

**The Past Is Never Dead, It Isn't Even Past: Maternal Environment
Affects Multiple Generations of Offspring via Hormone Provisioning**

by

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**A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Ecology and Evolutionary Biology)
in the University of Michigan
2018**

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DEDICATION

For Pascale, who is the best person I know and will ever meet.

For the rest of my family, especially my dad, all of whom I really can't thank enough.

Mi yette kadi beynguure nden ka nder Senegal, innde nden e yettore nden tinni Aissatou Diallo, Tamba Keita e bido mabbe Mariama Keita, tokara an--mi waawata hay fus onong

alaa.

And in memory of Marina Pepper.

ACKNOWLEDGEMENTS

During the past seven years I have had plenty of opportunities to consider how much I owe to how many. Every time I've tried to write this section of my dissertation, I've been overwhelmed by the amount of support, backup, help, and encouragement I've received. It would be nearly impossible to list everyone here, and to express how much they—and their support—have meant to me. Suffice to say, then, that if you are reading this, you are probably one of those people.

Wiblah.

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ABSTRACT

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 non-vertebrate 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.

In Chapter 2, I report that variation in the ecdysteroid hormones (ESH) provided by female house crickets (*Acheta domesticus*) to their eggs can be robustly, replicably measured using an Enzyme Immunoassay technique.

In Chapter 3, I show that variation in the ESH that female *A. domesticus* deposit into their eggs determines the early development rates of their offspring. I also show that variation in the active forms of ESH provided by a female house cricket to her eggs derives primarily from the quality of nutrition available to her mother and maternal grandmother, regardless of genetic background, age at oviposition, or any contribution of ESH by her mate.

In Chapter 4, I uncover a potential mechanism of generating delayed density dependence by showing that female *A. domesticus* alter their ESH provisioning strategy in response to social density.

In Chapter 5, I demonstrate that these effects are not limited to lab-raised species, but are relevant to and present in at least two species of wild Gryllid (*Gryllus veletis* and *G. pennsylvanicus*).

In sum, work reported here demonstrates the power of variable hormone provisioning by mothers to their offspring. These findings highlight the importance of distinguishing between phenotypic plasticity and genetic effects in observing field and laboratory organisms across time and space.

Chapter I Introduction

The past is never dead. It isn't even past.

- William Faulkner

Parents can influence the phenotype of offspring beyond the genes that they contribute. Non-Mendelian parental effects (hereafter, parental effects) may be a consequence of parenting behavior, parental physiology, the provisioning of resources to offspring, or a combination of these factors (Mousseau and Fox, 1998). For instance, female mice (*Mus musculus*) that experience relatively little interaction with their mothers as pups are less likely to thrive, and are inattentive mothers themselves upon reaching maturity (Champagne, 2013). However, their sisters can become highly attentive mothers if raised by an attentive (but unrelated) female (Champagne, 2013). Female gypsy moths (*Lymantria dispar*) detect increased social densities via altered phytochemicals in the leaves on which they feed, and respond to this physiological effect by epigenetically programming their offspring to become heavier at pupation (Rossiter, 1991).

As powerful as parental effects are in altering the phenotype of offspring, such effects are not always adaptive. In other words, though parental (predominantly maternal) effects may be adaptive for offspring at shorter time scales, they may be detrimental on the scale of the lifespan of offspring (Mousseau & Fox, 1998). The developmental (or fetal) programming hypothesis suggests that offspring will adapt to their fetal environment in order to survive until birth, but that these adaptations can act against an individual's fitness later in life. For example, in Japanese quail (*Coturnix coturnix*), increased glucocorticoid titers (a steroid hormone stress indicator) in mothers is transferred to eggs, reducing the growth rate of juvenile offspring and increasing the sensitivity of adult offspring to environmental stressors (Hayward and Wingfield, 2004). However, if a mother were able to program offspring in a way that increased their fitness for their specific environment, it would dramatically increase her fitness (Jablonka et al., 1995).

This is the case in spotted hyaenas: dominant females have higher lifetime reproductive success, and pass their rank to their daughters (Holekamp et al., 1996).

In order for maternal programming to develop as a reproductive strategy, mothers would require a mechanism for altering offspring phenotype, and a broadly variable environment, which could nevertheless be predicted on some scale (Carlisle, 1982). It is worth noting, however, that an adaptationist perspective is not necessary to support the developmental programming hypothesis. For example, with reference to Japanese quail (above), either the embryos that develop in an environment with high concentrations of glucocorticoids (a) make temporarily adaptive physiological compensations to cope with their endocrine environment (the adaptationist interpretation) or (b) are mechanistically driven to develop in a fashion that is detrimental later in life (the non-adaptationist interpretation). In either case, the developmental programming hypothesis states that an embryo follows the developmental program laid out for it by the combination of its environment and DNA, and that this program may render it ill-adapted to environments that it faces later in life.

Regardless of whether they function adaptively, parental effects impact ecological and evolutionary processes. Ecologically, they can shape animal abundance, developmental rate, and behavior. For example, female cattle egrets (*Bubulcus ibis*) vary the androgens that they provide to eggs, which affects the survival of their offspring: they provide higher concentrations to one egg than to its clutch-mates, enhancing the competitive ability of that hatchling, and decreasing the likelihood of its siblings' survival in years when resources are scarce (Schwabl et al., 1997). Female red squirrels (*Tamiasciurus hudsonicus*), respond to elevated social density by adaptively increasing the stress hormone titers that they provide to offspring, which in turn increases the growth rate (and thus, fitness) of their offspring (Dantzer et al., 2013). Parental effects can also dictate the reproductive behaviors of animals. For instance, female canaries (*Serinus canaria*) preferentially mate with males whose songs do not match that of a step-father (indicating a learned, rather than genetic, preference)(Trosch et al., 2017). Additionally, hybrid

Geospiza finches choose their mates depending on song, which is learned from their father (Grant and Grant, 1997).

Parental response to their environment can even dictate the sex ratio of the next generation. For example, female cockroaches (*Nauphoeta cinerea*), increase the number of male, but not female, offspring that they produce when they encounter few males in their developmental and adult environments (Moore et al., 2001). As a consequence, they increase the fitness of their sons in a female-biased environment. Similarly, female guinea pigs (*Cavea apera* f. *porcellus*) alter the sex ratio of their offspring to produce more daughters than sons in unstable environments (Kempe et al., 2009). In this species, females reach reproductive maturity earlier than males, so a female-biased sex ratio is interpreted to be adaptive in unstable conditions (Kempe et al., 2009). In both cases, the genetic makeup of the subsequent generation is affected, as is the social experience of offspring, which in turn produces its own parental effects.

Parental effects also impact evolution: when natural selection acts on genes via phenotypes that are altered by parental effects, these effects directly influence the survival of family lines, and thus the genetic composition of populations. For instance, exposure to a predator alters the parenting behavior of male threespine stickleback (*Gasterosteus aculeatus*), which in turn produces sons that are less colorful, and less bold (Stein and Bell, 2014). Female sticklebacks have been shown to prefer more brightly colored males (Milinski and Bakker, 1990). Thus, parental effects can be interpreted as having directly influenced the reproductive success of offspring, independent of genotype, via the predation risk experienced by fathers. A mother's social environment has been shown to affect her daughters' likelihood of reproductive success in the spotted hyena (*Crocuta crocuta*). In this species, maternal social position determines which pups are likely to receive more, higher quality food and, later in life, which females will be likely to successfully reproduce (Dloniak et al., 2006; Holekamp et al., 1996). Therefore, relatively ephemeral parental effects can influence the frequency of alleles in a population from one generation to the next.

Further, parental effects have been observed to act across multiple generations. 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 (hereafter “carryover effects”). However, 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 carryover effects in non-mammalian taxa. Notably, Magiafoglou and Hoffmann (2003) 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 decrease in response to maternal or paternal cold-shock, respectively, and all offspring develop more quickly if either their mother or their maternal grandmother experience cold shock (Magiafoglou and Hoffmann, 2003). Similarly, studies of *Gasterosteus aculeatus*, the threespine 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).

With growing appreciation of the widespread nature of parental and carryover effects, and their power to influence ecological and evolutionary interactions, comes increased interest in how they function. 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 occur via steroid hormone (SH) signaling pathways (Veenema, 2012). However, carryover effects are not limited to taxa that have DNA methylation (Takayama et al., 2014), nor is hormone-facilitated 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). Thus, to fully understand 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).

No mechanism of parental effects operates independently of the others, though interactions among different mechanisms are challenging to identify and measure. In work described here, I have chosen to focus on hormone-mediated maternal effects on offspring phenotype. Hormone-mediated maternal effects on offspring life history have been widely studied in vertebrates (Beach, 1974; Burton et al., 2011; Craft et al., 2009; Maestriperi, 1993; Stein and Bell, 2014), particularly in avian systems. In these studies, parental effects are often mediated by steroid hormone (SH) deposition to eggs, most often in the form of androgens and glucocorticoids (Love et al., 2005; McNabb and Wilson, 1997; Rutkowska and Badyaev, 2008; Tschirren et al., 2004). Notably, SH concentrations in eggs can reflect maternal social environment: both the composition and size of social groups affect adult endocrine status and reproductive strategy. For example, female great tits (*Parus major*) allocate higher concentrations of androgens to eggs when breeding at high social densities (Remeš et al., 2011), which increases the growth rate of their sons, but not of their daughters after hatching (Tschirren, 2015). Similarly, female house sparrows (*Passer domesticus*) vary the concentration of androgens that they deposit to their eggs according to their social environment (Mazuc et al., 2003). Their offspring that are exposed to elevated androgen concentrations are more competitive (Strasser and Schwabl, 2004). Although maternal steroid hormones have thus been shown to have adaptive effects on offspring phenotype, as discussed earlier, maladaptive impacts on offspring phenotype have been observed. Indeed, the negative consequences of maternal hormone provisioning for threespine stickleback (*Gasterosteus aculeatus*) embryos are strong enough to act as a selective pressure. Threespine stickleback embryos have evolved cellular machinery that they use to insulate themselves from the detrimental

effects of maternal hormones by removing those molecules from their cells (Paitz et al., 2016). Variable hormone provisioning, and its effects, are thus a product of maternal environment, physiology, and genotype, and its effects likely vary depending on context and species (Bonduriansky and Head, 2007; French et al., 2013).

Maternal hormone provisioning as a mechanism to modify the phenotype of offspring has a relatively well-developed research framework (Groothuis et al., 2005). However, it is a framework that has largely focused on the interaction between a mother and one, rather than many, generations of offspring, and in vertebrate, rather than invertebrate, species. Hormone-mediated maternal effects can bridge more than one generation of offspring and can be affected both by direct provisioning of hormones and by parenting behavior (Champagne, 2008; Champagne and Curley, 2009; Craft et al., 2009; Curley et al., 2011). To understand the extent to which the provisioning of hormones, rather than parental care and behavior can impact generations of descendants, we need to study offspring that are naturally isolated from interacting with their parents behaviorally and endocrinologically. Species that lay eggs that produce precocial offspring, rather than species that interact with their offspring throughout a pregnancy or after they hatch, are ideal for this purpose. In other words, an egg represents a finite, specific maternal investment, rather than an ongoing series of interactions by which a mother can constantly modify her offspring.

Moreover, insects systems provide an important alternative to vertebrate models with which to study hormone-based parental effects. Current ecological (Levins, 1969) and evolutionary (Dobzhansky and Pavlovsky, 1957) theory owes much to studies of insects, but relatively little is known about whether and how insects can program their offspring using hormones. Although insect hormones have been extensively researched in the contexts of biological control and embryonic development (Smagghe, 2009), their effects remain unconnected by a general model of the ontogenetic effects of early and embryonic hormone exposure. Ecdysteroid hormones (ESH), a major invertebrate hormone group, are important for embryonic development, molting, circadian rhythms, and reproduction in insects (Cymborowski, 1994; Deloof et al., 1984; Gabrieli et al., 2014; Sehna, 1989; Spindler et al., 2009). For example, in *Drosophila melanogaster* and *Manduca sexta*,

ESH are crucial for wing imaginal disk formation, without which neither insect would develop functional wing structures (Herboso et al., 2015; Nijhout et al., 2007). In *Drosophila melanogaster*, ESH also facilitate pre-metamorphosis larval light preference behavior (Yamanaka et al., 2013). The effects of ESH are not limited to development: mature female mosquitoes (*Anopheles gambiae*) post-mating behavior is altered allohormonally as a result of her mate transferring ESH to her during mating (Gabrieli et al., 2014).

ESH in insect eggs occurs in both active (“free”) and inactive (“conjugated”) forms (Dinan and Rees, 1981; Whiting, 1988). Free ESH are endocrinologically active, and are responsible for driving embryogenesis and cell differentiation; conjugated ESH are endocrinologically inactivated, but can be activated by embryos during development (reviewed in Smaghe, 2009). Most work on ESH has focused on the immediate mechanistic role of free ESH in model systems such as *Manduca* and *Drosophila*, which are often studied in isolation from the natural world. But there is evidence that ESH can link juvenile environment to adult behavior and to other ecologically relevant traits (Cooper et al., 2003; Oostra et al., 2014).

In this dissertation, I have focused on studying ESH-based parental and carryover effects in crickets. Female house crickets (*Acheta domesticus*) provide highly variable concentrations of ESH to their eggs, which are produced in sufficient quantities to measure. Environmental conditions are well known to affect orthopteran phenotype and fitness, as they affect endocrine status, reproductive decisions, and offspring phenotype in many species within the order (Bertram et al., 2012; Crankshaw, 1979; Tinghitella, 2014; Woodring et al., 1988). *Acheta domesticus* is also amenable to lab rearing, which makes it an expedient organism in which to investigate the long-term effects of variable maternal hormone deposition to eggs. This species has long been used as a model organism for the study of mate choice (Gray, 1997), acoustic signaling (Bertram et al., 2011), and male-male competition. During the first half (~ 5 days) of embryonic development, *A. domesticus* embryos are unable to synthesize ESH themselves and are thus dependent on that provided by their mothers (Dinan, 1997), as without ESH, they

cannot complete embryogenesis (Hoffmann and Gerstenlauer, 1997). The mechanistic effects of ESH on developing embryos are well-studied, but ESH in general are underappreciated in the context of longer-term “organizational” effects: I am aware of only one report of the long-term consequences of variable ESH titers in early life (Oostra et al., 2014).

For work presented here, I am indebted to the orthopteran species *Acheta domesticus* (Chapters 2-4), and *Gryllus veletis* and *G. pennsylvanicus* (Chapter 5). *Acheta domesticus* have a generation time of approximately three and a half months (thus, three to four generations per year), and are thought to have evolved in southwest Asia (Ghoury, 1961). As implied by its common name, scientists in the U.S. know little about the natural history of the house cricket. The two wild *Gryllus* species, on the other hand, are univoltine, native to Michigan and distributed broadly across what is currently the United States and Canada. *G. veletis*, (the spring field cricket) lays eggs in late spring, and hatch approximately 10 days later. Hatchlings develop through several nymphal stages throughout the rest of the summer, and undergo diapause as nymphs in small burrows or in leaf litter over the winter (Alexander and Bigelow, 1960; Cantrall, 1943). In early spring, nymphs emerge and continue their development. They reach sexual maturity in late spring. In contrast, *G. pennsylvanicus* (the fall field cricket) lays eggs in early- to mid-fall. Eggs enter hibernal diapause and hatch in early spring. Hatchlings develop over the summer, reaching sexual maturity in late summer (Alexander and Bigelow, 1960; Cantrall, 1943).

My dissertation research lays the foundation for investigating maternal hormone deposition strategies in insects. First, I validate an enzyme immunoassay capable of measuring very small masses of ESH (Chapter 2). Second, I show that variability in the provisioning of ESH by mothers to their eggs is directly linked to offspring fitness via offspring development rate (Chapter 3). Further, I show that a female cricket’s provisioning strategy reflects not only her genotype, but the environments experienced by multiple generations of her maternal ancestors (Chapter 3). I uncover a potential mechanism of generating delayed density dependence by showing that female crickets

alter their ESH provisioning strategy in response to social density (Chapter 4). Finally, I demonstrate that these effects are not limited to lab-raised species, but are relevant to and present in wild crickets (Chapter 5).

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Chapter II Enzyme Immunoassay: Validation of a robust, sensitive method for extracting and quantifying ecdysteroid hormones in insect tissues

published as a supplement to Chapter III

ABSTRACT

Animals use hormones to control development, regulate physiology, drive reproduction, and facilitate responses to changing environments. Because hormone titers reflect an animal's developmental, metabolic, physiological, reproductive, and behavioral status, measuring hormone concentrations has become increasingly popular in ecological and medical studies. However, it remains difficult to measure low concentrations of hormone molecules, such as those of small-bodied invertebrates, leading to a bias towards vertebrate taxa in our current theoretical framework of endocrinology. Here we report that commercially available Enzyme Immunoassay materials can be used to quantify concentrations of ecdysteroid hormones between 0.8 – 3.2 ng/mL with less than 7% error. This assay has been used in studies of diverse insect taxa in the past, but has not been validated previously. Our validation confirms its utility as a robust, sensitive assay for ecdysteroid hormones that requires less specialized equipment than either radioimmunoassay or chromatographic methods, and quantifies the sensitivity, accuracy, precision, and cross-reactivity of the assay materials. Robust and sensitive hormone assays will provide useful tools for comprehensive studies of endocrinology beyond vertebrates.

Key words: Enzyme Immunoassay, assay validation, ecdysteroid hormones, *Acheta domesticus*, insect eggs

INTRODUCTION

Animals commonly use hormones to adjust their physiology and behavior in response to their environment. Ecdysteroid hormones (ESH) regulate molting and control early

development in insects, and are ubiquitous in arthropod species (Chapman, 2012). Beyond orchestrating key life-stage transitions (Warren et al., 2006), ESH trigger alterations in growth (Mirth et al., 2009) and reproductive behavior (Gabrieli et al., 2014). ESH have also been linked to the maintenance of circadian rhythms (Truman, 1984) and the expression of ecological traits (Oostra et al., 2014). Although they are both crucial and ubiquitous to invertebrate development and fitness, some functions of ESH vary among species. For example, in the seasonal butterfly *Bicyclus anynana*, pupal ESH concentrations alter life history strategy (Oostra et al., 2014). However, other functions of ESH remain relatively constant across taxa: in *Drosophila melanogaster*, *Manduca sexta*, and *Precis coenia*, embryonic ESH control imaginal disc development (Herboso et al., 2015; Miner et al., 2000; Nijhout et al., 2007). To establish a cohesive theoretical framework of invertebrate endocrinology, and to better understand the evolution of steroid hormones in invertebrates, it is necessary to measure concentrations and effects of ESH across many species.

Compared to those of vertebrates, arthropod endocrine systems remain poorly characterized. Our knowledge of the functions of ESH (and other invertebrate hormones) is constrained by the logistical difficulty of measuring the small masses of ESH present in arthropods. Great successes have been achieved using molecular and genetic methods that quantify, silence, or knock down gene expression (Hannan et al., 2009). Unfortunately, the utility of these methods is constrained to species for which extensive genetic information exists and for scenarios in which genetic tools can be used. Further, many of these methods cannot be used to quantify natural variation, only to note extremes achieved in laboratory studies.

Quantifying hormone function and concentration is logistically challenging for species that are not model organisms, which have a small body size, or which must be sampled in the field. Chromatographic methods (such as reverse-phase high-performance liquid chromatography (RP-HPLC), reverse-phase ultra-performance liquid chromatography (RP-UPLC), gas chromatography (GC), and even liquid chromatography-mass spectrometry (LC-MS)) are powerful and flexible. Chromatography provides a detailed

summary of the relative concentrations of all molecules present in an extract, but often requires expensive specialized equipment and a larger mass of tissue than is realistic given the body size of many arthropod species (Snyder, 2009). In contrast, immunoassays (Enzyme or Radio Immunoassays: EIA and RIA, respectively) provide comparatively less detailed data, reporting only the concentration of molecules that interact with a specific antibody or antiserum. However, immunoassays offer a higher degree of sensitivity than chromatography while requiring less specific equipment, so they are well-suited to assay very small amounts of specific hormone molecules in solution.

Here we validate the commercially available EIA materials for use in quantifying 20-hydroxyecdysone (20E), the most active ESH molecule. Cayman Chemical's commercially available Enzyme Immunoassay (EIA) materials have been used to assay 20E in studies of diverse species, results from which form the basis of the current understanding of ESH functions (Hackney et al., 2012; Moeller et al., 2013; Yamanaka et al., 2015). However, because the assay has not been validated previously, the results obtained from its use may include unidentified inaccuracies. Using synthetic and native 20E, we measure the assay's linearity, sensitivity, and accuracy, as well as its inter- and intra-assay precision and the specificity of the antiserum. We also report a modified extraction technique for extracting 20E from insect eggs, and report the efficiency of that technique.

MATERIALS AND METHODS

Cricket rearing & obtaining eggs

We purchased juvenile *Acheta domesticus* from Reptilefood.com and Flukers Farms and housed them at 27°C, between 50% and 75% relative humidity, on a 14:10 light:dark cycle in large plastic bins covered by aluminum screen lids. We provided food and water *ad libitum* (Whattam and Bertram, 2011). During egg collection, low-sided 6" square trays of dampened coarse sand were provided and removed after 24 hours. By swirling an excess of water through the dampened sand, we could easily decant eggs, which we collected by filtration, counted or weighed, and stored under pure ethanol (EtOH) at -20°C (Warren et al., 2006).

Extraction of 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). For all samples, we ground eggs using a glass stirring rod in a glass culture tube with 0.5 mL cold methanol (MeOH), centrifuged for 5-8 minutes and collected the supernatant, repeating this process three times as described by Brent and Dolezal (2009). Discarding the pellet, we dried the pooled supernatant under vacuum using a SpeedVac, and redissolved in 1.0 mL of 0.5 M Tris/HCl Buffer (for details, see Dinan and Rees, 1981).

We incubated all samples (except those from which we calculated extraction efficiency, below) with Porcine Liver Esterase (PLE; Sigma Aldrich, activity ≥ 15 units/mg; we used up to 1 mg PLE for 15 samples of 100 eggs each) overnight at 35°C to hydrolyze ester bonds between ecdysteroid molecules and glycolipoprotein storage molecules (Dinan and Rees, 1981). After incubation (or immediately after drying, for extraction efficiency samples), we purified samples using a gravity-flow solid phase extraction (SPE) protocol. We used 100mg 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. During the extraction efficiency experiment only, we also eluted with 100% MeOH to ascertain whether additional ESH could be recovered from the SPE column after the 60% elution. We dried eluted samples under vacuum at 40°C and then redissolved them in EIA Buffer (Cayman Chemical Company, Ann Arbor, MI, USA).

To test the extraction efficiency of this method, we spiked egg samples with an aliquot of radiolabeled synthetic ecdysone and performed the extraction procedure (excluding PLE incubation, but including SPE purification). After each step of the extraction procedure, we isolated the products, measured the radioactivity present in each, and compared the radioactivity to that of an aliquot of pure radiolabeled synthetic ecdysone to assess the percent ESH in each product. We added 0.5 μ C of 3 H-ecdysone to each sample of 60

eggs, which we then crushed with a glass stirring rod, incubated for 2 hours at room temperature (~20°C), and then followed the extraction procedure as outlined above. After drying the eluted samples under vacuum, we dissolved them in deionized water and counted the radiation present in each sample (in disintegrations per minute, DPM) using a Tricarb 2100TR (Packard). We then calculated the percent efficiency of the extraction by comparing the DPM from 50 μ C of ³H-ecdysone to those present in the pellet (from the initial extraction) and in each SPE elution.

Antiserum cross-reactivity

To divide egg extracts into fractions for subsequent chemical analysis, we dissolved a dried extract from 40 g of eggs in 300 μ L pure MeOH, and fractionated a 30 μ L aliquot using reverse-phase high-performance liquid chromatography (RP-HPLC, Waters Inc., Milford, MA, USA) using a C-18 column (SUPELCO Discovery H5, 25 cm) on a linear acetonitrile (ACN)/ H₂O gradient from 5-60% ACN over 40 minutes, collecting fractions every 60 seconds (for a total of 40 fractions). We then washed the column with 90% ACN for 10 minutes, then with 70% ACN for 10 minutes (20 additional fractions; total fractions collected increased to 60). We dried all 60 fractions under vacuum to remove the ACN and H₂O, and redissolved them in EIA Buffer, and with assistance from Cayman Chemical, assayed each of the 60 fractions separately in triplicate using the Cayman Chemical EIA materials to determine which fractions contained immunoreactive molecules.

To determine which ESH molecules were present in immunoreactive fractions, we used the same RP-HPLC apparatus to run pure standards using the same ACN/H₂O gradient over 40 minutes; column washing was eliminated for runs of pure standards. Peaks were detected by absorption using a diode array detector between 200 and 300nm. We identified immunoreactive ecdysteroid species by comparing the retention times of immunoreactive peaks to the retention times of radioinert synthetic pure standards (Festucci-Buselli et al., 2008). We obtained pure authentic standards from Cayman Chemical (20-hydroxyecdysone), Sigma Aldrich (ecdysone), and by a generous gift from Dr. Hajime Ono (2-deoxyecdysone, University of Tsukuba).

To calculate the magnitude of cross-reactivity, we assayed two candidate cross-reacting species using the EIA materials individually in the presence of eight different concentrations of 20E (the molecule to which the assay is designed to react). In this way, we altered the relative concentration of cross-reacting molecule, and by comparing the concentrations reported by the assay to a standard curve, calculated the percent cross-reactivity.

Linearity & Sensitivity

We assessed the linearity of the EIA results by assaying a serial dilution of the extract of 0.2 g eggs, which we estimated to be a similar range of concentrations as the serial dilution of a radioinert synthetic standard used as a standard curve for the assay (Dinan, 1997). To measure the sensitivity of the assay to biological extracts of 20-hydroxyecdysone, we then compared the slopes of the two serial dilution curves (egg extract dilution and standard 20E dilution) using ANCOVA (Mills 2010). For analysis, we log-transformed the calculated concentration of ESH and exponentiated observed absorbance values to meet the assumptions of ANCOVA. We interpreted the assay as measuring equally the extracted and synthetic ESH if the slopes of the linear portions of the standard curves were statistically similar (Sebaugh and McCray, 2003).

Accuracy

An assay may respond linearly to similar concentrations of the synthetic and extracted analyte without being able to accurately distinguish between small differences in the concentration of the analyte. To assess the accuracy (or the minimum distinguishable difference) of the EIA we worked with Cayman Chemical to perform a spike-recovery experiment as described by Mills et al. (2010). Six egg extracts were dissolved in EIA Buffer. Each egg extract was assayed in triplicate with each of six different spikes of radioinert synthetic 20E standard ranging between 0 - 160 pg. We then compared the assay results from spiked and unspiked samples. By comparing the differences in 20E content, we calculated the minimum difference in 20E concentration necessary for the assay to distinguish between two concentrations.

Precision

To determine the inter- and intra-assay variability, or the precision of the assay within and between 96-well plates, we analyzed six samples in triplicate four times each (a total of 12 wells per sample, per plate) on two different 96-well plates at the University of Michigan. The concentrations of these samples were calculated to fall near the center of the linear range of the standard curves detailed in section 2.4. We discarded nine outliers (out of 150 wells) that fell more than two standard deviations from the overall mean result of each sample. Additional values of inter- and intra-assay variability were obtained independently by Cayman Chemical. For these values, Cayman Chemical scientists used cricket egg extract samples containing higher concentrations of ESH than those used at the University of Michigan. Cayman Chemical scientists assayed each sample 20 times in triplicate (60 wells per sample) using a single set of reagents.

RESULTS

Extraction

Mean total extraction efficiency (that is, combining all products of the extraction process) for the four extracted samples was 75.33% (SD = 3.77). Most of this was present in the 60% MeOH elution, which contained 53.58% (SD = 2.49) of the total radioactive tracer added (Table 1). For all subsequent analyses, we used only the ESH obtained using the 60% MeOH elution.

Antiserum cross-reactivity

The antiserum reacted to 12 of the 60 HPLC fractions, which were grouped into three separate peaks, indicating the presence of at least three cross-reacting species (Figure 1b). We compared the retention times of these cross-reacting peaks to those of three pure, synthetic standard ESH molecules, and identified two precursor ecdysteroids, E, and 2-deoxyecdysone (2dE), as the two cross-reacting species (Figure 1a). We tested these molecules (E and 2dE) for cross-reactivity with the antiserum, and determined by subtraction that compared to the reactivity of the antiserum with 20E, E and 2dE cross-react at 43.8% and 0.6% respectively.

Linearity & Sensitivity

The serial dilution curves of both extracts (linear regression, $F_{1,13} = 371.7$, $R^2 = .9636$, $p < 0.001$) and standards (linear regression, $F_{1,8} = 508.1$, $R^2 = .9826$, $p < 0.001$) were linear (Figure 2). The slopes of the serial dilution curves of both extracts and standards were not significantly different (ANCOVA, $F_{1,21} = 0.720$, $p = 0.406$), indicating that the assay responds identically to changes in the concentrations of synthetic and extracted ESH (Figure 2). The intercepts of the two lines differed (ANCOVA, $F_{1,22} = 714.65$, $p < 0.0001$), likely reflecting differences in the concentration of authentic standards and estimated concentration of ESH extracts from eggs.

Accuracy

The spike-recovery experiment indicated that, for concentrations between 0.2 - 20.0 μg 20E/mL the coefficient of variation was less than 25% for each sample (Table 2), and less than 15% for concentrations near the center of that range.

Precision

Results from the University of Michigan experiments showed that for this assay, intra-assay variation (the systematic variation of measurements within one 96-well plate) was less than 7% at concentrations within the linear range of the standard curve (Table 3). Likewise, inter-assay variation (the systematic variation of measurements between 96-well plates) was less than 25% at high concentrations, and less than 7% at concentrations within the linear range (0.8-3.2 ng ESH/mL) of the standard curve (Table 3). Cayman Chemical's experiments used concentrations of extracted ESH that were higher than the linear range of the assay's standard curve, and contained error of between 16-19% for intra-assay variation, and between 13-24% for inter-assay variation (Table 3).

DISCUSSION

Data from this study document the validity of commercially available EIA materials to measure ecdysteroid hormones in methanolic extracts of insect eggs.

Extraction

The extraction and purification methods described here consistently return an extraction efficiency of purified ESH of approximately 50%. This is not a highly efficient extraction method: we attribute the low efficiency to material lost in the purification steps.

However, without highly purified samples, the assay performs unreliably, likely because of steric interference from lipid-like molecules that are soluble in methanol (Crocker, unpublished data). Using purified material, the assay performs as reported. If the source of the ESH were hemolymph, which generally contains fewer potential lipophilic contaminants than eggs, it is possible that the purification (SPE) step could be skipped; this could increase the overall extraction efficiency, which would require a smaller mass of tissue for analysis.

Linearity, sensitivity, accuracy & precision

The slopes of the linear portions of the serial dilutions of synthetic 20E and extracted ESH were similar, indicating that the antiserum responds similarly to synthetic and naturally occurring ESH. For both synthetic and biological ESH, the assay cannot reliably measure concentrations of greater than 6 ng/mL or less than 0.02 ng/mL and performs with the highest precision between 0.8-3.2 ng/mL (Table 3). However, within this range, the assay returns coefficients of variation less than 10% between assays, and is capable of distinguishing within 20 pg of material with less than 25% variation, and within 40 pg with less than 15% variation (Table 2).

Comparing the mean results for a sample assayed in multiple wells between and within each of the two plates allowed us to calculate the inter- and intra-assay variability of the EIA, respectively. For the range of concentrations over which the assay can be meaningfully used, neither intra- nor inter-assay variation exceeds 7%, which is well within the < 20% guideline described by (Sukovaty et al., 2006).

Cross-reactivity

In the RP-HPLC spectra shown in Figure 1, the retention times of pure synthetic standards closely match the retention times of the immunoreactive peaks when eluted off of the same column using an identical gradient. We therefore conclude that these three chemical species (20E, E, and 2-dE) are the ESH molecules measured by the assay. We note that the cross reactivity that we measured between 2dE and 20E was roughly two orders of magnitude lower (0.6%, compared to 63%) for this antiserum than was reported previously (Polgar et al., 1996). However, Polgar et al (1996) used an EIA kit that was discontinued due to replication problems with the included radioinert synthetic 20E,

though it is unclear whether the problems were due to impurities or other reagents (including the antiserum) in the kit (Cayman Chemical, personal communication). That study analyzed whole-body hemipteran samples, and used different protocols for extraction and purification of the ESH analyzed. However, it is unlikely that the differences in reactivity are due to the study organism, tissue type, or extraction protocol. It is unclear why the immunoreactivity differs between the two studies. Further work is required to explain the disparity; one explanation may be that differences exist between the reactivity of the antiserum currently available and the antiserum that was available in the mid-1990s.

CONCLUSION

Here we validate the enzyme immunoassay commercially available from Cayman Chemical as a precise, accurate, and replicable method for quantifying the small concentrations of ESH available in insect tissues.

ACKNOWLEDGEMENTS

Elizabeth Hurst and her team at Cayman Chemical provided valuable assistance in developing the purification methods, measuring the cross-reactivity of the antiserum, and the precision and accuracy of the assay materials. A quantity of purified 2-deoxyecdysone was generously donated by Hajime Ono. Callie R. Chappell, Leslie E. Decker, Pascale R. Leroueil, Amanda R. Meier, Johanna Nifosi, and Hillary B. Streit contributed insightful comments and feedback on previous drafts of this paper. Raymond V. Barbehenn, Eran Pichersky, Reza Sohrabi, and Callie R. Chappell provided valuable technical expertise and an RP-HPLC machine with which we completed the cross-reactivity portion of this study. Funding for this work was provided by the University of Michigan Rackham Graduate School.

TABLES

Table 1: Extraction efficiencies of ^3H -ecdysone from four separate samples of *Acheta domesticus* eggs spiked with aliquots of ^3H -Ecdysone. Values are percent ^3H -ecdysone recovered after each step of a methanolic extraction and subsequent solid phase extraction (SPE) purification. For the purposes of enzyme immunoassay, we chose the 60% MeOH SPE elution, as it contains the majority of ESH and fewer contaminants. The 100% MeOH elution was performed to be certain that no additional ESH could be recovered from the SPE column after the 60% elution.

Sample	Pellet	Buffer	H ₂ O	15% MeOH	60% MeOH	100 % MeOH	Total recovered
<i>1</i>	2.1	3.7	4.9	8.6	55.4	2.1	76.8
<i>2</i>	3.2	4.5	5.7	9.2	56.0	1.5	79.9
<i>3</i>	2.3	2.6	3.9	8.9	51.0	2.8	71.5
<i>4</i>	2.1	3.9	5.2	8.5	51.9	1.5	73.1
<i>Average</i>	<i>2.4</i>	<i>3.7</i>	<i>4.9</i>	<i>8.8</i>	<i>53.6</i>	<i>2.0</i>	<i>75.3</i>

Table 2: Calculated accuracy values of Cayman Chemical’s 20-hydroxyecdysone enzyme immunoassay. Three extracts from *Acheta domesticus* eggs were spiked with radioinert synthetic 20-hydroxyecdysone (“20E spike”). The mean absorbance of each of three replicate trials is shown in columns named “Extract 1-3”. The %CV of all three combined is shown in the column labeled “%CV”. Shaded rows denote concentrations that are included in the linear portion of the serial dilution curves.

20E spike (pg)	Extract 1 (abs)	Extract 2 (abs)	Extract 3 (abs)	%CV
0	0	0	0	
10	310	200	470	41.56
20	490	490	720	23.43
40	1520	1529	1480	1.53
80	2270	2560	2880	11.87
160	4370	6530	3910	7.54

Table 3: Measurements of the inter- and intra-assay precision of Cayman Chemical’s 20-hydroxyecdysone enzyme immunoassay. Extracts from *Acheta domesticus* eggs were measured repeatedly on multiple 96-well plates; %CV of a sample on the same plate denotes intra-assay precision, the %CV of the same sample compared between plates denotes inter-assay precision. At the University of Michigan, four distinct extracts measured multiple times on each of two different plates; Cayman Chemical Company performed the same experiment using higher concentrations of *A. domesticus* egg extract, which are outside the linear range of the assay. Shaded rows denote concentrations that fall within the linear range of the assay’s serial dilution curves.

Assayer	<i>Intra-assay</i>		<i>Inter-assay</i>	
	Mean conc (ng/ml)	% CV	Mean conc (ng/ml)	% CV
<i>Cayman Chemical</i>	30.74	18.72	31.75	13.35
<i>Cayman Chemical</i>	22.52	16.01	25.15	19.02
<i>Cayman Chemical</i>	5.86	18.69	5.97	24.03
<i>Univ. of Michigan</i>	3.04	6.33	3.04	6.77

FIGURES

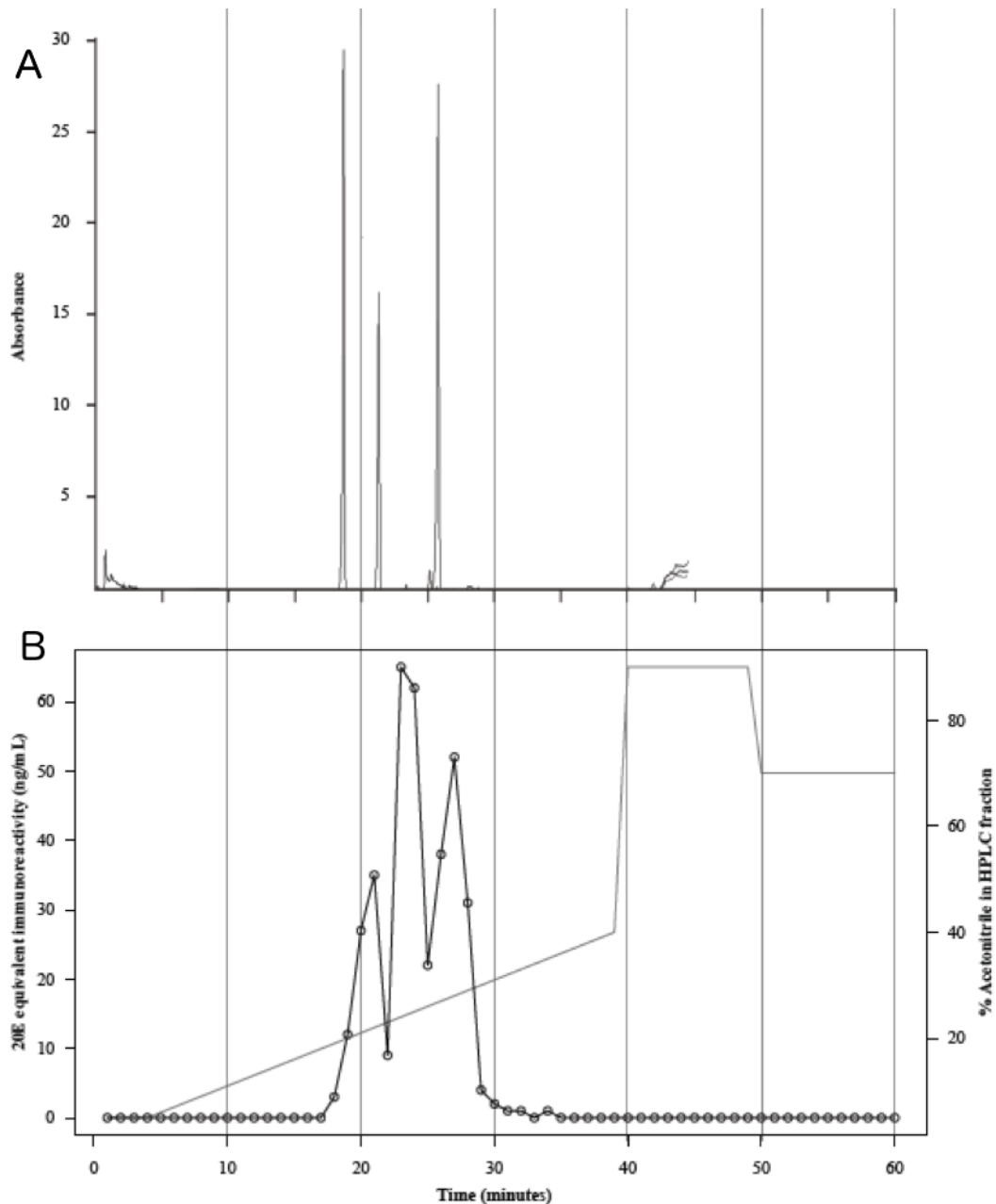


Figure 1: (A) Combined RP-HPLC spectra of 20-hydroxyecdysone (20E), ecdysone (E), and 2-deoxyecdysone (2-dE). (B) Combined depiction of the RP-HPLC solvent gradient used to fractionate *Acheta domesticus* egg extracts and the immunoreactivity of Cayman Chemical's 20-hydroxyecdysone enzyme immunoassay to each fraction. The immunoreactivity of all fractions is plotted against the left-hand y-axis (20-hydroxyecdysone equivalents (ng/mL)) in open black circles; the percent of the solvent gradient that is acetonitrile (the mobile phase) used to fractionate each fraction is shown as a gray line plotted against the right-hand y-axis.

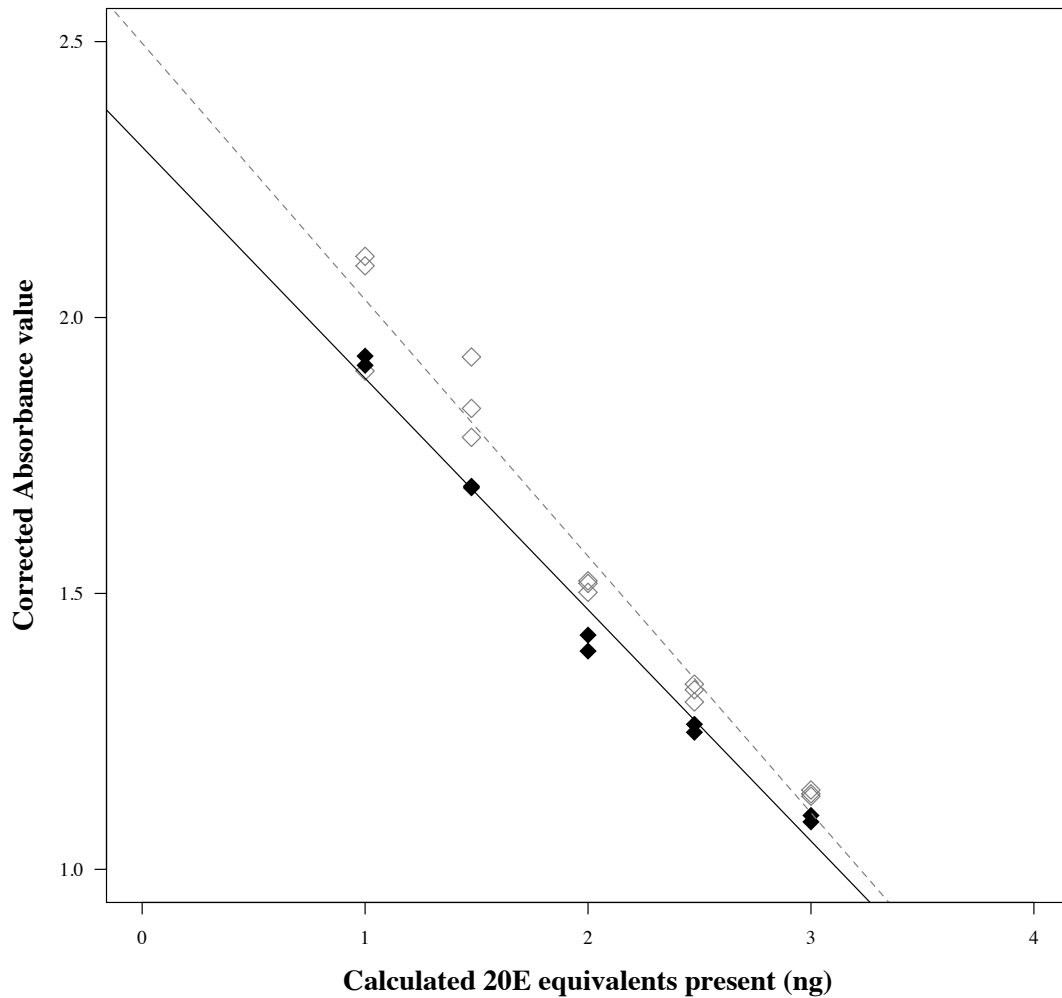


Figure 2: Scatterplot of serial dilutions of radioinert synthetic 20-hydroxyecdysone standard (solid points, unbroken line) and ecdysteroid hormones extracted from *Acheta domestica* eggs (hollow points, dotted line) assayed using Cayman Chemical's 20-hydroxyecdysone enzyme immunoassay. The corrected absorbance of all samples is plotted along the y-axis; the x-axis shows the calculated concentration of 20-hydroxyecdysone equivalents.

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

ABSTRACT

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 reveal that variation in the active ecdysteroid hormones provided by a female house cricket (*Acheta domesticus*) 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 most 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 the increased environmental variability that comes with global climate change, and that further study of hormones in this context could be profitable.

Key words: Nongenetic transgenerational effect, maternal effect, parental effect, hormone-mediated effect, ecdysteroid hormone, ecdysone, molting hormone, epigenetic effect, developmental programming hypothesis

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 Grootuis et al., 2005, Mar et al., 1999, Mousseau and Fox, 1998, and Weaver et al., 2004), Nongenetic parental effects are often mediated by hormones diverse taxa, either directly through variable hormone provisioning (e.g. barn swallows (*Hirundo rustica*) in Saino et al., 2005, cattle egrets (*Bubulcus ibis*) in Schwabl et al., 1997, and spotted hyaenas (*Crocuta crocuta*) in Dloniak et al., 2006) or indirectly through parenting behavior (e.g. studies on mice by Champagne and Curley, 2012 and Craft et al., 2009, and rats by Parent et al., 2005). Examples of direct effects 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 (Grootuis et al., 2005; Muller and Grootuis, 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 hatchling 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 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 threespine 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 (Veenema, 2012). However, carryover effects are not limited to taxa that have DNA methylation (Takayama et al., 2014), nor is hormone-facilitated 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). ESH actions and effects have been studied extensively with respect to their immediate, activational-type actions (listed above) (Phoenix et al., 1959) during early development (Kozlova and Thummel, 2003), molting and metamorphosis (Warren et al., 2006), and even reproductive behavior (Gabrieli et al., 2014). However, we are aware of only one study (Oostra et al., 2014) that investigates the long-term, organizational-type (Phoenix et al., 1959) effects of intraspecific variation in ESH throughout ontogeny, and we are unaware of any studies linking parental nutrition to ESH-mediated parental effects. Although hormone provisioning is far from the only mechanism of transmitting carryover effects, an expanded knowledge of the long-term effects of hormone provisioning is necessary to discover which mechanism may be operating in invertebrate species. Here we provide the first evidence that variability in ESH provisioning is linked to multiple generations of ancestral environment, and that this embryonic ESH has measurable organizational-type effects on offspring phenotype.

Specifically, the results of our two experiments combine to quantify the range of natural ecdysteroid hormone variation provisioned by female *A. domesticus* to their eggs, establish the strength of the effects of provisioned ecdysteroid hormones on offspring development, and measure the persistence of such hormone-mediated effects across three generations.

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 hatchlings 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 line 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).

METHODS

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"x6" 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 dechoriation, 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 (Dechoriation Control), or soaked for 24 hours at 30°C in a 1.0M Phosphate Buffered Saline (PBS) solution with varying concentrations of active ESH (20-hydroxyecdysone (Sigma Aldrich, St. Louis, MO, USA)) (0, 0.1, 0.5, 1, 5, 10, 50 μ M concentrations) (Kidokoro et al., 2006). We chose these concentrations for ESH treatment because a preliminary study showed that a 1mM solution was sufficiently concentrated to kill eggs. After 24 hours, 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 30C.

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 number of days, and used with measurements of structural size as estimates of development rate.

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

Raising the crickets

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 hours 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 thus 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 hours. We collected the eggs as described in experiment 1 methods, above. Daily clutches of eggs ranged in size from 1 - 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 11); eggs do not vary in size (Furneau 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 (Figure 3) (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 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, Figure 3) 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.

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 (Figure 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) (Figure 3). Daily clutches from this generation were divided into incubated and frozen subsets as described.

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) (Figure 3). 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-demarcated 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 (Figure 3).

Analyzing hormone content

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 minutes 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 redissolved 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, 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 redissolved 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 0.5M Tris/HCl Buffer for 25 samples: each sample was therefore reacted with between 0.6-1.2 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 was present in any sample, this variability in the concentration of PLE per sample was deemed unlikely to influence our end results (Whiting and Dinan, 1989). All samples (with and without PLE) were then incubated overnight at 35°C to hydrolyze ester bonds between ESH molecules and glycolipoprotein storage molecules (Dinan and Rees, 1981). After incubation, we purified samples using a gravity-flow solid phase extraction (SPE) protocol. We used 100mg 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 12x75mm disposable glass culture tubes.

The 60% MeOH fractions were then dried at 40°C under vacuum using a ThermoSavant SPD111V SpeedVac, and redissolved in 176 μ L EIA Buffer solution (Cayman Chemical product 400060). All samples were quantified using an Enzyme Immunoassay (EIA, Cayman Chemical products 400006, 482202, 482200, 16145, 400050, 400062, and 400035), using 50 μ L 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 Tables 4-6, Figure 9). 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. Although this approach limits our ability to distinguish inter-family effects from systematic error, it ensures that plate identity cannot drive our general conclusions.

All plates were read at 75 and 90 minutes, per Cayman Chemical product insert instructions, using a tunable plate reader set to 415nm 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 6). The linear-approximate portion of the curves was identified using the method developed by Sebaugh & McCray (Sebaugh and

McCray, 2003), and all samples falling outside those limits were discarded to be re-assayed.

Statistical Analyses:

Experiment 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 cricket responses to hormone application; we therefore included a quadratic term in all of the regression models. We applied these methods to all measures of 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.

Experiment 2

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 cricket responses to hormone application; we therefore included a

quadratic term in all of the regression models. We applied these methods to all measures of 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.

2. 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 effect of artificially reduced diet quality on estimating the range of natural hormone provisioning.

Heritability is often assessed by parent-offspring regression (Kempthorne and Tandon, 1953). Here, we used general linear models (SAS 9.4) to assess the heritability of the concentrations of free and conjugated ESH provided by mothers to their eggs. Specifically, the ESH in the eggs of mothers was a continuous variable, offspring diet was a class variable, and ESH in the eggs produced by offspring was the independent variable. By including offspring diet as a class variable, we can ask whether the heritability (slope) of the trait is influenced by dietary environment (i.e. a significant diet * maternal ESH interaction). We ran the analyses separately for free and conjugated ESH.

3. 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.

4. 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).

RESULTS

Experiment 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 7 and 8). Based on pronotum width, crickets grew most rapidly under intermediate levels of ESH application (quadratic term in regression model of all data points $F_{1,115} = 9.7$, $p = 0.002$, Figure 4). 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: $F_{5,112} = 3.63$, $p = 0.004$; weighted averages $F_{1,3} = 6.83$, $p = 0.0795$) (Tables 7-9, Figures 10 and

11). We also analyzed hatchling survival, hatch latency, and lifetime growth rate and found no additional relationships to exogenous ESH treatment (Table 9, Figures 12 and 13).

Experiment 2

*2. 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) ecdysteroids (ESH) provisioned to eggs. Levene's test indicated that the provisioning of free ESH varied more than that of conjugated ESH ($F_{1,128} = 35.26$, $p < 0.0001$, Figure 14) (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 (Figure 14), measured amounts of ESH do not overlap the upper limit of detection of the assay (Figure 9, Table 5). Similarly, although the amount of free ESH provisioned to eggs is closer to the lower limit of detection of the assay (Figure 9, Table 5), we would predict a floor effect to reduce, rather than elevate, the measured variance (Figure 14).

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: $F_{1,136} = 7.21$, $p = 0.0082$) (Figure 5). 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.

3. Natural variation in active, but not inactive, ESH provisioned to eggs predicts hatchling 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 and maternal diet on offspring phenotype were most striking 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) (Figure 6). Likewise, while higher quality parental nutrition was always positively related to offspring pronotum width, we measured a stronger relationship in early life (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 who 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 ($p = 0.0007$) (Figure 15).

4. 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

Grand-maternal diet quality strongly predicts the amount of free ESH that her granddaughters will deposit in their eggs in a nonlinear fashion ($F_{2,72} = 5.64$, $p = 0.0053$) (Figure 7). 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 ($F_{4,122} = 2.58$, $p = 0.0404$) (Figure 8). Specifically, F2 crickets whose grandmother (P generation) was raised on a low-quality diet could overcome this circumstance 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

(Figure 8). 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 (Figure 8).

The latency of egg production reflected a similar interaction, but between a cricket's own diet quality and that of her grandmother ($F_{4,118} = 2.73$, $p = 0.0322$) (Figure 16). In other words, granddaughters (F2 generation) 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 ($F_{4,118} = 2.73$, $p = 0.032$). Maternal diet quality was not significant in any model ($F_{2,118} = 1.10$, $p = 0.336$).

DISCUSSION

In the current study, we report that (i) varying concentrations of active (free) ESH available to embryos during early development alter offspring phenotype (Figures 4, 7), 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 (Figures 7, 8). 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 (Figure 7). 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 Wistar 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 (Benton 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 incentivizing the evolution of powerful transgenerational effects. For instance, 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. *Acheta domesticus* have a generation time of approximately three and a half months (thus, three to four generations 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. domesticus*, 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 reduced 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.

Because F2 descendants of reduced-quality diet P individuals lay eggs with less active ESH than do those descended from high-quality grandmothers, we 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 (Figure 7). 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 her first clutch was reduced if both her mother and grandmother (F1 and P generations respectively) also experienced low-quality diets (Figure 8). This interaction suggests that female crickets are more strongly influenced by the nutritional environment of their mothers and grandmothers than by their own nutritional environment when producing their eggs and provisioning them with ESH. 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 commences 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 (Figure 16).

Nongenetic grand-maternal effects have been noted in multiple taxa (Beemelmans and Roth, 2017; Hafer et al., 2011; Magiafoglou and Hoffmann, 2003; Shama and Wegner, 2014), but the molecular underpinnings 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 hormone 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 (Figure 4). In experiment 2, we show that the mass of ESH available to an egg is positively correlated with the hatchling's structural size during at least the first two weeks of its life (Figure 6). After this time, a hatchling from an egg with a low concentration of active ESH can transcend its initial disadvantage by eating a high-quality diet (Figure 6): neither diet quality nor active ESH concentration in

an egg affect a cricket's size at maturity, though crickets that eat a higher quality diet mature more quickly (Figure 15).

In general, nongenetic transgenerational effects are predicted to be highly adaptive, even outcompeting phenotypic plasticity under stochastic conditions (Jablonka et al., 1995). Although such effects may be developmentally ephemeral, natural selection operates throughout the lifetime 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.

ACKNOWLEDGEMENTS

This work was done on the occupied land of Odawa, Chippewa, Wendat, and Pottawatomi Nations, where Ann Arbor, Michigan, is currently located. Critical assistance in the laboratory was provided by Caroline A. Wilkinson, Gnyapti D. Majmudar, Megan S. Spencer, Alison M. Zachritz, Emily J. Lauderdale, Alena M. Olsen, and Jenna L. Dunbar. Preliminary studies referenced in the supplement were carried out by Daniel T. Nondorf (spermatophores) and Claire Freimark and Haley Kalis (provisioning of eggs over a female's lifetime). Valuable feedback on the manuscript and experimental procedures was provided by professor Robert J. Denver, Amanda R. Meier, Leslie E. Decker, Hillary B. Streit, Michelle L. Fearon, and two anonymous reviewers. Dr. Elizabeth Hurst and her team at Cayman Chemical provided crucial assistance in validating the Enzyme Immunoassay materials used to quantify ecdysteroid hormone. Many thanks also to Dr. Pascale R. Leroueil, and to the several thousand crickets who contributed their lives to these experiments.

Funding sources: This work was funded by the University of Michigan Department of Ecology and Evolutionary Biology and the Rackham Graduate School at the University of Michigan.

FIGURES

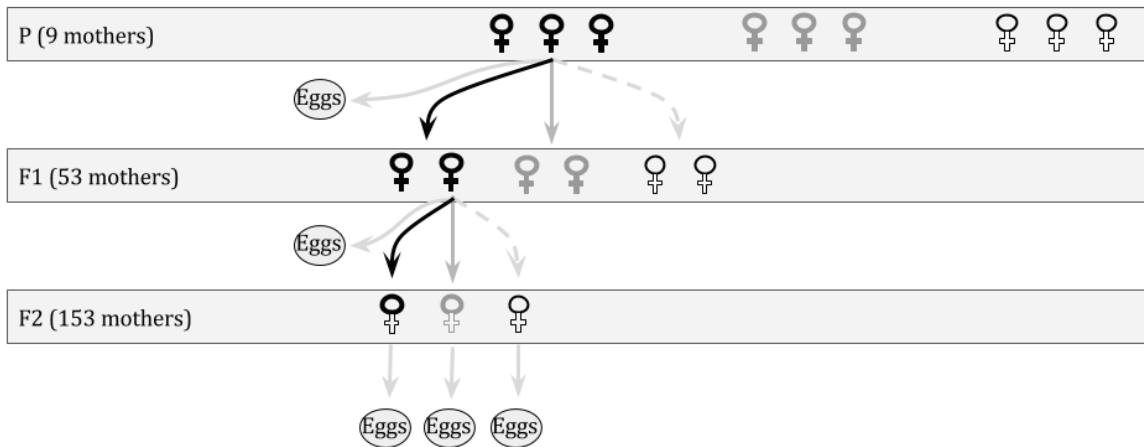


Figure 3: Diagram of experimental design. Females raised on high-, intermediate- and low-quality diet are shown in black, gray, and hollow/dotted figures and arrows respectively. For the P generation, three mated pairs per female diet quality were formed (N=9). For each female, half of all eggs produced were frozen (oval marked “Eggs”); the other half were divided into three groups to be raised on one of three diet qualities. For the F1 generation, six daughters per P generation pair (two each from high-, intermediate-, and low-quality diet) were mated with males raised on a high quality diet (N=53, as one family had only five F1 daughters survive). Half of the eggs produced by F1 females were frozen (oval marked “Eggs”); the other half were divided into three groups to be raised on one of three diet qualities. Of these F2 individuals, from each F1 pair, three daughters (one per diet quality) were raised (N=153). All F2 crickets were measured weekly for structural size, all eggs they produced were collected and frozen.

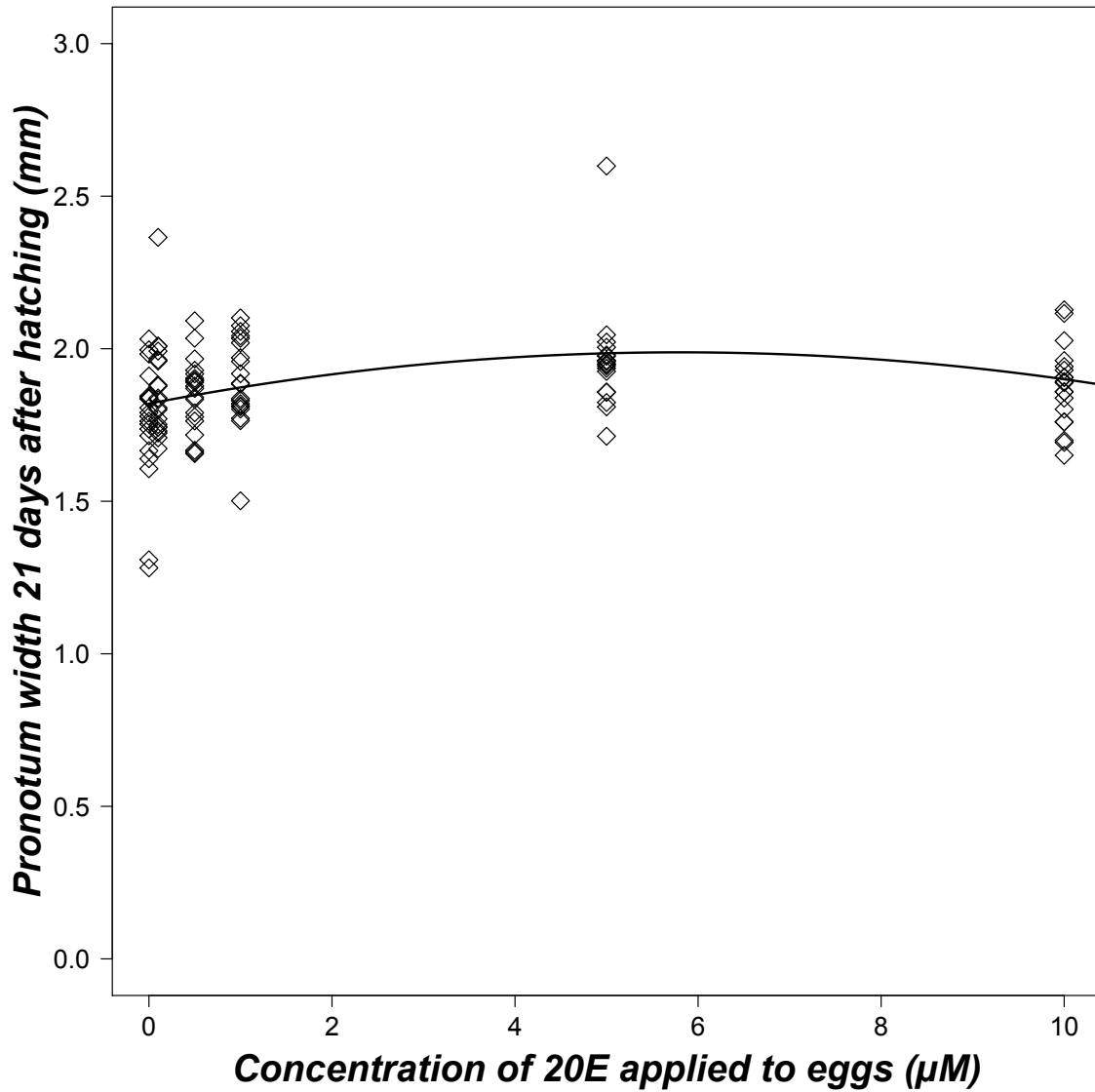


Figure 4: Quadratic relationship between hatchling phenotype (size,) and the concentrations of exogenous 20-hydroxyecdysone used to treat eggs. Crickets treated with intermediate concentrations of 20E (5µM) grew more quickly than did hatchlings from eggs treated with either lower or higher concentrations of 20E (General Linear Model, $F_{2,117} = 6.57$, $p = 0.002$).

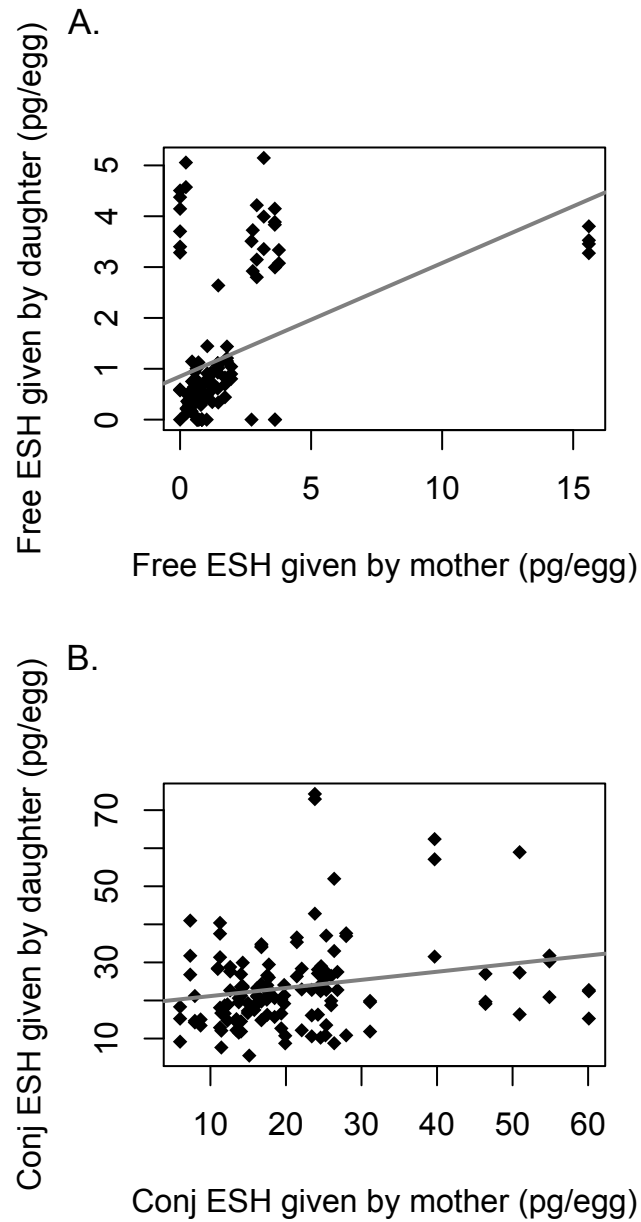


Figure 5: Relationships between ecdysteroid hormone (ESH) provisioning of eggs by female *Acheta domesticus* and the amount of ESH provisioned to them by their mothers (x-axes). The upper plot illustrates the mother-daughter correlation in provisioning of free ESH (ANCOVA $F_{1,136} = 30.63$, $p < 0.0001$; although there is no compelling biological reason to do so, removing the four extreme points yields a stronger trend); the lower plot shows the mother-daughter correlation in provisioning of conjugated ESH (ANCOVA $F_{1,136} = 7.21$, $p = 0.0082$).

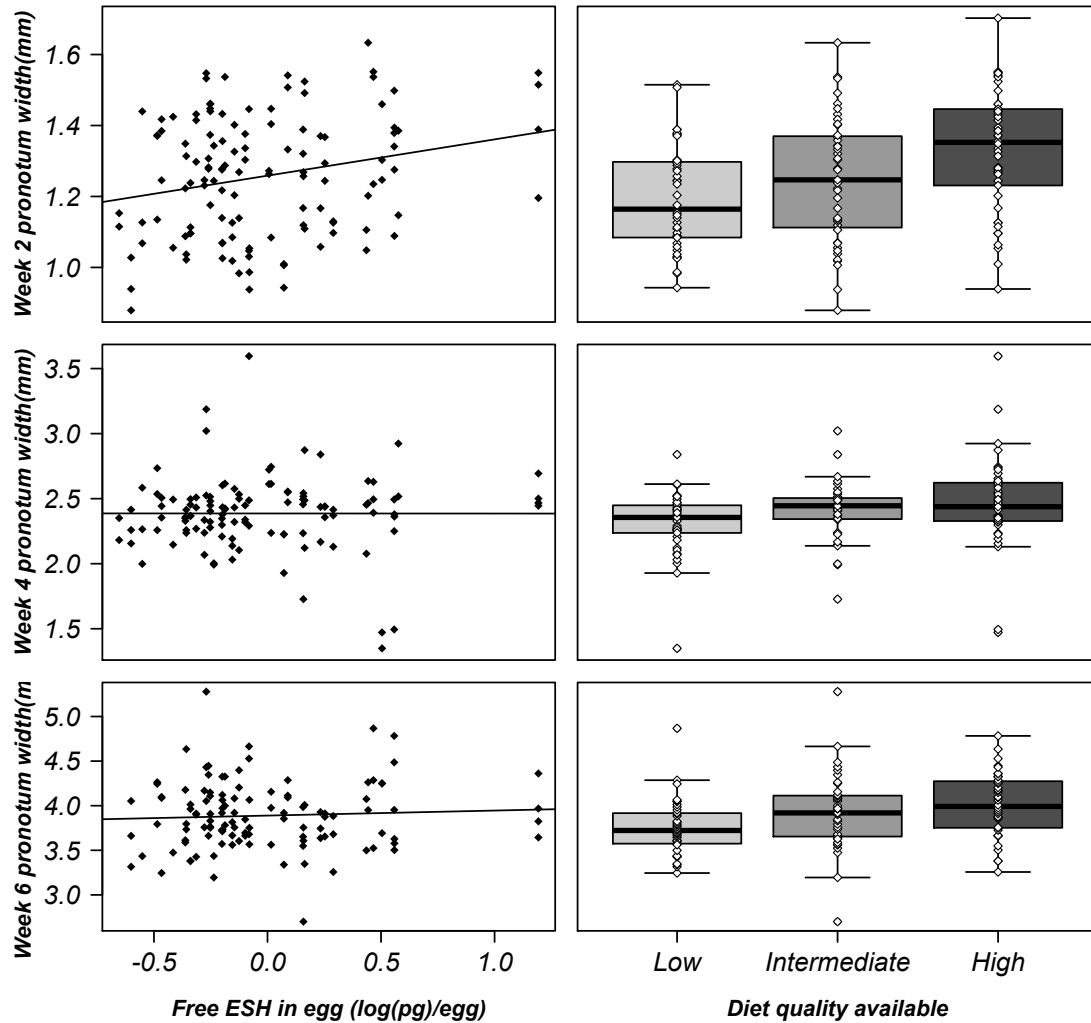


Figure 6: Correlations between *Acheta domestica* hatchling size two, four, and six weeks after hatching (different timepoints shown in rows) and (A) mass of free ESH in eggs (scatter plots, linear regressions, first column) and (B) hatchling diet quality (box plots, ANOVA, second column). At two weeks, both free ESH per egg ($F_{1,120} = 8.071$, $p = 0.0053$) and diet quality ($F_{2,120} = 8.647$, $p = 0.0003$) affect hatchling size. At four weeks, diet quality affects hatchling size ($F_{2,112} = 3.339$, $p = 0.039$) but free ESH mass ceases to have an effect ($F_{1,112} = 0$, $p = 0.993$). At six weeks, diet quality affects hatchling size ($F_{2,112} = 4.668$, $p = 0.0113$), though free ESH mass does not ($F_{1,112} = .394$, $p = 0.5316$). All error bars are SE.

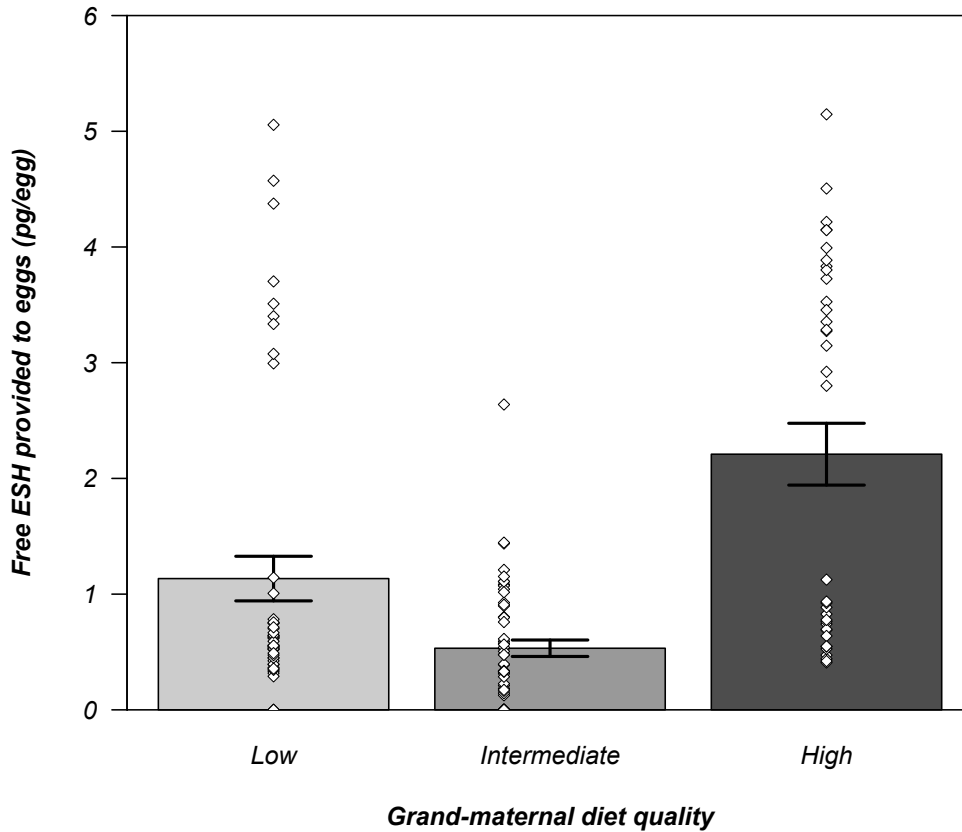


Figure 7: Relationship between the mass of free ecdysteroid hormones (ESH) provisioned by an *Acheta domesticus* female to her eggs and the diet quality of that cricket's grandmother. Crickets descended from grandmothers that were reared on low- or intermediate-quality diets provided less free ESH to their eggs than did crickets descended from grandmothers which ate a high-quality diet (Nested Mixed Model, $F_{2,72} = 5.64$, $p = 0.0053$).

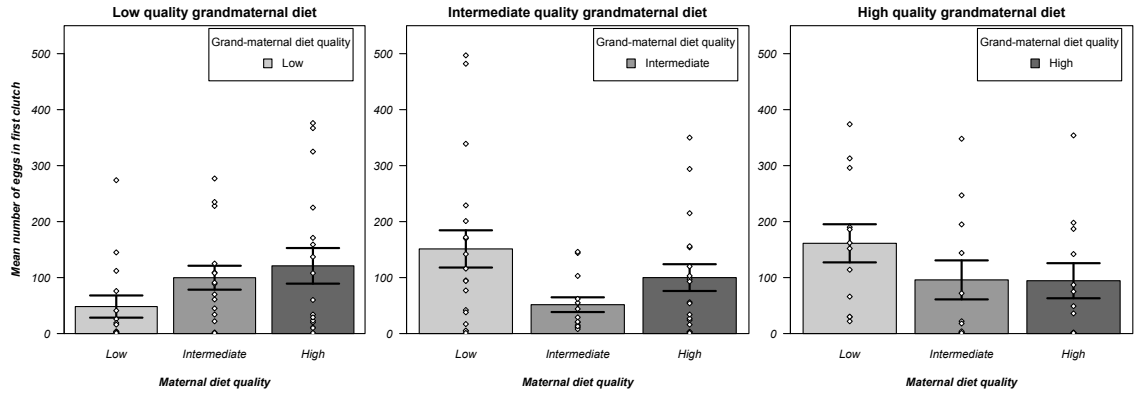


Figure 8: Effect of grand-maternal diet (low-quality diet is on the left, intermediate-quality diet is in the center, and high-quality diet is on the right) on the relationship between an *Acheta domesticus* female's first clutch size and the diet quality of her mother. Crickets whose mother and grandmother were both reared on a low-quality diet (left bar, left panel) laid fewer eggs than any other group (Nested Mixed Model, $F_{4,122} = 2.585$, $p = 0.0404$); all error bars are SE.

SUPPLEMENTARY MATERIAL

Methods validation: Confirming the extraction efficiency, linearity, sensitivity, accuracy, and precision of the Enzyme Immunoassay materials when used with cricket egg extracts.

Immunoassays (Radio and Enzyme) exploit the specificity of antibodies to quantify the concentration of material that interacts with those antibodies. However, because of the sensitivity of antibodies to their chemical environment, and because the Enzyme Immunoassay (EIA) materials we used had not been previously validated, we performed a validation of both our extraction method and our use of the EIA materials to quantify ecdysteroid hormones extracted from cricket eggs.

Extraction efficiency

We combined and modified existing extraction methods developed to extract ESH from whole insects, eggs, adult tissue, and larvae. To test the extraction efficiency of this method, we spiked egg samples with an aliquot of radiolabeled synthetic ecdysone and performed the extraction procedure (excluding PLE incubation, but including SPE purification). After each step of the extraction procedure, we isolated the products, measured the radioactivity present in each, and compared the radioactivity to that of an aliquot of pure radiolabeled synthetic ecdysone to assess the percent ESH in each product. We added 0.5 μ C of 3H-ecdysone to each sample of 60 eggs, which we then crushed with a glass stirring rod, incubated for 2 hours at room temperature (\sim 20 $^{\circ}$ C), and then followed the extraction procedure as outlined above. After drying the eluted samples under vacuum, we dissolved them in deionized water and counted the radiation present in each sample (in disintegrations per minute, DPM) using a Tricarb 2100TR (Packard). We then calculated the percent efficiency of the extraction by comparing the DPM from 50 μ C of 3H-ecdysone to those present in the pellet (from the initial extraction) and in each SPE elution (Table 4).

Table 4: Extraction efficiencies of ^3H -ecdysone from four separate samples of *Acheta domesticus* eggs spiked with aliquots of ^3H -Ecdysone. Values are percent ^3H -ecdysone recovered after each step of a methanolic extraction and subsequent solid phase extraction (SPE) purification. For the purposes of Enzyme Immunoassay, we chose the 60% MeOH SPE elution, as it contains the majority of ESH and fewer contaminants. The 100% MeOH elution was performed to be certain that no additional ESH could be recovered from the SPE column after the 60% elution.

Sample	Pellet	Buffer	H ₂ O	15% MeOH	60% MeOH	100 % MeOH	Total recovered
<i>1</i>	2.1	3.7	4.9	8.6	55.4	2.1	76.8
<i>2</i>	3.2	4.5	5.7	9.2	56.0	1.5	79.9
<i>3</i>	2.3	2.6	3.9	8.9	51.0	2.8	71.5
<i>4</i>	2.1	3.9	5.2	8.5	51.9	1.5	73.1
<i>Average</i>	<i>2.4</i>	<i>3.7</i>	<i>4.9</i>	<i>8.8</i>	<i>53.6</i>	<i>2.0</i>	<i>75.3</i>

Linearity and sensitivity

We assessed the linearity and sensitivity of the EIA results by assaying a serial dilution of the extract of 0.2 g eggs, which we estimated to be a similar range of concentrations as the serial dilution of a radioinert synthetic standard used as a standard curve for the assay (Dinan, 1997). To measure the sensitivity of the assay to biological extracts of 20-hydroxyecdysone, we then compared the slopes of the two serial dilution curves (egg extract dilution and standard 20E dilution) using ANCOVA. For analysis, we log-transformed the calculated concentration of ESH and exponentiated observed absorbance values to meet the assumptions of ANCOVA. We interpreted the assay as equally sensitive to concentrations of the extracted and synthetic ESH if the slopes of the linear portions of the standard curves were statistically similar.

The serial dilution curves of both extracts (linear regression, $F_{1,13} = 371.7$, $R^2 = .9636$, $p < 0.001$) and standards (linear regression, $F_{1,8} = 508.1$, $R^2 = .9826$, $p < 0.001$) were linear (Figure 9). The slopes of the serial dilution curves of both extracts and standards were not significantly different (ANCOVA, $F_{1,21} = 0.720$, $p = 0.406$), indicating that the assay responds identically to changes in the concentrations of synthetic and extracted ESH (Figure 9). The intercepts of the two lines differed (ANCOVA, $F_{1,22} = 714.65$, $p <$

0.0001), likely reflecting differences in the concentration of authentic standards and estimated concentration of ESH extracts from eggs.

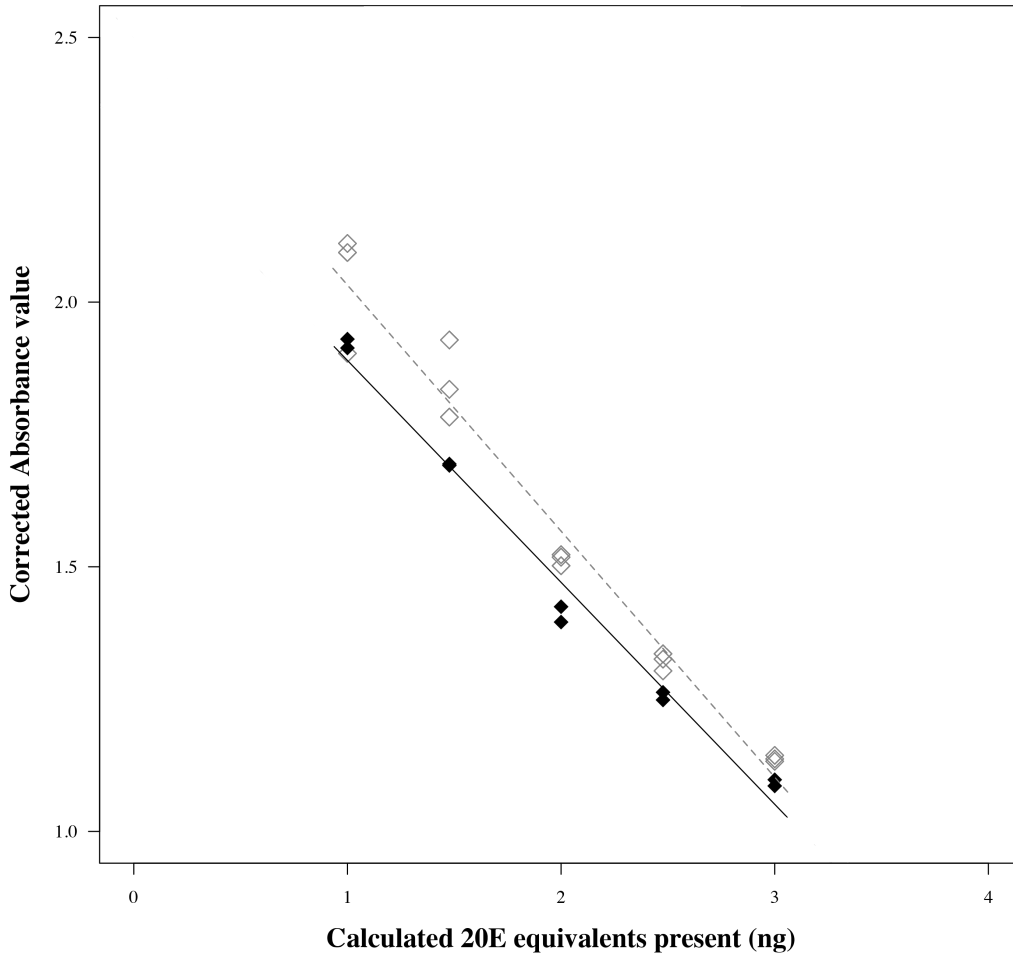


Figure 9: Scatterplot of serial dilutions of radioinert synthetic 20-hydroxyecdysone standard (solid points, unbroken line) and ecdysteroid hormones extracted from *Acheta domesticus* eggs (hollow points, dotted line) assayed using Cayman Chemical's 20-hydroxyecdysone Enzyme Immunoassay. The corrected absorbance of all samples is plotted along the y-axis; the x-axis shows the calculated concentration of 20-hydroxyecdysone equivalents.

Accuracy:

An assay may respond linearly to similar concentrations of the synthetic and extracted analyte without being able to accurately distinguish between small differences in the concentration of the analyte. To assess the accuracy (or the minimum distinguishable

difference) of the EIA we worked with Cayman Chemical to perform a spike-recovery experiment as described by Mills et al. (2010). Six egg were assayed in triplicate with each of six different spikes of radioinert synthetic 20E standard ranging between 0 - 160 pg. We then compared the assay results from spiked and unspiked samples. By comparing the differences in 20E content, we calculated the minimum difference in 20E concentration necessary for the assay to distinguish between two concentrations.

The spike-recovery experiment indicated that, for concentrations between 0.2 - 20.0 µg 20E/mL the coefficient of variation was less than 25% for each sample (Table 5), and less than 15% for concentrations near the center of that range.

Table 5: Calculated accuracy values of Cayman Chemical’s 20-hydroxyecdysone enzyme immunoassay. Three extracts from *Acheta domesticus* eggs were spiked with radioinert synthetic 20-hydroxyecdysone (“20E spike”). The mean absorbance of each of three replicate trials is shown in columns named “Extract 1-3”. The %CV of all three combined is shown in the column labeled “%CV”. Shaded rows denote concentrations that are included in the linear portion of the serial dilution curves.

20E spike (pg)	Extract 1 (abs)	Extract 2 (abs)	Extract 3 (abs)	%CV
0	0	0	0	
10	310	200	470	41.56
20	490	490	720	23.43
40	1520	1529	1480	1.53
80	2270	2560	2880	11.87
160	4370	6530	3910	7.54

Precision:

To determine the inter- and intra-assay variability, or the precision of the assay within and between 96-well plates, we analyzed six samples in triplicate four times each (a total of 12 wells per sample, per plate) on two different 96-well plates. The concentrations of these samples were calculated to fall near the center of the linear range of the standard

curves (above). We discarded nine outliers (out of 150 wells) that fell more than two standard deviations from the overall mean result of each sample

Results from the University of Michigan experiments showed that for this assay, intra-assay variation (the systematic variation of measurements within one 96-well plate) was less than 20% at very high concentrations, and less than 7% at concentrations within the linear range of the standard curve (Table 6). Likewise, inter-assay variation (the systematic variation of measurements between 96-well plates) was less than 25% at high concentrations, and less than 7% at concentrations within the linear range (0.8-3.2 ng ESH/mL) of the standard curve (Table 6). Cayman Chemical’s experiments used concentrations of extracted ESH that were higher than the linear range of the assay’s standard curve, and contained error of between 16-19% for intra-assay variation, and between 13-24% for inter-assay variation (Table 6).

Table 6: Measurements of the inter- and intra-assay precision of Cayman Chemical’s 20-hydroxyecdysone enzyme immunoassay. Extracts from *Acheta domesticus* eggs were measured repeatedly on multiple 96-well plates; %CV of a sample on the same plate denotes intra-assay precision, the %CV of the same sample compared between plates denotes inter-assay precision. Four distinct extracts measured multiple times on each of two different plates.

<i>Intra-assay</i>		<i>Inter-assay</i>	
Mean conc (ng/ml)	% CV	Mean conc (ng/ml)	% CV
3.04	6.33	3.04	6.77

Results of main study

Experiment 1: Disentangling causation and correlation by applying exogenous ecdysteroid hormone to eggs

In our first experiment, we applied multiple concentrations of exogenous 20-hydroxyecdysone (20E) to groups of cricket eggs. All eggs were less than 24 hours old, and were randomly selected from a communal lay dish provided to a laboratory colony that contained more than 300 laying females. We compared the body size of hatchings three weeks after they hatched to the concentration of 20E applied to that group. We used

both regression and ANOVA approaches to analyze these data (see main text for details), and show the full models and parameter estimates (Tables 7 & 8) below.

Table 7: Three analytical approaches were used to compare the size of crickets three weeks after hatching across treatment groups; shown are the models for each approach, with both type I and III sums of squares.

<u>Model</u>	<u>Source</u>	<u>DF</u>	<u>Sums of Squares</u>	<u>Mean Square</u>	<u>F value</u>	<u>p-value</u>
All points:						
	Model	2	0.324	0.162	6.57	0.002
	Error	115	2.832	0.025		
	Treatment	1	0.296	0.236	12.03	0.0007
	Treatment ²	1	0.239	0.239	9.7	0.002
Weighted average:						
	Model	2	0.507	0.253	4.17	0.136
	Error	3	0.182	0.061		
	Treatment	1	0.477	0.477	7.85	0.068
	Treatment ²	1	0.415	0.415	6.83	0.080
ANOVA:						
	Model	5	0.440	0.088	3.83	0.004
	Error	112	2.716	0.242		
	Treatment	2	0.440	0.088	3.63	0.004

Table 8: Parameter estimates for the two quadratic models shown in Table 7.

<u>Model</u>	<u>Parameter</u>	<u>Estimate</u>	<u>Standard error</u>	<u>t value</u>	<u>p value</u>
All points	Intercept	1.816	0.020	89.52	< 0.0001
	Treatment	0.058	0.017	3.47	0.0007
	Treatment ²	-0.005	0.002	-3.11	0.0023
Weighted average	Intercept	1.82	0.022	82.5	< 0.0001
	Treatment	0.06	0.020	2.80	0.068
	Treatment ²	-0.005	0.002	-2.61	0.080

We have also included plots of the two approaches not included in the main body of this paper (Figures 10 & 11).

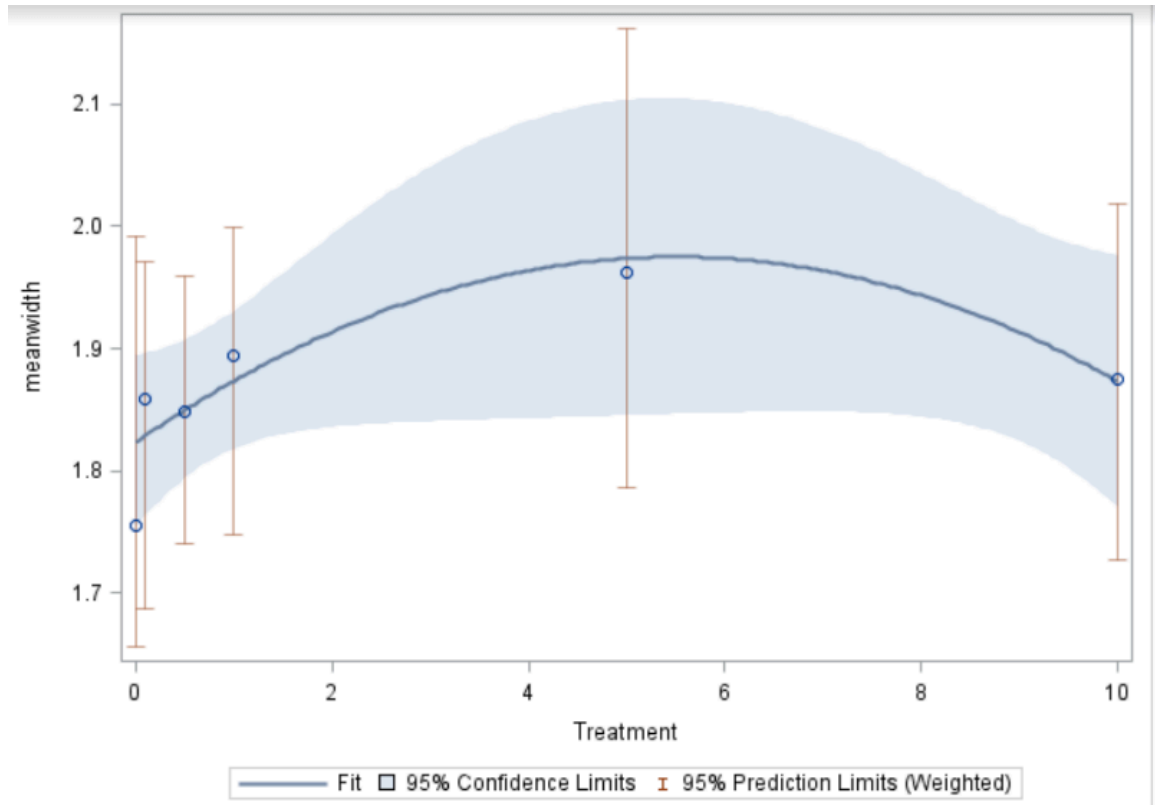


Figure 10: Plot showing weighted average fit from Table 7.

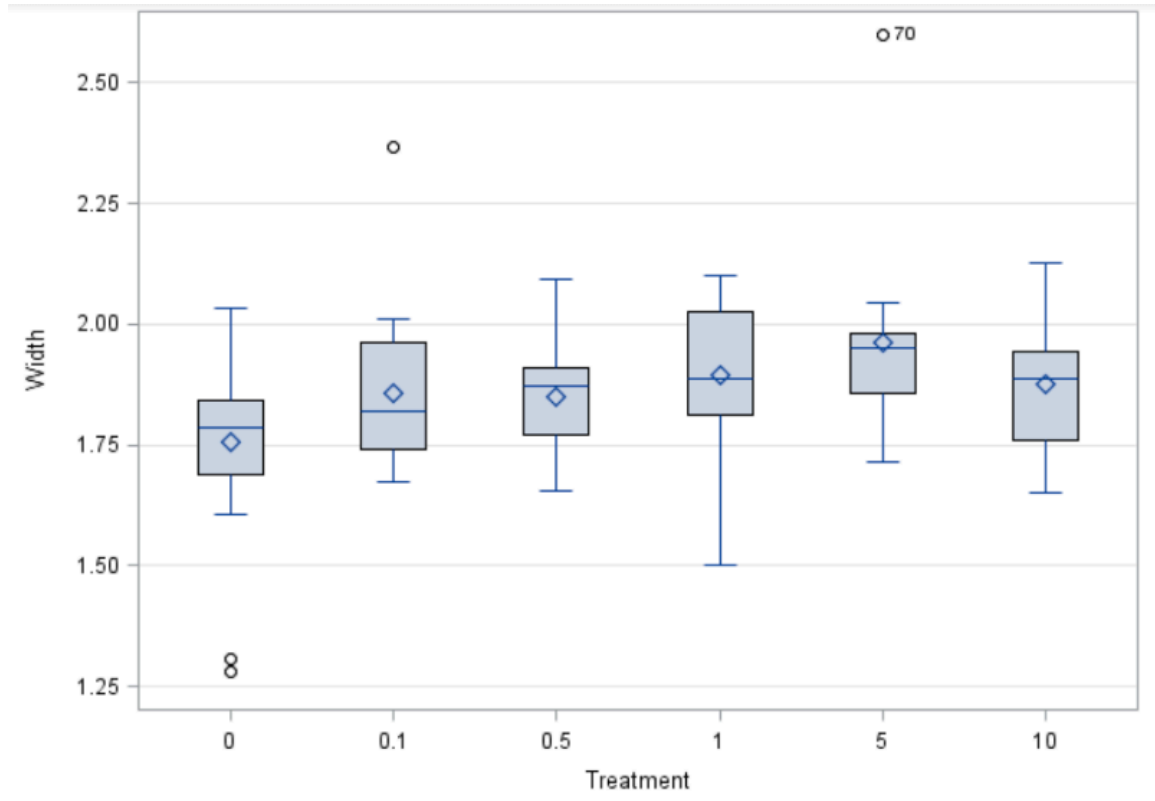


Figure 11: Plot showing ANOVA-approach fit from Table 7.

The conclusion of a quadratic response is robust to analysis method. The concentration of 20E that we applied did not affect the survival of eggs (Table 9), the hatch latency of eggs (Figure 7 & 8), or the change in size of hatchlings between three weeks of age and maturity (Table 10, Figure 13).

In experiment 1, survival rates of treated eggs did not differ among treatments, nor from untreated eggs (Pearson Chi-squared test, $\chi^2(18) = 21$, $p = 0.279$).

Table 9: Percent survival by treatment group in Experiment 1.

Treatment (μM ESH)	Survived (%)
0.1	21
0.5	20
1	21
5	21
10	23
0	21
Untreated	22

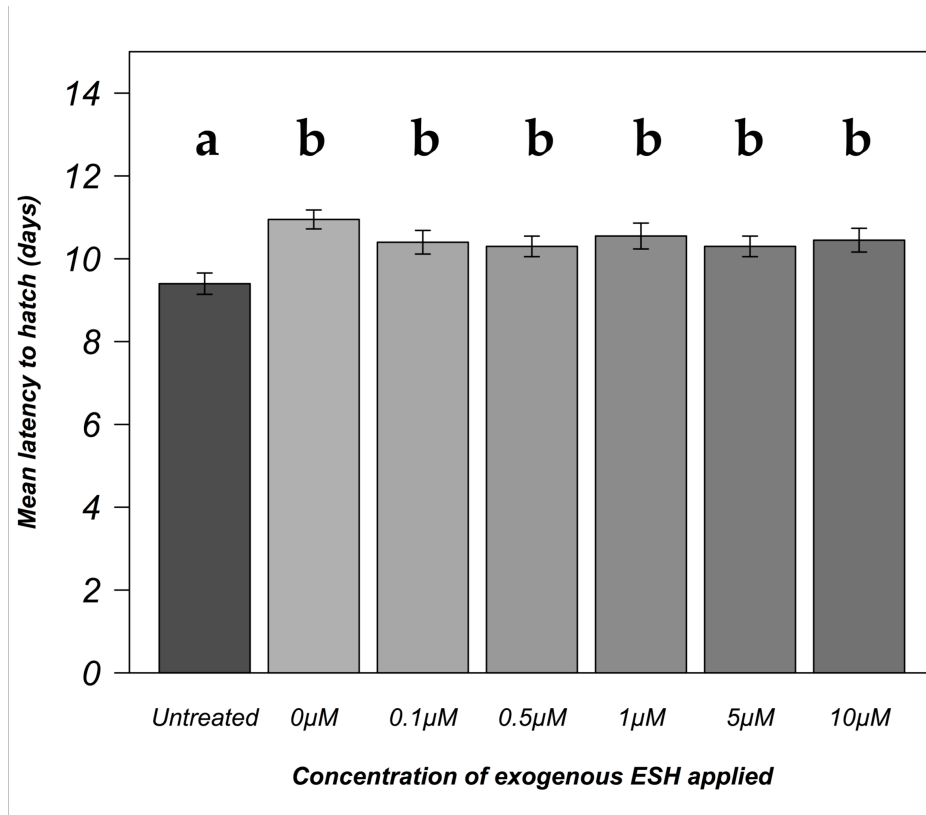


Figure 12: Plot of means (error bars are SE) of hatch latency by exogenous treatment group. No treatment groups differed in terms of hatch latency, though our treatment method delayed development (ANOVA, $F_{6,133} = 8.44$, $p < 0.0001$).

Table 10: Full model results of Multiple Ways ANOVA with change in size between three weeks after hatching and size at maturity (no interaction was significant, thus we excluded it from our final model). Intuitively, latency to maturity is related to change in size.

Factor	DF	Sum of squares	Mean square	F value	p value
Treatment	6	0.917	0.153	1.24	0.302
Maturity latency	1	1.336	1.336	10.82	0.002
Residuals	53	6.544	0.1235		

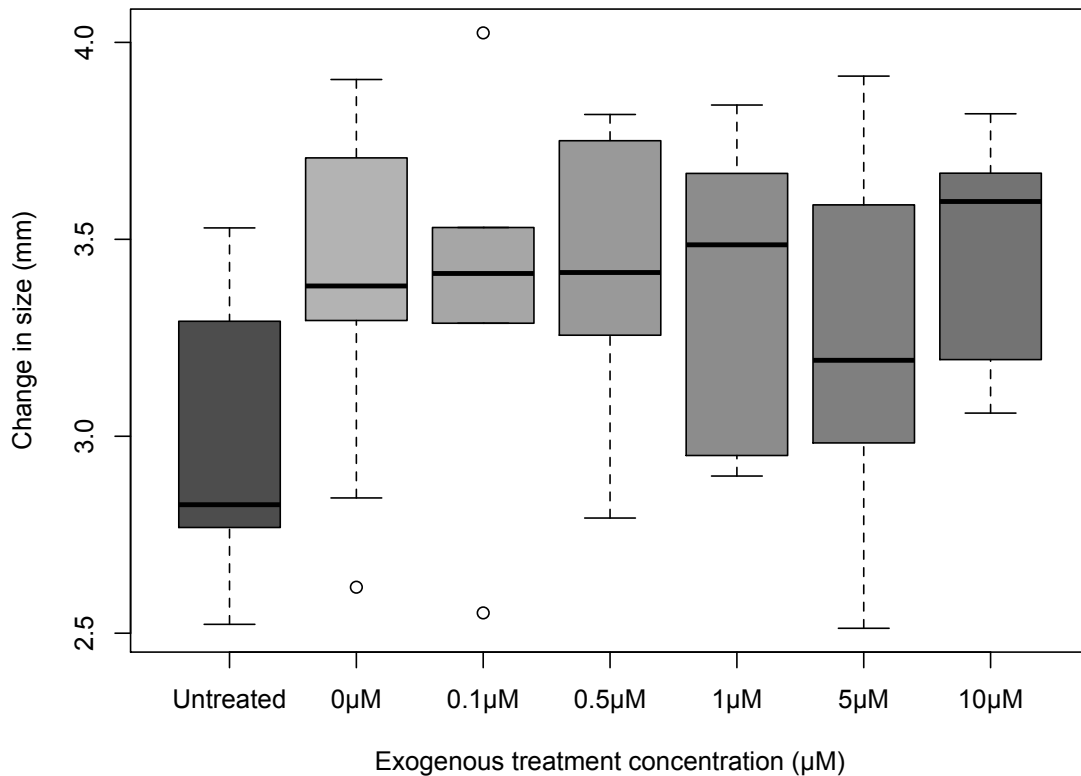


Figure 13: Plot of mean change in size (width) of hatchlings between 3 weeks of age and maturity. All groups were similar (Multiple Ways ANOVA, $F_{6,53} = 1.24$, $p = 0.302$).

Experiment 2

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 line and nutritional environment (high-, intermediate-, and low-quality diets) on hormone provisioning to eggs across three generations. All crickets were raised in a common environment, 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.

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 (see main text for details). Here we present additional data.

Natural variation exists in ESH provisioned to eggs of *Acheta domesticus*. Female crickets varied widely in both free (active) and conjugated (inactive) ecdysteroids (ESH) provisioned to eggs. Levene's test indicated that the provisioning of free ESH varied more than that of conjugated ESH ($F_{1, 128} = 35.26$, $p < 0.0001$, Figure 14) (CV for free ESH = 108.3%; for conjugated = 50.5%).

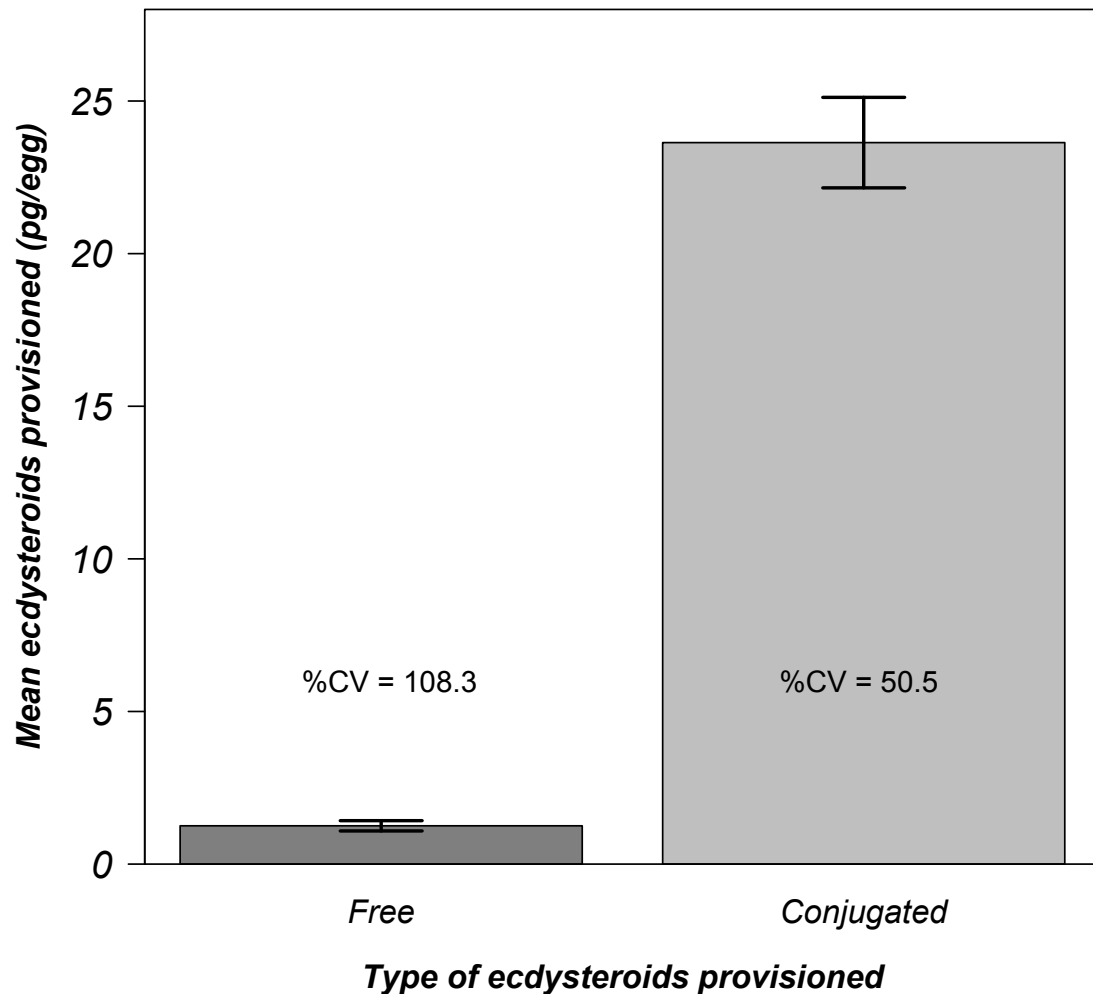


Figure 14: Variation present in provisioned ecdysteroid hormones (ESH) in eggs of *Acheta domesticus*. Two general forms of ESH exist in cricket eggs: free (plotted in a dark gray bar), and conjugated, (plotted in a light gray bar). Free ESH are biologically active and provisioned in relatively small amounts, while conjugated ESH are the inactive, stored form and make up the bulk of the ESH in eggs. Both varied substantially (CV for free ecdysteroids = 108.3%; for conjugated = 50.5%), but free ecdysteroids varied more (Levene's test, $F_{1,128} = 35.263$, $p < 0.0001$). All error bars are SE.

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

Size at maturity was determined by a cricket's sex, and unaffected by either egg ESH content or diet quality (see main text for details). Crickets that were fed a low quality diet

matured less quickly than did those who 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 ($p = 0.0007$) (Figure 15).

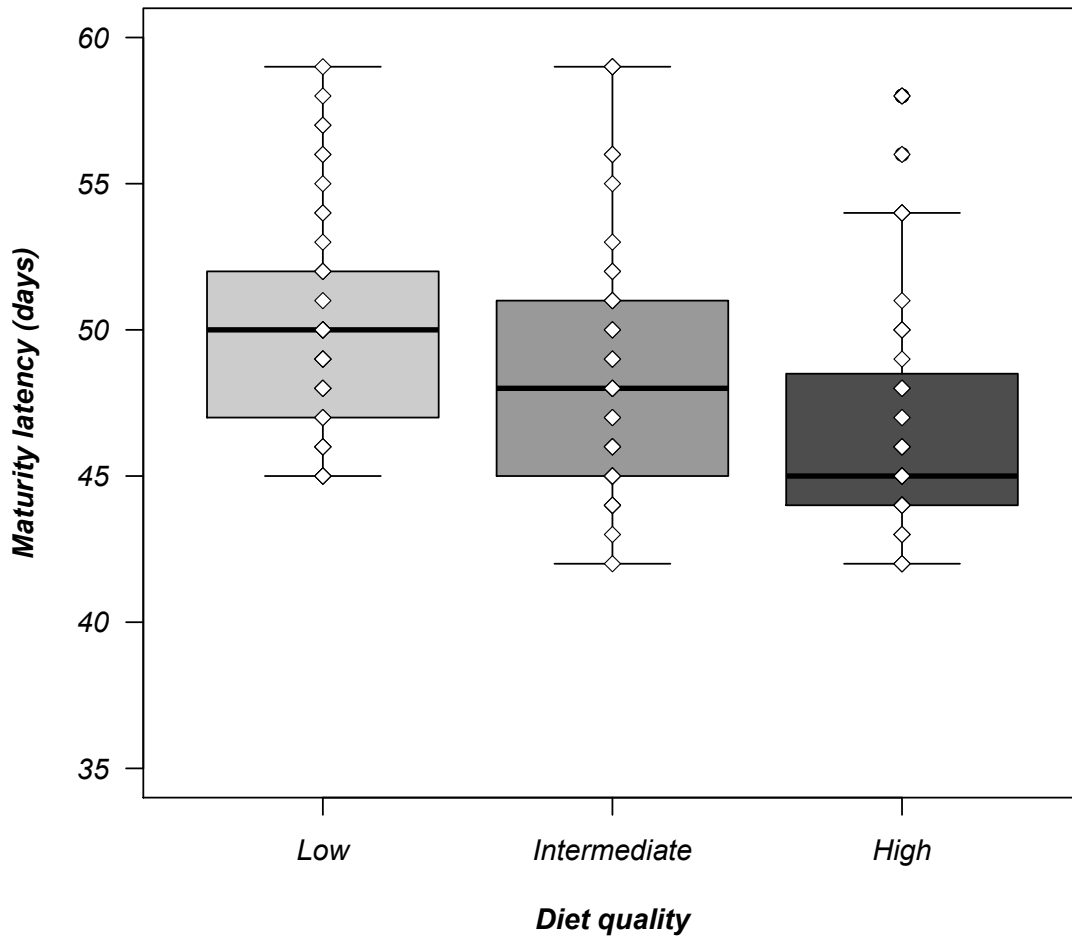


Figure 15: Relationship between maturity latency and diet quality for *Acheta domesticus* hatchlings. Crickets that were reared on a high quality diet matured earlier than did those reared on a low-quality diet (ANOVA, $F_{2,112} = 7.7845$, $p = 0.00065$); all error bars are SE; hollow points indicate datapoints used in our calculations.

Grand-maternal diet quality affects the number of eggs produced and the latency of egg production

The latency of egg production reflected a similar interaction, but between a cricket's own diet quality and that of her grandmother ($F_{4,118} = 2.73$, $p = 0.0322$) (Figure 16). In other words, granddaughters (F2 generation) 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 ($F_{4,118} = 2.73$, $p = 0.032$).

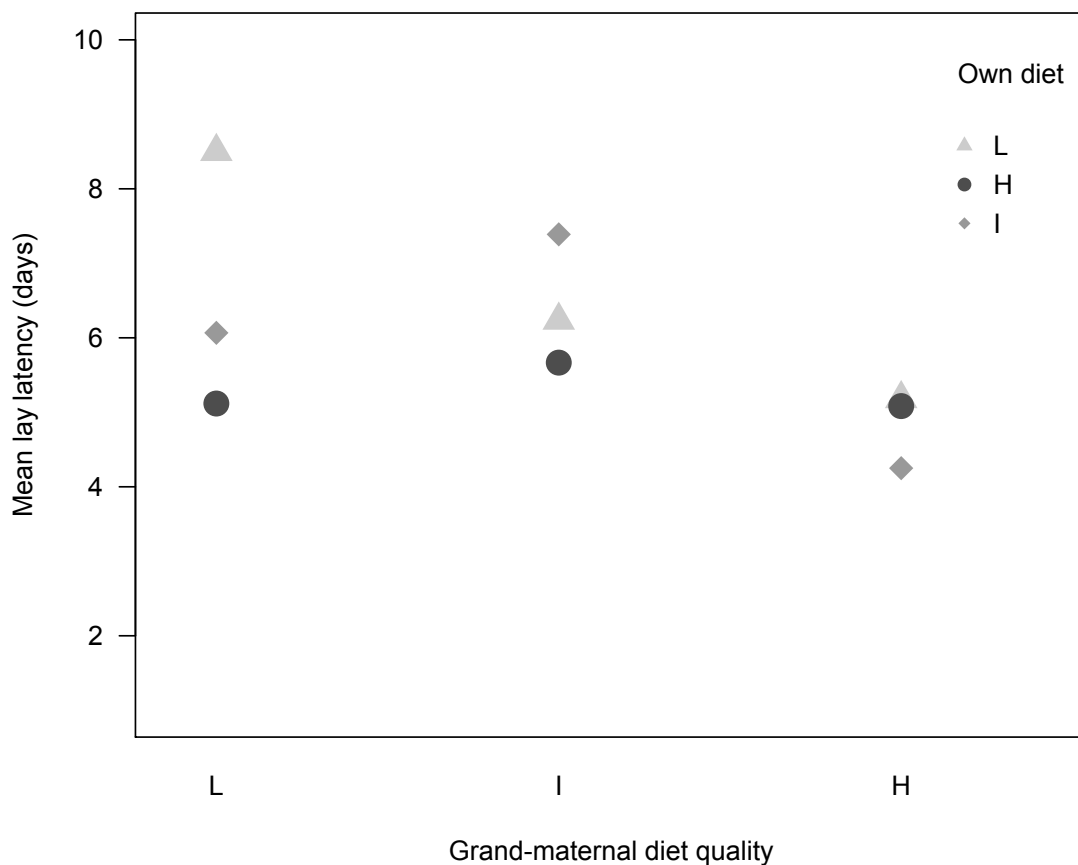


Figure 16: Relationship between an *Acheta domesticus* female's diet quality (low: light gray circles; intermediate: gray diamonds; high: dark gray triangles), her grandmother's diet (x-axis), and the delay in starting oviposition (measured in days between her imaginal molt and first clutch). Crickets descended from a grandmother reared on a low-quality diet and which were also reared on a low-quality diet laid their first clutches later than did any other combination of diet histories (ANOVA, $F_{4,118} = 2.733$, $p = 0.0322$), regardless of the diet quality of the intermediate generation.

Additional studies

Female response to male quality:

In this pilot study, we raised females *Acheta domesticus* on high quality diet, and males on either high- or low-quality diet (as described in Experiment 2, above). We measured both the ESH present in intercepted male spermatophores, from males raised on either high- or low-quality diet. Consistent with previously published work, male ESH contribution in the spermatophore was negligible (less than 10% by weight) compared to the ESH mass in eggs (Hoffmann and Wagemann, 1995), did not differ between males, regardless of diet quality (ANOVA, $F_{1,35} = 0.054$, $p = 0.817$) and did not vary with male body size (linear regression $F_{1,34}=2.228$, $p=0.145$).

Female variation of ESH provisioning over her lifetime:

In this pilot study, we raised mated pairs of *Acheta domesticus* on high- or low-quality diet (as described in Experiment 2, above). We collected eggs from females throughout their entire lifetime, then chose 30 eggs from the first clutch they produced, and 30 eggs from the last clutch they produced. Neither diet quality nor age influenced the concentration of either free or conjugated ESH that we measured in eggs (Table 11).

Table 11: T-test analyses comparing the concentration of ESH provided to eggs by individual females as a function of clutch number (first and last clutches) and diet quality (high and low). All analyses were conducted for both free and conjugated ESH.

Model	Model type	Free ESH			Conjugated ESH		
		<i>df</i>	<i>t value</i>	<i>p value</i>	<i>df</i>	<i>t-value</i>	<i>p value</i>
ESH ~ age	t-test	17.7	t-.422	0.68	13.5	-1.2	0.25
ESH ~ diet quality	t-test	7.26	2.04	0.07	4.43	0.02	0.98

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Chapter IV Social density, but not sex ratio, drives ecdysteroid hormone provisioning to eggs by female house crickets (*Acheta domesticus*)

ABSTRACT

Social environment profoundly influences the fitness of animals, affecting their probability of survival to adulthood, longevity, and reproductive output. For many species, the social conditions experienced by parents at the time of reproduction predict the social environments that offspring will face. Despite clear challenges in predicting future environmental conditions, adaptive maternal effects provide a mechanism of passing environmental information from parent to offspring, and are now considered pervasive in natural systems. Maternal effects have been widely studied in vertebrates, especially in the context of social environment, and are often mediated by steroid hormone (SH) deposition to eggs. In insects, although many species dramatically alter phenotype and life history traits in response to social density, the mechanisms of these alterations, and the role of hormone deposition by insect mothers into their eggs, remains unknown. In the experiments described here, we assess the effects of social environment on maternal hormone deposition to eggs in house crickets (*Acheta domesticus*). Specifically, we tested the hypotheses that variable deposition of ecdysteroid hormones (ESH) to eggs is affected by both maternal (1) social density and (2) social composition. We found that while maternal hormone deposition to eggs does not respond to social composition (sex ratio), it does reflect social density; females provision their eggs with higher ESH concentrations under low density conditions. This finding is consistent with the interpretation that variable ESH provisioning is an adaptive maternal response to social environment, and congruent with similar patterns of variable maternal provisioning across the tree of life. Moreover, our results confirm that maternal hormone provisioning may mediate delayed density dependence by introducing a time lag in the response of offspring phenotype to population size.

Key words: Hormone provisioning, maternal effects, density dependence, non-Mendelian parental effects

INTRODUCTION

Social environment profoundly influences the fitness of animals, affecting their probability of survival to adulthood, longevity, and reproductive output (McCullough, 1999; Nieberding and Holveck, 2017; Siracusa et al., 2017). However, social environment can vary substantially over relatively short time scales as population sizes fluctuate, as age structure changes, or as seasonal processes alter the types of groups to which animals belong (Clutton-Brock et al., 1992; Lewellen and Vessey, 1998; Randall et al., 2005; Tinghitella, 2014). For many species, the social conditions experienced by parents at the time of reproduction predict the social environments that offspring will face (Simpson and Miller, 2007). As a consequence, some adults express flexibility in their reproductive strategies in response to their social environment. For example, female cockroaches (*Nauphoeta cinerea*), increase the number of male, but not female, offspring that they produce when they encounter few males in their developmental and adult environments (Moore et al., 2001). As a consequence, they increase the fitness of their sons in a female-biased environment. Similarly, female guinea pigs (*Cavea apera f. porcellus*) alter the sex ratio of their offspring to produce more daughters than sons when social conditions are unstable (Kemme et al., 2009). In this species, females reach reproductive maturity earlier than males, so a female-biased sex ratio is interpreted to be adaptive in unstable conditions (Kemme et al., 2009).

Beyond simple changes in the sex ratio of their offspring, mothers may also alter the phenotypes of their offspring based on variation in social environment. Non-Mendelian parental effects (hereafter, maternal effects) provide one mechanism by which the social environment experienced by parents can influence offspring phenotype, life-history and fitness (reviewed in Mousseau and Fox (1998b) and Groothuis et al. (2005)). Social environment is a promising environmental variable to induce maternal effects, because while it can fluctuate within an animal's lifetime (Hamilton, 1966), a mother's social environment can also predict the social conditions her offspring will face (Dantzer et al.,

2013). For such maternal effects to be adaptive, mothers must be able to predict at least some aspects of their future environments, and influence offspring phenotype accordingly (Carlisle, 1982; Dantzer et al., 2013; Schwabl et al., 1997). Despite clear challenges in predicting future environmental conditions, adaptive maternal effects are now considered pervasive in natural systems (Mousseau and Fox, 1998b; Rossiter, 1996; Champagne, 2008; Chin et al., 2009; Shea et al., 2011).

Maternal effects on offspring life history have been widely studied in vertebrates (Beach, 1974; Burton et al., 2011; Craft et al., 2009; Maestripieri, 1993; Stein and Bell, 2014), particularly in avian systems, in which they are often mediated by steroid hormone (SH) deposition to eggs, most notably in the form of androgens and glucocorticoids (Love et al., 2005; McNabb and Wilson, 1997; Rutkowska and Badyaev, 2008; Tschirren et al., 2004). Notably, SH concentrations in eggs can reflect maternal social environment: both the composition and size of social groups affect adult endocrine status and reproductive strategy. For example, female great tits (*Parus major*) allocate higher concentrations of androgens to eggs when breeding at high social densities (Remeš, 2011), which increases the growth rate of their sons, but not of their daughters after hatching (Tschirren, 2015). Similarly, female house sparrows (*Passer domesticus*) vary the concentration of androgens that they deposit to their eggs according to their social environment (Mazuc et al., 2003). Their offspring that are exposed to elevated androgen concentrations are more competitive (Strasser and Schwabl, 2004). In Japanese quail (*Coturnix coturnix*), increased glucocorticoid titers (a SH stress indicator that responds to social density) in mothers are transferred to eggs, reducing the growth rate of juvenile offspring and increasing the sensitivity of adult offspring to environmental stressors (Hayward and Wingfield, 2004). Maternal steroid hormones can, therefore, have adaptive or maladaptive effects on offspring phenotype and fitness, depending on the concentrations and specific hormones deposited into eggs.

Beyond vertebrates, research on insect systems has greatly informed our understanding of the evolution of social behavior and adaptation to variable social environments (Lester et al., 2005; Tibbetts and Izzo, 2010; Wilson and Wilson, 2007). As with vertebrates, the

social environment experienced by parents can influence the phenotype of offspring. For example, females of both the *Delphacidae* species *Prokelisia marginata* and *P. dolus* respond to increased social density by altering the phenotype of their offspring: at low densities, offspring in both species are flightless, but in crowded conditions, they produce fully winged offspring that can disperse (Denno and Roderick, 1992). However, research on hormone-mediated effects of parental social experience on offspring reproductive strategy remains scarce, largely because of the logistical issues associated with measuring the very small quantities of hormones present in insects and their eggs. Of notable exception are studies on the infamously polyphenic desert locust species, *Schistocerca gregaria*: these studies are both numerous and extend to epigenetic maternal effects (reviewed in Maeno and Tanaka, 2010). Female desert locusts respond to social crowding by producing gregarious offspring, a polyphenic phase morphologically and behaviorally distinct from the solitary phase in desert locusts (Maeno et al., 2013). While variable hormone deposition to eggs is not responsible for the shift between phases (Simpson and Miller, 2007), substantially higher concentrations of ecdysteroid hormones (ESH, steroid hormones ubiquitous in invertebrate species) are found in the fresh eggs of crowded females, compared to ESH concentrations in fresh eggs produced by solitary females (Hägele et al., 2004). Further, the eggs produced by crowded females produce larger hatchlings than those laid by isolated females, although they take longer to mature (Uvarov, 1966). These findings are consistent with our previous work on house crickets (*Acheta domesticus*), which has shown that an elevated concentration of ESH available to fresh eggs (less than 24 hours old) results in larger hatchlings (Crocker et al, 2018).

Acheta domesticus is an excellent system for studying hormone-mediated maternal effects. Unlike those of the polyphenic *S. gregaria*, the general ecology and life history of *A. domesticus* do not depend on a polyphenic phase. Therefore, house crickets are suitable for investigating smaller-scale, nuanced effects of variable hormone provisioning on offspring phenotype. Female house crickets provision their eggs with highly variable concentrations of ESH in sufficient quantities to measure (Dinan, 1997). Moreover, variable social conditions influence orthopteran development, affecting adult phenotype, endocrine status and reproductive decisions in many species within the order (Bertram et

al., 2012; Crankshaw, 1979; Hägele et al., 2004; Tinghitella, 2014; Woodring et al., 1988). *A. domesticus* is amenable to lab rearing, which makes it an expedient organism with which to investigate trans-generational effects of variable maternal hormone deposition to eggs. Finally, we have already shown that variation in egg ESH provisioning translates into variation in offspring life history traits, whereby increased ESH provisioning results in larger, faster-growing hatchlings (Crocker et al., 2018). Here, we ask whether social environment is an important determinant of ESH provisioning in eggs.

In our study, we assessed the effects of social environment on maternal hormone deposition in house cricket eggs. We hypothesized that variable deposition of ESH to eggs is a predictive adaptive maternal response to environmental stressors. Specifically, we tested the hypotheses that variable deposition of ESH to eggs is affected by both (1) social density of the maternal environment (“social density”) and (2) the sex ratio of the maternal environment (“sex ratio”). Addressing our first hypothesis, we predicted that in high social density, female crickets would deposit more ESH to their eggs (resulting in larger, faster-growing hatchlings). Regarding our second hypothesis, we predicted that female house crickets reared in a female-skewed environment would deposit more ESH to their eggs (thus, producing larger, faster-growing hatchlings) than would females reared in male-skewed social environments. We made this prediction because we expected that the amount of resource competition experienced by offspring would be more closely linked to the number of females in their mother’s environment, rather than the density of all adult crickets.

We measured changes in ESH provisioning by female *A. domesticus* raised under varying social densities and compositions in a laboratory experiment. We found that while the ESH provisioned by mothers to their eggs does not reflect social composition (sex ratio), it does reflect social density. Specifically, female crickets reared at low population density provided higher concentrations of ESH to their eggs than did those raised in high population density, regardless of sex ratio. Maternal hormone provisioning of ESH to eggs thus links maternal social environment to the phenotype of her offspring.

METHODS

Experimental methods

We reared crickets following the general methods described by Crocker et al (2018) and Tinghitella (2014), briefly summarized here. We ordered 1500 juvenile crickets from reptilefood.com, and raised them to maturity in a communal bin. After approximately a third of the crickets had reached sexual maturity (estimated visually by the appearance of wings), we provided a shallow dish of damp coconut fiber into which female crickets oviposited. After four days, we removed the dish and incubated it at 30°C, checking daily for hatchlings. We separated 4000 hatchlings into 160 perforated 12-oz to-go salad containers: 120 containers had 30 hatchlings each (high density population), 40 containers had 10 hatchlings each (low density population) (both population densities were modeled after those described by Tinghitella (2014)). All containers were provided with food (ground Harlan Teklad Rodent Diet 8604) and water (in wet gravel-filled 1-oz plastic cups) *ad libitum* (modeled after Whattam and Bertram, 2011), and all densities were maintained throughout the experiment.

As crickets grew, we increased the size of their rearing containers, but maintained them at their appropriate experimental densities. Every week, we counted hatchlings in each salad container: containers that had fewer than the 30 or 10 crickets necessary were combined to result in fewer overall replicates, but consistent population density. At no time in the experiment were any crickets switched between high- and low-density treatments. When hatchlings were three weeks old, we transferred crickets from their original replicate containers to larger (32-oz) perforated to-go salad containers, maintaining their experimental densities. Six weeks after hatching, but before maturation, crickets can be sexed (Crocker, personal observation): we then transferred crickets into their final experimental containers. For these containers, we used screen-lidded 14-liter bins in which we maintained cricket densities, but also created three different sex ratios for the high-density crickets: equal, female-skewed, and male-skewed (Table 12). We created a total of 24 experimental bins, with the replicate number of bins per treatment provided in Table 12. We stored all bins in a 6 x 4 array of soundproofed, subdivided shelves insulated with 2” thick egg-crate acoustic foam and SoundBreak XP Drywall.

After transferring crickets to the screen-lidded bins, we checked all bins daily for crickets that had reached maturity. Six females from each high-density bin, and three females from each low-density bin, were chosen to serve as focal females in the experiment. To control for any confounding effects of a cricket's latency to mature relative to the population she interacted with, we chose focal females in all bins by the ordinal number in which they matured (e.g. in low-density bins, the first, third, and fifth females to mature). For each of these focal females, we then calculated her age at maturity as the number of days after hatching at which she reached her imaginal molt (assessed visually by the presence of wings and fully formed ovipositor).

To distinguish focal females, we marked each cricket's pronotum with a dot of paint when they reached maturity: focal females were marked with a unique color; all other crickets were marked with white. Every evening, we isolated focal females individually in small screen cages within their respective bins. Overnight, focal females were provided with individual oviposition dishes of damp sand, and water. Non-focal females were provided with a communal oviposition dish of damp sand, and water: food was removed from all bins overnight to control for any effect on focal females. Each morning after oviposition dishes were collected, focal females were released into the bin to eat and interact with conspecifics in their replicate bin.

We measured the size at maturity of each focal female by photographing her over a millimeter-demarked ruler. Then, in Adobe PhotoShop 7.0, we measured each cricket's pronotum width (in pixels) as an indicator of structural size (Kelly et al., 2014). To convert our pixel measurement to millimeters and standardize our measurements between crickets, we then measured the width of the photographed millimeter in pixels, and used this to convert the cricket measurement.

For each focal female, we recorded the length of time (days) between reaching maturity and laying their first batch of eggs (hereafter, lay latency). All eggs were collected as described by Crocker et al. (2018) and stored at -20°C (Warren et al., 2006). We used a

subset (N = 30) of each focal female's eggs to quantify the mass of active ESH present per egg using an enzyme immunoassay protocol described previously (Crocker et al., 2018). For each female, we used eggs from the first clutch laid. In under 10% of cases, crickets laid fewer than 30 eggs in their first clutch, and we supplemented the sample using eggs from the second clutch. *A. domesticus* do not vary in their ESH provisioning of eggs over their lifetime (Crocker et al., 2018).

Statistical methods

We used separate general linear models (SAS 9.4) to assess the impact of (1) social density and (2) social composition on maternal provisioning of ESH to eggs. When assessing effects of social density on egg ESH concentrations, we compared only those crickets reared under equal sex ratios (Table 12). When assessing effects of sex ratio on ESH, we compared only those crickets reared at high density (Table 12). ESH concentrations were log-transformed prior to analysis to meet conditions of homogeneity of variance and were the dependent variables in the models. Social density (high/low) or sex ratio (equal, female-skewed, male-skewed) were class variables in the separate models.

We asked whether a female's body size, age at maturity, or lay latency was associated with the amount of ESH that she provided to her eggs. We ran general linear models using adult body size, age at maturity, or lay latency as independent variables, and egg ESH concentration (log-transformed) as the dependent variable. Finally, we asked whether female body size interacted with either social density or social composition to influence ESH concentration in offspring eggs.

RESULTS

We provide the means for cricket traits (focal female size, ESH/egg, age at maturity, and lay latency) among treatment groups in Table 13, and the results of statistical models of variation in ESH/egg in Table 14. Female crickets provided higher concentrations of ESH to their eggs when raised in low density groups than they did when raised in high density groups ($F_{1,42} = 49.82$, $p < .0001$; Figure 17, Tables 13 & 14). In contrast, the sex ratio

under which female crickets were reared had no impact on the ESH concentrations that they allocated to eggs ($F_{2,70} = 2.52$, $p = 0.088$; Figure 18, Tables 13 & 14).

Indeed, social density was the only significant predictor of ESH concentration in eggs among those variables that we measured. Female size was not associated with ESH allocation to eggs, and neither age at maturity, nor the length of the period between reaching maturity and starting oviposition was linked with ESH concentrations in cricket eggs (Table 14).

DISCUSSION

Our results demonstrate that female house crickets reared at high density allocate lower concentrations of ESH to their eggs than do crickets reared at low density. Consistent with work on the related species, *Schistocerca gregaria* (Hägele et al., 2004), we predicted that we would observe a difference in provisioning strategy between crickets raised in high and low density treatments. Previous experiments in this same system have shown consistently that higher concentrations of ESH result in faster juvenile growth rates (Crocker et al 2018).

Given the accelerating effect of ESH on early juvenile growth rates, we can infer that crickets reared at low density will produce fast-growing offspring, whereas crickets reared at high density will produce slow-growing offspring. In this experiment, we expected that crickets reared at higher social densities would prime their offspring to encounter competition by depositing higher concentrations of ESH into their eggs, thus producing larger offspring and speeding their development (Crocker et al 2018). Our results indicate that while a female cricket's ESH provisioning strategy responds to her social environment, it does so in the opposite direction to that we predicted (Figure 17, Table 14).

In most cases reported previously, high conspecific density in the parental environment is associated with maternal effects that favor rapid offspring growth rates, presumably to accelerate consumption of declining resources (Burton et al., 2011; Dantzer et al., 2013; Denno and Roderick, 1992; Hayward and Wingfield, 2004; Kemme et al., 2009; Lester et

al., 2005). However, we note that a majority of these studies have been conducted with vertebrates (fish, birds and mammals) that have relatively little flexibility in their metabolic rates, irrespective of resource supply.

In contrast, the observed pattern of provisioning may be adaptive for populations that oscillate around an ecologically optimal density (Witting, 2000), especially in taxa that have metabolic flexibility. Female crickets under high density may program their offspring to grow more slowly in order to increase their likelihood of survival in resource-limited conditions. For example, in stressful conditions, a reduced metabolic budget (which is associated with slower growth rates) can increase an invertebrate's likelihood of survival (Nespolo et al., 2003; Wallace, 1973). This framework also explains the higher provisioned level of ESH to eggs laid by females that experienced the low-density social condition. At low population densities, fast-growing individuals are more likely to gain higher fitness than (and even cannibalize (Crocker, personal observation)) smaller conspecifics.

Density experienced by mothers has been found to alter the number of offspring they produce, in addition to the phenotype and growth of those offspring. For example, in the desert locust, crowded females produce fewer, larger eggs than solitary females, and optimize their offspring to survive starvation conditions (Maeno et al., 2013). Similarly, female gypsy moths (*Lymantria dispar*) detect increased social densities via altered phytochemicals in the leaves on which they feed, and respond by epigenetically programming their offspring to become heavier at pupation (Rossiter, 1991). Following this same pattern, females of both the *Delphacidae* species *Prokelisia marginata* and *P. dolus* respond to increased social density by altering the phenotype of their offspring: at low densities, offspring in both species are flightless, but in crowded conditions, they produce fully winged offspring that can disperse (Denno and Roderick, 1992).

Our data are consistent with variable ESH provisioning functioning as an adaptive maternal response to social environment, and congruent with similar patterns of variable maternal provisioning in response to environmental stressors that have been observed in

other taxa (Reviewed in Mousseau and Fox (1998), and Uvarov (1966); see Dantzer et al. (2013), Schwabl et al. (1997), and Rossiter (1991) for selected examples). However, more work is needed in this area. For example, if our conclusions are correct, we predict that an intermediate population density of crickets would result in an intermediate level of ESH provisioning to eggs. Variable maternal hormone provisioning is a promising context in which to investigate adaptation to variable environments (Dantzer et al., 2013;; Jablonka et al., 1995; Mousseau and Fox, 1998a; Schwabl et al., 1997; Witting, 2000), particularly given current rates of environmental change. Finally, density-mediated variation in egg hormone provisioning provides a potential mechanism underlying the time-lagged effects of parental environment on offspring life-history that can destabilize population dynamics and generate outbreaks (Rossiter, 1991; Ginzburg and Taneyhill, 1994).

ACKNOWLEDGEMENTS

This work was done on the occupied land of Odawa, Chippewa, Wendat, and Pottawatomi Nations, where Ann Arbor, Michigan, is currently located. Critical assistance in the laboratory was provided by Rebecca Schwutke, Rumi Deb, Else R. Peterson, and Abigail Potts. Valuable feedback on the manuscript and experimental procedures was provided by Amanda R. Meier, Leslie E. Decker, Hillary B. Streit, and Kristel Sanchez. Many thanks also to Dr. Pascale R. Leroueil, and to the several thousand crickets who contributed their lives to these experiments.

Funding sources: This work was funded by Doctoral Dissertation Improvement Grant IOS-1601455 from the National Science Foundation written by Katherine C. Crocker, awarded jointly to Katherine C. Crocker and Elizabeth A. Tibbetts.

TABLES

Table 12: Summary of experimental conditions. Group size refers to the number of crickets in each bin during an individual’s lifetime. Columns labeled “Males per bin” and “Females per bin” refer to the group composition formed at 6 weeks after hatching. The replicates column shows the number of experimental replicates (bins) that were used for each social condition. The low density treatment had three focal females per bin while all other treatments had six. The column labeled “Total” indicates the number of samples collected for each social condition.

Social condition	Group size	Males per bin	Females per bin	Replicates (N bins)	Focal females per bin	Total (replicates * focal females)
High density	30	15	15	6	6	36
Low density	10	5	5	12	3	36
High female	30	10	20	6	6	36
High male	30	20	10	6	6	36

Table 13: Effects of density and sex ratio (Composition) on female pronotum size, time to maturity, time between reaching maturity and initiating oviposition, and ESH provisioning per egg. Data are mean values and standard errors. We have included the results of post-hoc statistical comparisons among the groups in each column; statistical similarity ($\alpha = 0.05$) is denoted by the same letter superscript before each mean, while statistical difference ($\alpha = 0.05$) is indicated by differing superscript letters. Note that the sample sizes here (in the column labeled “N”) differs from that in Table 12, because we selected a subset of focal females from the groups described by Table 12 to use in our molecular analyses below.

Comparison	Treatment	N	Avg size (mm)	Avg ESH/egg (log(pg))	Maturity age (days)	Lay Latency (days)
<i>Density</i>	High	24	^a 4.83 ± 0.13	^a 0.655 ± 0.04	^a 70.59 ± 1.53	^a 18.68 ± 1.43
<i>Density</i>	Low	27	^a 5.01 ± 0.08	^b 0.998 ± 0.04	^a 68.04 ± 1.33	^a 18.93 ± 1.72
<i>Composition</i>	Equal	24	^a 4.83 ± 0.13	^a 0.655 ± 0.04	^a 70.59 ± 1.53	^a 18.68 ± 1.43
<i>Composition</i>	High female	26	^a 5.1 ± 0.08	^a 0.759 ± 0.04	^a 70.95 ± 1.82	^a 20.85 ± 1.39
<i>Composition</i>	High male	23	^a 4.7 ± 0.09	^a 0.722 ± 0.04	^a 69.10 ± 1.97	^a 19.4 ± 0.96

Table 14: Statistical models to assess potential drivers of variation in ESH provisioning of eggs by crickets. ‘Comparison’ refers to whether we were testing among groups that differed in density or in social composition (sex ratio). The column labeled ‘Model’ states the model formula we tested. The columns labeled ‘F value’ and ‘p value’ are the F and p values, respectively, for each model. We assessed any interactive effects between cricket size and (a) density and (b) sex ratio on ESH provisioning. We have reported the non-significant interactions here, but excluded them from the final models of size, sex ratio, and density.

Comparison	Model	F value	p value
Density	<i>ESH/egg ~ Density</i>	$F_{1,42} = 49.82$	$< .0001$
Density	<i>ESH/egg ~ Female Size</i>	$F_{1,37} = 1.84$	0.183
Density	<i>ESH/egg ~ Lay Latency</i>	$F_{1,42} = 1.01$	0.321
Density	<i>ESH/egg ~ Age at Maturity</i>	$F_{1,40} = 0.02$	0.880
Density	<i>ESH/egg ~ Density * Size</i>	$F_{1,35} = 0.13$	0.723
Composition	<i>ESH/egg ~ Sex Ratio</i>	$F_{2,70} = 2.52$	0.088
Composition	<i>ESH/egg ~ Size</i>	$F_{1,60} = 0.99$	0.324
Composition	<i>ESH/egg ~ Lay Latency</i>	$F_{1,71} = 0.54$	0.467
Composition	<i>ESH/egg ~ Age at Maturity</i>	$F_{1,60} = 3.17$	0.080
Composition	<i>ESH/egg ~ Sex Ratio * Size</i>	$F_{2,56} = 0.66$	0.523

FIGURES

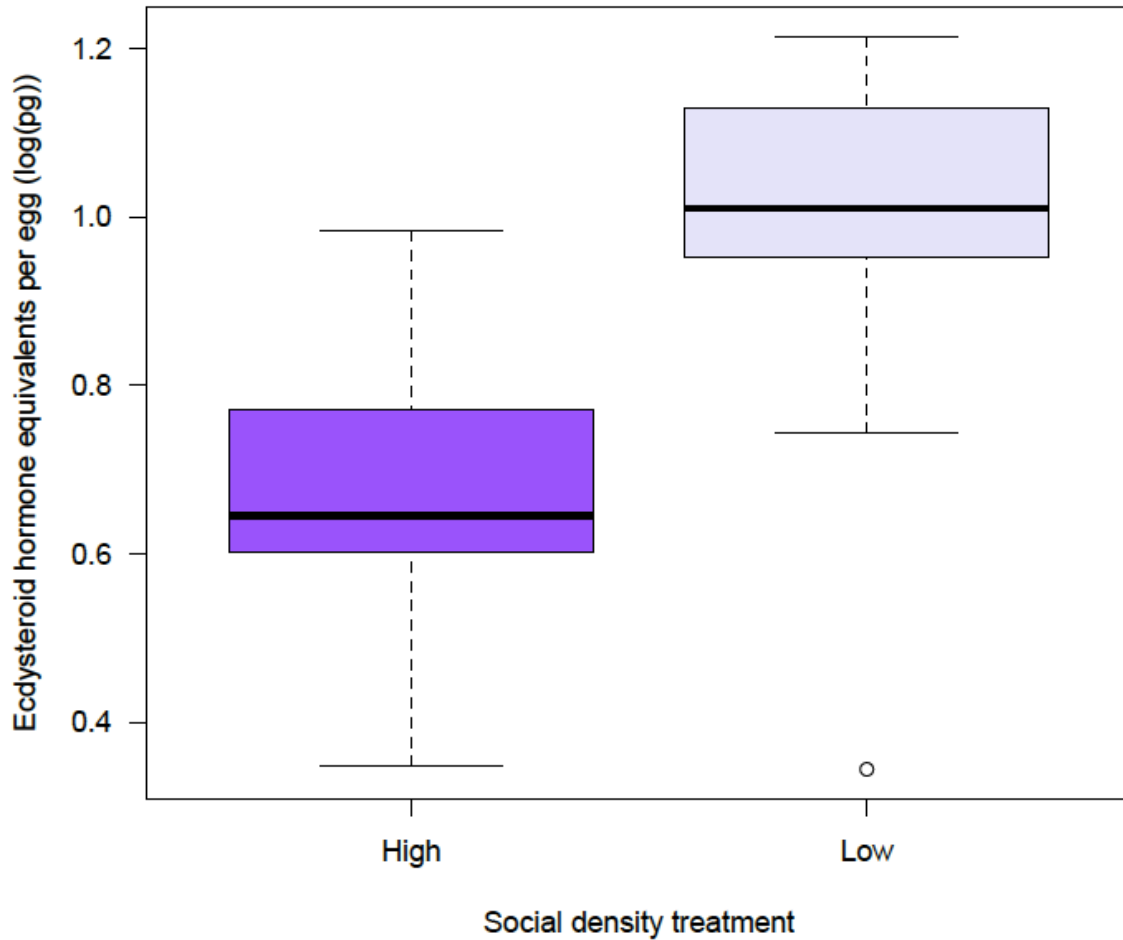


Figure 17: Boxplot depicting the mean ESH provided to eggs by *Acheta domesticus* females reared in high and low density environments. Females reared in high density environments deposited less ESH in their eggs than did females reared in low density environments ($F_{1,42} = 49.82$, $p < 0.0001$).

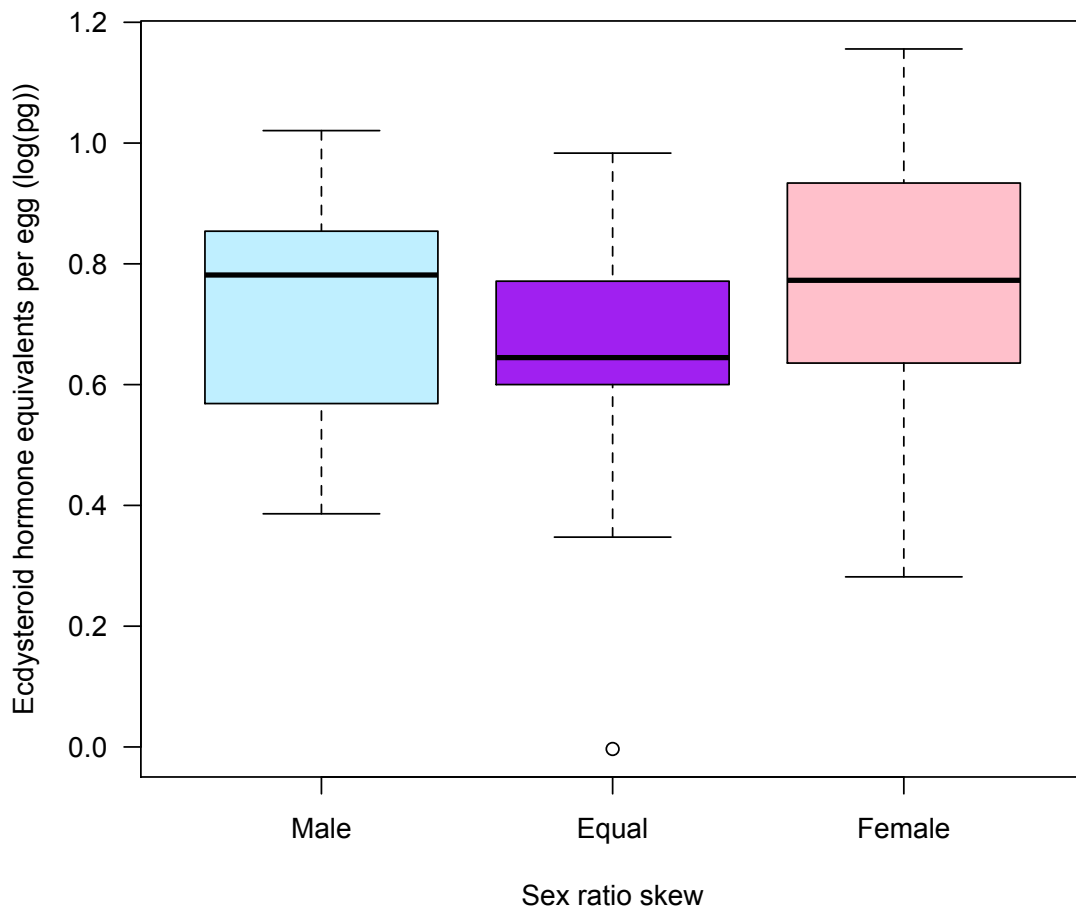


Figure 18: Boxplot depicting the concentrations of ESH that female *Acheta domesticus* deposited in their eggs under different social compositions (sex ratios). Neither Male-skewed (Male) nor Female-skewed (Female) sex ratios induced females to deposit ESH in their eggs in concentrations that differed from females in equal sex ratio (Equal) environments ($F_{2,70} = 2.52$, $p = 0.088$).

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Chapter V Population differences in maternal provisioning of eggs with ecdysteroid hormones in two wild Gryllid species

ABSTRACT

As climate change decreases environmental stability, understanding the physiological mechanisms by which multiple generations of organisms adapt to their environments has become increasingly urgent. There is growing evidence that nongenetic inherited characteristics and environments can powerfully affect animal fitness and ecology. Despite clear challenges in predicting future environmental conditions, adaptive maternal effects provide a mechanism of passing environmental information from parent to offspring, and are now considered pervasive in natural systems. Maternal effects have been widely studied in vertebrates, especially in the context of social environment, and are often mediated by steroid hormone (SH) deposition to eggs. To broaden the contexts and extend the taxonomic range in which transgenerational hormone-mediated maternal effects have been investigated, we captured individuals of two wild Gryllid species, each at two different latitudes. We then measured the concentration of ecdysteroid hormones (ESH) that females deposited into their eggs using organic extraction and enzyme immunoassay. We then raised their offspring in a common garden, and compared the developmental phenotypes of their offspring. Based on our previous lab studies, we hypothesized (1) that female field crickets from different latitudes would vary in the amount of ESH they provided to their eggs, and that (2) northern populations would use provisioning of ESH to speed the growth and development rate of their offspring in response to a shorter growing season. Here we report that while neither species responds to latitude in its provisioning of ESH to eggs, both exhibit substantial variation in provisioning among sites within latitudes. In both cricket species, the concentration of ESH provided to eggs impacts offspring phenotypes. We suggest that further study of maternal hormone provisioning would be profitable, as our results imply it is both a

mechanism of local adaptation and a potential compensatory response to climatic variability.

Key words: Ecdysteroid hormones, *Gryllus veletis*, *Gryllus pennsylvanicus*, carry-over effect, maternal effect, local adaptation

INTRODUCTION

How do parents prepare their offspring to cope with the environmental stressors they are likely to face? As climate change increases environmental variability, understanding the physiological mechanisms by which multiple generations of organisms adapt to their environments has become increasingly urgent (Parmesan and Yohe, 2003). Theoretical work predicts that parents that are able to fine tune their offspring to the future environments they face (via transgenerational “carry-over effects”) will outperform those relying solely on phenotypic plasticity within single generations to combat environments that are unpredictable (Jablonka et al., 1995).

There is growing evidence that nongenetic inherited characteristics and environments can powerfully affect animal fitness and ecology (Dantzer et al., 2013; Forbey and Hunter, 2012; Houde et al., 2015; Lefevre et al., 2010; Muriel et al., 2015; Shallow et al., 2015; Stein and Bell, 2014; Tao et al., 2016). For example, monarch butterflies and female red squirrels both act to increase their offspring’s fitness by altering their chemical environment during development (Dantzer et al., 2013; Forbey and Hunter, 2012). Monarch butterflies (*Danaus plexippus*), when infected with a vertically transmitted parasite, preferentially lay their eggs on *Asclepias* species with high concentrations of chemicals that, when ingested by offspring, reduce the growth rate of the parasite (Lefevre et al., 2010). Female red squirrels (*Tamiasciurus hudsonicus*) respond to increased competition for food and territory by elevating the concentration of glucocorticoids in their milk, which increases the growth rate of their offspring (Dantzer et al., 2013)

Varying the concentration of hormones and other chemicals available to offspring is a common mechanism of altering offspring phenotype (Grootuis et al., 2005), though others, such as maternal small RNAs, are also powerful (Babenko et al., 2015). Such variability in hormone provisioning can dramatically affect offspring phenotype and fitness (Champagne, 2011): effects may be immediate (Schoech et al., 2011) or delayed (Champagne, 2008); temporary or sustained (Schoech et al., 2011). For instance, in the spotted hyena (*Crocuta crocuta*), ambient concentrations of testosterone experienced by female embryos affect the competitive ability of those individuals throughout their lives (Dloniak et al., 2006; Drea et al., 1998).

However, hormone-mediated transgenerational effects are not always adaptive (Schoech et al., 2011). For instance, female mice (*Mus musculus*) that experience relatively little interaction with their mothers are less likely to thrive, and become inattentive mothers themselves upon reaching maturity (Champagne, 2013). More extremely, the negative consequences of maternal hormones for threespine stickleback (*Gasterosteus aculeatus*) embryos are strong enough to act as a selective pressure. Threespine stickleback embryos have evolved ATP-powered cellular machinery that they use to insulate themselves from the detrimental effects of maternal hormones by removing those molecules from their cells (Paitz et al., 2016). Variable hormone provisioning, and its effects, is thus a product of maternal environment, physiology, and genotype, and its effects likely vary depending on context and species (Bonduriansky and Head, 2007; French et al., 2013)

To broaden the contexts and extend the taxonomic range in which transgenerational hormone-mediated maternal effects have been investigated, we compared the reproductive and developmental phenotypes of two species of field cricket that differ in their hibernal diapause strategies, yet co-occur across a range of latitudes. Orthoptera provision their eggs with ecdysteroid hormones (ESH), a class of steroid hormone that drives embryonic development and life stage transitions in arthropod species (Smagghe, 2009). The concentration of ESH deposited in eggs by Orthopteran mothers has been found to vary with maternal environment (Dinan, 1997; Hägele et al., 2004). We studied *Gryllus veletis* and *G. pennsylvanicus*, which are broadly distributed across North

America (Alexander and Bigelow, 1960). Within each species, we compared northern Michigan to southern Michigan populations. Based on our previous lab studies of house crickets (Crocker et al., 2018), we hypothesized (1) that female field crickets from different latitudes would vary in the amount of ESH they provided to their eggs, and that (2) northern populations would use provisioning of ESH to speed the growth and development rate of their offspring in anticipation of oncoming winter. We also predicted that, in a common garden, offspring provided with more maternal ESH would grow more quickly than those that received less. Ours is the first study of variation in ESH provisioning to eggs of wild insects in temporally and spatially varying ecological contexts.

STUDY SYSTEM

Current ecological (Levins, 1969) and evolutionary (Dobzhansky and Pavlovsky, 1957) theory owes much to studies of insects, but relatively little is known about whether and how wild insects can program their offspring using hormones. Although insect hormones have been extensively researched in the context of biological control and embryonic development (Smagghe, 2009), their effects remain unconnected by a general model of the ontogenetic effects of early and embryonic hormone exposure to life history in the field.

Ecdysteroid hormones (ESH), a major invertebrate hormone group, are important for embryonic development, molting, circadian rhythms, and reproduction in insects (Cymborowski, 1994; Deloof et al., 1984; Gabrieli et al., 2014; Sehnal, 1989; Spindler et al., 2009). For example, in both *Drosophila melanogaster* and *Manduca sexta*, ESH are crucial for wing imaginal disk formation, without which neither insect would develop functional wing structures (Herboso et al., 2015; Nijhout et al., 2007). In *Drosophila melanogaster*, ESH also facilitate pre-metamorphosis larval light preference behavior (Yamanaka et al., 2013). The effects of ESH are not limited to development: mature female mosquitoes (*Anopheles gambiae*) post-mating behavior is altered allohormonally as a result of her mate transferring ESH to her during mating (Gabrieli et al., 2014). ESH in insect eggs occurs in both active (“free”) and inactive (“conjugated”) forms (Dinan and Rees, 1981; Whiting, 1988). Free ESH are endocrinologically active, and are responsible

for driving embryogenesis and cell differentiation; conjugated ESH are endocrinologically inactivated, but can be activated by embryos during development (Smagghe, 2009). Most work on ESH has focused on the immediate mechanistic role of free ESH in model systems such as *Manduca* and *Drosophila*, which are often studied in isolation from the natural world. But there is evidence that ESH can link juvenile environment to adult behavior and to other ecologically relevant traits (Cooper et al., 2003; Crocer et al., 2018; Oostra et al., 2014).

We studied two cricket species native to Michigan to investigate potential effects of latitude on hormone provisioning and offspring life history. Specifically, we chose the spring field cricket (*Gryllus veletis*) and the fall field cricket (*G. pennsylvanicus*). We chose these two cricket species because, while many aspects of their ecologies are nearly identical (their range and distribution overlap broadly), their life history traits are distinct (Alexander and Bigelow, 1960; Cantrall, 1943). Spring field crickets lay eggs in late spring, and hatch approximately 10 days later. Hatchlings develop through several nymphal stages throughout the rest of the summer, and undergo diapause as nymphs in small burrows or in leaf litter over the winter. In early spring, nymphs emerge and continue their development. They reach sexual maturity in late spring, when we collected mature individuals for our experiment. In contrast, fall field crickets lay eggs in early- to mid-fall. Eggs enter hibernal diapause and hatch in early spring. Hatchlings develop over the summer, reaching sexual maturity in late summer, when we collected mature individuals for our experiment.

METHODS

Collection and husbandry of wild-caught crickets

For each species, we collected mature crickets from three or four locations (replicate sites) at each of two latitudes (Table 1), hereafter referred to as upper and lower latitudes. Crickets were collected by both opportunistic hand-catching and by pitfall traps constructed of 32-oz polypropylene containers sunk into the ground and covered by a weathered 18" plywood square propped up on pebbles. No cricket was left in a trap for longer than 15 hours (overnight).

After capture, crickets were grouped into mating pairs (details below); each pair was housed in a perforated 12oz deli cup and provided with food (powdered Harlan Teklad Rodent Diet 8604) and water (in cotton-plugged 5-dram shell vials) ad libitum. Mating pairs remained constant throughout the experiment, though mature crickets are likely to have mated before capture. All mating pairs were housed in environments that matched the average day- and nighttime temperatures and photoperiods of their original latitude during the mating period (Tables S1, S2). For *G. veletis*, we used temperature controlled growth chambers for both latitudes (Norlake walk-in chamber with temperature controlled by a Watlow Digital Temperature Controller and monitored by a Honeywell Rotational Trace Monitor). For *G. pennsylvanicus*, as one of the two available chambers malfunctioned, we kept all lower-latitude mating pairs in a shaded, sheltered location outdoors at the appropriate latitude. In all chambers, we maintained humidity between 60-80% RH.

When assigning mating pairs, we prioritized individuals captured at the same location during the same three-day period, but in some cases pairings across locations were necessary. No mating pairs included crickets from different latitudes. At the lower latitude (southeast Michigan), crickets of both species were captured during a 3-week period; at the upper latitude (northern lower peninsula and Sugar Island), they were caught during a single week.

Before being placed in a mating pair, each cricket's pronotum width (an indicator of structural size) was measured as a proxy for parental condition (Kelly et al., 2014). Every day, all mating pairs were provided with a fresh 15mL cup of damp sand in which females deposited their eggs. All sand cups were checked for eggs every day. We identified eggs by swirling sand through excess water; eggs were easily collected by filtration. All eggs were counted, weighed, and divided into two equally sized groups: one group was frozen under pure ethanol at -20°C for later enzyme immunoassay analysis (EIA) as described elsewhere (Crocker et al., 2018), the other was incubated and reared.

Incubation of eggs and rearing of hatchlings

Some rearing conditions were specific to the ecologies of each species (below), whereas other rearing conditions were common to both species. For both species, eggs were incubated on a damp cotton pad in 60mm Petri dishes. We induced diapause for Winter 2016-17 by modifying the environment within a temperature-controlled growth chamber (Norlake walk-in chamber with temperature controlled by a Watlow Digital Temperature Controller and monitored by a Honeywell Rotational Trace Monitor). Specifically, day and nighttime temperatures were decreased beginning on October 4, 2016 at a rate of 1 degree C per day until the nighttime temperature reached 4°C; we continued to decrease daytime temperature at the same rate until they, too, reached 4°C. These conditions were maintained until May 4, 2017, when we began increasing temperature uniformly at a rate of 1 degree C per day until the temperature reached 27°C. Species-specific rearing protocols are described below:

Gryllus veletis:

For *G. veletis* eggs, all Petri dishes were incubated at 27.5°C and checked daily for moisture, mold, and hatchlings. All hatchlings were reared individually in 60mm Petri dishes and provided with food and water *ad libitum*. All hatchlings from both latitudes were raised in a common garden at 21°C and photoperiod corresponding to Clare, Michigan (halfway between our upper and lower latitudes) (Table 1).

Gryllus veletis nymphs spend the winter in hibernal diapause: during this time, *G. veletis* nymphs were checked biweekly for survival and mold. If we discovered mold we moved the nymph to a new Petri dish. Although the majority of *G. veletis* nymphs survived diapause, none survived the diapause termination; therefore no data conclusions are presented here regarding their phenotypes after October 4, 2016. Final sample sizes of eggs and nymphs from each site are provided in Table 1.

Gryllus pennsylvanicus:

For *G. pennsylvanicus* eggs, all Petri dishes were first kept at 27.5°C, then slowly introduced to overwintering temperature as described above. *G. pennsylvanicus* adults

produce diapause eggs, which hatch in the spring. During diapause *G. pennsylvanicus* eggs were checked biweekly for survival and mold. If we discovered mold we moved all unaffected eggs to a clean damp cotton pad in a fresh Petri dish using a small paintbrush sterilized in ethanol. We noted the number of affected eggs before discarding them. Final sample sizes of eggs and nymphs from each site are provided in Table 1.

Hormone quantification and statistical methods

For all hatchlings, we measured latency to hatch (in days), pronotum width (a proxy for structural size) each week, growth rate, and the average mass of ecdysteroid hormones (ESH) provided per egg by their mother (thus, one measure of ESH provisioning exists per wild-caught mother). To measure hatchling structural size, we photographed each cricket above a mm-demarcated ruler each week and used Adobe PhotoShop to measure each cricket's pronotum width and calculate its growth rate. We calculated growth rate as the percent increase in pronotum width across sample points (e.g. at N weeks of age, the growth rate = ((pronotum width at week N – pronotum width at week N-x) / pronotum width at week x)). We measured growth rate between one and two weeks after hatching to assess early growth rate of hatchlings. We wanted this separate measure of early growth rate because maternal effects on offspring life history traits often attenuate with offspring age (Mousseau & Fox, 1998). To assess longer-term growth rate, we calculated growth rate between one and eight weeks after hatching. We chose eight weeks for all crickets of both species, because the youngest cricket (*G. veletis*) was eight weeks old at the initiation of diapause. By terminating all estimates of long-term growth rate at week 8, we could standardize growth rate among crickets before any variation introduced by variable timing of diapause.

We quantified ESH mass using EIA methods described elsewhere (Crocker et al., 2018). Briefly, we extracted ESH from eggs by grinding them in cold methanol (MeOH), and decanting the MeOH from the pellet of solid egg material. We assayed both free (active) and conjugated (inactive) forms of ESH in each sample by incubating an aliquot of each sample with esterase overnight to hydrolyze the ester bond that inactivates conjugated ESH molecules (Dinan and Rees, 1981). We then used an Enzyme Immunoassay to

quantify the average mass of each form of ESH (free and conjugated) in the eggs of each female (Crocker et al., 2018).

Statistical analyses

We sought to answer two general questions with our analyses:

1. Is the extent of provisioning of either form of ESH linked to the latitude or site at which the mothers were collected?
2. For wild cricket hatchlings, is the variation in life history traits of hatchlings predicted by the mass of ESH provided to them by their mother?

To address our first question, we ran nested general linear models (SAS 9.4) using ESH contents (free, or conjugated) of eggs as response variables, and collection site and latitude as fixed effects. Specifically, we nested maternal collection site within the latitude at which she was collected. We ran analyses using the Satterthwaite approximation of denominator degrees of freedom (Littell et al., 2002).

To address our second question, we used a regression approach. The data gathered here provide replicate response (phenotype) values of hatchlings for each mass of ESH that we measured in a clutch of eggs. Such data can be analyzed by either regression or by ANOVA approaches (Sokal and Rohlf, 1995). ESH provisioning is a continuous variable, so we chose a regression approach to associate ESH concentration in eggs with offspring phenotype. To avoid pseudo-replication, we analyzed average phenotype values, weighted by the inverse of the variance (most conservative approach)(Sokal and Rohlf, 1995). We applied these methods to all measures of hatchling phenotype (the latency of each egg to hatch, offspring size after one week after hatching, initial growth rate (between weeks 1 and 2 after hatching) and overall growth rate (between weeks 1 and 8 weeks). Mean mass of ESH—either free or conjugated—provided by each cricket’s mother to her eggs was the independent variable in all models. Analyses were run using SAS (9.4).

Finally, we also ran the nested general linear models described above (nesting site within latitude, and using both as fixed effects) to assess any effects of latitude and site on hatch latency, structural size (one week and eight weeks after hatching), and both early and long-term growth rates of hatchlings.

RESULTS

1. Some, but not other, forms of ESH are provisioned variably with location

ESH provisioning by wild crickets to their eggs varied among collection sites, and between latitudes (Figure 19, Table 16). Specifically, spring field cricket eggs from northern latitudes had lower concentrations of conjugated ESH than did eggs from southern latitudes ($F_{1,5} = 21.57$, $p = 0.0051$, Figure 19, Table 16). Free ESH in the eggs of spring field crickets varied among collection sites ($F_{5,57} = 2.59$, $p = 0.0355$) but not between latitudes (Figure 19, Table 16). In contrast, concentrations of conjugated ESH in the eggs of fall field crickets did not vary between latitudes, but did vary among sites ($F_{5,55} = 5.88$, $p = 0.0002$, Figure 19, Table 16). The concentrations of free ESH in the eggs of fall field crickets varied neither among sites nor between latitudes (Figure 19, Table 16).

2. Variation in life history traits is predicted by variable ESH provisioning, but no clear pattern exists between species

For *G. veletis* hatchlings, the amount of conjugated ESH provided by mothers to their eggs was negatively associated with offspring size and growth rate (Table 3, Figure 20). Specifically, greater concentrations of conjugated ESH were associated with smaller size throughout life and a reduced early growth rate (Figure 20). In contrast, the concentration of free ESH provisioned to eggs was not associated with any of the offspring phenotypic traits that we measured in our study (size at one week old: $F_{1,61} = 1.14$, $p = 0.29$; size 8 weeks after hatching: $F_{1,59} = 0.44$, $p = 0.51$); early growth rate: $F_{1,61} = 2.65$, $p = 0.11$; overall growth rate: $F_{1,61} = 0.02$, $p = 0.89$).

As with *G. veletis*, the concentration of conjugated ESH in the eggs of *G. pennsylvanicus* was associated with offspring life history (Table 3, Figure 21). Specifically, high concentrations of conjugated ESH were associated with smaller size (Figure 21A), but

with increased nymphal growth rates (Figure 21B). Higher concentrations of conjugated ESH were also associated with more rapid egg hatch (Figure 22B). In notable contrast to the results for *G. veletis*, the concentration of free ESH provided to eggs was associated with multiple life history traits of offspring (Table 17). Increasing concentrations of free ESH were associated with faster egg hatch (Figure 22A), faster early growth (Figure 23A), and slower overall growth (Figure 23B).

Finally, of all offspring life history traits that we measured, only hatch latency was associated with either latitude or maternal collection site in either species (Figure 24). *Gryllus veletis* eggs varied in hatch latency according to the site from which their mothers were collected ($F_{8,54} = 2.87$, $p = 0.010$), but not according to the latitude of the site at which their mothers were collected ($F_{1,11} = 0.71$, $p = 0.420$) (Figure 24A). *Gryllus pennsylvanicus* eggs from mothers collected at sites located at lower latitudes hatched more quickly than eggs from mothers collected at sites located in upper latitudes ($F_{1,7} = 7.01$, $p = 0.033$), and also varied among sites independent of latitude ($F_{1,55} = 9.1$, $p < 0.001$) (Figure 24B).

DISCUSSION

Our results show that provisioning of ESH to eggs varies among populations of at least two species of wild Gryllid, and that this variation is associated with variation in life history traits of the resultant offspring. Variation in provisioning of ESH to eggs occurs across space much differently than we predicted. Because ESH provisioning can accelerate development in a lab-reared species (Crocker et al., 2018), we predicted that crickets in more northern latitudes would provide more ESH to eggs, as they must complete development during a shorter growing season than crickets in lower latitude sites.

In fact, we observed substantially more variation in ESH provisioning among sites than between latitudes with one exception: spring field crickets provided more conjugated ESH to their offspring in northern Michigan than they did in southern Michigan (Figure 19, Table 16). This general pattern is consistent with local adaptation to site-specific conditions. Such adaptation could be genetic or physiological (plastic, including maternal

effects). In laboratory populations of house crickets, we have observed significant, albeit relatively minor, genetic variation in provisioning of ESH (Crocker et al., 2018). However, wild field crickets should experience substantially greater variation in microclimate that could act to select for a particular provisioning strategy, or which generates a similar plastic ESH response (to variables we did not measure) that distinguishes crickets collected at various sites. We believe it is important to note that phenotypic plasticity cannot be said to be absent simply because organisms in a given environment respond similarly to that environment. In other words, local physiological adaptation may easily occur without reduction of gene flow, if all organisms have the phenotypic plasticity to respond similarly to an (in this case unmeasured) environmental pressure. Alternatively, wild cricket populations may experience low gene flow, or strong local selection, such that local genetic adaptation on a fine scale overwhelms any differences between latitudes. However, we do not believe that variation in the provisioning of ESH to eggs to be driven primarily by genotype (Crocker et al., 2018), and suggest that local physiological adaptation via maternal effects may be pronounced. The differences that we observed between species indicate that the natural history of ESH provisioning is related to the ecology of wild species, and that it is important to study organisms under ecologically relevant conditions. For instance, *Gryllus veletis* has a very similar life history to that of a laboratory-cultured species on which previous experiments on ESH provisioning have been done (*Acheta domesticus*). However, in stark contrast to those of lab crickets, the offspring of *G. veletis* respond obversely to elevated provisioning of ESH, and respond to the form (conjugated) that, in lab studies, has neither varied nor affected cricket development (Table 15, Figures 20 & 21) (Crocker et al., 2018). One potential explanation for this difference is that laboratory conditions may be such that all crickets in the laboratory population have responded plastically in order to provision their eggs with conjugated ESH at a constant level. Overall, this difference underscores the importance of testing laboratory-generated hypotheses in the natural world.

Similarly, although free ESH was observed to vary in the eggs laid by *G. veletis* according to site (Figure 19, Table 16), free ESH had no impact on the offspring life

history traits that we measured. This finding is in marked contrast to our previous laboratory experiments using the house cricket (*Acheta domesticus*), in which the concentration of free (but not conjugated) ESH provided by mothers to their eggs affected the phenotype of offspring (Crocker et al., 2018). Female house crickets provision their eggs with similar concentrations of ESH, and the eggs have a similar latency to hatch as the eggs of *G. veletis* (Crocker et al., 2018). We therefore expected to observe similarities between *G. veletis* and *A. domesticus* in terms of which form of ESH affected offspring phenotype, and what those effects were. We suggest that the differences we have described here are likely because ESH plays a more nuanced role in wild crickets than it does in laboratory cultured crickets. Again, the disparity in which form of ESH affects offspring, emphasizes the importance of validating the results of laboratory experiments by testing the same hypotheses in the field.

We were unable to explain variation in free ESH provisioning of eggs by *Gryllus pennsylvanicus* by either collection site or by latitude (Table 16). Nonetheless, variation in free ESH provisioning of *Gryllus pennsylvanicus* eggs was associated with rapid egg hatch (Figure 22A), and with nymphal growth rates (Figure 23). What drives individual variation in free ESH provisioning in *Gryllus pennsylvanicus* remains unclear. In other study systems, nongenetic maternal effects on offspring life history have been associated with the number of times a female has mated (horseshoe crabs (Johnson and Brockmann, 2013)), the quality of her mate or mates (hawkmoths (Levin et al., 2016)), whether any of her mates were virgin males (grapevine moths (Muller et al., 2016)), and by her body condition (northern leopard frogs (Bennett and Murray, 2014)). We were unable to collect immature crickets of either sex or species in this study, and so cannot account for these variables, but suggest that they merit further study.

Like *G. veletis*, *G. pennsylvanicus* nymphs that hatch from eggs with relatively large masses of conjugated ESH are smaller one week after hatching (Table 17, Figure 21A). However, *G. pennsylvanicus* offspring that received higher doses of conjugated ESH from their mothers generally hatched more rapidly and developed more quickly immediately after hatching (Table 16, Figures 22 & 23A). This particular finding is

consistent with previous findings (albeit, a response associated with provisioned free, rather than conjugated, ESH) in lab-reared house crickets (*Acheta domesticus*).

Here we report that variation in ESH provisioning to eggs in two wild cricket species is either constrained (via lack of gene flow) or caused (via universal plastic response to a habitat) by fine-scale geographic separation. Provisioning of both free and conjugated forms of ESH are generally associated with altered offspring life history traits, though both forms do not affect both species. More work is needed to explain causes of variation in ESH provisioning of eggs by wild Gryllids. We suggest that investigating the causes of such carry-over effects is key to understanding and predicting how species and ecosystems will respond to the increasingly variable environmental conditions. Maternal provisioning of varying concentrations of hormones to eggs is a promising potential mechanism for local adaptation and may become increasingly relevant to the survival of insect species, and the ecosystems that depend on their persistence (Wilson, 1987), under environmental change.

ACKNOWLEDGEMENTS

This work was done on the occupied land of Odawa, Chippewa, Wendat, and Pottawatomi Nations, where Ann Arbor and Pellston, Michigan, are currently located. Critical assistance in the laboratory and field were provided by Leslie E. Decker, Pascale R. Leroueil, Daniel T. Nondorf, Kendall Schissler, Rebecca Schwutke, Rumi Deb, Claire Freimark, and Haley Kalis. Valuable feedback on the manuscript and experimental procedures was provided by Amanda R. Meier, Leslie E. Decker, Hillary B. Streit, and Kristel Sanchez. Many thanks also to Dr. Pascale R. Leroueil, and to the several thousand crickets who contributed their lives to these experiments.

TABLES

Table 15: Latitudes and longitudes of all sites, the number of female crickets collected at each, the number of mothers from which a clutch of eggs was analyzed for ESH content, and the number of offspring from which life history characteristics were measured. Species codes are GV (for the spring field cricket, *Gryllus veletis*) and GP (for the fall field cricket, *G. pennsylvanicus*).

Species	Latitude	Longitude	N females collected	N clutches analyzed	N offspring measured
GV	42.301121	-83.664266	10	9	73
GV	42.305453	-83.751642	10	9	78
GV	46.357089	-84.135972	11	9	94
GV	42.309928	-83.75692	10	8	80
GV	45.557893	-84.649995	12	10	107
GV	45.561652	-84.679758	12	9	91
GV	45.557042	-84.66741	11	11	102
GP	42.286311	-83.743646	13	9	43
GP	42.297865	-83.663302	11	7	30
GP	42.303836	-83.662512	14	13	75
GP	45.560155	-84.668661	12	10	39
GP	46.351119	-84.141716	11	10	55
GP	45.557042	-84.66741	12	8	49
GP	45.561652	-84.679758	12	5	31

Table 16: Effects of collection site and latitude on free and conjugated ESH provisioning in the eggs of spring and fall field crickets. Species codes are GV (for the spring field cricket, *Gryllus veletis*) and GP (for the fall field cricket, *G. pennsylvanicus*).

Species	ESH form	F value site	p value site	F value latitude	p value latitude
GV	Free	$F_{5,57} = 2.59$	$p = 0.0355$	$F_{1,5} = 5.29$	$p = 0.0684$
GV	Conjugated	$F_{5,57} = 1.85$	$p = 0.1175$	$F_{1,5} = 21.57$	$p = 0.0051$
GP	Free	$F_{5,55} = 0.52$	$p = 0.7613$	$F_{1,5} = 0.03$	$p = 0.8782$
GP	Conjugated	$F_{5,55} = 5.88$	$p = 0.0002$	$F_{1,5} = 0.29$	$p = 0.6145$

Table 17: Associations between the free and conjugated ESH concentrations of cricket eggs and the average phenotypic traits of hatchlings. Species codes are GV (for the spring field cricket, *Gryllus veletis*) and GP (for the fall field cricket, *G. pennsylvanicus*).

Species	ESH form	Model	F value	p value
GV	Conjugated	<i>Growth rate weeks 1-2 ~ Conj ESH/egg</i>	$F_{1,61} = 4.16$	$p = 0.0457$
GV	Conjugated	<i>Structural size week 1 ~ Conj ESH/egg</i>	$F_{1,61} = 5.44$	$p = 0.0231$
GV	Conjugated	<i>Structural size week 8 ~ Conj ESH/egg</i>	$F_{1,59} = 4.13$	$p = 0.0466$
GP	Free	<i>Hatch latency ~ Free ESH/egg</i>	$F_{1,46} = 11.91$	$p = 0.0012$
GP	Free	<i>Structural size week 1 ~ Free ESH/egg</i>	$F_{1,54} = 39.48$	$p < 0.0001$
GP	Free	<i>Growth rate weeks 1-2 ~ Free ESH/egg</i>	$F_{1,61} = 4.16$	$p = 0.0457$
GP	Free	<i>Growth rate weeks 1-8 ~ Free ESH/egg</i>	$F_{1,48} = 126.54$	$p < 0.0001$
GP	Conjugated	<i>Hatch latency ~ Conj ESH/egg</i>	$F_{1,46} = 28.48$	$p < 0.0001$
GP	Conjugated	<i>Structural size week 1 ~ Conj ESH/egg</i>	$F_{1,54} = 57.93$	$p < 0.0001$
GP	Conjugated	<i>Growth rate weeks 1-2 ~ Conj ESH/egg</i>	$F_{1,53} = 6.14$	$p = 0.0164$
GP	Conjugated	<i>Growth rate weeks 1-8 ~ Conj ESH/egg</i>	$F_{1,48} = 109.36$	$p < 0.0001$

FIGURES

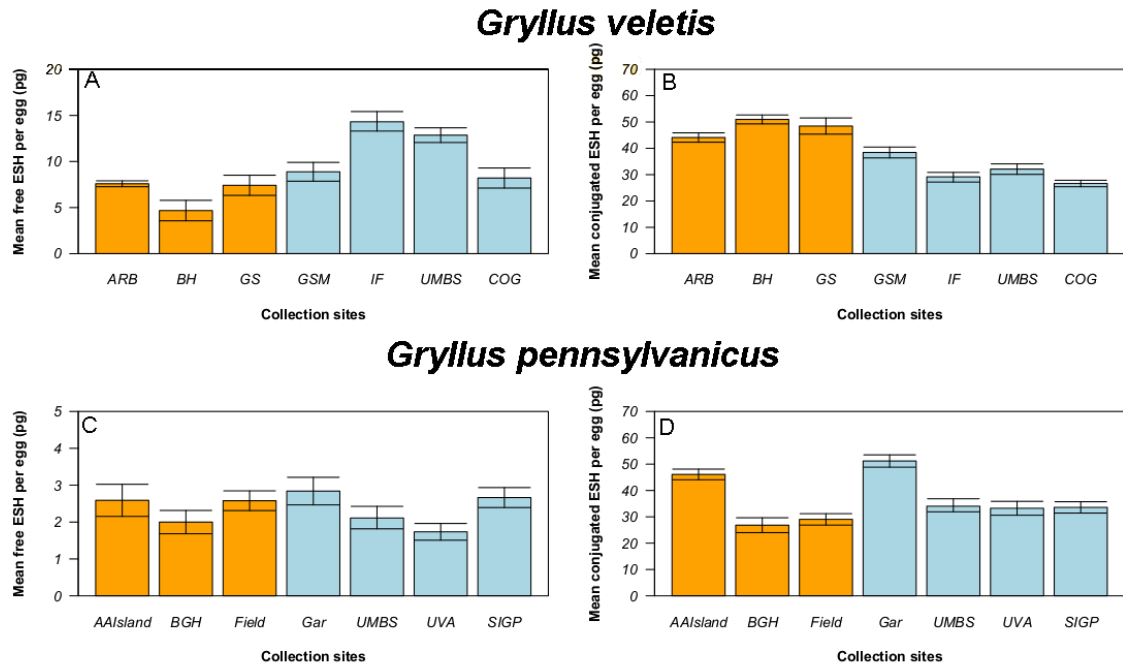


Figure 19: Bar plots showing the average mass of free and conjugated ESH deposited into eggs by females crickets of both species collected at different collection sites at two latitudes. Site codes are indicated on the x-axes of plots, mass and form of ESH are shown on the y-axes of plots. Collection sites in northern Michigan are shown in blue, while collection sites in southern Michigan are shown in orange. *Gryllus veletis* varied the concentration of (A) free ESH deposited per egg and (B) conjugated ESH deposited per egg among sites and latitudes. Specifically, they varied free ESH by site (Free: $F_{5,57} = 2.59$, $p = 0.0355$; Conjugated: $F_{5,57} = 1.85$, $p = 0.1175$), and conjugated ESH by latitude (Free: $F_{1,5} = 5.29$, $p = 0.0684$; Conjugated: $F_{1,5} = 21.57$, $p = 0.0051$). *Gryllus pennsylvanicus* did not vary the concentration of (C) free ESH deposited per egg by either site or by latitude (Site: $F_{5,55} = 0.52$, $p = 0.7613$; Latitude: $F_{1,5} = 0.03$, $p = 0.8782$), and (D) varied the concentration of conjugated ESH deposited per egg by site ($F_{5,55} = 5.88$, $p = 0.0002$), but not by latitude ($F_{1,5} = 0.29$, $p = 0.6145$).

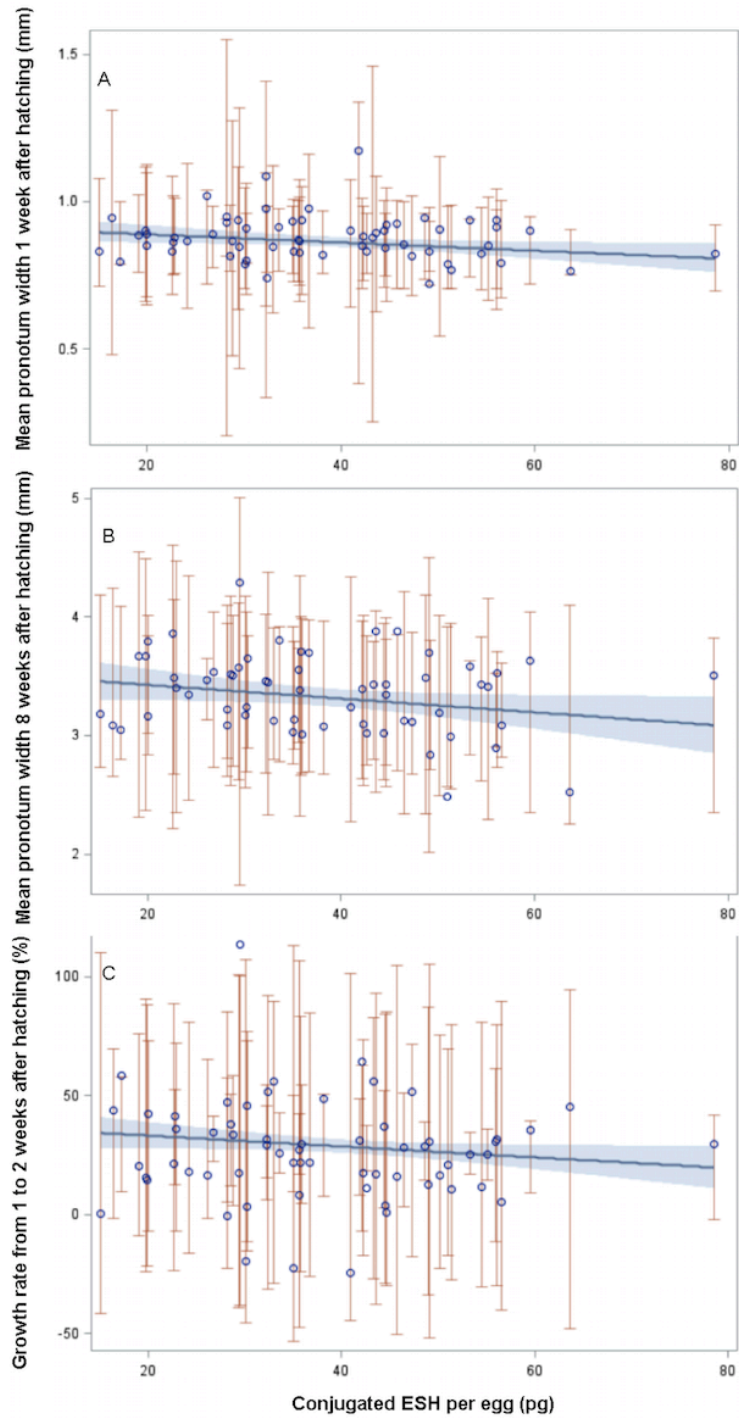


Figure 20: Weighted means regressions of spring field cricket (*Gryllus veletis*) life history traits as a function of the concentration of conjugated ESH per egg (in pg). (A) Hatchling structural size at 1 week after hatching, (B) hatchling structural size 8 weeks after hatching, (C) hatchling growth rate between one and two weeks after hatching.

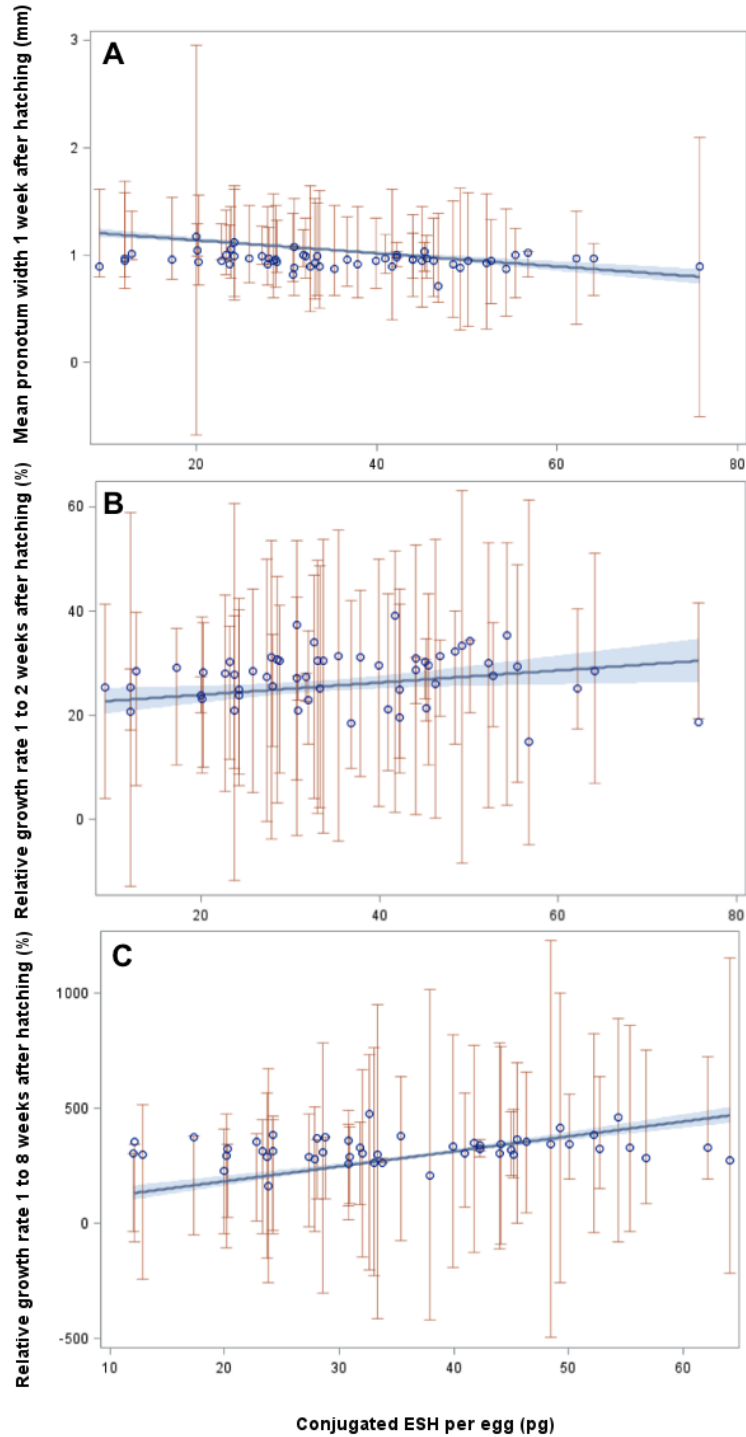


Figure 21: Weighted means regressions of fall field cricket (*Gryllus pennsylvanicus*) life history traits as a function of the concentration of conjugated ESH per egg (in pg). (A) Hatchling structural size at 1 week after hatching, (B) hatchling growth rate between one and two weeks after hatching, (C) hatchling growth rate between one and eight weeks after hatching.

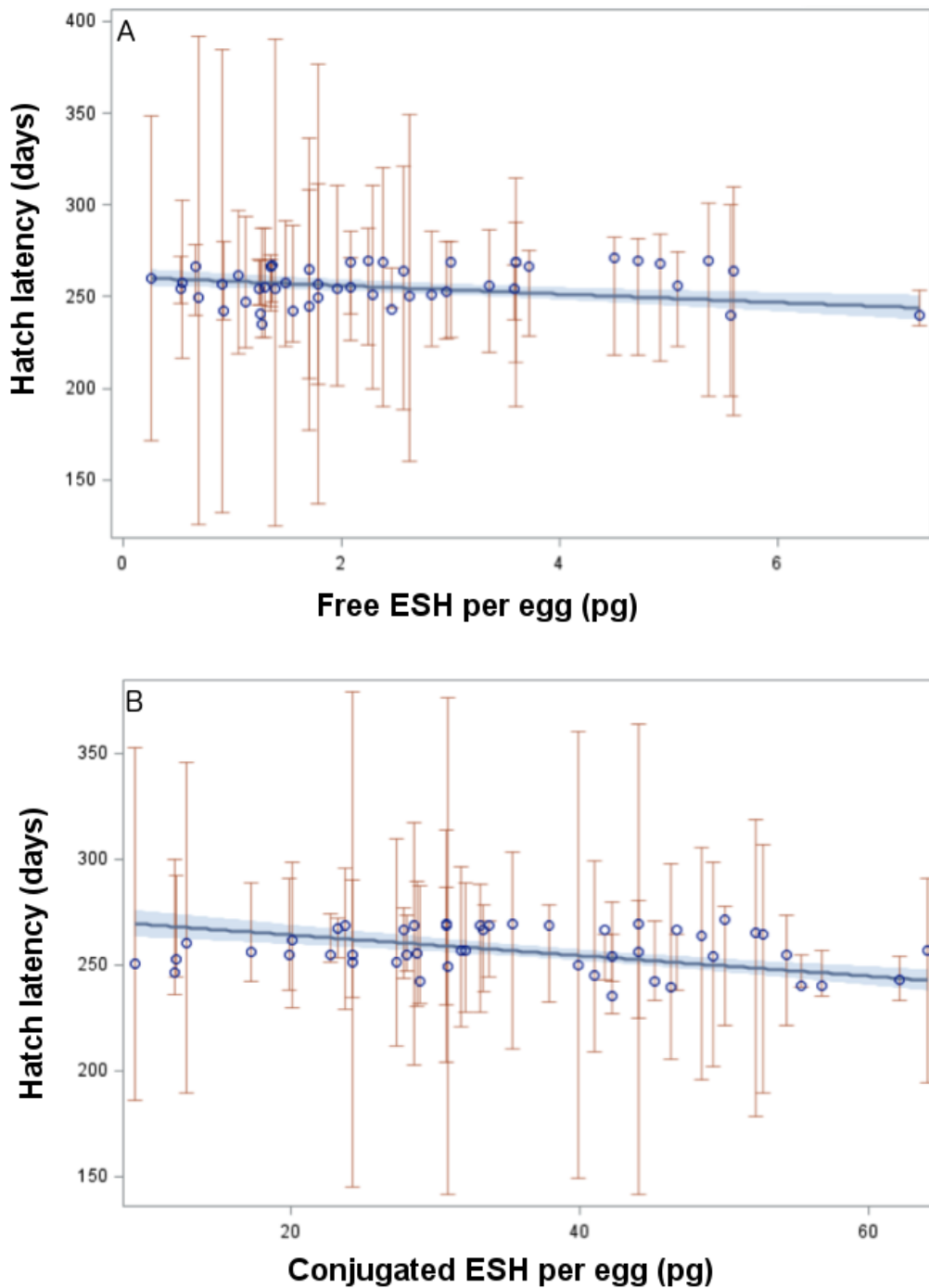


Figure 22: Weighted mean regressions of fall field cricket (*Gryllus pennsylvanicus*) hatch latency as a function of (A) the mass of free ESH provisioned per egg (in pg) and (B) the mass of conjugated ESH provisioned per egg (in pg).

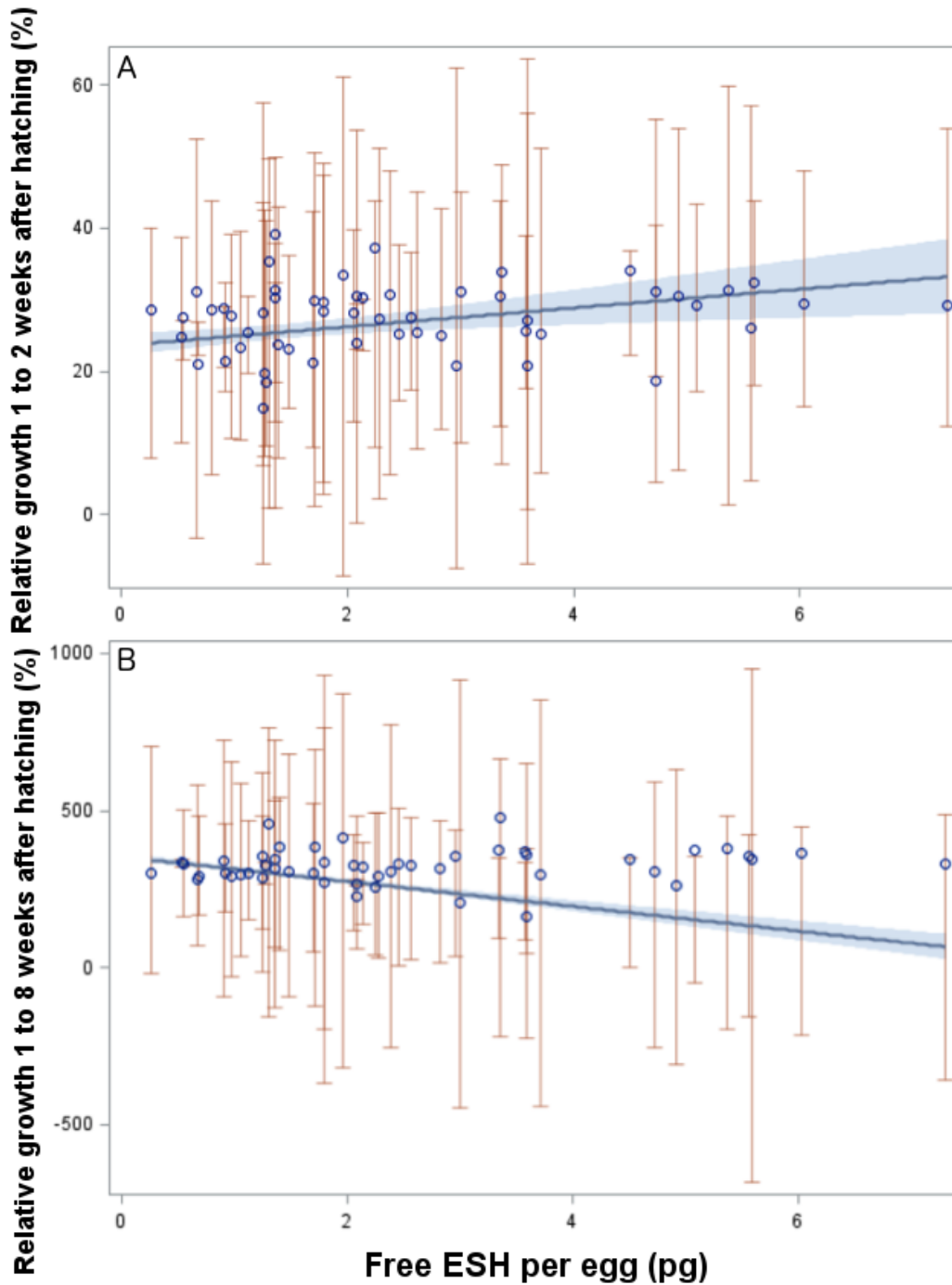


Figure 23: Weighted mean regressions of fall field cricket (*Gryllus pennsylvanicus*) relative growth rates as a function of (A) the mass of free ESH provisioned per egg (in pg) and (B) the mass of conjugated ESH provisioned per egg (in pg).

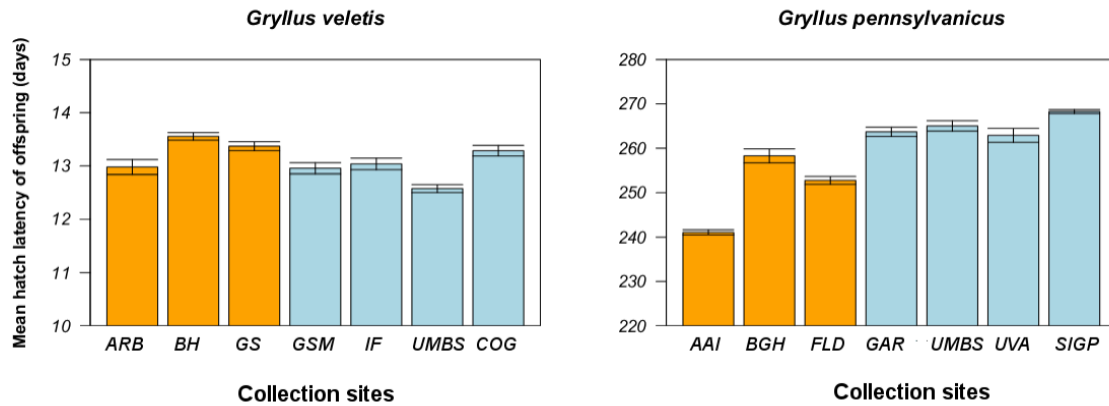


Figure 24: Bar plots showing the mean latency to hatch of eggs laid by female (A) *Gryllus veletis* and (B) *G. pennsylvanicus*. Collection site codes are indicated on the x-axes, mean latency to hatch (in days) is shown on the y-axes; northern Michigan collection sites are shaded blue, while collection sites in southern Michigan are shaded in orange.

(A) *Gryllus veletis* showed no difference in latency to hatch between latitudes ($F_{1,10} = 0.71$, $p = 0.420$), though females collected from different sites produced eggs that hatched at different speeds ($F_{8,54} = 2.87$, $p = 0.010$).

(B) *Gryllus pennsylvanicus* eggs laid by females from southern Michigan sites hatched more quickly than those laid by females collected from northern Michigan sites ($F_{1,7} = 7.01$, $p = 0.033$), though differences exist in hatch latency at the site level ($F_{6,55} = 9.10$, $p < 0.0001$).

SUPPLEMENTARY MATERIAL

Table 18: Day length, low, and high temperatures in both northern and southern Michigan growth chambers throughout the experiment. Beginning August 28, all crickets from southern Michigan were housed outdoors, see main text for details and Table 19 for daily photoperiod and temperature data.

Month	Day	Northern Michigan growth chamber			Southern Michigan growth chamber		
		Daylight/day	High temp (°F)	Low temp (°F)	Daylight/day	High temp (°F)	Low temp (°F)
April	1	12h45m	45.7	25.1	12h30m	53.1	33.3
April	7	12h50m	45.7	25.1	12h45m	53.1	33.3
April	14	13h	49.1	28.0	13h	56.0	35.7
April	21	13h50m	52.4	30.6	13h25m	59.0	38.0
April	28	14h10m	56.3	33.1	13h50	62.1	40.7
May	1	14h10m	56.3	33.1	13h50m		
May	7	14h30m	61.1	36.3	14h15m	66.0	44.0
May	14	14h50m	64.3	38.7	14h30m	68.9	46.7
May	21	15h	67.1	41.0	14h45m	71.3	49.0
May	28	15h10m	69.7	43.3	15h	73.7	51.4
June	1	15h10m	69.7	43.3	15h		
June	7	15h25m	72.3	46.3	15h	76.6	54.7
June	14	15h40m	73.7	48.1	15h15m	78.4	56.6
June	21	15h40m	75.4	50.1	15h15m	79.7	58.4
June	28	15h40m	76.6	51.6	15h15m	81.3	59.7
July	1	15h40m	76.6	51.6	15h15m		
July	7	15h30m	78.3	53.6	15h10m	82.7	61.1
July	14	15h20m	79.0	54.7	15h	83.0	62.0
July	21	15h10m	79.7	55.0	14h45m	83.3	62.1
July	28	14h50m	79.9	55.0	14h30m	83.0	63.0
Aug	1	14h50m	79.9	55.0	14h30m		
Aug	7	14h30m	79.0	54.9	14h30m	82.4	62.0
Aug	14	14h10m	77.9	54.0	14h15m	81.6	61.7
Aug	21	13h10m	76.6	52.9	13h45m	80.6	60.7
Aug	28	12h55m	74.9	51.4			
Sept	1	12h55m	74.9	51.4			
Sept	7	12h50m	71.6	49.0			
Sept	14	12h30m	69.0	47.0			
Sept	21	12h15m	66.7	45.0			
Sept	28	12h	64.1	42.7			
Oct	1	12h	61.0	39.7			

See supplemental table 19 for daily climate data

Table 19: Day length, low, and high temperatures each day in Ann Arbor, Michigan. *Gryllus pennsylvanicus* that were collected from low latitude sites (in and around Ann Arbor, Michigan) were housed outdoors from August 28 until the end of their lives (on or before October 4, 2016).

Date	Daylight/day	High temp (°F)	Low temp (°F)
August 28	13h16m	86	70
August 29	13h15m	84	64.9
August 30	13h12m	82	66
August 31	13h10m	79	69.1
September 1	13h9m	75	57
September 2	13h6m	75	54
September 3	13h3m	77	52
September 4	13h1m	80.1	54
September 5	12h58m	87.1	55.9
September 6	12h56m	91	68
September 7	12h54m	91.9	72
September 8	12h51m	80.1	73
September 9	12h48m	82.9	66
September 10	12h45m	80.1	66.9
September 11	12h43m	72	53.1
September 12	12h40m	75.9	53.1
September 13	12h37m	82.9	55
September 14	12h34m	79	61
September 15	12h30m	73.9	52
September 16	12h27m	79	52
September 17	12h24m	77	66.9
September 18	12h20m	81	61
September 19	12h17m	84.9	55.9
September 20	12h15m	82	62.1
September 21	12h12m	82.9	57
September 22	12h10m	88	63
September 23	12h7m	80.1	64.9
September 24	12h5m	70	54
September 25	12h3m	73.9	48.9
September 26	11h58m	69.1	60.1
September 27	11h55m	70	46.9
September 28	11h53m	66.9	51.1
September 29	11h50m	61	55.9
September 30	11h47m	60.1	57.9
October 1	11h45m	66.9	55.9
October 2	11h43m	66.9	51.1
October 3	11h40m	64.9	52
October 4	11h37m	73	50

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Chapter VI Conclusions

“...what everybody knows is true turns out to be what some people used to think”
–Ursula K. Le Guin

How we think about what we know is the most important confounding factor in biology. As humans, biologists—no matter how ruthlessly we reason—are prey to epistemic bias, which favors the abstract frameworks with which we are most familiar. This familiarity can prevent us from noticing patterns that would have been obvious long ago, to minds not enculturated into certain mental habits or which simply have a different set of cultural axioms. The relatively recent description of cryptic female choice is an excellent example, but the growing appreciation of transgenerational nongenetic effects is also striking in this regard.

People who are indigenous to the continent currently known as North America have had knowledge of nongenetic inheritance, most tragically exemplified as historical trauma, for thousands of years. This knowledge has been gained through observation, collection of data, and careful analysis. It is also highly replicated across time and space: tribes and traditions across the continent have come to similar conclusions across thousands of miles and years. The tradition of empirical investigation is not unique to Western European culture, but in the last 20 years Western European scientific thought has joined the hundreds of cultures that have discovered nongenetic inheritance.

As with all examples of confirmation bias, the more we search for nongenetic inheritance, the more we find. The work I have presented here is, I hope, of interest to entomologists, developmental biologists, physiologists, and animal behaviorists. But the most important result is the evolutionary implication, both for the presence of hormone-mediated transgenerational effects across the tree of life, and for the history of hormones and behavior.

Humans are vertebrates, and our view of behavioral endocrinology is correspondingly vertebrate-centric. However, all animals use hormones to adjust our phenotypes to match our environment. A minute change in the concentration of circulating hormones can alter behavior within fractions of a second, as with the fight-or-flight response, or maintain a sustained physiological shift, as with species that upregulate production of sex hormones during their breeding season. As such, hormones, rather than DNA or receptors, are the basic irreducible unit of animal behavior. While responsible for constant physiological and homeostatic adjustment, hormones—particularly steroid hormones—also govern embryonic development.

Behavioral endocrinology literature abounds with examples of the activational effects of steroid hormones on development in vertebrates (e.g. the masculinization or feminization of genitalia in mammals), and the organizational effects of those hormones on juvenile and adult animals (e.g. immune function up- or down-regulation in avian species). That the inevitable parent-offspring endocrine interaction can have transgenerational impact on the phenotype of offspring, is thus not surprising. Yet, with fitness at stake on both sides, how species and individuals navigate potential conflicts of interest is relatively under-investigated. In other words, how and whether a parent decides to dampen its endocrine response to an environmental stimulus, in order to prevent affecting its offspring (or, conversely, shows an accentuated response to environmental stimuli to engender an effect on offspring), while fascinating, remains poorly understood.

Particularly in light of our growing understanding of the transgenerational consequences of small differences in the provisioning of hormones to offspring, I suggest this general area merits further study.

Steroid hormones mediate transgenerational effects in vertebrates: work on species as diverse as mice, humans, bank voles, and pipefish has demonstrated this. Whether steroid hormone exposure is to parental hormones as an embryo, or to an individual's own circulating steroid levels as a juvenile, the physiological effects are far-reaching and profound. Ecdysteroid hormones are found only in invertebrates, and evolutionary

divergence, combined with very different life history characteristics, renders it difficult to compare vertebrate and invertebrate endocrinology. However, the results reported in this dissertation show that there are more parallels than previously thought between the functions of vertebrate and invertebrate steroid hormones. Most importantly, insects also use steroid hormones as at least part of the mechanism by which they mediate transgenerational nongenetic effects.

The presence of steroid-mediated transgenerational effects in species as distantly related as mice and crickets is suggestive of a general pattern across the tree of life. Steroid hormones, in both vertebrate and insect species, control embryonic development and cell differentiation, are at least implicated in reproductive success, coordinate life stage transitions, and mediate transgenerational effects. It is tempting to interpret these broad similarities as evidence of the adaptivity of, in particular, transgenerational effects. However, the most conservative statement is that steroid-mediated transgenerational effects are at least not sufficiently maladaptive to have been suppressed in vertebrates or insects, either before or after our evolutionary divergence.

This observation is important for our understanding of the evolutionary history of steroid hormones and behavior. Neither a circulating concentration of steroid hormones, nor specific behaviors, are likely to be fossilized. Therefore, the most useful path towards knowledge of the evolutionary and natural history of our endocrine systems is, to some extent, highly informed phylogenetic conjecture. Using information about similarities (and differences) between distantly related animals that use descendant hormones for important, fitness-related processes, may be the closest we come to obtaining a fossil of the ancestral molecules that facilitate our continued existence. Given the current evidence, both in this dissertation and elsewhere, we must either conclude that an extremely high degree of canalization exists in both vertebrate and insect endocrine systems, or that transgenerational effects have been extant longer than these phyla have been distinct.

If the former case, that evolution of the endocrine systems of modern vertebrates and insects is highly canalized, this opens doors for increased use of non-traditional model insects as model systems. More importantly, the constraints that would need to exist for this to be true would likely change the way we think about and research evolution and mutation. If the latter hypothesis is true, that transgenerational effects predate the divergence of vertebrate and invertebrate phyla, it is crucial to ground our understanding in a tree-of-life-wide approach, rather than continued focus on a handful of medically useful mammalian species. Without broad understanding of the mechanisms, persistence, and evolutionary history of such widespread phenomena, we are in danger of classifying important evidence as error. Continuing forward without the benefit of self-critical understanding is at best wasteful, and at worst dangerous, as the world changes at a speed with which we, so far, have been unable to keep pace.