

EFFECTS OF SPERM COMPETITION ON
RESOURCE ALLOCATION IN MALE
DROSOPHILA MELANOGASTER

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ABSTRACT

Evolution and sexual selection have favoured the development of morphological, physiological and behavioural adaptations in males to handle sperm competition risk. Some males however, are also able to respond to this risk within a much shorter time period. Their plastic phenotype allows them to adapt according to their ever-changing environment. There are, however, costs and limitations to plasticity and there is a trade-off between reproduction and survival. In addition, the investment into plastic sperm-competitive traits creates supplementary energy costs. The aim of this project was to study the trade-off between survival and male *Drosophila melanogaster* responses to sperm competition, and to look at the effect these responses have on both fitness and resource allocation. In a first experiment, the interactions between various nutrient treatments, sperm competition, and survival of virgin males were studied. It was found that males were only able to invest and benefit from sperm-competitive traits with a sufficient energy supply. Male survival was optimum at intermediate nutrient level and, when conditioned to sperm competition, they lived longer and were less active than males not conditioned to sperm competition. In a second set of experiments, using three nutrient levels and sperm competition risk, male survival and reproductive success were measured. It was found that males subjected to low nutrient treatment increased their mating duration and invested more in courtship, which suggests that terminal investment was taking place. This study has added evidence to the terminal investment concept, and has contributed to the research on the energy trade-off between reproduction, socio-sexuality and the individuals' experience of their environment.

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DECLARATION

I declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1.1 GENERAL INTRODUCTION

An individual's relative fitness is measured by their reproductive success relative to the reproductive success of other individuals; this is why there is such an important competitive element to reproduction (Emlen & Oring 1977). The aim of this project was to investigate and understand how the social and physical environment affects the reproductive fitness of male fruit flies, *Drosophila melanogaster*, and therefore their relative fitness.

To reproduce, males must first gain access to a mate, and in most cases, there is competition between males for females (Birkhead & Møller 1998). On top of pre-copulation competition (Payne 1979, Husak & Swallow 2011), males in most species also have to compete against rivals at a post-copulatory level because their reproduction system is polyandrous (Kvarnemo & Simmons 2013). Polyandry occurs whenever females mate with multiple males. There is evidence that this occurs in at least 14 major taxonomic groups (review by Taylor et al 2014).

However, within some of these groups, polyandry can range from 0 % to 100 % depending on the species. This is the case for example in birds, mammals, bony fish (*Osteichthyes*) and aquatic reptiles. Insects and cartilaginous fish (*Chondrichthyes*) show widespread polyandry in comparison to other taxonomic groups. This wider spread polyandry could be due to the fact that females from these two groups are

able to store sperm for extended periods of time (Parker 1970, Wourms 1977, Birkhead & Møller 1998).

Females obtain a range of direct and indirect benefits from mating multiply, which explains the prevalence of polyandrous species across most animal taxa (Birkhead & Møller 1998). For instance, re-mating may directly benefit the females through sperm replenishment and the supply of nutrients contained in the seminal fluids or nuptial gifts transferred by the male (Arnqvist & Nilsson 2000). There can also be indirect benefits from mating multiply such as the ability for females to fertilise their eggs with sperm from males with closer genomic compatibility (Tregenza & Wedell 2002), which can increase the fitness of their offspring (Arnqvist & Nilsson 2000).

A major implication of polyandry that has been singled out by Parker (1970) is that females will inevitably retain ejaculate from different males that will compete over the fertilisation of a set of ova. This is called sperm competition. This is why males have been driven to develop a wide variety of morphological, physiological and behavioural adaptations to outcompete the sperm of other males (Parker 1970, Birkhead 1995, Simmons 2001). One of the most common adaptations is the use of copulatory plugs to prevent other males from mating with a female by leaving a plug within her reproductive tract (Birkhead & Møller 1998). Evidence for this can be found in species such as snakes, spiders, bees, nematodes and mammals (Rothschild 1955, Devine 1975, Martan & Shepherd 1976, Matsumoto 1993, Hodgkin & Doniach 1997). Another common adaptation is the morphological change of the male's reproductive organs to help displace the sperm of rival males within the female reproductive tract; there are many examples of this in insects

(Waage 1979; Gage 1992; Harshman & Prout 1994; Von Helversen & Von Helversen 2004). The idea that testes size reflects promiscuity goes back as far as 1676 (Willoughby 1676), but it is only in 1970 that Parker re-affirmed this idea with theory and empirical data. He suggests that in response to sperm competition risk, male ejaculate expenditure should increase (Parker 1970, 1998). Consistent with this idea, there is evidence that there is a positive correlation between testes size and sperm competition risk (Gage 1994, Harcourt et al 1995, Birkhead & Møller 1998, Byrne et al 2002) and between testes size and sperm number per ejaculate (Møller 1988). Sperm competition game also predicts that sperm should increase in number but remain small (Parker 1982). Increasing sperm quantities increases males' chances of fertilisation (Parker 1970, 1990, Chargé et al 2016). However, not all studies support the idea that sperm should remain small. Several studies have demonstrated that as a response to sperm competition, males have evolved to produce longer and larger sperm (Pitnick 1993, Pitnick & Markow 1994, LaMunyon & Ward 1999).

Additional theories suggest that when levels of sperm competition are fluctuating, males may benefit by plastically adjusting accordingly their behaviour to the level of perceived sperm competition risk (Ball & Parker 1998, Delbarco-Trillo 2011, Kelly & Jennions 2011). The ability to produce a phenotype that matches a range of environments is called plasticity or plastic phenotype (DeWitt et al 1998, West-Eberhard 2003). Fruit flies, *Drosophila melanogaster*, are ideal to study plastic adaptations to sperm competition risk thanks to their versatility, fast reproduction rate and the fact that their mating behaviours are easily observed. Indeed, on top of adjustment of sperm and ejaculate quantity (Manier et al 2010, Lupold et al 2011, Garbaczewska et al 2013), male *Drosophila melanogaster* have been found to

exhibit a number of additional plastic responses to sperm competition. When males detect the presence of rivals through the combination of two of the following cues: sound, smell and touch (Bretman et al 2011a), they were found to increase their mating duration (Friberg 2006, Bretman et al 2009, Bretman et al 2010). As well as mate guarding, an increased mating duration may allow males to transfer more sperm to the females, which results in a higher volume of eggs being laid by the female and also increases offspring's survival to adulthood (Bretman et al 2011b). This extended copulation duration also allows males to strategically transfer seminal fluid proteins (Wigby et al 2009). Male *Drosophila melanogaster* transfer approximately 80 different proteins and peptides to females during copulation, which have various effects on female physiology and behaviour (Prout & Clark 2000, Chapman 2001, Chapman & Davies 2004, Harshman & Zera 2007).

However, mating is energetically expensive; in *Drosophila melanogaster*, once reproduction is initiated, survival probability begins to decline in both sexes (Partridge & Farquahr 1981, Partridge 1988, Cordts & Partridge 1996). In addition to this, there are also costs and limits to plasticity; individuals with a fixed development (no plasticity) only require 'production machinery' to express a given fixed phenotype. In the case of a plastic organism, it can require additional steps such as sensing the environment, processing information and regulating mechanisms (DeWitt et al 1998, Table 1.1).

TABLE 1.1. Adapted from DeWitt et al (1998). The potential costs to plasticity.

a) Costs of plasticity
<i>Maintenance cost</i> - plastic development requires sustenance of the sensing and regulating system.
<i>Production cost</i> – plastic characteristics production could exceed the production cost of a fixed individual's genotypes.
<i>Information acquisition cost</i> – the process of acquiring necessary information to adapt phenotype accordingly can be costly in terms of risks taken with regards to predation, this also means it is time not spent foraging for food or reproducing.
<i>Development instability</i> – imprecise development that could lead to reduced fitness.
<i>Genetic cost</i> – genes involved in producing plasticity could be linked with genes that decrease fitness (linkage), they could have a negative effect on top of the plastic characteristic (pleiotropy) or could alter the expression of other genes (epistasis).

Therefore, if a specific response to sperm competition is more costly than a “fixed” response, males will need to trade-off this extra cost with another life-history trait or else suffer effects detrimental to their survival (Williams 1957, 1966, Reznick 1985, 1992, Stearns 1989). It was predicted that, because there are costs to plasticity, male longevity should decrease because of sperm competition (Bretman et al 2009, Wigby et al 2009). However studies found that sperm competition increased virgin male survival (Bretman et al 2013, Moatt et al 2013). This means that the effects and costs sperm competition has on males are still not fully understood and that the trade-off between reproduction and survival in male *Drosophila* needs to be studied further.

An effective way to investigate the condition dependence of male sperm-competitive traits is through altering male food availability. General predictions are that when there is sufficient food available, males are able to balance energy requirements between somatic maintenance and reproduction (Kirkwood & Rose 1991, Cox et al 2010), which should lead to an increased level of trait expression.

However, when males are nutritionally stressed, males will have to trade-off between reproduction and survival (Kirkwood 1977), and this is where costs to sperm-competitive traits may become apparent. In addition to this, the majority of sperm competition-related experiments in fruit flies are carried out on an optimum standard laboratory yeast-sugar diet, which is atypical of actual environmental conditions. Natural fruit fly food sources are generally not as rich in protein as the diets found in laboratory cultures (Partridge 1988, Lushchak et al 2013). Therefore, on top of the fact that dietary restriction helps understand better the cost of sperm competition by forcing males into a trade-off, using this method would also allow me to look at the effect of sperm competition under more natural conditions.

The first nutritional stress experiments were performed on rats in the 1930s through restricting calorie intake (McCay et al 1935). These involved reducing the quantity of food, and directly controlling calorie intake. However, in invertebrates, this method is known as dietary restriction (DR). This is because the restriction is achieved through the dilution of nutrients, and there is no direct control of caloric intake (Min & Tatar 2006). Cotton et al (2004) found evidence in the stalk-eyed flies, *Cyrtodiopsis dalmanni*, to support the predictions that individuals maintained under dietary restriction will have to compromise reproductive capacity to increase allocation of nutritional resources into somatic maintenance (Piper & Partridge 2007). They investigated the effects dietary restriction had on the reproductive traits. When males were subjected to nutritional stress, they had to re-allocate energy originally spent on developing eye-span (a sexual signal for females) on necessities such as the development of wingspan. As nutritional stress

increased, the eye-span of males decreased (condition-dependence), whilst on the other hand, non-sexual traits indicated a much weaker condition-dependence. In *Drosophila melanogaster*, DR can be achieved by diluting the yeast and sugar of the standard food solution (Mair et al 2005). There are important effects and repercussions of the quality of fruit fly larval rearing environment on later mating success. In males, it was found that the number of sperm transferred and stored is depend on nutrient availability at the larval stage (Amitin & Pitnick 2007, McGraw et al 2007). When dietary restriction (DR) occurs at the adult stage of fruit flies, a number of studies have found that the reproductive fitness of females was affected and that the rate of egg-laying decreased (Chippindale et al 1993, Chapman & Partridge 1996, Lee et al 2008, Tatar 2011, Lee 2015). Some work has been carried out on the effects of DR on males, but only a few studies have looked at the effect on reproductive fitness. One of these studies was carried out by Fricke et al (2008) where they studied the condition-dependence of sperm-mediated traits. They found that, when two males mated with the same female, nutrition had no effect on mating duration or paternity share. However, in their experiments, there was no sperm competition conditioning prior to males being placed in a mating arena. The only sperm competition risk detected by the males was from chemical cues left on the female by the other male.

There is a well-documented increase in life span due to dietary restriction in a variety of species (yeast: Jiang et al 2000; nematodes: Houthoofd et al 2005; fruit flies: Mair et al 2005, Tatar 2011; rodents: McCay et al 1935). Therefore, it is important to carry out preliminary work in order to test what level of dietary restriction is needed to truly handicap males and achieve the wanted effect on fruit flies. When exposing flies to a range of different food dilutions, their life span is at

its highest at an intermediate food level (Partridge et al 2005). The results form a dome-shaped curve as can be seen in Figure 1.1, with a graded decline either side of the optimum food concentration level.

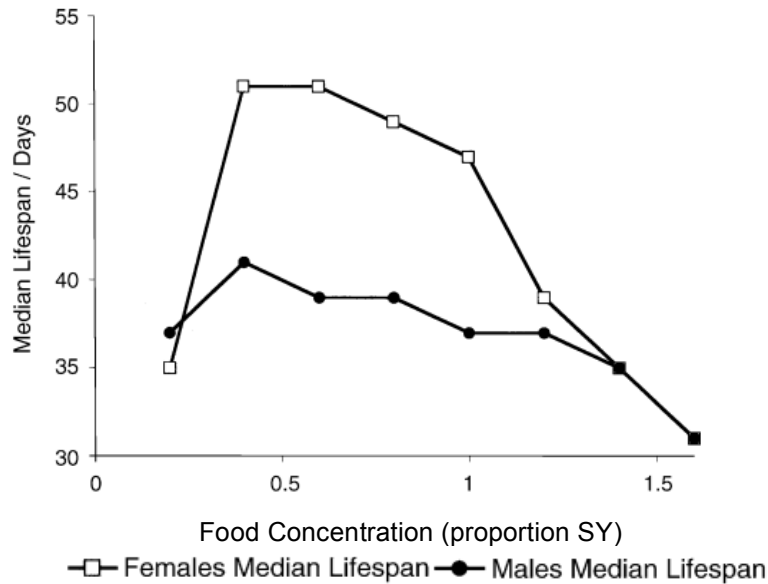


FIGURE 1.1. *Drosophila melanogaster* life span response to sugar and yeast dietary restriction, forming a dome-shaped curve with an optimum life span between 0.4 and 0.6 SY per litre (Figure from Partridge et al 2005, p 940)

This effect on life span was originally attributed to the reduction of carbohydrate intake and therefore the reduction of energy intake (Iwasaki et al 1988, Masoro et al 1989). But recently, researchers have associated this decrease in longevity to the intake of particular nutrients. A number of studies have shown that the intake of protein and particular amino acids plays a vital role in the modulation of life span (Chippindale et al 1993, Mair et al 2005, Piper et al 2005, Lee et al 2008, Fanson et al 2009).

In *Drosophila melanogaster*, an additional complication to this is that the carbohydrate:protein (C:P) ratio also has a critical effect. It was found that life span increases as the proportion of sugars increase in relation to proteins, but this also

results in low reproductive success. Males were also found to decrease ejaculate size when there are insufficient proteins (Gage & Cook 1994). Carbohydrate-rich diets result in high levels of somatic maintenance, but yield low reproductive success. As proteins are increased in the diets, resources are re-allocated from somatic maintenance towards reproduction and the investment of energy into building lipid reserves in eggs and sperm (Chippindale et al 1993, Mair et al 2005, Fanson et al 2009, Gage & Cook 1994). However, egg production dropped when females were maintained on food with a very high C:P ratio. This is because although proteins are vital for reproduction, they can also be detrimental due to the toxicity of some macromolecules (Min et al 2007, Piper & Partridge 2007, Lee et al 2008, Fanson et al 2012).

The aim of this project was to study the trade-off between survival and the responses of male *Drosophila melanogaster* to sperm competition, and to look at the effects these responses have on reproductive fitness and resource allocation. To do this, first I needed to determine what level of dietary restriction would force males into a nutritionally-restricted state. Therefore, in a first experiment covered in Chapter 2, I studied the effects sperm competition risk had on male life span and activity in a number of nutritionally restricted environments. I was able to identify three diets that handicapped males at different levels (high, medium and low nutritional stress). Following on from this, in a second experiment (covered in Chapter 3), males were conditioned to sperm competition and one of these three diets. This allowed me first to test the effects of sperm competition on male reproduction under various nutritional stresses, and secondly to investigate the cost of sperm competition and resource allocation in male *Drosophila melanogaster*. The results are discussed in Chapter 4.

2.1 INTRODUCTION

In order to identify a level of nutritional restriction that forced males to reduce their activity and/or longevity, I maintained newly-eclosed males on a medium that varied in concentration from 2.5% to 200% of normal yeast and sucrose concentration. I predicted that males would achieve optimum life span on intermediate levels of nutrient, with life span declining at either end of the scale. Counter-intuitively, I also predicted that exposure to sperm competition risk would increase male longevity. The effects of nutrition on activity are less predictable, but I expected that activity would decline at low nutrient levels.

2.2 METHODS

The flies used in this experiment were first generation (F1) virgin *Drosophila melanogaster* males, from a cross between four female virgin Oregon-R and four Canton-S males. They were kept in a 40 ml vial on 7ml of standard sugar-yeast medium (50 g of yeast per litre) (Bass et al 2007) at 25°C in a 12-hour light cycle. After 9 days, parent flies were removed and disposed of before the eclosion of their first offspring. Virgin F1 offspring were collected (up to six hours after eclosion), sexed under a light CO₂ anaesthesia and placed in their respective treatments.

For seven days, virgin F1 males were maintained in 40 ml vials that contained 5 ml of a given food treatment. They were placed on one of seven diets: 3.12, 6.25, 12.5, 25, 50, 100 and 200g of yeast and sugar per litre (refer to Appendix A. 1 to see the diet treatment recipe) called: nutrient level 1, 2, 3, 4, 5, 6, and 7 respectively. Males were further split into two sperm-competition-risk treatments: high sperm competition (+SC), which consisted of placing a group of five males into a food treatment vial, and low sperm competition (-SC), which consisted of placing a single male in a food treatment vial (Figure 2.1).

After seven days of being conditioned to treatments, flies were placed into individual 5 mm diameter glass tubes plugged with technical agar (1.5 g agar/ 100 ml) at one end and cotton wool at the other. The tubes were then placed in a *Drosophila* activity monitor (DAM2, Trikinetics, Waltham, MA, USA), where flies were monitored for movement using an infrared beam until their death (Figure 2.2). Male activity was measured by calculating the amount of time the infrared

beam had been activated whilst alive. In this experiment, resistance to starvation in the *Drosophila* activity monitor (DAM) was a proxy for male longevity under a range of stress levels. After an inactivity of 30 minutes in the DAM, flies were considered dead; longevity was measured by calculating the amount of time males took to die in their tubes in the DAM. The DAM was kept at 25 °C with 24 hour light. A total of 154 individuals were tested (see Table 2.1 for sample sizes).

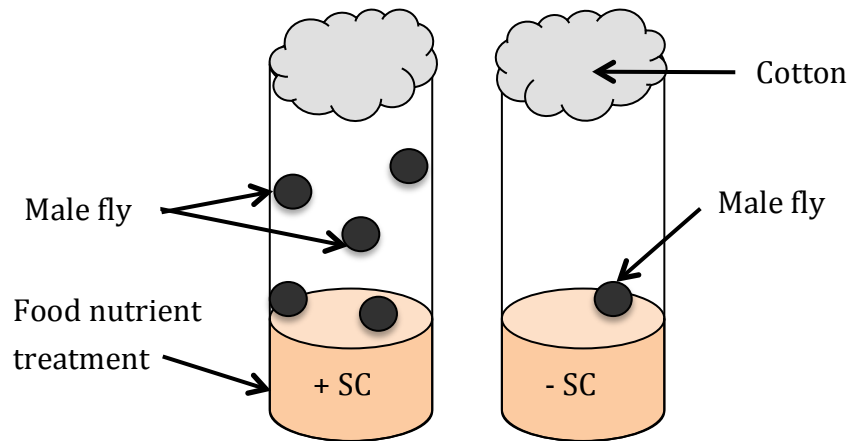


FIGURE 2.1. *Illustration of male sperm competition and nutrient treatment conditioning. Five males were placed together in a vial to create a high sperm competitive environment (+SC). A male was placed alone in a tube creating a low sperm competition environment (-SC). Food nutrient treatment corresponded to one of the seven experimental nutrient treatments.*



FIGURE 2.2. *A Drosophila Activity Monitor: 32 individual flies can be tested at the same time (one fly per hole) and, as a fly walks in the tube, it triggers an infrared beam. As the beam is*

interrupted by the fly's movement, this discontinuity is added to an overall fly activity count (Trikinetics catalogue).

TABLE 2.1. Nutrient levels and sample sizes for each treatment in Experiment 1.

Nutrient level	1	2	3	4	5	6	7
Yeast & sugar (g/L)	3.12	6.25	12.5	25	50	100	200
+SC	7	14	15	8	15	14	15
-SC	4	11	11	10	10	10	10

Only half the males maintained on nutrient level 1 survived to the DAM stage.

Statistical analysis:

Statistical analyses were performed on R (v. 3.2.1). Longevities (age in hours spent active in the DAM tube before death) were analysed using linear models, and survival patterns (proportion of individuals remaining alive) were analysed using Cox proportional hazard followed up with analyses of deviance. Survival pattern figures were performed on STATA (v. 12). Male activities (number of times the IR beam was activated) was also analysed using linear models. Differences between pairs of treatment were tested with *post hoc* Tukey test. Linear models were carried out with all factors and interactions, and then model simplifications were carried out by removing non-significant factors. Source vial for male was treated as a random effect in analyses. Results should be interpreted with caution due to small sample sizes.

2.3 RESULTS

Effects of treatments on male longevity and survival patterns.

There was a vial effect on male longevity in this experiment ($F_{74,66}=1.64$, $p<0.02$), which was accounted for in the following results by including vial identity as a random effect in all statistical models.

Sperm competition treatment had a significant effect on how longevity was influenced by nutrient treatments (interaction between sperm competition and nutrient: $F_{6,66}=4.13$, $p<0.001$; Figure 2.3). There was an equivalent interaction in the survival patterns ($X^2 = 18.10$, $p<0.001$). A *post hoc* test showed that sperm competition only had a significant effect on male's longevity at nutrient level 3 ($p<0.001$, all others $p>0.9$, clearly observed in Figure 2.3 and 2.4.).

Sperm competition had an effect on longevity ($F_{1,66}=5.52$, $p<0.01$). High sperm competition males overall lived significantly longer (+SC: 34.44 h) than the other males (-SC: 30.61 h) after being subjected to starvation conditions in the DAM, but, as mentioned before, this was only the case for +SC and -SC males on nutrient level 3. Male survival patterns were also affected by sperm competition ($X^2 = 4.65$, $p<0.05$; Figure 2.5). Males on the -SC treatment had a 1.44% higher risk of death per hour than +SC males. It was 5% more hazardous for males to be conditioned to -SC at nutrient level 3, as seen in Figure 2.4.C.

As expected there was a strong nutrient effect on longevity and survival patterns (longevity: $F_{6,66}=28.60$, $p<0.001$, Figure 2.3; survival pattern : $X^2 = 58.40$, $p<0.001$, Figure 2.6). No more than 25% of males exposed to nutrient levels 1 or 2 survived over 20 hours. From nutrient level 3 onwards, over 75% of flies survived for more

than 20 hours. A *Post hoc* test showed that only the longevity of males maintained on nutrient level 1 and 2 were significantly different from all other longevities at the other nutrient levels (Tukey test, $p < 0.001$, all other $p > 0.4$), even though mean male longevity appeared to be distributed in the form of a dome-shaped curve, as shown in Figure 2.3.

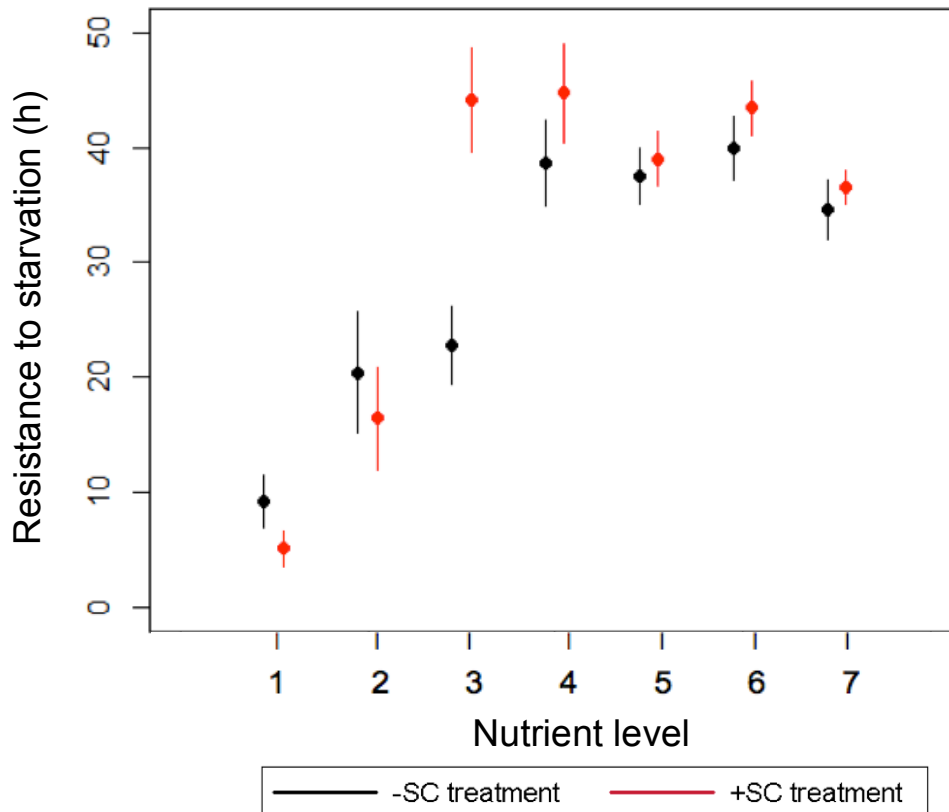


FIGURE 2.3. The effects of sperm competition and nutrient on male longevity. The interaction between the two factors was highly significant ($P < 0.01$). Longevity of males from the +SC treatments was longer than the one of -SC males at nutrient level 3 ($p < 0.01$). Mean \pm s. e.

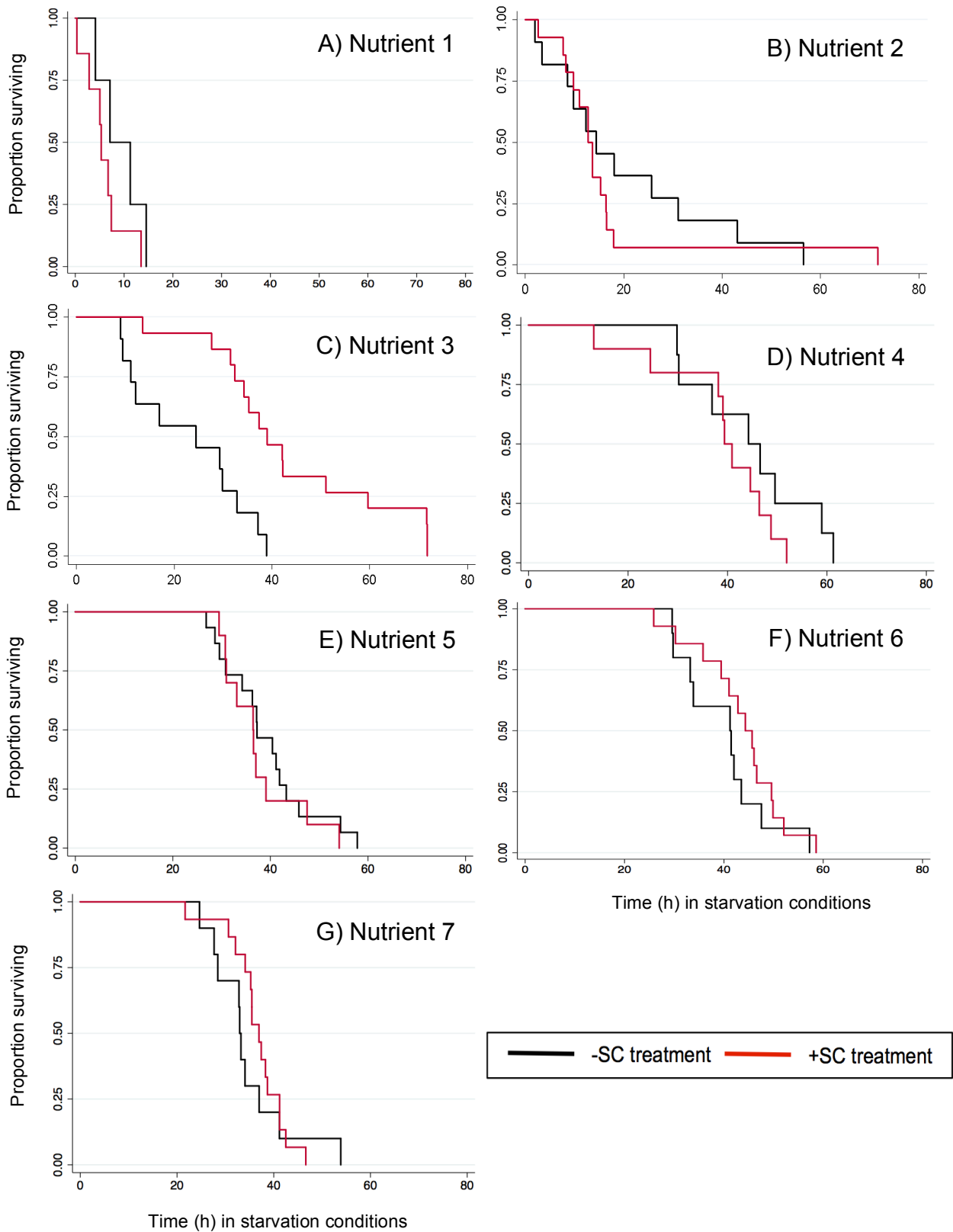


FIGURE 2.4. Survival curves showing the effects of sperm competition and dietary restriction on males' longevity. The different nutrients levels are presented here, showing the clear difference between the pattern of survival on poor quality nutrients (A and B) and better food quality (C, D, E, F, G). This also shows the contrast in longevity between +SC and -SC males at level 3 in comparison to other males at different nutrient levels.

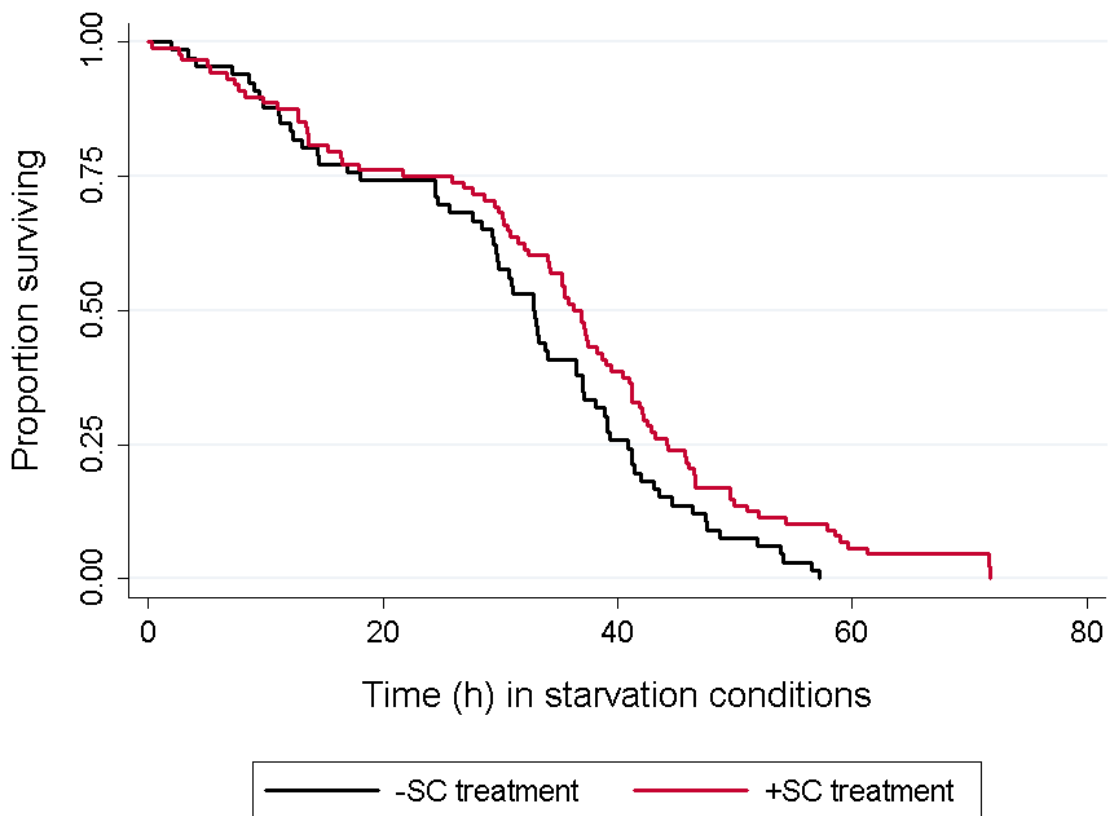


FIGURE 2.5. *The effect of sperm competition on males' resistance to starvation when all nutrient treatments are combined. Plot created using the Cox proportional hazards model. Males exposed to high risk (+SC, red) and low risk (-SC, black) of sperm competition were presented here. Survival was overall significantly better for +SC males (CoxPH: $p=0.030$).*

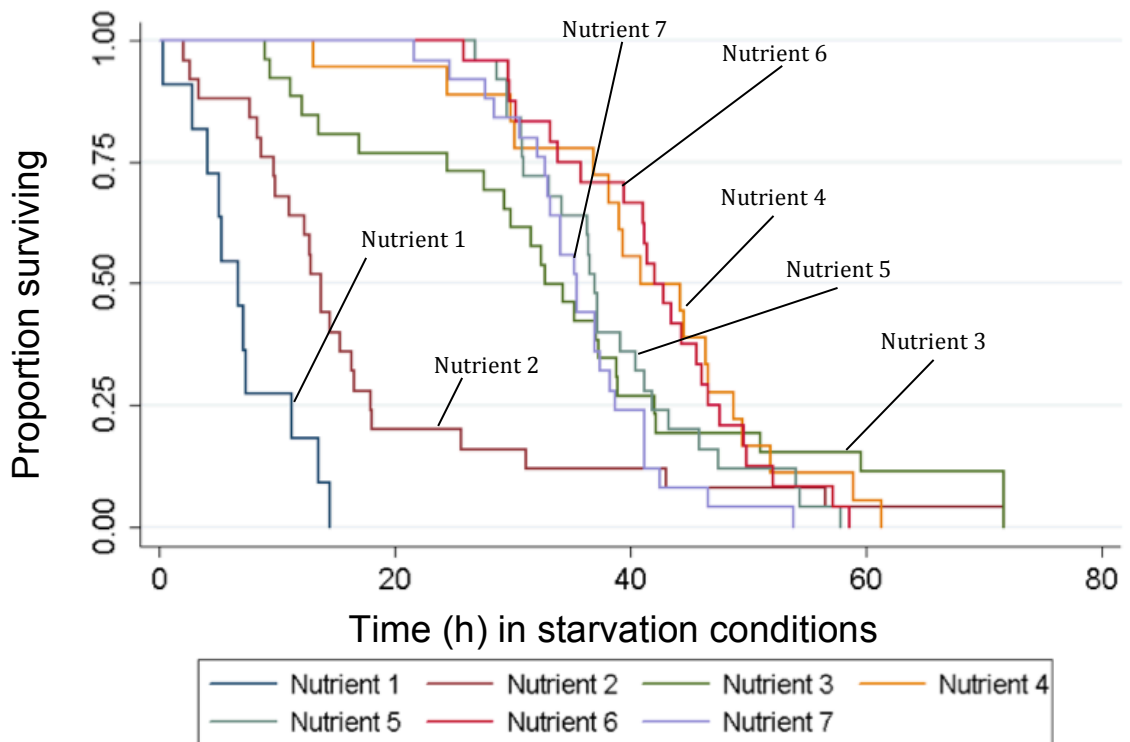


FIGURE 2.6. *Effects of dietary restriction on males' survival patterns (+SC and -SC males clustered together). Each nutrient treatment is represented by a different colour and this shows the clear nutrient effect ($P<0.001$).*

The effects of sperm competition and nutrient on male activity levels

The vial from which each male came from before being placed in the DAM had a significant effect on male activity levels ($F_{74,66}=2.724$, $p<0.001$). This effect was accounted for in the following results by including vial identity as a random effect in all statistical models.

The interaction between sperm competition and nutrient treatment here was not significant ($F_{6,66}=1.93$, $p=0.088$), although there seemed to be a non-significant trend where +SC males are less active towards higher quality nutrients (Figure 2.7).

Sperm competition treatment had a significant effect on male activity. Overall, -SC males triggered the IR beams in the DAM on average 97.30 times per hour. +SC males were less active and only triggered the laser on average 87.02 times per hour ($F_{1,66}=8.52$, $p<0.001$; Figure 2.7). When testing the effect of sperm competition within each nutrient level, sperm competition treatment only had a significant effect on activity at nutrient level 5 where -SC males were the most active ($F_{1,23}=4.96$, $p<0.01$).

Nutrient quality had a significant overall effect on male activity ($F_{6,66}=4.17$, $p<0.001$; Figure 2.7). This was due to a significant difference in activity between nutrients levels 2 and 7 (Tukey test, $p<0.01$; all other $p>0.4$).

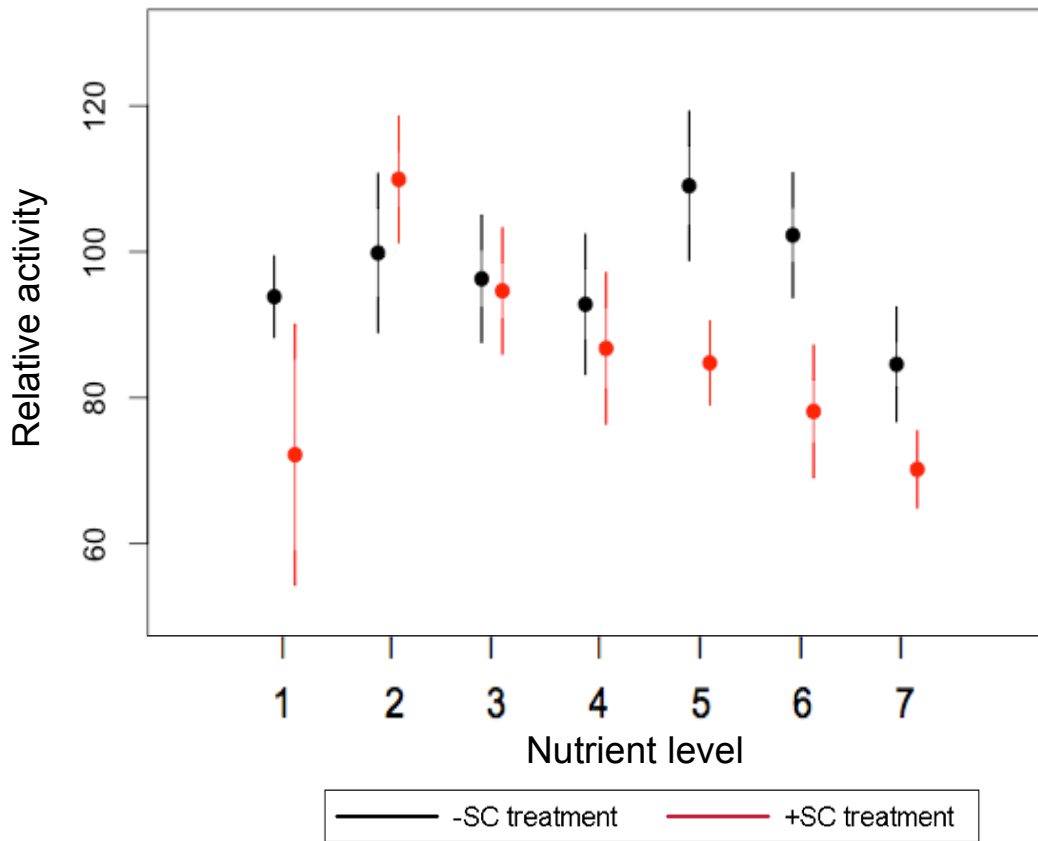


Figure 2.7. *The effect of nutrition and sperm competition on males' relative activity. Relative activity corresponds to the number of times the IR beam was triggered on average by each male per hour. Both nutrient ($p < 0.001$) and sperm competition treatment ($p < 0.01$) have affected male relative activity, and there is no interaction between the two variables. Mean \pm s. e.*

3.1 INTRODUCTION

In order to investigate the trade-off between survival and male responses to sperm competition, dietary restriction was used to force male *Drosophila melanogaster* into a trade-off situation. This also allowed me to study the effects these responses have on reproductive fitness and resource allocation.

In situations where nutritional and/or sperm competition stress occurs, males will be forced to alter reproductive behaviours according to the social and physical conditions of their environment. Using results from the first experiment, I chose three nutrient treatments ranging from 5% to 100% of normal yeast and sucrose concentration to implement different strengths of nutritional stress. Males were divided into four groups: ones who were only placed under nutritional stress, ones who were only placed under sperm competition stress, ones who were placed under both nutritional and sperm competition stress, and finally males who received no stress (control). I predicted that in comparison to the control group, males who were nutritionally stressed would have favoured survival over reproduction. I also predicted that in comparison to the control group, males who were conditioned to sperm competition, would have favoured reproduction over

survival. Males' behavioural reactions to both sperm competition and nutritional stress were unknown.

3.2 METHODS

The flies used in this experiment were first generation (F1) virgin *Drosophila melanogaster*, from a cross between four virgin female Oregon-R and four Canton-S males. Flies were kept in 40 ml vials with 7ml of standard sugar-yeast medium (50 g of yeast per litre) (Bass et al 2007) at a 12-hour light-cycle in a controlled-temperature room maintained at 25°C. After 9 days, parent flies were removed before the eclosion of their first offspring. Up to 6 hours after eclosion, virgin flies were collected and sexed under a light CO₂ anaesthesia and placed in their respective treatments.

Male mating behaviour

F1 females were collected and maintained in groups of 5-10 individuals in 40 ml vials with 7 ml of standard sugar-yeast medium. F1 males were placed in 40 ml vials containing 4 ml of either low (L), medium (M) or high (H) nutrient treatment (L: 6.25 g yeast/L; M: 12.5 g yeast/L; H: 50 g yeast/L; those respectively corresponding to nutrient levels 2, 3 and 5 in Chapter 2; Appendix A.1). Males were further assigned to one of two sperm competition risk treatments: the high sperm competition treatment (+SC) consisted of placing a male on each side of a central plastic divider perforated with small holes, permitting only exchange of olfactory and auditory cues (Bretman et al 2011a; Figure 3.1). The low sperm competition treatment (-SC) involved isolating a single male in a tube with a plastic divider (Figure 3.1). This resulted in a factorial design with six treatments as illustrated in Table 3.1. Males were kept in their treatments for seven days then placed with a randomly assigned virgin female of the same age in a 7 ml vial containing cotton wool and several yeast granules. When performing the

experiment, bijou vials were placed on a heating plate to keep the flies at 25-27 °C for optimum mating conditions (Imasheva et al 1998). Courtship and mating behaviours were recorded: The pair was given 2 hours to mate. The time at which they started courting was measured (courtship latency), as well as the intensity (courtship frequency). This was measured by counting the number of time the male orientated and vibrated his wing at a species-specific frequency (Ewing & Bennet-Clark 1968) towards the female in the first five minutes of courtship. The time males took to initiate mating was measured (mating latency). As a proxy for ejaculate investment, mating duration was measured because of the positive correlation between mating duration and seminal fluid transfer (Wigby et al 2009).

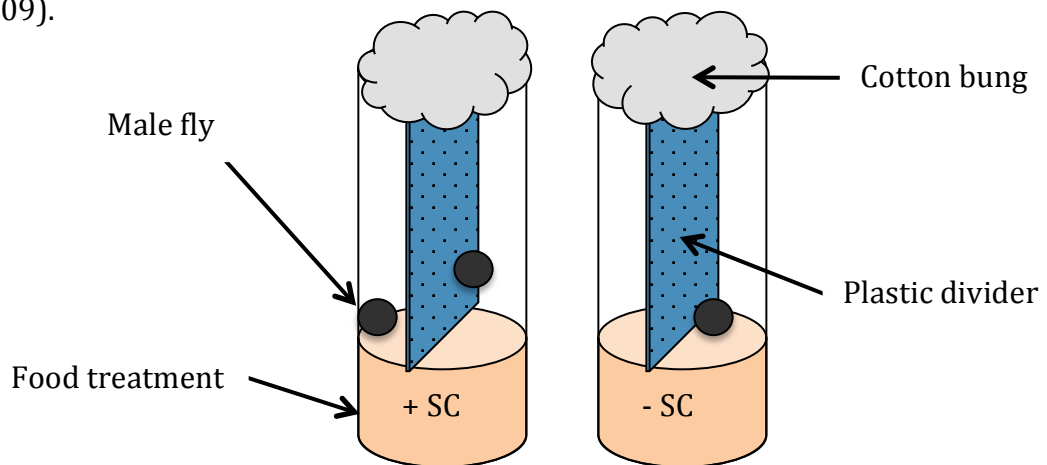


FIGURE 3.1. Male sperm-competition conditioning vials. Two males placed on either sides of a central rigid plastic divider with holes (left) created a high sperm competition environment (+SC). A male placed alone in a tube on one side of a divider (right) created a low sperm competition environment (-SC).

TABLE 3.1. Design testing the effect of DR on male sperm competition plasticity.

	Low nutrient quality (L) 6.25 g yeast/L	Medium nutrient quality (M) 12.5 g yeast/L	High nutrient quality (H) 50 g yeast/L
High sperm competition (+SC)	+SC/L	+SC/M	+SC/H
Low sperm competition (-SC)	-SC/L	-SC/M	-SC/H

Longevity and male activity levels

When copulation ended, males were extracted and placed into individual 5 mm diameter glass tubes plugged at one end with technical agar (1.5 g agar / 100 ml of distilled water) and cotton wool at the other. The tubes were then placed in a *Drosophila* activity monitor (DAM2, Trikinetics, Waltham, MA, USA), where flies were monitored for movement using an infrared beam (Chapter 2, Figure 2.2) until their death. Male activity was measured by measuring the number of times the infrared beam had been activated whilst alive. Resistance to starvation in the *Drosophila* activity monitor (DAM) was a proxy for male longevity under a range of stress levels. After an inactivity of 30 minutes in the DAM, flies were considered dead, and longevity was measured by calculating the amount of time males took to die in their tubes in the DAM (refer to Appendix B. 1 to view a sample of the data collected). The DAM was kept in a controlled temperature room at 25 °C in 24hr light.

Male fertility success

After copulation, females were taken aside and placed on a small flat circle of food medium (50 g Y:S per litre). To prevent the food from drying out, the agar circles were placed on a bed of damp sand. Beetroot dye was added to the agar to make the eggs more visible (Figure 3.2). After 24 hours, females were swapped to a second agar circle. The number of eggs was counted on the first and second day. The number of eggs that hatched on each agar circle was also measured. The number of eggs that were laid and that hatched on the first and second day, and in total, was also compared. The proportion of eggs that were laid and that hatched on the first and second day, and in total, was also compared. A total of 115

individuals were tested (with: +SC/L, n= 17; -SC/L, n= 19; +SC/M, n= 19; -SC/M, n= 20; +SC/H, n= 20; -SC/H, n= 20).

TABLE 3.2. *Experimental schedule to observe and measure female egg laying.*

Day 1	Day 2	Day 3	Day 4
Agar circle 1 (A1)	Eggs laid on A1 counted	Eggs unhatched on A1 counted	
	Agar circle 2 (A2)	Eggs laid on A2 counted	Eggs unhatched on A2 counted

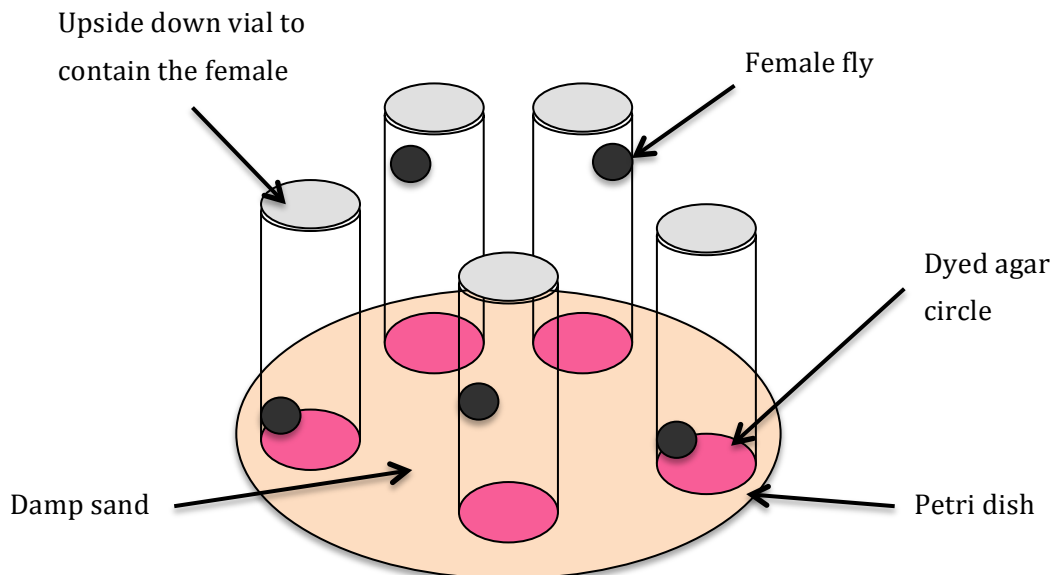


FIGURE 3.2. *Experimental design looking at female egg laying. Females were placed onto beetroot-died-agar circles in a petri dish filled with sand, allowing the counting of eggs laid by females, and 24 hours later, the number of unhatched eggs.*

Statistical analysis:

Statistical analyses were performed on R (v. 3.2.1). All measured mating behaviour (courtship latency, relative courtship frequency, mating latency and mating duration) were analysed using linear models. Male longevity (age in hours spent active in the DAM tube before death), male activity (number of times the IR beam was activated) and male fertility success (number of eggs laid and number of eggs hatched) were also analysed using linear models. Male survival patterns (proportion of individuals remaining alive) were analysed using Cox proportional hazard followed up with analyses of deviance. Survival pattern figures were performed on STATA (v. 12). Female egg laying behaviour (eggs laid on day 1 and day 2, eggs hatched on day 1 and day 2, proportion of eggs laid and hatched on day 1 and day 2, and proportion of eggs hatched) were analysed using linear models. Differences between pairs of treatments were tested with *post hoc* Tukey tests. Linear models were carried out with all factors and interactions, and then model simplifications were carried out by removing non-significant factors. Results should be interpreted with caution due to small sample sizes.

3.3 RESULTS

The effect of nutrient and sperm competition on male mating behaviour

The interaction between sperm competition risk and nutrient quality was not significant (courtship latency: $F_{2,109}=0.849$, $p=0.431$; courtship intensity: $F_{1,109}=0.282$, $p=0.755$). Male latency to start courtship was not affected by nutrient treatment ($F_{2,111}=0.496$, $p=0.611$) or sperm competition ($F_{1,111}=1.912$, $p=0.170$) (Figure 3.3.A). Male relative courtship intensity was also not affected (nutrient: $F_{2,111}=0.931$, $p=0.397$; sperm competition: $F_{1,111}=1.295$, $p=0.258$; Figure 3.3.B).

The interaction between sperm competition risk and nutrient quality was not significant (mating latency: $F_{1,109}=0.193$, $p=0.824$; mating duration: $F_{1,109}=1.298$, $p=0.277$). Males latency to mate was not affected by nutrient or sperm competition treatment (nutrient: $F_{2,111}=0.204$, $p=0.816$; sperm competition: $F_{1,111}=0.776$, $p=0.380$; Figure 3.4.A). Mating duration was affected by nutrients ($F_{1,1,9}=4.910$, $p<0.001$; Figure 3.5.B and Table 3.3): males maintained on low nutrient quality mated for significantly longer than males from the medium nutrient (Tukey test, nutrient M-H, $p=0.137$; L-H, $p=0.436$; L-M, $p<0.01$). Sperm competition did not have an effect on mating duration ($F_{1,109}=0.219$, $p=0.640$).

TABLE 3.3. Mean mating duration (in seconds) of males in each treatment.

	L	M	H
+SC	974	879	993
-SC	1068	890	944
Overall mean	1024	885	968

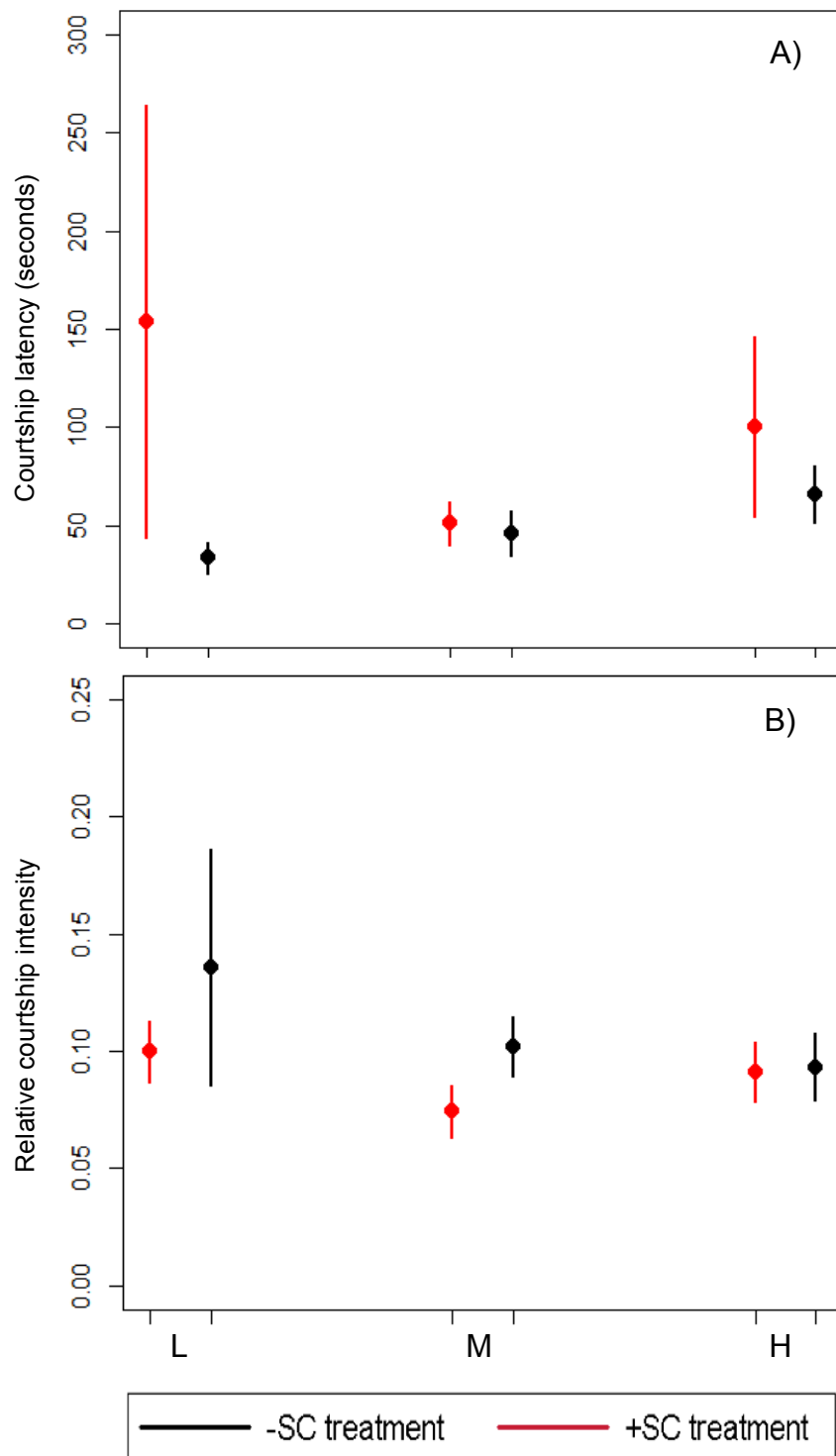


FIGURE 3.3. *The effect of sperm competition and nutrient restriction on male courtship latency (A) and intensity (B). Latency for males to initiate courtship and relative courtship intensity were not affected by nutrient treatments or sperm competition ($p>0.1$). Relative courtship intensity is the number of wing movements per second during courtship. Relative courtship intensity was presented here because courtship had different lengths. Mean \pm s. e*

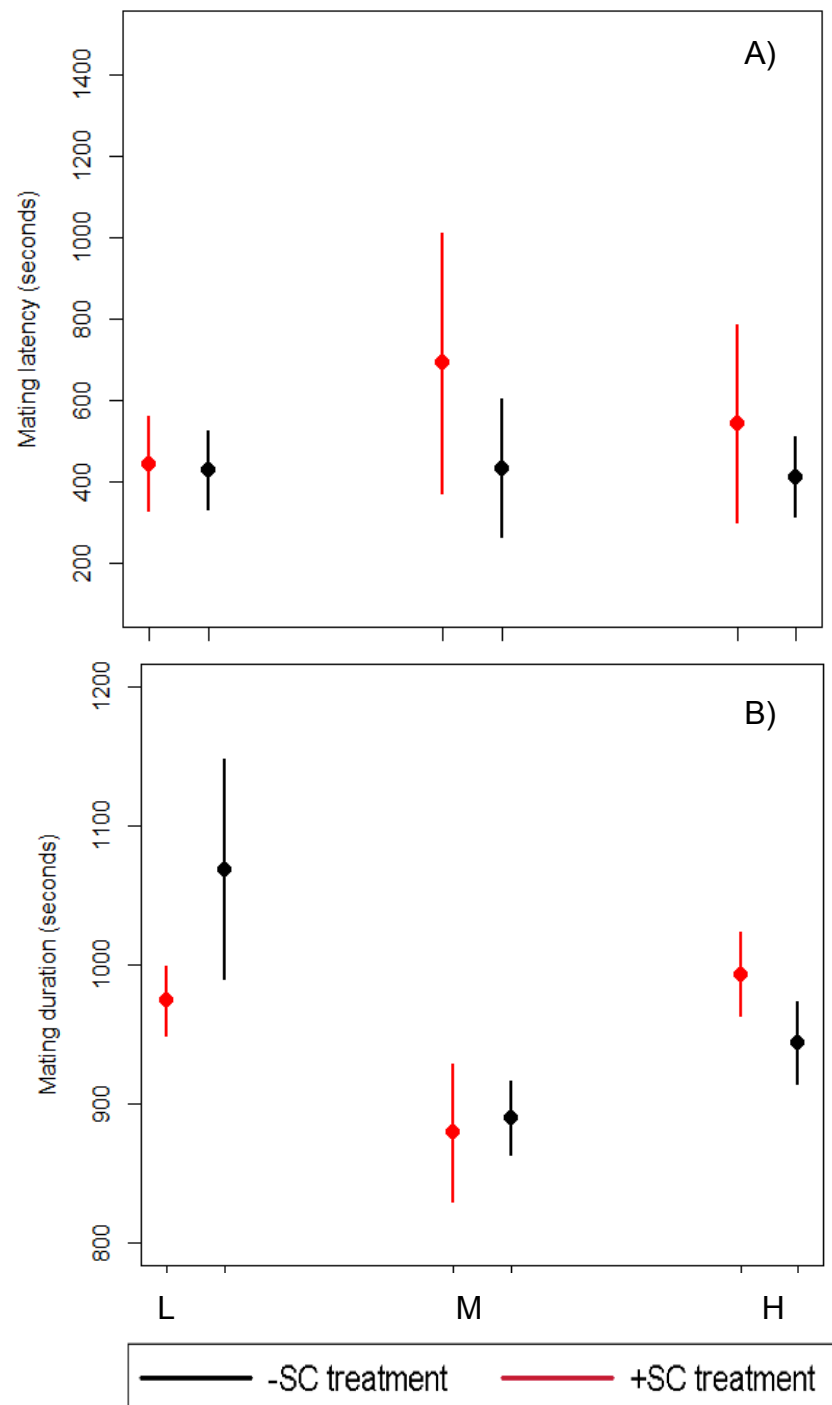


FIGURE 3.4. The effect of sperm competition and nutrient restriction on male mating latency (A) and duration (B). Mating latency (A) was not affected by nutrient or sperm treatments ($p>0.3$). Mating duration (B) was significantly affected by nutrient levels ($p<0.001$), males from the low nutrient treatment mated significantly longer than medium nutrient males (1024 versus 885 seconds). Mean \pm s. e.

The effect of male treatments on longevity and activity

a) Longevity and survival patterns

The interaction between sperm competition and nutrient was not significant ($F_{1,108}=0.129$, $p=0.879$), and therefore, only the main effects of sperm competition and nutrient treatment were analysed here. Longevity and survival patterns were significantly affected by nutrient treatments (longevity: $F_{1,110}=49.3$, $p<0.001$; survival patterns: $X^2 = 49.43$, $p<0.001$), with males from low, medium and high nutrient treatment surviving respectively for 20.57, 38.23, and 43.36 hours in the DAM (Figure 3.5). A *post hoc* test showed that low nutrient males lived for a significantly shorter period of time in comparison to males from both medium and high nutrient treatments (Tukey test, for both $p<0.001$; for nutrient M-H, $p=0.07$; Figure 3.6). Survival patterns and longevity in the DAM were not affected by sperm competition (survival pattern: $X^2 = 0.14$, $p>0.05$; longevity: $F_{1,110}=0.246$, $p=0.621$; Figure 3.6 and Figure 3.7).

b) Relative activity

The interaction between nutrient treatment and sperm competition on male activity was not significant ($F_{1,108}=0.601$, $p=0.550$). Male relative activity was not affected by nutrient treatment or sperm competition (nutrient: $F_{1,110}=0.588$, $p=0.557$; sperm competition: $F_{1,110}=1.653$, $p=0.201$; Figure 3.8).

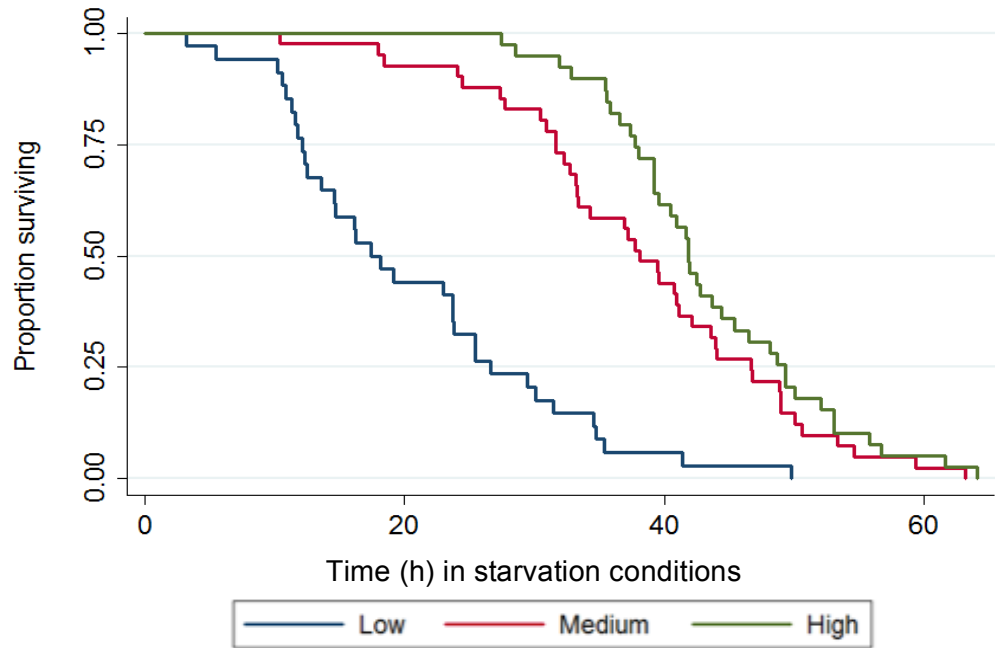


FIGURE 3.5. *Effects of nutritional stress on males' longevity (+SC and -SC males clustered together), created using the Cox proportional hazards model. Male's longevity from the low nutrient is significantly shorter than male's longevity who were maintained on medium and high quality nutrient ($P < 0.001$).*

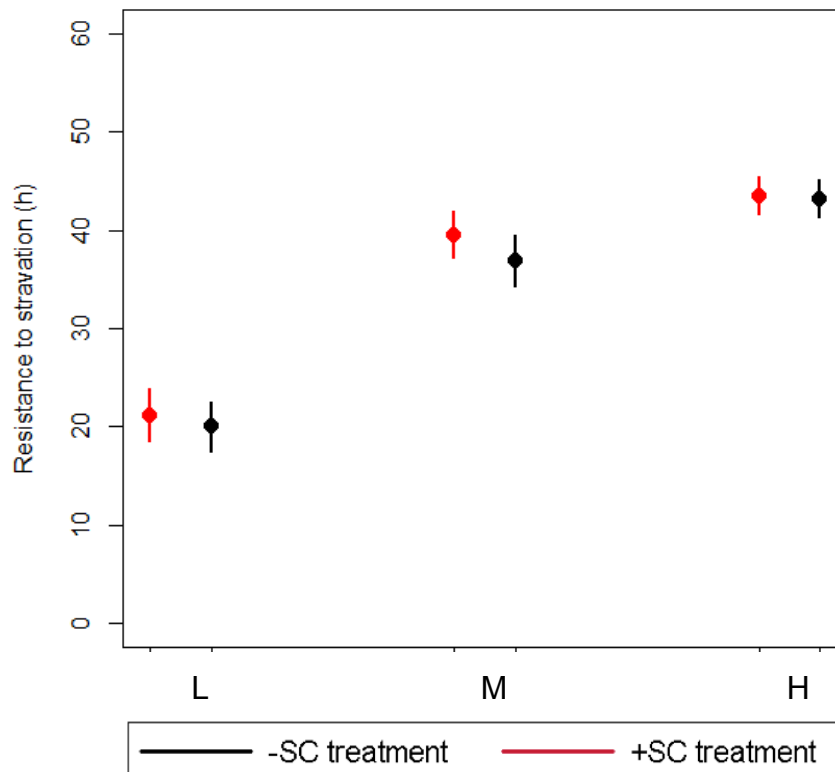


FIGURE 3.6. *The effects of nutrition and sperm competition treatment on male longevity: Survival was significantly affected by nutrient treatment ($p < 0.001$), but not by sperm competition ($p > 0.6$). Mean \pm s. e.*

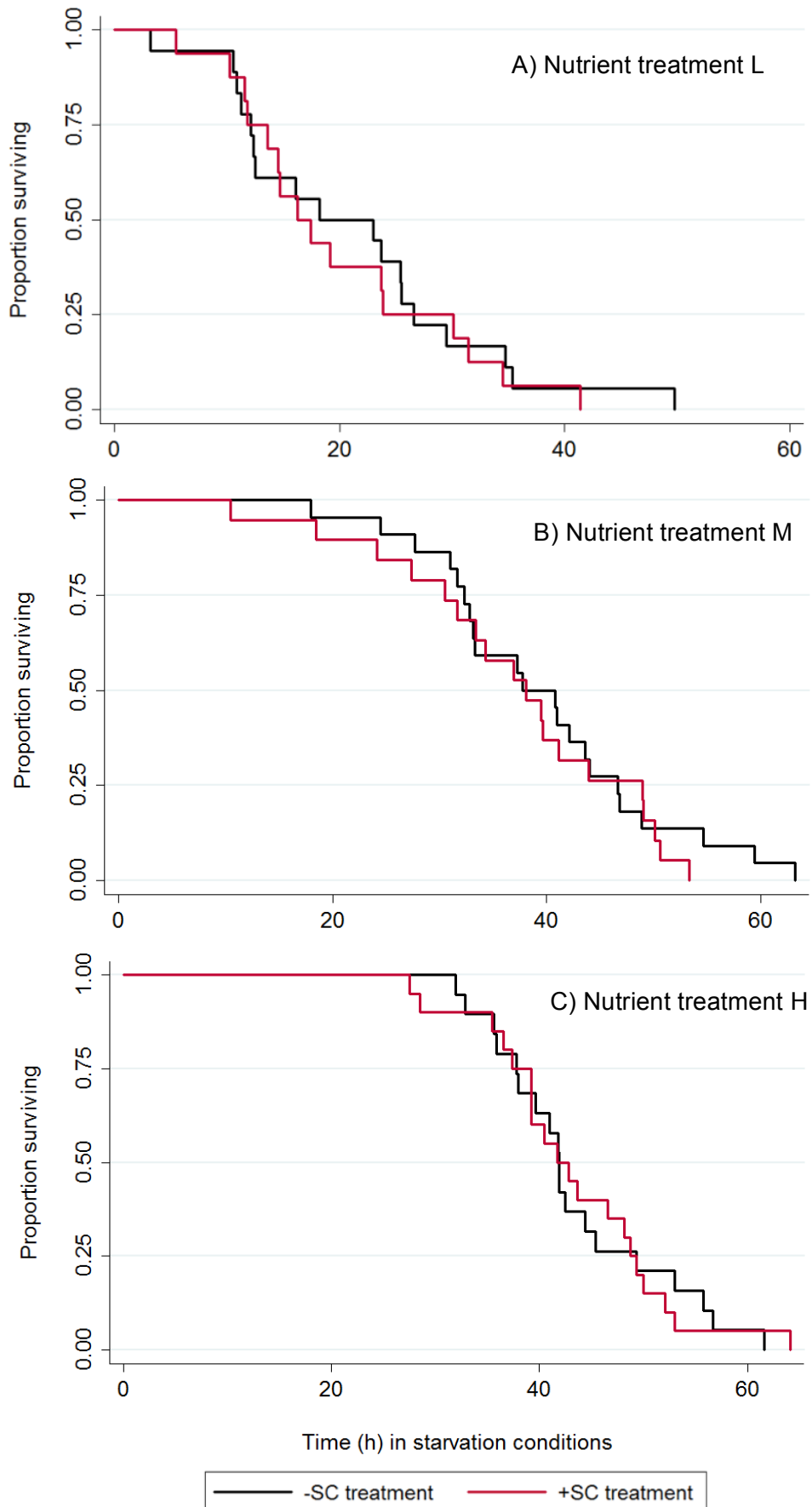


FIGURE 3.7. Survival curves showing the effects of sperm competition on males' longevity for males maintained on low (A), medium (B) and high (C) quality food treatment. There was no effect of sperm competition on male longevity ($p>0.06$).

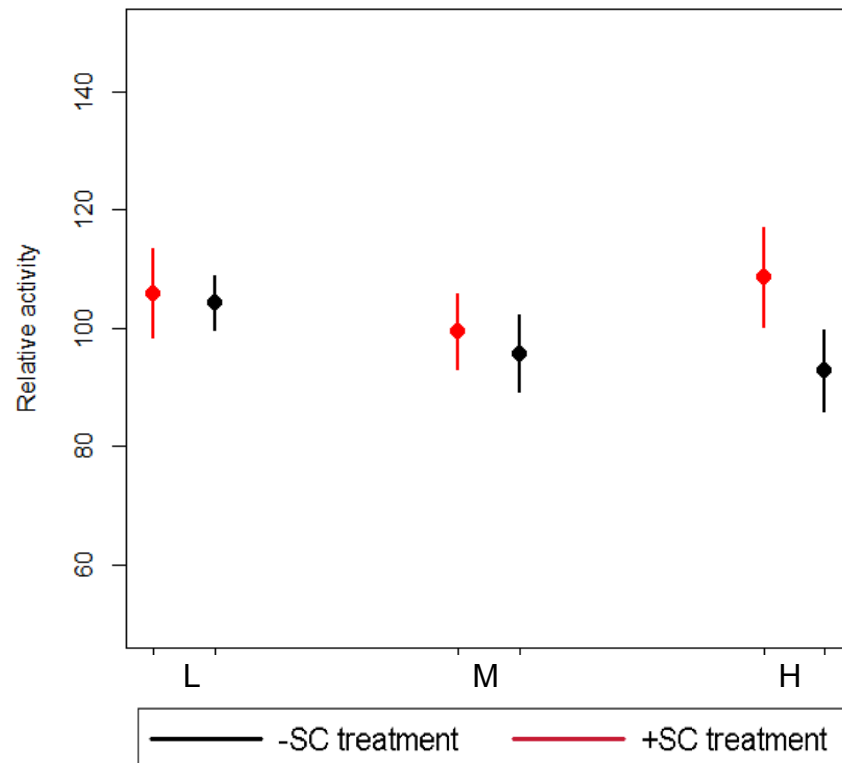


FIGURE 3.8. *The effect of nutrition and sperm competition on males' activity levels in the DAM. Relative activity corresponds to the number of times the IR beam was triggered on average by each male per hour. Nutrient or sperm competition did not affect male relative activity (both $p > 0.1$), and there was no interaction between the two variables ($P > 0.5$). Mean \pm s. e.*

The effect of male treatment on female egg laying

The total amount of eggs laid by females was not affected by male sperm competition treatment or nutrient treatment (sperm competition: $F_{1,111}=0.331$, $p=0.567$; nutrient: $F_{1,111}=0.124$, $p=0.884$; interaction: $p > 0.9$; Figure 3.9.A). This was also the case with the total amount of eggs that hatched (sperm competition: $F_{1,111}=2.075$, $p=0.153$; nutrient: $F_{1,111}=3.02$, $p=0.05$; interaction: $p > 0.8$; Figure 3.9.B).

When looking only at data from day 1, the number of eggs laid was not affected by male sperm competition or nutrient treatment (sperm competition: $F_{1,111}=0.231$,

$p=0.632$; nutrient: $F_{2,111}=0.157$, $p=0.856$; interaction: $p>0.5$; Figure 3.10.A). The same was true for the number of eggs hatched (sperm competition: $F_{1,111}=711.20$, $p=0.124$; nutrient: $F_{2,111}=0.255$, $p=0.0826$; interaction: $p>0.7$; Figure 3.10.C).

When looking only at data from day 2, the number of eggs laid was not affected by male sperm competition or nutrient treatment (sperm competition: $F_{1,111}=3.385$, $p=0.068$; nutrient: $F_{2,111}=0.110$, $p=0.896$; interaction: $p>0.1$; Figure 3.10.B). The same was true for the number of eggs hatched (sperm competition: $F_{1,111}=2.739$, $p=0.101$; nutrient: $F_{2,111}=0.826$, $p=0.441$; interaction: $p>0.4$; Figure 3.10.D).

The proportions of eggs that were laid on day 1 against the number that were laid on day 2 by each female were then compared. There was a significant interaction between sperm competition and nutrient treatment ($F_{2,109}=677.45$, $p<0.001$). The proportion of eggs laid on day 1 against the number of eggs laid on day 2 was significantly affected by sperm competition ($F_{1,113}=702.91$, $p<0.001$; Figure 3.11). Nutrient treatment did not have an effect on the proportion of eggs on day 1 and day 2 ($F_{2,111}=700.87$, $p=0.360$). See Table 3.4 for the mean number of eggs laid each day.

When comparing the proportions of eggs hatched on day 1 and day 2, the opposite was found. There was a significant effect of male nutrient treatment on the amount of eggs that hatched on day 1 and day 2: $F_{2,110}=702.92$, $p<0.05$ (Figure 3.11). Sperm competition did not affect the number of hatched eggs ($F_{1,112}=711.20$, $p=0.124$).

The interaction between sperm competition and nutrient treatment was significant ($F_{2,108}=687.47$, $p<0.001$). See Table 3.4 for the mean number of hatched eggs for each treatment in the two days.

Male fertility success was calculated by counting the number of eggs that hatched out of the total number each female laid (number of eggs hatching/total number of eggs laid). Male fertility was only affected by nutrient treatment (nutrient: $F_{1,111}=6.163$, $p<0.001$; sperm competition: $p>0.08$; interaction: $p>0.8$; Figure 3.12). The number of hatched eggs was only significantly different from one another when males were conditioned to medium and high nutrient treatment (Tukey test, $p=0.002$, all other $p>0.1$), with 91% of eggs hatching when fertilised by males from the medium nutrient treatment against 73 % from males of the high nutrient treatment (82% chances of eggs hatching with fertilisation by males from the low nutrient treatment).

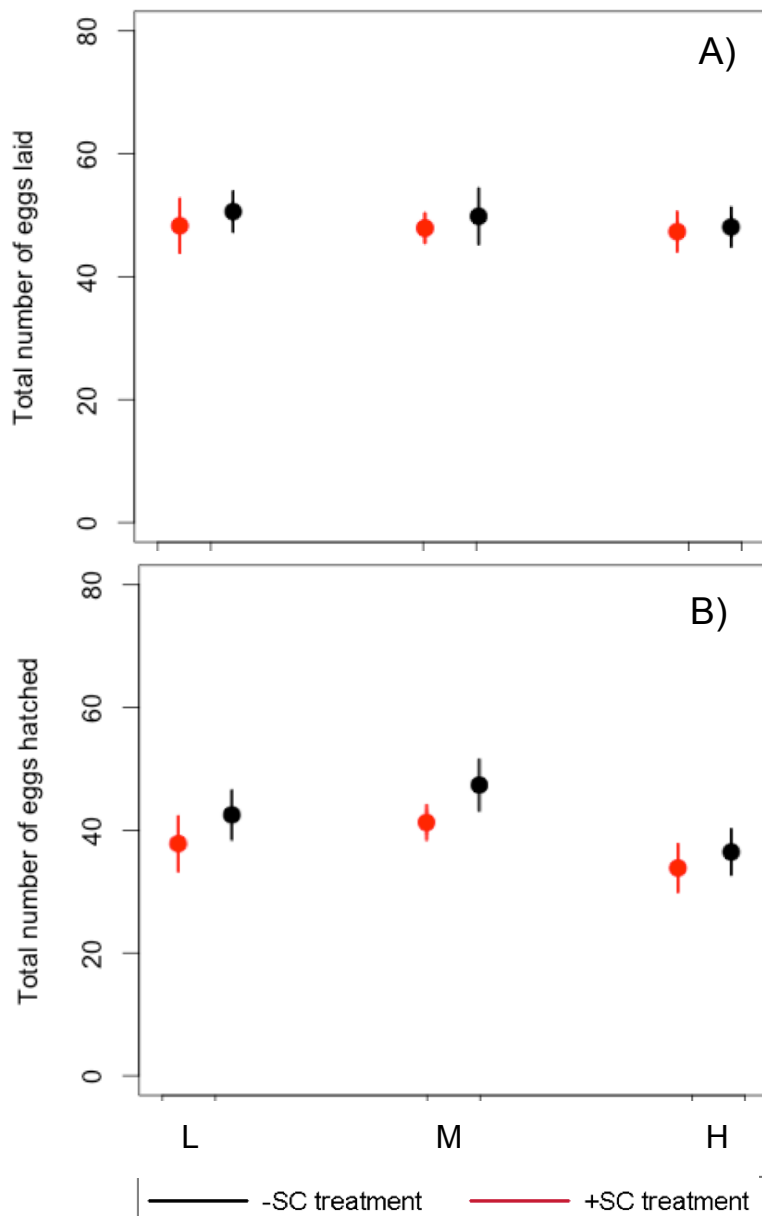


FIGURE 3.9. Mean total number of eggs laid by females (A) and number of hatched eggs (B). Sperm competition and nutrient had no significant effect on A or B (for all, $p>0.5$), the interaction between the two factors was not significant (for both: $p>0.8$). Mean \pm s. e.

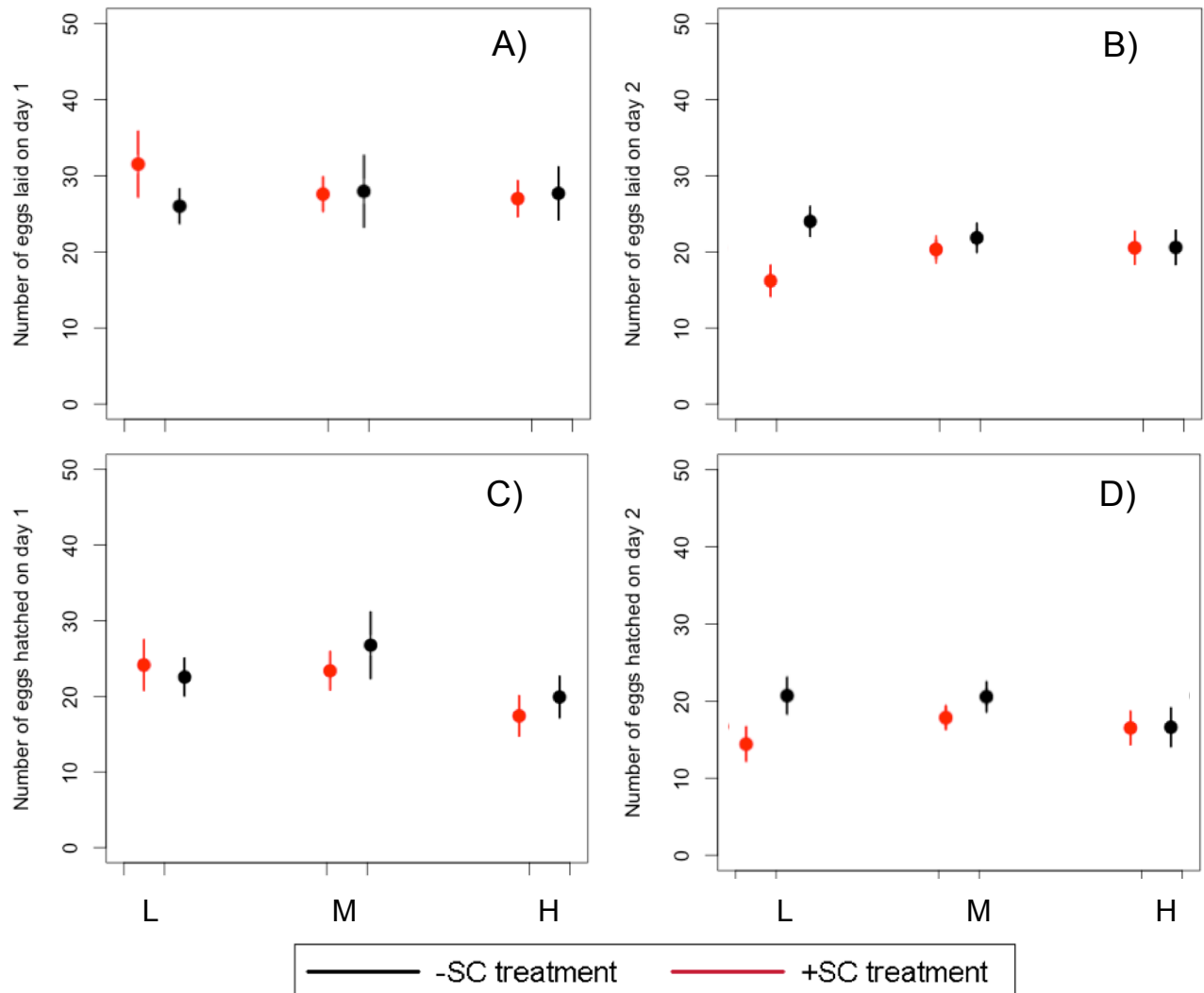


FIGURE 3.10. The number of eggs laid on day 1 (A) and day 2 (B), followed by the number of eggs that hatched on each of these days (Day 1 (C) and day 2 (D)). There was no effect of males' sperm competition or nutrient treatments on the amount of eggs females laid, and the number of eggs that successfully hatched (all $p > 0.05$).

TABLE 3.4. Mean number of eggs laid and hatched for each treatment on day 1 and day 2.

Number of eggs laid	Day 1		Day 2	
	+SC	-SC	+SC	-SC
Low	33.88	26.37	16.29	24.11
Medium	27.53	27.90	20.47	22.00
High	26.80	27.50	20.50	20.55

Number of eggs hatched	Day 1		Day 2	
	+SC	-SC	+SC	-SC
Low	33.88	26.37	16.29	24.11
Medium	27.53	27.90	20.47	22.00
High	26.80	27.50	20.50	20.55

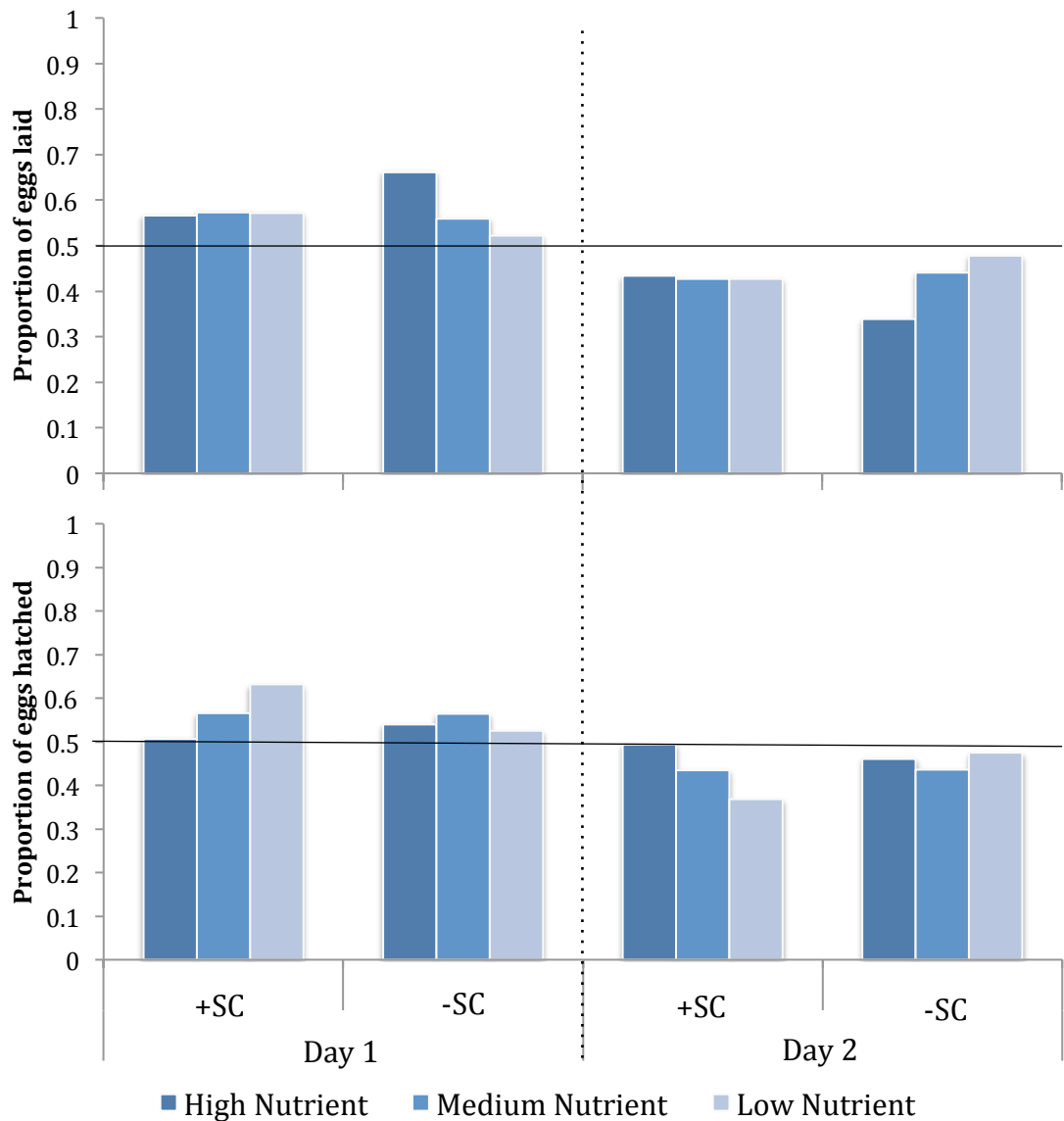


FIGURE 3.11. *The comparison of the proportion of eggs laid (top) and hatched (bottom) on day 1 and 2. The two horizontal lines represent the even proportion where eggs laid and hatched on the first and second day are the same. There was an effect of male sperm competition treatment on the proportion of eggs laid by females each day ($p < 0.001$; no effect of male nutrient treatment $p > 0.3$). Male nutrient treatment had an effect on the number of eggs that hatched ($p < 0.05$; no effect of sperm competition $p > 0.1$). In both variables, there was a significant interaction between male sperm competition and nutrient treatment ($p < 0.001$).*

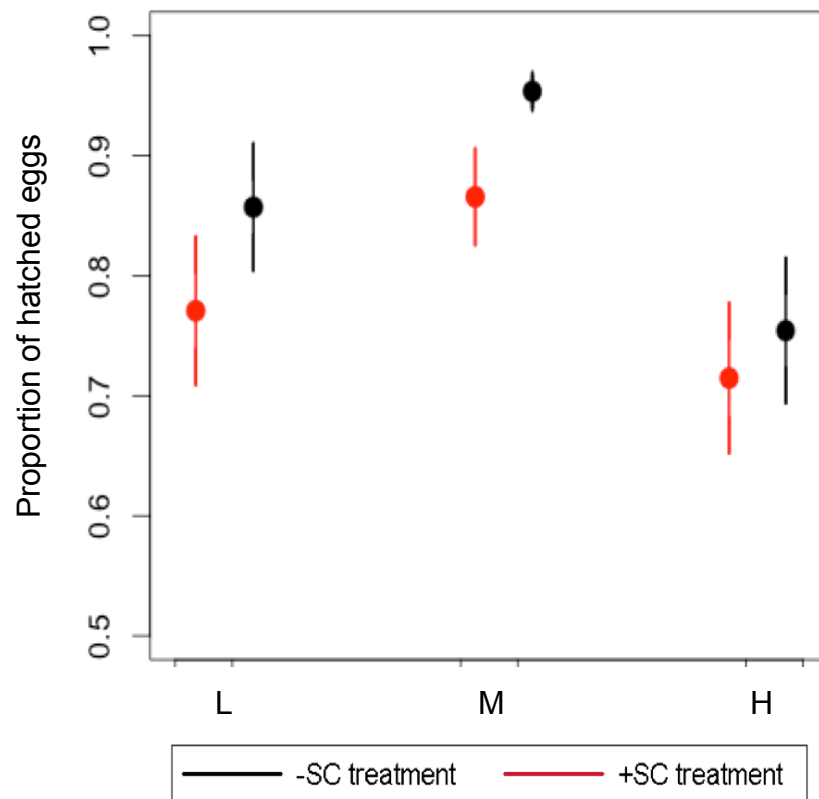


FIGURE 3.12. *Proportion of eggs hatched, giving an indication of male fertility success. There was a higher proportion of eggs hatching when females mated with male who were maintained on medium nutrient treatment than with males from the high nutrient treatment ($p < 0.01$)*

4.1 DISCUSSION AND CONCLUSION

The aim of this research project was to investigate the trade-off between survival and male *Drosophila melanogaster* responses to sperm competition, and to look at the effect these responses have on reproductive fitness and resource allocation. To do this, first I needed to determine what level of dietary restriction would force males into a nutritionally-restricted state. In order to identify a level of nutritional restriction that forced males to reduce their activity and/or longevity, I maintained newly-eclosed males on a medium that varied in concentration from 2.5% to 200% of normal yeast and sucrose (Chapter 2, experiment 1).

In previous studies, it has been reported that, under dietary restriction, male *Drosophila melanogaster* maximum life span occurs at an intermediate level of dietary restriction (Partridge et al 2005). They found that the median life span was highest with a yeast and sugar concentration of close to 50g per litre (equivalent to nutrient level 5 in experiment 1). The results of this study's first experiment appear to support this with an observed optimum survival at intermediate nutrient levels 4, 5 and 6 (25, 50 and 100 g of Y:S/L respectively; Figure 2.3). As expected, nutrient level 1 (3.12 g Y:S/L) proved inadequate for male fruit flies as none of the males survived for longer than 20 hours in the DAM (Figures 2.4.A and Figure 2.6). Nutrient level 2 (6.25 g of Y:S/L) also had a detrimental effect on the

life span of males, as only 25% of them survived over 20 hours (Figure 2.4.B). The survival curve of males on the most nutrient rich diet (Nutrient level 7, 200 g Y:S/L; Figure 2.6) appeared to be below those of the previous two nutrient levels. The start of the decline of male longevity at nutrient level 7 could be caused by the over-consumption of harmful proteins contained in the food. Indeed, a study found that *Drosophila melanogaster* increased their consumption of nutrients when on the highest yeast concentrated diets, suggesting that high yeast diets could stimulate feeding behaviour (Min et al 2007). The abrupt change between longevity at nutrient levels 1-2 and longevity at nutrient levels 4-6 appeared to be happening at nutrient level 3 (12.5 g Y:S/L; Figure 2.3). From that stage onwards, over 75% of the flies survived for at least 20 hours (in comparison to 25% at nutrient level 2). Nutrient level 3 appears to be critical as it appears to be the trigger point from which male survival increases, it is also the only treatment where sperm competition has a significant effect on survival.

Knowing that reproduction and investment in sperm competition traits is expensive (Bretman et al 2009, Wigby et al 2009, Parker et al 1997, 2013), it was predicted that males exposed to sperm competition risk would show reduced survival. However, in a previous study, where similar methods were adopted to examine sperm competition risk and life span, it was found that survival was greater for +SC males (Moatt et al 2013). My overall results from Experiment 1 in Chapter 2 adhere to Moatt et al's (2013) findings: sperm competition significantly affected survival, prolonging the lives of +SC males (overall mean survival in DAM for +SC males: 34.4 hours; -SC males: 30.6 hours). Yet it is only at nutrient level 3 that +SC males lived significantly longer than -SC males, which can be observed in Figure 2.4.C. Moatt et al (2013) used a diet with 50 g of yeast per litre,

corresponding to nutrient level 5 in this study. However, there was no significant difference in the survival of males on nutrient level 5 in my study. There appears to be a non-significant trend where -SC males displayed a higher resistance to starvation at lower nutrient levels (levels 1 and 2). From the trigger point nutrient level 3, the mean life span of +SC males was greater than that of -SC males. It can be presumed that males were able to invest and re-allocate resources into the development of plastic sperm-competitive traits from that point; these can include the development of seminal fluid proteins that help influence the outcome of sperm competition (Fedorka et al 2011). On top of this, under dietary-restricted conditions, it is possible for compensatory feeding to take place (Carvalho et al 2005, Lee et al 2008, Fanson et al 2009), and this could help explain why, from nutrient level 3 onwards, males showed similar mean longevities. Carvalho et al (p 814, 2005) found that “the life span of *D. melanogaster* is not exclusively determined by food source composition, but rather it is the product of the interaction between nutrient availability and active feeding behaviour”. In addition to this, there is evidence that individuals can regulate their protein and sugar intake to achieve optimal fitness (Simpson et al 2004). When presented with different food options, flies ingested nutrients that pushed them towards maximal lifetime egg production (Lee et al 2008). Regulation of nutrient intake and compensatory feeding means that the interpretation of life spans must be undertaken with care. Compensatory feeding and regulation of protein and sugar intake could explain why, in some cases, the difference between the life span of +SC and -SC males is not always clear.

A previous study by Ghimire & Kim (2015) in *Drosophila melanogaster* found that dietary restriction increased locomotor activity, which in turn increased stress resistance through the up-regulation of the expression of protective genes. In

agreement with these results, I found that overall nutrient treatment had a significant effect on male longevity. However, a *post hoc* analysis revealed that it was only longevity of males who were maintained on nutrient level 2 that was longer than the longevity of males maintained on nutrient level 7. I also found that male relative activity levels were affected by sperm competition. There was a non-significant tendency for relative activity levels of -SC males to stay within 90 to 110 IR beam activations per hour regardless of nutrient levels (Figure 2.7). Whilst on the other hand, relative activity levels of +SC males appeared to be decreasing as nutrient quality increased (if data points from nutrient level 1 are ignored due to small sample sizes). An explanation for this could be the fact that in a number of species, including in *Drosophila melanogaster*, males exposed to sperm competition have been found to invest more of their total energy into specific reproductive traits allowing them to compete against other males (Gage & Cook 1994, Fedorka et al 2001, Lupold et al 2011, Garbaczewska et al 2013). *Drosophila melanogaster* males might be expected to reduce their activity in order to re-allocate energy towards sperm-competitive-traits such as the development of larger sperm size and production of larger ejaculate quantities. This may have been the strategy adopted by -SC males in Experiment 1 presented in Chapter 2, as -SC males were found to be overall more active than +SC males (although only significant at nutrient level 5).

In the Experiment 2 described in Chapter 3, males were conditioned to sperm competition and one of three nutrient treatments (high quality: 50g Y:S/L; medium quality: 12.5 g Y:S/L; low quality; 6.25 g Y:S/L). This allowed me to both test the effects of sperm competition on male reproduction under various nutritional stresses, and investigate the cost of sperm competition and resource allocation in

male *Drosophila melanogaster*. I expected males that were nutritionally stressed would have favoured survival over reproduction. I also predicted that in comparison to the control group, males who were conditioned to sperm competition, would have favoured reproduction over survival. Males' behavioural reactions to both sperm competition and nutritional stress were unknown.

Throughout Experiment 2, there was no significant effect of sperm competition treatments on the male's pre-copulatory behaviours (courtship latency and courtship intensity) or mating behaviour (mating latency and mating duration). This could be due to there being no actual costs to expressing responses as a result of sperm competition. Costly forms of plasticity are only expected to persist through populations if they are compensated for by benefits (DeWitt et al 1998). Genes responsible for producing plastic responses should also gradually be replaced by genetic mechanisms promoting plasticity with or without reduced cost, if a given plastic trait is beneficial and outweighs the costs. The cost to plasticity should be reduced over time through natural selection (DeWitt et al 1998). In the wild, *Drosophila melanogaster* are very rarely found isolated and sperm competition risk is constantly present, or very common. This means that natural selection may have driven plastic sperm competition responses to become less costly over evolutionary time. Nonetheless there is a non-significant trend in mating duration (as seen in Figure 3.4.B), where high nutrient treatment +SC males had a higher mean mating duration than -SC males, whilst at the low nutrient treatment, +SC males had a shorter mating duration than -SC males. This non-significant trend could indicate that males, who have invested energy and resources into sperm competition traits, were unable to maintain a high mating duration with low resources. They had already expended too much energy in

response to sperm competition risk and had to prioritise survival in that case. This observation could also mean that an increased mating duration, as a response to sperm competition, might be a more subtle response than suggested in past studies (Bretman et al 2009) and only found at an excessively high nutrient availability.

While nutrients had no significant detectable effect on some mating components (courtship and mating latency; Figure 3.3, Figure 3.4), nutrients treatment had a strong effect on survival: Males who were maintained on low nutrient treatment quality were successfully handicapped and had shorter life spans than other males. The fact that there was no significant difference between the longevity of males maintained on medium and high nutrient treatment could be associated with compensatory feeding, where males were able to compensate for medium nutrient treatment quality by ingesting more nutrients (Carvalho et al 2005).

Despite the fact that males exposed to the low nutrient treatment had the shortest life spans, they mated for a significantly longer period of time when compared to males subjected to the medium nutrient treatment (Figure 3.4.B). Males on the medium nutrient treatment experienced a drop in mean mating duration, which then increased when maintained at high nutrient treatment (as seen on Figure 3.4.B, but no significant difference between mating duration of males maintained at medium and high nutrient treatments). This contradicts the findings of Fricke et al (2008) who found that nutrition had no effect on mating duration. The reason for this difference in results could be due to the fact that in their experiment there was no sperm competition conditioning prior to males being placed in a mating arena. The only sperm competition risk detected by the males was from chemical

cues left on the female by the other male. What is more, they did not take courtship into account, which is a key factor in reproduction and is energetically expensive (Jallon & Hotta 1979) which could play a major role in energy allocation and expenditure under nutritional stress. This could show that on the high nutrient treatment, males were able to comfortably invest energy into a longer mating duration, whilst when maintained on medium nutrient treatment, they did not possess the additional resources required for a longer mating duration.

Surprisingly then, in the case of males who were maintained on the low nutrient treatment, instead of a decrease in the mating duration, an increase was observed (Figure 3.4.B). This unexpected result posed the question whether mating duration is truly condition-dependent. The possession of features in various species, such as ornamental traits, songs or displays, is considered a handicap in day-to-day survival. They should therefore be a reliable signal of male vigour (survivorship or energetic state) and females should focus on male features that honestly indicate physical conditions (Zahavi 1975). Generally, the majority of traits associated with reproduction are condition-dependent, reflecting male quality (Cotton et al 2004), and females use these traits as an indicator to choose the best quality males (Simmons 1995). However, in some cases, some signals are not condition-dependent, and males are able to cheat thanks to terminal investment (Clutton-Brock 1984). This is where males in poor physical condition choose to maximise reproductive success when prospects of future survival and mating events are low (Kokko 1997). An example of this was found in the mealworm beetle, *Tenebrio molitor*, where males terminally invest in both short and medium-range pheromones to attract females when they perceive their future survival as being reduced (Nielsen & Holman 2012). In this case, males from the low quality treatment may have perceived that their probabilities of survival were low

because of the lack of protein and sugar in the nutrient treatment. Therefore, making one last attempt to increase reproductive success, they increased their mating durations.

Courtship is the most energetically expensive process in *Drosophila melanogaster* reproduction (Cordts & Partridge 1996), and was expected to be condition-dependent. However, in Experiment 2, courtship was not affected by nutrient treatment (Figure 3.3), and males from all treatments exhibited similar courtship behaviours. There are two possible explanations for this: it could mean that males from the high nutrient treatment did not expend as much energy as they could have on courtship, this being because they sensed their quality and health would ensure reproductive success. On the other hand, it may be due to low nutrient males investing more energy into courtship effort (terminal investment), deceiving females by demonstrating better health and quality. This type of behaviour has already been observed in *Drosophila nigrospiracula*, where males increased their courtship efforts when their life span was reduced by artificially-induced mite parasitism (Polak & Stramer 1998). In addition to this, the longer a male spends courting, the greater his reproductive success (Connolly et al 1969), which could explain why it is vital for males to court more intensely. That is why the second explanation seems more likely, where males exposed to low nutrient treatment terminally invested into courtship.

Despite the fact that low nutrient males appear to have terminally invested into mating and courtship effort, relative fertility does not seem to reflect this. It can be observed in Figure 3.11 that males who were maintained on low nutrient treatment, along with those from the high nutrient treatment, had lower fertility

success than males maintained on medium nutrient treatment. They fertilised a higher proportion of eggs than males from the high nutrient treatment, despite demonstrating the lowest mean mating durations. The decrease in sperm quality with nutritional stress (Gage & Cook 1994) may explain the lower egg fertility at low nutrient treatment, in spite (and maybe because) of the energy spent in terminal investment. Why the males from the high nutrient treatment achieved a significantly lower fertility than the males from the medium nutrient treatment remains unclear. This could be due to the high nutrient males not investing all their resources into one mating event, preserving their energy for future mating events.

Barbosa (2012) found that in the soldier fly, *Merosargus cingulatus*, sperm competition did not have an effect on clutch size. Sperm competition did, however, affect fertilisation success. Soldier fly males conditioned to sperm competition increased mating durations and fertilised a higher percentage of a female's egg clutch than -SC males. In Experiment 2, there was no effect of sperm competition on the total number of eggs laid, the number that hatched or relative male fertility. However, there was a significant effect of sperm competition on the proportion of eggs females laid on the first day, in comparison to the proportion laid on the second day (Figure 3.10). The proportions of eggs laid on the first day in comparison to the second day by females who were mated with +SC males from all three nutrient treatments were similar. In contrast, when females mated with -SC males, the better quality of nutrient treatment the male was subjected to, the higher the proportion of eggs laid on day 1. Overall, a higher proportion of eggs were laid on the first day than on the second day. With regards to fertilisation success, only male nutrient treatment significantly affected the proportion of eggs

that hatched on the first or second day (Figure 3.10). There was a significantly higher proportion of eggs hatching on the first day when females had mated with +SC males from the low nutrient treatment. The number of eggs hatching on the first and second day was similar for +SC males from the high nutrient treatment. No biological explanations could be found to explain these findings. I can only suggest that +SC males who were maintained on the most stressful nutrient treatment (low nutrient treatment) produced less sperm and could only fertilize a smaller proportion of the female's eggs, hence the drop in hatching success, but this is only an assumption.

This work has provided an insight in the trade-off between investment into reproduction and survival. It was not possible to conclude on the cost of sperm-competitive-traits. However, it demonstrated the importance diet has on reproduction investment, and has added some evidence to the terminal investment concept in *Drosophila melanogaster*. The problem proved to be more complex than was originally expected, and the whole process of sperm-competitive-trait investment may involve more variables and more interactions within this extended set of variables. This study has added evidence to the terminal investment concept, contributing to the research on the energy trade-off between reproduction, socio-sexuality and an individual's experience of their own environment.

4.2 FUTURE WORK

Chapman et al (1998) reviewed the literature for *Diptera* and found that in 10 species mating decreased life span and in 9 species it had no effect on life span. They point out that carefully controlled large scale or replicated experiments are required to show that mating can reduce survival. Looking back on methods used throughout this research, the fact that I found no cost to reproduction could be due to small sample sizes. Therefore, in future work, it would be interesting to see if results differed from those found in this research when increasing sample sizes. For example, in a similar sperm competition related experiment on *Drosophila melanogaster* (Bretman et al 2009), sample size was between 31-35 for each treatment, in contrast, sample sizes in my research averaged 8-15 in Experiment 1 (excluding samples from nutrient level 1 due to its high handicap) and 17-20 in Experiment 2. When analysing data from both experiments, in some cases, results were very close to being significant and it is possible that, by increasing sample sizes, the differences between treatments may become clearer and significant.

When a female *Drosophila melanogaster* is given the choice between a virgin male and an experienced male, she will mate with the virgin male (Markow et al 1978). When given the choice, females will choose mates based on their physical characteristics, the quality of the territory they control or their behaviour (Janetos 1980). If female fruit flies choose to mate with virgin males rather than experienced males, this means that they are of “higher” quality than mated males. This implies that if there is a cost to investment in sperm-competitive traits, it may not be visible within the time scale used for this work, and could start appearing after two or more mating events. In future work, investigating the costs of sperm

competition males should be mated with multiple females (2 or 3 additional mating events) to detect whether or not the cost to sperm competition only becomes apparent after two or more mating events. This experiment could be carried out using seed beetles, *Callosobruchus maculatus*. In this species, adults do not need nutrient or water to reproduce as they collect all necessary energy reserves during their larval stage (Fox & Tatar 1994). This means that they have a fixed amount of energy in their lifetime that they allocate into different activities during their adult life. This makes it ideal to manipulate and study the trade-off between sperm competition and survival (Paukku & Kotiaho 2005). Males would be conditioned to sperm competition and mated repeatedly with females. Longevities of males who were mated once, twice and three times would then be compared. A way to add a nutritional factor in this experiment would be to alter food availability of males at larval stage.

An effect of nutrient treatment on male resistance to starvation at nutrient level 3 (12.5 g yeast and sugar per litre) was found. There is an abrupt switch of male longevity and behavioural responses between nutrient levels 2 and 3. However, the nature of the changes remains unclear, whether it is a threshold or a more gradual switch. A possible method to investigate this further would be to perform the same experiment, but using a selection of nutrients ranging between nutrient level 2 (6.25 g yeast and sugar per litre) and nutrient level 4 (25 g yeast and sugar per litre). This would provide me with an understanding of what may triggers the switch in male behaviour and from what point males start re-allocating energy towards sperm-competitive traits, away from survival. As compensatory feeding and regulation of nutrient uptake can occur, a more accurate way to implement nutritional stress would be through the use of a capillary feeder (William 2007).

This method consists of using very fine glass tubes, which release small drops of food, allowing the exact measurements of how much each individual consumes.

Compensatory feeding (Carvalho et al 2005, Lee et al 2008, Fanson et al 2009), regulation of nutrient intake (Simpson et al 2004) and increased feeding behaviour when maintained on nutrient rich diets (Min et al 2007) are a few of many factors that can influence how male's life span is affected by dietary restriction. There are many complications that need to be taken into account when using dietary restriction to handicap males and therefore another method could be used to carry out future work on the trade-off between survival and reproduction. Another methods commonly used to handicap males is to trigger the immune system of individuals through parasitic infections or injections to force them in trading off between reproduction and immune response (French et al 2007, Simmons 2011, Nielsen & Holman 2012, Kivleniece et al 2010). A study has already demonstrated that the immune system in *Drosophila melanogaster* is closely linked to sexual activity (McKean & Nunney 2001). Indeed, males who increased their sexual activity showed reduced ability to tackle a bacterial infection. On top of this, terminal investment behaviour has been observed in male fruit flies that were infected by parasitic mites, *Macrocheles subbadius* (Polak & Starmer 1998). This method would be ideal to investigate further the link between investment into sperm-competitive traits, terminal investment and life span.

This work has provided an insight in the trade-off between investment into reproduction and survival. It was not possible to conclude on the cost of sperm-competitive-traits. However, it demonstrated the importance diet has on reproduction investment, and has added some evidence to the terminal investment

concept in *Drosophila melanogaster*. The problem proved to be more complex than was originally expected and the whole process of sperm-competitive-trait investment may involve more variables, and more interactions within this extended set of variables. This study has added evidence to the terminal investment concept, and has contributed to the research on the energy trade-off between reproduction, socio-sexuality and an individuals' experience of their environment.

APPENDIX

Appendix A- Nutrient recipe

1. *Drosophila* culture medium, using Zordan et al (2007) recipe.

Dried yeast, sugar and agar were combined, prior to the addition of water into a flask. The solution was brought to boiling point twice before it was allowed to cool down to approximately 50°C. At this point the nipagin was added and stirred in (which was previously dissolved in 95% ethanol - 10 g nipagin dissolved in 100 ml 95% ethanol). The following table shows the content for each nutrient treatment used.

TABLE 3.5. *Quantities of each ingredients in each nutrient treatment.*

Nutrient	Yeast (g)	Sugar (g)	Agar (g)	Nipagin (g)	Water (ml)
1	3.12	3.12	1.2	0.4	100
2	6.25	6.25	1.2	0.4	100
3	12.5	12.5	1.2	0.4	100
4	25	25	1.2	0.4	100
5	50	50	1.2	0.4	100
6	100	100	1.2	0.4	100
7	200	200	1.2	0.4	100

2. R script, created by Dr. M. D. Thom, which transforms the raw DAM data into output files. This produced data files with individual fly relative activity and life span.

```
setwd("") #insert appropriate working directory
nfiles<-9 #change this depending on how many DAM files you have
```

```
last<-function(s,column){
  flag<-0
  final<-NA
  prev<-NA
  startrow<-(s-min(dat[,1]))+1
  for(i in startrow:nrow(dat)){
    if(dat[i,column]==0 & flag==0){
      flag<-1
      prev<-final
      final<-dat[i,"index"]}
    else if(dat[i,column]!=0) flag<-0
  }
  final<-final-1
  prev<-prev-1
  if(!is.na(prev) &! is.na(final)){
    if(final-prev > 120) final<-prev
  }

  return(final)
}

for(set in 1:nfiles){
  dat<-read.table(paste("experiment1DAM",set,".txt",sep=""),sep="\t")

  dat<-dat[,-c(4:10)]
  y<-c("index","date","time",paste("hole",(1:32),sep=""))
  colnames(dat)<-y

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

Use previous time of death if the gap is over 30 minutes after the last movement detection.

Raw DAM data files should be name on the same template. Here: "experiment1DAM" and a number corresponding to data file number, "experiment1DAM1" up to "experiment1DAM9" (because 9 files).

Reads in a "layout" file that describes what flies were in what hole. See Appendix B.3

```

layout<-layout[layout$set==set, ]
layout$act<-0
layout$lastindex<-0
temp<-0

for(j in 1:length(layout[,1])){
  if(layout[j,"index"]>0){
    temp<-last(layout[j,"index"],j+3)
    layout$lastindex[j]<-temp
    layout$act[j]<-sum(dat[dat$index>=layout[j,"index"] &
dat$index<=temp,j+3])
  }
}

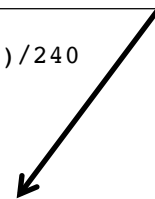
layout$lifespanh<-(layout$lastindex-layout$index)/240
layout$relact<-layout$act/layout$lifespanh

write.table(layout,paste("all_longevity_activity",set,".csv",sep=""),s
ep="," ,row.names=F)

}

```

Saves data in a separate output file for each input file.



3. "Layout" file.

(See figure below): Column A corresponds to the holes in the DAM, from 1 to 32 and should be repeated accordingly to the number of DAM set up. When starting a new DAM the number in the "set" column should change (here: set 1 for the first DAM and set 2 for the second). "index" corresponds to the time at which the flies were placed into the DAM, and index number of 1 means that fly was put in the DAM from the start. When a hole was not used and occupied by fly, the row was filled in with "-1". "nutrient" and "density" correspond to the experiments treatments.

	A	B	C	D	E	F
1	hole	set	date	index	nutrient	density
2	1	1	28/04/14	1	5	1
3	2	1	28/04/14	1	2	5
4	3	1	28/04/14	1	2	1
5	4	1	28/04/14	1	2	5
6	5	1	28/04/14	1	5	5
7	6	1	28/04/14	-1	-1	-1
8	7	1	28/04/14	1	-1	-1
9	8	1	28/04/14	1	3	5
10	9	1	28/04/14	-1	-1	-1
11	10	1	28/04/14	1	5	5
12	11	1	28/04/14	1	-1	-1
13	12	1	28/04/14	1	5	5
14	13	1	28/04/14	-1	-1	-1
15	14	1	28/04/14	1	3	5
16	15	1	28/04/14	1	2	5
17	16	1	28/04/14	-1	-1	-1
18	17	1	28/04/14	1	5	5
19	18	1	28/04/14	-1	-1	-1
20	19	1	28/04/14	1	2	5
21	20	1	28/04/14	1	3	5
22	21	1	28/04/14	-1	-1	-1
23	22	1	28/04/14	1	2	5
24	23	1	28/04/14	-1	-1	-1
25	24	1	28/04/14	1	5	5
26	25	1	28/04/14	1	2	1
27	26	1	28/04/14	-1	-1	-1
28	27	1	28/04/14	1	3	5
29	28	1	28/04/14	-1	-1	-1
30	29	1	28/04/14	1	3	5
31	30	1	28/04/14	-1	-1	-1
32	31	1	28/04/14	-1	-1	-1
33	32	1	28/04/14	1	3	1
34	1	2	05/05/14	1	2	1
35	2	2	05/05/14	1	2	5
36	3	2	05/05/14	1	3	1
37	4	2	05/05/14	1	5	1
38	5	2	05/05/14	1	5	1
39	6	2	05/05/14	1	2	5
40	7	2	05/05/14	-1	-1	-1
41	8	2	05/05/14	1	5	1
42	9	2	05/05/14	1	2	5
43	10	2	05/05/14	-1	-1	-1

FIGURE 3.13. *Illustration of how data should be presented within the “layout” file. This file is required in the R-script in order to translate data from the original DAM data output, into survival and relative activity data.*

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