black solid line = unmarked control

Black dashed line = water-marked control

Grey = grey-marked

Yellow = yellow-marked

Pink = Rhodamine B-marked

Marking females via ingestion of Rhodamine B significantly reduced their lifespan ($F_{1,17}$ = 13.3, p = 0.00198; Fig. 55) and survivorship (Cox proportional hazards model: X^2 = 14.1, d.f. = 1, p << 0.001; Fig. 56). Their mean average lifespan was ~28 days shorter than the control females (Rhodamine B: $\overline{x}$ = 49.8 days; control: $\overline{x}$ = 78.2 days).

4. iv. Discussion

None of the marking techniques, or handling, had a negative impact on male *D. melanogaster* longevity. Therefore, either of these marking techniques would be suitable for future studies investigating the impact of environmental and social factors on their longevity, and would be comparable to existing longevity studies.

There was no impact of thorax paint markings on the likelihood of the male to initiate courtship or to successfully copulate, so this marking technique could be used in studies where courtship or mating was needed. However, it did impact the duration of copulation so this would need to be considered. If being used in a future copulation duration investigation, all individuals would need to be marked using the same technique to avoid this impacting the results.

Male *Drosophila* court females by vibrating their wings to produce a song (Spieth, 1952, Spieth, 1974). Given that wing movements play such an important role in courtship, it is highly likely that any alterations made to the wings will have a detrimental effect on the male's ability to court a female. For this reason, I did not test the effect of wing markings on reproductive behaviours, but initial observations suggest that males with wing markings are

not able to effectively raise their wings, so this technique will likely not be optimal for studies investigating *Drosophila* reproductive behaviour.

There are a range of other marking techniques that could be used. These include via consumption, mutilation, tagging, dust marking and genetic marking (Hagler and Jackson, 2001, Dickens and Brant, 2014, Verspoor et al., 2015, Manier et al., 2010, Gill et al., 2012, Unruh and Chauvin, 2012). It is also possible to track and identify individuals in a laboratory setting without marking them, using computer tracking software. For example, Martin (2004) tracked the speed and direction of mated and virgin *Drosophila* using EthoVision software (Noldus, The Netherlands).

Marking via ingestion was tested in females in this study. Ingestion of Rhodamine B did reduce lifespan in females, although this was expected as it has previously been reported to be toxic (like many fluorescent dyes) (Kamimura, 2007, Marking, 1969, Alford et al., 2009). This marking technique could not be used for longevity studies, and if used for any behavioural studies it would need to be carefully balanced across the treatments to ensure this detrimental impact of the dye did not impact the results of the study. This would be necessary because unlike the other two techniques, it would not be possible to mark two individuals in different colour.

Interestingly, despite the thorax paint having no impact on male longevity, it did impact female longevity. Although there appeared to be no difference in longevity between the paint colours used so marking multiple females in different colours would be possible. It is unclear why this sex difference was found; however, this should be considered in any future studies

including female marking. Further investigation should be completed to understand why these sex differences exist, and to discover alternative marking techniques.

Appendix 5: An analysis of three density manipulation techniques

5. i. Introduction

Natural environments, and therefore population size and density, are constantly fluctuating and so it is important that we attempt to understand the potential impact that varying densities may have on individual behaviours and subsequent fitness. A number of studies have already tackled this issue (Flay et al., 2009, Masonjones and Rose, 2019, Toth et al., 2015, Liu et al., 2018, Bocci et al., 2013), but to my knowledge none have investigated the impact of population density of oviposition behaviours.

This study will help to provide a greater understanding of techniques that could be used to experimentally manipulate housing density. In this study I used two strains of *D. melanogaster,* including one where the females produce eggs that fluoresce Green Fluorescent Protein (GFP). Using this strain, alongside a wild-type (WT) strain, would enable me to directly compare oviposition decisions of two females in future studies.

5. ii. Methods

D. melanogaster were collected from LHm stock: two different stocks were used in this study – one wild type (WTs), and one with *D. melanogaster* ubiquitously fluorescing Green Fluorescent Protein (GFPs) transformed by Belote and Pitnick (*LHm pBac[Ubnls-EGFP, ProtB-EGFP]6A;* Belote and Pitnick (n.d.)). They were maintained in a 12hour light:dark cycle (08:00 – 20:00h GMT) at 25°C. Approximately 20–30 individuals were housed in a 40ml vial, containing 7ml of a standard agar-based medium (Appendix 1. i). All vials were mixed every seven days to minimise inbreeding.

Part one: Number of F0 parent flies

Parent flies were collected from vials with a standardised adult fly density: six females and six males were left to breed for three to four days. To ensure their virginity, F1 test flies were removed from these vials within six hours of eclosion. They were then anaesthetised using ice, so that they could be sexed and placed in the correct treatment.

For high density treatments, five males and five females were placed in a vial for 24 hours, and left to copulate and oviposit eggs. For low density treatments, just one male and one female were added to the vial and left for 24 hours. Number of offspring that successfully reached the adult life stage were counted 21 days after the parent flies were removed. This was repeated using both WT and GFP strains.

Part two: Egg counts

Ten males and ten females were placed in a Petri dish (9cm in diameter) and allowed to copulate and oviposit eggs into the 30ml of standard agar-based medium provided. After 18 – 22 hours, eggs were removed from these dishes using a pointed needle.

To set up the high-density treatments, 60 eggs were translocated to a standard 40ml vial with agar-based medium. Ten eggs were placed in each vial to set up low density treatments. Number of successful offspring were counted 21 days after the eggs were placed in vials. This method was also repeated for both strains of *D. melanogaster.*

Part three: Volume of eggs pipetted

Petri dishes for egg collections were set up as described above. Again, 20 hours later eggs were removed from the Petri dishes: they were suspended in phosphate buffer saline (PBS) and dislodged from the agar-based medium using a flat-edged spatula. The eggs were then washed into an Eppendorf tube and centrifuged so that that they rested at the bottom, then the excess PBS was removed (Clancy and Kennington, 2001).

Eggs were then pipetted (as described by Clancy and Kennington (2001)) into a standard vial – 10µl for low density treatments and 100µl for high density. Finally, as previously stated, this was repeated for both strains and number of successful offspring were counted 21 days after egg transfer.

Statistical analysis

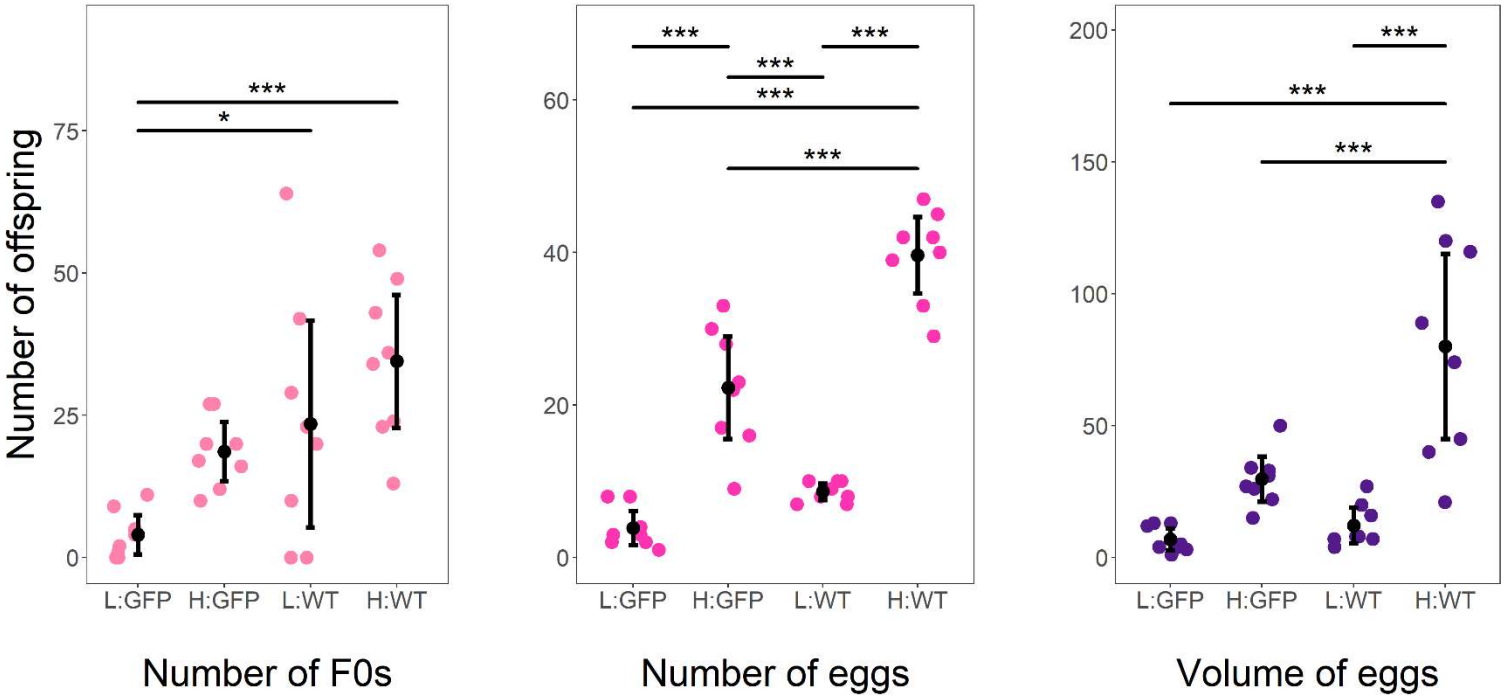
Statistical analysis was completed using RStudio version 4. 0. 3 (R Core Team, 2019). I analysed the effect of strain and density on offspring production using ANCOVAs and post-hoc TukeyHSD tests, then checked for normality using Anderson-Darling tests.

5. iii. Results

Part one: Number of F0 parent flies

High density vials with more F0 flies, produced significantly more successful offspring (offspring that reached adult life stage) that those containing fewer F0s ($F_{1,29}$ = 7.47, p = 0.0106; Fig. 57a). Strain also impacted fitness: WTs produced significantly more successful offspring than GFPs ($F_{1,29}$ = 14.2, p << 0.001; Fig. 58a). High density WTs produced significantly

more successful offspring than low density GFPs ($p << 0.001$), as did low density WTs ($p = 0.0344$).



a. b. c.

Figure 58. Number of offspring produced by wild type and GFP Drosophila, under high and low densities

a. Using different numbers of F0 parent flies

b. Counting out different numbers of eggs

c. Pipetting varying volumes of eggs

Black dots show means and 95% confidence intervals

L = low density

H = high density

GFP = ubiquitously GFP fluorescing *Drosophila*

WT = wild type *Drosophila*

Part two: Egg counts

Vials with a higher number of eggs transferred (set up for a high-density treatment) produced significantly more successful offspring than those with fewer eggs transferred (low density) ($F_{1,29} = 130$, $p << 0.001$; Fig. 58b). Once again, WTs also produced significantly more successful offspring than GFPs ($F_{1,29} = 26.2$, $p << 0.001$; Fig. 58b).

High density WTs produced significantly more offspring than low density WTs ($p << 0.001$). This same pattern was observed in GFPs ($p << 0.001$). High density WTs also produced significantly more offspring than high and low density GFPs (high: $p << 0.001$; low: $p << 0.001$). In addition to this, high density GFPs produced significantly more successful offspring than low density WTs ($p << 0.001$).

Part three: Volume of eggs pipetted

Vials with a larger volume of eggs transferred (for a high-density treatment) produced significantly more successful offspring than those with a smaller volume of eggs ($F_{1,29} = 135$, $p << 0.001$; Fig. 57c). As with the previous two studies, WTs also produced significantly more successful offspring than GFPs ($F_{1,29} = 33.8$, $p = 0.00131$; Fig. 58c).

High density WTs produced significantly more offspring than low density WTs ($p << 0.001$), however there was no significant difference between high- and low-density treatments in GFPs ($p = 0.188$). Plus, high density WTs produced significantly more offspring than low density and high density GFPs (high: $p << 0.001$; low: $p << 0.001$).

5. iv. Discussion

In both densities, and in all three techniques, there was often a difference between GFPs and WTs in the number of successful offspring they produced – with GFPs always producing fewer offspring. Chalfie et al. (1994) first discovered that GFP could be excited in living organisms (other than jellyfish where it originates); and since then it has been transformed into *Drosophila* and has been used in a vast range of studies, often as a marking technique at a whole organism level (Hagler and Jackson, 2001, Nakanishi et al., 1999, Marie-Orleach et al., 2014). However, GFP tagging should be used with caution as although it is rarely toxic to cells it can cause cell damage (Misteli and Spector, 1997), and therefore can be detrimental to lifespan, and can affect individual behaviours (Mawhinney and Staveley, 2011, Irvin et al., 2004, Rang et al., 2003). Given this, it is not surprising that fewer successful offspring are produced by *Drosophila* from the GFP strain – likely due to failure of sperm to fertilise eggs, and production of non-viable eggs.

With this difference, it would not be possible to directly compare the number or distribution of eggs oviposited by the two different strains – other techniques of identification between competitors would need to be considered. One such method would be to use a different sub-species of *Drosophila* as a competitor, as egg shape and size varies between sub-species (Markow et al., 2009, Lott et al., 2007). Although it is likely that there would be similar differences between behaviour and oviposition that would mean that direct comparisons between these two individuals would not be possible.

Rhodamine B has previously been used to stain sperm cells in *Drosophila,* via ingestion, to enable individual identification (Appendix 4, Kamimura, 2007). It is possible that this technique would also work for females' eggs, although further testing would need to be completed to confirm this.

A final alternative would be to place two marked *D. melanogaster* in the environment simultaneously, and film and track their movements so that it would be possible to work out which individual laid each egg at a later date. There are a range of marking techniques that could be used, including wing clipping, painting, and dust marking (Hagler and Jackson, 2001). This method would likely be highly effective, however a limiting factor would be the filming set-up, as only one replicate could be tested per available camera.

With such different advantages and disadvantages, if any future investigations also require two (or more) competing individuals, the suggested optimal competition technique would vary depending on the aims of the study and the limitations of those completing the study.

There were no differences in the number of offspring produced by parent flies, between low and high densities. As a result, it is likely that the female offspring that emerge from the high-density treatment would not have experienced a stressful environment, and therefore will not alter their oviposition behaviour when tested. In order for this method of density manipulation to be an effective stressor, the number of parent flies placed in each vial would need to be altered. With a higher number of parent flies in the high-density treatment, it is likely that there would be a significant difference between density treatments, but this requires further investigation.

Using the egg counting technique, in both GFPs and WTs low density vials produced fewer successful offspring than high density vials. Although this technique was time-intensive, it would be an effective method for controlling population density differences in future studies. Similarly, pipetting a set volume of eggs would also be an effective technique for the WT strain but not the GFP strain of *D. melanogaster*, as only WT low density vials produced fewer offspring. It is also worth noting

however, that there were significant differences between strains using both the counting and pipetting protocols, but this would not be an issue if any other of the aforementioned competition techniques were used – meaning the GFP strain was no longer needed.

In future investigations I would likely use the pipetting protocol to manipulate population density, as it was more effective than housing parent flies in vials, and less time-intensive than counting and transferring individual eggs. However, I believe with further investigation it would be possible to find the optimal number of parent flies required to generate a significant difference between low- and high-density treatments so that the high-density treatment would be an effect environmental stressor.

Appendix 6: Oviposition in virgins

6. i. Introduction

Wild *Drosophila melanogaster* lay on rotting fruits – a naturally patchy and ephemeral environment (Begon, 1982). Mated females are able to plastically adjust their oviposition behaviour in order to maximise their fitness by assessing potential level of competition their larvae may face (Malek and Long, 2020, Tait et al., 2020), and considering the energetic costs of larval travel (Schwartz et al., 2012).

Although they will not lay fertilised eggs, virgin females also regularly oviposit (Menon et al., 2014, Sheeba et al., 2001, Howlader and Sharma, 2006). However, without the drive to find suitable oviposition sites for potential offspring, it is likely that their oviposition decisions will vary from those of their mated conspecifics. Here I test this using a fully factorial design: virgin and mated females, placed on clustered and dispersed oviposition resources.

6. ii. Methods

Stock *D. melanogaster* originated from a Canton-S laboratory stock and were housed in 40ml vials containing 7ml of standard agar-based medium (Appendix 1. i). Around 20 – 30 *D. melanogaster* were housed in each in each vial, and all vials were pooled and randomly redistributed every seven days to minimise any effects of inbreeding they might otherwise occur within vials. All rearing and experiments were conducted on a 12hour light:dark cycle (08:00 – 20:00h GMT), at 25°C.

Parent generation vials were set up with six males and six females per vial. Female test flies (and standard mate flies) were collected from these vials under light ice anaesthesia, within six hours of eclosion to ensure virginity. They were then housed in vials, until aged to seven days.

Virgin versus mated females

After the seven-day ageing period, the flies were split into two groups: virgin and mated females. The half that were assigned to the mated treatment, were then paired with a standard seven-day old males, in a standard agar vial supplemented with ~0.003g of yeast granules. If the pair successfully mated, the female was transferred to a Petri dish (140mm in diameter). The virgin females were also transferred to a Petri dish at the same time.

Oviposition resource distributions

The Petri dishes contained four patches of agar-based medium (each 22mm in diameter) arranged in one of two different spatial distributions. These patches were arranged in either a dispersed pattern (four equidistant points around the circumference of the Petri dish, at an interpatch distance of 100mm), or placed in a clustered pattern (a square arrangement in the centre of the Petri dish) – as described in Chapter 5. To keep surface area constant between the two treatments, patches were placed at a 3mm distance from the edge of the Petri dish and other patches – where accessible surface area of patch edges would otherwise be reduced by adjacent edges. This arrangement resulted in four treatments with 12 females in each: virgin and mated females housed on clustered and dispersed oviposition resources.

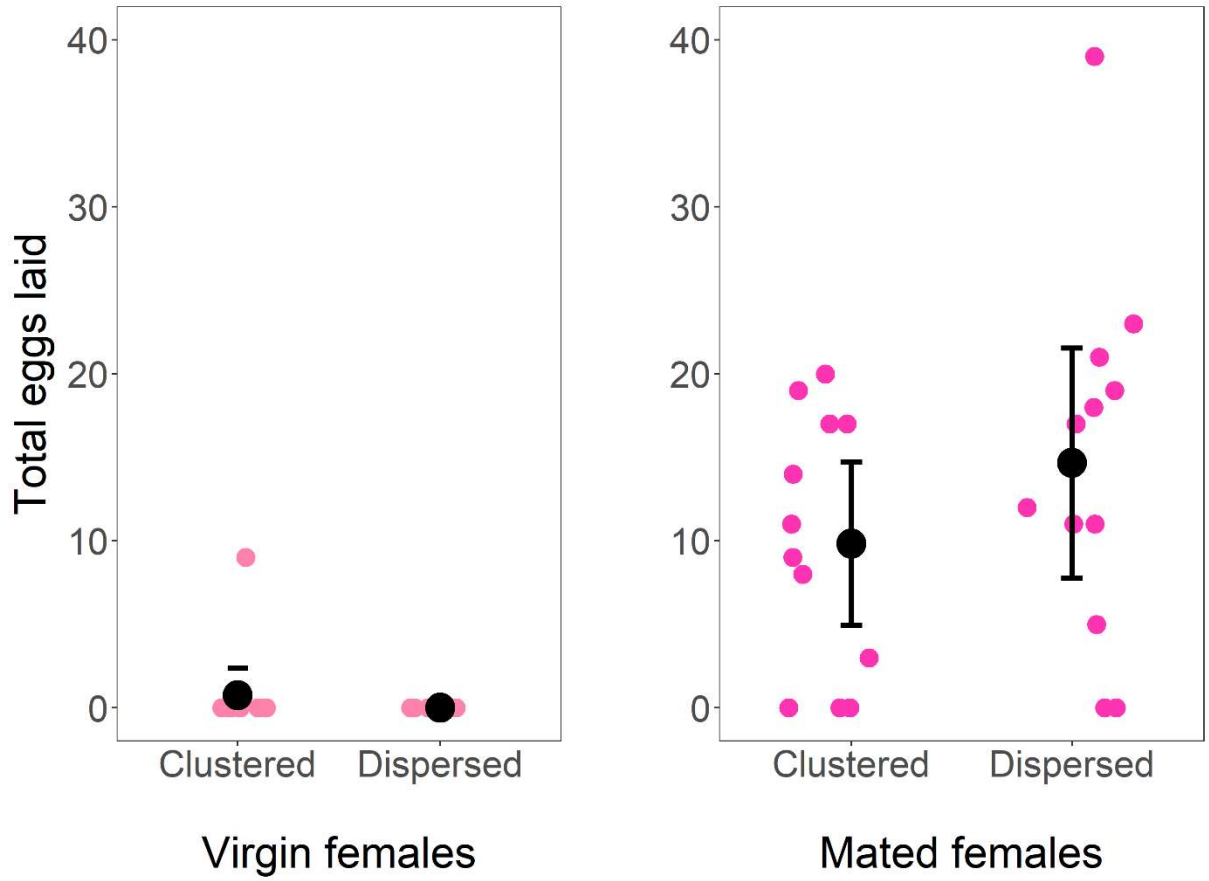
Females were left for 18 – 20 hours in constant light to oviposit eggs, before they were removed and killed by freezing to -18°C so that left wing measurements could be taken (as described in Chapter 2). The number of eggs that each female laid was counted, and the position that each egg was located was noted.

Statistical analysis

All statistical analysis was completed in R 4. 0. 3 (R Core Team, 2019). To analyse the effect of female mating status and oviposition resource distribution on likelihood to lay eggs, I used generalized linear models with a binomial error distribution. As all but one of the virgin females did not lay eggs, no further analysis was completed.

6. iii. Results

Not all females oviposited eggs; as expected mated females were significantly more likely to lay than virgin females on both clustered (general linear model with binomial errors; $X^2 = 20.4$, $p << 0.001$) and dispersed resources ($X^2 = 10.8$, $p << 0.001$); see Fig. 59. However, there was no effect of oviposition resource distribution on likelihood to lay in virgin females ($X^2 = 6.88$, $p = 0.232$), or in mated females ($X^2 = 24.3$, $p = 0.614$).



a. b.

Figure 59. The total number of eggs produced on clustered and dispersed oviposition resources

a. Virgin females

b. Mated females

Black dots show means and accompanying 95% confidence intervals

6. iv. Discussion

As expected, I found that fewer virgin females laid eggs than mated females. This is likely because virgins were not driven by the need to find suitable oviposition sites for their potential offspring. It was surprising that only one virgin female laid eggs, as Menon et al. (2014) found that virgin females regularly laid eggs after four to five days. But this could be an effect of the constant light,

interrupting the circadian regulation of oviposition (Sheeba et al., 2001, Howlader and Sharma, 2006).

As only one of the virgin females oviposited eggs, it was not possible to gain any insight into the difference in oviposition decisions between virgin and mated females. Further investigations with virgin females would need to be completed to gain a greater insight into the variation of their oviposition decisions.

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