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ABSTRACT

Virtually no published information exists on insect endocrine traits in natural populations, which limits our understanding of endocrine microevolution. We characterized the hemolymph titers of juvenile hormone (JH) and ecdysteroids (ECDs), two key insect hormones, in field-collected short-winged, flightless (SW) and long-winged, flight-capable (LW(f)) morphs of the cricket *Gryllus firmus*. The JH titer exhibited a dramatic circadian rhythm in the LW(f) morph but was temporally constant in the flightless SW morph. This pattern was consistent in each of three years; in young, middle-aged, and older *G. firmus*; and in three other cricket species. The ECD titer was considerably higher in SW than in LW(f) females but did not exhibit temporal variation in any morph and did not differ between male morphs. JH and ECD may control different aspects of the morph-specific trade-off between nocturnal dispersal and reproduction. Results confirm and extend laboratory studies on young female *G. firmus*; most, but not all, important aspects of morph-specific differences in JH and ECD titers can be extrapolated from field to laboratory environments and vice versa. Hormone titers in *Gryllus* are more complex than those proposed in evolutionary endocrine models. Directly measuring hormone titer variation remains a fundamentally important task of insect evolutionary endocrinology.

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Introduction

Evolutionary endocrinology is a newly developing subdiscipline that focuses explicitly on the microevolution of endocrine regulation per se and the endocrine basis of microevolutionary change in whole-organism traits. Recent studies in insects are contributing importantly to the development of this new subdiscipline; these studies currently comprise some of the most detailed investigations of phenotypic and genetic variation and covariation in endocrine regulators and their role in microevolutionary change in development, morphology, and life history (Zera and Zhang 1995; Zera and Huang 1999; Tatar et al. 2003; Hartfelder and Emlen 2005; Richard et al. 2005; Zera 2006; Nijhout and Davidowitz 2007; Zera et al. 2007).

A major gap in studies of insect evolutionary endocrinology, and indeed of insect endocrinology in general, is the paucity of data obtained in natural populations. With a few notable exceptions, such as field studies of the juvenile hormone (JH) titer in locusts (Botens et al. 1997) and studies of the JH titer in honeybees under seminatural conditions (Elekonich et al. 2001 and references therein), all insect endocrine studies have been undertaken in the laboratory. This paucity of information stands in stark contrast to the numerous ecological-endocrine field studies undertaken in vertebrates, most notably investigations of the endocrine control of various social behaviors (mate choice, parental behavior, sexual selection, territoriality), life-history traits, and body color (Marler 1988; Ketterson and Nolan 1999; Sinervo et al. 2000; Wingfield et al. 2001; Adkins-Regan 2005; Hoekstra et al. 2006; Reed et al. 2006; Zera et al. 2007). Because endocrine adaptations ultimately arise in natural populations, this paucity of field information in insects severely limits our understanding of the microevolution of endocrine regulation in this group. Numerous environmental variables that affect hormonal traits often differ significantly between the laboratory and the field (temperature, photoperiod, insect density, food availability and quality), and it is thus difficult to extrapolate from endocrine features documented in the laboratory to their existence under field conditions. Furthermore, traits are often measured on populations that have been kept in the laboratory for many years and may be adapted to specific laboratory conditions (Gibbs 1999; Harshman and Hoffmann 2000).

This study constitutes the first in-depth investigation of endocrine variation in natural populations of an insect species.

The foci are JH and ecdysteroid (ECD) titer variation in the wing-dimorphic cricket *Gryllus firmus*, one of the most intensively studied insects with respect to the endocrine regulation of dispersal polymorphism (reviewed in Zera 2004, 2006). This species exists primarily as a flight-capable morph that has fully developed (long) wings and fully developed, functional flight muscles (LW(f)) and as an obligately flightless morph with shortened wings and vestigial, nonfunctional flight muscles (SW). Importantly, in the laboratory, the SW morph begins reproduction earlier and produces substantially (100%–400%) more eggs than LW(f) females during early adulthood. Thus, early-age reproduction trades off with flight capability. JH and ECDs both regulate important aspects of development and reproduction and have been extensively studied in the laboratory with respect to the endocrine control of morph development and reproduction in insect polymorphism in general (Nijhout 1994; Klowden 2002; Gilbert et al. 2005) and dispersal polymorphism in *Gryllus* in particular (see above references).

Thus far, models of the hormonal regulation of insect polymorphism propose rather simple JH titer differences between phenotypes. For example, with respect to wing polymorphism, the JH titer in the flightless, reproductive morph is expected to be consistently higher than that of the dispersing morph with lower reproductive effort during adulthood (Nijhout 1994; Zera 2004, 2006). The basis for this expectation is that JH positively regulates reproduction and, in general, has negative effects on flight capability (e.g., causes histolysis of flight muscles; Nijhout 1994; Zera 2004, 2006). However, one of the first direct measurements of the hemolymph JH titer in a wing-polymorphic insect indicated a much more complex picture (Zera and Cisper 2001; Zhao and Zera 2004*a*). The JH titer exhibited a large-amplitude, genetically based circadian rhythm in the LW(f) morph of *G. firmus* that cycled above and below the relatively temporally invariant JH titer in the SW morph (see "Discussion"). Not only was this one of the first demonstrations of a JH titer circadian rhythm in an insect, it represents the first described case of a morph-specific polymorphism for a hormone titer and one of the largest-amplitude circadian rhythms yet reported for a hormone titer in insects (Zhao and Zera 2004*a*, 2004*b*; Vafopoulou and Steel 2005).

Thus far, all endocrine traits in *G. firmus* have been measured in females in artificially selected lines that had been kept in the laboratory under constant, benign conditions for at least a few years before endocrine characterization. Thus, the extent to which these endocrine traits are expressed to a similar degree in the field (or in males) was unknown. This issue is especially important for a novel feature, such as a morph-specific JH titer circadian rhythm. Consequently, we measured hemolymph JH and ECD titers in field-collected female and male morphs of *G. firmus* and in a few other LW or SW cricket species. Traits were measured in Gainesville, Florida, the locality where the founders of the laboratory lines of *G. firmus* had been collected, over a 3-yr period, to determine the temporal constancy of hormone titer profiles.

Material and Methods

Background on Species Studied

Gryllus firmus, the sand field cricket, was the major focus of the present study. Three other cricket species were studied to a lesser degree: *Gryllus rubens*, *Gryllus ovisopis*, and *Scapteriscus vicinus*. *Gryllus firmus* and *G. rubens* are wing polymorphic: each species exists as three morphs that trade off dispersal capability for egg production (Walker and Sivinski 1986; Zera 2004). The long-winged, flight-capable morph (LW(f)) has fully developed wings and large pink, functional flight muscles, while the short-winged morph (SW) has short wings and white, nonfunctional flight muscles. In the laboratory, SW females exhibit 100%–400% greater ovarian growth than LW(f) females during early adulthood (Zera 2004, 2006). In addition, some LW(f) females histolyze (degenerate) their flight muscles (denoted LW(h)) and become flightless, at which time ovarian growth increases to the level seen in SW females. These two wing-polymorphic species typically occur in temporary, early-successional habitats, such as pastures (Walker 1986). *Gryllus ovisopis* is a flightless, SW-monomorphic species that inhabits woodlands (Walker and Sivinski 1986), while *S. vicinus* (tawny mole cricket) is an LW-monomorphic species that occurs in temporary, successional pastures (Forrest 1986). The tawny mole cricket is an abundant, strong flier and could be easily collected in sound traps during the period of time when *Gryllus* species were sampled.

Population Sampling

The four species were sampled from several localities within 7 mi (11.26 km) of each other in and around Gainesville, Florida, during September–October 2002–2004. *Gryllus firmus* and *G. rubens* exhibit a major dispersal peak in September–October, and *S. vicinus* exhibits a minor dispersal peak during this time (Walker 1986). *Gryllus ovisopis* emerge as adults only during the fall (Walker 1974). *Gryllus firmus* were primarily collected using a sound trap (described below) at two pastures and, to a lesser degree, by hand in an organic garden. One of the collecting sites is illustrated in Figure 1. *Scapteriscus vicinus* and *G. rubens* were collected at pasture sites using sound traps. Males of the woodland species, *G. ovisopis*, do not call (Walker 1974), and thus male and female *G. ovisopis* were attracted to an oatmeal trail and were collected by hand at dusk.

Sound traps (described in Walker 1986) broadcast species-specific male calling songs, using an electronic sound synthesizer driven by a programmable microprocessor. The synthesizer and speaker were attached to a metal cone that broadcast the song upward (Fig. 1). The base of the cone emptied into a container or bucket, and a 20–40-ft^2 (1.84–3.68-m^2) area of

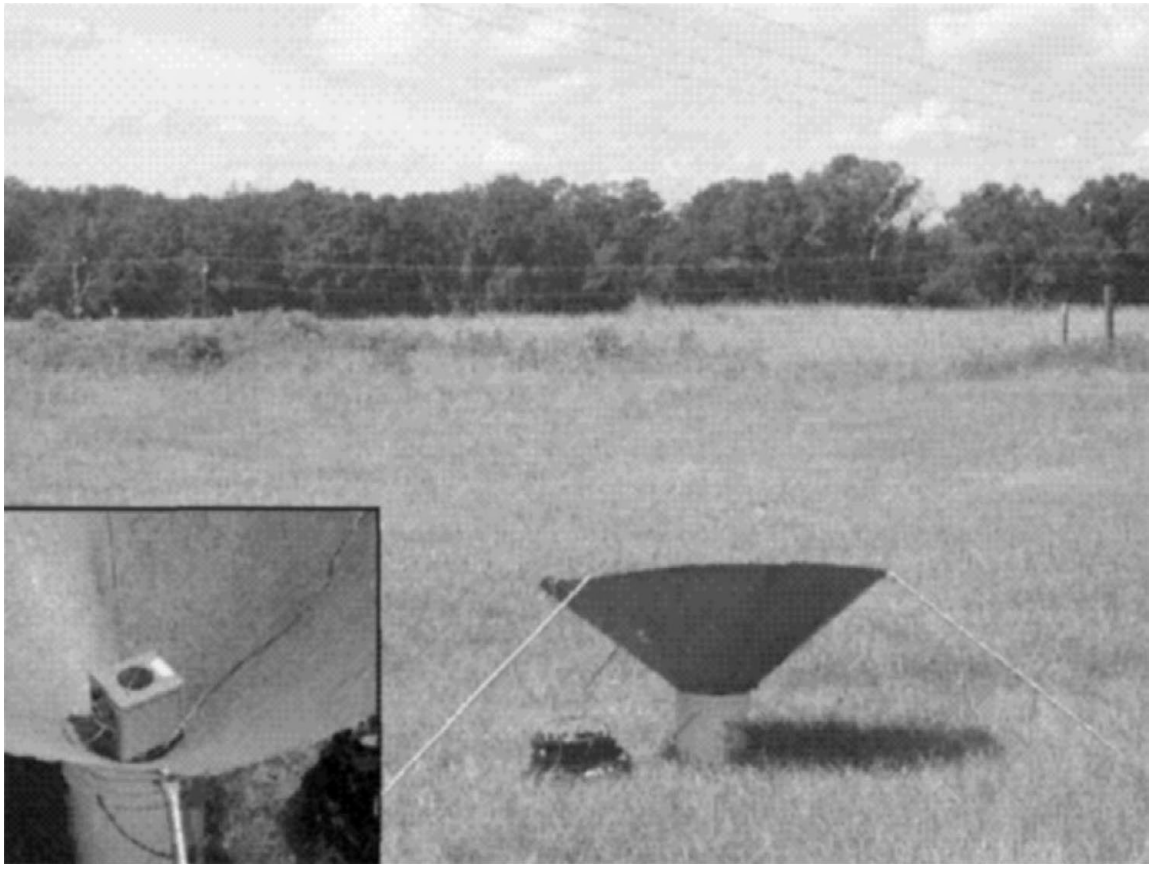

Figure 1. Pasture collecting site and sound trap. Inset illustrates the electronic song synthesizer. Normally, a 20–40-ft^2 area around the sound trap was covered with black plastic.

ground around the *G. firmus* and *G. rubens* traps was covered with black plastic. Flying male and female crickets attracted to the song flew into or near the metal cone and were trapped in the bucket or (more commonly) were found under the black plastic. Flightless individuals were also attracted to the trap and were found under the plastic. All mole crickets were trapped within the container attached to the broadcasting cone. In all cases, only individuals of the species whose song was broadcast were found in or around the sound trap. Traps were inspected daily in the morning. *Gryllus* were removed, placed in containers with a small piece of apple, and transported to the Entomology Department, University of Florida, Gainesville. Mole crickets were placed in containers with several inches of moist sand. Containers were kept outside in buckets or aquaria, on a shaded wooden platform, until blood samples were taken later that day or early the following morning. Additional *G. firmus* were collected by hand by turning over logs, boards, or pieces of black plastic in about a 100-m^2 area near the pasture traps and at the organic garden mentioned above.

Hemolymph Collection

Hemolymph (blood) was collected into graduated 50-μL micropipettes from cuts in the circi, legs, and antennae. In general, 10–20 μL of hemolymph were collected from a single individual. When less than 10 μL was obtained (almost always males), hemolymph was pooled from two individuals. Weight, gender, wing morph (LW or SW), and flight-muscle status (pink [functional] or white [nonfunctional]) was recorded for each individual that was subsequently placed in a test tube and frozen. Individuals were transported on dry ice to the University of Nebraska, where ovaries were removed and weighed, and the age of individuals was determined as described below.

Hemolymph was blown into 300 μL of 90% methanol in water, briefly vortexed to break up the clot, and kept on ice. After all hemolymph samples had been collected for a particular time period (ca. 45 min), they were processed essentially as described in Zhao and Zera (2004*a*). Briefly, the methanol-water mixture was vortexed with 600 μL hexane for 2 min to extract JH, and the mixture was centrifuged for a few minutes

at room temperature at low speed (ca. 1,000 rpm). About three-quarters of the upper hexane layer was removed using a pulled-glass pipette, with care taken not to remove solid material at the hexane-methanol/water interface, and was transferred to a 5-mL glass ampule. This extraction was repeated two more times, with all extracts of a single hemolymph sample being put into a single ampule. The ampules were flame sealed and were kept at −20°C for 1 wk to 1.5 mo, until transported on dry ice to the University of Nebraska. Of the 300 μL of methanol-water solution (containing ECDs), 250 μL was transferred to an Eppendorf tube and kept at −20°C until transferred on dry ice to Lincoln, Nebraska. JH and ECDs were quantified by radioimmunoassay as described in Zhao and Zera (2004*a*).

Hemolymph Sampling Schedule

The main purpose of the study was to determine whether JH and ECD titers differed between morphs in the field and whether a morph-specific circadian rhythm exists for the JH titer, as had previously been observed in the laboratory for *G. firmus* (Zhao and Zera 2004*a*, 2004*b*). Because the JH titer rose and fell dramatically a few hours before and just after lights-off in the laboratory, blood sampling was concentrated during the period just before and after sunset. During the sampling period (September 10–October 30, 2002–2004), sunrise occurred between 7:11 a.m. (September 10) and 7:41 a.m. (October 30), while sunset occurred between 7:44 and 6:44 p.m. (end of civil twilight: 8:05–7:09 p.m., all Eastern Daylight Time; U.S. Naval Observatory, Astronomical Applications Department, http://aa.usno.navy.mil). Blood samples were taken at 5:00 a.m. (before sunrise), 10:00 a.m., 3:00 p.m., 6:00 p.m. (before sunset), 9:00 p.m. (after sunset), and midnight (night). For each sampling period, 12–16 crickets were bled within a 45-min period starting about 15 min before the nominal sampling time. Thus, a blood sample designated 3:00 p.m. was taken between 2:45 and 3:30 p.m. For hemolymph samples collected after sunset, the following protocol was used to minimize the period of time that crickets were exposed to light in the laboratory before bleeding. Before sunset, crickets were divided, three or four per box containing a piece of apple, and individual boxes were brought into the laboratory, resulting in crickets being exposed to light no more than 1–15 min before bleeding.

Aging Crickets

Adult age (days after molt to adulthood) was estimated for individuals of each of the four sampled species by counting the daily growth layers of chitin, essentially as described by Zuk (1987). Chitin is laid down daily in a circadian rhythm, resulting in daily growth rings equal in number to the days after a molt. Cross sections of hind-tibial segments were examined under water under phase-contrast optics, and the number of growth rings was counted. Background studies using laboratory *G. firmus* of known age (unknown to the person counting the rings) demonstrated that age could be accurately estimated by this method for adult crickets that were 2–22 d post–adult molt, which bracketed the age of nearly all (>98%) crickets collected in the field (results of linear regression of number of growth rings on days post–adult molt: slope = 0.92 ± 0.02, $P < 0.001$, intercept = 0.09 ± 0.02, $r^2 = 0.97$, $n = 51$. Nearly identical slopes were obtained when linear regressions were performed separately on males or females or on LW or SW morphs.

Statistical Analyses

Linear regressions, χ^2 tests, ANCOVA, ANOVA, and correlational analyses were performed using Systat 8.0. Before ANOVA or linear regression, age was square root transformed and proportions were arcsine transformed. Because the ECD titer in females was associated with age, correlations involving this variable were performed on residuals derived from ANCOVA.

Results

Morph Frequencies, Ages, and Reproductive Characteristics of Gryllus firmus

Five hundred and fifty *Gryllus firmus* were collected during 2002–2004. The hemolymph JH titer was measured in 534 individuals, the ECD titer was measured in 130 individuals in 2002 and 139 individuals in 2004, and 140–160 individuals were aged in each of the three years of the study. The LW(f) and SW morphs were common in both males and females during each of the three years sampled (frequencies typically 0.4–0.5 for each morph; Table 1), while the frequency of the LW(h) morph typically ranged from 0.11 to 0.12. Morph frequencies did not differ across years in either sex (χ^2 tests, $P > 0.7$), and morph frequencies pooled across years did not differ between males and females (χ^2 test, $P > 0.6$).

Gryllus firmus ages ranged from 5 to 22 d post–adult molt, with the average ranging from 11 to 14 d for the various morphs measured in the three years (Table 1). Nearly all (445 of 450) crickets had ages that fell within the range of the standard curve (2–25 d post–adult eclosion; see "Material and Methods"), and the five that did not had ages (23–25 d) that were close to the upper limit of the standard curve. Cricket age did not differ between morphs overall (ANOVA, $P > 0.5$) or within sex (Table 1), although LW(h) individuals tended to be slightly older than those of the other two morphs. Average cricket age (pooled across morphs) was slightly higher in 2002 (13.6 ± 0.34 d, $n = 161$) than in the other two years (2003: 11.2 ± 0.47 d, $n = 147$; 2004: 11.5 ± 0.35 d, $n = 135$), and there was no morph × year interaction (ANOVA, $P > 0.5$ for all two- and three-way interactions). Average age and age distribution of cricket species sampled in the field in this study are similar to

Table 1: Age, morph frequencies, and ovarian mass of morphs of *Gryllus firmus* sampled in this study

Sex/Character	LW(f)	LW(h)	SW	Tests of Morph Differences (ANOVA)		
				F	df	*P*
Males:						
Morph frequency (*n*)	.49 (116)	.11 (26)	.40 (94)			
Adult age				.00	2, 182	>.9
Mean ± SEM (*n*)	11.8 ± .4 (87)	11.9 ± 1.0 (19)	11.7 ± .4 (77)			
Median (range)	12 (6–25)	12 (7–20)	11 (5–20)			
Females:						
Morph frequency (*n*)	.47(148)	.13(42)	.39 (108)			
Adult age				2.05	2, 66	>.1
Mean ± SEM (*n*)	11.9 ± .33 (125)	14.0 ± .784 (34)	12.1 ± .33 (108)			
Median (range)	12 (5–24)	13 (7–24)	12 (6–22)			
Ovarian mass (% whole-body wet mass, *n*)	6.0 ± .7 (84)	16.8 ± 1.3 (23)	14.8 ± .9 (75)	38.0	2, 176	.000[a]

Note. LW(f) = long wings with functional flight muscles; LW(h) = long wings with histolyzed flight muscles (flightless); SW = short wings with underdeveloped flight muscles (flightless). Data are pooled across years (did not differ significantly across years and exhibited no year × morph interaction; $P > 0.1$ in all cases); n = number of individuals. Age = number of days after molt to adulthood.

[a] See "Results" for pairwise comparisons between morphs.

values reported for other *Gryllus* species sampled in the field (Zuk 1987; Murray and Cade 1995).

Ovarian mass, scaled to whole-body wet mass, differed substantially among the three female morphs (Table 1) but did not differ between years ($P > 0.2$), and there was no morph × year interaction ($P > 0.2$; Table 1). In a priori pairwise comparisons (data pooled across years), ovaries were about 2–3 times heavier in each of the flightless female morphs than in flight-capable females (SW vs. LW(f) females: $t_{1,96} = 7.8$, $P < 0.001$; LW(h) vs. LW(f) females: $t_{1,105} = 7.2$, $P < 0.001$; Table 1). By contrast, ovarian mass did not differ between the two flightless morphs ($t_{1,96} = 1.12$, $P = 0.26$).

Effect of Age on Reproductive and Hormonal Traits

Linear regressions for JH titer or ovarian mass on age were nonsignificant (JH titer: $P > 0.2$, $n = 435$; ovarian mass: $P > 0.05$, $n = 173$). However, the ECD titer varied positively with age for the entire data set ($P < 0.005$; $n = 243$) and for females alone ($P < 0.005$, $n = 149$) but not for males alone ($P = 0.8$, $n = 94$). Thus, subsequent analyses of morph differences in the JH titer, ovarian mass, and male ECD titer involved ANOVA, while morph differences in the ECD titer in females were assessed by ANCOVA, with age as the covariate.

Hemolymph JH Titer

Hemolymph JH titer profiles as a function of morph and time of day for females obtained in each of the three sampled years and pooled across years are presented in Figure 2; comparable samples for males are presented in Figure 3, and titer profiles pooled across years and sexes are presented in Figure 4*A*. For visual clarity, and because only a few samples were obtained in any given year, titers for LW(h) individuals are presented only in Figure 4*A*. Substantial titer differences were observed between morphs that were consistent across the three sampled years. The JH titer in the SW morph exhibited relatively little diurnal change (typically about a onefold increase during the 24-h period) or variation that was not consistent from year to year. This relatively invariant diurnal pattern is most evident in SW males and females of 2003 and in data pooled across sexes and years (Fig. 4*A*). By contrast, the LW(f) morph exhibited a diurnal pattern consisting of a peak (six- to sevenfold increase) between 6:00 and 9:00 p.m. ($X = 0.75$–0.875, where X is the proportion of day passed from midnight) that was consistent between sexes and across years: the titer in the LW(f) morph was lower relative to the SW morph in the morning, 5:00–10:00 a.m. ($X = 0.2$–0.42), rose above the titer in the SW morph between 6:00 am and 9:00 p.m., and dropped below the SW titer by midnight (1.0). This pattern is also most evident in 2003 and in the samples pooled across years (Fig. 4*A*).

Results of ANOVAs verified the conclusions presented above. The JH titer in the SW morph did not vary diurnally in females alone ($F_{5,117} = 1.0$, $P = 0.44$) when both sexes were pooled ($F_{5,206} = 0.21$, $P = 0.2$), and it varied only marginally throughout the day in males alone ($F_{5,83} = 2.63$, $P = 0.05$). By contrast, the JH titer in the LW(f) morph varied significantly throughout the day in females alone ($F_{5,136} = 8.5$, $P = 0.000$), in males alone ($F_{5,106} = 4.70$, $P = 0.001$), and in both sexes ($F_{5,248} = 12.8$, $P = 0.000$). Most importantly, the JH titer exhibited a significant interaction between morph and time of day for females alone ($F_{5,253} = 3.25$, $P = 0.007$), males alone ($F_{5,189} =$

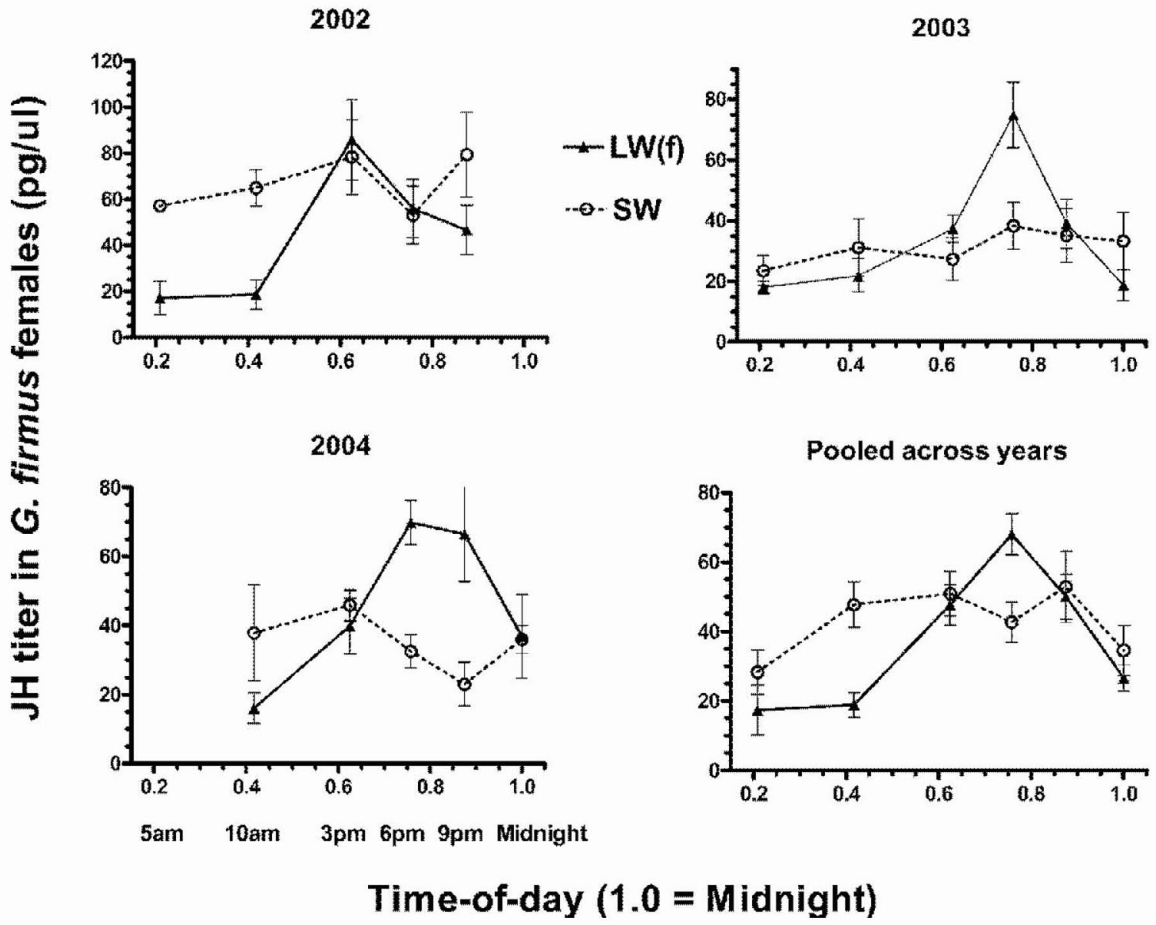

Figure 2. Hemolymph juvenile hormone (JH) titer (mean ± SEM) in LW(f) (long-winged, flight-capable; *triangles, solid line*) and SW (short-winged, flightless; *circles, dotted lines*) female *Gryllus firmus* as a function of time of day measured during September–October 2002–2004. Symbols represent mean ± SEM. Values on the *X*-axis represent proportion of the day passed, starting at midnight. Actual sampling times (all in Eastern Daylight Time) are given below the *X*-axis of the lower left panel. For visual clarity, and because many fewer LW(h) (long-winged, flightless, with histolyzed flight muscles) females were sampled, JH titer data for this morph are presented only in Figure 4*A*, where data are pooled across sexes and years. For individual years, means were based on sample sizes that ranged from five to 15 individual crickets (median $n = 9$), except for SW, 2002 at 5:00 a.m., where $n = 1$. For data pooled across years, sample sizes ranged from 13 to 39 (median $n = 20$), except for SW 5:00 a.m. ($n = 7$), and LW(f) 5:00 a.m. ($n = 5$). During the 1.5-mo sampling period (September 10–October 20), sunrise occurred between 7:11 and 7:41 a.m. ($X = 0.3$–0.32), and sunset occurred between 6:44 and 7:44 p.m. ($X = 0.78$–0.81).

2.36, $P = 0.04$), and the sexes pooled ($F_{5,454} = 5.13$, $P = 0.000$). The JH titer for the LW(h) morph pooled over years is presented in Figure 4*A*. The titer profile in this morph was intermediate between those of the LW(f) and SW morphs. The JH titer in the LW(h) morph was similar to that in the SW morph early (5:00–10:00 a.m.; $X = 0.21$–0.42) and late (midnight) in the 24-h cycle, but it rose to values equal to the peak values for the LW(f) morph at 3:00–6:00 p.m.

Hemolymph JH titers as a function of time of day in three age classes of *G. firmus* are presented in Figure 4*B*. Age classes were computed by ranking all crickets by age for each year and dividing them into three groups of equal size. Mean ± SEM ages (days post-adult eclosion) for these three classes are as follows: youngest (8.4 ± 0.13, $n = 150$), intermediate (12.0 ± 0.1, $n = 149$), oldest (15.0 ± 0.23, $n = 150$). JH titer profiles for the age classes clearly show that the morph-specific patterns of diurnal variation observed in the data pooled across ages (Fig. 4*A*) occur in each age class (Fig. 4*B*). In other words, the JH titer exhibits a morph-specific cycle in the LW(f) morph in the field. Because the scale on the *X*-axis in Figure 4*B* is compressed relative to that in the other plots of the JH titer, the cyclic pattern of the JH titer in the SW morph, which is of lower magnitude than that in the LW(f) (and which is not statistically significant; see above) is also apparent. JH titer by age class is not presented for the LW(h) morph in Figure 4*B* because of the much smaller number of samples obtained for this morph, which resulted in no or very few samples for several times of day for individual age classes.

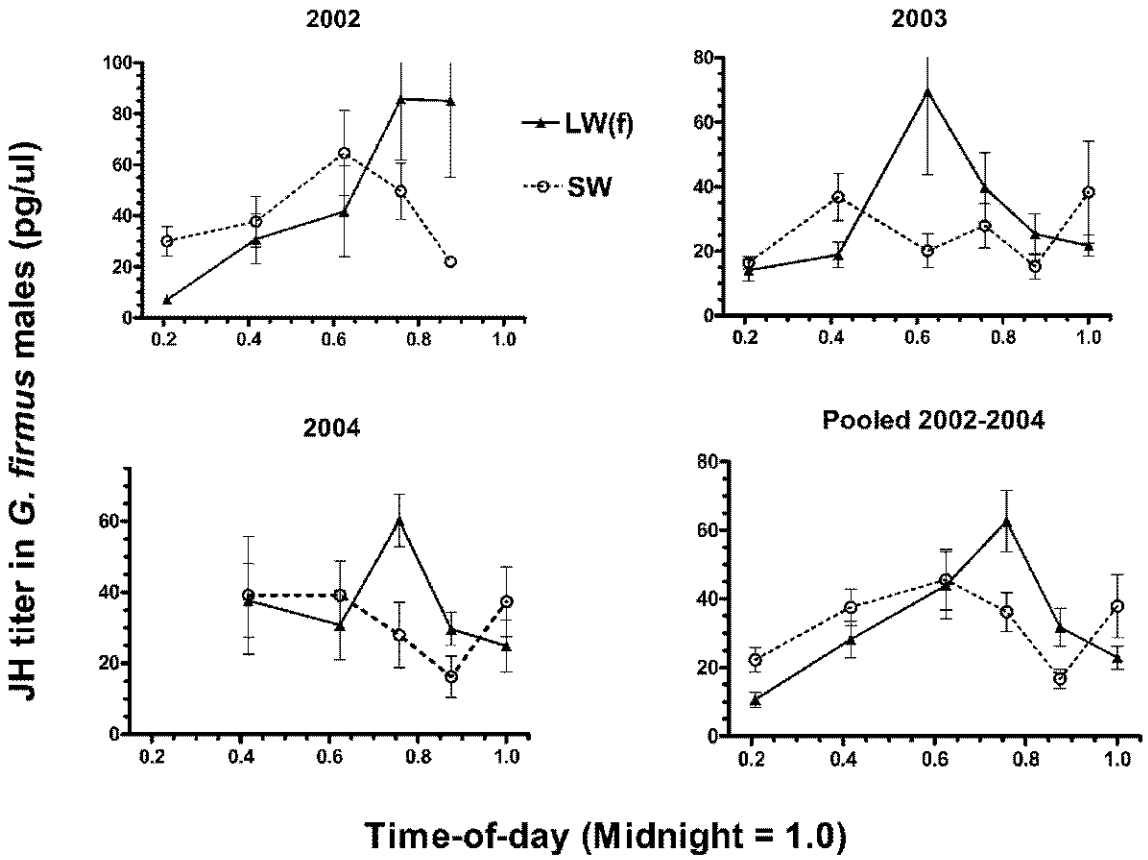

Figure 3. Hemolymph juvenile hormone (JH) titer (mean ± SEM) in LW(f) (long-winged, flight-capable) and SW (short-winged, flightless) male *Gryllus firmus* measured during 2002–2004. See Figure 2 for explanation of symbols, values on the *X*-axis, and timing of sunrise and sunset. Means within each year were based on sample sizes of 3–14 individuals (median $n = 6$), except for two cases where $n = 2$. For data pooled across years, means were based on samples of 6–24 individuals (median $n = 16$).

ECD Titer

The ECD titer profiles for females and males collected in 2002 and 2004 are presented in Figure 5; titer profiles pooled across years in each sex are presented in Figure 6. Because the ECD titer varied significantly with age in females but not in males (see above), data were analyzed separately by sex: by ANOVA for males and by ANCOVA for females. In addition, ECD titers in LW(h) morphs were not analyzed statistically; the ECD titer was measured in only a few individuals of this morph, resulting in very low ($n \leq 1$) sample sizes in each sex for more than half of the time periods.

In males, the ECD titer showed no significant variation for any factor (morph, time of day, year) or significant interaction between factors ($P > 0.1$ in all cases, typically $P > 0.3$). By contrast, in females, two highly significant interactions involving year and morph ($P < 0.03$) were observed in the overall ANCOVA. This was mainly because of the smaller difference between morphs in the ECD titer in 2004 compared with 2002 (Fig. 5, *left*). Because of these interactions, the ECD titer data in females were analyzed separately for each year. In each year, the ECD titer was significantly (2.5–3.2-fold) higher in SW than in LW(f) females ($P < 0.005$ in each year); there was no significant variation in time of day ($P > 0.15$) or morph × time interactions ($P > 0.1$) in either year.

Correlations between JH and ECD Titers and Ovarian Mass

The ECD titer (residuals from ANCOVA) was positively correlated with ovarian mass (arcsine-transformed percentage of body wet mass due to ovaries: $r = 0.46$, $P < 0.01$, $n = 64$, Bonferroni corrected for test of two independent variables). By contrast, the JH titer was not correlated with ovarian mass ($r = 0.28$, $P > 0.1$), nor were the JH and ECD titers correlated with each other ($r = 0.22$, $P > 0.1$).

Other Cricket Species: Ages, Ovarian Mass, and JH Titer

The hemolymph JH titer was measured in three other cricket species that differed in flight capability and reproductive out-

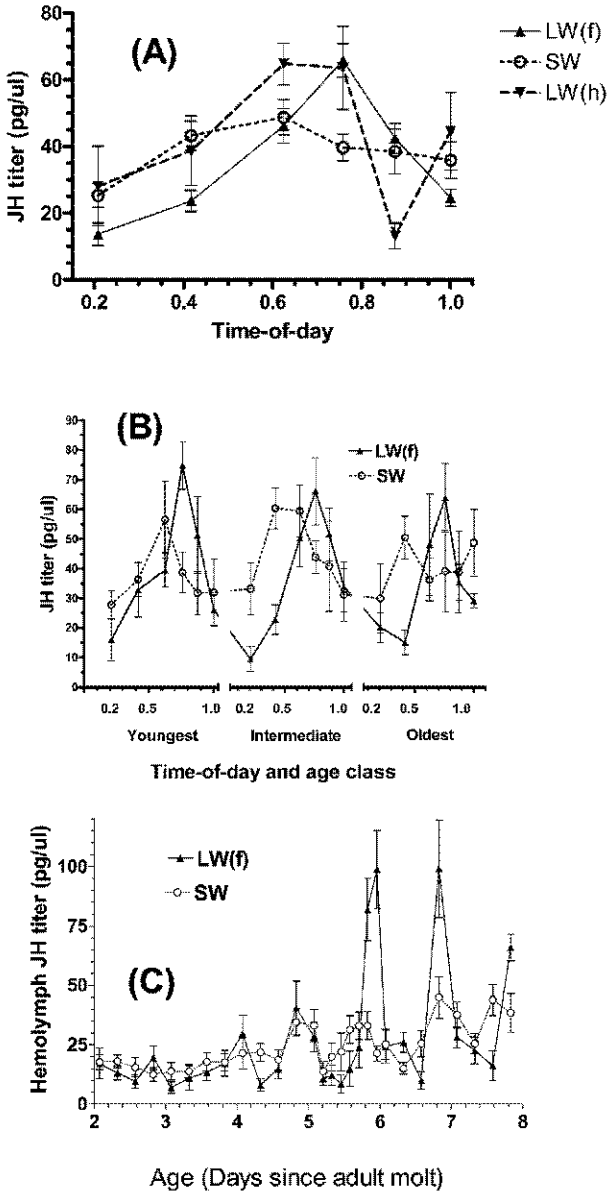

Figure 4. *A*, Hemolymph juvenile hormone (JH) titer (mean ± SEM) in LW(f) (long-winged, flight-capable), SW (short-winged, flightless), and LW(h) (long-winged, flightless, with histolyzed flight muscles), morphs, with data pooled across years and sexes. See Figure 2 for explanation of symbols, values on the *X*-axis, and timing of sunrise and sunset. Means for LW(f) and SW morphs were based on samples of 11–64 individuals (median $n = 40$). Sample sizes for means for the LW(h) morph ranged from 15 to 61 (median $n = 11$), except for 5:00 a.m. ($X = 0.21$), where the sample size was 4, and midnight, where the sample size was 7. *B*, Hemolymph JH titer in LW(f) and SW morphs as a function of age class (youngest [8.4 ± 0.2 d after adult eclosion], intermediate [12.0 ± 0.1 d], and oldest (15.0 ± 0.2 d]) and time of day. Because sample sizes were low or because no samples were available for LW(h) individuals at several time points when data were separated into age classes, JH titer data for this morph are not presented. See "Results" for construction of age classes. See Figure 2 for explanation of symbols and values on the *X*-axis. Means were based on sample sizes of 3–23 individuals (median $n = 11$), except for $X = 3.21$, where $n = 2$ for both SW and LW(f) morphs. *C*, Hemolymph JH titer measured in *Gryllus firmus* female morphs in the laboratory (Zhao and Zera 2004*a*). Note the similar morph-specific JH titer profile in laboratory crickets as was obtained from field-collected individuals (*A*).

put: SW-monomorphic (flightless) *Gryllus ovisopis*, LW *Gryllus rubens*, and LW-monomorphic *Scapteriscus vicinus*, the tawny mole cricket. All LW *G. rubens* and *S. vicinus* sampled for JH titer were LW(f). Although *G. rubens* is a wing-polymorphic species, only a very few (<10) SW individuals were collected, compared with hundreds of LW(f) individuals; thus, JH titers were measured only in the LW morph. Mean ± SEM age (days post–adult eclosion) for sampled individuals of the three species were as follows: *S. vicinus* males and females, 10.5 ± 0.42 d, $n = 69$; *G. ovisopis* females only, 11.0 ± 0.48 d, $n = 35$; *G. rubens* males and females, 11.4 ± 0.50 d, $n = 50$. No significant difference in age was observed between the sexes of *G. rubens* or *S. vicinus*. Ovarian mass (as percentage of body wet mass) for LW(f) *G. rubens* (6.3% ± 2.8%, $n = 42$) was only one-third that of SW *G. ovisopis* (14.7% ± 0.2%, $n = 39$). Ovarian masses of LW(f) or SW species were similar to those of the LW(f) or SW morphs, respectively, of *G. firmus* (Table 1). Ovarian mass was not measured in *S. vicinus* in this study, but it is known to be small in dispersing mole crickets (Forrest 1986), similar to that of LW(f) *Gryllus* females.

The JH titer exhibited no significant association with age in any species ($P > 0.1$ for all linear regressions), did not differ between sexes within any species ($P > 0.1$ for each ANOVA), and did not exhibit a sex × time of day interaction ($P > 0.1$, ANOVA). Thus, titers pooled between the sexes are presented in Figure 7. The JH titer in the two LW(f) species, *G. rubens* and *S. vicinus* (titer multiplied by 3 for *S. vicinus* in Fig. 7), exhibited dramatic diurnal variation similar to that in the LW(f) morph of *G. firmus*: the titer was low in the early part of the day (5:00–10:00 am), rose about sixfold between 10:00 a.m. and 3:00 p.m. ($X = 0.42$–0.63), and, by midnight, dropped (*G. rubens*), or began dropping (*S. vicinus*) to a low level. The JH titer in the SW-monomorphic *G. ovisopis* was similar to that in SW *G. firmus*: only minor diurnal variation was observed from morning until midnight. Also as in *G. firmus*, the JH titer was not significantly correlated with ovarian mass (scaled to whole-body wet mass) in either *G. ovisopis* ($r = 0.25$, $n = 30$, $P > 0.1$) or *G. rubens* ($r = -0.10$, $n = 42$, $P > 0.1$).

Discussion

Although endocrinology has been a major focus of research in insect physiology for more than 50 yr, with very few exceptions (see "Introduction"), all hormonal studies have been conducted in the laboratory. Consequently, data on endocrine variation

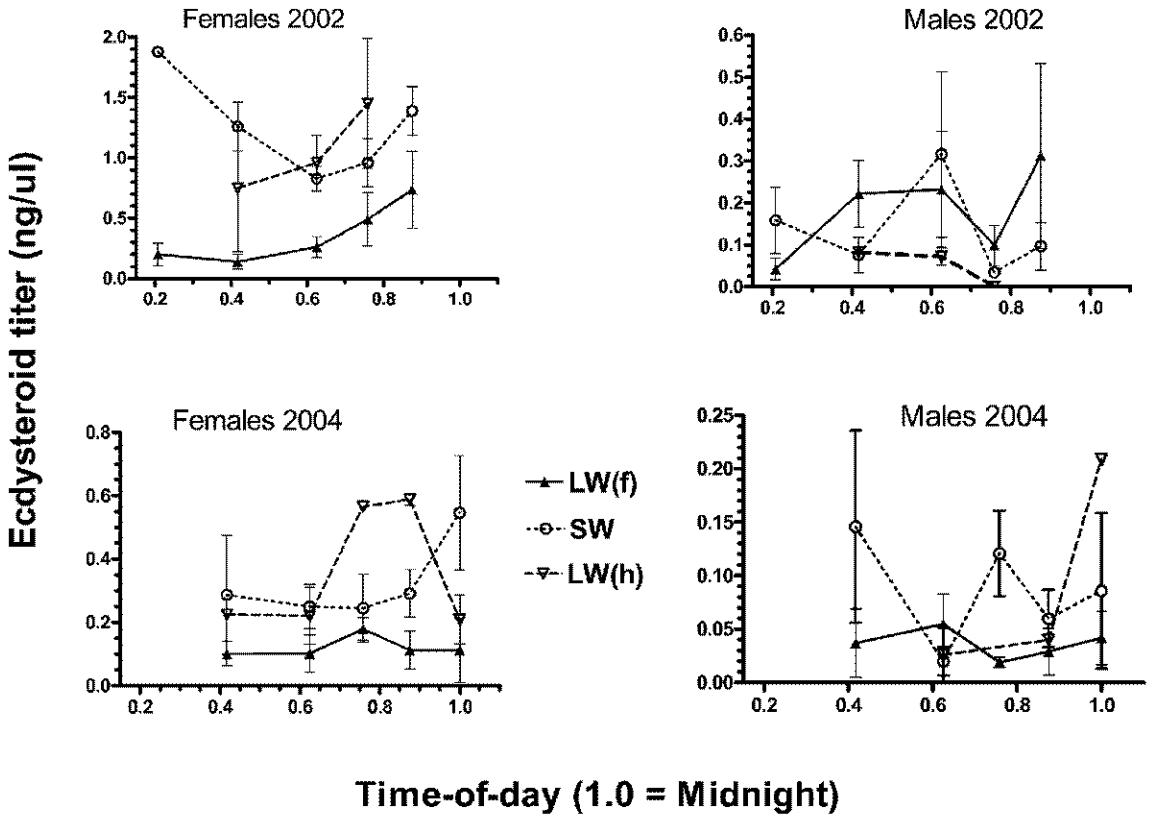

Figure 5. Hemolymph ecdysteroid titer (mean ± SEM) in male and female morphs of *Gryllus firmus* as a function of time of day, measured in 2002 and 2004. No samples were taken in 2003. See Figure 4*A* for explanation of symbols and lines and Figure 2 for values on the *X*-axis. Means for LW(f) (long-winged, flight-capable) and SW (short-winged, flightless) morphs were based on samples of 3–11 (median $n = 5$), except for SW female, 2002, $X = 0.21$ (5:00 a.m.; $n = 1$), and LW(f) male, 2004, $X = 0.42$ (10:00 a.m.; $n = 2$). Samples for LW(h) individuals ranged from $n = 1$ to $n = 5$ (median $n = 2$).

and adaptation in insects in the field are virtually nonexistent. This study represents the first comprehensive, multiyear investigation of endocrine variation in natural populations of an insect, focusing on the two preeminent hormones in insect endocrinology, JH and ECDs, in the context of the trade-off between dispersal and reproduction in morphs of *Gryllus firmus*. These results have important implications for insect endocrinology, evolutionary endocrinology, and life-history evolution.

JH Titer Differences between LW(f) and SW Morphs

The most important finding of this study is the dramatic difference between flight-capable (LW(f)) and flightless (SW) morphs of *G. firmus* with respect to the hemolymph titers of both JH and ECDs (Figs. 2–6) measured in the field. Morph difference in the hemolymph JH titer is unusual in that it consists of the presence versus absence of significant diurnal variation rather than of a consistent rank-order titer difference between morphs throughout the day. In both males and females, there was a clear pattern of daily change in the LW(f) morph, with a peak in the JH titer just before and after sunset that was consistent from year to year. The cyclic nature of the pattern was most evident in JH titers separated into three age classes (Fig. 4*B*). These data clearly indicate a morph-specific daily cycle that occurs repeatedly throughout much of adulthood in the LW(f) morph and a much smaller amplitude (and nonsignificant) cycle in the SW morph.

These field studies confirm and extend laboratory studies documenting a similar morph-specific pattern of JH titer diurnal variation, which had been exclusively studied in young (5–8 d post–adult eclosion) female *G. firmus* (Fig. 4*C*; Zera and Cisper 2001; Zhao and Zera 2004*a*). This concordance demonstrates that the morph-specific JH titer pattern is not an artifact of environmental conditions in the laboratory, the specific laboratory stocks characterized, or the specific sex or age distribution of crickets studied. As in the field, the JH titer rose and fell just before and just after the transition from the photophase to the scotophase (i.e., just before and just after lights-off) in the laboratory in crickets aged 4–8 d post–adult eclosion.

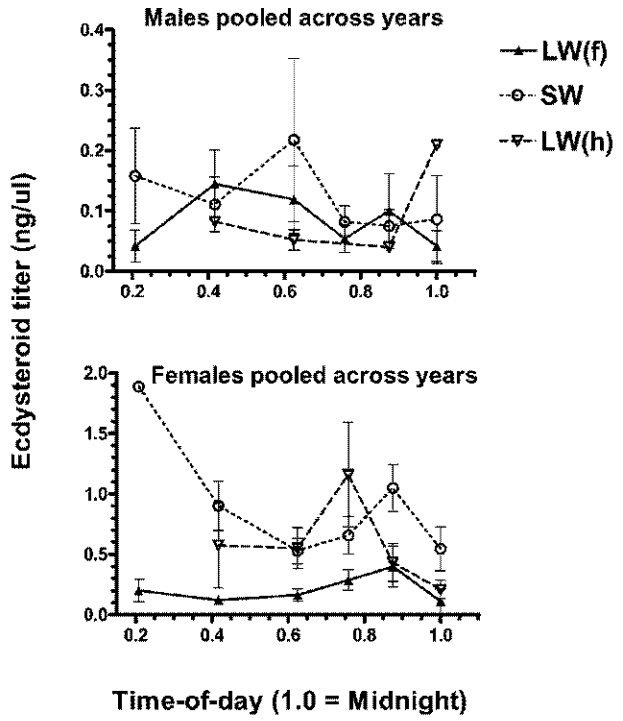

Figure 6. Hemolymph ecdysteroid titer (mean ± SEM) in male and female morphs of *Gryllus firmus* as a function of time of day, with data pooled across years. See Figure 4*A* for explanation of symbols and lines and Figure 2 for values on the *X*-axis. Means for LW(f) (long-winged, flight-capable) and SW (short-winged, flightless) morphs were based on sample sizes of 3–23 (median $n = 12$), except for SW females, $X = 0.21$ (5:00 a.m.; $n = 1$). Sample sizes for LW(h) (long-winged, flightless, with histolyzed flight muscles) ranged from 1 to 9 (median $n = 3$).

The titer was pronounced in LW(f) females and was of much lower amplitude in SW females. Importantly, the lower-amplitude titer in SW females appears to be primarily a passive consequence of cyclic changes in whole-organism blood volume, which contracts late in the photophase (Zhao and Zera 2004*b*). By contrast, the large-amplitude JH titer cycle in LW(f) females in the laboratory results primarily from diurnal change in hormone biosynthesis and, to a lesser degree, hormone degradation (Zhao and Zera 2004*a*, 2004*b*), in addition to diurnal contraction and expansion of the hemolymph volume. The only difference in the JH titer profile between laboratory and field *G. firmus* was the slightly greater amplitude of the JH titer cycle in the laboratory.

The increase in the JH titer in LW(f) individuals anticipates rather than follows lights-off (laboratory) or sunset (field). This pattern, in and of itself, strongly suggests an endogenous circadian rhythm, which has recently been directly verified in the laboratory. In laboratory stocks of *G. firmus*, the titer cycle persists under constant darkness, is abolished under constant light, and is temperature compensated (Z. Zhao and A. J. Zera, unpublished data), three key attributes of a circadian cycle (Saunders et al. 2002). The correlation between flight capability and a diurnal cycle in the hemolymph JH titer was also observed in three other species of cricket in the field, two *Gryllus* species and a more distantly related species of mole cricket (Fig. 7). At the very least, these data demonstrate that the morph-associated daily cycle for the JH titer is not restricted to *G. firmus* or indeed to field crickets (Gryllidae), because mole crickets are in a family outside of the Gryllidae. At present, only limited information is available on the phylogenetic distribution of this morph-specific cycle in orthopterans, and this remains an important topic for future research.

ECD Titer Differences between Morphs

The hemolymph ECD titer also differed dramatically between the morphs, but in a manner different from the hemolymph JH titer. First, the ECD titer did not vary temporally in either morph in either sex (Figs. 5, 6). Second, the ECD titer differed only between female morphs, where it was substantially higher in the flightless than in the flight-capable morph. As is the case for the JH titer, morph-specific differences in the ECD titer

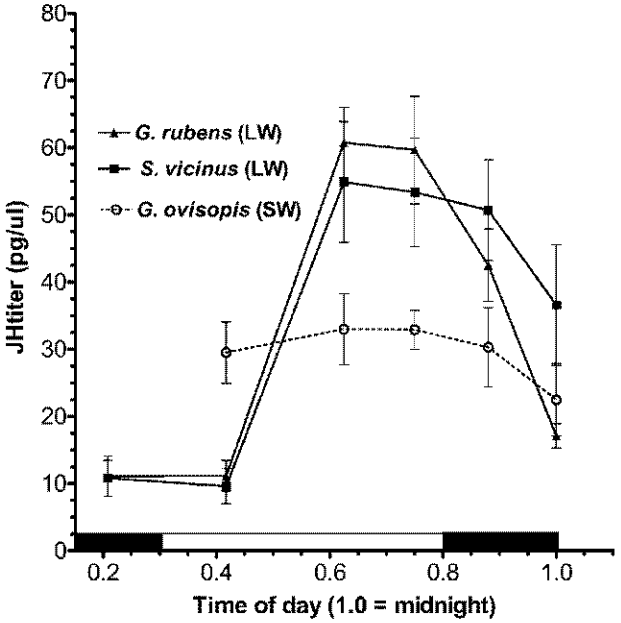

Figure 7. Hemolymph juvenile hormone (JH) titer (mean ± SEM) in field-collected adults of three cricket species: *Gryllus rubens*, *Gryllus ovisopis*, and *Scapteriscus vicinus* (tawny mole cricket). *Gryllus rubens* is wing-polymorphic, but only LW (long-winged, flight-capable) adults were sampled. *Gryllus ovisopis* is SW (short-winged, flightless) monomorphic, and *S. vicinus* is LW monomorphic. The JH titer in *S. vicinus* was multiplied by 3. Black bars on the *X*-axis indicate night. Values are exclusively for females of *G. ovisopis* and for both sexes of the other two species. Means were based on samples of 5–15 (*G. ovisopis*), 12–38 (*G. rubens*), and 7–11 (*S. vicinus*).

profiles were similar in the laboratory and the field. In both cases, the ECD titer was consistently higher in SW than in LW(f) females (not measured in males in the laboratory; Zera and Bottsford 2001; Zhao and Zera 2004*a*) and varied only minimally during the day or night (Zhao and Zera 2004*a*). In the laboratory, the subtle diurnal variation in the ECD titer (and the JH titer in SW females; Fig. 4*C*) was attributed to modest diurnal changes in the hemolymph (blood) volume. The major difference in the ECD titer between the laboratory and field was the much greater magnitude of difference between morphs in the field (240%, ANCOVA) compared with the laboratory (30%; Zhao and Zera 2004*a*). Thus, laboratory studies may understimate the functional importance of morph-specific differences in ECDs.

Hormone Titers in the Flightless LW(h) Morph

The above discussion focuses on hormone titer differences between the two main morphs studied: the LW(f) morph, which can fly but delays egg production, and the SW morph, which cannot fly but produces many more eggs than the LW(f) morph (Table 1). Another flightless morph (LW(h)) is produced from the LW(f) morph in the laboratory, and probably in the field, during adulthood by histolysis of the flight muscles and comprised about 11%–13% of sampled individuals (Table 1). Although retaining long wings, this morph is flightless and exhibits increased ovarian growth essentially identical to that of the obligately flightless SW morph. Zera and Cisper (2001) reported that the JH titer of flightless LW(h) females resembled that of flightless SW females in the laboratory. In this study, which involved more extensive sampling, the hemolymph JH titer temporal profile of the LW(h) morph was more intermediate between that of the LW(f) and SW morphs. As in the SW morph, the titer in LW(h) individuals was higher than that of the LW morph late in the evening (midnight) and throughout the morning (5:00–10:00 am). However, the titer in LW(h) females exhibited more substantial diurnal change (peak at 3:00–6:00 p.m.; Fig. 4) than that in the SW morph in the field. The ECD titer profile in LW(h) females was similar to that of the SW morph in that it was significantly higher than the titer in LW(f) females (Figs. 5, 6).

Functional Significance of Morph Differences in JH and ECD Titers

Morphs of *G. firmus* differ substantially in both flight capability and reproductive effort. Numerous laboratory studies have reported that ovaries of flightless SW and LW(h) *G. firmus* are substantially heavier (two to four times) than those of flight-capable LW(f) females (Zera and Bottsford 2001; Zera and Cisper 2001). Results of our study demonstrate similar enhanced reproductive effort in the flightless morphs in the field (Table 1). JH is thought to positively influence many aspects of reproduction and to negatively affect aspects of flight capability (e.g., reduces biosynthesis of flight fuel and causes histolysis of flight muscles; Zera and Denno 1997; Zera and Cisper 2001). However, in several nonpolymorphic insects, JH appears to positively affect aspects of flight per se (increases propensity to fly; Rankin 1989). Thus, JH titer differences between morphs could regulate any number of morph-specific differences in flight per se, preparation for flight, reproduction, or trade-offs between traits.

Zera and Cisper (2001) speculated that duration of elevation of the JH titer might be the key factor that regulates the trade-off between dispersal and reproduction. This hypothesis was based on the (modest) chronic elevation in the JH titer in SW compared to LW(f) females in the laboratory, coupled with the modest positive correlation between the JH titer and ovarian mass ($r = 0.21$, $P < 0.01$; Zhao and Zera 2004*a*). In other words, long-duration elevation of the JH titer above a threshold in the SW morph might be the cause of its elevated ovarian growth. By contrast, the short-duration peak in the JH titer in LW(f) females might be sufficient to induce the expression of traits associated with or necessary for daily flight while not being elevated long enough to express traits such as flight-muscle histolysis, which have negative effects on flight capability. Additional, more complex endocrine hypotheses involving multiple regulators have also been discussed previously (Zera and Cisper 2001; Zhao and Zera 2004*a*; Zera 2006).

New information obtained in this study suggests a modification of the aforementioned hypothesis, in which JH and ECDs might regulate different aspects of the dispersal-reproduction trade-off: morph differences in the JH titer (short-duration peak in the LW(f) but not the SW morph) might primarily regulate aspects of flight, while morph differences in the ECD titer might primarily regulate differences in egg production. This modification is suggested by the absence of a correlation between the JH titer and ovarian mass in females ("Correlations between JH and ECD Titers and Ovarian Mass") and by the strong association between the cyclic JH titer and flight capability (i.e., the LW(f) morph), which is independent of sex (i.e., occurs in both females and males; Figs. 2, 3, respectively). Thus, there is no evidence from this study and only moderate to weak evidence from previous studies (Zera and Cisper 2001; Zhao and Zera 2004*a*) that JH titer differences by themselves regulate morph-specific differences in egg production.

Although JH is a major gonadotropin in insects (Nijhout 1994; Klowden 2002), it has evolved to primarily regulate nonreproductive functions in some social insects. For example, in highly social bees, JH has a reduced role in caste-specific reproduction but an enhanced role in regulating other caste-specific activities, such as age-specific flight in workers (see "Discussion" in Hartfelder et al. 2002). Interestingly, the change of JH regulation from within-hive activities in young workers (nurse bees) to flight outside the hive in older workers (foragers) is correlated with a

change from a temporally constant JH titer to diurnal variation in the JH titer (Elekonich et al. 2001).

In crickets, JH could regulate either aspects of behavior directly involved in flight (takeoff propensity; Rankin 1989) or traits associated with flight. For example, JH decreases the phonotactic threshold in crickets (Stout et al. 1998), and thus an elevated JH titer might be involved in enhanced location of calling males by flying individuals (both males and females are attracted to calling males). Alternatively, the elevated JH titer might be associated with characteristics of the LW(f) morph independent of flight. Currently, there is no information on the timing of flight during the night in *G. firmus*. However, *Gryllus rubens* appears to fly throughout the night, while *Scapteriscus vicinus* flies only during 20–90 min after sunset (see "Discussion" in Walker 1986). Although these two species exhibit very different nocturnal flight profiles, they exhibit similar JH titer profiles from sunset to sunrise (Fig. 7).

In contrast to the JH titer, the ECD titer is correlated with and may primarily regulate reproductive differences between female morphs. The ECD titer is higher in the female morph that exhibits enhanced egg production (SW or LW(h); Fig. 6; Zhao and Zera 2004), is positively correlated with ovarian mass ("Correlations between JH and ECD Titers and Ovarian Mass"; Zhao and Zera 2004*a*), and does not differ between male morphs (Fig. 6). In some social insects, ECDs have taken on a greater role in regulating caste-specific reproduction as the role of JH has been reduced (see "Discussion" in Hartfelder et al. 2002).

Some data are problematic with regard to the modified hypothesis discussed above. For example, topical application of JH or a JH analog to LW(f) females caused enhanced ovarian growth, similar to that seen in the SW morph, suggesting that elevated JH titer may indeed regulate enhanced ovarian growth in the SW morph (Zera and Cisper 2001). However, this effect of exogenous JH could be a pharmacological effect or might have altered the ECD titer, an effect of applied JH that has been documented in some insects (Smith and Nijhout 1981). Furthermore, enhanced sensitivity of ovarian receptors to JH could cause enhanced ovarian growth in the SW morph even if the JH titer does not differ between the morphs. In short, although we have demonstrated substantial differences between LW(f) and SW morphs with respect to both the JH and ECD titers, which strongly suggest that these hormone titers regulate morph-specific aspects of the trade-off between reproduction and dispersal in the laboratory and in the field, the identity of the specific traits that are regulated and the specific mechanisms involved remain poorly understood. On the positive side, JH and ECD titers have now been documented more thoroughly in adult morphs of *G. firmus* than in any other polymorphic insect, indeed in any insect. Thus, *G. firmus* is now a powerful model to investigate the role of JH and ECDs in regulating aspects of adult polymorphism and the trade-off between dispersal and reproduction in insects.

Relevance to Endocrine Circadian Rhythms in Insects

Although endocrine traits in vertebrates, such as blood hormone titers, commonly exhibit circadian rhythms (Norris 1997; Adkins-Regan 2005), analogous reported examples in insects have, in general, been rare (Vafopoulou and Steel 2005). This situation has changed dramatically in recently years with reports of circadian rhythms or diurnal variation for a number of endocrine traits, such as titers of JH, ECD, prothoracicotropic hormone, and adipokinetic hormone; rate of JH biosynthesis and JH esterase activity; and ECD nuclear receptor expression (summarized in Zhao and Zera 2004*a*, 2004*b*; Vafopoulou and Steel 2005; Steel and Vafopoulou 2006). Thus, diurnal or circadian variation for hormonal traits is likely to be as common in insects as in vertebrates. In a number of cases, particular morphs, developmental stages, or physiological states differ dramatically in the magnitude or presence/absence of diurnal/circadian cycles for various endocrine traits (discussed in Kodrík et al. 2003; Zhao and Zera 2004*a*, 2004*b*; Vafopoulou and Steel 2005). Prominent examples include differences in the magnitude or presence/absence of cycles in the JH titer, JH titer regulators, or adipokinetic titer between dispersing and reproductive morphs of the wing-polymorphic insects *G. firmus* or *Pyrrhocoris apterus*, between mated and virgin females of *Heliothis virescens*, or between nurse and forager honeybees. These cases now provide the opportunity to investigate two poorly studied topics at the interface between evolutionary endocrinology and chronobiology: the microevolution of hormonal circadian rhythms per se and their role in the evolution of life-history traits (dispersal, oviposition, mating, etc.) that often vary significantly temporally (Saunders et al. 2002; Vafopoulou and Steel 2005). The morph-specific, circadian rhythm for the JH titer in *G. firmus* has several unique features that make it particularly powerful for investigating these issues. To our knowledge, the JH titer in *G. firmus* is the only hormonal trait that exhibits a genetically polymorphic circadian rhythm (Zera and Cisper 2001) that has been documented in natural populations, where it is strongly associated with a functionally important life-history polymorphism (this study). The high amplitude (20-fold titer change in 6 h; Zhao and Zera 2004*a*) of the JH titer cycle further contributes to its utility in functional/micoevolutionary studies.

There is an important practical reason why insect endocrinologists and evolutionary biologists need to pay attention to the possible existence of hormonal circadian rhythms: the failure to identify hormonal cycles can give rise to highly erroneous inferences regarding endocrine adaptations. This is especially the case if changes are of large magnitude and occur in a phenotype-specific manner. For example, the correlation between the blood JH titer and ovarian mass changes from $+0.9$ to -0.9 in *G. firmus* from early to late in the photophase, because of the substantial increase during the photophase in the blood JH titer in the LW(f) morph (with small ovaries) but

not in the SW morph (with large ovaries; Zera and Cisper 2001). Ignorance of this changing correlation could cause one to infer either a strongly positive or s negative influence of the JH titer on ovarian mass, depending on the specific time of day that the hormone titer was measured. Because studies of diurnal changes in endocrine traits have been so limited in insects, the extent of this problem is unknown at present.

General Implications for Empirical Studies of Evolutionary Endocrinology

Surprisingly, there are very few evolutionary endocrine studies of insects in which hormonal traits have been directly measured using well-validated analytical methods (Zera 2006; Zera et al. 2007). Indeed, direct measurement of JH and ECD titers and of regulators of the JH titer in species of *Gryllus* are the exception rather than the rule; documentation of hormonal profiles by direct measurement continues to be one of the most, if not the most, important tasks of insect evolutionary endocrinology (Zera 2004, 2006; Zera et al. 2007).

The unexpected complexity of the JH titer in *G. firmus* in this and previous studies (Zhao and Zera 2004*a*) underscores the importance of directly measuring hormonal traits, as opposed to indirectly inferring aspects of hormonal regulation using approaches such as topical hormone application. This superficial approach, which uses the ability of an applied hormone to convert one phenotype into another to infer aspects of endocrine regulation, is fraught with problems when used as the sole experimental approach to investigate the hormonal regulation of phenotypic expression (e.g., morphological or life-history differences between morphs of some polymorphisms; see Zera 2007 for a detailed critique of this approach). The dangers of using hormonal manipulation in this context are readily apparent in endocrine studies of *G. firmus*. Previous application of the JH analog methoprene to adult LW(f) *G. firmus* caused the expression of several important traits found in the SW morph, such as reduction of flight muscles and growth of the ovaries (Zera and Cisper 2001; Zhao and Zera 2004*a*). These results originally suggested that a chronically lower JH titer in the LW(f) morph, relative to the SW morph, might be required to suppress ovarian growth and to maintain large flight muscles for dispersal. However, as described in this and previous studies (Zera and Cisper 2001; Zhao and Zera 2004*a*), direct measurement of the JH titer demonstrates a much more complex morph-specific circadian rhythm for the JH titer and suggests that JH positively regulates the expression of traits associated with nocturnal flight. If the JH titer had not been directly measured in morphs of *G. firmus* but rather had been inferred solely from hormone manipulation, a functionally important hormonal difference between the morphs would have gone unidentified, and false conclusions would have been drawn regarding morph-specific hormone levels. Recent endocrine studies of locust phase polymorphism have also documented morph-specific hormone titers that are more complex than previously suspected (e.g., rank-order changes in JH titer phases; Botens et al. 1997), further illustrating the importance of direct measurement of hormone titers.

In summary, we have conducted the first detailed multiyear investigation of variation in the blood levels of two key insect hormones in the field. Importantly, hormone patterns are, in general, similar between studies undertaken in the laboratory and the field, and thus results of controlled laboratory studies can be extrapolated to natural populations and vice versa. However, in some cases, most notably the magnitude of ECD titer differences between morphs, important differences were observed between laboratory and field studies. Extending laboratory studies that had focused exclusively on young female *G. firmus* to investigations of hormonal variation in both sexes and to other cricket species has resulted in a new hypothesis of the hormonal control of dispersal polymorphism. The extensive database on JH and ECD titer variation in the laboratory and the field, especially the strong association between a genetic polymorphism for a hormonal circadian rhythm and a genetically based dispersal polymorphism, provides an unparalleled experimental model to investigate the microevolution of hormonal circadian rhythms and their influence on the microevolution of life histories.

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Literature Cited

Adkins-Regan E. 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton, NJ.

Botens F.W.W., H. Rembold, and A. Dorn. 1997. Phase-related juvenile hormone determinations in field catches and laboratory strains of different *Locusta migratoria* subspecies. Pp. 197–203 in S. Kawashima and S. Kikuyama, eds. Advances in Comparative Endocrinology. Monduzzi, Bologna.

Elekonich M.M., D.L. Schultz, G. Bloch, and G.E. Robinson.

2001. Juvenile hormone levels in honey bee (*Apis mellifera* L.) foragers: foraging experience and diurnal variation. J Insect Physiol 47:1119–1125.

Forrest T.G. 1986. Oviposition and maternal investment in mole crickets (Orthoptera: Gryllotalpidae): effects of season, size, and senescence. Ann Entomol Soc Am 79:918–924.

Gibbs A.G. 1999. Laboratory selection for the comparative physiologist. J Exp Biol 202:2709–2718.

Gilbert L., K. Iatrou, and S. Gill, eds. 2005. Comprehensive Molecular Insect Science. Vol. 3. Endocrinology. Elsevier, Amsterdam.

Harshman L.G. and A.A. Hoffmann. 2000. Laboratory selection experiments using *Drosophila*: what do they really tell us? Trends Ecol Evol 15:32–36.

Hartfelder K., M.M.G. Bitondi, W.C. Santana, and Z.L.P. Simones. 2002. Ecdysteroid titer and reproduction in queens and workers of the honey bee and a stingless bee: loss of ecdysteroid function at increasing levels of sociality. Insect Biochem Mol Biol 32:211–216.

Hartfelder K. and D. Emlen. 2005. Endocrine control of insect polyphenism. Pp. 651–703 in L. Gilbert, K. Iatrou, and S.S. Gill, eds. Comprehensive Molecular Insect Science. Vol. 3. Elsevier, Amsterdam.

Hoekstra H.E., R.J. Hirschmann, R.A. Bundy, P.A. Insel, and J.P. Crossland. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. Science 313: 101–104.

Ketterson E.D. and V. Nolan Jr. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. Am Nat 154(suppl.): S4–S25.

Klowden M. 2002. Physiological Systems in Insects. Academic Press, Amsterdam.

Kodrík D., R. Socha, and Z. Syrová. 2003. Developmental and diel changes of adipokinetic hormone in CNS and hemolymph of the flightless wing-polymorphic bug, *Pyrrhocoris apterus* (L.). J Insect Physiol 49:53–61.

Marler C.A. 1988. Evolutionary costs of aggression revealed by testosterone manipulations in free-living male lizards. Behav Ecol Sociobiol 23:21–26.

Murray A.-M. and W. Cade. 1995. Differences in age structure among field cricket populations (Orthoptera; Gryllidae): possible influence of a sex-biased parasitoid. Can J Zool 73: 1207–1213.

Nijhout H. and G. Davidowitz. 2007. The developmental-physiological basis of phenotypic plasticity. In D. Whitman and T.N. Ananthakrishnan, eds. Phenotypic Plasticity in Insects: Mechanisms and Consequences. Science Publishers, Plymouth, U.K. (forthcoming).

Nijhout H.F. 1994. Insect Hormones. Princeton University Press, Princeton, NJ.

Norris D.O. 1997. Vertebrate Endocrinology. Academic Press, San Diego, CA.

Rankin M.A. 1989. Hormonal control of flight. Pp. 139–163 in G.J. Goldworthy and C.H. Wheeler, eds. Insect Flight. CRC, Boca Raton, FL.

Reed W.L., M.E. Clark, P.G. Parker, S.A. Raoulf, N. Arguedas, D.S. Monk, E. Snajdr, V. Nolan Jr., and E.D. Ketterson. 2006. Physiological effects on demography: a long-term experimental study of testosterone's effects on fitness. Am Nat 167: 667–683.

Richard D.S., R. Rybczynski, T.G. Wilson, Y. Wang, M.L. Wayne, Y. Zhou, L. Partridge, and L.G. Harshman. 2005. Insulin signaling is necessary for vitellogenesis in *Drosophila melanogaster* independent of the roles of juvenile hormone and ecdysteroids: female sterility of the *chico*^{1} insulin signaling mutation is autonomous to the ovary. J Insect Physiol 51: 455–464.

Saunders D.S., C.G.H. Steel, X. Vafopoulou, and R.D. Lewis. 2002. Insect Clocks. Elsevier, Amsterdam.

Sinervo B., D.B. Miles, W.A. Frankino, M. Klukowski, and D. DeNardo. 2000. Testosterone, endurance, and Darwinian fitness: natural and sexual selection on the physiological bases of alternative male behaviors in side-blotched lizards. Horm Behav 38:222–233.

Smith W. and H. Nijhout. 1981. Effects of a juvenile hormone analogue on the duration of the fifth instar in the milkweed bug *Oncopeltus fasciatus*. J Insect Physiol 27:169–173.

Steel C.H.G. and X. Vafopoulou. 2006. Circadian orchestration of developmental hormones in the insect, *Rodnius prolixus*. Comp Biochem Physiol A 144:351–364.

Stout J., J. Hao, P. Kim, D. Mbungu, M. Bronsert, S. Slikkers, J. Maier, D. Kim, K. Bacchus, and G. Atkins. 1998. Regulation of the phonotactic threshold of the female cricket, *Acheta domesticus*: juvenile hormone III, allatectomy, L1 auditory neuron thresholds and environmental factors. J Comp Physiol A 182:635–645.

Tatar M., A. Bartke, and A. Antebi. 2003. The endocrine regulation of aging by insulin-like signals. Science 299:1346–1351.

Vafopoulou X. and C.G.H. Steel. 2005. Circadian organization of the endocrine system. Pp. 551–614 in L.I. Gilbert, K. Iatrou, and S.S. Gill, eds. Comprehensive Molecular Insect Science. Vol. 3. Elsevier, Oxford.

Walker T.J. 1974. *Gryllus ovisopis* n. species: a taciturn cricket with a life cycle suggesting allochronic speciation. Fla Entomol 57:13–22.

———. 1986. Monitoring the flights of field crickets (*Gryllus* spp.) and a tachinid fly (*Euphasiopteryx ochracea*) in northern Florida. Fla Entomol 69:678–685.

Walker T.J. and J.M. Sivinski. 1986. Wing dimorphism in field crickets (Orthoptera: Gryllidae: *Gryllus*). Ann Entomol Soc Am 79:84–90.

Wingfield J.C., S. Lynn, and K.K. Soma. 2001. Avoiding the "costs" of testosterone: ecological bases of hormone-behavior interactions. Brain Behav Evol 57:239–251.

Zera A.J. 2004. The endocrine regulation of wing polymor-

phism: state of the art, recent surprises, and future directions. Integr Comp Biol 43:607–616.

———. 2006. Evolutionary genetics of juvenile hormone and ecdysteroid regulation in *Gryllus*: a case study in the microevolution of endocrine regulation. Comp Biochem Physiol A 144:365–379.

———. 2007. Endocrine analysis in evolutionary-developmental studies of insects: hormone manipulation versus direct measurement of hormonal regulators. Evol Devel 9:499–513.

Zera A.J. and J. Bottsford. 2001. The endocrine-genetic basis of life-history variation: relationship between the ecdysteroid titer and morph-specific reproduction in the wing-polymorphic cricket, *Gryllus firmus*. Evolution 55:538–549.

Zera A.J. and G. Cisper. 2001. Genetic and diurnal variation in the juvenile hormone titer in a wing-polymorphic cricket: implications for the evolution of life histories and dispersal. Physiol Biochem Zool 74:293–306.

Zera A.J. and R.F. Denno. 1997. Physiology and ecology of dispersal polymorphism in insects. Annu Rev Entomol 42: 207–231.

Zera A.J., L.G. Harshman, and T.D. Williams. 2007. Evolutionary endocrinology: the developing synthesis between endocrinology and evolutionary genetics. Annu Rev Ecol Evol Syst (forthcoming).

Zera A.J. and Y. Huang. 1999. Evolutionary endocrinology of juvenile hormone esterase: functional relationship with wing polymorphism in the cricket, *Gryllus firmus*. Evolution 53: 837–847.

Zera A.J. and C. Zhang. 1995. Evolutionary endocrinology of juvenile hormone esterase in *Gryllus assimilis*: direct and correlated responses to selection. Genetics 141:1125–1134.

Zhao Z. and A.J. Zera. 2004*a*. The hemolymph JH titer exhibits a large-amplitude, morph-dependent, diurnal cycle in the wing-polymorphic cricket, *Gryllus firmus*. J Insect Physiol 50:93–102.

———. 2004*b*. A morph-specific daily cycle in the rate of JH biosynthesis underlies a morph-specific daily cycle in the hemolymph JH titer in a wing-polymorphic cricket. J Insect Physiol 50:965–973.

Zuk M. 1987. Age determination of adult field crickets: methodology and field application. Can J Zool 65:1564–1566.