Effects of early nutritional stress on physiology, life-histories and their trade-offs in a model ectothermic vertebrate

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Summary Statement

Early-life nutritional stress has immediate, but no lasting effects, on immune function or stress physiology and incites a compensatory growth response that has long-term effects on fitness and survival.

Abstract

Early-life experiences can have far-reaching consequences for phenotypes into adulthood. The effect of early-life experiences on fitness, particularly under adverse conditions, is mediated by resource allocation to particular life-history traits. Reptiles exhibit great variation in life-histories (e.g., indeterminate growth) thus selective pressures often mitigate the effects of early-life stress, particularly on growth and maturation. We examined the effects of early-life food restriction on growth, adult body size, physiology and reproduction in the checkered garter snake. Animals were placed on one of two early-life diet treatments: normal-diet (approximating *ad libitum* feeding) or low-diet (restricted to 20% of body mass in food weekly). At 15 weeks of age low-diet animals were switched to the normal-diet treatment. Individuals fed a restricted diet showed reduced growth rates, depressed immunocompetence and a heightened glucocorticoid response. Once food restriction was lifted, animals experiencing nutritional stress early in life (low-diet) caught up to the normal-diet group by increasing their growth, and were able to recover from the negative effects of nutritional stress on immune function and physiology. Growth restriction and the subsequent allocation of resources into increasing growth rates, however, had a negative effect on fitness. Mating success was reduced in low-diet males, while low-diet females gave birth to smaller offspring. In addition, although not a direct goal of our study, we found a sex-specific effect of early-life nutritional stress on median age of survival. Our study demonstrates both immediate and long-term effects of nutritional stress on physiology and growth, reproduction, and trade-offs among them.

Introduction

Early-life experiences can have far-reaching effects into adulthood, and ultimately fitness (Lemaitre et al., 2015; Lindstrom, 1999; Metcalfe and Monaghan, 2001). Early-life experiences can influence such life-history traits as growth, time to maturation, reproduction, and survival, and trade-offs among them (Roff and Fairbairn, 2007). Much of the work on the fitness effects of early-life experiences has focused on resource availability, but some have also focused on the effects of heightened glucocorticoid levels (Grace et al., 2017)) and thermal conditions (Lee et al., 2012). When considering resource availability in particular, poor nutrition in young animals can reduce fitness (Birkhead et al., 1999) or increase fitness (Ozanne and Hales, 2004; Shanley and Kirkwood, 2000), suggesting that the mechanism by which early-life experiences either positively or negatively impact fitness may depend on the environmental context or the homeostatic state of the individuals within a population. In general, food scarcity *per se* impacts growth, reproduction and survival in a variety of vertebrates, possibly through moderating energy allocation decisions to competing traits (Kubička and Kratochvíl, 2009). Specifically, early-life nutritional stress can have significant long-term consequences for adult traits (e.g., reduced body mass in zebra finches, Birkhead *et al.* 1999; impaired neural development in sparrows, MacDonald *et al.* 2006; reduced reproduction in lizards Kubička & Kratochvíl 2009; increased mortality in fish, Inness & Metcalfe 2008; delayed age and reduced size at maturation in guppies, Auer 2010). Even when nutritional stress is alleviated, lasting effects on adult phenotypes and ultimately fitness may occur (Marcil-Ferland et al., 2013).

Animals deploy different strategies to mitigate early-life nutritional stress on growth and maturation (Auer, 2010; Mueller et al., 2012; Vega-Trejo et al., 2016). Growth rates are plastic, particularly in species with indeterminate growth, and are usually regulated at optimal rather than maximal rates (Arendt, 1997). Growth can be increased when selective pressures favor an increase in overall size (Bronikowski, 2000; Metcalfe and Monaghan, 2003), and such plasticity can be important in environments where resources vary seasonally or annually. Moreover, in many species adult size is a strong determinant of fitness (Choudhury et al., 1996; Gaillard et al., 2000) and the ability to speed up or slow down growth becomes an important mechanism linking ecological conditions with fitness (Bize et al., 2006; Bjorndal et al., 2003). Ultimately, individuals should balance the costs and benefits of obtaining a larger body size (Charlesworth, 1994). Three distinct patterns of growth and sexual maturation are possible after alleviation of

early-life nutritional stress. Individuals may mature at a standard maturation size (but at a later age – Fig. 1a); they may mature at a standard maturation age (but smaller size – Fig. 1b), or they may increase their growth rate to mature at the standard size and age (Fig. 1c). In the first case, delaying maturation may be costly as it increases generation time and may decrease the overall reproductive lifespan of an individual (Roff, 1992). In the second case, maturing at a smaller body size may be costly if body size is positively correlated with survival and reproductive success (Roff, 1992). Given these potential fitness costs, selection might instead favor increasing growth to mature at normal age and size, provided the costs of doing so are not too high (Metcalfe and Monaghan, 2001). Compensatory growth has been reported across vertebrates: in mammals (Ryan, 1990), birds (Criscuolo et al., 2011), fish (Ali et al., 2003), amphibians (Hector et al., 2012), and reptiles (Radder et al., 2007). Compensatory growth is often achieved through a hyperphagic response to increases in resources (Ali et al., 2003; Gurney and Nisbet, 2004; Morgan and Metcalfe, 2001), but can also be achieved by allocating more energy into growth at the expense of other traits [e.g., locomotor performance (Criscuolo et al., 2011; Lee et al., 2010), immune function (Norris and Evans, 2000), cognition (Fisher et al., 2006), maturation (Auer et al., 2010; Hector et al., 2012; Vega-Trejo et al., 2016), adult body size (Auer, 2010), lifespan (Birkhead et al., 1999; Lee et al., 2013), and litter size (Auer et al., 2010)].

We tested among the competing hypotheses outlined in Fig. 1 to address how early-life nutritional environment influenced patterns of growth, maturation size, physiology and reproduction in the checkered garter snake, *Thamnophis marcianus*. To date, much of the literature on early-life experiences and the resultant effects on adult phenotypes has focused on birds and fishes (Ali et al., 2003; Lemaitre et al., 2015; Metcalfe and Monaghan, 2001). Our study species, the checkered garter snake, has indeterminate growth (in contrast to birds but in agreement with fishes), sexual size dimorphism, with females larger than males, and a positive correlation between body size and reproductive fitness for both males and females. We were further interested in whether measures of immune function and physiological stress would similarly reflect growth rate changes before and after the switch to normal food availability, as well as whether an effect of early-life nutritional stress would be evident on mating success and first reproductive effort. Finally, we tested whether any of these effects, and trade-offs among traits, differed between the sexes.

Materials and Methods

STUDY SYSTEM

We studied the effects of early-life food availability in checkered garter snakes. This species has a broad distribution throughout southwestern North America (Rossman et al., 1996; Seigel et al., 2000). Checkered garter snakes have female biased sexual size dimorphism (mean snout-to-vent length (SVL) for females: 579 mm; males: 477 mm). This species is viviparous and have litters that range from 5 to 31 neonates (Ford and Karges, 1987; Rossman et al., 1996).

Study subjects were offspring from paired breeding adults maintained at the Ophidian Research Colony (University of Texas at Tyler) where multiple generations have been bred from wild progenitors, subject to non-sibling matings. Seventy neonates from eight litters (i.e., eight different families) were randomly assigned to two experimental feeding groups with litters and sex split nearly evenly ("normal-diet female" $N= 15$; "normal-diet male" $N=18$; "low-diet female" $N=16$; "low-diet male" $N=21$). Neonates were weighed and measured at birth and housed in individual cages (35x21x13 cm translucent plastic storage boxes) each filled with 3-5cm of aspen bedding with water available *ad libitum* and maintained on a 14:10 light: dark cycle at $28\pm1°C$ to simulate a photoperiod and temperature regime commonly experienced throughout their range during the active season (Rossman et al., 1996).

DIET TREATMENTS, FOOD CONSUMPTION, BODY SIZE, AND GROWTH

Twice per week, for the first two weeks of life, all *T.marcianus* neonates were offered size appropriate pieces of tadpole tail to help ensure regular eating patterns, as this species' diet in the wild consists mainly of tadpoles, frogs and fish (Rossman et al., 1996). At two weeks of age, snakes were started on their diet protocol for *ad libitum*- and low-food availability. Each individual in the "*ad libitum* early-life diet" treatment (hereafter "normal-diet") was offered 60% of its body mass weekly in pinky mice over two feedings as established by an earlier pilot study (N. B. Ford, unpublished data). Each individual on the "low early-life diet" treatment (hereafter "low-diet") was offered 20% of its body mass in pinky mice weekly in one feeding. Previous work had shown that this amount results in reduced size, compared to unrestricted conspecifics, but is still adequate to facilitate growth and maturation (N.B. Ford, unpublished data). Feeding amounts were adjusted every 15 days to track increasing body weight.

Voluntary food consumption was measured at each feeding by weighing food before and after feeding. Snakes were maintained on these diet treatments until both (i) a significant difference in mass between the two diet treatments occurred, and (ii) the low-diet individuals had doubled their birth mass, so as to ensure individuals were large enough to obtain an adequate blood sample. These criteria were met at 15 weeks of age and animals on the low-diet were then switched to the normal-diet for 15 weeks (see Fig. 2 for experimental timeline). Animals were maintained on the normal-diet protocol for an additional 10 weeks (i.e., 40 weeks of age), fasted for two weeks, and placed into hibernation at 4C (Fig. 2). Over these first 38 weeks of the experiment (i.e. age 2-40 weeks) weekly food consumption was measured (g eaten), and animals were weighed (g) every 15 days, and measured (snout-to-vent length, mm) every 30 days.

IMMUNE FUNCTION AND PHYSIOLOGICAL STATE

To test for the effects of normal- and low-diet on immune function, blood was drawn at two time points for all animals – at 15 weeks of age (just prior to switching all animals to *ad libitum* food availability) – and again at the end of a doubling of that age (30 weeks of age). Approximately 100 µL of whole blood was collected from the caudal vein in heparin rinsed syringes; centrifuged to separate blood components, and plasma was snap-frozen and stored at -80C for subsequent physiological and immune assays.

We conducted three constitutive innate immunity assays: i) natural antibodies (NAbs), ii) complement-mediated cell lysis and iii) bactericidal competence of blood plasma to characterize and quantify non-specific and rapid responses to invading pathogens as a first line of defense. Both NAbs and complement-mediated cell lysis were measured using a hemolysis-hemagglutination assay (Matson et al., 2005), with modifications for use in garter snakes (Sparkman and Palacios, 2009). A series of eight serial two-fold dilutions of 10 µl of plasma were made with phosphate buffered saline (PBS) in a 96-well plate. Each well then received 10 µl of a 2% heterologous sheep's red blood cell (SRBC) suspension. All samples were run in duplicate; each plate had both positive (anti-SRBC) and negative (SRBC with no plasma sample) controls. Plates were incubated for 90 minutes at 28°C and then scored. For both hemagglutination and lysis, titres were estimated as the negative $\log_2$ of the highest dilution factor of plasma. Half scores were given for titres that appeared intermediate. Sheep red blood

cells were utilized as the foreign mitogen as has been previously demonstrated in snake/reptile studies (Kawaguchi et al., 1978; Sparkman and Palacios, 2009).

Bactericidal competence of snake plasma was assessed (Matson et al., 2006) with minor modifications for use in garter snakes (Sparkman and Palacios, 2009). A pellet of lyphophilized *Escherichia coli* (Microbiologics, Cat# 0483E7) was reconstituted using 40ml of phosphate buffered saline (PBS). This was further diluted with PBS to produce working solutions that yielded roughly 200 colony-forming bacteria per 10 µl. Plasma samples were diluted 1:10 with PBS. Sample reactions were prepared by adding 10 µl bacterial working solution to 100 µl of the diluted plasma samples. Replicate controls were prepared (one control per every 12 samples) by adding 10 µl of the bacterial working solution to 100 µl of PBS. Sample reactions were incubated for 20 minutes at 28°C, (i.e., the animal maintenance temperature) to provide adequate time for bacterial killing to occur. Duplicate controls and sample reactions were plated in 50 µl aliquots on 4% tryptic soy agar and incubated approximately 24 hours at 28°C, additional incubation at 37°C was used if bacterial colonies were not clearly visible. The number of bacterial colonies on each plate was counted and the percentage of colonies on each plate relative to the mean number of colonies in the control plates was calculated. This percentage was subtracted from 100 to obtain the percentage of bacterial colonies killed.

We measured two independent assessments of physiological state: (i) plasma concentration of corticosterone (Greenberg and Wingfield, 1987) – a hormone that is upregulated in both the fight-or-flight response and also under normal food conditions to facilitate foraging, and (ii) the ratio of heterophils-to-lymphocytes (H:L) in whole blood smears as a measure of stress. An increased H:L ratio is indicative of a higher number of heterophils in circulation and an increase in the movement of lymphocytes into peripheral tissues (Dhabhar et al., 1996; Dhabhar et al., 1994).

Circulating CORT (ngmL^{-1}) was quantified in blood plasma to characterize the relationship between baseline hormone levels and early-life condition. CORT levels were quantified with a radioimmunoassay (MP Biomedicals ImmuChem Double Antibody Corticosterone I-125 RIA kit, Irvine, CA; lowest detectable concentration for this kit is 7.7 ngmL^{-1}) following methodology previously adapted for use with snakes (Palacios et al., 2012; Robert et al., 2009; Sparkman et al., 2014). All measurements were made using a single kit with a provided control used to assess intra-assay variability. The control was included in each run

and yielded a coefficient of variation (%CV) of 13.0%. All samples were run in duplicate with a %CV < 10%. Time between first contact and bleeding was included as a covariate in the statistical analysis of CORT levels, as increases in CORT above baseline measures have been observed in garter snakes after 10 minutes of handling (Palacios et al., 2012).

To measure H:L Blood smears were prepared from a drop of freshly drawn blood from each animal at the time of bleeding. Smears were fixed in methanol and stained with Wright-Giemsa stain (Fisher Scientific, Cat#SDWG80) to determine heterophil-lymphocyte percentages. The number and type of leukocytes – lymphocytes, heterophils, eosinophils, basophils or monocytes (Strik et al., 2007) – were classified by scanning blood smears under 1000x magnification and classifying the first 100 leukocytes encountered. If a heterophil was not viewed within classification of the first 100 leukocytes, the count was extended until either a heterophil was viewed or 200 leukocytes were examined.

COURTSHIP AND REPRODUCTIVE OUTPUT

After hibernating for twelve weeks, animals were warmed up gradually over two weeks, offered food twice (once per week, *ad libitum*), and mating trials were begun (Fig. 2). To test for an effect of normal- and low-diet on both female and male reproductive output, females and males were randomly paired (subject to non-sibling matings) to construct all pairwise crosses of early-life diet treatment (normal-diet female x normal-diet male; normal-diet female x low-diet male; low-diet female x normal-diet male; low-diet female x low-diet male; Table 2). Each female and her designated male mate were placed in individual ten-gallon glass aquaria and observed for one hour. If copulation was not observed, females were paired with the same male five days later. If both mating opportunities were unsuccessful, a second non-sibling male of the same diet treatment group was paired with the female. If this third pairing did not result in copulation, we stopped mating attempts with this female. Data on successful (i.e., resulted in gravidity) and unsuccessful matings for each treatment combination were recorded and used to test for effects of early-life food availability on male and female mating success. Gravid females were monitored through parturition, and each female's reproductive effort (counts and masses of liveborns, stillborns, and unfertilized yolks) and reproductive success (counts and masses of liveborns) were measured. These data were used to test for effects of early-life food availability on maternal reproduction.

STATISTICAL ANALYSES

For our repeated measures of food consumption, body size, growth, and physiology, we conducted mixed-effects repeated measures general linear models (GLMM using Proc Mixed in SAS 9.4 (SAS Institute, Car, NC)). Data on food consumption (as grams eaten) were not normally distributed; no additional transformation achieved normality. Non-parametric tests of the main effects of interest were in agreement with our parametric general linear models approach, so we present only the GLMM results. Plasma CORT, natural antibodies and complement mediated cell lysis were $\log_{10}$-transformed, bactericidal competence was logit-transformed, and H:L ratios were square root transformed to achieve normality. For all analyses, fixed effects included diet treatment (early-life "normal"- versus early-life "low"-food availability), sex (female versus male), repeated time (weeks 2- 40), and the interactions of the three, as well as the random effect of individual nested within family utilizing the following general linear model

$$Y \sim \mu + diet + sex + time + diet*sex + diet*time + sex*time + diet*sex*time + cov(s) + \varepsilon$$

For the analysis of body size (SVL, mm), birth SVL was used as a covariate to account for the potential effect of birth size on subsequent body size. For the analysis of growth (change in SVL, mm), we used two time-varying covariates; food consumption during each interval between two measurements dates, and SVL at the start of each interval to account for slowing of growth with increasing length. For the repeated-measures analyses of physiology, we included the covariate of body condition at each measurement date (15 and 30 weeks). Body condition, calculated as the residuals of the regression of log body weight on log SVL (Weatherhead and Brown, 1996), is known to influence immunological responses of vertebrates, including snakes (Gangloff et al., 2017a; Palacios et al., 2011). Additionally, time between first contact and bleeding was included as a covariate in the statistical analysis of CORT levels, as increases in CORT above baseline measures have been observed in garter snakes after 10 minutes of handling (Palacios et al., 2012). Samples where blood collection occurred more than 30 minutes from time of initial handling (N=3) were removed. As in previous studies (Palacios et al., 2011; Sparkman and Palacios, 2009), natural antibodies and complement-mediated lysis were highly

correlated (*Pearson* r = 0.921, P < .0001, N = 136), thus only the results for natural antibodies are shown (see SI Table S2 for complement-mediated lysis results).

For our single measures of mating success for males and females, we tested for an effect of early-life diet within each sex with a G-test of goodness-of-fit (Sokal and Rohlf, 2012). Because paternal diet treatment was known but individual paternity was not always known with certainty, we could not test for individual reproductive success in males.

For the analysis of female first reproduction, we used the following general linear model

$$Y \sim \mu + diet + SVL + \varepsilon$$

Where diet is early-life normal- versus low-food availability and SVL is female body size prior to parturition. Dependent variables included measures of reproductive effort (total litter mass, and a count of total litter size), reproductive success (total mass of liveborn, and liveborn litter size), and individual offspring body mass and size.

Results

FOOD CONSUMPTION, BODY SIZE, AND GROWTH

Experimental animals consumed food voluntarily, so our first goal was to understand whether animals in the two early-life diet treatments were consuming different amounts of food. Food consumption (analyzed as grams eaten) was significantly affected by the interaction of Diet x Sex x Time (Table 1, plotted as percent of body mass eaten in Fig. 3a). Animals on the normal-diet consumed more food than low-diet animals during the 15 weeks of the experiment where low-diet animals were given less food. From ages 16 – 40 weeks, all animals were offered 60% of their body mass in food; low-diet animals initially increased their food consumption to surpass that of normal-diet animals, but by 40 weeks of age, all animals had slowed their consumption (Fig. 3a).

Repeated measures of body size (SVL) was also significantly affected by the interaction of Diet x Sex x Time (Table 1, Fig. 4). Females and males with early-life normal-food availability had significantly larger body sizes across time beginning with week 5 and continuing through the end of the experiment at week 40. Growth (change in SVL) was significantly affected by the interaction of Diet x Sex x Time (Table 1, Fig. 3b); the shapes of the body size

trajectories varied by sex and by diet over the course of the experiment. During the diet treatment, normal-diet males and females exhibited faster growth compared to low-diet individuals. From age 16 weeks onward (with all animals on the normal diet of 60% body mass) snakes previously on the low-diet increased their growth rates to equal or surpass those of the early-life normal-diet animals. Interestingly, they did not quite catch up in size; at hibernation, low-diet animals were still significantly smaller. In addition, following this experiment, animals were housed in standard lab conditions where we subsequently found long-term, sex-specific, effects of early-life nutritional stress on survival (median survival of low-diet females = 27.8 months; normal-diet females = 40.9 months; all males, regardless of early-life diet = 70+ months. See SI Fig. S1 for survival curves).

IMMUNE FUNCTION AND PHYSIOLOGICAL STATE

Early-life diet and time interacted to affect natural antibodies, bactericidal competence, and levels of plasma CORT (Table 1, Fig. 5). For measures of natural antibodies, at the end of the diet restriction (15 weeks of age) agglutination titres were higher in normal-diet animals than in low-diet animals. Whereas, at 30 weeks of age (all animals having been on normal diets for 15 weeks) normal-diet animals decreased their NAbs such that there was no difference between early-life treatments (Fig. 5a). For bactericidal competence, early-life normal-diet animals displayed higher killing capacity at 15 weeks of age. By 30 weeks of age, previously low-diet animals had increased their bactericidal killing capacity such that there were no differences between diet treatments (Fig. 5b). For measures of CORT, at 15 weeks of age, low-diet treatment animals had higher baseline levels of plasma CORT than normal-diet treatment animals. By 30 weeks of age, previously low-diet animals decreased their plasma CORT levels such that there was no difference between diet treatments (Fig. 5c). Heterophil-to-lymphocyte ratio was affected by time; all animals (regardless of early-life diet treatment) increased their H:L ratio between age 15 and 30 weeks. Additionally, diet and sex interacted such that normal-diet females had higher H:L ratios than all other Diet x Sex combinations at both time points (Table 1, Fig. 5d).

COURTSHIP AND REPRODUCTIVE OUTPUT

Of the 30 females that were used for the reproduction trials (one animal died during hibernation), six females did not mate with either their first assigned mate or their second assigned mate (Table 2; see SI for full details). Of the 53 unique female/male pairings, 24 resulted in copulation (Table 2). For male mating success, we found a significant effect of early-life diet, such that normal-diet males successfully mated more often than previously low-diet males (G = 4.797, df = 1, P = 0.029). For female mating success, we found no significant difference between early-life normal- and low-diet treatments (G =0.007, df = 1, P =0.932).

Of the 24 females that copulated with males, normal-diet females were on average larger than low-diet females (Fig. 4). After accounting for this diet-associated variation in mom's body size, we found no additional effect of early-life diet on reproductive effort (total litter mass, total litter size) or on reproductive success (total liveborn mass, total number of liveborn). However, early-life diet (after accounting for mom SVL) significantly affected both individual offspring mass and length – with normal-diet moms giving birth to heavier and longer offspring (Table 3, Fig. 6).

Discussion

We tested whether early-life nutritional stress leaves its mark on growth, body size, immune function, physiological stress, mating success, and female first reproduction. We found effects of early-life diet on growth, body size, mating success, and offspring size. We found that immune function and physiological state responded in an immediate manner to food limitation, but values returned to normal as animals were placed on normal diets, with limited support for a longer-term trade-off with growth and reproduction. For most life-history traits, sex significantly interacted with diet and age, which suggests a sex-specific effect of early-life nutritional stress in this species.

COMPENSATORY GROWTH

We found that an individual's early-life nutritional environment influenced their rate of growth not only during periods of reduced food availability, but also once resources were increased. Food consumption, growth rate, and body size were lower in low-diet snakes for the duration of the diet treatment. When nutritional environment improved, previously limited males and females increased their food consumption and growth rates such that they either matched or surpassed that of their non-stressed conspecifics (Fig. 3). This pattern of increased growth rates in the low-diet treatment group most closely reflects the strategy of compensatory growth (Fig. 1c).

Though low-diet individuals were able to increase their rate of growth above that of normal-diet individuals shortly after alleviation of nutritional stress, we found that low-diet animals of both sexes did not fully catch-up in size to normal-diet individuals by the end of the diet experiment (40 weeks of age). Although this absence of complete compensation could be an artifact of the length of the experiment, compensatory growth is not always accompanied by a convergence in growth trajectories. Compensation is often incomplete with individuals never fully attaining the size of their non-restricted conspecifics (Ali et al., 2003; Jobling, 2010; Mangel and Munch, 2005; Metcalfe and Monaghan, 2001). This plasticity in growth rates suggests that growth is typically regulated at optimal rates, below their potential maxima, such that when selective pressures favor a larger overall size growth rates can be increased (Arendt, 1997; Dmitriew, 2011; Metcalfe and Monaghan, 2001; Metcalfe and Monaghan, 2003; Nylin and Gotthard, 1998).

The ability to increase growth rates can potentially mitigate effects of poor early-life environment, as obtaining a larger size may minimize fitness loss (Mangel and Munch, 2005; Metcalfe and Monaghan, 2001), be advantageous in resource acquisition (Arendt and Wilson, 1997), aid in the avoidance of size specific predators , or in buffering individuals against harsh or variable environmental conditions. Although this is not always the case (e.g., advantages of smaller size discussed in Blanckenhorn, 2000) fitness benefits have been extensively studied in snakes (Ford and Killebrew, 1983; Ford and Seigel, 1989) and specifically, in our study species, it has been shown that large body size is positively correlated with measures of fitness (Ford and Karges, 1987; Ford and Seigel, 2015; Seigel et al., 2000). Individuals may also exhibit ontogenetic shifts in their energy allocation such that they preferentially allocate resources to

somatic growth during early-life and to reproduction when sexually mature (Bronikowski and Arnold, 1999; Norris and Evans, 2000; Stearns, 1989). This may explain why we found patterns of increased food consumption and growth in low-diet animals immediately after resources became readily available followed by all individuals (regardless of sex or early-life treatment) exhibiting a slowing in food consumption and growth as they reached maturation size (Fig. 3).

TRADE-OFFS WITH PHYSIOLOGY

We found that nutritional restriction during early-life had consequences on physiological state and constitutive innate immune function during the time of restriction; low-diet animals had lower immune function and higher circulating CORT levels (Fig. 5, Table 1). In wild garter snakes, we find the same patterns in years where food is scarce: lower innate immune function and higher CORT levels (Palacios et al., 2011; Palacios et al., 2012; Sparkman and Palacios, 2009). Interestingly, this is true across ages, sexes, and season. Studies examining immunocompetence in nestling birds (Birkhead et al., 1999; Brzek and Konarzewski, 2007; Hoi-Leitner et al., 2001) have also found reductions in various aspects of immune function under conditions of low quantity or quality resources.

In our study, once nutritional stress was alleviated (all animals on normal-diet), we found that as animals increased their growth rates, there was no concommitant reduction in immunocompetence. This lack of a trade-off between investment in compensatory growth and investment into immune function is in contrast to work on damselflies (Stoks et al., 2006) and poultry (van der Most et al., 2011), but is in accordance with work conducted on zebra finches (Killpack et al., 2014). In garter snakes (Palacios and Bronikowski, 2017), measures of adaptive immune function, but not innate, have been shown to trade-off with reproduction. This may suggest that the immune components measured here are maintained at the expense of other more energetically expensive components of immune function. Additionally, the lack of association between compensatory growth and immune function, such as natural antibodies, may not be altogether surprising as it has been suggested that their production is independent of internal and external stimuli (Ochsenbein and Zinkernagel, 2000). Moreover, a reduction in immunocompetence could negatively affect an individual's fitness and thus resource allocation to immune function may be maintained at the expense of other traits (Norris and Evans, 2000; Palacios and Bronikowski, 2017).

We found little evidence to suggest a trade-off between energy allocation to growth (i.e., compensatory growth) and the physiological parameters measured. In vertebrates, glucocorticoid hormones such as CORT mediate daily and seasonal metabolic processes such as energy acquisition, storage and utilization (Landys et al., 2006; Sapolsky et al., 2000). Upon exposure to adverse events such as increased risk of predation, changes in thermal conditions or food deprivation, activity of the hypothalamic-pituitary-adrenal (HPA) axis is up-regulated (Greenberg and Wingfield, 1987) increasing the secretion of CORT into circulation (Gangloff et al., 2016; Gangloff et al., 2017b; Palacios et al., 2012). Stress-induced levels of CORT mediate energy balance, such that self-maintenance and survival are prioritized over processes such as immunity and reproduction in the short-term (Greenberg and Wingfield, 1987; Romero et al., 2009). Chronically stressed individuals may show sustained increases in baseline CORT levels or changes in the magnitude of response to an acute stress which may impair reproduction or survival long-term (Angelier and Wingfield, 2013; Wingfield, 2013). In this study, after the period of food-restriction, low-diet animals exhibited higher circulating levels of CORT but once nutritional stress was alleviated, we found no association between increased growth rates and CORT levels.

Additionally, glucocorticoids can act to redistribute leukocytes, with heterophils released into circulation and an increase in the movement of lymphocytes into peripheral tissues (Dhabhar et al., 1996; Dhabhar et al., 1994; Vleck et al., 2000). H:L ratios have been shown to positively correlate with various environmental stressors (increases in H:L with increasing magnitude of a stressor: reviewed by (Davis et al., 2008)) and with levels of circulating glucocorticoids (Gangloff et al., 2017b; Goessling et al., 2015). However, levels of circulating CORT and H:L ratios are not always correlated within individuals (Davis and Maney, 2018; Goessling et al., 2015; Sparkman et al., 2014). We found no effect of early-life diet on H:L ratios and no trade-off with increasing growth rates in low-diet animals. This may be attributed to leukocyte numbers changing more slowly in response to stressors than do CORT levels; these changes are also less variable, longer lasting, and often multiple stressors tend to have an additive affect (Vleck et al., 2000) thus changes in H:L ratios might better be considered a "downstream" reaction to more chronically adverse environments (Davis and Maney, 2018).

EARLY-LIFE EXPERIENCES AFFECT FITNESS

Poor early-life environments can have negative consequences for fitness, with low resource availability leading to slower rates of growth and resulting in maturation at older ages or smaller sizes (as in Fig. 1A and 1B) (Auer, 2010). Delaying maturation may decrease fitness because it increases generation time and can also lead to a reduced reproductive lifespan (Roff, 2002) or a reduction in fecundity over time with subsequent reproductive events (Auer, 2010). When considering mating success, we saw no difference between low-diet females and those that had normal diets throughout the experiment. However, low-diet males were less likely to successfully mate (Table 2). This reduction in mating success for low-diet males may suggest that the allocation of resources to growth, following a period of growth depression, differentially trades-off with age of maturation for males. This is supported by Morgan and Metcalfe (2001) in which they found compensatory growth reduced the occurrence of sexual maturation in male salmon. Alternatively, this reduction in mating success may be due to a reduction in attractiveness, such that following a period of low food availability males exhibiting smaller size or a suppression in other sexually selected traits were not desirable to females (e.g., Kahn et al., 2012; Livingston et al., 2014; Ohlsson et al., 2002).

When considering the reproductive effort and success of females we found no effect of early-life diet on total litter size or mass; normal- and low-diet moms had equal litter sizes of equal mass. This is in accordance with findings in fish (Inness and Metcalfe, 2008), and in birds (Criscuolo et al., 2011) reporting that compensatory growth did not affect the latency to produce the first egg or mean clutch size (though did result in lower mean clutch mass). In our study, early-life diet was strongly correlated with female size (despite compensatory growth, normal-diet females were larger than low-diet females at first hibernation, Fig. 4). Variation in total litter size and litter mass was accounted for by mom size (Table 3) – thus we see an effect of early-life diet on female size at maturation and size in turn is a determinate of reproductive output. However, when accounting for differences in mom body size we still find a signature of early-life diet on offspring mass and SVL with normal-diet moms giving birth to larger babies (both longer and heavier, Table 3, Figure 6). This is in agreement with work done on Madagascar ground geckos (*Paroedura picta*) showing that food-limited females laid smaller eggs more infrequently (Kubička and Kratochvíl, 2009). Additionally, a large number of studies in both avian and non-avian reptiles (Janzen et al., 2001; Krist, 2011), including *Thamnophis* species

(Addis et al., 2017; Gangloff et al., 2017a), show that larger body size at birth or hatching increases early-life survival. Our results suggest that, in females, fitness is negatively affected by trade-offs between increased rates of growth and reproduction.

We demonstrated a compensatory growth response with individuals increasing their food consumption and rate of growth following a period of food restriction. Low-diet animals did not quite "catch-up" in size to their normal-diet conspecifics. We found immediate, but no lasting effects of early-life diet on immune function or physiological state. Traits in which the two sexes responded differently to early-life stress included mating success and long-term survival. Males differed in reproductive success in relation to early-life experiences such that low-diet males exhibited reduced success. Though females did not differ in their propensity to mate, low-diet females gave birth to smaller offspring. Early-life food restriction had long-term effects not only on female reproduction, but also on survival, with low-diet females exhibiting lower survival than their normal-diet conspecifics. Resource availability, especially during periods of development, is important in determining the life history trajectory of an organism. It has been widely hypothesized that accelerated growth rates, particularly when following periods of reduced resource availability, may trade-off with longevity (Metcalfe and Monaghan, 2001; Metcalfe and Monaghan, 2003). Our results suggest that, at least for females, current reproduction may be prioritized over future survival when exposed to adverse early-life conditions such resource limitation.

Authors' contributions

KGH, NBF and AMB designed the experiment; KGH conducted the experiment; KGH and DMR completed physiological assays; KGH performed data analysis and drafted manuscript. All authors contributed to the interpretation of results and final manuscript.

Competing interests

The authors declare no competing interests.

Data accessibility

Data from this study will be available on Dryad: doi:10.5061/dryad.mf1gm3p

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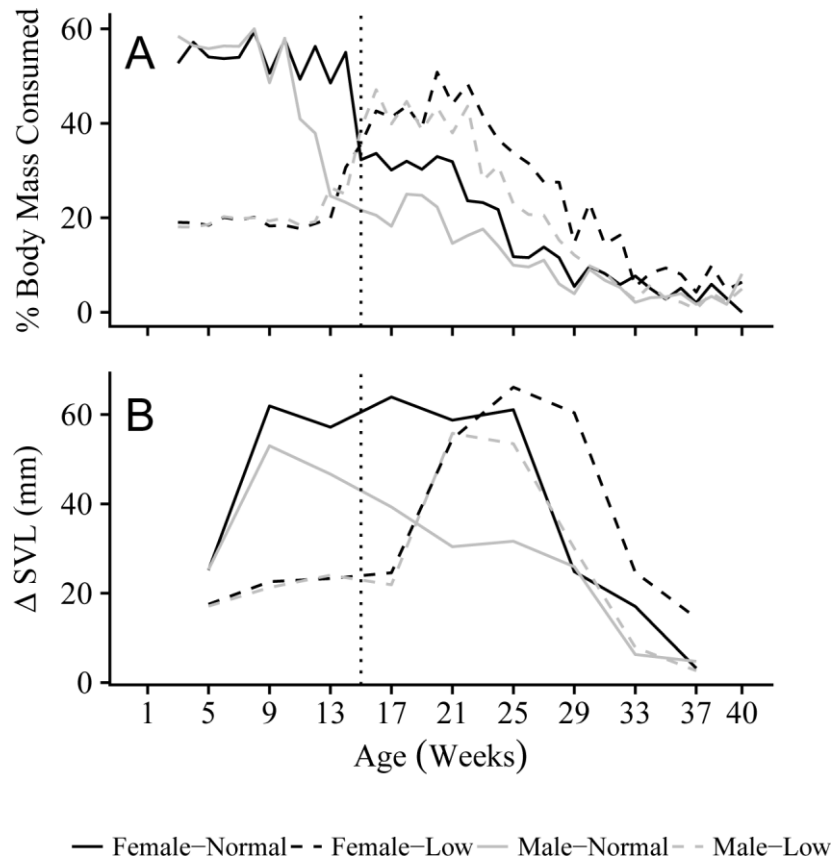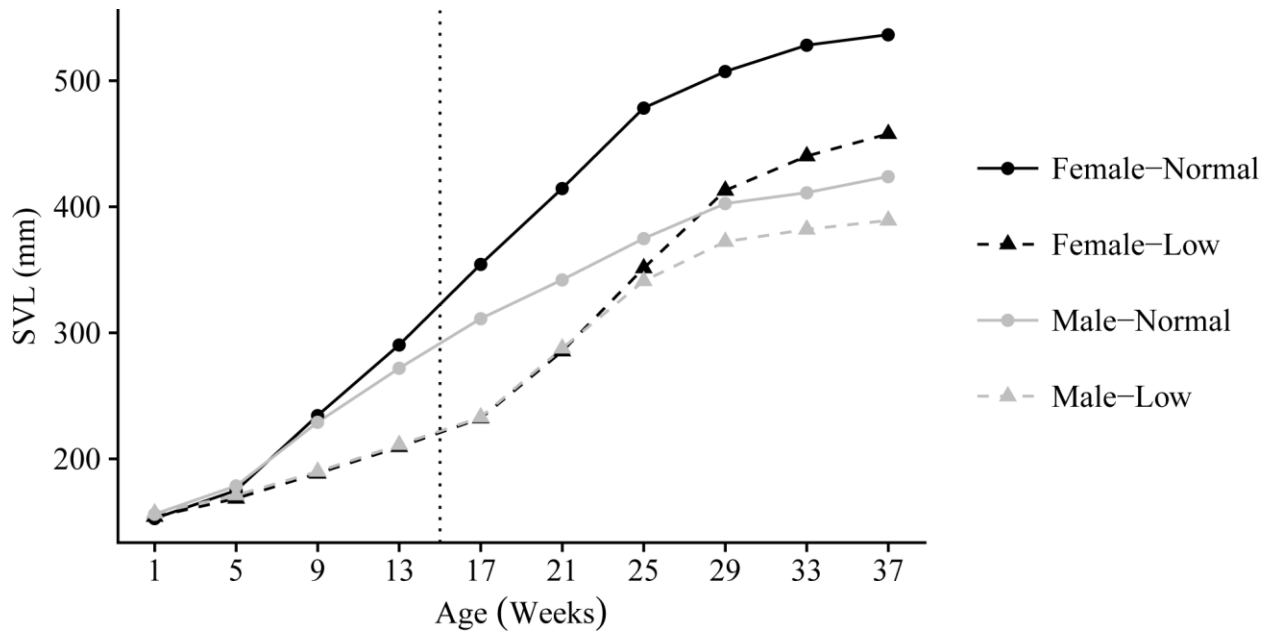
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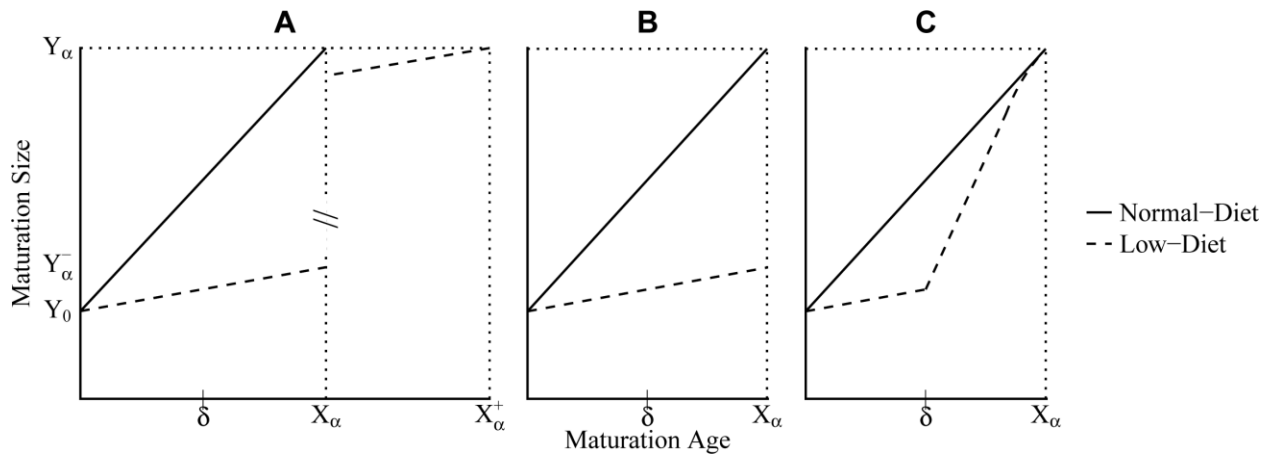
Figure 1. Hypothetical growth curves for resource-limited (dashed line) immature animals upon being switched to normal resource availability (δ), relative to animals that do not experience limited resources (solid line). X_α is the age of maturation for non-limited individuals; $X_\alpha^+ > X_\alpha$ represents a delayed maturation age; Y_0 is birth size; Y_α is the size of maturation for non-limited individuals; $Y_\alpha^- < Y_\alpha$ represents a smaller maturation size. The double hash mark at X_α in panel A represents passage of time. Individuals may (A) mature at a later age, but same size; (B) mature at a smaller size, but same age; or (C) may increase growth and mature at normal age and size – all relative to animals that have not experienced limited resources.
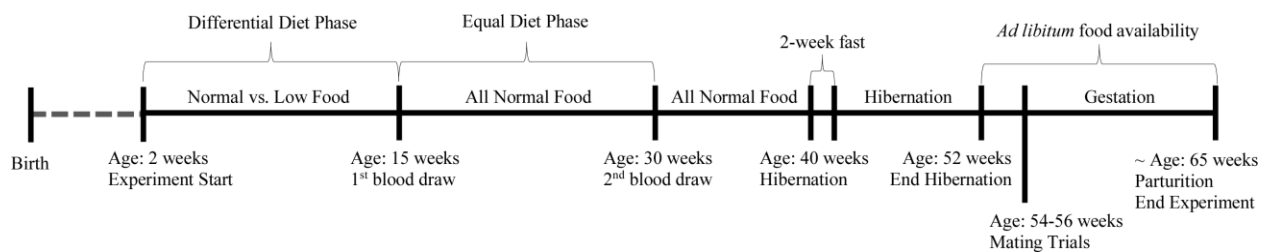


Figure 2. Experimental Timeline.

Figure 3. Food consumption from age 2 through 40 weeks, and growth (change in SVL) from ages 5-37 weeks. Data are plotted as (A) the proportion of body mass consumed in food weekly (*N*=2598 data points) and (B) the least squares mean from full model analyses (Table 1) of monthly increases in body size (change in SVL, mm; *N*=613 data points). Vertical dotted line at week 15 denotes end of diet treatment (alleviation of nutritional stress where all animals were offered normal diet thereafter). Error bars were removed from panels A and B for ease of viewing, standard error ranged from ±3.09-5.54.

Figure 4. Effect of early-life nutritional stress on snout-vent length (SVL). Data are plotted as the least squares mean from analysis of SVL for age 1-37 weeks (Table 1; N=692 data points). Vertical dotted line at week 15 denotes end of diet treatment (alleviation of nutritional stress where all animals were offered normal diet thereafter). Error bars were removed for ease of viewing, standard errors ranged from ±7.31-8.81.
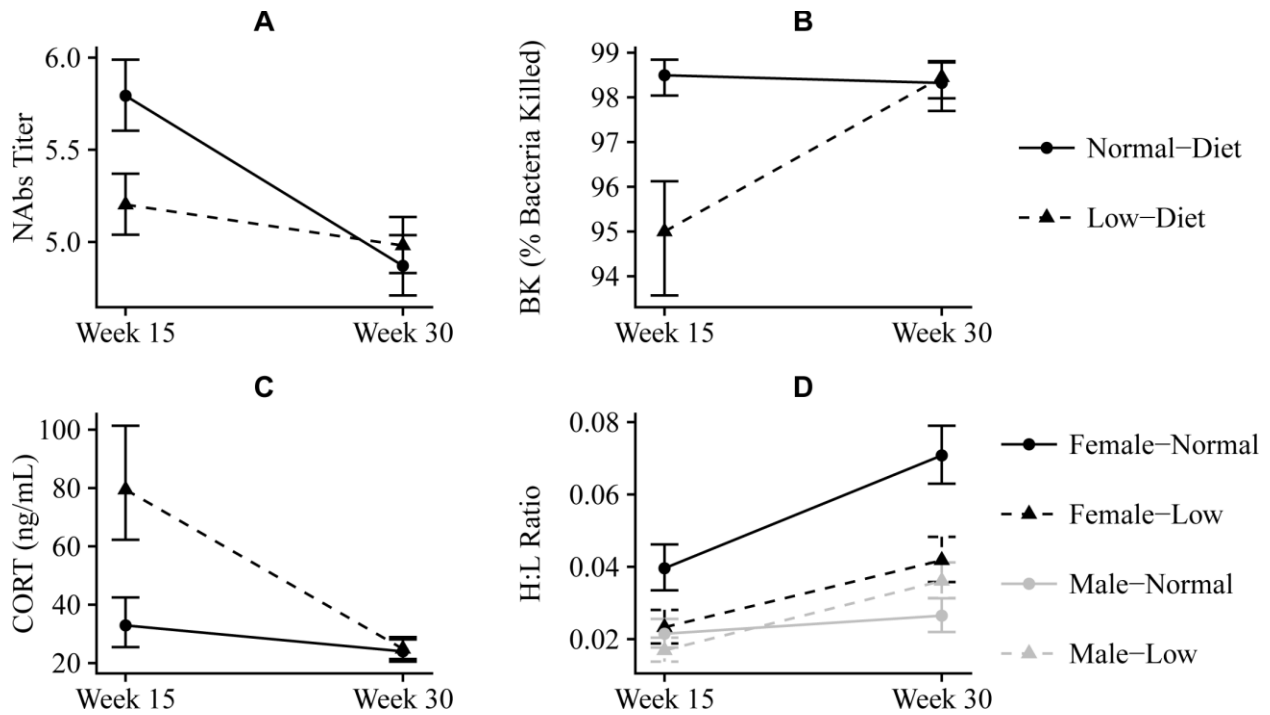
Figure 5. Measures of immune function and stress physiology pre and post diet treatment. Comparison of constitutive immune measures: (A) Natural antibodies (*N*=133 data points) and (B) Bactericidal competence (% bacteria killed; *N*=104 data points) and physiological markers of stress response: (C) Corticosterone (*N*=86 data points) and (D) Heterophil-Lymphocyte ratio (*N*=130 data points). Measurements were made at 15 and 30 weeks of age with data plotted as the least squares mean ± SE from analyses of immune and physiological function (see Table 1).
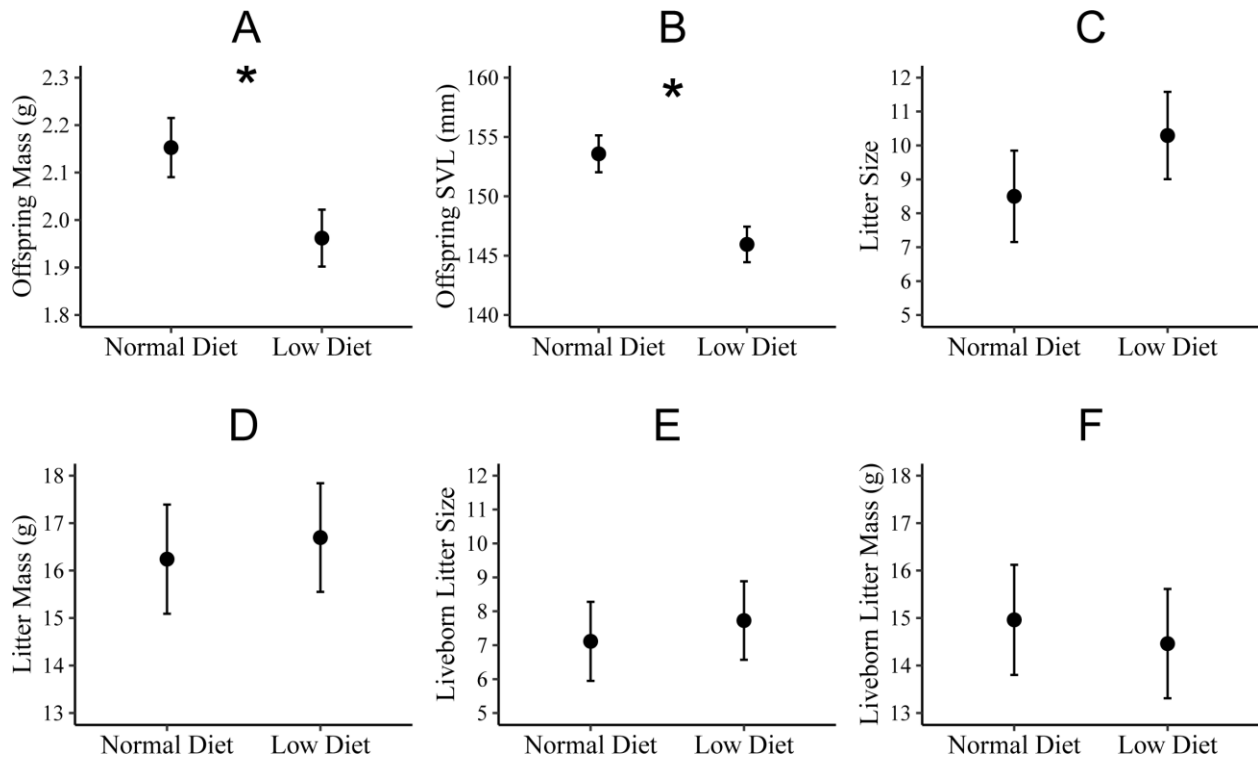
Figure 6. Effect of early-life diet on female fitness. Data are plotted as back-transformed least squares mean ± SE of offspring size (*N*=200 offspring): (A) offspring mass (g) and (B) offspring SVL (mm); Reproductive effort (*N*=24 litters from reproductive moms): (C) litter size (count of liveborn, stillborn, and unfertilized yolks) and (D) litter mass (g; total mass of liveborn, stillborn, and unfertilized yolks); Reproductive success (*N*=24 litters from reproductive moms): (E) liveborn litter size (number of liveborn offspring) and (F) liveborn litter mass (g; total mass of liveborn individuals in a single litter). Asterisks denotes significant pairwise differences in least squares mean.

Table 1. Repeated-measures mixed linear model analysis of food consumption, growth and physiology. Food consumption is the $\log_{10}$ of grams eaten weekly, body size (SVL in mm) and growth as the change in SVL (ΔSVL). Physiological measures are $\log_{10}$-transformed natural antibodies, logit-transformed bactericidal competence, $\log_{10}$-transformed corticosterone and square root-transformed heterophil-lymphocyte ratios. Treatment is normal or low early-life diet, sex is male or female, and time is weekly (food consumption), monthly (SVL) or for physiological measures at two timepoints: 15 weeks of age (where low-diet animals were switched to the normal-diet) and 30 weeks of age. Values are $F_{\text{dfn, dfd}}$. Significant effects are in bold; * is $Pr < 0.05$; ** is $Pr < 0.005$; *** is $Pr < 0.0001$. See Supporting Tables S1 and S2 for parameter estimates for size and physiological analyses, respectively.

	Food Consumption (Weekly)	SVL	ΔSVL	NAbs	BK	CORT	H:L
Condition	--	--	--	$0.04_{1,\ 123}$	$1.55_{1,\ 91.9}$	$0.44_{1,\ 76.3}$	$1.65_{1,\ 120}$
Birth SVL	--	$\mathbf{51.99}_{1,58.3}$**	--	--	--	--	--
Preceding SVL	--	--	$0.01_{1,\ 145}$	--	--	--	--
Food Consumed	--	--	$\mathbf{78.96}_{1,\ 323}$***	--	--	--	--
Treatment	$\mathbf{20.7}_{1,\ 2571}$***	$\mathbf{198.28}_{1,\ 61}$***	$\mathbf{5.18}_{1,\ 94}$*	$1.74_{1,\ 61.3}$	$\mathbf{4.76}_{1,\ 56.2}$*	$\mathbf{4.83}_{1,\ 63.6}$*	$2.55_{1,\ 68.1}$
Sex	$\mathbf{67.65}_{1,\ 2571}$***	$\mathbf{52.16}_{1,\ 63.1}$***	$\mathbf{5.29}_{1,\ 85.6}$*	$0.20_{1,\ 71.5}$	$1.13_{1,\ 55.4}$	$0.30_{1,\ 68.2}$	$\mathbf{12.21}_{1,\ 74.1}$**
Time	$\mathbf{31.77}_{39,\ 2571}$***	$\mathbf{2364.23}_{9,\ 586}$***	$\mathbf{35.13}_{8,\ 491}$***	$\mathbf{18.26}_{1,\ 64.3}$***	$\mathbf{4.36}_{1,\ 55.7}$*	$\mathbf{15.1}_{1,\ 56.7}$**	$\mathbf{21.64}_{1,\ 68.3}$***
Sex x Trt	$2.07_{1,\ 2571}$	$\mathbf{9.51}_{1,\ 60.5}$**	$\mathbf{3.92}_{1,\ 74}$*	$\mathbf{6.30}_{1,\ 65.4}$*	$\mathbf{5.95}_{1,\ 58}$*	$\mathbf{4.72}_{1,\ 57.1}$*	$1.67_{1,\ 69.9}$
Sex x Time	$\mathbf{3.5}_{39,\ 2571}$***	$\mathbf{29.24}_{9,\ 586}$***	$\mathbf{2.33}_{8,\ 493}$*	$1.17_{1,\ 58.5}$	$0.00_{1,\ 48.5}$	$1.41_{1,\ 66.2}$	$\mathbf{4.68}_{1,\ 66.2}$*
Trt x Time	$\mathbf{8.92}_{39,\ 2571}$***	$\mathbf{48.69}_{9,\ 586}$***	$\mathbf{12.55}_{8,\ 503}$***	$1.58_{1,\ 62.3}$	$0.06_{1,\ 54.6}$	$0.51_{1,\ 54.9}$	$0.25_{1,\ 65.8}$
Trt x Sex x Time	$\mathbf{2.45}_{39,\ 2571}$***	$\mathbf{4.24}_{9,\ 586}$***	$\mathbf{2.43}_{8,\ 489}$*	$0.65_{1,\ 60.1}$	$0.99_{1,\ 54.6}$	$2.29_{1,\ 54.9}$	$1.00_{1,\ 63.9}$

Table 2. Mating Attempts and Successes. (A) Number of unique female-male pairings for each Sex x Diet Treatment combination, and (B) the number of successful (resulting in gravidity) female-male mating pairs representing each combination of Sex x Diet Treatment.

A

	Male (normal diet)	Male (low diet)
Female (normal diet)	12	12
Female (low diet)	17	12

B

	Male (normal diet)	Male (low diet)
Female (normal diet)	8	3
Female (low diet)	10	3

Table 3. Parameter estimates and repeated-measures mixed linear model analysis of female reproductive output. Significant effects (P<0.05) are in bold.

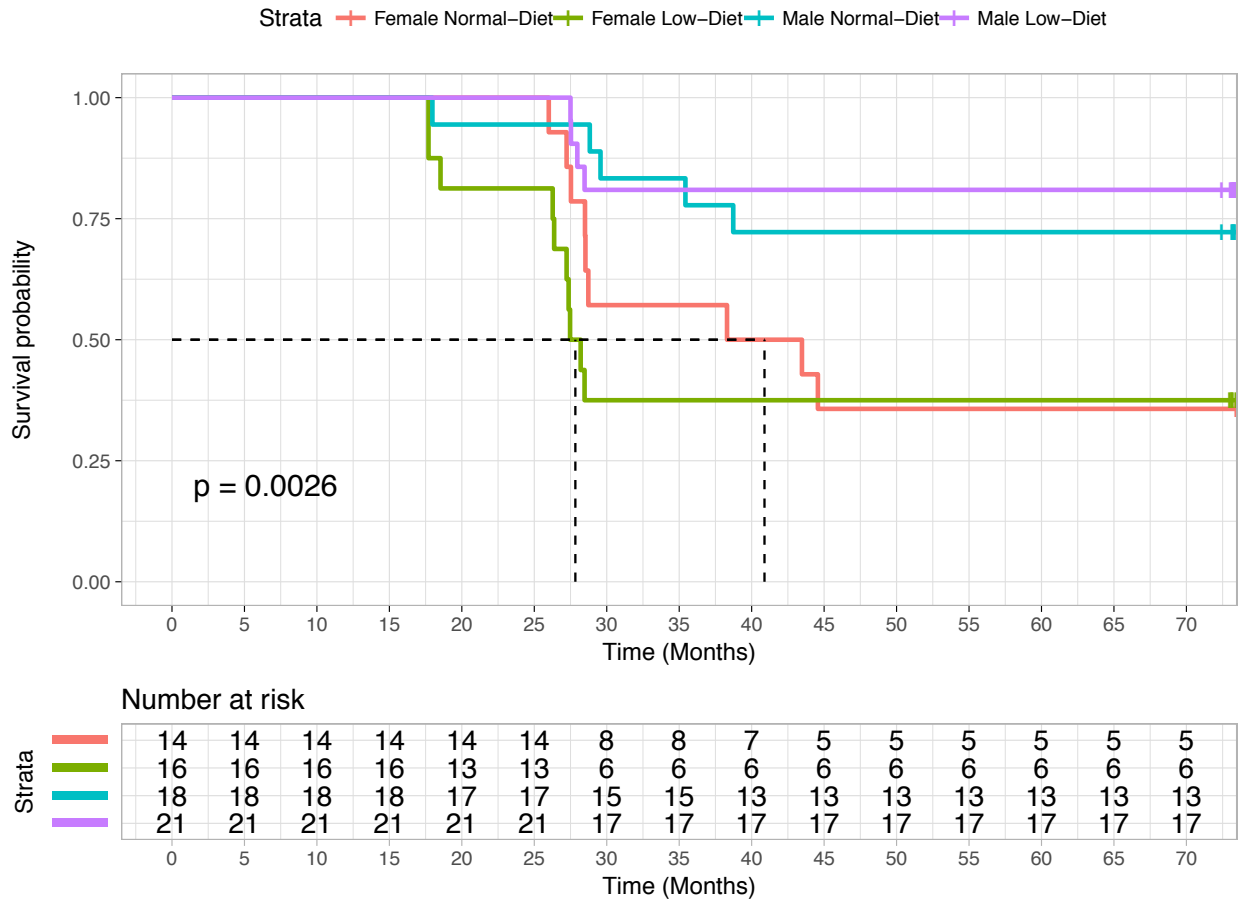|	Offspring Mass	Offspring SVL	Reproductive Effort		Reproductive Success	
			Total Litter Size	Total Litter Mass	Liveborn Litter Size	Liveborn Litter Mass
Mom SVL						
$F_{dfn, dfd}$	33.62 (1, 192)	33.07 (1, 192)	4.82 (1, 20)	10.41 (1, 20)	5.21 (1, 20)	10.82 (1, 20)
$P_r > F$	**< .0001**	**< .0001**	**0.0400**	**0.0042**	**0.0335**	**0.0037**
Treatment						
Estimate	0.191	7.63	-1.52	-0.01	-0.04	0.01
$F_{dfn, dfd}$	4.49 (1, 192)	11.53 (1, 192)	0.64 (1, 20)	0.02 (1, 20)	0.14 (1, 20)	0.03 (1, 20)
$P_r > F$	**0.0354**	**0.0008**	0.4340	0.8921	0.7094	0.8737

Table S1. Parameter estimates and repeated-measures mixed linear model analysis of food consumption as the $\log_{10}$ of grams eaten weekly, body size (SVL in mm) and growth as the change in SVL (ΔSVL). Significant effects (P<0.05) are in bold.

	Food Consumption (Weekly)	SVL	ΔSVL
Birth SVL			
F (d.f.$_n$, d.f.$_d$)	--	51.99 (1, 58.3)	--
$P_r >$ F	--	**< .0001**	--
Preceding SVL			
F (d.f.$_n$, d.f.$_d$)	--	--	0.01 (1, 145)
$P_r >$ F	--	--	0.9083
Food Consumed			
F (d.f.$_n$, d.f.$_d$)	--	--	78.96 (1, 323)
$P_r >$ F	--	--	**< .0001**
Treatment			
Estimate	0.12	0.0806	0.0532
F (d.f.$_n$, d.f.$_d$)	20.7 (1, 2571)	198.28 (1, 61)	5.18 (1, 94)
$P_r >$ F	**< .0001**	**< .0001**	**0.0251**
Sex			
Estimate	0.22	0.0437	0.045
F (d.f.$_n$, d.f.$_d$)	67.65 (1, 2571)	52.16 (1, 63.1)	5.29 (1, 85.6)
$P_r >$ F	**< .0001**	**< .0001**	**0.0239**
Time			
F (d.f.$_n$, d.f.$_d$)	31.77 (39, 2571)	2364.23 (9, 586)	35.13 (8, 491)
$P_r >$ F	**< .0001**	**< .0001**	**< .0001**
Sex x Trt			
F (d.f.$_n$, d.f.$_d$)	2.07 (1, 2571)	9.51 (1, 60.5)	3.92 (1, 74)
$P_r >$ F	0.1503	**0.0031**	**0.0513**
Sex x Time			
F (d.f.$_n$, d.f.$_d$)	3.5 (39, 2571)	29.24 (9, 586)	2.33 (8, 493)
$P_r >$ F	**< .0001**	**< .0001**	**0.0183**
Trt x Time			
F (d.f.$_n$, d.f.$_d$)	8.92 (39, 2571)	48.69 (9, 586)	12.55 (8, 503)
$P_r >$ F	**< .0001**	**< .0001**	**< .0001**
Trt x Sex x Time			
F (d.f.$_n$, d.f.$_d$)	2.45 (39, 2571)	4.24 (9, 586)	2.43 (8, 489)
$P_r >$ F	**< .0001**	**< .0001**	**0.0139**

Table S2. Parameter estimates and repeated-measures mixed linear model analysis of physiological function: log$_{10}$-transformed natural antibodies and lysis, logit-transformed bactericidal competence, log$_{10}$-transformed corticosterone and square root-transformed heterophil-lymphocyte ratios. Treatment is normal or low early-life diet, sex is male or female, and time is 15 weeks (age where low-diet animals were switched to the normal-diet) and 30 weeks of age. Significant effects (P<0.05) are in bold.

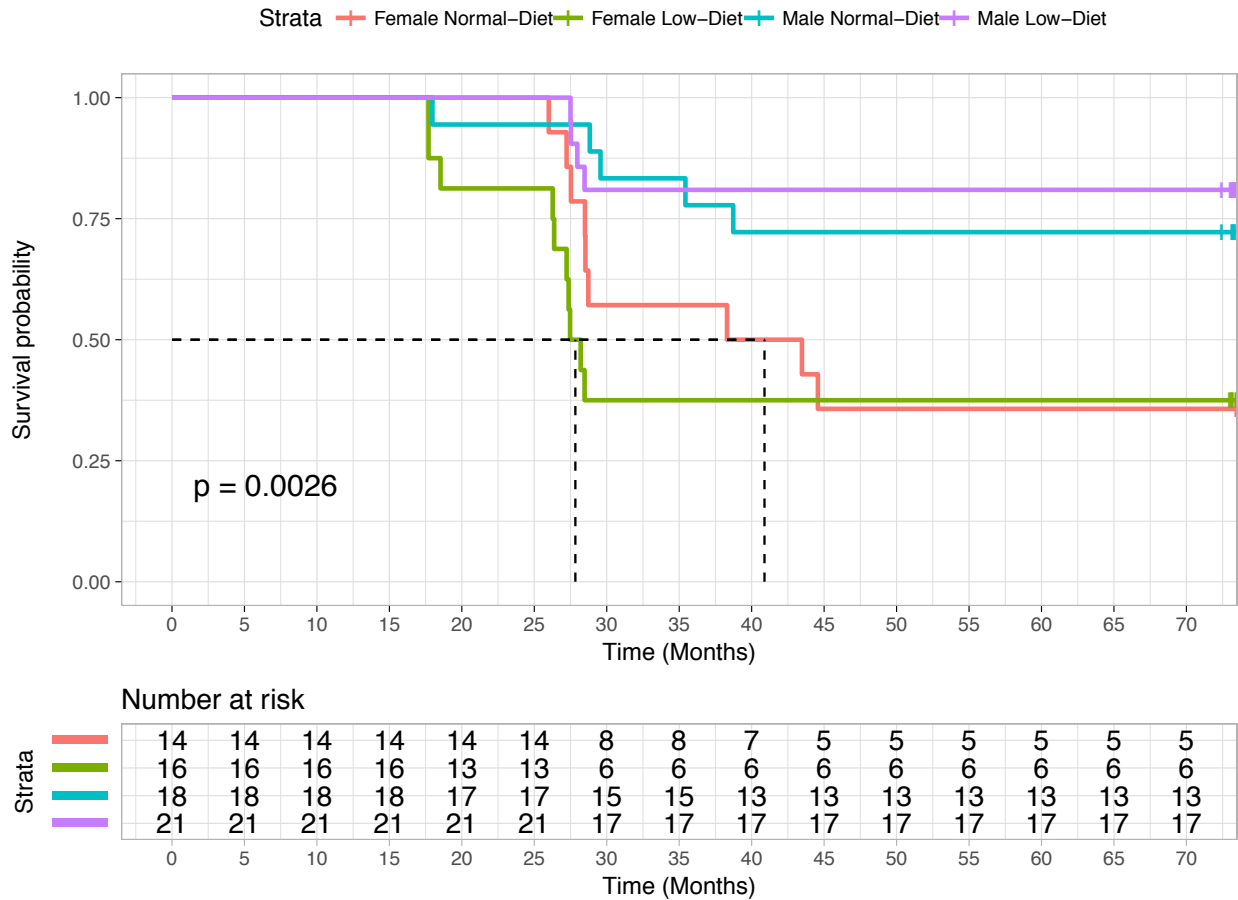|	NAbs	Lysis	BK	CORT	H:L
Condition					
F (d.f.$_n$, d.f.$_d$)	0.04 (1, 123)	0.01 (1, 123)	1.55 (1, 91.9)	0.44 (1, 76.3)	1.65 (1, 120)
P_r > F	0.8517	0.9432	0.2170	0.5115	0.2013
Treatment					
Estimate	0.02	0.02	0.58	-0.20	0.02
F (d.f.$_n$, d.f.$_d$)	1.74 (1, 61.3)	2.78 (1, 62.5)	4.76 (1, 56.2)	4.83 (1, 63.6)	2.55 (1, 68.1)
P_r > F	0.1918	0.0100	**0.0333**	**0.0317**	0.1150
Sex					
Estimate	-0.007	0.002	0.30	-0.05	0.04
F (d.f.$_n$, d.f.$_d$)	0.20 (1, 71.5)	0.02 (1, 71.8)	1.13 (1, 55.4)	0.30 (1, 68.2)	12.21 (1, 74.1)
P_r > F	0.6543	0.8923	0.2921	0.5863	**0.0008**
Time					
Estimate	0.05	0.06	-0.55	0.32	-0.04
F (d.f.$_n$, d.f.$_d$)	18.26 (1, 64.3)	24.79 (1, 64.9)	4.36 (1, 55.7)	15.1 (1, 56.7)	21.64 (1, 68.3)
P_r > F	**< .0001**	**< .0001**	**0.0415**	**0.0003**	**< .0001**
Trt x Time					
F (d.f.$_n$, d.f.$_d$)	6.30 (1, 65.4)	4.84 (1, 66.1)	5.95 (1, 58)	4.72 (1, 57.1)	1.67 (1, 69.9)
P_r > F	**0.0145**	**0.0314**	**0.0178**	**0.0340**	0.2007
Trt x Sex					
F (d.f.$_n$, d.f.$_d$)	1.17 (1, 58.5)	1.01 (1, 59.2)	0.00 (1, 48.5)	1.41 (1, 66.2)	4.68 (1, 66.2)
P_r > F	0.2839	0.3200	0.9502	0.2388	**0.0342**
Sex x Time					
F (d.f.$_n$, d.f.$_d$)	1.58 (1, 62.3)	1.17 (1, 63)	0.06 (1, 54.6)	0.51 (1, 54.9)	0.25 (1, 65.8)
P_r > F	0.2140	0.6837	0.8000	0.4796	0.6168
Trt x Sex x Time					
F (d.f.$_n$, d.f.$_d$)	0.65 (1, 60.1)	0.17 (1, 60.8)	0.99 (1, 54.6)	2.29 (1, 54.9)	1.00 (1, 63.9)
P_r > F	0.4238	0.6837	0.3236	0.1363	0.3208

Figure S1. Survival curves and risk table for each sex by early-life diet treatment group.
Dashed lines denote median survival. Median survival of normal-diet females = 40.9 months
(N=14); low-diet females = 27.8 months (N=16); all males, regardless of early-life diet = 70+
months (normal-diet N=18, low-diet N=21).

Table S1. Parameter estimates and repeated-measures mixed linear model analysis of food consumption as the $\log_{10}$ of grams eaten weekly, body size (SVL in mm) and growth as the change in SVL (ΔSVL). Significant effects (P<0.05) are in bold.

	Food Consumption (Weekly)	SVL	ΔSVL
Birth SVL			
F (d.f.$_n$, d.f.$_d$)	--	51.99 (1, 58.3)	--
$P_r >$ F	--	**< .0001**	--
Preceding SVL			
F (d.f.$_n$, d.f.$_d$)	--	--	0.01 (1, 145)
$P_r >$ F	--	--	0.9083
Food Consumed			
F (d.f.$_n$, d.f.$_d$)	--	--	78.96 (1, 323)
$P_r >$ F	--	--	**< .0001**
Treatment			
Estimate	0.12	0.0806	0.0532
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F (d.f.$_n$, d.f.$_d$)	67.65 (1, 2571)	52.16 (1, 63.1)	5.29 (1, 85.6)
$P_r >$ F	**< .0001**	**< .0001**	**0.0239**
Time			
F (d.f.$_n$, d.f.$_d$)	31.77 (39, 2571)	2364.23 (9, 586)	35.13 (8, 491)
$P_r >$ F	**< .0001**	**< .0001**	**< .0001**
Sex x Trt			
F (d.f.$_n$, d.f.$_d$)	2.07 (1, 2571)	9.51 (1, 60.5)	3.92 (1, 74)
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Trt x Time			
F (d.f.$_n$, d.f.$_d$)	8.92 (39, 2571)	48.69 (9, 586)	12.55 (8, 503)
$P_r >$ F	**< .0001**	**< .0001**	**< .0001**
Trt x Sex x Time			
F (d.f.$_n$, d.f.$_d$)	2.45 (39, 2571)	4.24 (9, 586)	2.43 (8, 489)
$P_r >$ F	**< .0001**	**< .0001**	**0.0139**

Table S2. Parameter estimates and repeated-measures mixed linear model analysis of physiological function: log$_{10}$-transformed natural antibodies and lysis, logit-transformed bactericidal competence, log$_{10}$-transformed corticosterone and square root-transformed heterophil-lymphocyte ratios. Treatment is normal or low early-life diet, sex is male or female, and time is 15 weeks (age where low-diet animals were switched to the normal-diet) and 30 weeks of age. Significant effects (P<0.05) are in bold.

		NAbs	Lysis	BK	CORT	H:L
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	F (d.f.$_n$, d.f.$_d$)	0.04 (1, 123)	0.01 (1, 123)	1.55 (1, 91.9)	0.44 (1, 76.3)	1.65 (1, 120)
	P_r > F	0.8517	0.9432	0.2170	0.5115	0.2013
Treatment						
	Estimate	0.02	0.02	0.58	-0.20	0.02
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	P_r > F	0.1918	0.0100	**0.0333**	**0.0317**	0.1150
Sex						
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	P_r > F	0.6543	0.8923	0.2921	0.5863	**0.0008**
Time						
	Estimate	0.05	0.06	-0.55	0.32	-0.04
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	P_r > F	**< .0001**	**< .0001**	**0.0415**	**0.0003**	**< .0001**
Trt x Time						
	F (d.f.$_n$, d.f.$_d$)	6.30 (1, 65.4)	4.84 (1, 66.1)	5.95 (1, 58)	4.72 (1, 57.1)	1.67 (1, 69.9)
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	F (d.f.$_n$, d.f.$_d$)	1.58 (1, 62.3)	1.17 (1, 63)	0.06 (1, 54.6)	0.51 (1, 54.9)	0.25 (1, 65.8)
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