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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

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Abstract
A previous study documented a high amplitude, morph-specific daily cycle in the hemolymph JH titer in the wing-polymorphic cricket, *Gryllus firmus*. The JH titer rose and fell 10–20 fold in the flight-capable [LW(f), long-winged] morph during the late-photophase-early scotophase, while it was relatively constant during that time in the flightless (SW, short-winged) morph. In the present study we documented a dramatic morph-specific daily cycle in the *in vitro* rate of juvenile hormone (JH) biosynthesis that was tightly correlated with the hemolymph JH titer on days 5–7 of adulthood. Biosynthetic rates rose and fell 1–2 fold between the late photophase-early scotophase on each of days 5–6 and 6–7 of adulthood in the LW(f) morph, while biosynthetic rates were relatively constant during this period in the flightless, short-winged morph (SW), except for a slight dip in the rate of biosynthesis late in the photophase on these days. Similar morph-specific patterns of JH biosynthesis were observed whether rates were measured on corpora allata attached to corpora cardiaca in males or females, or on corpora allata alone. Hemolymph juvenile hormone esterase activity was significantly higher in the LW(f) vs. the SW morph during the beginning of scotophase, when the JH titer is decreasing rapidly in the LW(f) morph. Results indicate that the morph-specific daily cycle in the JH titer in *G. firmus* is primarily regulated by a morph-specific daily cycle in the rate of JH biosynthesis and to a lesser degree by hemolymph JH esterase activity. This is the first documentation of a diurnal cycle in the rate of JH biosynthesis in any insect, or a daily cycle in the rate of JH biosynthesis that is correlated with a specific morph in a polymorphic species. Results have important implications for the endocrine regulation of dispersal polymorphism, circadian rhythms of insect hormone titers and their regulators, and general studies of the JH titer and its regulation in insects.

Keywords: wing polymorphism, juvenile hormone, JH biosynthesis, trade-off, dispersal, life history

1. Introduction

The hormonal control of complex (multi-trait) polymorphism is a fundamental problem in insect endocrinology. At issue are the endocrine processes that regulate the expression of traits that define phases, castes, or wing/flight muscle morphs in polymorphic species. One of the most intensively studied complex polymorphisms is wing polymorphism, which consists of morphs adapted for flight at the expense of reproduction or vice versa (Wigglesworth, 1961; Hardie and Lees, 1985; Dingle, 1996; Zera and Denno, 1997; Nijhout, 1994 and Nijhout, 1999; Zera and Harshman, 2001; Zhao and Zera, 2002; Zera, 2004). One morph has fully developed wings and flight muscles, is capable of flight, and prioritizes triglyceride flight fuel biosynthesis over egg production during early adulthood. The alternate flightless morph has underdeveloped wings and flight muscles, and produces considerably more eggs, but a reduced level of triglyceride reserves, relative to its flight-capable counterpart during early adulthood.

Juvenile hormone (JH) has long been proposed to be a key regulator of morph development and reproduction in wing-polymorphic species. The traditional view has been that a JH titer above some threshold causes the expression of traits found in the flightless morph, while a JH titer below that threshold specifies an alternate set of traits found in the flight-capable morph (Wigglesworth, 1961; Nijhout and Wheeler, 1982; Hardie and Lees, 1985; Nijhout, 1994 and Nijhout, 1999; Zera and Denno, 1997; Zera, 2004). However, recent studies in the wing polymorphic cricket, *Gryllus firmus*, indicate that differences in the JH titer between wing morphs is more complex than proposed by this model (Zera and Cisper, 2001; Zhao and Zera, 2004).

Rather than simply differing in concentration, the JH titer in morphs of *G. firmus* differs dramatically in the presence/absence of a daily cycle (Zera and Cisper, 2001; Zhao and Zera, 2004). The JH titer in the SW morph exhibits very little diel variation throughout the first week of adulthood. By contrast, the JH titer in the long-winged (LW(f)) morph exhibits a 10-20-fold spike near the end of the photophase-beginning of the scotophase on each of several days of early adulthood (see Discussion). Not only is this the first example of a morph-specific daily cycle in the JH titer in a phase, caste, or wing-polymorphic insect species, it is the most dramatic example of diel change in the JH titer found in any insect to date (Zhao and Zera, 2004).

The existence of a daily cycle in the JH titer that is restricted to the flight-capable morph raises a number of intriguing questions concerning the function of these morph-specific titer differences and the regulatory mechanisms that are responsible for their existence. As a first step in addressing the latter question, we compared the *in vitro* rate of JH biosynthesis and hemolymph JHE activity in morphs of *G. firmus* during a period of early adulthood when the JH titer cycles dramatically in the flight-capable, but not in the flightless morph. Rate of JH biosynthesis and hemolymph JHE activity were measured because they are considered the primary regulators of the hemolymph JH titer in insects in general, and in complex-polymorphic insects in particular (Hardie and Lees, 1985; Tobe and Stay, 1985; Roe and Venkatesh, 1990; Nijhout, 1994 and Nijhout, 1999; Huang and Robinson, 1995; Zera and Denno, 1997; Zera, 2004).

2. Materials and methods

2.1. Chemicals and medium

All chemicals and solvents used in the present study were at least reagent or HPLC grade and were purchased from Sigma Chemical Company or Fisher Scientific. Racemic, unlabeled JH III and silica gel thin-layer chromatographic plates were purchased from Sigma Chemical Co. L-[methyl-^{14}C]-methionine (59 mCi/mmol; 2.18 Gbq/mmol) was purchased from Perkin Elmer, and Medium 199 with Hanks salts, 25 mM HEPES buffer and L-glutamine was purchased from GIBCO BRL. The juvenile hormone esterase inhibitor OTFP (3-octylthio-1,1,1-trifluoropropan-2-one) was a generous gift of Dr. Bruce Hammock, Deptartment of Entomology, University of California, Davis 95616.

2.2. Insects, morph designations and rearing conditions

G. 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 obligatorily flightless (Veazy *et al.*, 1976). Except for a few rare cases, all SW females have white, non-functional flight muscles, which never fully develop. All LW females initially have large, pink flight muscles at the adult ecdysis or shortly thereafter and are denoted as LW(f). After about 5–6 days of adulthood, some LW(f) individuals begin to histolyze their flight muscles thus becoming flightless (denoted as the LW(h) morph; see Zera *et al.* 1997). Because virtually all *G. firmus* investigated in the present study had large, pink flight muscles, juvenile hormone (JH) biosynthetic rates and juvenile hormone esterase (JHE) activities were not measured in the LW(h) morph. The *G. firmus* used in the present study were taken from a pair of LW- and SW-selected lines (Block-2; see Zera and Cisper, 2001) that were derived from a colony founded from 30 gravid females collected in Gainesville, Florida during the summer of 1995. These are the same lines in which JH titers were measured by Zhao and Zera (2004).

Crickets were reared as in Zhao and Zera (2004), that is, under a 16:8 L:D cycle at 28° C and were fed the standard (100%, High) wet diet described in Zera and Larsen (2001). Other details of rearing can be found in Zera and Cisper (2001). Crickets used for experiments were checked for ecdysis at 24-h intervals and equal numbers of newly ecdysed adult males and females were housed together at a density of 6 per 1 gallon box or 12 per 3 gallon box with oviposition substrate added on day 6. Presence or absence of oviposition substrate does not significantly alter the JH titer of day 6-8 female crickets (Zhao and Zera, 2004).

2.3. In vitro assay of JH biosynthesis

In vitro rate of juvenile hormone III biosynthesis was measured on a pair of corpora allata (CA) alone or a pair of corpora allata with attached corpora cardiaca (CC) obtained from a single 5–7 day-old adult *G. firmus* (day 0 = day of adult ecdysis). These days were chosen because this is the period of adulthood when the hemolymph JH

titer exhibits a strong morph-specific daily cycle in *G. firmus* (Zhao and Zera, 2004). Rate of JH biosynthesis was measured essentially as described previously for rates measured on nymphal *G. rubens* (Zera and Tobe, 1990) with a few modifications. Most importantly, we used L-[methyl^{14}C]-methionine in the present study rather than L-[methyl^{3}H]-methionine used previously, because of technical problems encountered with the latter compound (Yagi and Tobe, 2001). Radiolabelled methionine (600,000–650,000 DPM) was added to100 µl of Medium 199, containing 100 µM unlabelled L-methionine, resulting in a total concentration of L-methionine of 145-150 µM. In the standard assay, crickets were chilled on ice, heads were removed, and glands were dissected under sterile Medium199 containing 20 mg/ml Ficoll and 5 mM $CaCl_2$ but without radiolabelled L-methionine. Glands were placed in a fresh drop of medium, extraneous tissue was removed, glands were transferred to a new drop of medium and held on ice until a sufficient number of glands were obtained for an assay (usually 40–60 min). Individual pairs of CA or CA–CC were then transferred to test tubes (one pair per tube) containing 100 µl of Medium 199, but containing radiolabelled L-methionine, and preincubated for 45 min. Glands were then transferred to fresh medium, and (except for time-course studies) incubated for 3 h. Preincubation was required because of the 45 min lag time in the biosynthesis of radiolabelled JH (see below). After the incubation period, JH was extracted from medium plus glands as described in Zera and Tobe (1990). Extracts were run on plastic-backed thin-layer chromatographic plates, and spots containing JH-III were cut out and DPM measured (as in Zera and Tobe, 1990). Because less than 4% of biosynthesized JH-III was retained within the glands (see below), extraction of the medium containing CA or CA–CC essentially quantified both rate of JH biosynthesis and release into the medium. All incubations and preincubations were performed at 28 ± 1°C in the dark.

2.4. JHE assay

Juvenile hormone esterase activity was measured using the standard radiochemical assay of Hammock and Sparks (1977) as modified slightly for *Gryllus* (Zera and Huang, 1999).

2.5. Validation of biosynthetic assay

2.5.1. Time course and release of biosynthesized JH-III into the medium

CA–CC complexes were incubated as described above, but for 10 h. Medium was changed at 1, 2, 4, 6, 8, and 10 h after the start of the assay. At each of these times, CA–CC were rinsed with medium, and the rinse was combined with the medium taken from the tube at that time point. CA–CC complexes were removed from the assay tubes at the end of the assay. Biosynthesized JH-III was extracted and quantified from each time aliquot of medium, and from the CA–CC complexes separated from the last temporal aliquot of medium. Rate of JH release exhibited an initial 45 min lag, after which it was essentially linear for 10 h, well beyond the standard 3 h incubation period (Figure 1). When incubated for 3 h, 2798 ± 93 DPM attributable to JH was found in the medium while only 107 ± 61 DPM (3.8% of total) attributable to JH was found within the CA-CC. That is, essentially all biosynthesized JH was released from the CA-CC complexes.

2.5.2. Effect of Ca^{++} concentration on rate of JH biosynthesis

Rate of JH-III biosynthesis in *G. bimaculatus* changed significantly when Ca^{++} concentration was varied from 1 to 10 mM (Klein *et al.*, 1993). We measured the rate of JH III biosynthesis as a function of Ca^{++} concentration to determine if this also was the case for female CA–CC complexes from *G. firmus*. The Ca^{++} concentration of Medium 199 (1.26 mM) was elevated to 5 mM, or 10 mM, respectively, by addition of $CaCl_2$, and rates of JH biosynthesis were determined. Rates were slightly lower at 1.26 mM Ca^{++} (34.4 ± 5.5 pmol h^{-1} per pair) than at 5 mM Ca^{++} (49.7 ± 4.0 pmol h^{-1} per pair) or 10 mM Ca^{++} (46.4 ± 7.5 pmol h^{-1} per pair; N = 6 in all treatments). Five millimolar Ca^{++} was chosen as the Ca^{++} concentration in the standard assay, as was the case for our earlier studies of JH biosynthesis in nymphal *G. rubens* (Zera and Tobe, 1990).

2.5.3. Effect of JH-esterase on JH III biosynthesis

To determine whether rate of JH-III biosynthesis is reduced because of degradation of radio-biosynthesized JH by endogenous juvenile hormone esterase, biosynthetic

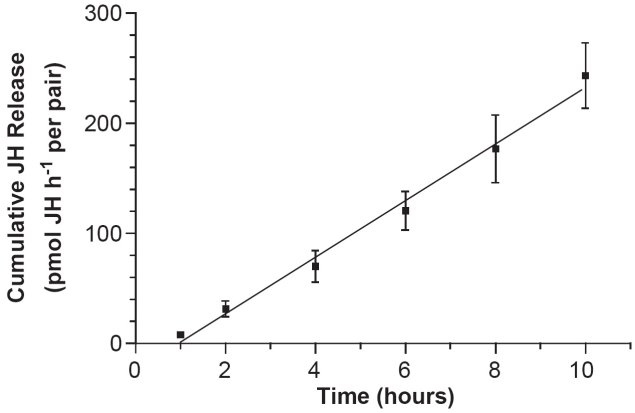

Figure 1. Time-course of JH biosynthesis in *G. firmus*. Rates were measured on pairs of corpora allata with attached corpora cardiaca (one pair per assay derived from a single SW female obtained early in the photophase. Means (± SEM) were based on assays of four pairs of glands at each time point.

rates were compared in the presence or absence of 10^{-6} M OTFP (3-octylthio-1,1,1-trifluoropropan-2-one), a potent inhibitor of JHE in *Gryllus* (Zera *et al.*, 1992 and Zera *et al.*, 2002). For either LW(f) or SW morphs, no difference in the rate of JH biosynthesis was observed between assays with or without OTFP (Table 1). Thus, degradation of biosynthesized JH by endogenous JH esterase does not complicate measurement of *in vitro* JH biosynthetic rates.

2.6. Standard Assay of JH biosynthesis

Based on these and other background experiments, the standard *in vitro* assay of JH biosynthesis in the present study consisted of a pair of CA or CA–CC from a single individual preincubated for 45 min followed by a 3 h incubation period. We used Medium 199 with 5 mM Ca^{++}, without OTFP, a total L-methionine concentration of 145–150 μM and a specfic radioactivity of L-[methyl-^{14}C]-methionine of 19–20 mCi/mmol. CA or CA–CC were not removed from the medium prior to JH extraction. JH-III was assumed to be the only JH biosynthesized by CA from *G. firmus* because this has been documented in two closely related *Gryllus* species (*G. bimaculatus* and *G. rubens*) using either single-label (Koch and Hoffman, 1985) or dual-label protocols (Zera and Tobe, 1990).

3. Results

Rates of JH biosynthesis ranged from about 10–35 pmol h^{-1} per pair CA for day 5–7 adult female *G. firmus* (Figure 2, top and middle panels). Temporal profiles of biosynthetic rates obtained from the corpora allata (CA) alone were similar to profiles obtained using corpora allata and attached corpora cardiaca (CC). Thus, no evidence was obtained indicating that regulators of JH biosynthesis emanate from the corpora cardiaca. Biosynthetic rates in flightless SW females were, in general, temporally constant during these days, except for the slight but consistent drop in the biosynthetic rate near the end of the photophase on days 5 and 6. By contrast, rate of JH biosynthesis from CA of flight-capable [LW(f)] females exhibited a striking daily cycle. Rates rose sharply (ca.100–200%) a few hours before lights-off and decreased during the early scotophase on each of days 5–6 and 6–7. Biosynthetic rates were significantly higher in corpora alata alone or corpora allata plus corpora cardiaca from LW(f) vs. SW females during the peak in the JH biosynthetic cycle on both days 5 and day 6 of adulthood, but not at other times (Figure 2, top and middle panels).

JH biosynthetic profiles in male morphs were only measured on CA–CC complexes, and only during one daily cycle (Fig 2, bottom panel). Biosynthetic rates ranged from 10 to 17 pmol h^{-1} per pair, which were slightly lower than rates in females (Fig 2, top panel). Morph-specific temporal profiles were similar in males and females (Figure 2): Like LW(f) females, JH biosynthetic rates in LW(f) males increased significantly during the latter portion of the photophase, and dropped back to pre-peak levels during the early portion of the scotophase (within 2 h after lights off; Figure 2, bottom panel). Also, no peak was observed in SW males, and JH biosynthetic rate exhibited a slight dip near the end of the photophase in this morph.

On days 5–7, hemolymph JHE activity cycled roughly in parallel in LW(f) and SW female *G. firmus*, with activities increasing about 50–100% in both morphs during the latter portion of the photophase and decreasing during the first half of the scotophase (Figure 3, bottom panel). Activities did not differ significantly between morphs on either day 5 or 6 during the rise in JHE activity. However, early on day 6 and day 7 (beginning of scotophase), JHE activity continued to rise in LW(f) when activities began to drop in SW females and differed significantly between morphs during this time (*t*-test; $^{*} = P < 0.05$; $^{***} = P < 0.005$).

For comparative purposes, temporal profiles of JH biosynthetic rate, hemolymph JHE activity, and hemolymph JH titer (JH titer data from Zhao and Zera, 2004) for female morphs on days 5–7 of adulthood are presented together in Figure 3. Morph-specific temporal profiles of JH biosynthesis strongly co-varied with profiles of the hemolymph JH titer. In the flight-capable LW(f) morph, peaks of JH titer and JH biosynthesis

Table 1. Rates of juvenile hormone biosynthesis (pmol h^{-1} per pair) by corpora allata-corpora cardiaca complexes from flight-capable [LW(f)] and flightless (SW) female *G. firmus* in assay medium with or without the juvenile hormone esterase inhibitor OTFP

Without OTFP		With OTFP[a]	
LW(f)[b]	SW	LW(f)	SW
26.1 ± 3.9 (8)	22.9 ± 4.3 (7)	28.2 ± 2.5 (8)	25.6 ± 4.5 (8)

[a] 10^{-6} M OTFP ((3-octylthio-1,1,1-trifluoropropan-2-one) in the assay medium; 10^{-5} M OTFP resulted in a significant decrease in rates of hormone biosynthesis (data not shown).

[b] Glands were taken from day 5–6-adult females during the first hour after lights-on and subjected to the standard *in vitro* assay for JH biosynthesis (see Methods).

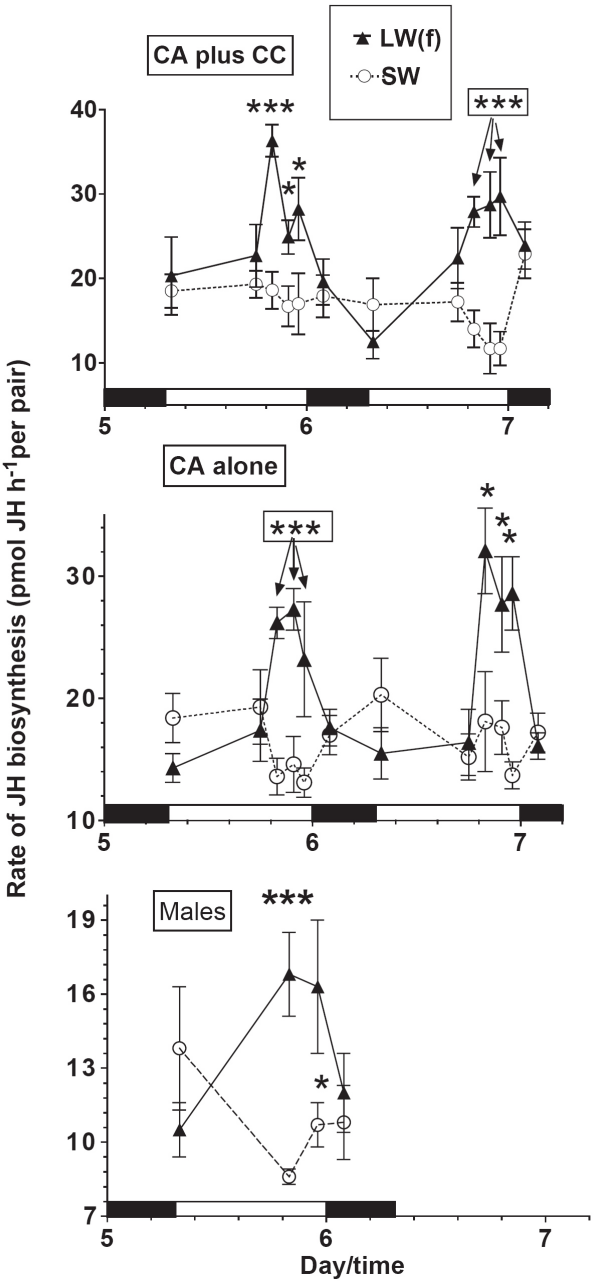

Figure 2. Morph-specific daily cycle in the rate of JH biosynthesis on days 5–7 of adulthood. LW(f) denotes the flight-capable morph with long wings and large, functional flight muscles, while SW denotes the flightless, short-winged morph of *G. firmus*. Rates (mean ± SEM) were measured on female corpora allata (CA) plus attached corpora cardiaca (CC) (top panel), corpora allata alone (middle panel), or male corpora allata with attached corpora cardiaca (lower panel). In all cases, biosynthetic rate was measured on a pair of glands derived from a single individual. Numbers on the *x*-axis refer to day of adulthood (0 = day of adult ecdysis). Light and dark bars on the *x*-axis denote the photophase (16 h) and scotophase (8 h), with time zero arbitrarily denoted as the beginning of the scotophase. Asterisks denote significant differences between biosynthetic rates in LW(f) vs. SW individuals at a particular time (*t*-test; * = $P < 0.05$, *** = $P < 0.005$), while means without asterisks did not differ statistically. Mean biosynthetic rates for each time point were based on assays of the following number of individual pairs of CA or CA-CC: 7–11 (top panel), 6–8 (middle panel), and 6–8 (lower panel), respectively.

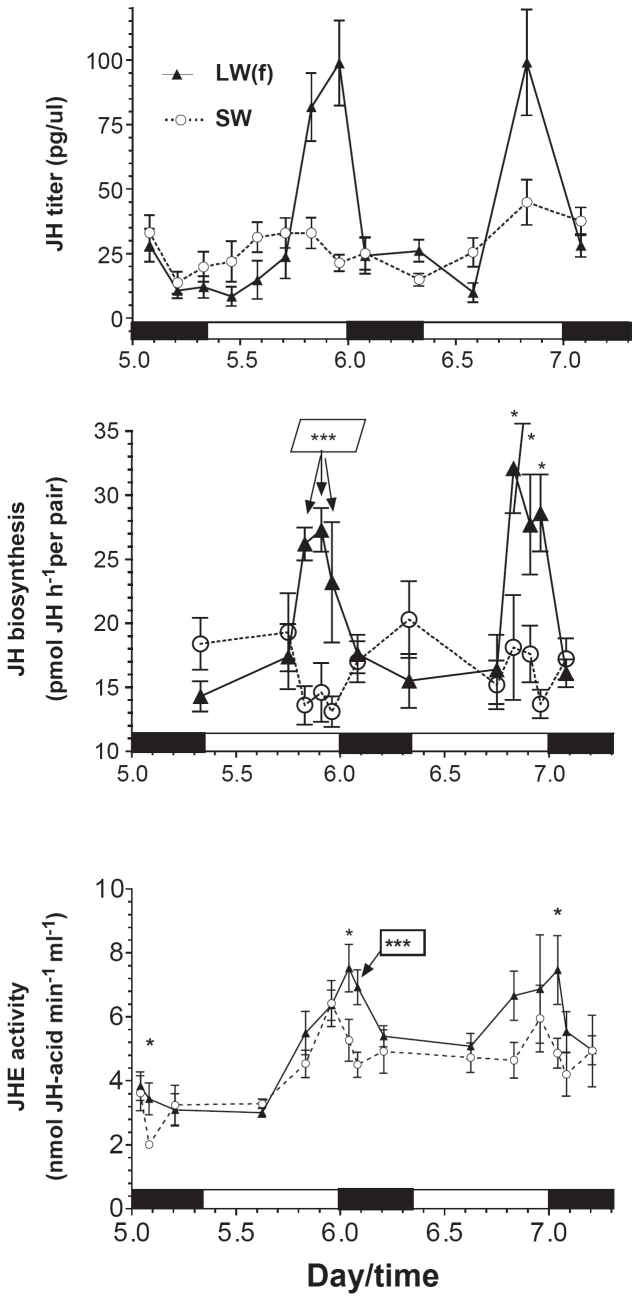

Figure 3. Relationships among morph-specific temporal profiles of the hemolymph JH titer (top panel), rate of JH biosynthesis (middle panel), and hemolymph juvenile hormone esterase (JHE) activity (lower panel) in adult female *G. firmus*. JH titer data are from Zhao and Zera (2004), rates of JH biosynthesis are from Figure 2 (middle panel). See legend of Figure 2 for explanation of bars and values on *x*-axis. Times at which JHE activities differed significantly between LW(f) and SW morphs are denoted by asterisks (* = $P < 0.05$, *** = $P < 0.005$).

occurred synchronously near the end of the photophase-beginning of the scotophase, while in the SW morph, JH titer and JH biosynthetic temporal profiles were both relatively flat during this time, except for the slight dip in the rate of JH biosynthesis at the end of the photophase on either day 5 or 6. Although the phases of the

cycles of JH biosynthesis and hemolymph JH titer were synchronous for LW(f) females, the amplitude of the JH titer cycle was greater than the amplitude of JH biosynthetic rate. That is, JH titers increased about 10–20 fold, while rates of JH biosynthesis increased only 1–2 fold on days 5 and 6 of adulthood.

JHE activity did not differ between morphs when the JH titer rose dramatically in LW(f) but not SW females during the photophase (Figure 3). However, JHE activity was significantly higher in LW(f) vs. SW females during the beginning of the scotophase on both days 5 and 6, precisely when the JH titer JH dropped precipitously in the LW(f) morph.

4. Discussion

Results of the present study strongly imply that the morph-specific daily cycle of JH biosynthesis is an important cause of the previously documented (Zhao and Zera, 2004), morph-specific daily cycle in the hemolymph JH titer in adult *G. firmus*. Rate of JH biosynthesis in the flight-capable LW(f) morph, measured on corpora allata alone, or on corpora allata and attached corpora cardiaca, exhibited a strong daily cycle that closely paralleled the daily cycle of the hemolymph JH titer (Figure 2 and Figure 3). By contrast, temporal profiles in both the rate of JH biosynthesis and hemolymph JH titer in SW females were relatively constant, except for the slight dip in the rate of JH biosynthesis near the end of the photophase (Figure 2 and Figure 3). To our knowledge, the present study not only represents the first documentation of a daily cycle in the rate of JH biosynthesis in any insect, it also is the first documentation of a cycle in JH biosynthesis that is morph-specific. In addition, we have recently shown that the cycles in the JH titer and rate of JH biosynthesis in LW(f) females persist in constant darkness (Z. Zhao and A. J. Zera, unpubl. data), and thus are endogenous circadian rhythms.

In vitro rates of JH biosynthesis in adult female and male *G. firmus* (Figure 2) were very similar to biosynthetic rates reported in adult females and males of the congener, *G. bimaculatus* (Koch and Hoffmann, 1985; Klein *et al.*, 1993). Rates of JH biosynthesis by *G. bimaculatus* were presumably measured during the photophase and diel variation in JH biosynthesis was not investigated. The existence of similar diel variation in the rate of JH biosynthesis in both male and female *G. firmus*, (Figure 2), suggests that both sexes also exhibit a corresponding morph-specific daily cycle in the JH titer, which thus far has only been measured in females (Zhao and Zera, 2004). JH biosynthetic rates in a more distantly related cricket, *Teleogryllus commodus* (Ruegg *et al.*, 1986), a species which consists only of long-winged adults, were about 10-fold lower than those measured in the two *Gryllus* species. Interestingly, the study of Ruegg *et al.* (1986) is the only study other than the present one in which rates of JH biosynthesis were measured every few hours over a 24 h period. In contrast to the present study, no temporal cycle was observed, which could have been due to a variety of reasons. For example, Ruegg *et al.* (1986) used day 3 virgin females, in contrast to mated day 5–7 females which were used in the present study. In *G. firmus*, the daily cycle in the JH titer is barely perceptible on day 3, and the large-amplitude cycle begins on day 5 (Zhao and Zera, 2004). Thus, a diel cycle in JH biosynthesis may exist in adult *T. commodus* that are older than the young adults used by Reugg *et al.* (1986). Thusfar, morph-specific cycles of JH biosynthesis or the JH titer have only been reported in *G. firmus* (Zhao and Zera, 2004). However, we have recently measured a corresponding, large-amplitude diel cycle in the hemolymph JH titer in day-5 and older LW(f) males and females of a number of *Gryllus* species, either in lab populations, or in field populations bled in the field (A. J. Zera, Z. Zhao and Y. Mori, unpubl. data).

JH biosynthesis is thought to be one of, if not the most important, regulators of the hemolymph JH titer (Tobe and Stay, 1985; Feyereisen, 1985; Nijhout, 1994). A positive association between the hemolymph JH titer and *in vitro* rate of JH biosynthesis has been reported in several insect species, both non-polymorphic species (*e.g.* Tobe *et al.*, 1985; Couillaud *et al.*, 1985; Renucci *et al.*, 1990; Klein *et al.*, 1993; Scott *et al.*, 2001), as well as social insects exhibiting complex polymorphism (Rachinsky and Hartfelder,1990; Rachinsky *et al.*, 1990; Huang and Robinson, 1995). However, with few exceptions, correlations between the JH titer and *in vitro* rate of JH biosynthesis have been measured on a time scale of days. In addition to the present study, rapid parallel changes (within a few hours or less) in the *in vitro* rate of JH biosynthesis and the hemolymph JH titer have been observed in the burying beetle, *Nicrophorus orbicollis* (Trumbo *et al.*, 1995; Scott *et al.*, 2001). The rapid change in the JH titer in this species is thought to regulate behaviors associated with the discovery and processing of a carrion carcass. Similarly, Zera and Cisper (2001) speculated that the large-magnitude change in the JH titer in LW(f) *G. firmus* late in the photophase may regulate behaviors associated with nocturnal flight. Studies of Trumbo *et al.* (1995), and Scott *et al.* (2001), together with the present investigation, provide compelling evidence that rapid, large-magnitude, functionally important changes in the JH titer can occur via rapid modulation of the rate of JH biosynthesis.

A number of studies also have reported strong negative associations between the hemolymph JH titer and JHE activity, which suggests that JHE can be an impor-

tant regulator of the hemolymph JH titer. For example, Scott et al (2001), found that the rapid increase in the JH titer in adult *N. orbicollis* was correlated with a decrease in hemolymph JHE activity, in addition to an increase in JH biosynthesis. Similarly, JH titers and JHE activity are negatively correlated in adult mated vs. virgin *Heliothis virescens* (Ramaswamy *et al.*, 2000) and in reproductive vs. diapausing adult *Leptinotarsa decemlineata* (Kramer and de Kort, 1978; de Kort and Granger, 1996). JHE activities are elevated 3–6 fold in the LW(f) vs. the SW morph during the *last juvenile stadium* in *G. rubens* and *G. firmus*, and appear to be involved in the regulation of alternate morph development (Zera and Tiebel, 1989; Zera and Huang, 1999; reviewed in Zera, 2004). However, prior to the present study, no differences in JHE activity have been observed between *adult* morphs of either of these two species (Zera *et al.*, 1993; Zera and Huang, 1999). Like most other insect endocrine studies, the investigations of Zera *et al.* (1993) and Zera and Huang (1999) only measured JHE activities during the photophase.

JHE activity exhibited a low amplitude (0.5–1-fold change) parallel daily cycle in LW(f) and SW *G. firmus* (Figure 3, bottom panel) with activities rising during the late photophase and dropping during the early scotophase. The majority of this cycle was likely a passive consequence of the cyclic daily contraction (25–30% during the late photophase) and expansion in the hemolymph volume which occurs to a similar degree in both LW(f) and SW *G. firmus* (Zhao and Zera, 2004). However, over and above this parallel daily cycle, there was a significant elevation in JHE activity in LW(f) vs. SW females during the beginning of the scotophase, which was negatively correlated with the JH titer and which may be functionally important (Figure 3, top and bottom panels). That is, the significantly elevated JHE activity in LW(f) vs. SW females at the beginning of the scotophase may act in concert with the dramatic decrease in the rate of JH biosynthesis to cause the precipitous drop in the JH titer in LW(f) females. By contrast, JHE activity does not appear to be involved in the substantial increase in the JH titer in LW(f) females during the late photophase, since JHE activities rose in parallel in both morphs (Figure 3). The contribution of other aspects of JH degradation (*e.g.*, by JH-epoxide hydrolase; Roe and Venkatesh, 1990), sequestration, or excretion, to the daily cycle of the JH titer in LW(f) females has not been investigated in *G. firmus*.

An unexpected, but interesting, finding of the present study was the consistent dip in the rate of JH biosynthesis during the late photophase in SW females (Figure 2). This dip might be a homeostatic response to the expected modest rise in the JH titer during the late photophase, due to the 25–30% contraction of the hemolymph volume in both morphs during this time (Zhao and Zera, 2004; discussed above). A temporary reduction in the rate of JH biosynthesis might be important in stabilizing the hemolymph JH titer in the SW morph during the late photophase.

Although the phases of the diel cycles of JH biosynthesis and hemolymph JH titer in the LW(f) morph of *G. firmus* were synchronous, the amplitude of the cycle of JH biosynthesis (approx 1–2 fold change) was considerably less than that of the JH titer (ca. 10–20-fold change). This discrepancy could be due to the *in vitro* measure of JH biosynthetic rates not accurately corresponding to *in vivo* rates, as has been proposed by Bloch *et al.* (2000) for *Bombus terrestris*. Rate of JH biosynthesis in many insects, including crickets, is strongly influenced by fast-acting peptide regulators (allatostatins and allatotropins; Stay *et al.*, 1994; Stay, 2000; Neuhauser *et al.*, 1994; Lorenz and Hoffmann, 1995; Wicker, 1987). For example, Neuhauser *et al.* (1994) found that an exogenous allatostatin decreased the rate of JH biosynthesis in *G. bimaculatus* within 1.5 h after introduction into the *in vitro* assay, and that its effect completely wore off within 3 h of introduction. As is typical of many *in vitro* studies of JH biosynthesis, measurement of JH biosynthetic rates did not begin in our study until about 1–1.5 h after dissection of the first CA. This was due to the time period required to obtain a sufficient number of glands for assay (40–60 min), and the 45 min preincubation period, which was required because of the lag time in radiobiosynthesis of JH (see Methods). Thus, it is possible that the effects of endogenous regulators might have worn off to some extent before or during the assay.

In summary, we have identified a morph-specific daily cycle in the rate of JH biosynthesis that is tightly correlated with and is a likely major cause of a morph-specific daily cycle in the hemolymph JH titer in adult *G. firmus*. A morph-specific negative association between JHE activity and the JH titer during the early scotophase, may also contribute to the morph-specific decline in the JH titer. These results not only provide a proximate explanation for the daily cycle in the hemolymph JH titer that is restricted to a particular morph, they also set the stage for investigations to identify the morph-specific neurohormonal regulators of JH biosynthesis and JHE activity. Such studies will be important in identifying the endocrine mechanisms underlying morph specialization for flight vs. reproduction in wing polymorphic species. These neuroendocrine studies may also provide important insights into the proximate mechanisms by which a circadian clock regulates daily fluctuations in a hormone titer in an insect species (in a morph-specific manner), a fascinating but poorly understood area of insect endocrinology (Steele and Vafopoulou, 2002).

Finally, insect endocrinologists, especially those working on dispersal (*e.g.* phase-, wing-, or flight muscle-) polymorphisms, or on long-winged cricket spe-

cies, should determine whether the strong daily cycles in the JH titer and rate of JH biosynthesis that we have observed in *G. firmus* also occur in their experimental organism. The failure to identify such cycles can result in highly misleading inferences concerning causal relationships between endocrine and whole-organism traits (*e.g.*, JH titer or rate of JH biosynthesis and ovarian mass). For example, we observed a strong positive correlation between the JH titer and ovarian mass early in the photophase which changed to a strong negative correlation late in the photophase (Zera and Cisper, 2001).

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