SYMPOSIUM

Insights from Stable Isotopic Tracers on Reproductive Allocation under Stress

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Synopsis

Fecundity is affected by changes in the nutritional and energetic environment, as a result of changes in
acquisition, assimilation, or allocation of macro-nutrients and micro-nutrients. Stable isotopes of carbon and nitrogen
offer a window into the processes underlying these changes. In insects that feed on nectar as adults, carbon isotopes can
be used to trace allocation of carbon to eggs from larval (capital) and adult (income) sources. If adults are fed sugar-
water, there is no source of nitrogen from the adult diet. Thus, nitrogen isotopes in eggs reflect fractionation of larval
nitrogen due to protein catabolism and anabolism. We subjected adult females of two butterfly species, Speyeria mormonia
and Colias eurytheme, to dietary restriction (DR), larval female S. mormonia to DR, and adult female S. mormonia
to extra flight. Females subjected to extra flight were previously found to eat more as adults and to have a higher resting
metabolic rate. As predicted, significantly less carbon obtained by feeding as adults was incorporated into eggs in both
species under DR when adult.

Introduction

Animal life histories vary in response to variation in the nutritional environment (reviewed by Boggs 2009). We have a growing understanding of the mechanisms underlying allocation and phenotypic
or genotypic life-history trade-offs observed among individuals within a species under standard nutritional conditions (reviewed by Zera and Harshman 2001), particularly in insect model organisms such as Drosophila (e.g., Flatt 2011), parasitic hymenoptera
(e.g., Casas et al. 2005), and Gryllus with wing polymorphism (e.g., Zera and Zhao 2006; Vellichirammal et al. 2014). Likewise, studies are accumulating on the life-history effects of dietary restriction (DR) at
diverse life stages in insects ranging from Drosophila (e.g., Lee et al. 2008; Gibbs and Reynolds 2012) to parasitic hymenoptera (e.g., Ellers et al. 2011) to Lepidoptera (e.g., Bauerfeind et al. 2009; Gibbs et al. 2012; Saastamoinen et al. 2013; van den Heuvel et al. 2013), and more. The organismal
diversity of these studies is important. If we are to understand what drives differential responses among species, we need studies of species with differing life styles, timing of the need for specific nutrients, ovarian dynamics, and type and amount of fuel for flight. Likewise, study both of the effects of DR on life-history traits and of the mechanisms underlying those effects is needed in order to develop a predictive understanding of insect nutritional ecology.

We ourselves have examined survival and reproductive responses to a variety of environmental stresses, using a set of butterflies with similar diets as adults (nectar, which is primarily carbohydrate), fuels for flight (carbohydrates) and ovarian dynamics, but differing in the requirements for diapause and in egg size (Boggs and Ross 1993; Boggs and Freeman 2005; Niitepöld et al. 2014). Using a resource allocation framework (Fig. 1), we earlier asked how investment in reproduction and survival changed under quantitative DR, either as larvae or as adults, or under an additional expenditure for flight. To begin addressing the underlying mechanisms of metabolism and allocation, we here report the results of studies of stable isotopes, using $^{13}$C to examine age-specific investment of carbon derived from larval and adult feeding into eggs and $^{15}$N to develop hypotheses concerning changes in metabolism of nitrogenous compounds under stress.

Stable isotopes of carbon and nitrogen

Stable isotopes of carbon and nitrogen have been used extensively to infer trophic position or dietary shifts in a diverse array of organisms, both in the wild and in the laboratory (reviewed by Layman et al. 2012). Dietary shifts generally parallel our interest in changes in allocation to reproduction both across the lifespan and under stress. Previous work (e.g., O’Brien et al. 2004) took advantage of the fact that plants using C3 and C4 photosynthetic pathways fractionate $^{13}$C differentially. Plants eaten by larvae (C3) have a significantly different isotopic signature than does cane sugar (C4), whereas beet sugar’s (C3) signature is similar to the signature of the plants eaten by larvae. These sources were used to test a mixing model, in which larval-derived carbon is gradually replaced by adult-derived carbon in eggs laid throughout a female’s life, until an equilibrium balance of larval-derived and adult-derived carbon is reached. Here, we generate and test predictions concerning how the age-specific incorporation of larval-derived versus adult-derived carbon in eggs changes in response to quantitative reduction in adult or larval food, or to increased expenditure of resources during flight.

In nectar-feeding insects, stable isotopes of nitrogen will not follow a mixing model, since the adults’ diet contains little—and in our experiment, no—nitrogen. Nonetheless, in general, nitrogen isotopes are fractionated during metabolic processes. First, starvation or semi-starvation can result in protein catabolism to produce other compounds or in protein anabolism. Both activities often result in $^{15}$N enrichment in the affected tissues, since $^{14}$N is selectively excreted under protein catabolism and selectively not taken up in protein anabolism (reviewed by Lee et al. [2012] and Hatch [2012]). Second, theory predicts that rapid growth should result in depletion of $^{15}$N (Martinez del Rio and Wolf 2005) and several studies have found this to be the case (Williams et al. 2007; Sears et al. 2009). Third, when the diet contains protein, assimilation of those dietary proteins can result in $^{15}$N enrichment. This enrichment, as N moves up the trophic chain, is the basis for use of stable nitrogen isotopes in determining diet trophic level in the wild. Such enrichment is also seen in Gromphadorhina portentosa, the Madagascar hissing cockroach, as a maternal effect (McCue 2007). At birth, nymphs showed enrichment of 1.8 $^{15}$N compared with their mothers. After 30 days, the offspring’s $^{15}$N was depleted, and showed the same signature as their mothers. The author did not suggest a mechanism for this effect, but since the diet contained protein, presumably a gradual replacement of maternal-derived protein occurred. Finally, previous work by O’Brien et al. (2002) showed that carbon in essential amino acids was derived entirely from feeding by larvae in one of the butterfly species studied here (S. mormonia). However, carbon in non-essential amino acids
had mixed origin from adults and larvae in eggs from older females, specific to each amino acid, but consistent between *S. mormonia* and a moth, *Amphion floridensis* (O’Brien et al. 2002). These data indicate that non-essential amino acids are made de novo by the adult female, using nitrogen derived from the larva’s feeding. Here, we generate and test predictions as to how age-specific 15N signatures in the egg change in response to stresses from semi-starvation stress or increased demand of resources for flight.

**Study system**

*Speyeria mormonia* (Lepidoptera: Nymphalidae) is a western North American montane species. Larvae diapause over winter as unfed first-instar larvae, and feed on *Viola* spp. in spring. Adults fly in late June–early September at our study site near the Rocky Mountain Biological Laboratory, Gunnison County, Colorado, USA (38°57′N, 106°58′W, 2900 m.a.s.l.). Adults fed on nectar from a diversity of flowers, but preferred *Erigeron speciosus* (Compositae) when available in our study site (Boggs and Inouye 2012). Females mate on average 1.1 times in the field, and mean wet egg mass is approximately 0.24 mg (Boggs 1986). *Speyeria mormonia* eggs have a high carbon content, and 79% of the egg’s carbon is derived from food eaten by older females (O’Brien et al. 2002). These conclusions. Flight is critical to all the egg’s carbon is derived from food eaten by older eggs have a high carbon content, and 79% of ammonia approximately 0.24 mg (Boggs 1986).

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**Predictions**

**DR as adults: *S. mormonia* and *C. eurytheme**

Boggs and Ross (1993) and Niitepöld et al. (2014) fed adult female *S. mormonia* a restricted diet of 50% of the amount imbibed by females fed *ad libitum*, using either a 25% honey-water or 25% sugar-water solution. Fecundity of DR females was about 50% of that of females fed *ad libitum* in both studies. Lifespan was not significantly altered. Dry mass of eggs declined with age, but was unaffected by restricting the adults’ diet. However, the C:N content of eggs was significantly lower for females on restricted diets as adults.

This experiment was repeated using *C. eurytheme* (Niitepöld et al. 2014). Fecundity was about 75% of that of females fed *ad libitum*. The dry mass of eggs declined with age, but was unaffected by restricting the adults’ diet. Eggs’ C:N content was unaffected by diet, but the eggs’ protein content increased significantly when adult were subjected to DR.

Based on this background, we predicted that 13C in eggs should increase with females’ age, to a plateau, in both species and treatments. This reflects a mixing of larval-derived and adult-derived carbon, as carbon from nectar is added to the resource pool after adults emerge. Further, DR females should incorporate less adult-derived carbon into eggs each day than do females fed *ad libitum*, resulting in lower 13C in eggs at all ages from DR females than from females fed *ad libitum*. We did not expect differences between species in the age-specific pattern of 13C in eggs.

Likewise, we predicted that 15N should be relatively enriched in eggs from DR females when compared with those from females fed *ad libitum*, resulting in greater 15N in eggs from DR females. We predicted that enrichment should increase with age in eggs from DR females. Such a pattern would result from protein catabolism generating relatively scarce carbohydrates and lipids and recycling of that nitrogen into amino acids in eggs. Note that butterflies are capable of degrading flight muscle with age, apparently using it in reproduction (e.g., Stjernholm et al. 2005).

**Restriction of the diet of larvae (DR): *S. mormonia***

Boggs and Freeman (2005) fed last-instar larvae of *S. mormonia* half the amount of leaves eaten by sibling larvae allowed to eat *ad libitum*, resulting in repeated periodic bouts of starvation. This treatment occurred during the second half of the instar. Resulting adults from DR larvae were smaller, but showed no effect of treatment on fecundity independent of the adults’ body size. However, the lifespan of adults was significantly reduced in DR females, about 25% in comparison with females fed *ad libitum*. In subsequent work, the dry mass and C:N of eggs were both significantly lower in stressed
individuals, indicating proportionately more nitrogen invested in smaller eggs (K. Niitepöld and C. L. Boggs, unpublished data).

Based on this background, we predicted that $\%_{00}^{13}$C in eggs should again increase with age of the female, to a plateau, both in species and in treatments. This reflects a mixing of larval-derived and adult-derived carbon. However, unlike the situation for DR adults, eggs from females stressed as larvae should show a higher content of adult-derived carbon, across all ages, than would eggs from control females, given that less carbon is available from larval stores. This should result in greater $\%_{00}^{13}$C in eggs from DR females than from females fed ad libitum at all ages.

In contrast to adult DR females, we expected that females subject to DR as larvae, with less total protein in reserve, should minimize protein catabolism to the extent possible. This should result in lower $\%_{00}^{15}$N in eggs of stressed females than in eggs from females fed ad libitum. Note, however, that protein reserves may be proportional to body size and fecundity between treatments. In that case, we expect no effect on $\%_{00}^{15}$N in eggs between treatments.

**Adult flight stress: S. mormonia**

Niitepöld and Boggs (2014) found that forcing females to fly for an additional time each day had no effect on lifespan, fecundity, or on dry mass of eggs. However, stressed females imbibed significantly more sugar-water, particularly in the critical first week, than did control females. C:N ratio in eggs was greater from flight-stressed females, indicating proportionately more carbon invested in eggs. Resting metabolic rate was also higher in flight-stressed females. In short, adult females were able to adjust their food intake in response to increased expenditures, and maintain lifespan and fecundity, but their allocation of carbon and nitrogen and their metabolic rate changed.

Based on this background, we again predicted that $\%_{00}^{13}$C in eggs should increase with the female’s age, to a plateau, both in species and in treatments. This reflects a mixing of larval-derived and adult-derived carbon sources. We expected that eggs from flight-stressed females should have a greater $\%_{00}^{13}$C at each female age, compared with eggs from control females. This reflects increased intake of sugar by adults and increased expenditure of carbohydrates for functions, other than reproduction, in flight-stressed females.

In this case, we predicted that $^{15}$N should be enriched in eggs from flight-stressed females, reflecting increased turnover of protein (i.e., catabolism and anabolism) in flight muscle and increased consumption of energy, reflected in an elevated resting metabolic rate. Thus, $\%_{00}^{15}$N should be greater in eggs from treated females than from control females.

**Methods**

**Common methods**

*S. mormonia* were offspring of field-caught females from our study site in Colorado. First-instar larvae were held for 4–6 months at 4°C prior to breaking diapause and rearing on potted *Viola sororia*. After mating, females were kept in cages made from glass lantern globes with large plastic petri dishes at each end and lined with wax paper (Boggs and Ross 1993). A sprig of *V. sororia* was provided to promote oviposition.

*Colias eurytheme* came from a laboratory colony maintained by Ward Watt at Stanford University. The colony originated from individuals caught near Tracy, California (37°46’N, 121°25’W; 5 m.a.s.l.). The colony was outbred, with no first-cousin or sib mating, and with roughly 40 pairs forming the basis for each new generation. New individuals were bred into the colony each fall, or roughly every 9–10 generations. We reared larvae on *Viola villosa*, which was grown hydroponically. After mating, females were kept in screen cages (10 x 10 x 15 cm) over a few potted sprigs of *V. villosa* in moist vermiculite.

All insects of both species were reared and maintained on a 16:8 L:D cycle and a 27°C:15°C temperature cycle in the Stanford greenhouses. Males were fed an aqueous solution of 25% beet sugar daily. Females were mated on the day of emergence (or in a few cases on the day after emergence) to a non-sib male. All females were fed twice daily on an aqueous solution of 25% cane sugar, except for *C. eurytheme* females, which were fed a 25% solution of beet sugar in water (see below). Batch sugar solutions were made for each experiment, aliquotted into 1.5 mL Eppendorf tubes and stored in a freezer at −20°C until needed. Eggs were counted and collected after 16:00 h each day. Eggs for analysis were immediately dried at 50°C for 3 days.

For another purpose, metabolic rate of butterflies in flight was measured once every third day for all females in all experiments. In the course of measuring metabolic rates while in flight, females were forced to fly for 7 min by agitating the respirometry chamber (see Niitepöld et al. [2009] for details).
Restriction of adults’ diet

Methods followed those of Boggs and Ross (1993). The experiment using C. eurytheme was performed in spring 2010, and that using S. mormonia in spring 2011. Females were paired by forewing length in order to control for reserves carried through metamorphosis. One member (A) of each pair was fed a 25% solution of cane sugar ad libitum twice daily using a 25 or 50 µL Hamilton syringe. The other member (B) of the pair was fed half of the 2-day running average of A’s intake at the same time of day.

Female C. eurytheme were accidently fed beet sugar instead of cane sugar. Beet sugar has a $\delta^{13}C$ value much closer to that of the host plant of the larvae than does cane sugar (beet: $\sim -25\%^{13}C$; cane: $\sim 11$ to $12\%^{13}C$; V. villosa: $\sim 26$ to $27\%^{13}C$; V. sororia: $\sim -33\%^{13}C$) (O’Brien et al. 2004), which should make patterns of carbon incorporation more difficult to distinguish. However, the adults’ food did not include nitrogen, so patterns of $\%^{15}N$ should be unaffected.

We analyzed eggs from 13 pairs of S. mormonia plus one additional female for whom no eggs were available from her paired female. Eggs were analyzed for ages 3, 4, 6, 8, 10, 12, 14, 16, and 18 days after emergence. For C. eurytheme, we analyzed eggs from 15 pairs of females plus five additional females for whom no eggs were available from their paired female. Eggs were analyzed for ages 2, 3, 4, 6, 8, 10, and 12 days after emergence.

Restriction of the diet of larvae

Methods followed those of Boggs and Freeman (2005). This experiment was conducted in spring 2012. Larvae were raised by family in sleeves on potted V. soraria. When larvae reached 25 mm in length, they were weighed and members of each pair with similar mass were randomly assigned within families to control (ad libitum) and stress treatments. Five larvae were placed in each sleeve. Sleeves were moved over new leaves half as often for stress treatments as for control treatments, resulting in periodic starvation.

We analyzed eggs from 18 pairs of S. mormonia. Eggs were analyzed for ages 4, 6, 8, 10, 12, 16, and 20 days after emergence.

Adults subjected to increased flight

This experiment was performed in spring 2013. Larvae and adults were reared according to standard procedure. In general, we used a split-sib design, with one female assigned to the control group and one female assigned to the treatment group from each family. However, for five families, only one female was available. For the additional flight-treatment, females were placed in a cage ($30 \times 30 \times 30$ cm$^3$) lined with transparent plastic film and stimulated to fly by gently brushing their legs with a fine paintbrush. Each female was forced to fly three times for 4 min, with 5 min between bouts of flight. This treatment was applied each morning, prior to feeding the females.

We analyzed eggs from 38 female S. mormonia, including 20 control females and 18 treated females. Eggs were analyzed from females aged 4, 6, 8, 10, 12, 16, and 20 days after emergence.

Analysis of stable isotopes

We analyzed pooled groups of five to eight dried eggs for S. mormonia and 25 dried eggs for C. eurytheme from each date and female for $\%^{13}C$ and $\%^{15}N$. Determination of carbon and nitrogen was performed at Stanford University’s Stable Isotope Biogeochemistry Laboratory using a Thermo Finnegan Delta plus XL isotope-ratio mass spectrometer interfaced with a Costech Elemental Analyzer. The $\%^{13}C$ and $\%^{15}N$ are relative to a Pee Dee Belemnite and air standard, respectively. Precision was 0.1% for both $\%^{13}C$ and $\%^{15}N$ in most runs.

Analysis of data

We used analysis of variances to analyze the data, with $\%^{13}C$ or $\%^{15}N$ as the dependent variable, and treatment and age as category variables. Treatment $\times$ age interactions were also tested. Female was nested within treatment to account for repeated measures. We used Akaike Information Criteria for final selection of the model.

Results

Restriction of adults’ diet

As predicted, $\delta^{13}C$ values in eggs increased significantly with females’ age for all treatments and species, leveling off at older ages. Values were significantly lower in both S. mormonia and C. eurytheme for females fed half of an ad libitum diet than for those fed ad libitum (Fig. 2A, B). This reflects less incorporation of adult-derived carbon into the eggs under a restricted diet when adult. Speyeria mormonia exhibited a significant age $\times$ treatment interaction. The difference in $\delta^{13}C$ values for eggs from treated versus control females was greater for younger females than for
older ones. This indicates that a greater fraction of eggs produced at younger ages is made from larval reserves under adult DR. The age \times treatment interaction was not significant in \textit{C. eurytheme}, although such an interaction may have been harder to detect, given that females of this species were fed beet sugar.

Effects of age, treatment, and treatment \times age were significant for $\delta^{15}$N in both species, although the age \times treatment pattern was more extreme in \textit{S. mormonia} than in \textit{C. eurytheme} (Fig. 2C, D). For females of both species fed \textit{ad libitum}, eggs became more depleted in $^{15}$N, but recovered slightly in eggs from the oldest females. This pattern is consistent with high rates of provisioning eggs at females’ early ages associated with high age-specific fecundity of young females, but with an increasing relative influence of nitrogen re-cycling in older females (e.g., in flight muscles). As predicted, the age-specific pattern for $\delta^{15}$N in eggs in DR females of \textit{C. eurytheme} resembled that of females fed \textit{ad libitum}, except that $^{15}$N was increasingly enriched at each age in eggs from DR females relative to eggs from females fed \textit{ad libitum}. Such a pattern is consistent with increased protein catabolism at all ages in adult DR females. \textit{Speyeria mormonia} DR females exhibited a similar, but much more extreme, pattern of increasing enrichment of $^{15}$N in eggs with female’s age initially, but which then declined at older ages. This pattern was not predicted, but could be consistent with catabolism of protein beginning at an early age, with the products used in carbohydrate and lipid metabolism. Such differences between species are
consistent with difference in C:N ratios in eggs, hence the relative importance of C and N to egg production.

**Restriction of the diet of larvae**

Values of $\delta^{13}C$ in eggs increased with the age of the female producing them, leveling off at older ages as predicted for *S. mormonia* females (Fig. 3A). As predicted, $\delta^{13}C$ was significantly higher in eggs from females who had been subject to DR when larvae, than in eggs from females who had been fed *ad libitum*. This result is consistent with an increased relative allocation of adult-derived carbon to eggs. We found no significant age $\times$ treatment interaction, unlike the case for adult DR in this species.

Age or age $\times$ treatment had no effect on $\delta^{15}N$ of eggs (Fig. 3B). As predicted, eggs from females who had restricted diets as larvae were depleted in $^{15}N$ relative to eggs from females fed *ad libitum* as larvae. This result is consistent with minimization of protein catabolism.

**Adults subjected to additional flight**

Values of $\delta^{13}C$ in eggs increased with the age of the female that produced them, as predicted both for control and for flight-stressed *S. mormonia* females (Fig. 4A). The $\delta^{13}C$ was significantly greater in eggs from females that had been subjected to flight stress, when compared with controls. As for larvae on restricted diets, we found no significant age $\times$ treatment interaction.
The isotope $^{15}$N was again depleted in eggs laid early in the female’s life, leveling off at older ages (Fig. 4B). Eggs from females subject to extra flight were significantly more depleted in $^{15}$N than were eggs from control females. We found no significant age × treatment interaction. This result was not expected, and suggests that increased metabolism overall had the same effect as increased growth on the fraction of $^{15}$N by increasing retention of the lighter nitrogen isotope.

**Discussion**

The stable isotopic signatures of carbon and nitrogen in eggs of two butterfly species generally followed our predictions. We found a decreased signature of adult-derived carbon in eggs of females under DR in the adult stage, but an increased signature of adult-derived carbon in eggs of females who had restricted diets as larvae, or who were subjected to additional flight, relative to eggs from control females fed ad libitum. The increased signature of adult-derived carbon in female *S. mormonia* under DR as adults is more impressive in light of the decreased C:N ratio in those eggs (C. L. Boggs, K. Niitepöld, and A. Perez, submitted for publication). These results shed light on the use of carbon income versus carbon reserves for egg production in a nectar-feeding insect. Most work to date has focused on cross-species comparisons. The work here highlights changes in response to environmental conditions. Additional cross-species comparisons of responses to environmental conditions would be particularly interesting for species with different fuels for flight, or with different timing of oogenesis.

The isotope $^{15}$N was enriched in eggs from females who had restricted diets as adults, but was depleted in eggs from females that had restricted diets as larvae, a result consistent with expected differences in protein catabolism based on work on other animals. Unexpectedly, $^{15}$N was also depleted in eggs of females subject to extra flight, perhaps due to an overall increase in resting metabolic rate and the resultant conservation of the lighter N isotope. Our results thus suggest hypotheses for further tests of the response of protein metabolism to nutritional stressors and flight stress in organisms with an incomplete life-history.

As expected, these females showed increased investment of adult-derived carbon in the eggs. However, the depletion of $^{15}$N in eggs indicated that flight had effects beyond simply competing for incoming glucose. This result indicates that the framework for allocation of single macro-nutrients is oversimplified. That is, stressors that affect the same macro-nutrient (in this case low glucose in adults’ diet or expenditure of glucose for flight) can result in different underlying physiological processes with consequent differing effects on diverse life-history traits (in this case, composition of the eggs).

Our experiments utilized single stressors, of types commonly encountered by butterflies in the field (e.g., Boggs and Inouye 2012). The stable isotope patterns described here, with the implied underlying physiological mechanisms, can be used to predict the life-history results of combinations of stressors. In particular, restricted diets of adults and increased stress impose by flight likely co-occur regularly as females disperse to find nectar during times of scarcity (e.g., Fred and Brommer 2009; Ponisio 2010).

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**References**


