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Juvenile hormone titer and morph-specific reproduction in the wing-polymorphic cricket, *Gryllus firmus*

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Abstract

Juvenile hormone titers and reproductive characteristics were measured in adult wing and flight-muscle morphs of the wing-polymorphic cricket, *Gryllus firmus*, during the first week of adulthood. This species has three morphs: one flight capable morph with fully-developed wings and fully-developed flight muscles [LW(F)], one flightless morph with fully-developed wings and histolyzed (non-functional) flight muscles [LW(H)], and another flightless morph with underdeveloped (short) wings and underdeveloped flight muscles (SW). Both flightless morphs [LW(H) and SW] had larger ovaries which contained a greater number of postvitellogenic eggs compared with the flight capable [LW(F)] morph. The juvenile hormone titer was significantly higher in SW compared with LW(F) females on days 3–7 of adulthood. On these days, the JH titer also was significantly higher in the other flightless morph, LW(H), compared with flight-capable [LW(F)] females as determined by one statistical test, but did not differ significantly by another test. The JH titer was positively correlated with ovarian mass or terminal oocyte length, but not with the number of post-vitellogenic eggs. This study is the first direct comparison of juvenile hormone titers in adult wing morphs of a wing-polymorphic insect. Results indicate that an elevated juvenile hormone titer may be at least partly responsible for one of the most distinctive features of wing-polymorphic species, the increased early fecundity of flightless females.

Keywords: juvenile hormone, JH, JH III, wing polymorphism, reproduction, *Gryllus*, crickets

1. Introduction

Wing polymorphism occurs widely in the Insecta (Harrison, 1980; Roff, 1986; Dingle, 1996; Zera and Denno, 1997). The polymorphism involves discontinuous variation in a wide variety of traits involved in dispersal and reproduction. Polymorphic species contain a flightless morph with underdeveloped wings and flight muscles. An alternate morph has fully developed wings, fully developed flight muscles shortly after the adult molt, and is capable of flight. Importantly, flight capability trades off with ovarian growth: flightless adults begin egg development earlier and typically produce more eggs, especially during the early stages of reproduction, than their flight-capable counterpart (Harrison, 1980; Roff, 1986; Zera and Denno, 1997). Wing polymorphism has been used extensively by evolutionary biolo-

gists as a model to study the evolution of dispersal, development, and life histories and by insect physiologists to study the endocrine regulation of alternate developmental pathways within species (Hardie and Lees, 1985; Nijhout, 1994; Dingle, 1996; Zera and Denno, 1997).

Despite decades of study, the endocrine regulation of wing-polymorphism remains a largely unresolved problem in insect endocrinology (Hardie and Lees, 1985; Zera and Denno, 1997). At issue are the endocrine mechanisms that regulate the differential development and reproduction of alternate morphs within a species. The central focus of this topic has been juvenile hormone (JH), a key regulator of insect development and reproduction (Nijhout, 1994; Wyatt and Davey, 1996). During the standard molting cycle, JH, above some threshold, causes retention of juvenile features by blocking the metamorphic effect of ecdysone. During the last sta-

dium, the JH titer drops to a very low level and ecdysone-mediated metamorphosis ensues. In most insect orders, JH takes on a different function in adults, where it positively regulates many aspects of reproduction such as the synthesis of vitellogenin and the uptake of this molecule into developing oocytes (Nijhout, 1994; Wyatt and Davey, 1996).

Decades ago, Wigglesworth (1961) and Southwood (1961) proposed that the short-winged (or wingless) morph of wing polymorphic species is produced by an elevated JH titer during some critical stage in development. This elevated titer purportedly blocks the full development of wings, flight muscles and associated structures thus giving rise to a short-winged or wingless morph. Since JH positively affects reproduction, an elevated JH titer in the short-winged adult would also account for its earlier and greater egg production (Roff, 1986; Zera and Denno, 1997).

Numerous studies have attempted to indirectly test this hypothesis. Variation in endogenous JH titers between morphs has been inferred from the effect of gross-level experimental manipulations of the JH titer (Hardie and Lees, 1985; Mittler, 1991; Zera and Denno, 1997, and references therein). For a variety of reasons, results from these indirect studies have been equivocal (Zera and Denno, 1997; see Section 4). Clearly, more definitive evidence on the role of JH in regulating aspects of wing polymorphism requires direct quantification and comparison of JH titers between morphs during juvenile and adult stages. Thus far, no such direct comparisons of JH titers have been performed for adult morphs and only one comparison has been performed for juvenile morphs (Zera et al., 1989; Zera and Denno, 1997).

The present investigation represents part of a comprehensive study of the hormonal, energetic and biochemical bases of wing polymorphism in crickets of the genus *Gryllus*. One of our major goals is to identify the endocrine mechanisms responsible for the elevated egg production of the flightless morph relative to the flight-capable morph. Egg production is especially enhanced during early adulthood in flightless females of wing-polymorphic species (Roff, 1986; Zera and Denno, 1997). This elevated early fecundity typically is the major contributor to the overall greater fecundity of flightless females (Roff, 1986, 1986; Zera, 1984; Denno et al., 1989; Zera and Rankin, 1989; Zera and Denno, 1997). Hence, in the present study we focused on the endocrine regulation of the first stages of reproduction. Specifically, we compared juvenile hormone titers between long-winged and short-winged females of *Gryllus firmus* during the first week of adulthood when these morphs differ dramatically in ovarian growth. JH titers were measured using a well established radioimmunoassay (Hunnicut et al., 1989) that is sensitive enough to quantify hemolymph titers in single individuals.

Importantly, in the present study, we not only quantified endocrine and reproductive characteristics of long-winged and short-winged adults, but also characterized these traits separately in the two functionally-differentiated types of long-winged individuals. In many wing polymorphic species, including *G. firmus*, long-winged adults are a composite group consisting of individuals that retain fully-developed flight muscles and those that histolyze their flight muscles when ovarian growth commences (Zera and Denno, 1997). These two types of long-winged morphs typically have not been distinguished in previous studies of wing polymorphism (e.g. Roff, 1984; Zera and Rankin, 1989). However, a recent study has shown that long-winged individuals with histolyzed flight muscles are much more similar to short-winged individuals in a variety of key physiological and reproductive characteristics than they are to long-winged individuals that retain their flight muscles (Zera et al., 1997; see Section 4). Consequently, in the present study, endocrine and reproductive features were measured separately for each of the two types of long-winged morphs, in addition to the short-winged morph.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents used in the present study were at least reagent or HPLC grade and were purchased from Sigma Chemical Company, Mallinckrodt, or Fisher Scientific. Silica gel Prep-Sep extraction columns were purchased from Fisher Scientific. Racemic unlabeled JH III and labeled [10^{-3}H] JH III (19.5 Ci/mmol; 721.5 GBq/mmol) were purchased from Sigma Chemical Co. and New England Nuclear, respectively.

2.2. Insects, morph designations and rearing conditions

Gryllus firmus, the sand cricket, occurs in the southeastern United States as a long-winged (LW) morph, some of which are capable of flight, or as a short-winged (SW) form that is obligatory flightless (Veazy et al., 1976). All SW females have white, non-functional flight muscles which never fully develop. All LW females have fully-developed, functional (pink) flight muscles at or shortly after the adult molt and are denoted as LW(F). During adulthood, some LW(F) individuals histolyze their flight muscles thus becoming flightless [denoted as LW(H); for review see Zera et al., 1997]. The *G. firmus* used in the present study were derived from a colony founded from 30 gravid females collected in Gainesville, Florida during the summer of 1995. This base population was used to initiate a selection exper-

iment on wing morph, the details of which will be reported elsewhere. Results presented here are for two unselected control stocks (C-1 and C-3) that were derived from this base population. Studies were done on crickets of the third-fifth laboratory generations. Frequencies of the LW morph in females of these stocks during these generations ranged from 0.60 to 0.69 in C-1 ($N=120-148$ each generation) and from 0.49 to 0.75 in C-3 ($N=95-212$ each generation).

Crickets were reared approximately 40–60 per 10 gallon aquarium at 30°C under a 16L:8D photoperiod until late in the last instar after which they were subsequently held at 28°C under the same photoperiod. Approximately 100–150 males and 100–150 females were used as breeders each generation. All individuals were fed a standard wet diet (Mole and Zera, 1994). Crickets used for experiments were checked for molting at 24-h intervals and equal numbers of newly molted males and females were housed together at a density of 6 per 1-gallon box or 12 per 3-gallon box. No oviposition material was provided and hence females retained their eggs.

2.3. Sampling schedule and hemolymph collection and processing

Juvenile hormone titers were measured in females that were 3, 5, and 7 days old (molt to adult=day 0). These days were chosen because they span the period of development during which ovaries first begin to grow to a time when ovarian mass is substantially greater in flightless females [either SW or LW(H)]. All hemolymph samples were collected within the first 10 h of the 16-h light cycle. 12.5–25 μ l hemolymph from individual females were collected in micropipettes from cuts in the cerci and legs of *G. firmus*. Hemolymph was blown into a test tube containing 300 μ l 90% methanol in water. Samples were sonicated for 3–5 s and JH was extracted twice with 600 μ l hexane. Hexane extracts were stored in glass vials at –80°C until radioimmunoassay (RIA). Bled females were stored at –80°C until dissection, when reproductive characteristics and flight muscle phenotype were determined. Background experiments showed that these features did not change during the time crickets were frozen. JH titers were measured in 3–20 individual females of each morph from each line on each day.

2.4. Juvenile hormone RIA: assay conditions

Juvenile hormone titers were determined using a RIA specific for the naturally occurring (10R) enantiomer of juvenile hormone (Hunnicutt et al., 1989; Huang et al., 1994). JH III has been identified as the only juvenile hormone in *G. firmus* (Yadlowski, 1994) and other gril-lids (Loher et al., 1983; Koch and Hoffman, 1985; Zera et al., 1989). Prior to RIA, individual hemolymph sam-

ples were first dried in a vacuum centrifuge and 50 μ l of methanol was added to each sample. A 4- μ l aliquot was transferred to another tube containing 200 μ l of pre-mixed antiserum in gel-PBST (0.1% Knox's gelatin in 0.05 M phosphate buffer, pH 7.3; 0.9% NaCl; 0.01% Triton X-100) and 4000 dpm of [$10-^3$ H]-JH III. The final dilution of anti-JH III rabbit serum was 1:16,000. Tubes were incubated for 8–12 h at room temperature. After cooling in an ice bath for 5 min, unbound radiolabelled JH III was separated from bound JH III by adding 500 μ l dextran-coated charcoal, incubating for 5 min, and centrifuging for 5 min at 1500 g at 4°C. Radioactivity in the supernatant was due to radiolabeled JH III bound to antiserum while radioactivity in the pellet was due to charcoal adsorption of unbound radiolabeled hormone. The supernatant was transferred to scintillation vials and dpm (disintegrations per minute) quantified by liquid scintillation spectrometry (RackBeta 1219). Under the assay conditions described above, antiserum bound approximately 25% radiolabeled JH III (50% 10-R JH III) in the absence of unlabelled ligand, which is optimal for this assay.

2.5. Juvenile hormone RIA: validation

A standard curve based on seven concentrations of racemic JH III ranging from 1.5 to 1500 pg was constructed for each assay (see Figure 1 for representative curve). Parameters of the standard curve were estimated by non-linear regression (Prism 2.0, GraphPad Inc.). Data points fit the standard curve very well (mean $r^2=0.9959\pm0.0003$, $N=8$ curves), enabling accurate estimates of the juvenile hormone titer in hemolymph samples of individual *G. firmus*. The concentration of racemic JH III standards was multiplied by 0.5 since they contain only 50% of the 10R enantiomer that is recognized by the anti-JH antibody (Hunnicutt et al., 1989).

To determine whether RIA of *G. firmus* hemolymph extracts is affected by hemolymph lipids, 28 individual hemolymph extracts were divided in half and one half of each sample was passed through a Prep-Sep column to remove lipids. The extract to be passed through the column was dried in a vacuum centrifuge and re-suspended in 1 ml 100% methylene chloride. The silica gel Prep-Sep column was washed with 2 ml methanol followed by 2 ml methylene chloride. JH extracts were passed through the column and the column was washed with 1 ml methylene chloride. The column was then washed with 2 ml 10% methylene chloride in methanol which elutes JH but retains lipids. Radiolabeled JH III (5000 dpm) was added to three samples to monitor JH recovery during Prep-Sep purification. More than 97% of JH III was recovered in the 10% methylene chloride wash. JH titers estimated in the purified and unpurified sample halves were very similar ($r = 0.90$, $P < 0.0001$; see

Figure 1). This strong correlation indicates that determinations of the hemolymph JH titer of *G. firmus* using this RIA are only minimally affected by the presence of hemolymph lipids. A similar situation as has been demonstrated for honey bees (Huang et al., 1994) and burying beetles (Trumbo et al., 1995).

To check the specificity of the RIA, four separate extracts of hemolymph from adult female *G. firmus* and two JH III standards (5 ng) were individually fractionated by normal phase HPLC as described in Trumbo et al. (1995). Prior to HPLC, a known amount of radio-labeled JH III was added to the sample. A portion of each sample was saved and the remainder was injected into the column and seven fractions that bracketed the

elution of JH III from the column were collected. Aliquots of the unfractionated portion and each HPLC fraction were individually counted to determine the elution time of JH and its recovery. Other aliquots of these samples were analyzed by RIA to determine their JH content. The amount of ^3H -JH III (added to monitor recovery) present in each sample was taken into account when calculating the amount of JH. These data were then used to calculate the percentage of the immunoreactive material in the unfractionated sample that co-migrated with JH III, after correcting for the recovery of JH from HPLC. In all of the samples ($n=4$), immunoreactive material was only detected in the two HPLC fractions that also contained ^3H -JH III. The immunoreactive material in these fractions accounted for all ($102.0 \pm 17.4\%$) of the activity observed in the unfractionated sample. The co-migration of all immunoreactivity with JH III in normal phase HPLC is similar to results obtained by Huang et al. (1994) and Trumbo et al. (1995).

2.6. Dissections

Ovarian mass, number of postvitellogenic eggs, longest terminal oocyte length (average of five terminal oocytes in each individual) and flight muscle phenotype (pink, fully developed vs. white, reduced) were determined for individuals whose JH titers were determined. Muscle color is strongly correlated with muscle size and can be used as an indicator of muscle histolysis in LW females (see Zera et al., 1997).

2.7. Statistical analyses

Variation in the JH titer between the three morphs (MORPH), three days of adulthood (DAY) and the two control stocks (BLOCK) was determined by Analysis of Variance (ANOVA; Sokal and Rohlf, 1981). BLOCK was a random effect and DAY and MORPH were fixed effects. Distributions of JH titers were significantly skewed and titers were normalized prior to analysis by transforming them to $\log[\text{JH titer}+1]$ (pg/ μl). One was added to the estimated JH titer in order to circumvent the technical problem of log transforming 0 values (see Sokal and Rohlf, 1981). The initial ANOVA showed no significant effect of BLOCK or any two- or three-way interactions involving BLOCK. Thus, BLOCK was removed from the model (i.e. data from the two control lines were pooled) and data were analyzed by two-way ANOVA. In addition, untransformed JH titer data were analyzed by the non-parametric Mann-Whitney U-test. The overall comparison of JH titers among the three morphs was followed by two-way comparisons of titers between the various morphs [LW(F) vs. SW, LW(F) vs. LW(H), and LW(H) vs. SW]. Since these comparisons were planned contrasts, probabilities were not adjusted for multiple comparisons.

Figure 1. Top panel: typical standard curve for RIA of racemic juvenile hormone-III. Values are means ($\pm$ SEM) of three replicates. SEMs are smaller than the symbols. See Section 2 for details. Bottom panel: effect of Prep-Sep purification on the estimation of juvenile hormone in extracts of hemolymph from *G. firmus*. Symbols represent individual hemolymph extracts that were divided in half and subjected to RIA with or without passage through a Prep-Sep column. Units on the X- and Y-axes are picograms of juvenile hormone. The dotted line indicates values that are equal for purified and unpurified portions of hemolymph extracts.

Associations between reproductive traits (which could not be normalized by various transformations) and the JH titer were analyzed using the nonparametric Spearman rank correlation (Sokal and Rohlf, 1981).

The various reproductive traits (e.g. % ovarian mass), in addition to not being normally distributed, also differed between the two control stocks. Because of these complications, reproductive data were analyzed by Mann-Whitney U-tests in which traits were compared between pairs of morphs of each of the two control lines separately for each day of adulthood. As was the case for the JH titers, analyses of reproductive characteristics were planned contrasts and were not corrected for multiple comparisons. Finally, comparisons between LW(F) and SW or LW(H) were one-tailed with the a priori expectation that reproductive traits or JH titers would be higher in LW(H) or SW. This expectation is based on many studies in other insects, as well as a previous study in *G. firmus*, which have documented enhanced reproduction in the morph with reduced flight muscles compared with the flight-capable morph (Zera et al., 1997; Zera and Denno, 1997). By contrast, we had no expectation concerning the directionality of differences for various reproductive traits or the JH titer between LW(H) and SW morphs. Hence these comparisons were two-tailed.

3. Results

3.1. Reproductive characteristics of wing and flight-muscle morphs

Ovarian masses (mean $\pm$ SEM % body wet mass) as a function of age for the three morphs of *G. firmus* from the two unselected (control) stocks are presented in Figure 2. Statistics for longest terminal oocyte length and number of postvitellogenic eggs are reported in Table 1. Ovarian mass and number of postvitellogenic eggs consistently increased over days 3, 5, and 7 for all three morphs. Two distinct profiles were evident for these reproductive traits. One profile of enhanced reproduction was found in SW and LW(H) morphs. This profile differed markedly from that of delayed reproduction in LW(F) females. On day 3, ovarian mass was significantly higher in females with reduced flight muscles [LW(H) or SW] compared with females with pink flight muscles [LW(F)] for both C-1 and C-3 lines ($P < 0.018$ for three of four Mann-Whitney U-tests). The one exception was day 3, C-3 crickets in which ovarian mass did not differ between LW females with white [LW(H)] and pink [LW(F)] flight muscles ($P > 0.10$; one-tailed Mann-Whitney U-test). Postvitellogenic eggs were not observed in any morph on day 3. On days 5 and 7, all females with reduced flight muscles [SW or LW(H)] had significantly larger ovaries and held a significantly greater number of

postvitellogenic eggs in their ovaries than individuals with functional flight muscles [LW(F)] ($P < 0.01$ for each one-tailed Mann-Whitney U-test). The two morphs with white flight muscles [SW and LW(H)] did not differ in ovarian mass or number of postvitellogenic eggs on any day measured ($P > 0.20$ for each two-tailed, Mann-Whitney U-test).

Patterns of terminal oocyte growth differed between the two control lines (Table 1). C-1 morphs with reduced flight muscles [LW(H) and SW] had significantly longer terminal oocytes than LW(F) females on days 3 and 5 ($P < 0.02$ for each one-tailed Mann-Whitney U-test), but not on day 7 ($P > 0.40$ for each one-tailed Mann-Whitney U-test). In contrast, morphs of C-3 did not differ significantly in oocyte length on any day ($P > 0.10$ for each one-tailed Mann-Whitney U-test). Examination of the data indicates that, in general, terminal oocytes approached maximum length by day 5 for females with reduced flight muscles [LW(H) and SW] and that terminal oocyte length in LW(F) females on day 7 was slightly less than or equal to that of day 5 LW(H) or SW females.

Figure 2. Ovarian mass (as % body wet mass) for wing and flight muscle morphs of *G. firmus* from two control lines as a function of adult age. Day 0=molt to adult. LW(F) = long-winged with functional flight muscles, LW(H) = long winged with histolyzed flight muscles, SW=short-winged with underdeveloped flight muscles. See Section 2 for line designations. For each control line on each day of adulthood, sample size ranges were 5–20 (median=14), 3–14 (median=5) and 10–20 (median=15) for LW(F), LW(H), and SW females, respectively.

Table 1. Terminal oocyte length and number of postvitellogenic eggs in wing and flight muscle morphs of the two control stocks of *G. firmus*

	Control-1			Control-3		
	LW(F) ^a	LW(H)	SW	LW(F)	LW(H)	SW
<i>Longest terminal oocyte length (mm)</i>						
Day-3	1.3±0.1b (1.2, 8)c	1.9±0.1 (1.9, 3)	1.7±0.1 (1.6, 13)	1.1±0.1 (1.1, 10)	1.2±0.1 (1.2, 3)	1.1±0.1 (1.1, 9)
Day-5	2.3±0.2 (2.2, 16)	3.3±0.2 (3.4, 4)	3.1±0.7 (3.3, 16)	3.2±0.2 (3.3, 10)	3.6±0.0 (3.6, 5)	3.3±0.0 (3.3, 10)
Day-7	3.1±0.0 (3.2, 12)	3.2±0.0 (3.3, 7)	3.3±0.0 (3.3, 13)	3.3±0.1 (3.4, 10)	3.5±0.1 (3.5, 5)	3.5±0.0 (3.5, 10)
<i>Number of post-vitellogenic eggs</i>						
Day-5	33±8 (23, 9)	77±13 (74, 3)	56±5 (52, 15)	22±8 (18, 10)	110±17 (114, 5)	84±19 (74, 10)
Day-7	41±9 (36, 11)	136±23 (138, 7)	170±15 (181, 14)	90±9 (81, 10)	270±35 (250, 5)	212±19 (221, 10)

a LW(F) = long-winged with functional flight muscles, LW(H) = long-winged with histolyzed flight muscles, SW = short-winged with underdeveloped flight muscles.

b Grand mean±(SEM) of the mean of five longest terminal oocytes per cricket.

c Numbers in parentheses are the median followed by sample size (number of individual crickets).

3.2. Juvenile hormone titers

Median JH titers and mean log[JH+1] titers as a function of age for the three morphs of *G. firmus* are presented in Figure 3. Titters did not differ significantly between the two control lines and hence titer data from these lines were combined for presentation and further analysis (see Section 2). Median JH titers ranged from 2 to 13 pg/μl in the morphs across the 3 days measured. Except for two cases discussed below, statistical analyses of the log-transformed JH titers by ANOVA or the untransformed titers by the non-parametric Mann-Whitney U-test gave identical results. The three morphs differed significantly in their JH titers (Table 2). LW individuals with pink flight muscles [LW(F)] had the lowest JH titers and SW individuals had the highest JH titers. LW individuals with white flight muscles [LW(H)] had intermediate JH titers that were closer to the SW than the LW(F) morph on most days (Figure 3). When tested separately, the SW morph had a significantly higher JH titer than the LW morph with pink flight muscles across all days and on days 5 and 7, but not on day 3 (Table 2). Titer differences between the LW morphs with white and pink flight muscles [LW(H) vs. LW(F)] were nonsignificant across all days when tested by ANOVA (Table 2) but differed significantly when tested by a one-tailed Mann-Whitney U-test ($U=1096$, $n_1=81$, $n_2=35$, $P<0.03$). Titters did not differ between these two morphs on any single day when tested by ANOVA (Table 2) and only differed marginally on day 5 when tested by a one-tailed

Figure 3. Median (panel A) and mean (±SEM) (panel B) hemolymph JH titer in wing and flight muscle morphs of *G. firmus*. Symbol designations are the same as those in Figure 2. Since JH titers did not differ significantly between the two control lines, pooled data for both lines are presented. Sample sizes on each day were 25–28 [LW(F)], 6–21 [LW(H)], and 30 (SW). Note that JH titers in panel B were transformed to log(JH+1) values (see Section 2).

Table 2. Analysis of JH titer variation among morphs of *G. firmus* measured on different days of adulthood

Morphs compared	Source of variation		
	Morphs	Days	
A. Variation among morphs and among all days (Days 3, 5, and 7)			
All [LW(F), LW(H), SW] ^a	7.1 (2, 197) ^{b***}	4.0 (2, 197) ^{**}	
LW(F) vs. SW	13.1 (1, 165) ^{***}	7.5 (2, 75) ^{***}	
LW(F) vs. LW(H)	1.3 (1, 110), ns	1.0 (2, 110), ns	
LW(H) vs. SW	2.7 (1, 119), ns	3.2 (2, 119) [*]	
B. Variation between morphs tested on individual days			
	Day 3	Day 5	Day 7
LW(F) vs. SW	2.6 (1, 56), ns	6.3 (1, 53) ^{**}	5.2 (1, 56) ^{**}
LW(F) vs. LW(H)	0.8 (1, 32), ns	0.8 (1, 31), ns	0.0 (1, 47) ns
LW(H) vs. SW	0.0 (1, 34), ns	1.0 (1, 36), ns	8.1 (1, 49) ^{**}

a Morph designations: LW(F) = long-winged with functional flight muscles; LW(H) = long-winged with histolyzed flight muscles; SW = short-wings with underdeveloped flight muscles (see Section 2).

b Values outside of parentheses are *F*-ratios from ANOVA of (log+1)-transformed titers while values inside parentheses are degrees of freedom. Comparisons of all morphs and LW(H) vs. SW were two-tailed while comparisons between LW(F) and SW or LW(H) were one-tailed (see Section 2). No significant morph $\times$ day interactions were observed.

*** $P < 0.005$; ** $P < 0.025$; * $P < 0.05$; ns = non-significant ($P > 0.1$)

Mann-Whitney U-test ($U=134$, $n_1 = 33$, $n_2 = 12$, $P = 0.05$). Titers did not differ between the two morphs with white flight muscles [LW(H) and SW] across all days, or on any individual day with one exception. On day 7, the SW morph had a significantly higher titer than the LW(H) morph (Table 2).

3.3. Correlations between the juvenile hormone titer and reproductive characters

Scatterplots of JH titers and reproductive traits [ovarian mass (as % body mass), terminal oocyte length, and number of postvitellogenic eggs] are presented in Figure 4 and Figure 5. Data points in Figure 4 are measurements in individuals while Figure 5 represents the mean values for each of the three morphs calculated for each of the two stocks on each of the 3 days of adulthood ($N = 18$ means). The JH titer was significantly correlated with ovarian mass and terminal oocyte length for all individuals ($r = 0.31$, $P < 0.001$ and $r = 0.26$, $P = 0.001$, respectively; $N = 206$) but was not significantly correlated with the number of postvitellogenic eggs ($r = 0.13$, $P = 0.22$; $N = 206$) (Figure 4). These correlations were essentially the same when LW(F) females were tested with only one of the types of flightless morph. For example, the correlation between JH titer and ovarian mass for the sample without LW(H) females was $r = 0.38$, $P < 0.001$ ($N = 171$), while the correlation for the sample without SW females was $r = 0.26$, $P < 0.005$ ($N=116$). Correlations were substantially higher between mean JH titers and mean values for the various reproductive traits (values averaged over individuals of a particular morph of particu-

lar stock for a particular day of adulthood) [$r = 0.62$, $P = 0.004$ for ovarian mass, $r = 0.58$, $P = 0.011$ for terminal oocyte length, and $r = 0.57$, $P = 0.014$ for number of postvitellogenic eggs (Figure 5)].

4. Discussion

4.1. General aspects

4.1.1. JH titer in *G. firmus* compared with other insects

Median JH titers in *G. firmus* females derived from unselected control lines during the first week of adulthood (3–14 pg/ul; Figure 3) are at the lower end of the range of titers reported for females of the cricket species *Teleogryllus commodus* (8–60 pg/ul) and *Acheta domesticus* (20–70 pg/ul) and the locust, *Locusta migratoria* (4–30 pg/ul) (Loher et al., 1983, 1987; Renucci and Strambi, 1983; Dale and Tobe, 1986). As mentioned previously (see Section 2), JH III is the only juvenile hormone found in *G. firmus*. JH titers reported in the present study and in studies mentioned above have been quantified by a variety of methods all of which appear to accurately measure JH. The RIA used in the present study (Hunnicuttt et al., 1989) has been used successfully to quantify JH levels in unpurified hemolymph from bees (Huang et al., 1994) and a beetle (Trumbo et al., 1995). Furthermore, the Hunnicutt RIA has been validated against two other JH-RIAs (Goodman et al., 1990; Strambi et al., 1981) which, in turn, have been validated against the gas-chromatographic/mass spectrophotometric (GC/MS) method [see Methods of Huang et al. (1994) for details]. The Strambi

Figure 4. Scatterplots illustrating the relationship between ovarian mass (top panel), mean terminal oocyte length (middle panel), and number of postvitellogenic eggs (bottom panel) vs. JH titer in wing and flight muscle morphs of *G. firmus*. Symbol designations are the same as those in Figure 2 and refer to values for individual crickets.

RIA was used to quantify the JH titer in *Acheta* (Renucci and Strambi, 1983), while the GC/MS method was used to quantify JH in *T. commodus* (Loher et al., 1983, 1987) and *L. migratoria* (Dale and Tobe, 1986). The similar JH titers in *G. firmus*, *Teleogryllus*, *Acheta* and *Locusta*, combined with the results of our background RIA studies of HPLC-fractionated and Prep-Sep-purified hemolymph from *G. firmus* (see Section 2), indicate that the RIA used in the present study accurately measures JH in unpurified hemolymph from individual *G. firmus*.

Figure 5. Scatterplots illustrating the relationship between mean ovarian mass (top panel), grand mean terminal oocyte length (middle panel) and mean number of postvitellogenic eggs (bottom panel) and hemolymph JH titer in wing and flight muscle morphs of *G. firmus*. Symbol designations are the same as those in Figure 2. Values represent means of crickets of a particular morph, day of adulthood, and line (control-1 or control-3) (see Section 2).

4.2. JH titer and reproduction in *G. firmus*

JH plays an important role in regulating oocyte growth and maturation in all crickets studied thus far (reviewed in Strambi et al., 1997). Prior to the present study, no information was available on the endocrine regulation of oocyte growth in *G. firmus*. Our results

clearly implicate JH as a regulator of oocyte growth in this species. Ovarian mass, the number of postvitellogenic eggs, and JH titer all increased from day 3 to day 7. Furthermore, the JH titer was positively correlated with ovarian mass and length of terminal oocytes in individual *G. firmus* (see Section 3). We also have recently documented that topical application of either methoprene or JH III to the abdomen of intact LW *G. firmus* significantly increases ovarian mass (A. J. Zera, G. Cisper, and R. Koltz, unpublished data). On the other hand, the only moderate correlations between JH titer and various measures of reproduction in wing and flight muscle morphs indicate that JH is not the sole regulator of ovarian growth in this species. This is to be expected since numerous studies have identified hormones other than JH that influence ovarian growth (e.g. ecdysteroids and various neurohormones; Renucci and Strambi, 1981; Behrens and Hoffman, 1983; Nijhout, 1994; Strambi et al., 1997; Pener and Yerushalmi, 1998).

4.3. Morph-specific reproduction and its endocrine regulation: long-winged, flight capable [LW(F)] vs. short-winged (SW) females

Elevated ovarian growth during early adulthood is one of the distinguishing features of the flightless morph of wing polymorphic insects (Harrison, 1980; Roff, 1984, 1986; Zera and Rankin, 1989; Zera and Denno, 1997; Zera et al., 1997). This elevated egg production during early adulthood is a major contributor to the greater overall fecundity of flightless vs. flight-capable females in many wing-polymorphic species (Roff, 1984, 1986; Zera and Rankin, 1989; Zera and Denno, 1997). Results obtained in the present study clearly document that SW females have substantially elevated ovarian mass and a greater number of post-vitellogenic eggs, compared with long-winged, flight-capable [LW(F)] females during the first week of adulthood (Figure 2; Table 1). The elevated ovarian mass of flightless females did not simply result from an earlier onset of ovarian growth. Rather, the growth rate of ovaries was about twice as high in flightless compared with flight-capable females during the first week of adulthood (Figure 2). The existence of larger ovaries in SW females during this period of adulthood is similar to results of previous studies of morph-specific reproduction in *G. firmus* (Roff, 1984; Zera et al., 1997).

For many years, an elevated JH titer has been proposed as the proximate cause of the elevated ovarian growth in flightless females of wing polymorphic species (Southwood, 1961; Wigglesworth, 1961; Lees, 1966; Nijhout and Wheeler, 1982; Hardie and Lees, 1985; Nijhout, 1994; Zera, and Denno, 1997). Although many studies have investigated this topic, all previous studies have been indirect. That is, an elevated JH titer in the flightless morph has been *inferred* from the results of ex-

perimental manipulations, such as application of exogenous JH or JH analogues, or implantation of corpora allata into LW females. These manipulations have typically caused ovarian mass in long-winged females to increase in size to that of unmanipulated, flightless females, suggesting that an elevated JH titer causes the enhanced ovarian growth in flightless, unmanipulated females (Tanaka, 1994; Iwanaga and Tojo, 1986). However, these results are also consistent with other explanations, such as the induction by exogenous JH of unidentified hormones which are the true regulators of enhanced ovarian growth. The many feedback loops that typify endocrine physiology (Nijhout, 1994) make this a likely possibility, thus severely limiting the inferences about endogenous hormone titer variation that can be drawn solely from experimental manipulations (Zera and Tiebel, 1988; Zera and Denno, 1997).

The present study is the first to directly quantify JH titers in flight capable and flightless adult morphs of a wing polymorphic insect species. Moreover, titers were measured separately in the two LW morphs that differ dramatically in ovarian growth: long-winged females with pink functional flight muscles [LW(F)] and long-winged females with histolyzed, non-functional flight muscles [LW(H)]. Our results show that the JH titer is significantly higher in flightless, SW females compared with flight-capable [LW(F)] females during the first week of adulthood (Figure 3 and Table 2). These results are clearly consistent with the idea that the greater ovarian growth in SW females during early adulthood is due, at least in part, to an elevated JH titer [JH titers in LW(H) females will be discussed below]. The positive correlation between JH titer and ovarian mass and size of terminal oocytes in individual *G. firmus* (Section 3) is also consistent with this idea.

Because no JH titer data are available for other wing polymorphic species, the extent to which the elevated JH titer in flightless adult *G. firmus* (Figure 3) also occurs in flightless females of other wing polymorphic species is unknown. Indeed there are very few data on JH titer variation in functionally similar types of polymorphism. For example, in the only other report of JH titer variation in aphids, Hardie et al. (1985) found that the JH III titer was elevated two-fold in whole-body extracts of the adult aphid, *Megura viciae*, that produced vivipara (parthenogenic females) vs. those that produced oviparae (sexual females). This suggested that JH might be involved in the regulation of sexual/parthenogenic polymorphism in this aphid species. However, the JH III titers were very low (<0.2 ng/g) and it is unclear whether JH-III is the main juvenile hormone in this species (Hardie et al., 1985). Juvenile hormone has been discussed for over four decades in the context of the endocrine regulation of phase polymorphism in locusts (Nijhout and Wheeler, 1982; Pener, 1991; Pener and Yerushalmi, 1998). However, other than the early

study of Joly et al. (1977), which was done before the development of reliable methods for quantifying JH titers (see above), only very limited data on JH titer variation between phases has been reported. For example, Dale and Tobe (1986) found a two-fold elevation in the JH III titer in solitary vs. gregarious female *L. migratoria* during the first week of adulthood. Solitary females also had significantly larger ovaries than gregarious females during this time. However, the study of Dale and Tobe (1986), which primarily focused on phase-dependent differences in JH biosynthesis, only contained four individual JH titer measurements.

Although our results are consistent with the long-standing JH-morph reproduction hypothesis, they also indicate that an elevated JH titer does not appear to be the only factor responsible for the larger ovaries found in flightless females. Variation in the mean JH titer does account for a large proportion (35–40%) of the variation in mean ovarian mass between morphs from the two control lines on various days of adulthood (Section 3 and Figure 5). However, variation in the JH titer among individuals accounts for only 10–15% of the total variation in ovarian mass ($r^2 = 0.096$ – 0.144 ; r = Spearman correlation between JH titer and % ovarian mass; Section 3 and Figure 4). There are several possible explanations for the low-moderate correlation between JH titer and ovarian mass. One likely possibility is that variation in the JH titer is only one of several factors that causes the difference in ovarian growth between the flight-capable and flightless morphs. As mentioned above, there are numerous endocrine factors such as ecdysteroids and various neuropeptides which influence ovarian growth (see references above). Thus, an assessment of the importance of JH in morph-specific ovarian growth must await studies of these other potential regulators of ovarian growth.

Very recently, we have found that the JH titer varies diurnally in *G. firmus* (A. J. Zera, G. Cisper, and D. Katz, unpublished data). Diurnal change in the JH titer was unknown when the present study was conducted, and, there is no published report of diurnal variation in the JH titer except for Walker and Denlinger (1980). The existence of diurnal JH titer variation complicates an assessment of the existence, magnitude and functional significance of JH titer differences between wing and flight muscle morphs of *G. firmus*. For example, if hemolymph samples were taken during a period of time during which JH titers were changing, then titer differences between morphs may have been masked to some extent and hence underestimated. If this were the case, correlations between the JH titer and reproductive traits might also have been underestimated. Furthermore, observed titer differences between the morphs (Figure 3) may only exist during the restricted period of time during which the titers were measured. The extent of these complications can only be determined by more compre-

hensive studies of *G. firmus* in which JH titers are measured during short periods of time throughout the photophase and scotophase. Such studies are currently in progress. Hence, we emphasize that JH titer variation among morphs of *G. firmus* reported in the present study (Figure 3) may underestimate actual titer differences between the morphs. Furthermore, differences among the morphs in the JH titer (Figure 3) may not exist during all periods of the photophase and scotophase. We also emphasize that our studies have focused exclusively on the endocrine regulation of morph-specific reproduction during the first week of adulthood.

4.4. LW morphs with fully-developed [LW(F)] and histolyzed [LW(H)] flight muscles

Until very recently, ecological and physiological studies of wing polymorphism have involved comparisons between morphs that vary in wing length (Zera et al., 1997). However, as mentioned previously (see Section 1), in many insect species, including *G. firmus*, LW females are a composite group of individuals some of which retain their flight muscles [LW(F)] and others of which histolyze their flight muscles when ovarian growth begins [LW(H)]. LW(H) females are more similar to SW than to LW(F) females in a variety of key characteristics including ovarian growth, respiration rates of flight muscles and activities of enzymes in flight muscles (Figure 2; Zera et al., 1997, 1999). Because of these marked differences, it is essential to obtain measurements separately for these two types of LW morphs. Indeed, Zera et al. (1997) recently documented that the degree of enhancement of egg production due to flightlessness can be underestimated by as much as 50% when reproductive differences between flight-capable LW(F) and flightless LW(H) morphs are not distinguished. The existence of two types of flightless morphs in the same species, that are produced by different mechanisms (blockage of flight muscle growth vs. muscle histolysis) during different life cycle stages (juvenile vs. adult), provides the opportunity to compare physiological features of flightless phenotypes that are the products of different evolutionary pathways to flightlessness (this point is discussed in more detail in Zera et al., 1997).

As was found in a previous study (Zera et al., 1997), ovarian growth is essentially identical in LW(H) and SW females and is substantially higher in each of these morphs than in LW(F) females (Figure 2). This corroborates our previous argument that reduction in flight muscles is more strongly associated with enhanced reproduction than is reduction in wings (Zera et al., 1997). By contrast, differences in the JH titer were greater between LW(F) and SW females than between LW(F) and LW(H) females (Section 3 and Figure 3). This suggests that the enhanced reproduction in the two differ-

ent flightless morphs may be caused, in part, by modification of different endocrine mechanisms that regulate ovarian growth.

4.5. Conclusions and future prospects

In summary, we have documented significant variation in the JH titer among morphs of *G. firmus* that is correlated with variation in several reproductive measures (Figures 3–5 and Table 2). These results suggest that the increased JH titer during the first week of adulthood, at least in part, causes the elevated ovarian growth of the SW morph. To more precisely identify the role of JH titer variation in morph-specific reproduction in *G. firmus*, we are currently quantifying variation in the JH titer, variation in the ecdysteroid titer, and variation in the activity of regulators of the JH titer (juvenile hormone esterase) between adult wing morphs at specific points of the photophase and scotophase. Our previous studies of the endocrine regulation of wing polymorphism have focused on the regulation of morph development by JH during the last stadium in *G. rubens* and *G. firmus*. Despite considerable study, the importance of JH in regulating morph development remains uncertain (reviewed in Zera and Denno, 1997; Zera, 1999; Zera and Huang, 1999). JH titers are 10–100 fold higher in adult compared with juvenile *Gryllus* (e.g. compare titers in Figure 3 of this study with those of Figure 8 of Zera et al., 1989). Because of the much higher JH titer in adults, hormone titers can be quantified in individuals, in contrast to last-stadium nymphs where hemolymph from at least 20 crickets must be pooled for a single titer measurement. Furthermore, titers clearly differ between adult morphs (Figure 3), in contrast to the only subtle titer variation between last-stadium morphs (Zera et al., 1989; Zera and Denno, 1997; Zera, 1999). Thus, JH regulation of morph-specific reproduction will likely be a more-tractable experimental problem than JH regulation of morph-specific development.

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