

1 *Drosophila melanogaster* females prioritise dietary sterols for producing high  
2 quality eggs

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7 **Abstract:**

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9 Limiting calories or specific nutrients without malnutrition, otherwise known as dietary  
10 restriction (DR), has been shown to extend lifespan across a broad range of taxa. Our recent  
11 findings in *Drosophila melanogaster* show that supplementing flies on macronutrient-rich  
12 diets with additional cholesterol can extend lifespan to the same extent as DR. Macronutrient-  
13 rich diets drive high levels of egg production and in doing so deplete the mothers of somatic  
14 sterols that are essential for survival. Thus, DR may be beneficial for lifespan because it  
15 reduces egg production which in turn reduces the mother's demand for sterols. If this is true,  
16 mothers must be prioritising their available sterols, whether from the diet or from their own  
17 bodies, to sustain high quality egg production. To test this, we measured the quality of eggs  
18 laid by mothers fed either cholesterol-sufficient or cholesterol-depleted diets. We found that  
19 even when the mother's diet was completely devoid of cholesterol, high quality egg  
20 production persisted. Furthermore, we show that sterol-supplemented flies with long lives  
21 continue to lay high quality eggs that give rise to healthy offspring. Thus, in our assays, long  
22 life does not require a fecundity cost.

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35 **Introduction:**

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37 Changing the nutritional balance of an animal's diet can extend lifespan in a manner that  
38 accounts for the full effects of dietary restriction (DR) (Mair *et al.*, 2005; Lee *et al.*, 2008;  
39 Simpson and Raubenheimer, 2007; Skorupa *et al.*, 2008; Grandison *et al.*, 2009; Solon-Biet  
40 *et al.*, 2014, 2015; Regan *et al.*, 2020). Specifically, across a broad range of species higher  
41 protein : carbohydrate (P:C) ratios are associated with short lifespan and high reproduction,  
42 while low P:C diets are associated with longer life, but lower levels of reproduction  
43 (Maklakov *et al.*, 2008; Piper *et al.*, 2011; Fanson and Taylor, 2012; Simpson *et al.*, 2012,  
44 2017). Our recent work provides a new nutritional explanation for this phenomenon by  
45 demonstrating that while P:C ratio does positively associate with female reproduction in  
46 *Drosophila*, it is the abundance of a different nutritional component, dietary sterols, that  
47 dictates length of life (Zanco *et al.*, 2021). Importantly, the dietary P:C ratio manipulates the  
48 availability of sterols accessible for somatic maintenance, by either inhibiting or driving  
49 reproductive output, of which sterols are required for.

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51 Based on our previous data, we proposed that in *Drosophila*, dietary P:C ratio is monitored to  
52 set the level of egg laying, while sterols, which determine length of life, are constitutively  
53 prioritised for reproduction (Zanco *et al.*, 2021). Thus, when reproduction is low (on low P:C  
54 food) the diet supplies sufficient sterols to meet the relatively low demands for reproduction  
55 and this spares the mother from drawing on her own sterol reserves, which leaves her soma  
56 intact to sustain longer life. By contrast, on higher P:C diets that contain a proportionally low,  
57 inadequate, supply of sterols (e.g. high yeast food), the flies preferentially supply sterols to  
58 sustain reproduction at a faster rate than can be replenished from the diet. Thus, they draw on  
59 their own somatic reserves, which eventually depletes key tissues of sterols, reducing  
60 lifespan.

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62 The constitutive prioritisation of limiting sterols to reproduction on both low and high protein  
63 food is consistent with the Nutrient Recycling Hypothesis, which argues that it is more  
64 advantageous for flies to maximise reproduction with whatever resources they have available,  
65 even if it means reducing maternal lifespan (Adler and Bonduriansky, 2014). This drive to  
66 meet the short term needs for reproduction is preferred because any strategy that involves  
67 protecting the soma to enhance future possible reproduction would rarely be beneficial in the  
68 wild due to the high risk of dying from extrinsic hazards (Adler and Bonduriansky, 2014). If  
69 true, we would expect that egg quality should not be compromised on sterol depleted diets.

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71 An alternative hypothesis, the “Direct constraints” model of reproduction, can also explain  
72 our previous observations, but in this model, cholesterol is constitutively prioritised for use  
73 by the soma for maintenance rather than for reproduction (O’Brien *et al.*, 2008; Tatar, 2017).  
74 This hypothesis argues that reproduction induces damage to the somatic tissues due to a  
75 multitude of factors. For instance, changes in metabolic activity during the reproductive  
76 phase can lead to an increase in oxidative damage while mating effort has been shown to  
77 induce immunosuppression (Nordling *et al.*, 1998; Fedorka *et al.*, 2004; Dowling and  
78 Simmons, 2009; Latta *et al.*, 2019). Thus, restricted diets support longer lifespan because  
79 reproduction is low on these diets, meaning that even the limited dietary resources available  
80 are sufficient to repair damage. By contrast, on nutrient rich diets, elevated reproduction leads  
81 to an increase in damage above the levels that the diet can counter, and this shortens lifespan.  
82 If this were true in flies, when dietary yeast is increased, the resultant increase in P:C would  
83 cause greater reproduction-related damage than what the sterol supply could counter. In  
84 contrast, when dietary yeast is low, the fall in P:C ratio would lower reproduction to a level  
85 such that the damage it inflicts could be met by the small amounts of sterols that are provided  
86 in the food. Under this scenario, because limiting sterols are prioritised for somatic  
87 maintenance over reproduction, the higher P:C diets that elevate egg laying and shorten  
88 lifespan should be accompanied by the production of sterol depleted eggs, which  
89 compromises their viability (Heier *et al.*, 2021).

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91 Under both of these models, supplementing sterols in a high P:C (high yeast) diet should  
92 increase the mothers’ lifespan, which is what has been observed (Zanco *et al.*, 2021), but they  
93 predict different effects on egg-to-adult viability. In the Nutrient Recycling Hypothesis  
94 (Adler and Bonduriansky, 2014), because the flies already prioritise sterols for reproduction,  
95 adding more to the diet would not markedly increase egg-to-adult viability, as it should  
96 already be high. Instead, the added sterol should create a surplus over what is required for egg  
97 production that mothers can use to preserve the soma to sustain longer life. In the direct  
98 constraints model (O’Brien *et al.*, 2008; Tatar, 2017), sterol-supplemented flies would live  
99 longer because they would have increased their capacity to repair somatic damage, and  
100 because the mothers were already prioritising sterols for use by the soma, any excess from the  
101 addition of sterols to the diet should increase what is available for egg production and so we  
102 should see that egg-to-adult viability increases from low to high. Distinguishing between  
103 these possibilities is important as it gives us insights into the likely way in which nutrients  
104 and their depletion may cause death – the very key to understanding the beneficial effects of

105 diet on lifespan. To distinguish between these possibilities, we manipulated the major  
106 nutritional determinant of lifespan (sterols) independently of the major nutritional  
107 determinant of egg production (P:C ratio) and monitored egg-to-adult viability and offspring  
108 development.

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140 **Results**

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142 **Maternal sterol supplies are preferentially used to produce high quality eggs when**  
143 **dietary intake is limited**

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145 If egg quality is prioritised by mothers, then the egg-to-adult viability of eggs from young  
146 mothers should be sustained at a high level when fed either a cholesterol-sufficient or  
147 cholesterol-depleted diet. In contrast, if the flies prioritise somatic maintenance then we  
148 expect that egg-to-adult viability will be low for mothers on cholesterol-depleted diets, and  
149 high when they are fed a cholesterol-sufficient diet. To test this, we used our completely  
150 defined (holodic) diet (Piper et al., 2014) to manipulate cholesterol independently of all other  
151 nutrients. Mated females were placed on experimental diets containing either 0g/l or 0.3g/l  
152 (sufficient) cholesterol for 16 days. We scored the total number of eggs laid and egg-to-adult  
153 viability daily.

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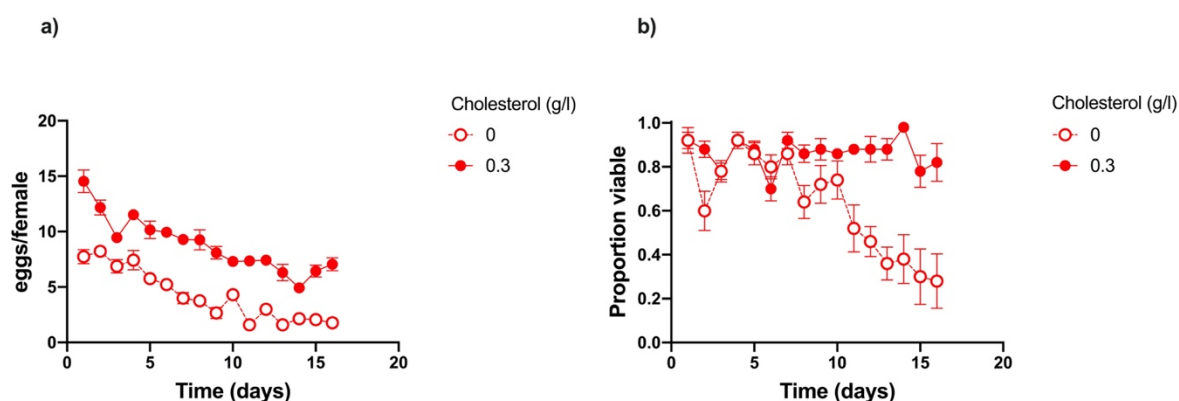
155 We found that while the number of eggs laid declined over time for flies on both sterol  
156 sufficient and depleted foods (Figure 1a), flies from both treatments were able to sustain  
157 maximum egg-to-adult viability for approximately 10 days (Figure 1b), at which point, egg  
158 laying had dropped to approximately four eggs per day for non-supplemented mothers  
159 (Figure 1a). This pattern of sustaining high egg-to-adult viability, which then drops off  
160 rapidly as egg production nears zero (as opposed to a slow linear decline in viability) is what  
161 we would predict if mothers prioritised egg quality over somatic maintenance.

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163 While egg-to-adult viability is sustained, it is possible there is a cost during development for  
164 larvae from sterol depleted mothers. To assess this, we monitored egg-to-adult viability, egg-  
165 to-adult development time, and final adult body size of larvae that emerged from eggs laid on  
166 day 10 - the time point right on the verge of when mothers on the sterol-depleted food started  
167 to lay inviable eggs. As before, we found that there was no effect of maternal cholesterol on  
168 egg-to-adult viability, but interestingly, adding cholesterol to the larval diet did significantly  
169 increase the egg-to-adult viability of offspring, indicating a rescue of a sterol shortfall for  
170 development (Figure 2a). Larvae from eggs laid by sterol-supplemented mothers developed  
171 quicker than those from mothers whose diet did not contain sterols (Figure 2b), and they  
172 achieved a smaller final body size as adults than offspring from mothers without sterols  
173 (Figure 2c). Finally, supplementing the larval medium with cholesterol brought the  
174 development time and adult body size of offspring to the same level, irrespective of the

175 mothers' dietary sterol condition (Figure 2b, c). Interestingly, these values were intermediate  
176 to the differences found between treatments when larvae did not receive a cholesterol  
177 supplement. Together, these data show that mothers match egg production to the number of  
178 high-quality eggs they can produce, for ~10 days after encountering a sterol-depleted diet.  
179 Furthermore, any effects of the mothers' diet which modify egg-to-adult viability, offspring  
180 development time, and adult body size, are normalised to a common level when the larvae  
181 ingest sterol-replete food.

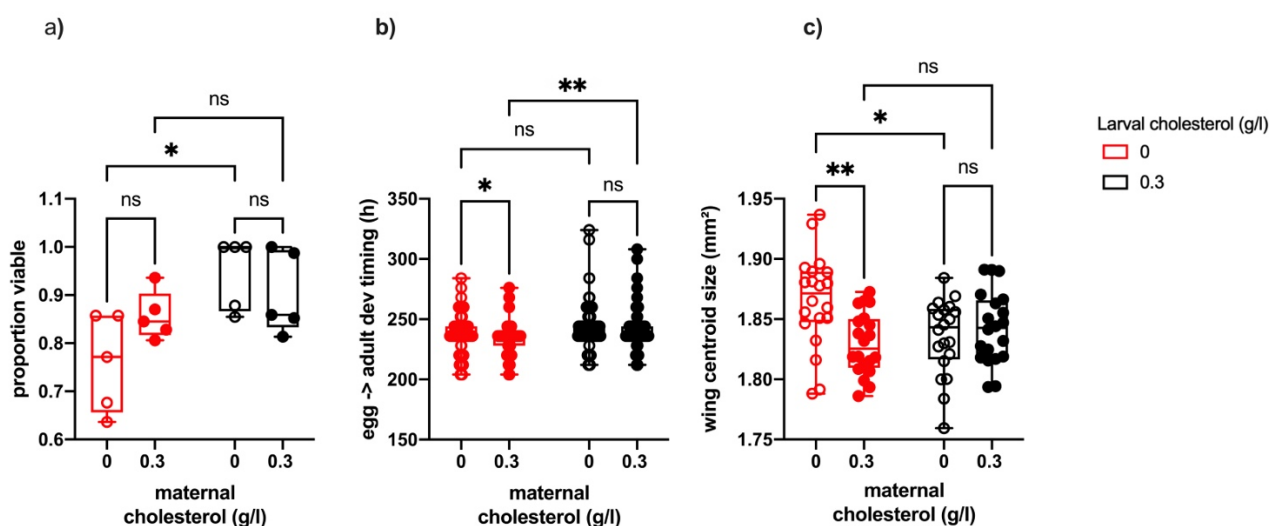
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186 Figure 1. When flies were fed a fixed P:C ratio, removing cholesterol from the diet resulted in  
187 a sustained reduction in daily egg production (a). Egg-to-adult viability remained high across  
188 treatments until day 10, with no significant difference in mean viability when compared  
189 against the mean viability of all previous days ( $P > 0.05$ ). After day 10 the total number of  
190 eggs laid by mothers fed 0g/l cholesterol dropped to 4 eggs per female and viability began to  
191 fall (a, b), at which point the mean viability on all successive days were significantly lower  
192 than the mean viability on day 10 ( $P < 0.05$ ), except for day 11 which was not significantly  
193 different ( $P = 0.123$ ). Each data point is the average ( $\pm$  s.e.) of eggs from five vials, each  
194 containing ten females (a). Viability (b) was assessed by transferring ten eggs into each of  
195 five replicate vials. Each data point is the average ( $\pm$  s.e.) of viable individuals across these  
196 vials.

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201 Figure 2. The amount of cholesterol present in either the maternal diet, larval diet or both,  
202 alters offspring fitness, however the direction of this variation differs between traits (a – c).  
203 Egg-to-adult viability was not significantly altered by maternal cholesterol levels, however,  
204 when mothers were fed a diet with 0g/l cholesterol, egg-to-adult viability increased  
205 significantly when the larval diet was supplemented with 0.3g/l cholesterol (a). Offspring  
206 developed significantly faster when mothers were fed 0.3g/l cholesterol (b), and their final  
207 body size (measured as wing centroid size) was significantly smaller (c). These effects  
208 disappeared when the larval diet was supplemented with 0.3g/l cholesterol (c, d). Ns = non-  
209 significant, \* = < 0.05, \*\* = < 0.005, \*\*\* = < 0.001

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### 213 **Maternal cholesterol levels do not significantly impact egg quality despite limiting** 214 **maternal lifespan**

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216 The previous experiment used diets without sterols to assess the mothers' commitment to  
217 producing high-quality eggs when faced with extreme nutrient stress. To test the extent of  
218 maternal sterol prioritisation under more natural conditions, we exposed flies to a standard  
219 sugar/yeast diet, which we have previously shown to be sterol limiting for female egg  
220 production and lifespan (Zanco *et al.* 2021), and supplemented it with sufficient sterols to  
221 overcome these limitations (0.3g/l cholesterol).

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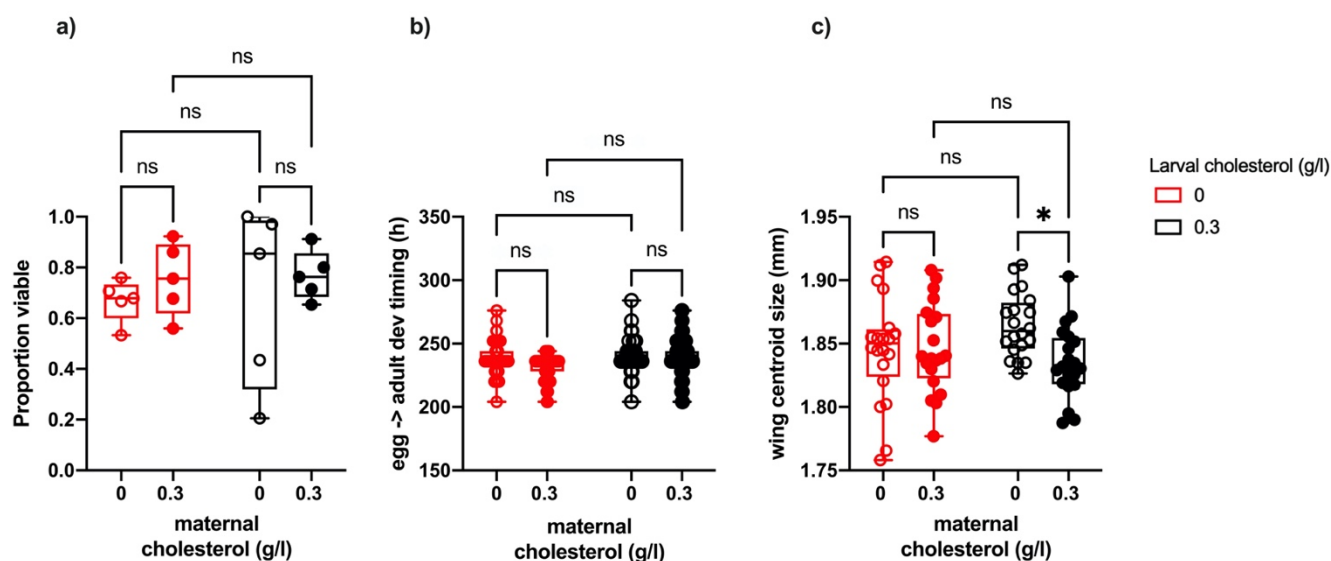
223 The same 10-day timepoint and parameters of egg quality were used as above. We found that  
224 there was no significant effect of either maternal or larval cholesterol supplementation on  
225 egg-to-adult viability, which remained at the highest level across all experimental conditions  
226 (Figure 3a). Furthermore, offspring development time (Figure 3b) was not affected by sterol  
227 addition (Figure 3c). In line with our earlier experiment, offspring achieved a smaller final  
228 body size when the maternal diet was supplemented with additional sterols, however here this

229 only occurred when the larval diet was also supplemented with 0.3g/l of cholesterol (Figure  
230 3c).

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232 These findings indicate that the responses of the traits to cholesterol supplementation on the  
233 holidic diet (Figure 2) are not apparent when some dietary sterols are available to the mother  
234 (Figure 3) – even though those levels that are available are insufficient to support full lifespan  
235 (Zanco *et al.*, 2021). Together, these data indicate that mothers prioritise sterols for use in  
236 producing viable eggs rather than maintaining the mother's soma, supporting the Nutrient  
237 Recycling Hypothesis.

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241 Figure 3. Egg-to-adult viability and egg-to-adult development timing were not significantly  
242 different across treatments (a-b). There was however a significant negative effect on  
243 offspring body size when both the maternal and larval diets were supplemented with  
244 cholesterol (in addition to naturally occurring sterols present in yeast) (c). Ns = non-  
245 significant, \* = < 0.05, \*\* = < 0.005, \*\*\* = < 0.001

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## 260 **Discussion**

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262 The mechanism by which DR increases animal lifespan is often attributed to the differential  
263 allocation of resources between reproduction and somatic maintenance (the disposable soma  
264 theory) (Kirkwood, 1977; Shanley and Kirkwood, 2000). Specifically, reproduction is  
265 repressed upon dietary restriction to redirect scarce nutrients to somatic maintenance, which  
266 increases the animal's chances of surviving and reproducing at a later date. Here we show  
267 that during sterol limitation, the longer lifespan of flies during DR relative to those on higher  
268 nutrient diets cannot be explained by a strategic investment of the lifespan-limiting nutrient  
269 (sterols) into somatic maintenance and away from reproduction. Instead, our data indicate  
270 that flies constitutively invest sterols into reproduction to the maximum extent possible,  
271 which enhances early offspring viability. This is supported by the fact that any apparent  
272 enhancement of fitness as a result of sterol supplementation to the larval diet is only evident  
273 when mothers experience severe sterol limitation. Thus, flies on DR are longer lived because  
274 their commitment to reproduction is reduced, which spares the mothers from an early death  
275 caused by sterol depletion.

## 276 277 **Flies prioritise high quality egg production when fed a nutritionally deficient diet**

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279 In recent work to advance the Disposable Soma theory, the Nutrient Recycling Hypothesis  
280 (Adler and Bonduriansky, 2014) proposed that animals on restricted diets will attempt to  
281 utilise all available nutrients to maximise reproductive output rather than allocating it to  
282 somatic maintenance to prolong survival. The reason for employing this strategy is that  
283 prolonging survival is not likely to be advantageous when the chances of dying from extrinsic  
284 hazards are high, as is the case for most organisms in the wild. Our data support this concept  
285 since they indicate that female flies prioritise their supply of sterols to sustain high-quality  
286 reproduction whatever the cost to maternal lifespan. Flies likely maintain this level of optimal  
287 fecundity by first utilising as many resources from the diet as possible and secondly, by  
288 supplementing it from sterols stored in body tissues (Heier *et al.*, 2021). Such a concept is  
289 curious as it suggests that DR may alter lifespan by stalling a program of tissue repurposing,  
290 which is in stark contrast to traditional models of ageing that envisage death as a side effect  
291 of random molecular damage (Kirkwood, 2005). Thus, we propose that the mechanisms for  
292 lifespan responses to DR in *Drosophila* are different from the mechanisms of ageing. This  
293 observation is consistent with the data of Mair *et al.* (2005) who showed that DR modifies a  
294 reversible risk of dying at any given age, and that this risk is different from, and additive to,  
295 the underlying, irreversible risk of dying that increases with advancing age (i.e. ageing).

296 Importantly, our data also indicate that the mechanisms leading to earlier death on nutrient-  
297 rich diets should proceed in a reproducible (programmed) fashion; thus, if we can identify the  
298 process that causes lethal damage resulting from sterol depletion, we will discover the  
299 mechanism by which DR enhances *Drosophila* lifespan.

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342 **Methods**

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344 Fly Husbandry:

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346 All experiments were conducted using a wild type *Drosophila melanogaster* strain called  
347 Dahomey (Mair *et al.* 2005). These flies have been maintained in large numbers with  
348 overlapping generations to maintain genetic diversity. Flies were reared, mated prior to  
349 experiments and maintained under the same conditions described in Zanco *et al.* (2021).

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351 Experimental Diets:

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353 *Holidic medium*

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355 To examine the effects of maternal cholesterol on developmental traits a fixed protein (amino  
356 acid): carbohydrate (sucrose) ratio of 1:3.6 was chosen (Zanco *et al.*, 2021). This diet was  
357 made using the holidic medium described in Piper *et al.* (2014), in which free amino acids are  
358 used to make up protein equivalents. In this case, an amino acid ratio matched to the exome  
359 of adult flies (Flyaa) was utilised (Piper *et al.*, 2017; Ma *et al.*, 2020; Zanco *et al.*, 2021). One  
360 of two cholesterol concentrations (0 and 0.3g/l) (Glentham Life Sciences, GEO100, #100IEZ)  
361 were then added to the otherwise identical media. We used cholesterol in the diet as opposed  
362 to ergosterol, because it is easily accessible, and has been shown to support *Drosophila* adult  
363 nutrition to the same extent as a yeast-based diet (Piper *et al.*, 2014).

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365 *Yeast based diets*

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367 To examine the effects of both maternal and larval cholesterol on developmental traits using a  
368 standard laboratory medium, a fixed protein sugar/yeast (SY) diet was created using 50 g/l of  
369 sucrose (Bundaberg Sugar, Melbourne Distributors) and 100 g/l whole yeast autolysate (MP  
370 Biomedicals, LLC, #903312), previously described in Mair *et al.* (2005) and Zanco *et al.*  
371 (2021). We then added cholesterol (Glentham Life Sciences, GEO100, #100IEZ) at a  
372 concentration of either 0 or 0.3 g/l to both maternal and larval diets. This gave us a total of  
373 two maternal diets and two larval diets. Cholesterol was added as a powder which was mixed  
374 in with the sugar and yeast prior to cooking.

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376 Developmental trait assays:

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378 *Egg-to-adult viability assay*

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380 Females were mated for two days post eclosion before being placed in vials (FS32, Pathtech)  
381 containing 3mL of treatment food with 5 replicate vials per diet and ten flies per vial. Diets  
382 utilised for this experiment included the holidic (fully defined synthetic) diet described  
383 above. Flies were transferred onto fresh food daily for 16 days so that egg and viability scores  
384 could be conducted. Eggs were imaged using a Zeiss Axiocam ERc 5s dissecting microscope  
385 and then counted manually. Eggs were then transferred to a standard sugar/yeast medium and  
386 left to develop (five vials of 10 eggs each). Egg-to-adult viability was scored as the number  
387 of eclosed adults.

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389 *Egg-to-adult viability, embryo to adult development timing and final body size assays*

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391 Females were mated for two days post eclosion and placed in vials (FS32, Pathtech)  
392 containing 3mL of treatment food with 10 replicate vials per diet and ten flies per vial. Diets  
393 utilised for this experiment included the holidic (fully defined synthetic) diets described  
394 above and the sugar/yeast diets described above (Bass *et al.* 2007; Piper *et al.* 2014; Katewa  
395 *et al.* 2016). Flies were transferred to fresh vials every two to three days. On day 10 (from the  
396 start of the experiment), adult flies were anaesthetised using CO<sub>2</sub> and transferred to vials  
397 containing 3mL of our standard SY food (Bass *et al.* 2007; see below) either with or without  
398 cholesterol supplementation (0g/l vs. 0.3g/l) for five hours to lay eggs.

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400 After laying, adults were discarded and eggs were imaged immediately using a Zeiss  
401 Axiocam ERc 5s dissecting microscope. Eggs were then counted manually. Scoring for  
402 developmental timing began on day 6 from egg lay and was done every 8 hours thereafter.  
403 Newly emerged flies were recorded and then stored in glycerol for wing analysis. To assess  
404 egg-to-adult viability, the number of eclosed adults were transferred out of experimental vials  
405 and counted. Viability was measured by allocating each individual a score of 1 if they  
406 survived to eclosion or 0 if they died. The assay continued until four consecutive days passed  
407 without any new emergences.

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409 Wing size was calculated as a proxy for body size (David *et al.*, 1997). The left wing of 20  
410 individuals per treatment was dissected and mounted on a glass microscope slide (Clemson *et*  
411 *al.*, 2016). Wing centroid size was calculated following the methodology described by  
412 (Clemson *et al.*, 2016)

413 Statistical analyses:

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415 All statistical analyses were performed using R (version 3.3.0, available from [http://www.R-](http://www.R-project.org/)  
416 [project.org/](http://www.R-project.org/)) and Graphpad Prism (version 8.4.2). Linear mixed effect models included egg  
417 count as a covariate, maternal and larval cholesterol levels as fixed effects and replicate vial  
418 as a random effect. The emmeans package was also used for pairwise comparisons, except for  
419 pairwise comparison of the daily viability data, in which case a two-way ANOVA was  
420 applied using Graphpad Prism (version 8.4.2). Plots were made in Graphpad Prism (version  
421 8.4.2).

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