**The effects of dietary adaptation to life history traits in the Mediterranean fruit fly (*Ceratitis capitata*)**

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# Abstract

Nutritional variation influences many traits including developmental times and adult phenotypes, including body mass. Responses to dietary variation is key to the evolution of dietary specialism and thus, speciation. This study assessed the effects of proximate diet versus the effects from evolved diet-mediated divergence between medflies reared on a low-calorie Starch larval diet and a high-calorie ASG larval diet. This study built on findings from earlier generations of the same fly lines by Leftwich *et al.* in 2019, to see whether new adaptations were shown. Different nutritional environments were found to cause divergence between the fly lines. Body mass, egg number and pupal showed evidence of diet-mediated adaptation. Development times and egg volume exhibited divergence between lines. A trade-off between egg number and egg volume was observed. Flies long reared on Starch were shown to have adapted to minimise negative effects of their low-calorie diet, benefitting from: faster development time and higher pupal survival. This furthers understanding of medfly trait plasticity, which could help improve mass-rearing programmes, eradicating populations of the agricultural pest. Understanding medfly adaptation to their nutritional environment could also help identify invasive risks of medfly populations.

# Introduction

## Dietary adaptation

Diet affects many biological mechanisms, influencing major life-history event timings and adult phenotypes, such as body mass and copulation success [1][2]. Therefore, individual’s responses to nutritional variation are key in driving divergent selection and thus, the evolution of dietary specialism and speciation [3].

Previous studies on insects show that larval diet can affect plastic and evolved responses including ageing, stress tolerance and fecundity [3][4]. Whereas evolved responses are heritable, plastic responses are non-heritable and their phenotype is flexible and can be affected by their environment, including their dietary conditions [5]. Nutrient availability for insect larvae affects larval development but is also vital for amassing nutrient reserves for pupae and has knock-on effects on adult phenotypes, including body mass [6][7].

Many studies have looked at proximate responses of life-history traits to specific ratios of nutrients, that maximise life-history traits, such as effects of different combinations of simple and complex carbohydrates [4][8]. However, less is known about the effects of long-term dietary selection, which is explored in this study.

### Parental stress hypothesis vs the adaptive hypothesis

Traits such as development time and body mass can be influenced by parental responses to diet [9]. The parental stress hypothesis predicts that poor parental diet produces poor-quality offspring due to poor-quality females having fewer resources for reproduction and, if applicable, child-rearing [9]. The adaptive hypothesis, however, suggests that parents on a poor-quality diet, under nutritional stress, would allocate resources to improve their offspring’s tolerance of poor nutrition [10]. One mechanism of resource allocation is a trade-off between offspring quality and number, with organisms in poor conditions investing more resources per offspring, producing fewer offspring but with improved fitness [11]. Studies on *Drosophila*, cockroaches, seed beetles and *Daphnia* show egg size increases when parents experience poor nutrition [9][12][13][14]. Female *Drosophila* reared on poor-quality diets laid 3–6% heavier eggs and produced offspring that developed 4% faster than those on high-quality diets, but those raised on poor nutrients had lower body mass; suggesting both adaptive plasticity and maladaptation result from parental nutritional stress [9]. Females in poor nutritional environments that invest more resources per offspring (environments the offspring are also likely to develop in), would be favoured under natural selection.

## Medfly – an agricultural pest

The study explores the effect dietary selection on the Mediterranean fruit fly, *Ceratitis capitata*, an experimentally tractable organism native to sub-Saharan Africa. It survives better in colder climates than many other fly species and, therefore, can be found across the world in a range of habitats [15]. Females oviposit beneath the skin of a variety of fruits and vegetables including plums, mangos, and pears. When these eggs hatch, the larvae cause major damage to the fruits. This makes medfly one of the most harmful agricultural pests, as it can damage over 300 different crops globally [16]. Infestations dramatically decrease crop yields, which has a large social and economic impact with many people relying on the food and the income. Crops from infested areas undergo expensive sorting processes to reduce spread of the pests and sometimes cause trade embargoes [17]. The establishment of medfly in California could cause a loss of $493 million to $875 million loss in revenue and a loss of over 14,000 jobs [17].

Development in medfly stops in temperatures below 10˚C, however, with climate change affecting habitats, medfly population sizes and distribution are predicted to increase within ten years [15][18]. With increasing food security issues, agricultural losses from medfly infestation are significant, and their possible effect will continue to grow.

Medlfy could also be a vector of human pathogens to crops. A study showed that a medfly could carry *E. coli* for 7 days following inoculation with the GFP-tagged bacteria [19]. If medflies infected fruit with human pathogens, they could cause global disease outbreaks [19][20]. Therefore, due to the increasing current impacts of this pest and its possible effects in the future, controlling medfly abundance and distribution is vital.

Traditional pest control methods, including pesticides, biological control and integrated pest management have been successful, but also cause problems, including economic cost, effectiveness and environmental and social issues. For instance, synthetic insecticides are non-selective and therefore kill other non-harmful or beneficial organisms, which can affect whole ecosystems [21]. An approach called the Sterile Insect Technique (SIT) encompasses the mass-rearing of irradiated, infertile male medflies. Large quantities of sterile males are released into wild populations of medfly, decreasing the size of the next generations by reducing successful copulations [22]. This method is not harmful to other species, does not require the use of chemicals on, or near, crops and in the long-term is more economically feasible [23]. This has been shown to be effective, including in western Australia in 1984 and in Guatemala and Mexico from 1979-1982 where SIT not only eradicated the flies, but also prevented the pest’s spread [24][25]. With this process happening on a large scale, and its importance increasing due to climate change, the approach needs to be as efficient as possible. Many factors, including larval diet, need to be optimised [26]. Shorter development time, higher egg-to-adult survival rate and higher fertility rate, would mean many high-quality flies can be produced quicker in the mass-rearing programmes, increasing rate at which males could be released. However, the male flies also need to have high fitness when released into wild populations, so their longevity, copulation rate and tolerance to environmental conditions, such as diet, also needs to be increased for optimal results. Increasing the efficiency of the SIT by optimising these traits would decrease the economic cost and could irradicate infestations faster, reducing their effects on crops. Therefore, understanding the effects of proximate diet and the effects of long-term dietary adaptation could help improve the mass-rearing technique and the efficiency of the male flies once released.

Nutrient effects on medfly survival, copulation success and development have been well studied to improve mass-rearing efficiency, but predominantly the proximate responses [27][28][29]. Long-term adaptation to divergent diets is less understood. A study by Leftwich *et al.* in 2019 used earlier generations of the same fly lines used in this experiment and found evidence of evolved divergence between lines reared on different larval diets for egg to eclosion development times and egg to adult survival [30].  The study also found evidence of adaptation, with flies exhibiting higher adult body mass when reared on their ‘own’ diet.

Understanding medfly adaptation to nutrition could also be vital in understanding the pest’s invasive potential. Development time and survival has been seen to differ in geographically isolated populations of medfly, this could be due to adaptation to their local nutritional environment and could impact their invasive potential; population-specific medfly control methods may, therefore, be needed [31].

Hypotheses and aims

To assess the effects of proximate diet versus effects from evolved diet-mediated divergence, the following traits were compared across medfly lines either reared on their own long-term larval regime diet or crossed onto the opposite diet: development time, survival, adult female weight, egg number and egg size. Two diets were used in this experiment, a high-calorie ASG diet, composed of simple and complex carbohydrates; and a low-calorie Starch diet composed of complex carbohydrates [30]. The lines of flies used were later generations of those used in an experiment by Leftwich *et al.* (2019) on larval dietary adaptation in medfly, so I will compare my results to those from earlier generations, as any changes could suggest that adaptations to the different diets have occurred or that long-term laboratory conditions negate diet effects [30].  This earlier study showed that medfly on ASG obtained more successful copulations than those on Starch, which is likely due to increased available nutrition reserves from the larval stage in ASG flies [32]. However, males and females on from the regime Starch lines developed from an egg to an adult quicker than those on ASG and had a higher egg-to-adult survival rate. Proximate larval Starch diet lowered pupa to adult survival rate.

Therefore, I investigated multiple similar hypotheses. This first set of hypotheses are:

1. ASG regime flies will have longer development times than Starch regime medfly and
2. Medfly on ASG proximate larval diet will have a higher pupa to adult survival probability.

Whether the results follow the trends observed in the earlier generations and if any adaptations are observed will also be investigated.

The earlier study by Leftwich *et al*. (2019) found that flies reared on ASG had a higher body mass than those on Starch, and that both lines were heavier when reared on their own diet; this suggests adaptation to their respective diets [31]. The effect of larval diet on body mass many generations later is investigated to see whether this still follows the same observations that:

1. Regime ASG flies will have a higher body mass than the Starch lines and
2. both Starch and ASG lines will be heavier when raised on their own diet.

To see if a trade-off exists between egg number and egg size, possibly providing evidence for the adaptive hypothesis, the effect of diet on egg volume and egg number was measured [11]. Previous studies in other insects, including *Drosophila* [19], led to the hypotheses that:

1. Regime Starch flies would produce eggs of a larger volume than the regime ASG flies and
2. the regime ASG flies would produce a higher egg count than the Starch regime flies.

# Method

## Fly stocks and maintenance:

Fly lines were obtained from the Guatemalan TOLIMAN wildtype strain, which has been laboratory reared since 1990 [33]. Derivatives were raised on a wheat bran diet (24% wheat bran, 16% sugar, 8% yeast, 0.6% citric acid, 0.5% sodium benzoate) since, at least, 2015 with adults fed on a 3:1 sucrose: yeast hydrolysate mix [2]. These lines were then established, by Leftwich *et al.* in 2017, on two different larval diets, either:

* An ASG (A) diet (7.4% sugar, 6.7% maize, 4.75% yeast, 2.5% Nipagin (10% in ethanol), 1% agar, 0.2% propionic acid);
* Or a Starch (S) diet (5% yeast, 3% starch, 1.5% agar, 0.5% propionic acid).

Three experimental lines for each regime diet (A1, A2, A3, S1, S2, S3) were long reared on their respective larval diet and maintained separately by Leftwich *et al.* (2017 and 2019). In this current study, three egg collections were taken for each experimental line (over 3 days) and raised separately. Temperature was controlled at 25˚C for both diets and they were maintained under a 12:12 hr light-dark cycle with 50% relative humidity. All adult flies were fed weekly and given water biweekly.

## Development times/pupae to adult survival

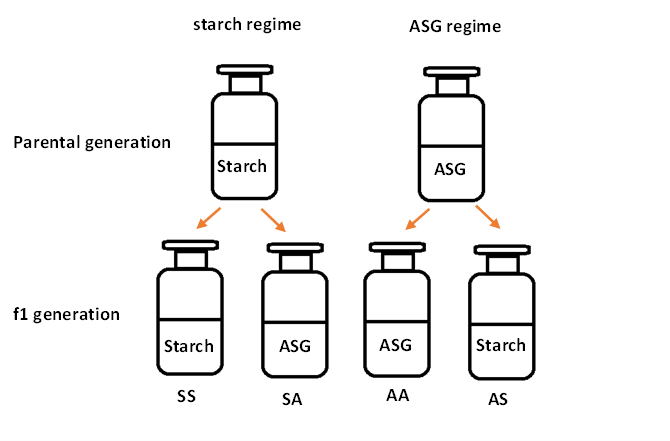
A pilot study was carried out to determine peak egg-laying for the diet regimes and to determine the best method for observing adult eclosion, which was found to be eclosion plates with a cover of perforated adhesive PCR film (Supplemental Method 1; *Supplemental Figure 1*).

To analyse effects of regime diet, proximate larval diet and to observe any possible adaptations, diet crosses were completed, producing four treatments (regime larval diet/proximate larval diet: AA, AS, SS and SA) (*Figure 1*). A daily egg collection from the flies in the pilot study were carried out for three days (following Supplemental Method 2). From each day of egg collections ~250 eggs from each Starch and ASG replicate were transferred to wet Fisher Scientific filter paper (QL100, size 90mm) and placed into glass bottles containing 100ml of their regime larval food. ~250 eggs were also swapped to the reciprocal diet. S1 lines produced a low egg number, so on the first date of collections, eggs from the S1 line were only put in a bottle with ASG food. For the other two egg collection dates, S1 eggs from my study were combined with S1 eggs from a colleague’s study (kept under the same conditions and developed from the same time) to enable enough eggs for Starch and ASG bottles. 35 bottles were produced in total.

Each bottle was laid down in a box with sand seven days post egg collection, at around the time of the third larval instar. Boxes were checked daily at 14:00, and any pupae found were counted and placed into wells in eclosion plates, then covered with perforated PCR adhesive film. 60 pupae from the peak pupation date of each pupation cage were placed into petri dishes (15 pupae per petri dish) to be used for the adult cages once eclosed.

The plates and petri dishes containing pupae from the first of the three F1 egg collection dates were checked for eclosions and their sex at 9:00AM (at lights on), 12:00, 15:00 and 18:00 to see if a pattern in eclosion exists (*Supplemental Figure* *2* & Supplemental Table 1). The plates and petri dishes from the other days were checked for eclosions and sex at 18:00.

At 3pm, eclosions in the petri dishes were checked and eclosed flies were transferred into cages until 20 females and 10 males were present in each. Flies were transferred by placing the petri dishes into ice until unconscious, then transferred with soft forceps by the wings. Flies were not on ice for longer than 7 minutes. First egg-laying was noted for each cage.



***Figure 1.*** Reciprocal diet crosses between a Starch (S) line and an ASG (A) line (both long reared on a regime of their respective diet) to produce four diet regimes (regime larval diet, proximate larval diet: SS, SA, AA and AS) to allow effects of diet to be observed.

## Egg number

Eggs were collected on the fourth day of egg-laying for each cage (pilot study found this was the day with highest egg count for most lines) using my egg collection protocol (Supplemental Method 1) and were counted under a dissecting microscope using a paint brush. The mesh size for each cage was kept constant.

## Egg volume

Whilst counting the eggs laid by the parental generation, I noticed that the S replicates appeared to lay larger eggs than the ASG lines, so I decided to test this by sealing and freezing spare eggs laid. Eggs were removed from the freezer and 30 eggs were aligned in the same orientation on damp filter paper, this was done for each of the 6 lines. The programme ToupView (version x64, 3.7.5660) was used to take measurements and a Brunel microscope (model N-300M) at 4 X magnification was used and calibrated. Length was taken from the centre of the anterior end to the centre of the posterior end; width was taken at half the length (*Figure* 2). Egg volume was then calculated using the formula [34]. Starch eggs were found to be on average 17.6% larger than ASG eggs with Starch eggs having a mean volume of 21309 [95% CI: 21023 – 21594] and ASG eggs having a mean volume of 18124 [95% CI: 17839 – 18409]. A linear model (LM) showed that the Starch regime flies produced significantly larger eggs (LM: *df* = 358, *t* = 15.52, *P* < 0.001). Therefore, eggs produced by F1 generation were measured to assess the effect of regime A screenshot of a computer

Description automatically generated with medium confidencediet compared to proximate larval diet.

***Figure* 2.** Medfly eggs through a camera attached to a microscope. Using the programme ToupView, the microscope was calibrated with the programme, and length was measured to 0.01µm from the centre of the anterior end to the centre of the posterior end. Width was measured at half the length of the egg.

## Female weight

Flies from the cages were frozen after egg collections were taken. The dry weights of females from the development tests were taken by desiccating the flies at 60˚C for 24hrs in a drying oven. A plastic weigh boat was weighed, and the balance zeroed before 5 female flies from each cage were weighed individually in the tray on a AND BM-20 microanalytical balance.

## Data analysis

R programming software v4.0.2 [35] was used to run analyses on the data collected. Egg to adult eclosion development time was calculated for each individual fly and modelled using Poisson log-link generalised linear-mixed models (GLMMs) to compare across diet treatments and males and females. Fly replicate, F1 egg collection date, eclosion plate and eclosion well were treated as random effects. Egg to eclosion was then split into larval development time (egg to pupation) and pupae development time (pupation to eclosion) and modelled in the same way.

A binomial log-link GLMM was used to compare pupae to adult survival across diet treatments. Female body mass, eggs per female and egg volume was compared across diets using linear-mixed models. The estimated marginal means (EMMs) for each model were then calculated.

All analyses were carried out in R (v4.0.2) [35] with the following packages: lme4 [36], lmerTest [37] and emmeans [38] for modelling and analysis; and tidyverse [39], and DHARMa [40] for checking model assumptions.

# Results

## Development time

### Egg to Eclosion

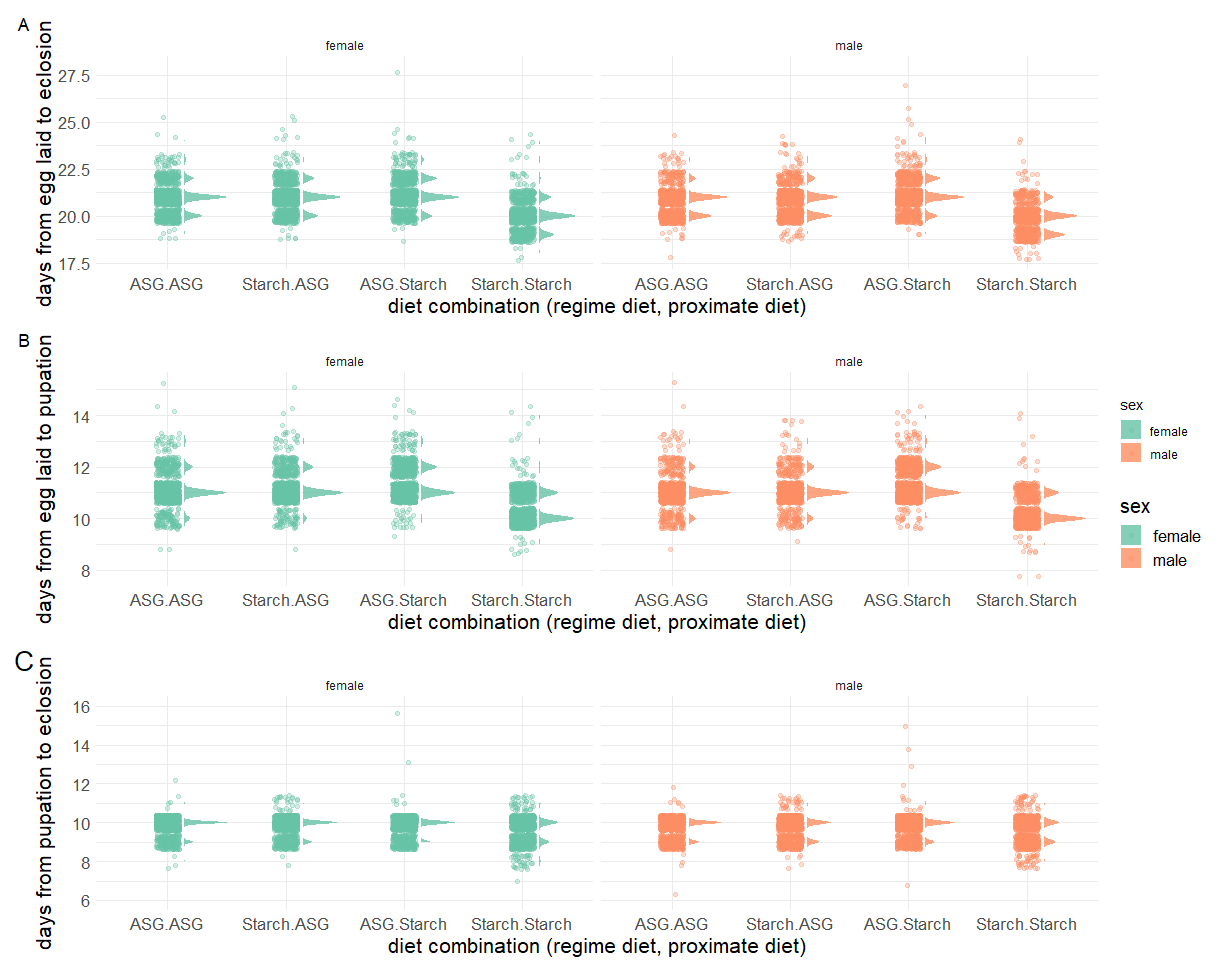
Egg to eclosion time was calculated for each fly to determine the relative effects of sex, dietary adaptation, and immediate larval diet on development rates. (*Figure 3A*). Development time appears to be affected by an interaction between the evolutionary diet regime and the proximate larval diet (Poisson GLMM: N = 7999, z = -5.265, P < 0.001, Supplemental Table 2). Flies reared on the immediate larval diet that matched their evolutionary diet regime developed faster than flies switched onto the reciprocal diet; this effect was much more pronounced in flies from the Starch regime (*Figure 3A*). ASG flies reared on a Starch diet (AS) had the longest development time at 21.2 days [95% CI; 20.8-21.6], while AA and SA flies both had a mean development time of 21 days [AA 20.6-21.4; SA 20.6-21.5]. The Starch flies reared on a Starch diet (SS) had the fastest development time at 20 days [19.6-20.4]. While males trended towards a faster development time than females, the effect was small and non-significant at this sample size (Supplemental Table 2).

I then investigated whether differences in development time from egg to adult eclosion followed the same pattern at all stages of development, by analysing egg to pupation, and pupation to adult eclosion separately.

Egg to pupation development time also appears to be affected by an interaction between the evolutionary regime diet and the proximate larval diet (Poisson GLMM: *N* = 8683, *z* = -7.229, *P*< 0.001, Supplemental Table 3). Both the ASG and the Starch regime flies developed quickest on their ‘own’ proximate diet. This effect was more distinct in the Starch regime flies (*Figure 3B*). Proximate larval Starch diet increased development time by 3%, compared the ASG flies on larval ASG diet (Poisson GLMM: N = 8683, z = 2.755, P = 0.006, Supplemental Table 3). Starch regime flies reared on a larval Starch diet (SS) had the fastest egg to pupation time at 10.3 days [95% CI: 10.1 – 10.5] followed by AA and SA which both with means of 11.1 days [95% CI: AA 10.9 - 11.3; SA 10.9 – 11.3]. Again, males trended towards developing faster than females, but the effect was small and non-significant (Supplemental Table 3).

Flies on a Starch proximate diet trended towards faster pupation to eclosion development, and Starch regime flies trended towards faster development, but the effects were small and non-significant (*Figure 3C*) (Supplemental Table 4). ASG regime flies reared on ASG proximate diet (AA) developed the slowest with a mean development time of 9.81 days [95% CI: 9.64 – 9.98]. SA had a mean development time of 9.79 days [9.61 – 9.98] and AS had a mean development time of 9.56 days [9.6 – 9.94]. SS flies had the shortest development time at 9.56 days [9.38 – 9.74].

Adult Eclosion to Egg-laying was also compared across the diet treatments, however, very little effect of regime or proximate diet was shown (*Supplemental Figure 3*; Supplemental Table 5).



***Figure 3.*** **Developments times of Medfly individuals from lines long reared on either a ASG or Starch regime larval diet, which have been maintained on their evolutionary regime diet or crossed onto the reciprocal larval diet for their proximate diet.** *Figure (A)* shows a density plot and the distribution of the number of days from egg to adult eclosion for males and females. *Figure (B)* shows a density plot and the distribution of the number of days from egg to pupation for males and females. *Figure (C)* shows a density plot and the distribution of the number of days from pupation to adult eclosion for males and females. (Each dot represents an individual fly).

## Pupae to Adult Survival

The relative effects of dietary adaptation and proximate larval diet on development pupae to adult survival was measured by determining the probability of eclosion for each diet (*Figure 4*). Pupal to adult survival appears to be affected by an interaction between the regime diet and the proximate larval diet (Binomial GLMM: *N* = 8683, *z* = -3.876, *P* < 0.001, Supplemental Table 6). Flies reared on the proximate larval diet that matched their regime diet had a higher probability of surviving from pupation to eclosion than flies switched onto the reciprocal diet, an effect which was more pronounced in the ASG regime lines.

Starch regime flies on proximate Starch diet (SS) had the highest probability of surviving from pupation to eclosion, with a survival probability of 0.933 [95% CI: 0.855 – 0.970], which is 7.1% higher than the diet with the lowest probability, AS, with a 0.871 probability of eclosion [0.746 – 0.939]. SA pupa had a 0.92 chance of eclosing [0.832 – 0.964] and AA had a 0.921 probability of surviving from pupation to eclosion [0.835 – 0.964]. Proximate Starch diet significantly lowered the probability of survival for ASG regime flies by about 5.4% (Binomial GLMM: N = 8683, z = -4.780, P < 0.001, Supplemental Table 6).

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***Figure 4*.** **The probability of adult eclosion from Medfly individuals from lines long reared on either a ASG or Starch larval regime diet which have been maintained on their regime diet or crossed onto the reciprocal larval diet for their proximate diet.** (Circles = the estimated marginal means produced by binomial general linear-mixed models on the data and the coloured lines from them represent the 95% confidence intervals).

## Female weight, egg volume and egg number

### Egg volume

Egg volume () compared across diets (*Figure 5A*). Egg volume appeared to be affected by an interaction between the evolutionary diet regime and the proximate diet, greatly increasing the egg volume produced by Starch regime flies on Starch larval diet (LMM: *df* = 474.91, *t* = 7.253, *P* < 0.001, Table 6). Starch regime flies on their matching proximate diet (SS) had the produced eggs of the largest volume with a mean volume of 22866[95% CI: 22131 - 23602]. Egg produced by SA flies had a mean volume of 19548 [18812 – 20284], followed by AS flies which produced a mean egg volume of 18561 [17853 – 19270]. AA flies produced the eggs with the smallest volume at 17689 [16981 – 18397].

Proximate Starch diet significantly increased the volume of ASG regime flies by 4.9% (LMM: *df* = 461.23, *t* = 3.601, *P* < 0.001, Supplemental Table 7). But regime Starch diet produced a greater effect on egg volume, increasing egg volume by 10.5% (compared to AA flies) (LMM: *df* = 6.46, *t* = 5.241, *P* < 0.001, Supplemental Table 7).

### Egg number per female

To account for differences in female number, total egg count per cage from the 5hrs of collection was divided by female number. Flies on the regime ASG diet (AA) produced the largest mean number at 35.7 eggs per female [95% CI: 29.8 – 41.7], followed by AS flies at 32.4 eggs per female [26.4 – 38.4]. SS flies produced a mean egg count of 27.9 eggs per females [21.9 – 33.9]. Flies from the Starch regime reared on proximate ASG larval diet laid the fewest eggs with a mean of 26.5 eggs per female [20.6 – 32.5], which was 26% fewer eggs than the AA flies.

The experimentally evolved Starch lines had the lowest rate of egg-laying regardless of their proximate diet (LMM: *N* = 35, *t* = -2.636, *P* = 0.0347, Supplemental Table 8). Flies from both diet regimes laid more eggs when on a proximate diet that matched their regime diet (*Figure 5B*). This difference as especially pronounced between AA and AS lines, with egg number decreasing by 9.3% when ASG regime flies were switched to a proximate Starch larval diet.

### Adult female weight

Adult female dry weight (mg) was compared across diets and appeared to be affected by an interaction between the evolutionary diet regime and the proximate larval diet (LMM: *df* = 167.336, *t* = 4.294, *P* < 0.001, Supplemental Table 9). Flies from both regime diets were heavier when reared on their ‘own’ proximate larval diet than when switched to the reciprocal diet and this effect was most pronounced in the ASG regime flies (*Figure 5C*).

Flies from the ASG regime reared on a proximate ASG larval diet had that largest mean dry female body weight at 3.46 mg [95% CI: 3.27 – 3.65]. AS flies had a mean body weight of 3.16 mg [2.97 – 3.34], then SS at 3.07 mg [2.88 – 3.26]. SA flies had the lowest mean body weight at 3.01 mg [2.82 – 3.20].

Larval Starch diet significantly decreased female body weight by 0.31 mg (compared to AA flies) (LMM: *df* =167.051, *t* = -5.122, *P* < 0.001, Supplemental Table 9). However, regime Starch diet caused a greater weight decrease of 0.45 mg (compared to AA flies) (LMM: *df* = 5.632*,* *t* = -4.247, *P* = 0.0062, Supplemental Table 9).

Chart, box and whisker chart

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***Figure 5*.** **Life history traits of Medfly individuals from lines long reared on either a ASG or Starch larval diet regime which have been maintained on their regime diet or crossed onto the reciprocal larval diet for their proximate diet.** Plot (A) shows the effect of diet on egg volume (). Plot (B) shows the effect of diet on the number of eggs laid per female. Plot (C) shows the effect of diet on adult female dry weight (mg). The circles show the estimated marginal means produced by linear-mixed models of the data, and the coloured lines from them represent the confidence intervals.

# Discussion

## Development time and pupae to adult survival

Development times from egg to eclosion and egg to pupation showed that flies from both regimes developed faster when reared on their ‘own’ proximate larval diet, suggesting adaptations to their respective diets. Generation 30 from the earlier study by Leftwich *et al*., showed that these development times were only influenced by proximate diet, with ASG regime flies and Starch regime flies developing slower when reared on Starch [30]. Now SS flies pupate quicker, suggesting the adaptation of Starch regime flies to Starch food (in respect to quicker larval development time) is recent. However, generation 5 of this study showed that SS flies developed faster than SA flies which is the same as our results. This could suggest that the lines responses to the larval diet fluctuate regarding development time or that the divergence that appeared to evolve in the generation 30 lines was inaccurate. Therefore, further testing on more generations would be needed to confirm whether an adaptation is present.

It would be interesting to look at the development times of F1 generation’s offspring to see whether AS offspring would show a reduced development time due to parental effects of being reared on the lower quality Starch diet, which would support the parental adaptive hypothesis.

Flies from the ASG regime on proximate ASG larval diet had the fastest mean pupal development time, however no significant differences were observed between pupation to eclosion time across the diets at this sample size. Generation 30 of the previous study lines showed that ASG regime flies had a significantly longer development time, regardless of proximate diet [30]. As ASG regime flies now show no significant difference to Starch regime flies, this could suggest SS flies are adapting to allocate more diet reserves to early development. This would be beneficial as the longer the larval development time, the more nutrients would become unavailable due to competing larvae, build-up of waste materials and from desiccation of food [9]. However, generation 5 of the study by Leftwich *et al.* showed that AA flies had the fastest pupation to eclosion rate, following a similar pattern to our results [30]. This adds further evidence that development rate fluctuates between the lines, requiring further investigation as to whether this is the case.

Males trended towards faster development times than females in both egg to pupation and pupation to eclosion time. Although I found no evidence for differences in the development rate between the sexes. Male medfly have been shown to develop to reproductive maturity faster post-eclosion than females [41] and a review on sex differences in invertebrate development times found that in 76% of the invertebrates studies females had a longer larval development period [42]. Further study could provide evidence for this divergence and a higher sample size might allow the effects of sex on development time to be clearer

Pupae to adult survival was highest in Starch regime flies but flies on both Starch and ASG had a higher probability of surviving from pupation to eclosion on their own diet, suggesting adaptation to their respective diets. Earlier generations showed lower pupal survival when reared on a proximate larval starch diet, regardless of their regime diet [30]. As SS now displays a higher survival rate than SA flies, it suggests recent adaptation within Starch regime flies to the low-quality Starch diet.

## Egg number, egg size and female weight

Flies from the Starch regime reared on proximate larval Starch diet (SS) produced the largest eggs (volume ) whilst AA produced the smallest. Both regime Starch diet and proximate Starch diet increased egg volume, with SS showing evidence for adaptation to the low-quality Starch diet. A study by Vijendravarma *et al.* in *Drosophila* found parents raised on poor-quality arval food laid 3–6% heavier eggs than parents raised on a higher-quality diet [9]. This increase in egg could be due to increased egg provisioning, meaning Starch eggs are higher quality [9]. This could provide evidence for the parental adaptive hypothesis as poor parental diet can have an immediate, possibly adaptive, effect on egg size in just one generation. However, large volume might not correlate with high quality and could be due to increased water weight rather than nutrients, so weighing desiccated eggs and measuring the hatching success across the diets could help determine whether the larger Starch eggs are higher quality. As the Starch regime flies showed higher pupal to adult survival and faster development times, this could provide evidence that Starch regime flies (which laid larger volume eggs) have higher fitness than ASG flies. A study by Azevedo *et al*. found that in a variety of invertebrates 62% of the species showed that as egg size increased, development rate increased, which agrees with our results [43]. The study also showed that 61% of Arthropoda tested showed a positive correlation between egg size and survival, also suggesting larger egg size correlated with increased fitness.

Both regimes reared on their ‘own’ proximate larval diet had increased egg-laying rate compared to when reared on their reciprocal diets, and Starch regime flies produced fewer eggs regardless of proximate diet. This suggests that both regimes have adapted to their respective diet, with starch regime flies adapting to their poor-quality diet to produce a lower egg count.

Egg size and egg number appear to be inversely correlated suggesting a trade-off between egg number and egg volume, with regime Starch flies adapting to produce fewer but larger eggs. This could provide evidence for the adaptive hypothesis that flies reared on a less nutritious diet would produce fewer but higher quality eggs, but further investigation would be required to determine whether larger volume does correlate with higher fitness [9].

A previous experiment has shown that flies reared on ASG had more copulations than those on Starch (especially AA flies), as resources acquired during larval development provide energy reserves in adults [2][44]. This increased copulation could cause the higher egg counts produced by ASG regime flies, but further investigation would be needed to look at this directly.

Earlier generations of the fly lines showed that flies from both diet regimes had higher body mass when reared on their own diet, suggesting nutrition-mediated adaptation to their respective diet [30]. Many generations later, these results remain the case, as both lines were still heaviest on their own diet, and AA flies were heavier than SS flies. As starch regime flies are smaller than ASG regime flies, this suggests body mass does not fit the adaptive parental effect hypothesis, instead it conforms to the maternal stress hypothesis. Exploring the adult body mass of the F2 generation would provide further insight as to which hypothesis body mass fits.

The lower body mass of those with parents on a low-quality diet could reflect a trade-off between development time and adult body mass, by having a lower critical size for metamorphosis, which carries on to adulthood [6]. A study on *Drosophila* found that flies long-term reared on a poor-quality larval diet evolved a 18% smaller critical size than those on a high-quality diet [6]. If medfly evolved a lower critical size threshold for metamorphosis initiation, like *Drosophila*, this could be producing the lower larval development time in Starch lines and the lower adult body mass. Therefore, it would be interesting to explore the critical size threshold of these medfly lines to see whether divergence in critical size thresholds exists. Another reason for the lower body mass of Starch regime flies could be that adults from a poor larval diet could appoint more resources to reproduction than adult growth and maintenance.

## Future research directions

This experiment showed that lines of medfly long reared on different diets can diverge and adapt to their respective diet over time, affecting adult phenotypes and development times. Exploring the effect of long-term diet regimes on similar traits, with respect to adult diet, and whether this allows for any adaptation could be beneficial. As poor larval diet can lead to adaptation in traits such as pupal survival and development time, maybe adult diet could also cause some divergence. If so, lines long reared on a poor adult diet could be better in mass rearing programmes as males could be better adapted to survive in the wild, especially if released into poor nutritional conditions.

The best choice for larval diet may not be as intuitively predictable as expected as despite low nutrients, experimental lines long reared on Starch showed many benefits, including higher pupal survival and faster development. These traits could be beneficial in mass rearing programmes, increasing their efficiency. It would be important to see the effects of dietary adaptation on overall longevity of the flies, as infertile male which sexually active for longer would be more efficient at decreasing medfly population sizes. It would also be beneficial to see how flies long reared on Starch handle poor adult diets compared to lines on high-quality larval diets, as being able to survive better on a poor-quality adult diet would be beneficial to males once released.

It would be beneficial to explore egg to eclosion and egg to pupation development times in further generations to see whether development time does fluctuate between the lines, or whether results from this study or from earlier generations of the fly lines in the previous study by Leftwich *et al.* are inaccurate [30].

This study aids the understanding of diet-mediated divergence in medfly, and the plasticity of different traits, which could be vital to understanding divergence between isolated medfly populations and their invasive potential [45][46]. Divergence in body size, for instance, could affect invasiveness as larger flies may be unable to colonise new host crops [47]. This could help to identify populations which pose higher invasive risks [30].

To investigate whether egg volume does correlate with high fitness, desiccated egg weight and hatching probability could be measured as higher hatching rate and heavier desiccated egg weight would suggest higher maternal provisioning and higher egg quality. The organic content of the eggs could also be studied by dichromate oxidation against a glucose standard, a method which is described by McEdward and Carson in 1987 [48]. This study by McEdward and Carson, on Starfish, found that egg volume was not a reliable predictor of egg contents [48]. Therefore, despite divergence in egg volume between our lines, egg contents and thus, egg provisioning might show no divergence. It would also be interesting to see the effect of diet on pupae weight, and whether this follows the pattern of egg volume or adult body mass.

Overall, the study showed that the two larval diets supplied enough variation in nutrients to cause divergence between the fly lines. Body mass, egg number and pupal survival showed evidence for diet-mediated adaptation, with regime Starch lines evolving to minimize the negative effects of their poor-quality diet. Development time and egg volume require further research to determine whether the divergences observed are adaptations. There is evidence of a trade-off between egg number and egg volume, with parents on poor-quality diet adapting to produce fewer offspring but larger eggs with possibly a higher fitness. This helps understanding the divergence of medfly traits to help improve mass-rearing programmes and to help identify the invasive risks of medfly populations.

**Data/code:** https://github.com/AmyButler1/Medfly-Development-Study.git

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# Supplemental Materials:

## ***Text Description automatically generated with medium confidence***Supplemental Figures

***Supplemental Figure 1:*** Eclosion plates, each with 96 wells for individual pupae to be placed within. Plate A is covered with parafilm, however this provided poor visibility and eclosed flies could escape into neighbouring wells. Plate B was covered with Thermo Scientific adhesive PCR, this was the most efficient method. Plate C was covered with a lid, however, this some flies escaped into neighbouring wells. Both plate A and B had to had small holes poked into the top of each well.

*Chart, bar chart, waterfall chart

Description automatically generated****Supplemental Figure 2:*** **Probability of eclosion over 24 hours for males and females on different larval diet treatments (regime diet, proximate larval diet).** (Grey blocked areas represent lights off; White blocked areas represent lights on.

Chart, histogram

Description automatically generated ***Supplemental Figure 3.*** **First adult eclosion to first egg-laying development times of medfly cages from lines long reared on either a ASG or starch larval diet regime which have been maintained on their regime diet or crossed onto the reciprocal larval diet for their proximate diet.** The figure shows a density plot of the number of days from first eclosion within the cage to first egg-laying within the cage. (Lines on graph represent the estimated marginal means produced by a Poisson general linear model and the corresponding 95% confidence intervals; the values on the graph are the estimated marginal means). Little evidence was found for differences in the rate of eclosion to egg-laying between the diet regimes and the proximate diets. But this could be due to low sample size as, unlike the earlier development times, this was done per cage rather than per individual. A study by Kaspi *et al.* showed medfly reared on a better-quality larval diet sexually matured quicker than those on a low-quality diet [32]. This suggests that ASG flies should have a faster eclosion to egg laying development time. ASG flies did show the fastest mean sexual maturation development time, but the differences between diets were very small. Further experiments, looking at sexual maturation time per mating pair instead of per cage, on a larger scale, would be beneficial. This would help see if the lack of significance was due to absence of effect, low sample size or because starch flies evolved a faster sexual maturation time, decreasing the divergence between the lines. As a trend was shown that both lines developed faster on their respective larval diet, Starch may have adapted to their low-nutrient diet.

Supplemental Tables

**Supplemental Table 1:** A Poisson log-link General Linear Mixed Model to analyse the effects of sex, regime diet, and proximate diet on hour of eclosion, and to see whether there was an interaction effect between regime diet and proximate diet. The model shows that sex and proximate diet have no effect on hour of eclosion and that there is no significant interaction between regime and proximate diet. However, regime diet does produce a significant affect, with more SA flies eclosing later in the day.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Time of eclosion:  Fixed effects N = 2687 | Estimate (log odds) | Std. Error | t - value | P - value |
| (Intercept) | 2.247360 | 0.018626 | 120.657 | <2e-16 |
| Sex = male | 0.020768 | 0.012245 | 1.696 | 0.0899 |
| Regime diet = starch | 0.064136 | 0.025471 | 2.518 | 0.0118 |
| Proximate diet = starch | 0.007607 | 0.016829 | 0.452 | 0.6513 |
| Proximatel diet starch: Regime diet starch | 0.042789 | 0.024812 | 1.725 | 0.0846 |
|  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |
| Fly line | 0.0005178 | 0.02275 |  |  |

**Supplemental Table 2:** Poisson log-link GLMM of time from egg to eclosion across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Egg to Eclosion:  Fixed effects – N = 7999 | Estimate (log odds) | Std. Error | z - value | P - value |
| (Intercept) | 3.047186 | 0.010636 | 286.491 | <2e-16 |
| Sex = male | -0.006463 | 0.005717 | -1.130 | 0.258 |
| Regime diet = starch | 0.001554 | 0.012620 | 0.123 | 0.902 |
| Proximate diet = starch | 0.010685 | 0.007719 | 1.384 | 0.166 |
| Proximate diet starch: Regime diet starch | -0.060493 | 0.011489 | -5.265 | 1.4e-07 |
|  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |
| Eclosion plate | 4.102e-04 | 0.0203 |  |  |
| Plate well | 0 | 0 |  |  |
| Fly line | 7.186e-05 | 0.0085 |  |  |
| Egg collection date | 1.030e-04 | 0.0101 |  |  |

**Supplemental Table 3:** Poisson log-link GLMM of time from egg to pupation across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Egg to Pupation:  Fixed effects N = 8682 | Estimate (log odds) | Std. Error | z - value | P - value |
| (Intercept) | 2.407790 | 0.009347 | 257.600 | <2e-16 |
| Sex = male | -0.006716 | 0.006758 | -0.994 | 0.32033 |
| Regime diet = starch | 0.003960 | 0.010261 | 0.386 | 0.69951 |
| Proximate diet = starch | 0.025256 | 0.009168 | 2.755 | 0.00587 |
| Proximate diet starch: regime diet starch | -0.098270 | 0.013594 | -7.229 | 4.86e-13 |
|  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |
| Fly line | 2.533e-05 | 0.0050 |  |  |
| Egg collection date | 8.109e-05 | 0.0090 |  |  |

**Supplemental Table 4:** Poisson log-link GLMM of time from pupation to eclosion across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Pupation to Eclosion:  Fixed effects N = 7999 | Estimate (log odds) | Std. Error | z - value | P - value |
| (Intercept) | 2.286851 | 0.009695 | 225.875 | <2e-16 |
| Sex = male | -0.007756 | 0.008332 | -0.931 | 0.352 |
| Regime diet = starch | -0.001327 | 0.011595 | -0.114 | 0.909 |
| Proximate diet = starch | -0.003829 | 0.011297 | -0.339 | 0.735 |
| Proximate diet starch: regime diet starch | -0.020573 | 0.016675 | -1.234 | 0.217 |
|  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |
| Eclosion plate | 0.000e+00 | 0.0000 |  |  |
| Plate well | 0.000e+00 | 0.0000 |  |  |
| Fly line | 0.000e+00 | 0.0000 |  |  |
| Egg collection date | 4.971e-05 | 0.00705 |  |  |

**Supplemental Table 5:** Poisson log-link GLM of time from first eclosion to first egg-laying per cage, across starch and ASG fly lines reared on either their own larval diet or the opposite diet (each cage contained flies from their respective peak pupation date). ASG regime flies reared on a proximate ASG larval diet developed the quickest at 2.78 days [95% CI: 1.88 – 4.11]. AS flies had the second quickest development time at 3.00 days [2.06 – 4.37], followed by SS flies with a mean develop time of 3.12 days [2.11 – 4.62]. SA flies took the longest to develop from adult eclosion to egg-laying with a mean development time of 3.44 days [2.42 – 4.90]. Flies from both evolutionary diet regimes developed slowest on their reciprocal diet, however, at this sample size the effect was small and non-significant.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Eclosion to egg laying:  Fixed effects N = 35 | Estimate (log odds) | Std. Error | z - value | P - value |
| (Intercept) | 1.02165 | 0.20000 | 5.108 | 3.25e-07 |
| Regime diet = starch | 0.21511 | 0.26881 | 0.800 | 0.424 |
| Proximate diet = starch | 0.07696 | 0.27756 | 0.277 | 0.782 |
| Proximate diet starch: Regime diet starch | -0.17429 | 0.38639 | -0.451 | 0.652 |

**Supplemental Table 6:** Binomial log-link GLMM of probability of pupa to adult survival across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Pupae to adult survival:  Fixed effects N = 8682 | Estimate (log odds) | Std. Error | z - value | P - value |
| (Intercept) | 2.4594 | 0.4280 | 5.747 | 9.09e-09 |
| Regime diet = starch | -0.0169 | 0.3722 | -0.045 | 0.9638 |
| Proximate diet = starch | -0.5536 | 0.1158 | -4.780 | 1.75e-06 |
| Proximate diet starch: Regime diet starch | 0.7379 | 0.1903 | -3.876 | 0.0001 |
|  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |
| Eclosion plate | 2.500e-01 | 5.000e-01 |  |  |
| Plate well | 2.133e-10 | 1.461e-05 |  |  |
| Fly line | 1.799e-01 | 4.241e-01 |  |  |
| Egg collection date | 8.479e-02 | 2.912e-01 |  |  |

**Supplemental Table 7:** *t*-test results from a linear-mixed model of egg volume across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Egg volume  Fixed effects N = 480 | Estimate (log odds) | Std. Error | df | t - value | P - value |
| (Intercept) | 17689.29 | 250.90 | 6.46 | 70.503 | 1.43e-10 |
| Regime diet = starch | 1858.79 | 354.66 | 6.46 | 5.241 | 0.0015 |
| Proximate diet = starch | 872.16 | 242.23 | 461.23 | 3.601 | 0.0004 |
| Proximate diet starch: Regime diet starch | 2446.16 | 337.27 | 474.91 | 7.253 | 1.67e-12 |
|  |  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |  |
| Fly line | 102507 | 320.2 |  |  |  |
| F1 Egg collection date | 0 | 0.0 |  |  |  |
| Residual | 3304472 | 1817.8 |  |  |  |

**Supplemental Table 8**: *t*-test results from a linear-mixed model of number of eggs laid per adult female across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Egg number per female:  Fixed effects N = 35 | Estimate (log odds) | Std. Error | df | t - value | P - value |
| (Intercept) | 35.739 | 2.472 | 6.764 | 14.457 | 2.46e-06 |
| Regime diet = starch | -9.214 | 3.496 | 6.764 | -2.636 | 0.0347 |
| Proximate diet = starch | -3.332 | 2.413 | 27.070 | -1.381 | 0.1786 |
| Proximate diet starch: Regime diet starch | 4.712 | 3.470 | 27.146 | 1.358 | 0.1856 |
|  |  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |  |
| Fly line | 9.601 | 3.099 |  |  |  |
| F1 Egg collection date | 0.000 | 0.000 |  |  |  |
| Residual | 26.199 | 5.118 |  |  |  |

**Supplemental Table 9:** *t*-test results from a linear-mixed model of adult female dry weight across starch and ASG fly lines reared on either their own larval diet or the opposite diet.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Adult female weight (mg):  Fixed effects N = 175 | Estimate (log odds) | Std. Error | df | t - value | P - value |
| (Intercept) | 3.46160 | 0.07532 | 5.63189 | 45.958 | 1.80e-08 |
| Regime diet = starch | -0.45240 | 0.10652 | 5.63189 | -4.247 | 0.0062 |
| Proximate diet = starch | -0.30647 | 0.05984 | 167.05057 | -5.122 | 8.27e-07 |
| Proximate diet starch: Regime diet starch | 0.36967 | 0.08608 | 167.33647 | 4.294 | 2.96e-05 |
|  |  |  |  |  |  |
| Random effects | *Variance* | *Std. Dev* |  |  |  |
| Fly line | 0.01165 | 0.1079 |  |  |  |
| F1 Egg collection date | 0.00000 | 0.0000 |  |  |  |
| Residual | 0.08056 | 0.2838 |  |  |  |

**Supplemental Table 10:** A Binomial Generalised Linear Model to analyse the effect of plate cover on probability of eclosion. The model shows that the type of lid on the eclosion plate has no effect on probability of eclosion.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Eclosion to egg laying:  Fixed effects N = 275 | Estimate (log odds) | Std. Error | z - value | P - value |
| (Intercept) (Plastic lid) | 2.6856 | 0.4219 | 6.365 | 1.95e-10 |
| Adhesive PCR film | 0.7484 | 0.7226 | 1.036 | 0.300 |
| Parafilm | -0.6707 | 0.5398 | -1.242 | 0.214 |

## Supplemental Methods

**Supplemental Method 1:** Pilot study

To gauge techniques and a timeline, I completed a pilot study from pupae to egg collections (parental generation). Pupae were sieved from sand 14 days after egg collection, and six cages were produced (one for each experimental line: A1, A2, A3, S1, S2, S3); each with 100 pupae.

To test efficiency of possible plates for eclosion, 288 pupae were put into three 96-Well plates. To test which cover for these plates allowed for the highest accuracy in identifying eclosion date and sex for my later experiment, one plate was covered with a plastic lid, one with parafilm and one with adhesive PCR film (*Supplemental Figure 1*). The parafilm and PCR films were perforated with a needle to allow for air circulation. Eclosions and their respective sex were noted daily at 3pm. Parafilm provided poor visibility, and both the parafilm and the lid allowed for some eclosed flies to escape to other wells, posing difficulties in distinguishing which daily eclosions and sex. Some pupae had stuck to the adhesive PCR film. Analysis of the number of eclosions per plate found that the probability of eclosion for the PCR film was the highest at 0.9638 [95% CI: 0.0101 – 0.0924], then the plastic lid (0.9362) [95% CI: 0.0290 – 0.1349] and then the parafilm (0.8824) [95% CI: 0.0645 – 0.2050]. A general linear model found no significant difference in probability of eclosion between the plates (*P* > 0.05) (Supplemental Table 10). Adhesive PCR film was deemed most efficient as was easiest to identify sex and eclosion date and had the highest probability of eclosion.

I noted the first day of egg-laying for each cage and on the third, fourth and fifth day from the start of egg-laying, for each cage, I followed my egg-laying protocol (Supplemental Method 2). Eggs were collected from lights on (9:00AM) for five hours (till 14:00) each day to produce 3 replicates of each regime replicate. Eggs (F1 generation) were counted using a dissecting microscope and the day with the most replicates having their peak egg-laying was found to be four days post first egg-laying.

**Supplemental Method 2:** Egg collection protocol

At lights on eggs were discarded from egg pots, which were then rinsed round to ensure no eggs left within and refilled with water. This ensures eggs collected are fresh. Egg pots were then collected 5 hours later, and plastic pipettes were used to transfer eggs onto damp filter paper folded within a funnel. The damp filter paper was then placed into a petri dish upon another piece of damp filter paper to ensure the eggs did not dry out. The eggs were then ready for counting before the filter paper was folded up into a cone to be placed into bottles of larval food.