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Age-dependent changes in reproductive allocation in a facultative ectoparasite, the blowfly *Lucilia sericata* (Meigen) (Calliphoridae)

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Abstract

Organisms trade-off limited resources between life-history traits to maximize fitness. In particular, costs associated with reproduction are balanced against somatic maintenance and this can result in age-dependent changes in the optimal allocation of resource to reproduction. Changes in the allocation of resources to reproduction with age were considered in the facultatively parasitic blowfly *Lucilia sericata* (Diptera: Calliphoridae), using biochemical analysis of lipids in the body and ovary, and lipid and protein in individual eggs. Resource allocation to reproduction, measured as lipid content in the ovary, declined over time. This decline was associated with the production of fewer and smaller eggs per batch. The lipid content of the residual body did not change. A decrease in lipid and increase in protein contents of individual eggs over time, although statistically significant, were relatively slight, suggesting that age-related changes in nutritional allocation to individual eggs were more subtle than changes in egg batch number or size. This study highlights the insights to be gained from considering both biochemical measures of nutritional allocation, and observable measures of reproductive effort, when evaluating how females balance allocation across competing life-history traits. Future work should explore how allocation patterns might vary under conditions of resource constraint and whether age-dependent allocation in laboratory flies is representative of that found in wild populations.

KEYWORDS
eggs, fitness, investment, lipids, nutrition, ovary, protein, reproduction, senescence

INTRODUCTION

Life-history theory predicts that organisms trade-off limited resources among life-history traits to maximize fitness (Stearns, 1992). In particular, costs associated with reproduction are balanced against somatic maintenance, and individuals are thus expected to optimize their age-specific allocation between reproduction and survival (Bell, 1980; McNamara et al., 2009). In insects, egg production is metabolically demanding and the evolutionarily stable strategy for females will be to optimize the trade-off between somatic maintenance, the risk of death associated with senescence and the unpredictable availability of resources and reproduction (Hanski et al., 2006; Partridge & Harvey, 1988; Reznick, 1985). Such allocation may have a number of potential outcomes. Patterns of declining reproductive allocation might be a direct consequence of somatic deterioration (Kirkwood & Austad, 2000; Zajitschek et al., 2020), and thus be considered

reproductive senescence. However, it has also been widely predicted that reproductive allocation should decline with age where adult females are subject to an elevated mortality associated with the production of successive clutches of offspring (Begon & Parker, 1986; Kirkwood, 1977; McNamara et al., 2009). Support for this prediction is found in a wide variety of insect species (Pöykkö & Mänttäri, 2012). Alternatively, young individuals with high opportunity for future reproduction might allocate less to current reproduction to maximize future reproduction, and organisms may increase their investment in reproduction as they approach the end of their life (termed 'terminal investment') and have low prospects of future survival (Creighton et al., 2009). These various drivers of age-dependent allocation are not mutually exclusive and can combine to form an inverse U-shaped pattern of increase and then decline (Barreaux et al., 2022), as observed across multiple taxa (Monaghan et al., 2020).

A wide variety of factors may influence the trade-off between reproduction and survival, however, and as a result they may be difficult to quantify. Furthermore, while female insects may change their investment by altering the number of eggs in each clutch or individual egg size, less commonly considered is the possibility that they may alter the nutritional composition of each egg, an understanding of which may be important in defining reproductive allocation (Fox & Czesak, 2000; Pöykkö & Mänttäri, 2012). For example, a decrease in resource allocation to eggs with age was noted for the solitary parasitic wasp *Eupelmus vuilletti* (Hymenoptera: Eupelmidae) both in terms of egg size but also in the sugar, protein, lipid and energy contents of each egg (Giron & Casas, 2003). Lipids are a particularly important energy resource in insects (Van Handel, 1985) so the amount of lipid in each egg may influence both embryonic development and survival of larvae prior to finding a feeding site after hatching. In *E. vuilletti*, lipid content in eggs decreased with maternal age to a stable lower value of about 0.24 μg per egg, suggesting that this may be the minimum required for successful embryonic development; however, it was suggested that protein was a better predictor of offspring fitness than lipid because it correlated most strongly with egg size (Giron & Casas, 2003). This indicates that measuring both lipid and protein in eggs can lead to a more comprehensive overview of age-dependent patterns of allocation, and consequences for offspring survival.

The way insects allocate and partition resources can change flexibly with the changing requirements and challenges of age and, particularly, with changes in resource availability: for example, some newly emerged insects invest resources into flight before reproduction, because of the importance of seeking out resources (Boggs, 1981). In some cases, often where survival probability is low and unpredictable and resource availability is uncertain, an increase in reproductive output may be the optimum strategy. For example, an exponential increase in reproductive output with age was observed in the beetle *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) (David et al., 2015) and it was suggested that there was no trade-off between energy allocated to dispersal and reproduction, due to short periods of flight. Resource availability can also affect the trade-off between survival and reproduction, in some instances making it more

pronounced, in others obscuring it. For example, in the parasitoid *Venturia canescens* (Hymenoptera: Ichneumonidae), resource restriction led to relatively more energy being allocated to flight and egg production, with a consequent decline in adult longevity (Pelosse et al., 2011). No trade-off between dispersal and fecundity was seen in the Glanville butterfly *Melitaea cinxia* (Lepidoptera: Nymphalidae) that had access to unlimited resources (Hanski et al., 2006).

Given that the optimum strategy for allocation to reproduction is likely to be influenced by the nature of limiting food or oviposition sites (Boggs, 1981; Rodríguez-Muñoz et al., 2019), any age-dependent change in reproductive allocation might be particularly pronounced in insects that rely on highly patchy and ephemeral resources either for oviposition or for specific nutritional resources required for reproduction, since the probability of finding suitable patches is low and this may favour higher levels of early reproductive effort at the cost of future survival (Wall et al., 2002). Here, the study aimed to identify changes in allocation to reproduction with age in the blowfly, *L. sericata* (Diptera), by quantifying age-dependent changes both in the number and size of eggs matured and in maternal nutritional allocation to eggs, in terms of lipid and protein. *Lucila sericata* is a valuable laboratory model species, an ecologically important member of the decomposer insect community (Smith & Wall, 1997) and an economically important facultative ectoparasite, principally of sheep (Wall et al., 1992a).

MATERIALS AND METHODS

L. sericata rearing and egg dissection

Populations of *L. sericata* were reared under laboratory conditions in a cooled incubator at 25°C, 60% relative humidity and subjected to a photoperiod of 17:7 L:D. They were given ad lib access to sucrose and water and provided with raw lamb liver for 24 h 3 days after emergence, to allow egg maturation. Under these conditions, females would be expected to mature eggs and be ready to oviposit at about 5–6 days after emergence (Wall, 1993; Wall et al., 1992b). Hence, at 7 days after emergence, six females were removed and killed by chilling. For each female, the left wing was removed and the length of the posterior cross vein between the fourth and fifth longitudinal veins (dm–cu, between veins CuA1 and M) was measured using an eyepiece graticule under a binocular microscope to give an index of size (Hayes et al., 1998). These females were then placed on a microscope slide in a small volume of Ringer's insect solution (0.9% saline) (OXOID, Fisher Scientific Ltd) and dissected using forceps. Their ovaries were carefully removed and eggs teased out under a dissection microscope (Leica S6E, Germany); 30 eggs were taken from each of the six females dissected. Ten eggs from each female were used for lipid, 10 for protein and 10 for carbohydrate analysis. Each egg was weighed to the nearest microgram using an ultrasensitive microbalance (Sartorius-CPA26P, Germany) and then placed individually into a clean glass test tube (16 × 100 mm) prior to biochemical analysis.

Egg lipid, protein and carbohydrate extractions

Lipid analysis using spectrophotometry was undertaken based on a microquantity colorimetric sulphovanillan method (Alqurashi et al., 2020; Van Handel, 1985). Each egg was thoroughly crushed using a glass rod after which 0.5 ml of chloroform-methanol (1:1) was added. Next, 0.25 ml of supernatant was transferred to a clean glass tube, and this tube was then placed in a dry bath at 100°C in a fume cupboard to evaporate off the solvent. Once the solvent had evaporated, 0.1 ml of sulphuric acid was added, and the test tube placed again in the dry bath for 10 min. The test tube was removed and left to cool for 2 min, after which 2.4 ml of vanillin/phosphoric acid reagent was added. A vortex mixer was then used to spin the sample thoroughly following which 1 ml of the solution was transferred into a cuvette and read immediately in a spectrophotometer (WPA Biowave UV/Vis Spectrophotometer, Biochrom, UK) at 525 nm. The lipid content was then read from a standard curve (as explained below).

For protein measurement, the technique was as described by Bradford (1976). Individual eggs were crushed and 1 ml of aqueous lysis buffer solution was then added. The lysis buffer was prepared by dissolving 110 mg of monopotassium phosphate (KH_2PO_4), 3.1 mg of dichlorodiphenyltrichloroethane (DDT) and 5.9 mg of ethylenediaminetetraacetic acid (EDTA) in 20 ml of distilled water and a dipotassium phosphate (K_2HPO_4) solution was then added gradually into the previous solution to reach a final aqueous lysis buffer solution with pH 7.4. This dipotassium phosphate solution was prepared by dissolving 6270 mg of dipotassium phosphate (K_2HPO_4) in 600 ml distilled water. This aqueous lysis buffer solution was placed on crushed ice for up to 7 h until required. After adding 1 ml of lysis buffer, 1 ml of the Bradford Reagent was added and mixed thoroughly. A vortex mixer (Fisher Scientific, Bibby Scientific™, Stuart™) was used to spin the sample thoroughly, a blue colour appeared in the test tube. The final step was to transfer 1 ml of the solution into a cuvette; the samples were allowed to incubate at room temperature for 5 min, then the absorbance reading was taken from the spectrophotometer at 595 nm. The protein content was then read from a standard curve.

For carbohydrate extraction, individual eggs were crushed with 200 μl of solution of 2% sodium sulphate (Na_2So_4) (VWR International, Leicestershire, UK), and 1 ml of chloroform-methanol (1:1) added. A vortex mixer was then used to spin each sample thoroughly following which 2 ml was transferred into a 2 ml Eppendorf tube and centrifuged for 15 min at 180g and 4°C. Next, 200 μl of supernatant from individual samples were moved into new tubes and put in a water bath at 90°C for 40 s to allow the solvent to evaporate to around 20 μl. After that, 1 ml of anthrone reagent (Sigma Aldrich, UK) was added to each sample, then incubated for 15 min at a temperature of 25°C, then followed by heating at 90°C for 15 min and then cooled for 15 min at room temperature. The last step was to transfer 1 ml of the solution into a cuvette, then the absorbance reading was taken from the spectrophotometer at 625 nm. The carbohydrate content was then read from a standard curve.

Standard curves

Absolute values were extrapolated from the spectrophotometer RFU (Relative Fluorescence Unit) reading using standard curves. The standard curves were obtained by using serial dilutions of analytical standards. For lipid analysis, a standard curve was obtained with dilutions of analytical oil solution (0.917 g/mL) (Sigma Aldrich, UK). For protein concentration, a standard curve was generated by using dilutions of analytical protein standard (2 mg/mL) (Sigma Aldrich, UK). For carbohydrate estimation, a standard curve was obtained with using a range of dilutions of glucose (1 mg/mL) (Sigma Aldrich, UK). All serial dilutions were treated as the samples described above. Dilutions were triplicated and the average of the three readings was used to produce the standard curve.

Lipid allocation to eggs with age

Twelve fly cages (15 × 15 × 15 cm), each containing 10 newly emerged *L. sericata*, five males and five females, were maintained as described previously. Seven days after emergence, when female flies would be expected to be ready to lay the first egg batch, four adult females were removed from one cage and killed by chilling. The remaining flies in the cage from which they came were discarded. For each female killed, the ovaries were removed as described above. The degree of oocyte maturation was assessed and, where yolk deposition was complete, the number of mature oocytes present was counted. The average length of mature oocytes was measured to the nearest micrometre using a Leica eyepiece graticule. The ovaries plus oocytes and the remainder of the body were then dried separately for lipid analysis, as described above, but with reagent volumes increased to reflect the larger tissue mass (Alqurashi et al., 2020). Only lipid was measured in the body or ovary of adults. In the 11 cages remaining, lamb liver was provided for 12 h to allow oviposition and to ensure that they had sufficient dietary protein to mature further batches of eggs. In these cages, the number of egg batches oviposited was counted and matched to the number of females in the cage to ensure that all females had oviposited. If all females had not oviposited, the liver was left in the cage for longer, but generally all females oviposited in the time available. Subsequently, four females were removed from a further cage and killed at 10, 13, 17, 21, 25, 30, 35 and 40 days and subjected to the same dissection procedure, and liver was provided to the remaining cages to allow females to oviposit. Hence females were able to mature multiple egg batches in cages that were samples later. A duration of 40 days was selected as it represents the expected lifespan in a laboratory population of *L. sericata* (Wall, 1993) and in field cages (Prinkkilä & Hanski, 1995). The entire experiment was replicated.

In addition, to compare the differences between lipid and protein content in eggs in relation to the age of the females that oviposited them, 12 eggs were taken from each of the four females dissected at each timepoint: of these, six eggs were used for lipid analysis and the other six for protein analysis.
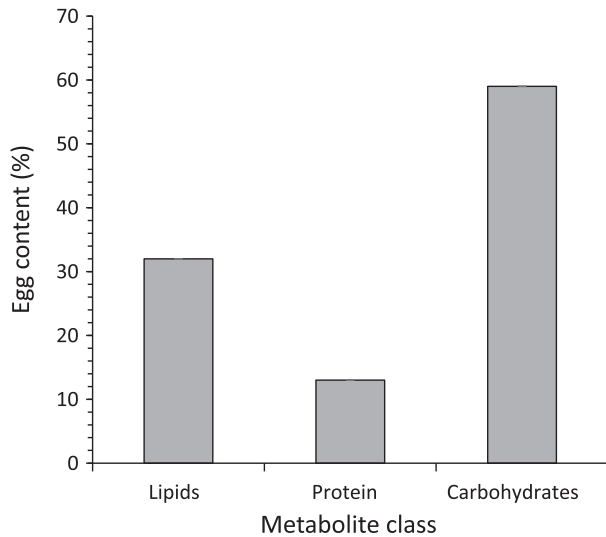
FIGURE 1 The mean (±SE) percentage of lipids, protein and carbohydrates in individual eggs of the blowfly *Lucilia sericata*, extracted from six females that were 7-days old ($n = 30$ eggs per female). Error bars too small to be visible.

Data analysis

Where multiple eggs were taken from the same female for biochemical analysis, the mean value for these eggs for each individual was used in the statistical analysis to avoid issues associated with repeated measures and because within female variation is thought to be small relative to between female variation (Wall, 1993). For each female, the amount of lipid recorded was divided by its wing vein length to remove the effects of absolute variation in fly size (Hayes et al., 1998). This is described as the corrected lipid content, expressed as mg/mm with all means presented ± their standard deviation (SD) or ± their standard error (SE). The relationships between the corrected lipid contents of fly ovaries or their bodies minus their ovaries and over time and the lipid and protein contents of eggs over time, were assessed by regression, as were the relationships between the number of eggs matured in an ovary and their lengths (mm). Linear or second order polynomial regression models were fitted as appropriate to maximize the r^2 of the fitted line. All analyses were undertaken using SPSS for Windows (IBM, Version 24) and all figures prepared using Microsoft Excel.

RESULTS

In eggs produced by 7-day old females, a mean and (±S.D.) of their body size were measured as 1.30 mm (±0.07); lipid represented a mean (±S.D.) of 32% (±0.05) of the mass, protein 13% (±0.01) and the largest component was carbohydrate with a mean of 59% (±0.03) (Figure 1).

There were slight but significant non-linear changes in the amounts of lipid and protein in individual eggs across female age. Lipid
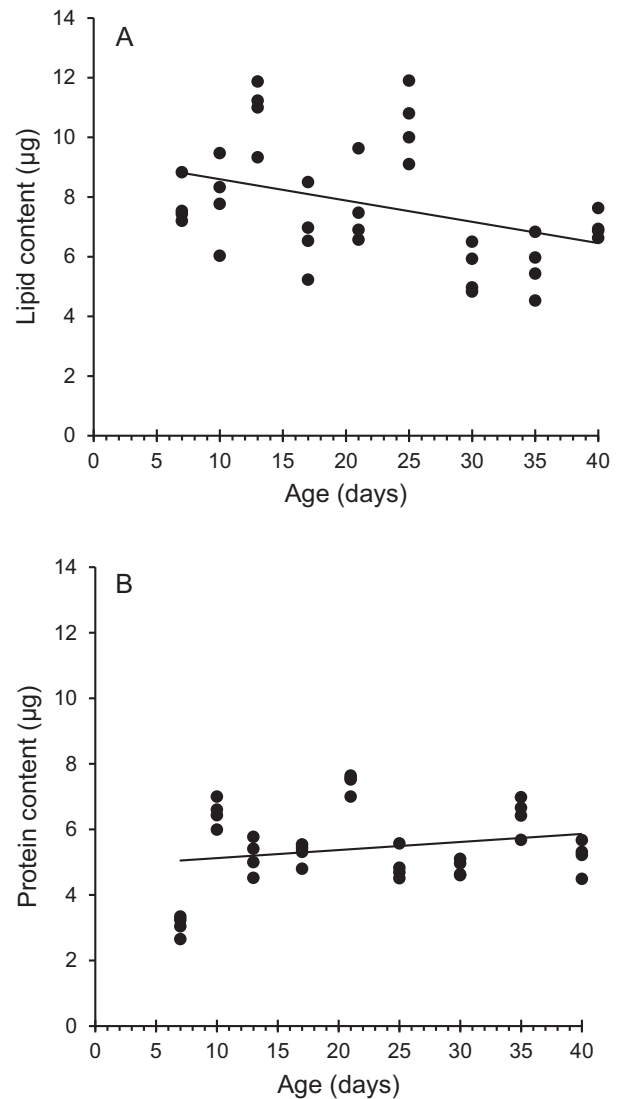

FIGURE 2 The content of (A) lipids (µg) and (B) protein (µg) of eggs in female *Lucilia sericata* across female age (days). Best fit linear regressions (A) Lipid content $= -0.0714 \times$ Age $+ 9.3108$, $p < 0.001$, $r^2 = 14.97\%$ and (b) Protein content $= 0.02464 \times$ Age $+ 4.897$, $p < 0.01$, $r^2 = 4.58\%$.

values in individual eggs declined with female age up to 40 days ($F_{2,33} = 3.40$, $p = 0.046$; Figure 2a) whereas protein concentrations appeared to increase significantly over this period ($F_{2,33} = 3.72$, $p = 0.035$; Figure 2b).

The number of eggs matured in gravid flies declined significantly across female age in a non-linear manner ($F_{2,69} = 19.53$, $p < 0.001$; Figure 3a), while the length of mature oocytes also decreased significantly ($F_{2,69} = 13.51$, $p < 0.001$; Figure 3b), both falling particularly over the last 10–15 days of the reproductive period measured.

There was no significant change in the lipid mass in the residual body of females after removal of the ovary over 40 days ($F_{2,69} = 0.61$, $p = 0.55$). However, in the ovary, the lipid content corrected for female size showed a significant non-linear change over 40 days
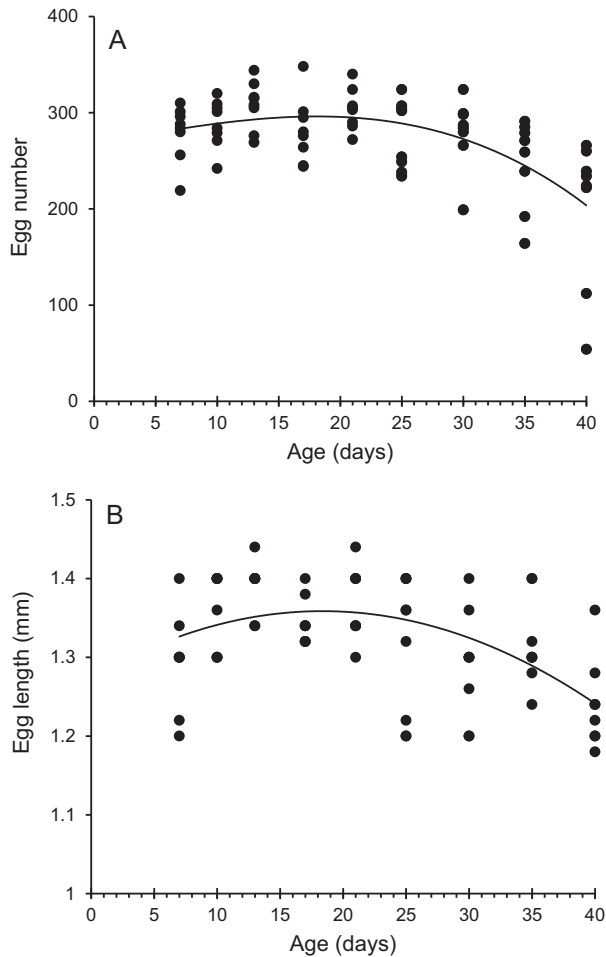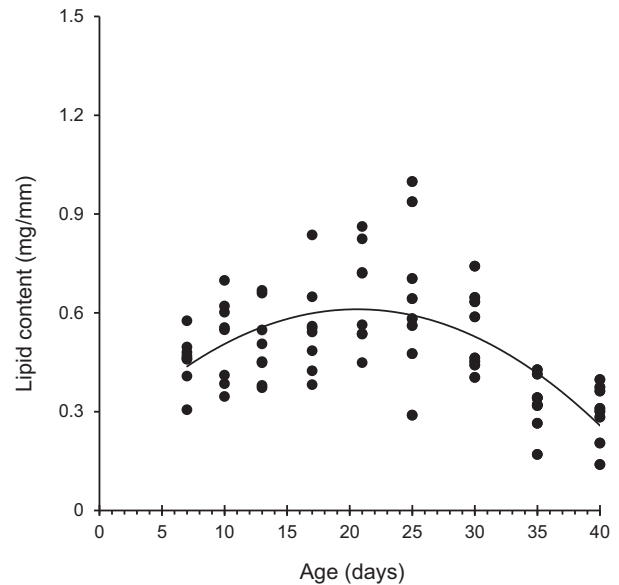
FIGURE 3 The (A) number and (B) length of eggs (mm) matured by female *Lucilia sericata* across female age (days). Best fit second order polynomial regressions (A) Egg number $= -0.1769 \times Age^2 + 6.086 \times Age + 246.2$, $p < 0.001$, $r^2 = 36.4\%$ and (B) egg length $= -0.0003 \times Age^2 + 0.0092 \times Age + 1.274$, $p < 0.001$, $r^2 = 30.8\%$.

FIGURE 4 The lipid content of the ovary of female *L. sericata* corrected for body size (mg/mm) across female age (days). Best fit second order polynomial: Lipid content $= -0.0009 \times Age^2 + 0.0386 \times Age + 0.213$, $p < 0.001$, $r^2 = 39.0\%$.

$(F_{2,69} = 22.25, p < 0.0001)$, rising initially between Days 7 and 21 and then declining from Day 25 to Day 40 (Figure 4).

DISCUSSION

The current study has shown that the lipids in the residual body of individual *L. sericata* did not change over 40 days, which is considered to be their expected lifespan in laboratory conditions. This is in contrast with the lipid allocation seen in studies of fruit flies *Ceratitis capitata* (Diptera: Tephritidae) (Nestel et al., 2005) and *Anastrepha serpentina* (Diptera: Tephritidae) (Jacome et al., 1995) where the total lipid content decreased significantly with advancing age. However, in *L. sericata* the energetic allocation to eggs, measured as lipid in the entire ovary after controlling for female size, declined, particularly over the latter half of the time period, indicating a decline in the allocation of resource with increasing age (Kirkwood & Shanley, 2005).

The study then investigated consequences of this declining allocation in terms of reproductive output and showed that it was associated with the production of fewer and smaller eggs.

In general, we find the allocation towards the number and size of eggs across female age followed an inverse U-shaped pattern, in line with combined theories about senescence and adaptive reproductive allocation (Barreaux et al., 2022; Kirkwood, 1977; McNamara et al., 2009), and has been shown in a range of taxa (Monaghan et al., 2020). A decline in the number and size of eggs with increasing age has been reported in the moth, *Cleorodes lichenaria* (Lepidoptera: Geometridae) and this was considered to reflect a trade-off between various competing life-history traits (Pöykkö & Mänttäri, 2012). Calliphorid blowflies are predominantly saprophages, with a small number of species such as *L. sericata* also acting as facultative ectoparasites (Wall et al., 1992a). Given the ephemeral and patchy nature of the resource on which they depend both for the initiation of egg production and oviposition, it might have been expected that relatively early high reproductive investment would be more pronounced than observed here (Wall et al., 2002). However, it is possible that the amount of flexibility in reproductive investment is subject to physiological constraint, dictated by ovariole number, and, although females are able to reduce the number and size of eggs in a batch when protein is limiting (Wall et al., 2002), when resources are plentiful, over the early stages of life females simply mature the maximum numbers of eggs at every oviposition, with little flexibility for increase in either the maximum number or size of oocytes matured. Repeating the study under conditions of resource constraint might allow plasticity in age-dependent allocation decisions to be seen more readily.

The observation that lipids made up 30%–40% of the mass of an individual egg, whereas protein composed only 14%–12% is of particular note because protein is the limiting resource for egg development (Wall et al., 2002): female *L. sericata* given access to sucrose only are unable to synthesize egg yolk, only flies provided with protein mature eggs (Alqurashi et al., 2020) and egg batch size is correlated with the amount of protein available (Wall et al., 2002). Here, the changes in lipid and protein contents of individual eggs over time, although statistically significant, were relatively slight and the fitted regressions explained only a small proportion of the variance; the high between age-group variance relative to the within age-group variance is anomalous and may be associated with the fact that measurement at the individual egg level is approaching the resolution of the biochemical method employed. A constant composition in levels of protein and lipid over time was recorded in *Cleorodes lichenaria* eggs (Pöykkö & Mänttäri, 2012). In the spider, *Argiope radon* (Araneae: Araneomorphae) the amount of protein in the eggs declined slightly, but not significantly, with maternal age (Ameri et al., 2019). A significant decrease in egg size, lipid and protein contents with age was recorded in the parasitic wasp *E. vuilletti* (Giron & Casas, 2003; Muller et al., 2017). Hence, a general pattern within arthropod studies appears to suggest that resource allocation to reproduction is affected primarily through changes in the number and size of eggs rather than changes in resource content within eggs.

One problem that studies of age-dependent reproductive investment face when undertaken in the laboratory is that life expectancy in laboratory conditions is likely to be very different to that observed in the field (Lord et al., 2021). In a study of adult mortality and oviposition in populations of *L. sericata* in the field, mortality rates of around 2% per day-degree and mean life expectancy of 51.5 and 47.9 day-degrees above a threshold of 11°C, were recorded, giving a life expectancy of only a few days at expected summer temperatures and a mean lifetime reproductive output of 159.6 and 138.4 eggs per female, that is on average less than one egg batch per female (Pitts & Wall, 2004). Similarly, mean life expectancy in the field of 9 days was estimated at an ambient temperature of 26.5°C for the Old World screwworm fly, *Chrysomya bezziana* (Diptera: Calliphoridae) (Spradbery & Vogt, 1993) and 7.5–9.9 days, with a corresponding mean lifetime reproductive output of 320–460 eggs per female, in the New World screwworm fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae) (Thomas & Chen, 1990). Mean life expectancy was estimated as 109 day-degrees for wild populations of *Lucilia cuprina* (Diptera: Calliphoridae) (Vogt et al., 1991). In contrast, *L. sericata* in the laboratory survive more than twice as long as flies in the field (Pitts & Wall, 2004). It was notable that the declines in egg number and egg size observed here largely predominated in the second half of the life of the laboratory reared flies, outside the expected life-expectancy in the field. The differences between the field and laboratory populations highlight the caution required when extrapolating life-history parameters from artificial to natural habitats and the fact that costs associated with flight, and resource location may outweigh physiological impacts associated with senescence (Pitts & Wall, 2004; Prinkkilä & Hanski, 1995; Zajitschek et al., 2020).

Here, we use both biochemical analysis of nutritional components of eggs and observed reproductive output to document age-dependent patterns of reproductive allocation in a facultatively parasitic insect which relies on ephemeral, patchy resources for survival. While the number and size of eggs shows the typical increase and then decline with female age as documented in many other taxa, the nutritional composition of the eggs only changes slightly. This suggests that females might maximize offspring survival, by ensuring that even if fewer or smaller offspring are produced, these remain well-provisioned with nutrients. Future work could explore how these patterns might vary under conditions of resource constraint and whether age-dependent allocation in laboratory flies is representative of that found in wild populations which have lower survival and a less predictable food supply. In conclusion, the study demonstrates the value and insights to be gained from considering both biochemical measures, in this case the measurement of lipid and protein, and observable measures of reproductive effort, when evaluating how females balance allocation across competing life-history traits.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available.

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