Environmental causes and transgenerational consequences of ecdysteroid hormone provisioning in *Acheta domesticus*

Katherine C. Crocker*, Mark D. Hunter

1105 North University Ave, Kraus Natural Sciences Building, Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109–1085, USA

**A R T I C L E   I N F O**

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**A B S T R A C T**

An animal’s phenotype may be shaped by its genes, but also reflects its own environment and often that of its parents. Nongenetic parental effects are often mediated by steroid hormones, and operate between parents and offspring through mechanisms that are well described in vertebrate and model systems. However, less is understood about the strength and frequency of hormone mediated nongenetic parental effects across more than one generation of descendants, and in nonmodel systems. Here we show that the concentration of active ecdysteroid hormones provided by a female house cricket (*Acheta domesticus*) affects the growth rate of her offspring. We also reveal that variation in the active ecdysteroid hormones provided by a female house cricket to her eggs derives primarily from the quality of nutrition available to her maternal grandmother, regardless of genetic background. This finding is in stark contrast to previous work that documents a decline in the strength of environmentally based parental effects with each passing generation. Strong grandparental effects may be adaptive under predictable, cyclical changes in the environment. Our results also suggest that hormone-mediated grand-maternal effects represent an important potential mechanism by which organisms can respond to environmental variability, and that further study of hormone-mediated carryover effects in this context could be profitable.

1. Background

An animal’s phenotype may be shaped by its genes, but also reflects its own environment and often that of its parents (Hargitai et al., 2009; Holekamp et al., 1996). Numerous studies have addressed the mechanisms and fitness consequences of nongenetic parental effects (reviewed in Groothuis et al., 2005; Mar et al., 1999; Mousseau and Fox, 1998; Weaver et al., 2004). Nongenetic parental effects are often mediated by hormones in diverse taxa, either directly through variable hormone provisioning (barn swallows, *Hirundo rustica*, cattle egrets, *Bubulcus ibis*, and spotted hyaenas, *Crocuta crocuta* (Dloniak et al., 2006; Saino et al., 2005; Schwabl et al., 1997)) or indirectly through parenting behavior (*Rodentia* species, (Champagne and Curley, 2012; Craft et al., 2009; Parent et al., 2005). Examples of direct effects (variable hormone provisioning to the offspring) include female red squirrels that respond to high social densities by increasing their circulating concentrations of glucocorticoids, which adaptively increases offspring growth rate (Dantzer et al., 2013). Likewise, many avian species increase the concentration of androgens provided to eggs under stressful conditions (Groothuis et al., 2005; Muller and Groothuis, 2013). Additionally, the quality of parental care can affect juvenile offspring development (Capodeanu-Nagler et al., 2016) which can indirectly alter adult reproductive and predator avoidance behaviors (Craft et al., 2009; Stein and Bell, 2014), often via steroid hormone pathways (Monk et al., 2012).

The quality of nutrition provided during parental care can also influence the subsequent development of offspring and their performance as adults. For example, the quality of nutrition that zebra finch (*Taeniopygia guttata*) hatchlings receive from their parents affects both their growth rate and their size at adulthood, with better-nourished hatchlings growing more quickly, into larger adults, than their undernourished counterparts (Boag, 1987), which in turn influences their attractiveness as mates (Jones et al., 2001). Notably, the quality of diet available to a hatching zebra finch can also alter the phenotype of its own offspring, regardless of the diet quality it provides to those offspring (Krause and Naguib, 2014). In other words, nutritionally mediated parental effects can cross multiple generations to affect offspring phenotype, with important ecological and evolutionary consequences (e.g. number, quality and survival of offspring) (Mousseau and Fox, 1998; Rossiter, 1994). Nongenetic parental effects, such as the ones described above, are commonly referred to as ‘developmental programming’ (Harris and Seckl, 2011), and while such effects can be

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* Corresponding author.
E-mail address: crockerk@umich.edu (K.C. Crocker).

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adaptive for offspring (Dantzer et al., 2013), they may also be deleterious (Braun et al., 2013).

To date, most work examining transgenerational effects—both genetic and nongenetic—has focused on the relationship between parents and offspring. Much less is understood about the strength of nongenetic effects across more than one generation of descendants. Humans provide an important example of a nongenetic effect that spans more than two generations: individuals who survive extreme physiological and emotional stress have grandchildren who may be more vulnerable to the effects of stress (Yehuda et al., 2016), and more susceptible to some medical conditions (Bygren et al., 2014; Voland and Beise, 2002). A few studies have observed nongenetic grand-parental effects in non-mammalian taxa. Notably, Magiafoglou and Hoffmann found that Drosophila serrata Malloch offspring have different responses to cold shock based on which generation, and whether maternal or paternal line, were exposed to cold (Magiafoglou and Hoffmann, 2003). Specifically, the viability of daughters and sons decreased in response to maternal or paternal cold-shock, respectively, and all offspring developed more quickly if either their mother or their maternal grandmother experienced cold shock (Magiafoglou and Hoffmann, 2003). Similarly, studies of Gasterosteus aculeatus, the three-spine stickleback, have shown that both grand-parental and parental environment can substantially impact the phenotype of their progeny by altering development rate and reproductive success of their offspring and grand-offspring differentially according to environmental stress (Shama and Wegner, 2014). Though not well understood, the mechanisms by which such carryover effects operate are sensitive to both the ancestral generation (parent or grandparent) and line (maternal or paternal) that experienced environmental perturbation (Magiafoglou and Hoffmann, 2003; Shama and Wegner, 2014). Epigenetic modification of the genome (Champagne, 2013) is one generally accepted mechanism of transferring carryover effects in vertebrates, and may act via steroid hormone (SH) signaling pathways (Veeneema, 2012). However, carryover effects are not limited to taxa that have DNA methylation (Takayama et al., 2014), nor is hormone facilitate DNA methylation the only mechanism by which carryover effects can occur. For example, sperm content may differ based on female phenotype, and maternal small mRNAs can be transferred to embryos (Babenko et al., 2015). Beyond developmental temperature, nutritional restriction has been shown to affect more than one generation of offspring (Carone et al., 2010; Lillycrop et al., 2005; Lillycrop et al., 2008). Thus, to fully understand both the mechanisms and the relevance of transgenerational nongenetic effects for organisms in the natural world, we need to investigate the relative strength of such effects across more than two generations, and in taxa extending beyond popular model systems (Beemelmanns and Roth, 2016, 2017; Hales et al., 2017).

To elucidate potential hormonal mechanisms underlying transgenerational nongenetic effects in nonmodel invertebrate systems, we investigated the strength and mechanistic basis of non-genetic parental effects in Acheta domesticus (the house cricket). Like vertebrate species, arthropods use steroid hormones (SH) to facilitate early development and life stage transitions: ecdysteroid hormones (ESH) are ubiquitous in invertebrate species and control embryogenesis, orchestrate cell differentiation, and are crucial for molting (Festucci-Buselli et al., 2008). In our first experiment, we verified the causative effect of maternally provisioned ESH on offspring phenotype by treating A. domesticus eggs with exogenous ESH and measuring the development rates and mature size of hatching from those eggs. In our second experiment, we assessed the strength of nongenetic effects on variation in hormone provisioning across multiple generations of offspring. Specifically, we used a factorial design to investigate the relative strength of maternal genetic and nutritional environment (high-, intermediate-, and low-quality diets) on hormone provisioning to eggs across three generations. In our analyses, we distinguish nonadditive effects from additive effects between factors by the presence or absence, respectively, of interaction terms in our models. In other words, a lack of interaction terms suggests that additive effects are present in the model, whereas the presence of an interaction term indicates the obverse.

We predicted that female crickets would provision their eggs with ESH in proportion to the quality of nutrition available to them (Mar et al., 1999; Weaver et al., 2004), and that daughters of females that experienced low nutrition would provide less ESH to their eggs than would daughters of females that experienced high nutrition (Mousseau and Dingle, 1991; Zehnder and Hunter, 2007). We predicted that environmental effects on ESH provisioning would be strongest between a mother and her offspring, and decrease in strength after additional generations (Mousseau and Dingle, 1991; Zehnder and Hunter, 2007) because of direct endocrine contact between a mother and her eggs. We predicted that the amount of ESH provisioned to eggs would be heritable (that is, detectable by regression analysis of mother and daughter provisioning levels of ESH) (Kirkpatrick and Lande, 1989). Finally, we predicted that first- and second-generation effects of diet quality would be stronger than any effects of genetic line on ESH provisioning, because plastic responses to environmental quality are crucial to reproductive success (Hahn and MacDougall-Shackleton, 2008). Overall, our predictions were in line with a balanced view of both genetic (heritable) and non-genetic parental effects on hormone provisioning, under an expectation of declining importance of non-Mendelian effects across multiple generations.

2. Methods

2.1. Experiment 1: Disentangling causation and correlation by applying exogenous ESH to eggs

We ordered 1000 Acheta domesticus juveniles from reptilefood.com and raised them to maturity in a communal bin on a 10:14 dark:light regime. They were provided with food ad libitum in the form of ground Harlan Teklad Rodent Diet 8604 (Rodent Diet) and water in cotton-plugged 50 mL conical tubes. Once crickets were sexually receptive (estimated both auditorily by the sound of courtship song and visually by the presence of wings on mature adults), we provided a shallow (2° deep, 6° × 6° square) dish of damp sand overnight to the colony. We collected eggs deposited into the sand by swirling the sand through excess water, and then decanting all eggs to collect them by filtration. We counted, but did not weigh or measure eggs, as egg size did not vary in a subset of eggs that we both measured and weighed.

After collection, nine groups of 100 eggs were randomly chosen from the > 20,000 eggs produced by the colony. We stored each group of 100 eggs in individual 2 mL microcentrifuge tubes for the following procedure. Eggs were either untreated and placed on a damp cotton pad in one of four Petri dishes for incubation (Control), or soaked in 5% hypochlorite solution for three minutes to dechorionate them in order to allow penetration by hormone treatment solutions (below) (Kidokoro et al., 2006). After dechorination, eggs were rinsed three times with room-temperature distilled water, and either placed on a damp cotton
pad in one of four Petri dishes for incubation (Dechorionation Control), or soaked for 24 h at 30 °C in a 1.0 M Phosphate Buffered Saline (PBS) solution with varying concentrations of active ESH (20-hydroxycyecdysone (Sigma Aldrich, St. Louis, MO, USA)) (0, 0.1, 0.5, 1, 5, 10, 50 μM concentrations) (Kidokoro et al., 2006). We chose these concentrations for ESH treatment because a preliminary study showed that a 1 mM solution was sufficiently concentrated to kill eggs. After 24 h, eggs were rinsed with room-temperature distilled water, and each treatment was divided into four equal groups. Each group was placed on a damp cotton pad in a Petri dish for incubation, and then incubated at 30 °C.

Each day, Petri dishes were checked for hatchlings; any hatchlings were recorded and housed individually with food and water in a Petri dish. All hatchlings were photographed above a millimeter-increment ruler every two weeks, and their pronotum widths were measured to compute growth rate (Adobe PhotoShop 7.0). The latency of each egg to hatch and of each hatchling to mature were recorded in a whole ruler every two weeks, and their pronotum widths were measured to compute growth rate (Adobe PhotoShop 7.0). The latency of each egg to hatch and of each hatchling to mature were recorded in a whole

2.2. Experiment 2: Evaluating transgenerational effects of nutritional quality on egg provisioning

2.2.1. Raising the crickets

2.2.1.1. General methods for all generations. For our second experiment, all crickets (sample sizes for all generations are provided in following sections) were raised in a common environment (10:14 dark:light, humidity 60–90%), on one of three diet qualities. Food (in open dishes) and water (in cotton-plugged conical tubes) were available ad libitum. High-quality diet was 100% ground Rodent Diet; intermediate- and low-quality diets were the same ground diet, cut with 15% and 30% w/w Alphacel Non-Nutritive Cellulose. A preliminary study showed that a 25% decrease in nutrition was sufficient to generate difference in egg production, but usually insufficient to induce sexually cannibalistic behavior in females. Thus, we chose these diet compositions to be similar to the preliminary study, but chose a slightly more extreme treatment as our low-quality diet in order to maintain straightforward calculations for our diet formulations to reduce the likelihood of experimenter error during the nine month experiment (decreases of 15% being more straightforward than decreases of 12.5%). All diets were treated with 1% propionic acid to control mold growth.

All crickets were raised communally during early development, and separated either upon reaching maturity (but before either sex became sexually receptive) or at six weeks of age, whichever came first. Separated crickets were placed individually in 12-oz perforated deli cups with the applicable diet and a cotton-plugged 5-dram water vial. Four to ten days after maturity, mating pairs were formed using females raised in the experiment, and males obtained by mail from Fluker’s Farms (Port Allen, Louisiana, USA). Males were all fed a high-quality diet, and were virgins between four and ten days after their imaginal molt at the time that they were first paired with a female. For each mating pair, individuals were housed separately for all but between two to three hours at the beginning of the dark period of their light cycle every day. All mating pairs were consistent (the same two individuals), but separated for most of the day because, in previous experiments, females on low-quality diets cannibalized their mates at a low but problematic frequency (approximately 30% cannibalism in the first 36 h after pairing, Crocker, unpublished data). We separated pairs regardless of their diet quality to control for any effects of separation on egg production, hormone provisioning, or mating latency. Because all males experienced the same environment, this experiment will not allow us to detect paternal effects or compare the relative strength of paternal and maternal transgenerational effects. However, any differences observed between any treatment group can likely be ascribed to maternal effects.

All females were provided with a 15-mL dish of damp, coarse sand in which to lay their eggs; we replaced these dishes every 24 h. We collected the eggs as described in experiment 1 methods, above. Daily clutches of eggs ranged in size from 1 to 300 eggs. Although females produced multiple clutches, preliminary data show that even under extreme dietary stress, female provisioning of ESH to eggs does not vary throughout her lifetime (Table 7S); eggs do not vary in size (Furneaux et al., 1969). After collection, we counted the eggs, stored half of the daily clutch at −20 °C under ethanol, and incubated the other half in Petri dishes at 27.5 °C (Fig. 1) (a lower temperature was used to
incubate all eggs in experiment 2, because of an incubator malfunction at the beginning of the experiment). After each pair had produced 60 eggs (30 incubated, 30 frozen), all subsequent eggs produced were counted and frozen. For the incubated portion of each daily clutch, the number of incubated eggs was divided into three equal-sized groups, which we distributed among three Petri dishes to control for the effect of any individual dish on incubation. Each incubated Petri dish contained a damp cotton pad to prevent eggs from desiccating during incubation. All Petri dishes were checked daily for hatchlings, and the cotton dampened as necessary. If any mold began growing on the cotton, eggs were removed to a new cotton pad using a fine paintbrush.

We checked all incubated Petri dishes each day for hatchlings. Those that we found were divided into three equally sized groups (one group per diet, Fig. 1) to control for any interaction effects of hatch latency or incubation dish with diet quality. We raised all hatchlings (N = 20–30 per pair) with any same-age clutch-mates sharing their diet quality until they were six weeks old, and then raised them individually in 12-oz perforated deli cups to prevent extra-pair mating at maturity.

2.2.1.2. P generation (grandmothers). We collected eggs laid by the colony of 1000 crickets purchased from reptilefood.com (as above), and raised ~200 hatchlings per bin in three communal 14-liter bins (one bin each for high-, intermediate-, and low-quality diets). Each day, we checked all bins for mature individuals, which we separated into individual deli cups before either sex was sexually receptive. For this generation, we formed three mating pairs per diet quality for a total of nine mating pairs (Fig. 1).

2.2.1.3. F1 generation (mothers). From each of the nine pairs of the P generation, we raised offspring (N = 20–30 per pair) on each of the three diet qualities (N = 6–10 offspring per diet per pair). We checked all crickets (~250) daily for maturity. Between four and ten days after maturity, two females per diet quality per mother (for a total of six daughters per P-generation female) were paired with a virgin male (obtained from Fluker’s Farms) (Fig. 1). Daily clutches from this generation were divided into incubated and frozen subsets as described.

2.2.1.4. F2 generation (granddaughters). From each of 53 pairs of the F1 generation, we raised offspring (N = 20–30 per pair) on each of three diet qualities (N = 6–10 offspring per diet quality per F1 generation pair) (Fig. 1). We checked all crickets (~1400) daily for maturity and estimated their growth rates by measuring pronotum width, a structural measurement that is a proxy for body size (Kelly et al. 2014). Specifically, we photographed all crickets 2, 4, and 6 weeks after hatching above a mm-diamonded ruler and used Adobe Photoshop 6.0 to quantify the width of the pronotum (as above). Between four and ten days after maturity, one female per diet quality per mother (for a total of three daughters per F1-generation female) were paired with a virgin male (obtained from Fluker’s Farms). Daily clutches from this generation were all stored at ~20 °C under ethanol for analysis; none was incubated (Fig. 1).

2.3. Analyzing hormone content

2.3.1. Extraction

To extract ecdysteroid hormones (ESH) from eggs, we combined and modified existing extraction methods developed to extract ESH from whole insects, eggs, adult tissue, and larvae (Brent and Dolezal, 2009; Dinan and Rees, 1981; Warren et al., 2006). Specifically, we ground 30 eggs per female in a 10x75mm glass culture tube with 0.5 mL cold methanol (MeOH) using a glass stirring rod. We then centrifuged at 8000 rpm for 5–8 min and collected the supernatant, repeating this process three times as described by Brent and Dolezal (Brent and Dolezal, 2009). Discarding the pellet, we dried the pooled supernatant under vacuum using a SpeedVac, and redisolved in 1.5 mL of 0.5 M Tris/HCl Buffer (for details, see (Dinan and Rees, 1981)).

ESH in insect eggs occurs in both active (“free”) and inactive (“conjugated”) forms (Dinan and Rees, 1981; Whiting and Dinan, 1988). Briefly, free ESH are endocrinologically active, and are responsible for driving embryogenesis and cell differentiation; conjugated ESH are endocrinologically inactive, but can be activated by embryos during development (reviewed in (Smagghe, 2009)). To determine the masses of both free and conjugated ESH per egg, we analyzed each sample twice. To do this, we divided the resolved ESH extract into two aliquots: an untreated 1.0 mL aliquot and an 0.5 mL aliquot which we incubated with esterase to release conjugated ESH molecules. To the 0.5 mL fraction of the extract, we added 0.5 mL solution of Porcine Liver Esterase (PLE; Sigma Aldrich, activity ≥15 units/mg). We used between 1 and 2 mg PLE in 14 mL 0.5 M Tris/HCl Buffer for 25 samples: each sample was therefore reacted with between 0.6 and 1.2 units of enzymatic activity, which are sufficient to catalytically release 0.3–0.6 mg ESH per minute (Lopez et al., 2010). As this is sufficient to release at least eight orders of magnitude more conjugated ESH than the maximum concentration previously observed in eggs of this species (Whiting and Dinan, 1989), this variability in the concentration of PLE per sample was deemed unlikely to influence the concentrations of ESH we subsequently measured. All samples (with and without PLE) were then incubated overnight at 35 °C to hydrolyze ester bonds between ESH molecules and glycolipidprotein storage molecules (Dinan and Rees, 1981). After incubation, we purified samples using a gravity-flow solid phase extraction (SPE) protocol. We used 100 mg bed weight, 3 mL Isolute C-18 SPE columns, and conditioned the columns with 3 mL pure MeOH and 3 mL Milli-Q water before loading each sample using a Pasteur Pipet. Columns were then washed with 3 mL Milli-Q-water, 2.8 mL 15% MeOH in Milli-Q water, and eluted with 2.8 mL 60% MeOH in Milli-Q water into 12 × 75 mm disposable glass culture tubes.

The 60% MeOH fractions were then dried at 40 °C under vacuum using a ThermoSavant SPD111V SpeedVac, and redisolved in 176 µL EIA Buffer solution (Cayman Chemical product 400060). All samples were quantified using an Enzyme Immunoassay (ELA, Cayman Chemical products 400006, 482202, 482200, 16145, 400050, 400062, and 400035), using 50 µL of each sample per well, and assaying each sample in triplicate as directed by Cayman Chemical product inserts (validation information of this Enzyme Immunoassay for quantifying ESH extracts from cricket eggs is available in supplementary Tables 1S–3S, Fig. 1S). To minimize the impact of inter-assay error on our results, for each family, we assayed all free ESH samples on a single plate, and all conjugated ESH samples on an additional plate. Because each family is limited to a single plate for each assay, it necessarily confounds family line and plate identity. Therefore, if there is large inter-plate variation in the assay, it might obscure any effects of family line on our results. We note that (a) inter-plate variation in the assay is generally small (see validation in supplementary materials), and (b) we used family line as a random effect in our statistical models (below). The latter ensures that any variance introduced by a particular family-plate combination is accounted for when we quantify the effects of our diet treatments on hormone provisioning.

All plates were read at 75 and 90 min, per Cayman Chemical product insert instructions, using a tunable plate reader set to 415 nm absorbance. All samples with a %CV greater than 10 were discarded to be re-assayed; standard curves were interpolated using a four-point logistic fit analysis using GraphPad Prism software (average intra- and inter-assay %CVs are each 7%; Table 3S). The linear-approximate portion of the curves was identified using the method developed by Sebaugh and McCray (2003), and all samples falling outside those limits were discarded to be re-assayed.
3. Statistical analyses

3.1. Experiment 1

3.1.1. Relationships between exogenous ESH applied to eggs and hatchling phenotype

The results from Experiment 1 provide replicate independent response (phenotype) values for each concentration of ESH that we applied. Such data can be analyzed by either regression or by ANOVA approaches (Sokal and Rohlf, 1995); we therefore analyzed data both ways. Additionally, when using regression approaches, we used each response value as an independent replicate (least conservative approach) but we also analyzed averages of those replicates, weighted by the inverse of the variance (most conservative approach) (Sokal and Rohlf 1995). Plotting of the data revealed evidence of a quadratic trend in hatchling phenotype (the latency of each egg to hatch, and of each hatchling to mature, as well as pronotum width three weeks after hatching). Exogenous ESH treatment was the independent variable in all models. Analyses were run using SAS (9.4). However, when using regression approaches, we used each point as an independent replicate. Such data can be analyzed by either regression or ANOVA approaches (Sokal and Rohlf, 1995); we therefore analyzed data both ways. Additionally, when using regression approaches, we used each response value as an independent replicate (least conservative approach) but we also analyzed averages of those replicates, weighted by the inverse of the variance (most conservative approach) (Sokal and Rohlf 1995). Plotting of the data revealed evidence of a quadratic trend in hatchling phenotype (the latency of each egg to hatch, and of each hatchling to mature, as well as pronotum width three weeks after hatching). Exogenous ESH treatment was the independent variable in all models. Analyses were run using SAS (9.4). For ANOVA analyses, data were checked for normality using the Shapiro-Wilk test, and log-transformed as necessary.

3.2. Experiment 2

To determine whether ESH provisioning reflected maternal or grand-maternal environment, we calculated mass of free, conjugated, and total ESH per egg for eggs produced by all three generations. Free ESH per egg was determined by analyzing the aliquots of each sample not treated with PLE, and dividing by the number of egg equivalents in that fraction of the extract. Total ESH per egg was calculated by analyzing the aliquots of each sample treated with PLE, and dividing by the number of egg equivalents in that fraction of the extract. Conjugated ESH per egg was calculated by subtracting the mass of free ESH from the total ESH value. During the F1 generation, one family line did not produce enough daughters for a balanced sample; this family line was excluded from our statistical analyses. In all models that follow, we used family line as a random effect unless genetic identity or genetic variance was the subject of the hypothesis test (Littell et al., 2002).

3.2.1. Assessing natural variation in ESH provisioning, and its correlation with family line

We used Levene’s test (R 3.2.4, version Very Secure Dishes) to compare the variance in free, conjugated, and total ESH per egg within and among family lines. We included only the eggs of individuals raised on high-quality diet in this analysis to prevent any effects of a ceiling or floor effect (Everitt, 1998). Although there is less variation in the amount of conjugated ESH that is provisioned to eggs (Fig. 6S), measured amounts of conjugated ESH do not overlap the upper limit of detection of the assay (Fig. 1S, Table 2S). We ran ANOVAs and Tukey post-hoc tests (R 3.2.4) to examine whether development rates or size were associated with the following fixed effects: a cricket’s sex, the quantity of hormone present in an individual’s egg during its embryonic development, diet quality, or the quantity of hormone provided by that individual to her own eggs, or affected by the family line (a proxy for genotype). We measured development speed in the form of hatch and maturity latencies, and growth rate in the forms of both change in pronotum width over time and size at maturity. For all F1 and F2 crickets, we defined hatch and maturity latency as the number of days between when the eggs were laid and when they hatched, and the number of days between hatching and the imaginal molt, respectively. We calculated average growth rate (to avoid pseudoreplication) by pooling all hatchlings that were reared in the same Petri dish and were therefore full siblings with identical hatch latencies raised on the same diet. We subtracted their average pronotum width 21 days after hatching from their average pronotum width at maturity. Each cricket’s size at maturity was individually measured and recorded.

3.2.2. Correlating the concentration of endogenous ESH in eggs with offspring development rate

We ran ANOVAs and Tukey post-hoc tests (R 3.2.4) to examine whether development rates or size were associated with the following fixed effects: a cricket’s sex, the quantity of hormone present in an individual’s egg during its embryonic development, diet quality, or the quantity of hormone provided by that individual to her own eggs, or affected by the family line (a proxy for genotype). We measured development speed in the form of hatch and maturity latencies, and growth rate in the forms of both change in pronotum width over time and size at maturity. For all F1 and F2 crickets, we defined hatch and maturity latency as the number of days between when the eggs were laid and when they hatched, and the number of days between hatching and the imaginal molt, respectively. We calculated average growth rate (to avoid pseudoreplication) by pooling all hatchlings that were reared in the same Petri dish and were therefore full siblings with identical hatch latencies raised on the same diet. We subtracted their average pronotum width 21 days after hatching from their average pronotum width at maturity. Each cricket’s size at maturity was individually measured and recorded.

3.2.3. Examining which factors predict the provisioned concentration of ESH in eggs

We ran nested mixed models (SAS 9.4) using ESH contents (free, total, and conjugated) of eggs, latency (in days) to lay eggs, and number of eggs in a cricket’s first clutch as response variables, and nesting F2 (grand-daughter) within F1 (mother) within P (grandmother) generation individuals. We used diet qualities of all three generations of crickets as fixed effects, and ran analyses using the Satterthwaite approximation of denominator degrees of freedom (Littell et al., 2002). Because family line was a random effect (above), this approach accounted for nested effects of maternal and grandmaternal identity (Littell et al., 2002).

4. Results

4.1. Experiment 1

4.1.1. Application of exogenous free ESH to eggs alters the growth rate of juvenile crickets in a dose-dependent manner

In assessing effects of experimental ESH application on offspring phenotype, ANOVA and regression approaches provided qualitatively similar results. Results of all analyses are provided in Supplementary Material (Tables 4Sa and b). Based on pronotum width, crickets grew most rapidly under intermediate levels of ESH application (quadratic term in regression model of all data points F1,115 = 9.7, p = 0.002). Analyses in which we used either an ANOVA or weighted average approach were consonant with this result, though the weighted average approach indicated a weaker trend (ANOVA: F5,112 = 3.63, p = 0.004; weighted averages F1, 128 = 35.26, p < 0.0001, Fig. 6S and 3S). We also analyzed hatchling survival, hatch latency, and lifetime growth rate and found no additional relationships to exogenous ESH treatment (Table 5S, Figs. 4S and 5S).

4.2. Experiment 2

4.2.1. Natural variation exists in ESH provisioned to eggs of Acheta domesticus; daughters follow a general provisioning template provided by their mothers in provisioning ESH to their eggs

Female crickets varied widely in both free (active) and conjugated (inactive) ecysteroids (ESH) provisioned to eggs. Levene’s test indicated that the provisioning of free ESH varied more than that of conjugated ESH (F1, 128 = 35.26, p < 0.0001, Fig. 6S) (CV for free ESH = 108.3%; for conjugated = 50.5%). We interpret these results as biologically valid, rather than artifacts of a ceiling or floor effect (Everitt, 1998). Although there is less variation in the amount of conjugated ESH that is provisioned to eggs (Fig. 6S), measured amounts of ESH do not overlap the upper limit of detection of the assay (Fig. 1S, Table 2S). Similarly, although the amount of free ESH provisioned to eggs is closer to the lower limit of detection of the assay (Fig. 1S, Table 2S), we would predict a floor effect to reduce, rather than elevate, the measured variance (Fig. 6S).
Levels of hormone provisioning of eggs appear to be heritable. Specifically, daughters provided ESH to eggs in direct proportion to the concentrations of ESH that were provided to them by their mothers (Free: $F_{1,136} = 29.55, p < 0.0001$; Conjugated: $F_{1,136} = 7.21, p = 0.0082$). This apparent heritability in ESH provisioning may reflect other egg traits, such as egg size, which might also be heritable. Although we did not collect data on egg size in our experiment, we note that previous work reports negligible variation in the size of house cricket eggs (Furneaux et al., 1969).

There was no effect of offspring diet (Free: $F_{2,161} = 0.45, p = 0.64$; Conjugated: $F_{2,136} = 0.18, p = 0.84$) nor an interaction between offspring diet and maternal provisioning (Free: $F_{2,136} = 0.1, p = 0.9$; Conjugated: $F_{2,136} = 0.42, p = 0.66$) on offspring provisioning of eggs.

4.2.2. Natural variation in active, but not inactive, ESH provisioned to eggs predicts hatching size during early life

At no time in a cricket’s life was size or growth rate predicted by its family line (a proxy for genotype). Effects of maternal hormone provisioning on offspring phenotype were strongest early in offspring development. Specifically, in two-week-old offspring, concentrations of free (active), but not conjugated (inactive), ESH in eggs correlated positively with pronotum width ($F_{1,120} = 8.07, p = 0.0053$); in 4- and 6-week-old offspring, this effect had disappeared ($F_{1,112} = 0, p = 0.993$; and $F_{1,112} = 0.394, p = 0.5316$ respectively) (Fig. 2). Offspring diet quality was always positively related to offspring pronotum width (week 2: $F_{2,120} = 8.65, p = 0.0003$; week 4: $F_{2,112} = 3.34, p = 0.039$; week 6: $F_{2,112} = 4.668, p = 0.0113$).

Size at maturity was determined by a cricket’s sex, with females larger than males ($F_{1,225} = 19.16, p < 0.0001$). Size at maturity was unaffected by either egg ESH content or diet quality (Free ESH: $F_{1,225} = 1.28, p = 0.26$; Conjugated ESH: $F_{1,225} = 0.160, p = 0.69$; Diet quality: $F_{4,225} = 2.22, p = 0.068$), though crickets that were fed a low quality diet matured less quickly than did those that ate high-quality food ($F_{2,112} = 7.78, p = 0.0006$). Tukey post-hoc test indicated that the intermediate diet quality group did not differ from high- or low-quality groups ($p = 0.187$ and $p = 0.118$ respectively), but that crickets that ate a high-quality diet matured faster than did those that...
ate a low-quality diet (p = 0.0007) (Fig. 7S).

4.2.3. Grand-maternal diet quality predicts the amount of free, but not conjugated, ESH that a cricket provisions to her eggs and affects the number of eggs produced and the latency of egg production.

Of the three generations of individuals for which we varied diet quality, only grand-maternal diet quality affected ESH provisioning in eggs. The diet of a cricket’s grandmother (P generation) strongly predicts the amount of free ESH that her granddaughters will deposit in their eggs in a nonlinear fashion, whereby the intermediate diet quality results in the lowest level of provisioning (F_{2,73} = 5.64, p = 0.0053) (Fig. 3). The number of eggs produced in a cricket’s first clutch (which were among the eggs we analyzed for ESH; the amount of ESH provisioned to eggs remains constant throughout a female’s life, and is not supplemented by her mate (supplementary material)) reflected an interaction between the diet qualities of both a cricket’s mother and grandmother (F_{1,122} = 2.58, p = 0.0404) (Fig. 4). Specifically, F2 crickets whose grandmother (P generation) was raised on a low-quality diet could compensate for the effects of their grandmother’s environment if their mothers (F1) ate either an intermediate- or high-quality diet. However, if their mothers (F1) also experienced a low-quality diet, the F2 generation produced fewer eggs than any other combination of diet qualities across generations (Fig. 4). Grandmothers (P generation) who ate high- and intermediate-quality diets had grand-daughters whose first clutch sizes were similar in size, regardless of their mothers’ (F1) diets (Fig. 4).

The latency of egg production reflected a similar interaction, but between a cricket’s own diet quality and that of her grandmother (F_{3,118} = 2.73, p = 0.0322) (Fig. 8S). In other words, granddaughters (F2 generation) that were raised on a low-quality diet, and whose grandmothers (P generation) were raised on a low-quality diet took more time to lay their eggs than any other combination of diet qualities across generations (F_{3,118} = 2.73, p = 0.032). Maternal diet quality was not significant in any model (F_{2,118} = 1.10, p = 0.336).

5. Discussion

In the current study, we report that (i) varying concentrations of active (free) ESH available to embryos during early development alters offspring phenotype (Fig. 2, Table 4S), and (ii) the concentrations of active ESH provided by a mother to her eggs reflect her lineage and the nutritional environment of both her mother and her grandmother (Figs. 3 & 4). We acknowledge that plate effects might have contributed to the resemblance among relatives, potentially inflating heritability (see Fig. 6S). Because each family line was analyzed on a single plate, family line and plate identity are confounded.

Documenting the long-term consequences of maternal effects across a broad range of taxa is important for our understanding of the ecology and evolution of maternal hormone provisioning across the tree of life. Ecdysteroid hormones (ESH) control embryonic development and regulate molting in insects (Festucci-Buselli et al., 2008). In some insects, they are provisioned in widely varying concentrations: both the causes of this variation, and any long-term effects it may have on offspring phenotype, remain largely unexplored (but see (Oostra et al., 2014)).

Here we reveal that variation in the active ESH provided by a mother to her eggs derives strongly from the quality of nutrition available to her maternal grandmother (Fig. 3). This strong grandparental effect in our study is consistent with previous findings of studies examining nongenetic grandparental effects in vertebrates. For instance, Zambrano and colleagues reported that restricting the dietary protein available to pregnant female albino Winstar rats adversely affected the metabolisms of F2 progeny (grand-offspring) (Zambrano et al., 2005). Although in our experiment we restricted general nutrition, rather than protein alone, we interpret the resulting effects on F2 progeny to be similar. Similar findings are not limited to terrestrial vertebrates: work on guppies (Poecilia reticulata) has shown that variation in temperature (warm or cool) results in sex-dependent adaptive phenotypic matching in F2, but not F1 progeny (Le Roy et al., 2017), and studies of Daphnia ambigua show strong effects of embryonic predator exposure on F2 progeny (Walsh et al., 2015). However, D. ambigua also exhibits opposite effects within generations as between generations of progeny, indicating that canalization of transgenerational effects across multiple generations of progeny is unlikely (Walsh et al., 2015).

In spite of the increasing general appreciation for the power and ubiquity of transgenerational effects, extensive previous work documents a decline in the strength of environmentally based parental effects with each passing generation (Mousseau and Dingle, 1991; Mousseau and Fox, 1998; Zehnder and Hunter, 2007). The longer the time period between the environmental cue (in the case of our experiment, nutrition) and its effect on phenotype, the greater the likelihood of a mismatch between organism phenotype and current environmental conditions (Turchin, 1990). As a consequence, time lags in the expression of non-genetic effects can destabilize population dynamics, facilitating wide fluctuations in animal numbers (Bentont et al., 2005; Ginzburg and Taneyhill, 1994; Rossiter, 1994; Rossiter, 1991).

It is necessary to reconcile these broadly substantiated ecological observations of diminishing environmental signal over multiple generations with observations of powerful, delayed transgenerational effects. We suggest that both may work in concert in the presence of cyclical variation in environmental cues, which reduce the danger of a
mismatch between progeny phenotype and environment, while in-
centivizing the evolution of powerful transgenerational effects. For in-
stance, if environmental variation is associated with cyclical change
(for example, seasonality), transgenerational effects may then be an
artifact of (in the case of house crickets) ancestral trivoltinism (that is,
an effect of descending from ancestors whose species reproduced
roughly three times per year). Acheta domestica has a generation time
of approximately three and a half months (thus, three to four genera-
tions per year), are thought to have evolved in southwest Asia (Ghouri,
1961). In this part of the world, the climate is strongly seasonal and
thus could pose predictable, but distinct, environmental challenges to
A. domestica, which breed year-round (Peel et al., 2007). If the strength
of carryover effects is indeed an adaptive mechanism for coping with a
predictably varying environment, we suggest that carryover effects
would be weaker in univoltine species, each generation of which will
likely face a similar environment.

Surprisingly, the mass of active ESH per egg is more strongly re-
duced in the eggs of granddaughter (F2) crickets descended from
grandmothers reared on intermediate quality diet than it is in the eggs
of F2 crickets descended from grandmothers (P) reared on low quality
diet, regardless of the diet quality of the intervening F1 generation. This
result shows that although F2 descendants respond to grand-maternal
dietary stress by altering their provisioning behavior, the effects of F2,
F1, and P generations are not additive in the sense of accumulating to
surpass a given threshold. Rather, granddaughters seem to pay more
attention to nutritional stress experienced by their grandmothers when
provisioning their eggs with ESH.

Generally, we observed that F2 descendants of reduced-quality diet
P individuals lay eggs with less active ESH than do those descended
from high-quality grandmothers. Although F2 descendants provided the
least free ESH to their eggs when descended from P-generation indi-
viduals that ate an intermediate-quality diet, the most striking result is
that F2 descendants of grandmothers that ate a low-quality diet neg-
ogatied an apparent trade-off differently. Specifically, F2 descendants
of grandmothers that ate a low-quality diet reduced the number of eggs
they produced (Fig. 4), but increased their provisioning of ESH to their
eggs (Fig. 3), while F2 descendants of grandmothers that ate an inter-
mediate-quality diet reduced their provisioning of ESH, but not the
number of eggs that they produced. We therefore suggest that a stress
threshold exists between the intermediate- and low-quality groups, the
crossing of which caused the descendants of P generation crickets that
experienced a low-quality diet to alter their reproductive strategy
(Figs. 3, 4). We have interpreted a low-quality diet to constitute an
environmental stressor, in response to which, organisms across the tree
of life have been found to decrease the number of offspring produced,
but increase their investment per offspring unit (Goutte et al., 2010;
King, 1993; Tammaru et al., 1996). In support of this hypothesis, the
number of eggs an F2 cricket produced in its first clutch was reduced if
both her mother and grandmother (F1 and P generations respectively)
also experienced low-quality diets (Fig. 4). This interaction suggests that
female crickets are more strongly influenced by the nutritional envi-
environment of their mothers and grandmothers than by their own
nutritional environment when producing their eggs and provisioning
them with ESH, although a cricket’s own environment affects her la-
tency to lay eggs (Fig. 85). However, whether F2 crickets descended
from grandmothers of varying diet quality produce lower numbers of
eggs in the wild is not known.

Similarly, as reproductive delay can act as an environmental stressor,
we also assessed whether the length of time between a female’s
imaginal molt (when she reaches sexual maturity) and when she com-
menes to oviposit could explain any of the variation we observed in
the concentration of ESH in her eggs. Because there is evidence that the
interval between a female’s imaginal molt is positively correlated with
egg size, we reasoned that an increased egg size could be associated
with increased provisioning of both hormones and nutrition (Cherrill,
2002). Therefore, we asked whether the length of time between when a
female matured and when she laid her first eggs (lay latency) could explain
any of the variation observed in hormone concentration in the
eggs she produced: at least in our study, it could not (Fig. 85).

Nongenetic grand-maternal effects have been noted in multiple taxa
(Beemelmanns and Roth, 2017; Hafer et al., 2011; Magiafoglou and
Hoffmann, 2003; Shama and Wegner, 2014), but the molecular under-
pinnings of these effects are not yet well understood (Takayama
et al., 2014). In several cases, they appear to be facilitated by steroid
hormone signaling (Yehuda et al., 2016), though maternal small RNAs,
and the modification of histones by methylation are also important
mechanisms (Babenko et al., 2015; Curley et al., 2011). In experiment
2, we manipulated diet quality, observed subsequent changes in hor-
moneme provisioning to eggs, and associated those changes with the
phenotypes of offspring and grand-offspring in the generations that
followed. We feel justified in imputing a causal link from diet, through
egg ESH, to phenotype based on the results of experiment 1, in which
exogenous treatment of eggs with ESH had a dose-dependent effect on
the phenotype of hatchlings (Table 4S). In experiment 2, we show that
the mass of ESH available to an egg is positively correlated with the
hatching’s structural size during at least the first two weeks of its life
(Fig. 2). After this time, a hatching from an egg with a low con-
centration of active ESH can transcend its initial disadvantage by eating
a high-quality diet (Fig. 2): neither diet quality nor active ESH con-
centration in an egg affect a cricket’s size at maturity, though crickets
that eat a higher quality diet mature more quickly (Fig. 75).

In general, nongenetic transgenerational effects are predicted to be
highly adaptive, even outcompeting phenotypic plasticity under sto-
chastic conditions (Jablonska et al., 1995). Although such effects may
be developmentally ephemeral, natural selection operates throughout the
time of offspring. As a consequence, even transitory maternal- or
grand-maternal effects may still increase the fitness of genetic lines that
express those effects, if selection pressures are strong during those
critical life stages or transitions. Weathering stressful environments,
and reproducing successfully while stressed, is particularly important
when conditions change rapidly. Steroid hormones mediate both stress
and reproduction across a broad range of taxa (Goutte et al., 2010;
Harris and Seckl, 2011; Ping et al., 2015). Together with the powerful
effects that steroid hormones may have on both mothers and embryos
(Saino et al., 2005; Sheriff et al., 2015), our results imply that further
study of steroid hormones in the context of carryover effects could be
profitable.

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