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Increased juvenile hormone levels after long-duration flight in the grasshopper, *Melanoplus sanguinipes*

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Abstract

Although, in many insects, migration imposes a cost in terms of timing or amount of reproduction, in the migratory grasshopper *Melanoplus sanguinipes* performance of long-duration flight to voluntary cessation or exhaustion accelerates the onset of first reproduction and enhances reproductive success over the entire lifetime of the insect. Since juvenile hormone (JH) is involved in the control of reproduction in most species, we examined JH titer after long flight using a chiral selective radioimmunoassay. JH levels increased on days 5 and 8 in animals flown to exhaustion on day 4 but not in 1-h or non-flier controls. No difference was seen in the diel pattern of JH titer, but hemolymph samples were taken between 5 and 7 h after lights on. Treatment of grasshoppers with JH-III mimicked the effect of long-duration flight in the induction of early reproduction. The increased JH titer induced by performance of long-duration flight is thus at least one component of flight-enhanced reproduction.

To test the possibility that post-flight JH titer increases are caused by adipokinetic hormone (AKH) released during long flights, a series of injections of physiological doses of Lom-AKH I were given to unflown animals to simulate AKH release during long flight. This treatment had no effect on JH titers. Thus, although AKH is released during flight and controls lipid mobilization, it is not the factor responsible for increased JH titers after long-duration flight.

Keywords: Juvenile hormone; Titer determination; JH esterase; Radioimmunoassay; Migratory flight; Adipokinetic hormone

1. Introduction

It has often been observed that, in insects, development of the anatomical structures and physiological mechanisms for flight and/or the utilization of energy reserves in performance of migration are correlated with reduced egg production and delayed age at first reproduction (Dingle, 1996; Zera and Denno, 1997; Zera and Harshman, 2001), suggesting that there is a significant reproductive "cost" to being a migrant. It is also often the case that migration is restricted to the post-teneral, pre-reproductive or inter-oviposition period in insects, and once significant ovarian development has occurred, the tendency to make migratory flight is greatly reduced or absent ("oogenesis-flight syndrome", Johnson, 1969), implying that migration and reproduction are alternate physiological states.

However, for migrant colonizers or highly dispersive species, natural selection might be expected to favour individuals that are able to begin reproductive development while dispersing so that they might begin reproducing immediately upon arrival. An insect colonist might be expected to display a suite of life history characters that involve both adaptations for long flight *and* rapid and prolific reproduction (Lewontin, 1965; Palmer, 1985; Rankin and Burchsted, 1992). Indeed, in some insects, long-duration flight has no observed deleterious effect on reproduction and even *stimulates* it in a few species (Rankin and Burchsted, 1992; Highnam and Haskell, 1964). However, this is rare, and the underlying mechanisms by which long-distance flight stimulates reproduction are not understood in any species.

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Melanoplus sanguinipes, a grasshopper that can be a highly mobile and successful colonist and a very serious pest of rangeland and crops, is one such species (McAnelly and Rankin, 1986; Rankin and Burchsted, 1992). Indeed, among the offspring of field-collected animals from the desert southwestern US, the performance of even one long, tethered flight to exhaustion (i.e., voluntary cessation) accelerates reproduction and enhances reproductive success over the entire lifetime of the insect. This paper investigates the mechanism by which performance of migratory flight, clearly an energetically demanding behavior, could accelerate and enhance rather than delay and diminish reproduction in this species.

One possible link between migration and reproduction may be juvenile hormone (JH), an important control factor in insect reproduction (Engelmann, 1970) and migration (Rankin, 1980). There is evidence that JH production is cyclical in acridids including *M. sanguinipes*, and the level of JH may act as an on/off switch in the process of oocyte maturation and oviposition (Wyatt et al., 1996; McCaffery and McCaffery, 1983). In locusts, cyclical changes in the volume of the corpora allata (CA) are associated with oocyte growth and also affected by flight activity (Highnam and Haskell, 1964). We, therefore, hypothesized that the enhanced reproduction observed in *M. sanguinipes* after migration could be caused in part by a flight-induced change in JH titer. Titer determinations were done using a well-established, chiral selective radioimmunoassay (RIA) (Huang et al., 1994; Hunnicutt et al., 1989) using hemolymph collected between 5 and 7 h after lights on. We also checked to determine whether there was a diel periodicity to JH secretion because we thought it possible that flight performance might change that periodicity if a rhythm existed.

2. Materials and methods

2.1. Grasshopper collection and rearing

M. sanguinipes adults used for this study were reared as described previously (Kent et al., 1997). The adults were the first-generation offspring of grasshoppers collected on the San Carlos Apace Indian Reservation in Arizona.

2.2. Flight assay and hemolymph collection

Grasshoppers were evaluated for flight propensity using the approach of McAnelly and Rankin (1986). Briefly, a small stick was attached to the pronotum of each grasshopper with wax, and the insect was then suspended in front of a fan. The fan, an electric heater, and incandescent lamps were used to simulate the conditions of wind speed, illumination, and temperature associated with migratory flights in the field (Parker et al., 1955). On day 4 after emergence, grasshoppers were either flown for 1 h and stopped or flown to voluntary cessation, here termed exhaustion. All hemolymph samples were taken between noon and 2 PM to control for possible circadian changes in JH titer. Hemolymph was collected by puncturing the dorsal neck membrane between the head and pronotum. Hemolymph (5 μl) bleeding freely from the wound was collected with a graduated glass micropipette.

In tethered flight tests *M. sanguinipes*, like a number of other insect migrants, fly for only a few moments or for several hours at a time. Thus, if an individual flies at least 60 min in a single flight test, it is very likely to fly much longer. One can, therefore, classify 1-h fliers as migrants (McAnelly, 1985) in terms of behavioral proclivity without actually allowing the animals to perform flights to "exhaustion". Three 60-min flight tests on succeeding days, can reliably distinguish all migrants from non-migrants in a population (Kent and Rankin, 2001), but these 1-h flights do not elicit the accelerated or enhanced reproduction seen after flights to exhaustion. By comparing animals that have made 1-h flight tests with those that have flown to exhaustion (i.e., voluntary cessation) and those that have refused to make a long flight, we can compare characteristics of migrants with non-migrants, and among the migrants we can distinguish the effects of performance of flight from the characteristics of migrants generally. Thus, in the experiments described herein, animals that had actually *performed* flight to exhaustion were compared with animals that had made a 1-h flight, and therefore could be identified as migrants, as well as with those of non-migrant controls.

2.3. Radioimmunoassay (RIA) for JH-III

Hemolymph samples were immediately added to 0.5 ml acetonitrile. 1.0 ml of 0.9% NaCl was added, and the sample was extracted twice with 1.0 ml hexane. The pooled hexane fractions were stored at 80 °C until analyzed by radioimmunoassay (Huang et al., 1994; Hunnicutt et al., 1989). At the time of analysis, we dried each hexane extract and re-suspended it in 20 μl methanol. About 4000–5000 dpm [^{3}H] JH-III was added to each RIA tube in 100 μl gel-PBST (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; 0.1% gelatin; 0.01% Triton X-100; pH 7.2). Then, 4 μl of the methanol re-suspension of an unknown sample was added to duplicate assay tubes. Next, 100 μl of a rabbit anti-10R-JH-III serum (1:16,000) diluted in gel-PBST was added to each assay tube. The assay tubes were vortexed and then incubated for 2 h at room temperature. After the incubation period, the samples were chilled on ice for 5 min and then 0.5 ml of cold, stirred dextran-coated charcoal (5 mg dextran; 5.6 mg

EDTA; 0.2 mg sodium azide; 1.0 ml PBS; 100 mg charcoal; 23 ml dH_2O) was added to each tube. The samples were incubated for 5 min and then centrifuged at 2000*g* at 4 °C for 5 min. The supernatant (containing [^{3}H] JH-III bound to antibody but no free [^{3}H] JH) was poured into a scintillation vial and counted in a liquid scintillation counter. The radioactivity in each unknown sample was compared to a standard curve to determine the amount of JH-III in the sample.

Methanol dilutions of racemic JH-III (Sigma Chemical Co., St. Louis, MO, USA) ranging from 0 (=total bound) to 3000 pg were used to generate a standard curve; radioactivity from each sample was expressed as a percentage of total bound (=% TB). The standard curve was plotted as log JH versus % TB and fit to a quartic equation used to estimate the JH levels in each experimental sample. Because this assay is chiral selective, results are expressed as ng 10R-JH-III/ml hemolymph; detection limit was approximately 5 ng 10R-JH-III/ml hemolymph.

2.4. RIA validation

Small amounts (about 5000 dpm) of [^{3}H] JH-III were added to hexane extracts of hemolymph samples ($n = 4$). Half of each sample was fractionated by normal phase high performance chromatography (HPLC) as described by Trumbo et al. (1995). One-minute fractions were collected, dried, and re-suspended in 20 μl methanol. An aliquot of each fraction was analyzed for radioactivity to determine the recovery of JH-III. Another aliquot was analyzed for immunoreactivity using RIA. Similar aliquots of unfractionated half of each sample were also analyzed for radioactivity and immunoreactivity. The amount of [^{3}H] JH-III (added to monitor recovery) present in each sample was taken into account when calculating the amount of JH. The amount of immunoreactivity detected in the HPLC fractions of each extract was similar to the amount present in the unfractionated material ($109 \pm 19\%$, $n = 4$). In all samples, immunoreactive material was only detected in two HPLC fractions that also contained [^{3}H] JH-III.

2.5. JHE assay method

Juvenile hormone esterase assay was performed by the partition method of Hammock and Sparks (1977) as modified by Lefevere (1989). The substrate [^{3}H] JH-III was dissolved in ethanol and stored at −20 °C prior to assay. 0.5 μl hemolymph samples were collected on sequential days post-flight and diluted in phosphate buffer pH 7.4. JH-III was added to a final concentration of 5×10^{-6} M and the solution incubated at 30 °C for 15 min.

2.6. JH-III treatment and oviposition check

JH-III (NEN) was dissolved in MeOH to make final concentration of 50 μg/μl. One microliter of methanol with or without JH-III was applied to the abdominal sternites of female grasshoppers on days 5 and 8. After the second topical application of JH-III on day 8, each female was mated with two 8-day-old males. Each trio was placed in an individual cage with a sand cup for oviposition that was examined daily for egg pods.

2.7. AKH I injections

On day 4 after eclosion females received 2 μl of synthetic Lom-AKH I (Peninsula Labs) at a concentration of 0.25 pmol/μl in distilled water at time zero ($T = 0$) and then 1 μl of the AKH solutions at 1-h intervals for the next 6 h to simulate AKH release during flight (Min et al., 2004). Hemolymph samples were collected on days 5 and 8 after eclosion and analyzed by RIA for JH titer.

3. Results

3.1. Diel JH titers

Even though sample collection times were controlled to minimize the effect of any diel periodicity of JH production, we thought it necessary to check for such a rhythm. Thus, hemolymph samples were collected on day 5 after eclosion in the morning (9–10 AM, 2 h after light on), late afternoon (5–6 PM) and late night (1–2 AM, 2 h after light off) to check for any possible diel rhythm of JH titers. There was no significant difference in JH titer with time of hemolymph collection ($F_{(2,23)} = 1.45$, one-way ANOVA $p = 0.25$, Table 1).

3.2. JH titers after long flight

JH titers of unflown grasshoppers were quite stable, and were in the range of 10–20 ng/ml from day 5 to day 15 after emergence (Fig. 1A). Similarly, animals that had been identified as migrants via a 1-h flight test, but had not made a flight to exhaustion showed no significant difference in JH titer when compared with unflown controls.

In contrast, animals that had made a flight to exhaustion (total duration: 5–12 h) on day 4 after eclosion had elevated JH titers on day 5 (30.9 ± 6.5 ng/ml, mean ± S.E.; $F_{(2.20)} = 4.40$, one-way ANOVA $p = 0.026$) above those values observed in unflown controls

Table 1
Hemolymph JH titers (ng/ml) by time of collection (mean ± S.E., $N = 8$)

Collection time	9–10 AM	5–6 PM	1–2 AM
JH titer	10.1 ± 1.8	14.6 ± 3.1	9.6 ± 1.7

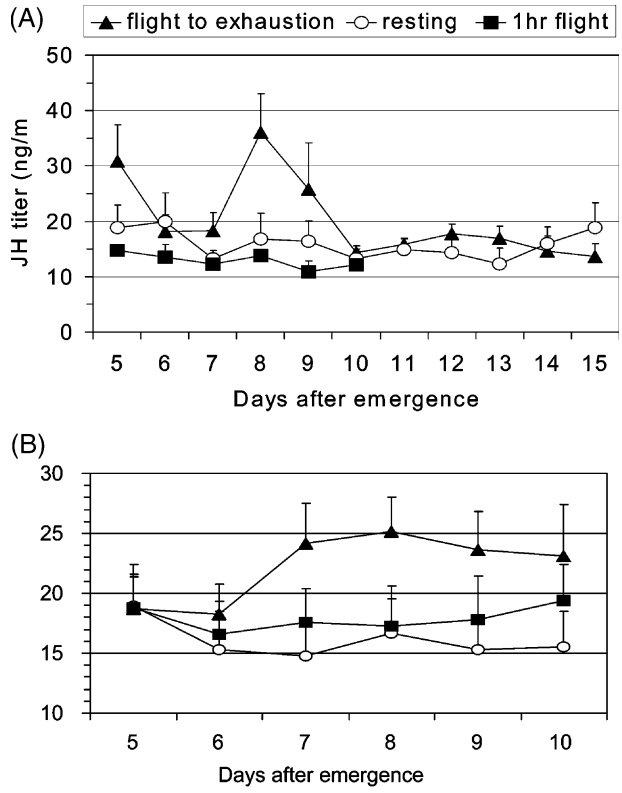

Fig. 1. Long-duration flights cause an increase in hemolymph JH levels. (A) JH titer after long flight. Animals were flown to exhaustion on day 4 and bled from days 5 to 15 after eclosion. The animals were divided into three groups. Individuals from each group were bled once every three days to reduce the effect of repeated bleeding. $N = 6$ each group. (B) JH esterase determination (as nM of JH-III converted to JH acid/min/ml) after long flight performed on day 4. Same legend as (A). $N = 6$ for flown to exhaustion, 9 for 1-h controls and 12 for unflown controls. For both experiments, controls were animals that had not been flight tested and those that made only a 1-h flight. Each point represents the mean ± S.E.

(18.8 ± 4.1 ng/ml) and 1-h flier controls (14.7± 0.9 ng/ml) and again on day 8 (36 ± 6.9 ng/ml; $F_{(2.13)} = 7.89$, one-way ANOVA $p = 0.006$) above those observed in controls (16.8 ± 4.63 ng/ml) and 1-h flier controls (13.8 ± 0.7 ng/ml).

3.3. JH esterase activity

JH esterase (JHE) levels (Fig. 1B) were elevated on day 7 in animals that had flown to exhaustion on day 4 and remained higher than control levels throughout the remainder of the experiment, but it was significantly different only on day 7 ($F_{(1,14)} = 6.51$, one-way ANOVA $p = 0.023$) (JHE and JH titer determinations were made in different groups of animals).

3.4. Correlation between increased JH-III levels and early oviposition

To determine whether the rise in JH titer after flight actually causes or is merely correlated with flight-enhanced reproduction, 50 μg JH-III was topically applied in 1 μl methanol on days 5 and 8, to mimic the increase in JH that follows long-duration flight. JH-III was used in preference to a JH mimic such as methoprene because we wanted to induce transient increases in JH similar to those observed after flight. When hemolymph levels of JH-III were checked after topical applications, it was clear that application of 50 μg JH-III increased JH titer, though not to the extent observed after a long-duration flight (Table 2). Nevertheless, oviposition occurred earlier in the treated than in the control group ($F_{(1.10)} = 11.78$, one-way ANOVA $p < 0.01$) and indeed the timing was similar to that observed in animals that had performed a flight to exhaustion (Fig. 2).

3.5. Effect of AKH on JH titer

AKH levels are elevated during long flight in *M. sanguinipes* (Min et al., 2004) and might conceivably be the stimulus for increased JH production after flight. Thus, we tested the hypothesis that the elevation of AKH during flight might affect subsequent JH production by the CA. Unflown grasshoppers were treated with repeated injections of 0.5 and then 0.25 pmol AKH, a physiological dose as determined by AKH radioimmunoassay (Min et al., 2004) for 6 h on day 4 to mimic the AKH levels in animals during a long

Table 2
Hemolymph JH titers (ng/ml) after topical application of 50 μg JH-III in methanol on day 5 (mean ± S.E., N= 5 each group)

Treatments	$T = 0$ h	$T = 12$ h	$T = 24$ h
MeOH	7.0 ± 1.0	8.2 ± 2.4	8.7 ± 1.7
50 μg JH-III	7.1 ± 1.1	19.4 ± 2.6	12.7 ± 1.8

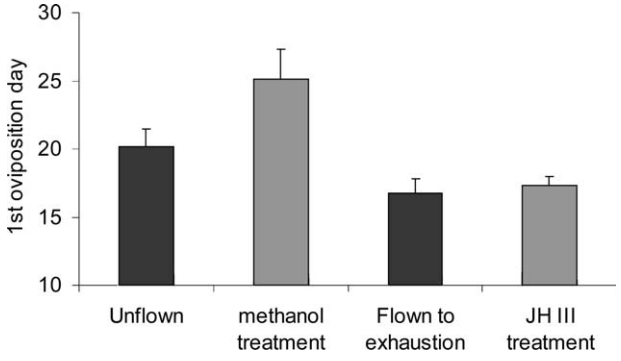

Fig. 2. Change in age at first oviposition after JH treatment. Topical application of 50 μg JH-III induced early oviposition as did flight to exhaustion. $N = 10$ for unflown and flown to exhaustion groups and 6 for each treatment group. JH-III was used rather than the JH mimic, methoprene, because we were hoping to achieve a transient JH peak as that which occurs after flight, using the natural hormone that could be degraded by JHE.

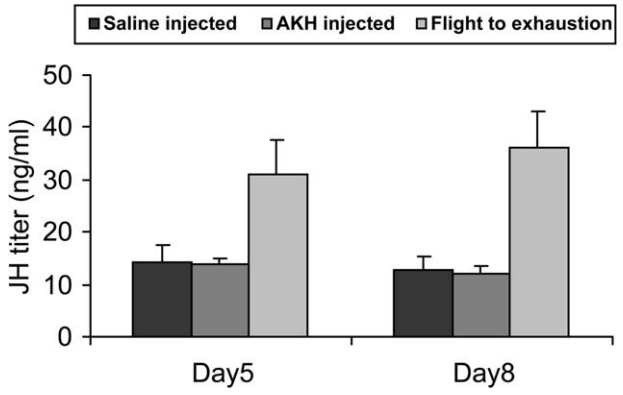

Fig. 3. Hemolymph JH titers after treatment with physiological doses of AKH I. 0.5 or 0.25 pmol Lom-AKH I was injected for 6 h at 1-h intervals to simulate the rise in AKH observed during a long-duration flight on day 4 after eclosion. Sample size: 9 for saline-injected group and 12 for AKH I injected group. Error bars are S.E.

flight. JH titers were measured on days 5 and 8 in saline-injected animals, AKH-injected animals, and animals flown to exhaustion. AKH treatment did *not* induce the JH titer increase seen after flight to exhaustion (Fig. 3) nor did it significantly affect the age at oviposition ($F_{(1,10)} = 0.76$, one-way ANOVA $p = 0.4$, data not shown).

4. Discussion

The RIA used in this study has successfully been employed to measure JH levels from bees (Huang et al., 1994), beetles (Trumbo et al., 1995), crickets (Cisper et al., 2000) and lubber grasshoppers (Hatle et al., 2000), and has been validated against two other JH-RIAs (Goodman et al., 1990; Strambi et al., 1981) that have, in turn, been validated against gas-chromatographic/mass spectrophotometric (GC/MS) JH titer determinations (Huang et al., 1994). In *M. sanguinipes*, it delivered consistent results in both control and treated animals. The JH titer of unflown animals was in the range of 10–20 pg/μl from day 5 to day 15. After flight to voluntary cessation, JH titers reached 30 or 36 pg/μl on day 5 and day 8, and were significantly higher than those observed in unflown controls or 1-h fliers. The JH titer of *M. sanguinipes* at rest or after a 1-h flight is similar to that reported for *L. migratoria* (4–30 pg/μl) (Dale and Tobe, 1986). Long-duration flight, on the other hand, results in two transient increases in JH titers. This is the first report that JH titer is altered after long-duration of flight. Furthermore, when unflown grasshoppers were treated with topical applications of JH-III in methanol, we observed a decrease in age at first reproduction that was similar to that observed after a long flight. Together these observations indicate that the increased JH titer that occurs after long-duration flight is at least one component of flight-induced enhancement of reproduction in this species.

Although we did not see significant changes in diel rhythm of JH titer, our sampling schedule (collected every 6 h) used in this study might not have picked up a diel cycle of short duration. Zhao and Zera (2004) have shown that there is a large-amplitude JH titer cycle in the cricket *G. firmus* that is short duration (4 h). Indeed, the titer *in M. sanguinipes* was about 50% higher at 5–6 PM compared with 9–10 AM. It is thus possible that *M. sanguinipes* has a diel cycle of JH titer that our sampling times missed.

The fact that JH esterase levels seem to be somewhat elevated after long flight is also interesting. The timing of the JHE increase in long fliers suggests that it may be induced by the first JH peak on day 5, but we have not tested that possibility. Whether or not JHE is playing a role in JH homeostasis after flight is not clear from our data, but it is tempting to suggest that it may return JH titers to lower levels after the second peak. More work is necessary to look at long-term effects of flight on JH titers and JHE levels.

After exogenous application of JH, we actually measured the induced in vivo JH titer change. This study showed that the artificially enhanced JH titer was physiological and was sufficient to accelerate the day of first oviposition. This combination of titer-manipulation/titer determination has not often been used in physiological–ecological studies in insects and is more powerful than the more common practice of simply applying or injecting hormone and observing its effects without any certain knowledge of how the exogenous hormone has affected endogenous hormone levels.

The locust adipokinetic hormones (AKHs—at least three have been isolated) are members of a family of short peptides with similar amino acid sequences that have various metabolic functions in different insects (Van der Horst et al., 2001). In locusts and in *M. sanguinipes*, AKH I is responsible for lipid mobilization during flight (Kent et al., 1997). The AKHs are produced in the glandular lobes of the CC, a neurohemal/endocrine gland that both stores brain neurosecretions and produces neurohormone.

AKH levels are elevated during long flight in *M. sanguinipes* (Min et al., 2004) and might conceivably be the stimulus for increased JH production after flight. The amount of AKH I in *M. sanguinipes* hemolymph after 30 min flight is approximately 0.3 pmol (Min et al., 2004). To mimic the AKH experience during flight, we first injected 0.5 pmol Lom-AKH I that is identical with *M. sanguinipes* AKH I (Taub-Montemayor et al., 2002). The half-life of AKH I in resting locusts has been calculated to be 53 min (Oudejans et al., 1996). Assuming that the half-life of AKH I in *M. sanguinipes* is similar to that of the locust, the amount of AKH I that remains 1 h after the first injec-

tion (0.5 pmol) will be about 0.25 pmol. The degraded amount of the peptide in an hour was supplemented with hourly injection of 0.25 pmol AKH I. These treatments had no effect on JH titer or age of first oviposition. Thus, it seems that flight-induced increased JH levels are *not* caused by the release of AKH during flight.

In *M. sanguinipes*, as in locusts, JH, produced by the CA, is necessary for reproductive development. Either cautery of the MNSC or removal of the CA prevents vitellogenesis. It appears that the MNSC are necessary for general protein synthesis, that both the CA and these cells are necessary for synthesis of vitellogenin, and that the CA are necessary for the oocyte–follicle cell complex to become competent to sequester yolk (Gillott and Elliott, 1976). The specific effect of JH on the *M. sanguinipes* ovary has not been examined as yet, but in *L. migratoria* JH is required for patency (withdrawal of the follicle cells from the ovarian surface that allows uptake of Vg and other hemolymph proteins to proceed (Davey et al., 1993). As in locusts, a peak of JH synthesis occurs in *M. sanguinipes* with the onset of previtellogenic growth in each cycle of oocyte development. A decline in JH synthesis is correlated with onset of oviposition (McCaffery and McCaffery, 1983) (note that this work was done with a non-diapausing lab strain of *M. sanguinipes* in which onset of reproduction was much earlier than in our field-derived animals). Increasing JH levels at key times in the ovarian cycle could have a major impact on timing of oogenesis, and thus we believe that the increases in JH titers after performance of long flight are a critical part of the phenomenon of flight-enhanced reproduction. At the same time, it is unlikely to be the whole story. Little is known about control of JH production in *M. sanguinipes* or the occurrence of neurohormones affecting the ovary directly. However, in *L. migratoria* (Girardie et al., 1991) and in *S. gregaria* (Girardie et al., 1998) a protein produced by the pars intercerebralis called ovary maturating parsin (Lom/Scg-OMP) appears to act directly on the ovarian follicle cells to induce production of 20-hydroxyecdysone (20HE) which in turn stimulates fat body production of Vg even in the absence of JH (Girardie et al., 1991, 1998; Girardie and Girardie, 1996). Furthermore, in *L. migratoria*, increase in size of the CA occurs after flight on a roundabout (Highnam and Haskell, 1964) and there is some evidence that neurosecretory cells (BNCS) in the locust brain release material during flight. The CA are known to be under control of the brain or brain neurohormones in most insects, thus a key link between flight and reproduction may involve control of one or more neuroendocrine factors from the BNSC and possibly a reprogramming of the brain-CA system. These possibilities will be the subject of future investigations.

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