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Evolutionary genetics of juvenile hormone and ecdysteroid regulation in *Gryllus*: A case study in the microevolution of endocrine regulation

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Review

Evolutionary genetics of juvenile hormone and ecdysteroid regulation in *Gryllus*: A case study in the microevolution of endocrine regulation

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Abstract

During the past 15 years the first detailed synthesis of endocrinology and population genetics has begun, in which natural genetic variations for endocrine regulators have been characterized, almost exclusively in species of the cricket genus *Gryllus*. Artificial selection studies have documented that regulators of the juvenile hormone titer can rapidly evolve and exhibit levels of genetic variability similar to other physiological traits. Strong genetic correlations exist between some but not all regulators of the JH titer during the juvenile stage. No genetic correlation exists between regulators functioning in juvenile and adult stages, and thus, endocrine regulation can evolve independently in these stages. Genetic variation in the JH titer, the ecdysteroid titer, and JHE activity, in adult and juvenile stages, have been documented in genetic stocks of wing-polymorphic crickets; morph-specific differences in these endocrine traits are potentially responsible for genetically based differences in aspects of wing and flight muscle development, adult egg production, and adult dispersal. An unexpected morph-specific, genetic polymorphism for a circadian rhythm for the JH titer was observed in both the laboratory and field. Few comparable studies exist in non-*Gryllus* species, in which in vivo endocrine-genetic variation has been directly quantified using reliable analytical methods; many reported cases of endocrine variation in these species have been obtained using an inappropriate method and thus should be considered unsubstantiated. Obtaining basic information on the characteristics of natural genetic variation for endocrine regulators still remains one of the most important tasks of the fledgling subdiscipline of evolutionary endocrinology. Single gene endocrine mutants in *Drosophila* are promising candidates for investigating molecular-genetic variation in natural populations. Future studies should also focus on endocrine traits studied in the field and geographic variation in endocrine regulation.

Keywords: Juvenile hormone, Ecdysteroid, Evolutionary endocrinology, Life history, Wing polymorphism, Development

Contents

1. Introduction

Evolutionary modification of endocrine regulation is an important topic for the fields of endocrinology and evolutionary biology. At issue are the mechanisms by which endocrine traits such as hormone titers, activities of hormone-regulating enzymes, or receptor characteristics are modified by natural selection, thus giving rise to the diversity of endocrine regulatory mechanisms observed in organisms. An equally important topic is the extent and mechanisms by which evolutionary changes in hormonal regulation contribute to adaptive changes in organismal structure and function. Until very recently, endocrine evolution has been studied primarily from an interspecific or phylogenetic perspective (Baker, 2003, Bern, 1983, Bertrand et al., 2004 and Sherwood et al., 1994). However, adaptive modification of endocrine regulation begins with natural selection affecting the transmission of genetically based endocrine variants within natural populations. We know relatively little about this aspect of hormonal evolution.

During the past 15 years the first detailed synthesis of population genetics and endocrinology has begun, resulting in the fledging subdiscipline called Evolutionary Endocrinology (Zera, 1999 and Zera and Zhang, 1995). Evolutionary-endocrine studies conducted during this time have focused on various physiological aspects of the two broad topics mentioned above (discussed in detail below). One group of studies has attempted to quantify and characterize genetically based variation and covariation (correlations) in endocrine traits found in natural populations. The motivation for this approach is the long-held view that understanding the mechanisms of evolutionary change requires detailed information on naturally occurring genetic variation, the raw material for evolution (Brakefield et al., 2003, Lewontin, 1974 and Zera and Zhang, 1995). The second group of studies has focused on the role played by the modification of endocrine regulation on the evolution of life history traits, key organismal traits that contribute importantly to Darwinian fitness. Life history traits determine the pattern of reproduction and survivorship during the life cycle of a species (e.g., age at which reproduction begins, trade-offs between growth, longevity and reproduction), and have been intensively studied by evolutionary biologists for decades (Roff, 2002 and Stearns, 1992). However, the physiological–genetic mechanisms underlying variation in life history traits have only recently been investigated in any detail. Because hormones regulate the expression of all major life history traits, evolutionary modification of endocrine regulation has been increasingly viewed as a critically important aspect of life history evolution (Ketterson and Nolan, 1999, Ricklefs and Wikelski, 2002, Zera and Harshman, 2001, Zera and Zhao, 2004 and Harshman and Zera, in press).

Physiological–genetic studies of endocrine variation have been undertaken in a variety of organisms using a variety of approaches (Brakefield et al., 2003, Dingle and Winchell, 1997, Emlen and Nijhout, 1999, Emlen and Nijhout, 2001, Flatt et al., 2005, Moczek and Nijhout, 2002, Richard et al., 2005, Zera, 1999, Zera, 2004 and Zera and Cisper, 2001). A key first step in studies of hormonal microevolution is to characterize naturally occurring genetic variation for endocrine traits using well-established analytical methods. Thus far, this approach has largely been limited to studies of natural variation in juvenile hormone and ecdysteroid regulators in species of *Gryllus*. The present review will focus primarily on these studies. In addition, two related topics will be briefly discussed: (1) recent investigations of life history trade-offs using a relatively new approach employing laboratory-induced endocrine mutants in *Drosophila* (Flatt et al., 2005, Richard et al., 2005, Tatar et al., 2003 and Tatar et al., 2001) and (2) the continued use of the dubious technique of hormone manipulation to indirectly infer endocrine variation.

Most of the physiological-genetic studies of endocrine regulators in *Gryllus*, as well as in other organisms (Brakefield et al., 2003 and Emlen and Nijhout, 1999) have employed quantitative-genetic methods, most notably artificial selection. Because these methods are not familiar to many endocrinologists and physiologists, I will first provide some brief background information on this topic to illustrate the rational, strengths, and limitations of various quantitative-genetic approaches. I will also provide some background information on the endocrinology of juvenile hormone, and to a lesser degree ecdysteroids, since these hormones have been the major foci of studies on endocrine microevolution.

2. Background in evolutionary quantitative genetics

Heritable variation provides the raw material required for adaptive evolutionary change and is the primary focus of evolutionary quantitative genetics (Brakefield et al., 2003, Lynch and Walsh, 1998 and Roff, 1997). In addition, traits are often genetically correlated, because of pleiotropy and other factors, and the magnitude of genetic correlations can strongly influence the evolution of a particular trait (Falconer and Mackay, 1996 and Roff, 1997). The reason is that selection favors or disfavors a particular variant based on the combined direct effects of the variant itself on

fitness, as well as the indirect effects on fitness of genetically correlated variants. Thus, identifying genetic correlations represents another key aspect of evolutionary quantitative genetics (Falconer and Mackay, 1996 and Roff, 1997).

Like most other physiological traits [e.g., performance measures, enzyme activities (Clark, 1990, Clark et al., 1990, Garland, 1994 and Garland and Carter, 1994)], phenotypic variation for endocrine traits at the biochemical/physiological level (e.g., hormone titers or activities of hormone-regulating enzymes) is continuous rather than polymorphic. That is, phenotypes of individuals typically differ along a continuum and cannot be grouped into a few discrete classes (see Fig. 1 for a typical example). This type of variation results from the interaction among multiple genetic and environmental factors. Genetic analysis of quantitative traits, in large part, focuses on populational parameters such as means, variances and correlations that describe the genetic and environmental components of overall variation in the trait of interest, and the degree of correlation between traits. Important goals of quantitative-genetic analysis are to quantify the extent to which overall variation in a trait has a genetic basis (and thus has the capability to evolve), and the extent to which that trait is genetically correlated with other traits. In addition, of special importance in physiological-genetic studies of quantitative variation, is functional information on quantitative trait variation, such as the following: (1) the identity of variable genes (at least classes of genes) that contribute to overall phenotypic and genetic variation, and their mode-of-action, (2) the identity and mode-of-action of pleiotropic genes that are responsible for genetic correlations, and (3) the identity of mechanisms that link variation in endocrine regulation with variation in the expression of higher-level traits (e.g., the effect of altered endocrine regulation on duration of development, reproductive characteristics, etc.). Additional techniques such as QTL (quantitative trait loci) mapping and mutational studies can also be applied to identify and study individual genes that contribute to a composite quantitative trait in model genetic organisms (Mackay, 1995, Mackay, 2001, Richard et al., 2005, Tatar et al., 2003 and Tatar et al., 2001). These techniques are especially useful when mutants are used as candidate genes to investigate naturally occurring genetic variation (Brakefield et al., 2003, Mackay, 2001 and Stern, 2000).

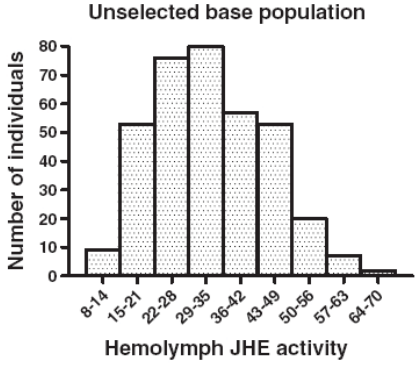

Figure 1. Hemolymph juvenile hormone esterase (JHE) activities (nmol min^{-1} mL $hemolymph^{-1}$) in the base population of *Gryllus assimilis* used in the selection study of Zera and Zhang (1995). Note the continuous distribution of enzyme activities. Distribution was based on assays of 385 females.

Traditional quantitative-genetic analysis consists of various breeding designs (half-sib breeding, parent–offspring regression, artificial selection) to estimate genetic parameters that describe the amount of genetic variation/correlation for traits of interest (Falconer and Mackay, 1996 and Lynch and Walsh, 1998). The most detailed evolutionary-genetic studies of endocrine regulation have employed artificial selection, which essentially involves selective breeding of individuals of a population that have the highest or lowest phenotypic values for the trait of interest (truncation selection). If phenotypic variation for the selected trait has a genetic component, then the mean value (i.e., mean enzyme activity) will increase in the high-selected lines, and will decrease in the low-selected lines (i.e., the trait will respond to selection; Fig. 2, top panel). The rates of increase or decrease over successive generations can be used to estimate the heritability of the trait (h^2), which is the proportion of phenotypic variation of the selected trait that is due to variation in genetic as opposed to environmental factors (i.e., the proportion of phenotypic variation that is available for selection to act upon; for experimental methods see Clark et al. (1990), Falconer and Mackay (1996), Gibbs (1999), and Zera and Zhang (1995)). The degree to which other non-selected traits, also increase or decrease in the selected lines, reflects the magnitude of genetic correlations between these traits and the trait directly selected (e.g., Fig. 2, middle panel).

Artificial selection has a number of advantages over other breeding designs such as parent–offspring regression or sib analysis. Most importantly for physiological studies, artificial selection produces stocks that differ genetically for the trait selected and other genetically correlated traits. These lines can be used to investigate the functional causes of both genetic variation and genetic correlation. Much of this review will deal with biochemical and endocrine characterizations of lines that have been artificially selected for either endocrine or life history traits.

Whichever method is employed, quantitative-genetic studies are typically very labor intensive, which is a likely reason for the paucity of data on naturally occurring endocrine-genetic variation. Selection is often required for at least 5–10 generations to produce genetic stocks that are sufficiently divergent for functional analyses. Furthermore, replicate selected lines are required, both to assess the reproducibility of the direct and indirect responses to selection and for adequate statistical estimation of genetic parameters. Thus, sample sizes in the thousands are not uncommon in selection studies. For example, the *initial* artificial selection study on hemolymph juvenile hormone esterase activity in *Gryllus assimilis* (seven generations of selection; three up-selected, three down-selected, and three unselected controls) involved measurement of enzyme activity in more than 10,000 individually reared crickets (Zera and Zhang, 1995).

Artificial selection also has some important limitations which need to be born in mind (Gibbs, 1999, Harshman and Hoffmann, 2000 and Roff, 1994). Most importantly, the relationship between the trait selected and fitness is typically not considered in artificial selection. Thus, although genetic factors that have negative effects on fitness can still be positively

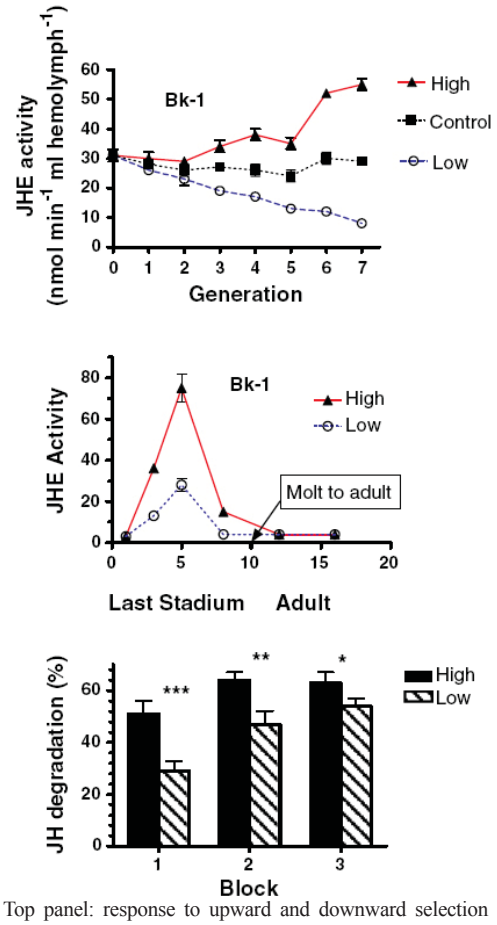

Figure 2. Top panel: response to upward and downward selection on hemolymph JHE activity in *G. assimilis*. Data are for one of the three blocks (independent selection trials); response to selection was very similar in the other two blocks (Zera and Zhang, 1995). Middle panel: correlated responses to selection on hemolymph JHE activity in *G. assimilis* measured during the fifth generation of selection. Data are for one of three blocks (controls not shown); virtually identical patterns were obtained in the other two blocks (Zera and Zhang, 1995). Note the similar shape of the JHE activity developmental profile in high- and low-selected lines during the last juvenile stage indicating the existence of strong genetic correlations between JHE activities throughout this stadium. Also note the absence of differences in JHE activity between high- and low-selected lines in adults indicating no detectable genetic correlation between JHE activities in adults and juveniles. Lower panel: elevated in vivo juvenile hormone (JH) degradation in high- vs. low-JHE activity lines of *G. assimilis* (Zera et al., 1996). JH degradation was measured during the seventh generation of selection.

selected during artificial selection, these factors may not contribute significantly to the adaptive evolution of the same trait in natural populations. A classic example of this point is artificial selection for negative phototaxis in *Drosophila melanogaster* (Markow and Clark, 1984 and Roff, 1994). Analysis of the selected lines indicated that the response to selection (increased incidence of negative phototaxis) was due to selection on deleterious alleles that produced visually impaired flies. Because such flies likely have substantially reduced fitness, it is questionable whether the evolution of negative phototaxis in natural populations would evolve by selection on these genetic factors. Thus, artificial selection, in and of itself, should be viewed only as an experimental manipulation that is used to analyze standing genetic variation for a quantitative trait. Additional information must be obtained to evaluate the relevance of genetic variation and correlations identified by artificial selection with respect to their role in the microevolution of the selected trait in natural populations.

3. Background on juvenile hormone and ecdysteroid endocrinology

During the past 15 years, two hormones, juvenile hormone (JH) and 20-OH ecdysone, have been the primary foci of micoevolutionary studies of endocrine regulation. These hormones have been intensively studied by insect endocrinologists (Klowden, 2002 and Nijhout, 1994), and, long before they were investigated in a microevolutionary context, they were the primary foci of speculation concerning the role of hormones in the evolution of development and life histories (Matsuda, 1987, Nijhout and Wheeler, 1982, West-Eberhard, 2003 and Zera, 2004). The juvenile hormones are a group of about a half-dozen structurally related (16–19 carbon) isoterpenoids (Klowden, 2002 and Nijhout, 1994). Many insect groups (e.g., grasshoppers, locusts, crickets, social insects) contain only a single JH (JH-III), while moths and butterflies contain four or more JHs in addition to JH-III. Some dipterans (flies) also contain a unique JH (JH bisepoxide), which is the major JH in some species (e.g., *Drosophila melanogaster*). The presence of multiple JHs complicates studies of endocrine microevolution in lepidopterans and in *D. melanogaster*. JH bisepoxide is especially problematic because of its instability, which has precluded quantification of its in vivo titer in *Drosophila*. Ecdysteroid is the generic name applied to a group of structurally related polyhydroxylated steroid hormones (Nijhout, 1994). There are a wide variety of ecdysteroids in insects, the most important being 20-OH ecdysone.

JH and 20-OH ecdysone control many important aspects of reproduction and development (Klowden, 2002, Nijhout, 1994 and Wyatt and Davey, 1996). With respect to development, 20-OH ecdysone induces the molt and regulates the expression of genes that specify adult characteristics (metamorphosis). Juvenile hormone (JH), on the other hand, antagonizes the metamorphic action of ecdysone, while allowing the molt to occur. A high JH concentration thus causes a juvenile-to-juvenile molt, while a drop in the JH titer to a low or imperceptible level during the early last juvenile instar, allows ecdysone-induced gene expression to occur, resulting in a metamorphic molt [i.e., a molt from a juvenile to an adult in hemimetabolous insects (e.g., crickets, aphids, waterstriders), or to a pupa in holometabolous insects (beetles, butterflies, moths, flies)]. During the adult stage, JH and ecdysone, take on new roles as gonadotropins, regulating, among other things, the synthesis of vitellogenin (yolk protein), and uptake of this molecule into the developing oocyte. In some insect groups (e.g., crickets, cockroaches, grasshoppers, bees, and wasps), JH is the main regulator of both vitellogenin synthesis by the fat body (insect analogue of the vertebrate liver) and vitellogenin uptake by the ovaries. In the Diptera (e.g., *Dro-*

sophila, mosquitoes) 20-OH ecdysone primarily controls vitellogenin synthesis, with JH being necessary for the fat body to respond to JH. In addition to these hormones, a variety of other hormones (e.g., various neuropeptides, insulin-like peptides) also regulate various aspects of reproduction (Klowden, 2002, Nijhout, 1999, Nijhout, 1994 and Richard et al., 2005). JH and ecdysone also regulate numerous other important organismal traits, such as various behaviors involved in mating, foraging, and flight; the induction and maintenance of diapause, and the expression of many morphological, behavioral, and reproductive traits of ecologically important "complex" polymorphisms (caste-, phase-, and dispersal-polymorphism (Klowden, 2002, Nijhout, 1994 and Zera, 2004).

Studies of JH endocrinology and microevolution have focused on variation in the JH titer as well as regulators of the JH titer, most notably JH-esterase, an enzyme that degrades JH, and rate of JH biosynthesis (Nijhout, 1994, Wyatt and Davey, 1996 and Zera, 2004). It is widely assumed that the molecular mode of JH action involves a nuclear receptor. However, despite considerable study, the receptor has not been identified, although several candidates have been proposed (Wilson, 2004). The absence of an identified nuclear receptor has limited studies of JH endocrinology. By contrast, the ecdysteroid receptor has been cloned in several insects and the molecular basis of 20-OH ecdysone-induced metamorphosis has been extensively studied (Klowden, 2002 and Thummel, 1996). However, no microevolutionary studies have investigated genetic variation for any aspect of the ecdysteroid receptor in natural populations. Virtually all studies of genetic variation in ecdysteroid regulation have focused on the ecdysteroid titer (discussed below).

4. Microevolution of endocrine regulators: artificial selection on juvenile hormone esterase activity in juvenile *G. assimilis*

The first and still the most detailed study of naturally occurring endocrine variation in any organism is that of hemolymph JHE activity in the cricket *G. assimilis* (Zera et al., 1996, Zera et al., 1998 and Zera and Zhang, 1995). As mentioned above, hemolymph JHE degrades JH and regulates its hemolymph titer (Wyatt and Davey, 1996). JHE activity was chosen as a model endocrine trait for artificial selection, because, unlike most other endocrine traits (e.g., the JH titer) hemolymph JHE activity can be rapidly and accurately quantified in a small hemolymph sample, the removal of which does not significantly damage the insect. Thus, individuals, whose hemolymph JHE activity have been determined, can be directly bred in artificial selection experiments.

Artificial selection was conducted on a laboratory population that had recently (few generations) been founded from *G. assimilis* collected in the field in Homestead, Fla [see Zera and Zhang (1995) for details]. Hemolymph JHE activities in the base population were continuously distributed, as expected for a quantitative trait (Fig. 1). Nine lines were derived from the base population: three lines were selected for high JHE activity (i.e., 60 individuals with highest JHE activity were selected out of 240 assayed individuals each generation), three lines were selected for low JHE activity (using a similar selection protocol), and three lines were unselected (controls). Hemolymph JHE activity in *G. assimilis* selected for high or low activity exhibited a strong response to selection: high- and low-selected lines differed 6–7 fold by the 6th–7th generations of selection (Fig. 2 top panel; (Zera et al., 1996 and Zera and Zhang, 1995)) and further selection resulted in additional genetic divergence between the lines (Zera et al., 1998). Heritability estimated from the response to selection was approximately 0.2–0.3 ± 0.04; a similar heritability was obtained for hemolymph JHE activity, as well as hemolymph JH binding, using a different breeding design (single generation, half-sib breeding; see (Gu and Zera, 1996)). Heritabilities for hemolymph JHE and JH binding (JHB) are similar to values obtained for activities of enzymes of intermediary metabolism (Clark, 1990 and Clark and Keith, 1988), and for other physiological traits (Garland and Carter, 1994). Thus there is no indication, at least for JHE and JH binding activity, that endocrine characters have unusually high or low levels of genetic variability compared with other physiological/biochemical traits. To my knowledge, hemolymph JHE and JHB activities in *G. assimilis* are still the only endocrine traits whose heritabilities have been estimated in a laboratory population recently derived from the field-collected individuals. High- and low-selected lines differed in JHE activity to a similar degree when raised under a variety of temperatures and cricket densities (i.e., no genotype X environment interaction was observed; A.J. Zera, unpubl.). Thus the expression of genetic variation for JHE activity does not appear to be strongly contingent upon the particular laboratory environment under which artificial selection was conducted. This increases the probability that roughly similar JHE genetic variation exists in field populations as was observed in the laboratory. At present no species has been screened for genetic variation for an endocrine trait in the field, and only one insect species has been screened for phenotypic variation in the field [JH titer in *Gryllus firmus* (A. J. Zera, unpubl.); see below].

4.1. Correlated responses to selection: developmental profiles of hemolymph JHE activity

As mentioned previously, one of the most important characteristics of a quantitative trait is the degree to which it is genetically correlated with other traits. Prior to the artificial selection studies on JHE in *G. assimilis*, no information was available on the extent or magnitude of genetic correlations among endocrine traits in any non-domesticated organism. One important type of genetic correlation is that between the same trait measured during different periods of development (Arnold, 1987 and Cheverud et al., 1983). A high genetic correlation between the same endocrine regulator across different stages of development would indicate that evolutionary changes in endocrine regulation during one stage of development would also produce changes in regulation in other stages. This, in turn, would cause the developmental profile of the regulator to evolve as a tightly integrated unit and would constrain the independent evolution of traits during different periods of development that are controlled by the regulator. The

extent and magnitude of this type of genetic correlation has important implications for the evolution of development and life histories (Zera et al., 1998 and Zera and Zhang, 1995).

One of the most important findings of the JHE selection experiment is the strong genetic correlations between JHE activity during different periods of the juvenile stage in *G. assimilis*, but a complete absence of a genetic correlation between adult and juvenile stages (Fig. 2, middle panel). Although directly selected only on day 3 of the last juvenile instar, JHE activities differed between the high and low-selected lines to the same degree on days 1, 5 and 8 of the last stadium as on day 3, thus demonstrating a very strong genetic correlation between JHE activity on these days. Because different regulators are thought to control JHE activity during the beginning and end of the last juvenile instar in some moth species (Jones et al., 1981), genetic correlations between JHE activities during the beginning and end of the last instar in *G. assimilis* were not necessarily expected. On the other hand, no differences in JHE activity were observed between selected lines during two days of adulthood, thus indicating no observable genetic correlations between JHE activity during juvenile and adult stages. Strong positive genetic correlations among JHE activities during different times of the last stadium, were also found using a different quantitative-genetic approach [half-sib breeding] (Gu and Zera, 1996). The genetic independence of JHE activities between adult and juvenile stages also was confirmed by a complimentary artificial selection experiment in which JHE activity was selected upwards and downwards during the *adult* stage: selected lines differed in JHE activity during the adult stage, but not during the last juvenile instar (Zera et al., 1998). These results collectively constitute the first and remain the most detailed investigation of genetic correlations among endocrine traits in natural populations of any organism. These results indicate that evolutionary changes in JHE regulation can occur during the juvenile stage of *G. assimilis*, without modifying JHE regulation in the adult stage. JHE correlations identified by artificial selection have proven useful for dissecting mechanisms of JHE microevolution in natural populations of a related species (discussed below). Because genetic correlations have not been measured for traits other than JHE in *Gryllus*, it is unknown whether the results obtained for *Gryllus* are representative of endocrine traits in other organisms. It would be particularly interesting to know whether hormone titers and receptor characteristics, also are uncorrelated between adults and juveniles.

4.2. Genetic correlations: JHE activity and other endocrine traits

Direct selection on hemolymph JHE activity resulted in substantial correlated changes in hemolymph JH binding (JHB; a measure of the activity of the JH binding protein). By generation seven, JHB activity (% JH bound per unit hemolymph) was 50–100% higher in high- vs. low-JHE-selected lines (Zera et al., 1996). A strong positive genetic correlation between hemolymph JHE and JHB activities (0.6 ± 0.2) was also quantified using a half-sib breeding design (Gu and Zera, 1996), thus corroborating the results of artificial selection. Thus, variable genes producing phenotypic variation in hemolymph JHE activity also produce phenotypic variation in JH binding, probably via pleiotropic effects of variable co-regulators. This genetic coupling causes JHE activity and JH binding activity to evolve in concert [see discussion in (Gu and Zera, 1996) on the functional importance of this correlation]. In contrast to JHB activity, activity of JH epoxide hydrolase (JHEH), the other major JH-degrading enzyme, did not differ between high- and low-selected lines (Zera and Zhang, 1995 and Zera et al., 1996) (Table 1). The absence of a genetic correlation between JHE and JHEH indicates that activities of the two main JH-degrading enzymes have the capacity of evolve independently of each other. At present it is unclear why some JH regulators are strongly correlated genetically, while others are not.

4.3. Functional causes and consequences of genetic variation in hemolymph JHE activity

4.3.1. Physiological, biochemical and molecular causes

One of the most important reasons for conducting artificial selection on hemolymph JHE activity was to obtain genetic lines that could be used to identify the functional causes of genetic variation in JHE activity. High- and low-JHE activity lines were compared for a variety of physiological, biochemical, and molecular factors that potentially contribute to variation in hemolymph JHE activity. One important finding was that the selected lines differed in both *whole-organism* JHE activity and in JHE tissue distribution (% whole-body JHE found in the hemolymph vs. the rest of the organism) (Table 1). Thus, the divergence in *hemolymph* JHE activity (the trait directly selected) between the high- and low-activity lines was caused by artificial selection on two different classes of variable loci: one that controls whole-organism JHE activity and the other that controls the amount of JHE secreted into the hemolymph. The natural population from which the base pop-

Table 1.
Endocrine differences between lines of *Gryllus assimilis* selected for high or low hemolymph juvenile hormone esterase (JHE) activity and lines of *G. firmus* selected for the long-winged (LW) or short-winged (SW) morph

Trait	*G. assimilis*		*G. firmus*	
	High	Low	LW	SW
Hemolymph JHE activity[a]	57±2.6	8±0.63	69±4.6	11±1.5
Whole-body JHE activity[b]	11±1.5	5±0.55	28±1.7	16±2.0
% JHE in hemolymph[c]	72±2.0	28±1.5	55±5.3	20±4.3
JHEH activity[d]	11±1.0	10±0.3	4±0.2	5±0.4
JH degradation (%)[e]	61±4.8	43±7.1	44±0.5	34±1.6
Adult hemolymph JHE[f] activity (day-2/3 of adulthood)	5±0.8	3±0.2	8±0.7	7±0.8

Values are means(± SEM) of three replicate selected line means. Modified from Table 4 of Zera and Huang (1999).
[a,f]nmol JH acid min^{-1} mL $hemolymph^{-1}$; [b]nmol JH acid min^{-1} mg wet $weight^{-1}$; [c]percentage whole-body JHE activity found in the hemolymph; [d]nmol JH diol min^{-1} mg wet $weight^{-1}$; [e]percentage injected radiolabelled juvenile hormone that was degraded during a standard incubation period; for experimental details see Zera and Zhang (1995), Zera et al., 1996 and Zera et al., 1998, and Zera and Huang (1999). [a–e]Refer to measurements made during the last juvenile instar.

ulation for the selection experiment was derived is therefore variable for these two classes of JHE-regulating loci.

JHE enzymes from the high- and low-selected lines did not differ in any of a variety of biochemical traits such as Michaelis constant (K_M) for JH, inhibition by general esterase or JHE-specific inhibitors, or thermostability (Zera and Zeisset, 1996). These data indicate that the response to selection was unlikely produced by selection on genetic variants of JHE that differ in kinetic or physical properties (allozymes). These data further imply that the response to selection on JHE activity was due to variable loci that regulate the activity and tissue distribution of JHE activity. Current research is focusing on identifying the molecular causes of genetic variation for JHE activity. JHE from *G. assimilis* has been purified to homogeneity (Zera et al., 2002). Using amino-acid sequence information from the purified protein, a JHE gene has recently been cloned and a full-length sequence, minus the signal peptide, obtained (E. J. Crone, A. J. Zera, R. Russell, J. G. Oakeshott, and L.G. Harshman, unpubl. data). Molecular studies using probes designed from the gene sequence have recently documented line differences in JHE transcript abundance that correlate with line differences in JHE activity (E. J. Crone, A. N. Anand, and A. J. Zera, unpubl.).

4.3.2. Consequences of altered JHE activity

4.3.2.1. In vivo JH metabolism Is the difference in JHE activity between the high- and low-selected lines sufficient to alter whole-organism, in vivo JH metabolism? This question is important for a variety of reasons. In order for JHE activity to evolve by natural selection, variation in activity must be sufficient to cause a change in fitness. This can only occur if a change in JHE activity is sufficient to alter the rate of JH degradation, the JH titer, and the expression of JH-regulated traits that contribute to fitness. Metabolic studies have documented that, in many cases, enzyme activities must be altered considerably before they have "higher level" physiological effects, such as altering the flux through the pathway in which they function (Dykhuizen, 1990 and Fell, 1997). In vivo rate of JH degradation was compared between the selected lines during different stages of artificial selection to determine whether changes in JHE activity were sufficient to alter in vivo JH metabolism. By generation 5, when JHE activity was 3–4 fold higher in high- vs. low-selected lines, in vivo rate of JH degradation only differed significantly between one of the three pairs of high- and low-selected lines (Zera and Zhang, 1995). One–two generations later, when lines differed 6–8 fold in JHE activity, each of three pairs of lines differed significantly with respect to in vivo JH degradation [(Zera et al., 1996); Fig. 2, Table 1]. These data suggest that JHE activity must differ somewhere between 4–7 fold before the differences can effect JH-regulated traits and hence fitness, and that by generations 6–8 of artificial selection, JHE activity had diverged sufficiently to potentially affect the expression of JH regulated traits.

4.3.3. Whole-organism traits

Did artificial selection on JHE activity in fact alter the in vivo rate of JH degradation sufficient to modify the expression of various whole-organism traits? Three traits, duration of the last stadium, muscle mass, and wing length, were measured in the high- and low-activity lines during generation 6 of selection. Each of these traits is known or thought to be regulated by JH (Nijhout, 1994, Wyatt and Davey, 1996, Zera, 2004 and Zera and Denno, 1997). Lines differed significantly in duration of the last stadium, and in the mass of flight muscles, but not in the length of the wings (Table 2). Thus, JHE activity, and in vivo rate of JH degradation, appeared to differ to a sufficient degree between selected lines to alter some whole-organism traits. These results have important implications for mechanisms underlying the evolution of dispersal polymorphism in insects (discussed below).

5. Naturally occurring JHE activity polymorphism in juvenile *Gryllus*: the endocrine developmental basis of a dispersal/life history polymorphism

The artificial selection experiment described above was an experimental manipulation used to investigate the nature, underlying causes, and consequences of standing genetic variation for JHE activity in *G. assimilis*. Another central issue in evolutionary endocrinology is the extent, and mechanisms by which endocrine regulation has been modified by natural selection as a means of evolving changes in organismal structure/function. In insects, there are many examples of ecologically important polymorphisms consisting of morphs (discontinuous phenotypes) that differ in numerous traits that constitute adaptations for flight, reproduction, defense, or camouflage (Nijhout, 1999, Nijhout, 1994, West-Eberhard, 2003, Zera, 2004 and Zera, 2006). Because hormones are known to have many pleiotropic effects, modification of hormonal regulation has long been suspected as the cause of the evolution of these complex, multi-trait polymorphisms such as caste-polymorphism in social insects, and dispersal and phase polymorphisms. Because of their obvious adaptive importance, and the large phenotypic differences between the morphs, these "complex polymorphisms" are very powerful experimental models to study the endocrine basis of adaptive evolutionary change

Table 2.
Morphological and developmental traits measured in lines of *G. assimilis* selected for high or low juvenile hormone esterase (JHE) activity

Trait	*Lines*		Results of AN(C)OVA
	Low	High	
Duration of last juvenile instar (days)	8.9±0.08	9.5±0.06	$P<0.001$
Flight muscle mass (% of whole-body wet mass)	16.7±0.4%	18.1±0.3%	$P<0.025$
Wing length/pronotum length	5.0±0.04	4.9±0.03	$P>0.1$, ns

Data are for lines of one of the three blocks (independent selection trials). Essentially the same results were obtained for lines of the other two blocks (data not shown). Measurements were made on 12 individuals of each line during generation six of artificial selection; flight muscles and wings were measured on newly molted (day-1) adults. Duration of development was tested by ANOVA, while muscle wet mass and wing length were tested by ANCOVA with whole-body wet mass and pronotum length (a measure of body length) as the covariate.

within species. In most endocrine studies of these polymorphisms, environmental modulation of endocrine function (environmental polyphenism) has been the primary focus of attention (Nijhout, 1999). Dispersal polymorphism in crickets (*Gryllus*) is unique in that it is the only "complex polymorphism" that has been extensively studied from an endocrine-genetic perspective, and thus is especially useful for investigating how natural selection has modified endocrine regulatory mechanisms to alter a suite of whole-organism traits to produce within-species variants (morphs) that are specialized for particular tasks.

The most common type of dispersal polymorphism is wing polymorphism, which occurs commonly in many insect groups (Zera, 2004 and Zera and Denno, 1997). The polymorphism consists of a flight-capable morph (LW, long-winged) with fully developed wings and flight muscles and a flightless morph (SW, short-winged), with vestigial, non-functional wings and flight muscles [(Zera and Denno, 1997); see (Zera, 2004) for illustrations of *Gryllus* morphs]. Importantly, the flightless morph exhibits substantially enhanced ovarian growth and fecundity relative to the flightless morph (100–400% greater in SW than LW *G. firmus*); wing polymorphism thus represents the most dramatic example of the trade-off between flight (capability) and reproduction, a trade-off that occurs to some extent in all insects. For decades wing polymorphism in insects has served as a focus of hypotheses concerning the mechanisms by which endocrine control is altered during adaptive evolution (Gould, 1977, Matsuda, 1987, Nijhout and Wheeler, 1982, Zera, 2006, West-Eberhard, 2003, Wigglesworth, 1961 and Zera, 2004). However, only during the past 15 years have detailed endocrine-genetic studies begun to identify the specific aspects of endocrine regulation that have been modified (Roff et al., 1997, Zera, 1999, Zera, 2004, Zera et al., 1989 and Zera and Teibel, 1989).

Endocrine-genetic studies have focused on two closely related species of *Gryllus*, *G. rubens* and *G. firmus*, both of which are wing-polymorphic in natural populations. Importantly, although wing polymorphism is a discontinuous polymorphism, like many ecologically important polymorphisms, it is a quantitative trait with a threshold [see (Falconer and Mackay, 1996 and Roff, 1997) for a general discussion of quantitative traits with a threshold]. That is, flight-capable or flightless morphs are specified by variation in multiple genetic and environmental factors. A particular morph is produced depending upon whether the combined genetic and environmental factors specify morph-inducing factors are above or below a threshold value. Wing morph has a high heritability (Roff et al., 1997) and genetic stocks nearly pure-breeding for LW or SW morphs can be rapidly produced by artificial selection (Zera, 2005 and Zera and Cisper, 2001).

Artificial selection was conducted on a laboratory population of *G. firmus* recently founded from crickets collected from a population in Gainesville, Florida that contained high frequencies of both the LW and SW morph [see (Zera and Cisper, 2001) for details]. Artificial selection resulted in three pairs of genetic stocks nearly pure-breeding for LW or SW morphs. These selected lines, and those in the congener, *G. rubens*, have been extensively characterized with respect to endocrine variation during both the juvenile and adult stages to identify the factors responsible for the differential expression of wings, flight muscles, and reproductive characters in alternate morphs.

With respect to the endocrine-genetics of morph development, JHE in *G. firmus* and *G. rubens*, has been the endocrine factor most intensively studied (Fig. 3; Table 1). Hemolymph JHE activity exhibits a striking activity polymorphism that is very tightly correlated with wing morph in both *G. firmus* and *G. rubens*: Hemolymph JHE activity was approximately 4–6 fold higher in LW vs. SW lines of *G. firmus* and *G. rubens* (Roff et al., 1997, Zera and Huang, 1999 and Zera and Teibel, 1989) (Fig. 3). The strong divergence in JHE activity between lines of *G. firmus* and *G. rubens* selected for wing morph, the nearly perfect co-segregation between wing morph and JHE activity in crosses and backcrosses between LW and SW lines of *G. rubens*, and results of a half-sib quantitative-genetic study of *G. firmus*, all documented a strong genetic correlation between wing morph and JHE activity (Roff et al., 1997, Zera, 2004, Zera and Harshman, 2001, Zera and Huang, 1999 and Zera and Teibel, 1989).

Endocrine-genetic data obtained from artificial selection on JHE in *G. assimilis* has provided important insights into the mechanism of JHE microevolution in natural populations of *Gryllus*. Conversely, studies of the naturally occurring JHE activity polymorphism in *G. firmus* provide information on the microevolutionary significance of genetically variable endocrine traits identified in laboratory populations of *G. assimilis*. First, variation in the same factors contributed to differ-

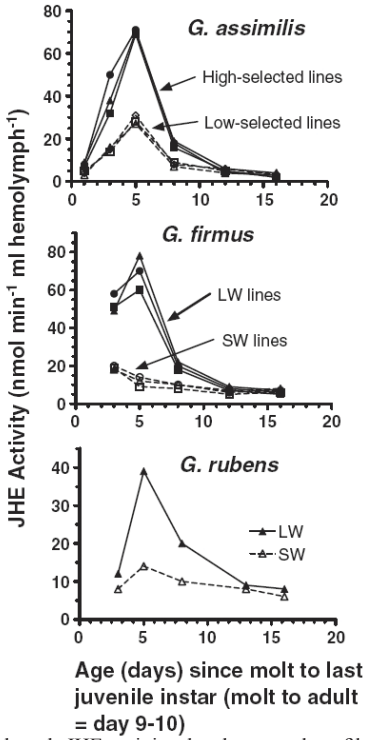

Figure 3. Hemolymph JHE activity developmental profiles in lines selected for JHE activity (*G. assimilis,* a long-winged (LW)-monomorphic species) or wing morph (*G. firmus and G. rubens*). Note the similar developmental profiles in the three species. In *G. rubens*, only one line was selected for the LW morph and one line was selected for the SW (short-winged) morph. Data are from Zera and Huang (1999).

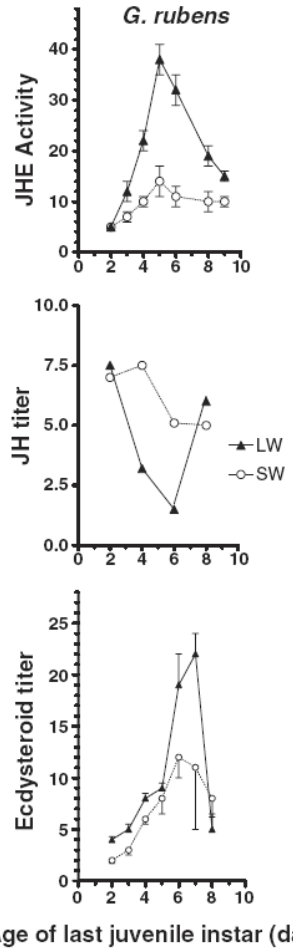

Figure 4. JHE activity, JH titer, and ecdysteroid titer in LW- and SW-selected lines of *G. rubens* (data from Zera (2004)). Measurement units: nM (JH titer); nmol min^{-1} mL $hemolymph^{-1}$ (JHE activity); nmol 20-OH ecdysone equivalents/mL hemolymph (ecdysteroid titer).

ences between LW and SW lines of *G. firmus* and high- and low-selected JHE activity lines in *G. assimilis* (Table 1). For example, about half of the elevated hemolymph JHE activity in LW vs. SW lines of *G. firmus* and in high- vs. low-JHE activity lines of *G. assimilis* resulted from elevated whole-organism JHE activity, while the other half was due to increased secretion of JHE into the hemolymph. Similarly, selected lines of *G. firmus* and *G. assimilis* did not differ in JH-epoxide hydrolase activity, and elevated JHE activity was associated with a elevated in vivo rate of JH degradation (Table 1). These data suggest that the same or similar variable genes that were responsible for the response to artificial selection on JHE activity in the laboratory in the non-polymorphic *G. assimilis* have been the targets of selection during the evolution of the JHE activity polymorphism and dispersal polymorphism in natural populations of *G. firmus.* Second, the large differences in JHE activity between LW and SW morphs of *G. firmus* throughout the juvenile stage but not during the adult stage, are very similar to the JHE developmental profiles produced by artificial selection on JHE in *G. assimilis* during the juvenile stage (Fig. 3). This suggests that (1) there is a similar JHE correlational structure in *G. firmus* (i.e., strong genetic correlations between hemolymph JHE activities on different days of the last stadium, but no correlation between adult and juvenile JHE activities), as was documented in *G. assimilis*, and that (2) divergence in JHE activity in *G. firmus* during the juvenile stage was due to the direct action of natural selection on that stage (similar to artificial selection on juvenile JHE activity). Third, experimental studies of the relationships among JHE activity, in vivo JH degradation, and modification of the expression of whole-organism traits in *G. assimilis* (see above) indicate that the 4–6 fold difference in JHE activity observed between juvenile morphs of *G. firmus* was sufficient to alter the JH titer and influence the expression of whole-organism traits.

In addition to comparing LW and SW stocks with respect to the activity of JHE and related traits, lines of *G. rubens* have also been compared with respect to variation in the JH and the ecdysteroid titers (Fig. 4) (Zera, 2004 and Zera et al., 1989). Because JH titer differences during the juvenile stage of LW and SW lines of *G. rubens* have recently been reviewed in detail, only a brief summary of this topic will be given [see (Zera, 2004) for extensive discussion of this topic]. In a nutshell, JHE activity, the JH titer, and the ecdysteroid titer all differ substantially between morphs of *G. rubens*, in a manner consistent with a role for these endocrine factors in regulating the differential expression of LW and SW morphs (Fig. 4 and Fig. 5). Relative to the LW morph, JHE activity is lower, the JH titer is higher, and the ecdysteroid titer is lower in the SW morph during the last stadium. These relationships suggest the following scenario (Fig. 5): reduced JHE activity delays the drop in JH titer in the SW morph during the last juvenile instar; the prolonged elevated JH titer, in turn, inhibits the development of wings/flight muscles. The significantly reduced ecdysteroid titer in the SW morph may also inhibit development of wings and flight muscles by itself or in combination with the elevated JH titer (Zera, 2004). (Recall that an increased JH titer inhibits the expression of adult genes during metamorphosis while an increased ecdysteroid titer induces their

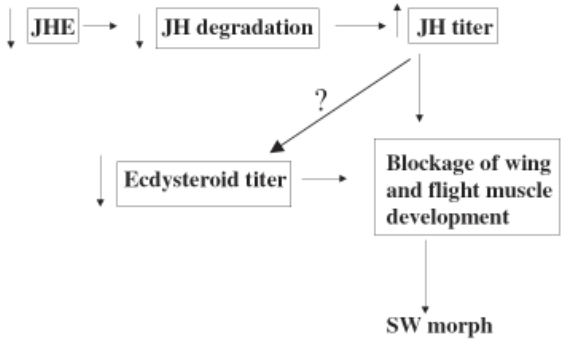

Figure 5. Diagram of postulated endocrine regulation of wing morph in juvenile LW and SW *G. rubens* due to alterations in the JH and ecdysteroid titers. Reduced JHE activity is associated with and potentially causes a prolonged elevation of the JH titer which may inhibit full development of wings and/or flight muscles in the SW morph. Reduced ecdysteroid titer, caused by the elevated JH titer, or by factors independent of the JH titer, may also inhibit wing and flight muscle development, either by itself, or in conjunction with the elevated JH titer [see (Zera, 2004) discussion of alternate hypotheses of JH regulation and morph development].

expression; see above). JH inhibits release of ecdysteroids from the prothoracic gland (Nijhout, 1994). Thus the reduced ecdysteroid titer in the SW morph may be caused by its elevated JH titer, or by factors that regulate the ecdysteroid titer independent of the JH titer [see (Zera, 2004) for additional hypotheses on the endocrine control of morph development]. For over 4 decades, modulation of the JH titer has been proposed as an important factor in the production of alternate morphs in various dispersal polymorphisms (e.g., in aphids and locusts; Gould, 1977, West-Eberhard, 2003, Wigglesworth, 1961 and Zera, 2004). However, only in *G. rubens* have differences in the JH titer, and regulators of the JH titer, in juveniles been directly documented using reliable analytical techniques (Zera, 2004).

As mentioned above (Table 2), high- and low-JHE lines in *G. assimilis*, which differ > 6 fold in activity, do not differ in wing length. This finding has important implications for the model of the endocrine regulation of wing polymorphism discussed above (Fig. 5). The data on *G. assimilis* imply that alteration of JHE activity by itself is insufficient to alter wing length, and that variation in additional regulators is required. A likely candidate is the ecdysteroid titer which differs considerably between LW and SW morphs of *G. rubens* (Fig. 4; titer not measured in *G. firmus*), but which does not differ between high- and low-JHE-selected lines of *G. assimilis* (A.N. Anand and A.J. Zera, unpubl. data). By contrast, flight muscle mass was significantly reduced in the low-selected vs. high-selected JHE activity lines of *G. assimilis*, similar to the reduction in muscle mass in the SW lines of *G. firmus* and *G. rubens* (which have low-JHE activity). This observation raises the interesting possibility that the expression of some key components of wing polymorphism (flight muscles) may be regulated by JHE and JH, while other components (wings) may be regulated by other factors (e.g., ecdysteroids alone or in concert with JHE and JH). This is another illustration of the utility of laboratory artificial selection which can provide information useful to unravel the mechanisms underlying adaptive variation in endocrine regulation.

Although most of the attention concerning the endocrine control of ecologically important polymorphisms has focused on juvenile hormone, ecdysteroid variation may be at least as, or more important (Zera, 2004). Horn size in dung beetles and eyespot size in *Bicyclus* butterflies are adaptively important morphological polymorphisms in natural populations (Brakefield et al., 2003). The ecdysteroid titer also differs between lines artificially selected for horn or wing spot size (Emlen and Nijhout, 1999 and Koch et al., 1996). Similarly, LW- and SW-selected lines of *G. firmus* that differ in adult ovarian growth also differ in the ecdysteroid titer during the adult stage (Zera and Bottsford, 2001) (discussed below).

6. Microevolution of JH and ecdysteroid titers in adults: endocrine basis of life history evolution

Evolutionary biologists have become increasingly interested in identifying the endocrine basis of genetic variation in and covariation (correlation; trade-offs) between life history traits to understand the physiological-genetic mechanisms of life history evolution (Ketterson and Nolan, 1999, Ricklefs and Wikelski, 2002 and Zera and Harshman, 2001). Because juvenile hormone and ecdysteroids control many aspects of reproduction (Klowden, 2002 and Nijhout, 1994), most discussion of the endocrine-genetic basis of life history evolution in insects has focused on these two hormones. However, only in the past few years has genetically based variation been directly documented in adults that differ in life history traits. Hormonal variation itself has proved to be more complex than previously suspected.

6.1. JH titer in adults

Thus far, naturally occurring genetic variation in the JH and ecdysteroid titers have been extensively studied in the context of life history evolution in only one insect species: the wing-polymorphic cricket *G. firmus*. As mentioned previously, flightless SW females exhibit dramatically increased egg production (100–400% higher during the first week of adulthood) relative to their flight-capable counterpart. Because JH is thought to play the primary role in regulating reproduction in othopterans (crickets, locusts, and grasshoppers) (Nijhout, 1994), this hormone has been the focus of attention with respect to evolutionary modification of reproduction in dispersal-polymorphic orthopterans (crickets and locusts). Topical application of JH or JH agonists typically causes enhanced egg production in orthopterans (Zera and Cisper, 2001); when applied to the LW morph of dispersal-polymorphic species, these hormones or analogues increase egg production in the low fecundity LW morph to levels seen in the more fecund SW morph (Zera and Cisper, 2001). These data initially suggested that an elevated JH titer in the SW morph is responsible for the increased fecundity in that morph. However, more detailed investigations revealed a much more complex and interesting picture.

The hemolymph JH titer exhibits a completely unexpected morph (genotype)-specific circadian rhythm, in both laboratory and field populations of *G. firmus* (Fig. 6; Zera and Cisper, 2001 and Zhao and Zera, 2004a; A. J. Zera and Z. Zhao, unpubl. field data). Starting on about day 5 and continuing through at least day 8 of adulthood, the JH titer in the LW morph rises 10–50 fold during the last few hours of the photophase, and drops back down to baseline levels a few hours after the beginning of the scotophase (dark period) (Fig. 6). By contrast, the JH titer in the SW morph is relatively constant during this period, exhibiting only a slight daily cycle due to the cyclic contraction and expansion of whole-cricket blood volume. The morph-specific daily cycle is seen in each of the three pairs of LW and SW lines of *G. firmus* (Fig. 7; Zera and Cisper, 2001) and the JH titer cycle is primarily driven by a morph-specific cycle in the rate of JH biosynthesis (Zhao and Zera, 2004b). Moreover, the morph-specific cycle persists in constant darkness, is eliminated under constant light, and is temperature compensated (Z. Zhao and A. Zera, unpubl. data). Thus, in *G. firmus* there is a genetic polymorphism for a JH titer circadian rhythm that is strongly correlated with wing morph. Finally, a similar morph-specific cycle is observed in laboratory populations of *G. firmus* raised in the field, as well as in the laboratory; in field populations of *G. firmus* sampled in the field; and in other *Gryllus* species sampled in ei-

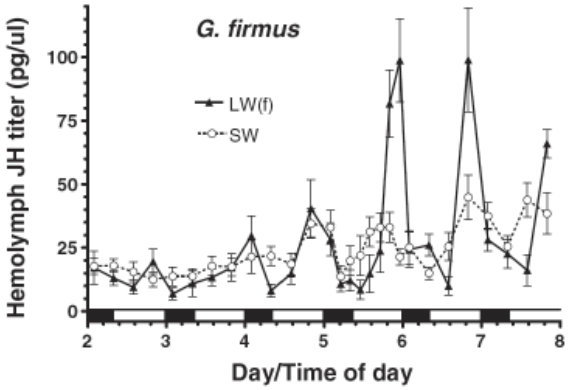

Figure 6. Morph-specific circadian rhythm for the hemolymph JH titer in *G. firmus*. Light and dark bars on the *x*-axis denote light and dark phases of the daily photocycle. Note the high-amplitude titer cycle in the LW(f) morph beginning on about day 5 of adulthood, but only low-amplitude cycle in the SW morph (due to a daily cycle in whole-cricket blood volume). LW(f) denotes LW morph with functional flight muscles (i.e., flight-capable morph) and is synonymous with LW used in other figures.

ther the laboratory or field (A. J. Zera and Z. Zhao, unpubl. data). Thus, the morph-specific JH titer cycle is not restricted to laboratory stocks, or specific laboratory conditions, but is a general characteristic of *Gryllus* species in natural populations. To my knowledge this is the first example of a naturally occurring, functionally important, genetic polymorphism for a circadian rhythm for a hormone titer in any organism.

Several pieces of evidence suggest that the daily cycle in the JH titer appears to be involved in the regulation of some aspect of dispersal in the LW morph. First, the large-amplitude JH titer cycle is only seen in the morph that is capable of flight, and the cycle starts on the day of adulthood when individuals first become competent to fly (Zera and Cisper, 2001 and Zera et al., 1999). Second, topical application of JH to a number of insects enhances propensity for (migratory) flight (Rankin, 1989). Third, some LW adults histolyze (degenerate) their flight muscles and become flightless individuals with enhanced reproduction (ovarian mass is very similar to that of SW females). These LW, but flightless, females loose the JH titer cycle concomitant with flight muscle histolysis (Zera and Cisper, 2001). Finally, fight in crickets is cyclic and only occurs during the night.

The existence of the morph-specific JH titer circadian rhythm raises some intriguing questions regarding mechanisms by which JH regulates the expression of morph-specific traits in dispersal-polymorphic species. Although JH positively affects flight by increasing the propensity to fly in many insects, including some orthopterans (Rankin, 1989), the most widely recognized effects of JH on insect flight are negative: exogenous hormone causes degeneration of the flight muscles, reduces the production and accumulation triglyceride flight fuel, and increases ovarian mass, which increases wing-loading, in many insects including *Gryllus* (Zera, 2005, Zera, 2006, Zera and Cisper, 2001, Zera and Denno, 1997 and Zera and Zhao, 2004). How can the substantially increased JH titer in the LW morph positively regulate aspects of flight without causing the negative effects listed above? This is an intriguing question that is currently under study. Clearly, the endocrine regulation of ovarian growth and dispersal characteristics by JH must be more complex than previously expected, and cannot be due solely to a morph-specific difference in the hormone titer. One intriguing possibility is that the duration of JH titer elevation may be as important as the JH titer itself. Thus, the short-duration (a few hours) elevation in the JH titer in the LW morph, near the end of the photophase and beginning of the scotophase, may be sufficient to release flight behaviors but of insufficient duration to cause histolysis of flight muscles or enhanced ovarian growth. This and other hypotheses concerning morph-specific JH titer cycles and the endocrine control of the trade-off between dispersal and reproduction are discussed in more detail in Zhao and Zera, 2004a and Zhao and Zera, 2004b. Whatever the solution to this paradox, the first direct documentation of naturally occurring genetic variation for the JH titer in adults has identified an unanticipated degree of complexity. Importantly, the morph-specific circadian rhythm could only have been identified by detailed, direct in vivo JH titer measurements. A disturbing development in endocrine-evolutionary studies of insects is the increasing number of studies that rely on indirect measures

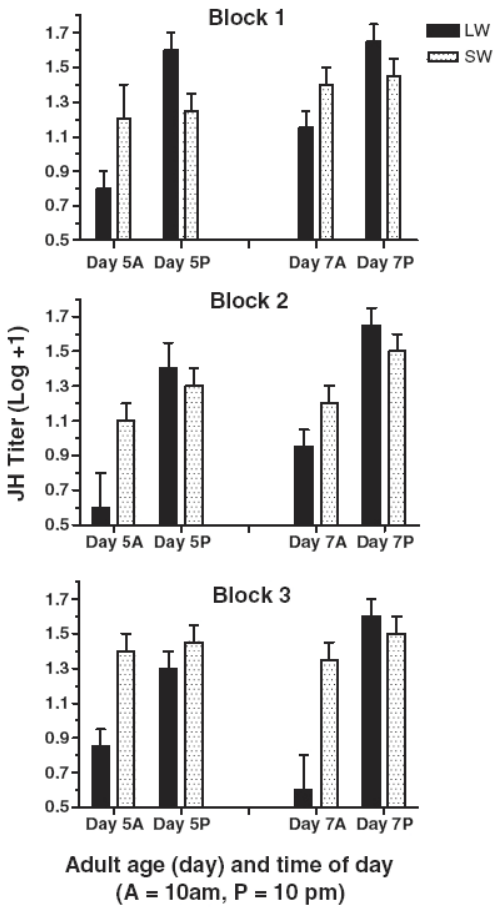

Figure 7. Genetic polymorphism for the morph-specific cyclic increase in the JH titer in three pairs of LW- and SW-selected lines of *G. firmus*. The JH titer exhibited a significantly greater increase during the photophase (AM vs. PM samples) in the LW vs. the SW morph in each of the three blocks (morph × time-of-day interaction: $P < 0.01$ for each ANOVA of each of the three blocks. (Lights on: 8 AM; lights off: 12 AM; measurements were made at 10 AM and 10 PM.

of JH titers that are derived solely from gross level, hormone application experiments (discussed below).

6.2. The ecdysteroid titer in adults

Genetic variation in the adult ecdysteroid titer exhibits a much more straightforward relationship with wing morph than does the JH titer (Zera and Bottsford, 2001; Fig. 8). The ecdysteroid titer is consistently higher in the SW reproductive vs. the LW dispersing morph, both in the laboratory (Fig. 8) and in the field (A. Zera and Z. Zhao, unpubl.), and no strong diurnal variation was observed in the ecdysteroid titer in either morph (Zhao and Zera, 2004a; A. J. Zera and Z. Zhao, unpubl. data). Ecdysteroid titer differences between the morphs strongly parallel differences in ovarian growth and egg production [(Zera and Bottsford, 2001); Fig. 8]. These data suggest that morph-specific differences in the ecdysteroid titer may be involved in the regulation of morph-differences in ovarian growth and egg production. An intriguing possibility is that ecdysteroids may have taken on a more important role in regulating reproductive differences between morphs, thus allowing JH to assume a greater role in regulating flight in the LW morph. An analogous situation has been proposed in honeybees, where JH has been implicated in the regulation of age-dependent division of labor in the worker caste, and does not appear to regulate ovarian development (Bloch et al., 2000).

7. Related topics: endocrine mutants in *Drosophila* and the inappropriate use of endocrine manipulation

Two experimental approaches used to investigate endocrine variation, one relatively new and promising, the other relative old and problematic, merit brief discussion. First, in a promising new approach, laboratory-induced endocrine mutants are beginning to be used to dissect the mechanisms underlying life history trade-offs. The most notable example of this approach has been endocrine-genetic investigation of the fecundity–longevity trade-off in *Drosophila melanogaster*, using single-gene mutants with severely disrupted components of insulin-like signaling (Clancy et al., 2001, Flatt et al., 2005, Richard et al., 2005, Tatar et al., 2003 and Tatar et al., 2001). Some insulin-like signaling mutants are associated with alterations in JH and ecdysteroid regulation (Tatar et al., 2003 and Tatar et al., 2001), while others are not (Partridge et al., 2005 and Richard et al., 2005). Because of the power of *Drosophila* molecular genetics and genomics, this approach will be especially useful for investigating molecular-genetic variation in endocrine regulation. On the other hand, the small size of *Drosophila*, and the instability of its major JH (JH bisepoxide), which has thus far precluded quantification of the JH titer, are significant impediments for integrating molecular and physiological-genetic studies. For example, because of its very small volume, even obtaining a few estimates of the *hemolymph* ecdysteroid titer in adult *D. melanogaster* required a Herculean effort (bleeding ca. 2500 individual flies to obtain 6 titer points; Richard et al., 2005). It is important to note that, while laboratory-induced mutations of large-effect are very useful for investigating proximate mechanisms of endocrine regulation (Flatt et al., 2005, Partridge et al., 2005 and Thummel, 1996), only very limited conclusions can be drawn from such mutants with respect to evolutionary processes in natural populations (Brakefield et al., 2003 and Stern, 2000). Although *Drosophila* laboratory mutants hold great promise as candidate genes to investigate naturally occurring genetic variation, thus far, no study has characterized natural genetic variation for any endocrine regulator in *Drosophila*.

Finally, it is important to mention a commonly used, but inappropriate, technique for investigating endocrine variation that has lead to in a number of questionable reports of functionally important variation in endocrine regulation (e.g., (Dingle and Winchell, 1997, Emlen and Nijhout, 1999, Emlen and Nijhout, 2001 and Moczek and Nijhout, 2002). The technique is hormone manipulation, which involves assessment of the effect of exogenous hormones, agonists, or antagonists, on the expression of some trait. Inferences are then drawn from these experiments concerning the existence of variation in hormonal regulation that controls the trait (typically a morphological polymorphism) in unmanipulated individuals. For example, Emlen and Nijhout, 1999 and Emlen and Nijhout, 2001 constructed a model of variation in JH regulation that controls the expression of an ecologically important horn polymorphism in a beetle. Moczek and Nijhout (2002) have gone on to report populational differences in the threshold sensitivity to JH required to induce horn growth in this species. Importantly, in these studies, conclusions regarding the existence of endocrine variation were based solely on results obtained from hormone manipulation. No aspect of in vivo JH regulation (JH titer, JH titer regulators) was directly measured and shown to differ between genetic stocks that differ in horn morphology. Because applied JH or JH analogues can influence numerous hormones (ecdysteroids, neurohormones; Zera, 2004),

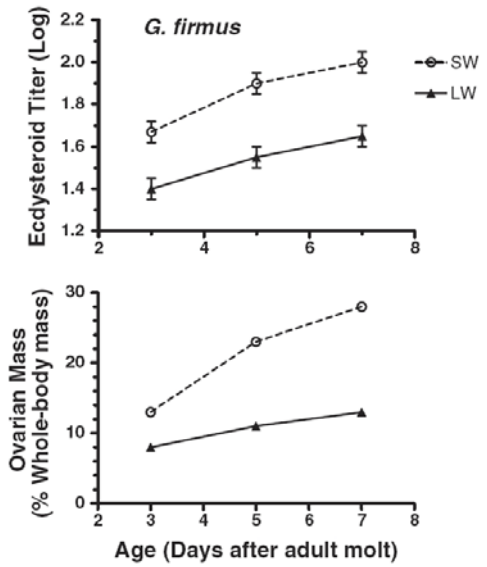

Figure 8. Top panel: elevated ecdysteroid titer in SW-selected vs. LW-selected lines of *G. firmus*. Symbols denote the average (± SEM) of the three LW-selected or the three SW-selected lines. Bottom panel: elevated ovarian mass in SW-selected vs. LW-selected lines of *G. firmus*. Note the association between elevated ecdysteroid titer and elevated ovarian mass in the selected lines.

no reliable information can be obtained regarding variation in a particular hormone, *when hormone manipulation is used as the sole or primary experimental technique*. Of course, when used in conjunction with direct in vivo measures of hormonal variation, hormone manipulation is a powerful experimental tool in endocrine studies (Zera, 2004 and Zera and Cisper, 2001). Assertions of variation in JH regulation reported in studies only using hormonal manipulation (Dingle and Winchell, 1997, Emlen and Nijhout, 1999, Emlen and Nijhout, 2001 and Moczek and Nijhout, 2002); numerous other examples cited in Zera (2004) should be considered as unsubstantiated until purported endocrine variation is verified by direct in vivo measures.

8. Summary, synthesis, and future directions

Characterizing naturally occurring, genetically based endocrine variation, using appropriate analytical techniques, is an essential first step in microevolutionary studies of endocrine regulation. During the past 15 years, the first direct and reliable identification of naturally occurring genetic variation for specific endocrine traits (e.g., JH and ecdysteroid titers, juvenile hormone esterase) has been reported in species of *Gryllus.* Differences in JH regulation between genetic stocks, selected lines, or geographically separated populations, reported in numerous other species (e.g., (Dingle and Winchell, 1997, Emlen and Nijhout, 1999, Emlen and Nijhout, 2001 and Moczek and Nijhout, 2002), should be considered unsubstantiated for reasons discussed above. Natural variation in the ecdysteroid titer, estimated using reliable methodologies, has been reported in selected lines of a few non-*Gryllus* species (Emlen and Nijhout, 1999 and Koch et al., 1996). Given the restricted number of reliably documented cases, obtaining baseline characterizations of natural genetic variation for endocrine regulators remains one of the most important tasks of evolutionary endocrinology.

Although virtually all studies of endocrine variation in *Gryllus* were conducted in the laboratory, there are reasons to believe that, at least to a first approximation, results can be extrapolated to field populations: Laboratory populations were characterized within a relatively short period of time after initiation (< 10 generation of selection) and thus genetic characteristics were likely similar to those of the base population from which selected lines were derived. In the case of JHE activity, no genotype × environment (G × E) interaction was observed. Thus, JHE activity differences between selected lines were independent of specific environmental conditions. Most importantly, in the case of the hemolymph JH titer in adult *G. firmus*, morph-specific patterns of JH titer variation in laboratory populations measured in the laboratory (Zera and Cisper, 2001 and Zhao and Zera, 2004a) were similar to patterns observed in (1) the same laboratory populations raised and characterized in the field, and (2) in field populations of *G. firmus* and other *Gryllus* species directly sampled in the field (Zera and Zhao, unpubl.).

Studies of *Gryllus* have provided the first estimates of genetic correlations between endocrine regulators and the information on the nature of genes responsible for evolutionary changes in endocrine regulation (e.g., hemolymph JHE activity). At this point it is unclear why certain functionally related endocrine regulation are strongly correlated (JHE and JH binding activity), while others are not (JHE activity and JH epoxide hydrolase activity). Data from artificial selection in the laboratory has provided important insights concerning the microevolution of endocrine adaptations in natural populations. As was the case for artificially selected lines of *G. assimilis* produced in the laboratory, JHE activity profiles in LW and SW morphs of *G. firmus* from natural populations differed in magnitude but not in shape during the juvenile stage, and no difference in activities were observed between juveniles and adults (Fig. 3). These parallel patterns suggest that selection on JHE in the field to produce an adaptive endocrine polymorphism was similar to artificial selection on JHE in the laboratory. The response to artificial selection on JHE activity in the laboratory (*G. assimilis*) and divergence in JHE activity between wing morphs in natural populations (*G. firmus*) also appeared to be due to selection on similar types of variable genes: those that regulate whole-organism JHE activity, and those that regulate JHE tissue distribution (Table 1). These parallel analyses further illustrate how laboratory selection studies can provide important insights into microevolutionary mechanisms that produce endocrine adaptations in the field. Roff and Fairbairn (1999) also have used results of laboratory quantitative-genetic studies of JHE in *G. firmus* to predict characteristics of JHE variation in related island populations of this species. Finally, endocrine-genetic studies of life history trade-offs in *Gryllus* have provided the first detailed analysis of the contribution of evolutionary alterations of endocrine regulation to the evolution of life history traits involving specializations for dispersal and reproduction.

Thus far, microevolutionary studies of naturally occurring genetic variation have focused on a restricted set of topics, specifically variation and covariation in juvenile hormone and ecdysteroid titers, and regulators of the JH titer. At present, there are no published biochemical or molecular investigations of natural variation in juvenile hormone or ecdysteroid receptors, or components of cellular signaling of these hormones. Nor have characterizations of naturally occurring genetic variation in other hormones been reported. However, this situation will almost certainly change dramatically in the near future. For example, in the Zera laboratory, JHE gene has been cloned in *Gryllus,* (E. Crone, A. J. Zera, R. Russell, J. Oakeshott, L. G. Harshman, unpubl. data), molecular studies of genetic variation in JHE regulation in selected lines of *Gryllus* are in progress (see above), and surveys of molecular variation of the JHE gene in natural populations will shortly commence. Similarly, the ecdysteroid receptor has been cloned in many organisms, as has various genes of the insulin-like signaling pathway (Thummel, 1996, Flatt et al., 2005, Klowden, 2002 and Partridge et al., 2005), which will stimulate investigations of natural variation in these genes. Clearly, the field of evolutionary endocrinology is poised for explosive development in a molecular direction.

There are also many other central non-molecular aspects of evolutionary endocrinology that have yet to be explored, and which are promising areas for future research. Understanding the microevolution of hormonal regulation requires

endocrine data to be obtained under field conditions. Yet no information on genetic variation in endocrine regulation measured in the field is available for any organism. The only direct measurements of hormonal variation in the field for any insect (other than JH studies in domesticated honeybees), are recent measurement of phenotypic variation in the JH and ecdysteroid titers in field populations of several *Gryllus* species (A. J. Zera and Z. Zhao, unpubl. data). As mentioned above, the morph-specific JH and ecdysteroid titer profiles, which are qualitatively similar when measured in the laboratory or in the field, is reassuring. These data suggest that laboratory endocrine studies may, in fact, provide reliable information concerning endocrine variation in field populations.

Another central topic in microevolution is geographic variation of genetically variable traits, which provides important information on adaptation. There currently are no reliable data on populational differences in any endocrine trait in any organism [studies of Moczek and Nijhout (2002) on geographical variation in JH regulation used the unreliable technique of hormone topical application; see above]. Many important life history traits that are hormonally regulated, such as the onset of reproduction, diapause, etc., vary geographically, and could be powerful experimental models for studies of geographic variation in endocrine control. This topic should be a major focus of future studies of evolutionary endocrinology.

Finally, studies of endocrine variation are likely to have many surprises in store. A good example is the completely unexpected finding of a large-amplitude, morph-specific, genetic polymorphism for the JH titer circadian rhythm in species of *Gryllus* (Fig. 6). This finding raises fundamental questions concerning the mechanisms by which JH regulates important organismal functions, and the microevolution of hormonal circadian rhythms. Studies in *Gryllus* have provided a solid foundation for the development of the nascent field of evolutionary endocrinology, which is emerging as an important subdiscipline at the interface of endocrinology and population genetics.

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