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Influence of juvenile hormone on territorial and aggressive behavior in the Painted Lady (Vanessa cardui) and Eastern Black Swallowtail (Papilio polyxenes)

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The Influence of Juvenile Hormone on Territorial and Aggressive Behavior in the Painted Lady (*Vanessa cardui*)and Eastern Black Swallowtail (*Papilio polyxenes*)

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by

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Abstract

Competition is important in environments with limited resources. Males of many insect species are territorial and will defend resources, such as a food source or egg-laying site, against intruders, or even compete to attract a mate. In insects, evidence suggests that juvenile hormone acts as an aggression mediator, much like testosterone in other animal species. In this study I tested this idea using a group of male Painted Lady butterflies, *Vanessa cardui*, and Eastern Black Swallowtail butterflies, *Papilio polyxenes*, that were treated prior to metamorphosis with either a high or low dose of methoprene acid (a juvenile hormone mimic). Male-male encounters were staged with treated and untreated adults or males treated with a high dose or low dose to observe effects of hormone augmentation on aggression and territoriality. Hemolymph was sampled from larvae and analyzed for JH content using gas chromatography. In each encounter a female was positioned to witness the encounter; after a winner was determined, she was released and allowed to choose a male. Male *V. cardui* treated with either a high or low dose of methoprene were significantly more likely to win an encounter over a control male, and males given a high dose of methoprene were more likely to win an encounter over a male given a low dose. This result suggests that juvenile hormone influences the aggressive potential of male *V. cardui* butterflies. High mortality among adult *P. polyxenes* limited the experimental data collection for that species. Finally, females were not interested in males of any dosage and showed no preference. This suggests that females of *V. cardui* are not choosing males based on JH level or perhaps not in captivity.

Introduction

Aggressive and territorial behaviors are exhibited by many different animals, and are characteristic behaviors in environments with limited resources. Food resources are particularly important for energy and reproduction, and individuals often compete for the available resources. Males of many insect species are territorial and will defend food sources or egg-laying sites against intruders. For example, males of the speckled wood butterfly will compete for small patches of sunlight on the forest floor, where they may encounter receptive females (Alcock, 2005). Successful residents then have ready access to food for themselves as well as control over resources for potential mates and offspring. Males of many species may also compete to attract and monopolize a mate, and as suggested by the theory of sexual selection, the outcome of male-male encounters in many species is an indicator of which animal is the fitter mate (Lederhouse, 1978). For example, Lederhouse (1982) showed that male butterflies chased other males away from a perch and mated repeatedly at that perch site. Females judged the quality of the male by the quality of his territory and preferentially mated with those males occupying the higher quality territory (Lederhouse, 1978).

To facilitate these competitive interactions, males of many species have evolved large body size or weapons, such as horns or tusks, as an outward show of their ability to win these encounters, and an indication of their subsequent "fitness" as a mate. For example, both large body size and large horn size are important in determining contest outcome in male dung beetles, *Euoniticellus intermedius* (Pomfret and Knell, 2006). Sexual selection is the process proposed by Darwin (1871) to explain the evolutionary acquisition of these traits which do not lend any advantage to survival, but rather give one individual an advantage over another member of the same sex in terms of mating success. In species that lack dimorphic size or weaponry, however,

males often resort to prolonged passive displays, termed wars of attrition, to indicate their fitness and determine the outcome of a conflict between two competing males (Takeuchi, 2006). A war of attrition is a tactic used by males to assess the strength and fitness of another before engaging in any potentially costly physical encounter. Fighting ability is revealed through behaviors performed in a series of steps, beginning with low-risk behavior such as display of weapons, and if necessary, proceeds to higher-cost behaviors (Kelly, 2006). For example, in Fiddler Crabs (*Uca pugilator*), males will engage in war-of-attrition encounters in order to determine who will gain residence in breeding burrows; they begin with extending their claws towards one another, lunging at each other, and finally interlacing claws and tossing the other crab on its back (Pratt et al., 2003).

Males of many butterfly species engage in war-of-attrition encounters, and several species are known to exhibit territorial behavior. For example males of the Painted Lady (*Vanessa cardui*) and the Eastern Black Swallowtail (*Papilio polyxenes*) will engage other males of their species in conspicuous aerial maneuvers when competing for a resource or mating site (Lederhouse 1978). Since they have virtually no means of forcing another male to leave their territory, simple persistence and endurance is often the determining factor in outcome (Kemp and Wicklund, 2001). Encounters are generally initiated when a resident male rises to engage another male encroaching on his perceived territory. A series of escalating maneuvers typically follows. First, males pursue each other in a circular formation, followed by horizontal and vertical chases or hovering flights, lasting 10 minutes or more. If after this point, a male has not been driven off, both males will engage in ascending or non-ascending spiraling flights, followed by horizontal chases resulting in one male being chased from the disputed territory (Kemp and Wicklund, 2001). Kemp (2000) demonstrated that the spiraling flights represented the

component of encounters that was most likely to decide the outcome. Recent experiments have thus shown that aggressive behaviors are associated with territoriality and resource defense in butterflies (Kemp, 2000; Takeuchi, 2006; Kemp et. al, 2006a, b), but little is known about whether aggression and territorial behavior in butterflies is mediated by hormones, as it is in other insects such as the the honey bee, *Apis mellifera* (Pearce et. al, 2001), and the burying beetle, *Nicrophorus orbicollis* (Scott, 2006a). In addition, female choice for male butterflies may be largely influenced by witnessing the outcomes of these encounters.

Female butterflies, unlike males in most species, may only get a few chances to mate in their lifetime (Rutowski, 1998), and so must be choosy in their selection of a mate. In some species, such as those in the heliconiid family, the female mates as soon as she emerges from her chrysalis. Competition for females is intense, so males will often remain close to and defend a chrysalis to monopolize that female and mate with her immediately upon eclosion (Lederhouse, 1978). In other species female-locating is categorized as perching, as exhibited by male *Vanessa cardui,* and patrolling, as exhibited by male *Papilio polyxenes* (Lederhouse 1978). A perching male will sit and wait at a strategic vantage point such as the top of a hill, flying only to inspect females coming within his field of vision or to chase away potential rivals for territory. Patrolling males, on the other hand, spend almost their entire adult lives flying in search of females. In many species, including both *V. cardui* and *P. polyxenes*, males will collect on hilltops to wait for receptive females, termed hilltopping, and then form leks that females visit for the sole purpose of mating (Lederhouse, 1978; Boggs et. al., 2003). In *P. polyxenes*, however, the lek system differs from the traditional form in which males perform courtship displays in competition for females. Instead, *P. polyxenes* females mate with males that defend particular neighboring territories (Lederhouse 1978). Courtship displays precede copulation; during these

displays, males will rub pheromones on the females' antennae to entice them to mate. However, unreceptive females will attempt to discourage the suitor by flapping their wings rapidly if perched, or by fleeing in a rapid vertical flight. It is not clear at all, though, what element influences females to reject these males.

Studies have shown that both chemical and visual signals from male butterflies entice females to mate. Pheromones produced by males serve as cues for the females about the health of the male, the quality of the male's diet (Dussourd et al., 1991; Iyengar et al., 2001), and the ability to provide chemical protection for eggs against predators (Dussourd et al., 1988). For example, the mating pheromone of male Queen butterflies (*Danaus gillipus*) is produced only after the male has fed on certain plants (Rutowski, 1998), and the moth *Utetheisa ornatrix* produces a more attractive pheromone if it can transfer more protective chemical to the female during mating (Dussord et al., 1988, 1991).

Visual signals can also indicate fitness of a male. Bright color in butterflies is often an indicator of overall health or age; the coloring of males tends to fade with age, particularly in the ultraviolet spectrum due to normal wear-and-tear of wing scales (Rutowski, 1998). Size is also an indicator of fitness, as larger males can produce larger spermatophores with which the female can fertilize her eggs (Iyengar et. al, 2001), and females can selectively use sperm from larger males even when they have mated multiple males before laying eggs (LaMunyon and Eisner, 1993). In stalk-eyed flies, males with wider eye-spans are better competitors in male-male encounters (Panhuis and Wilkinson, 1999) and are preferred by females over males with smaller eye-spans (Wilkinson and Reillo, 1994).

In addition to physiological and morphological characters, females may also rely on aggressive and territorial behaviors. Very little is known about the influence of territorial or

aggressive behavior on mating success in insects, but in other organisms, hormones such as testosterone are often linked with male aggression, strength, and overall mate fitness which females find attractive (Roney, 2006). In humans, testosterone levels in males has been shown to correlate with the physical features that women consider attractive, with a male's immunocompetence, and with his interest in parental care (Roney, 2006). In the Dark-eyed Junco (*Junco hyemalis)*, testosterone levels mediate the intensity and frequency of courtship displays, and females prefer those males with higher testosterone and more elaborate displays (Enstrom et. al, 1997). This suggests that hormones play a role in determining the physical attractiveness of a male and his ability to provide for his offspring, either through parental care or through good genes.

Insects do not produce testosterone, but juvenile hormone (JH) mediates behavior in a similar way, as in the burying beetle, *Nicrophorus orbicollis*, where hormone levels are associated with increased parental care (Scott, 2006b) and aggressive behavior (Scott, 2006a). Juvenile hormone is also important in the molting process for arthropods that produce a rigid exoskeleton. The larva's epidermis produces a series of cuticles that differ with life-stage, determined by the concentration of JH within the insect's hemolymph (Riddiford and Truman, 1993). The presence of JH at particular levels and at critical periods of the life cycle prevents developmental switches between alternative pathways of development, prevents the initiation of pupation and metamorphosis, determines polymorphic traits (Nijhout, 1994), and stimulates the development of reproductive organs in adult insects (Gilbert et al., 2000). JH has also been shown to influence the development of secondary sexual characteristics: the treatment of stalk-eyed flies with the JH-mimic methoprene resulted in increased eye-span (Fry, 2006).

Juvenile hormones, five homologues of which have been identified in approximately 100 species, are produced by the corpora allata, paired ganglion-like organs in the head of an insect. After secretion by the corpora allata, JH binds to specific proteins called JH-binding proteins (JHBP), which transport JH and protect it from degradation because of its chemical instability and enzymes present in the hemolymph (Minakuchi and Riddiford, 2006). The synthesis of JH is controlled by the nervous system, in particular by neurosecretory hormones called allotropins, which stimulate the corpora allata, allostatins, which inhibit the production of JH by the corpora allata; and by a variety of neurotransmitters (Gilbert et al., 2000).

The molecular mechanism of action of juvenile hormone is rather complicated, and still largely unclear; at different stages of life it has both suppressive and stimulatory effects (Jones, 1995). However, the presence of JH in the hemolymph of a fifth instar larva, the last instar before pupation, inhibits the production of prothoracicotropic hormone (PTTH) (Minakuchi and Riddiford, 2006). This hormone stimulates ecdysteroid secretion to initiate metamorphosis. The combined effect of the cessation of JH secretion by the corpora allata and the breakdown of JH by JH-specific esterases stimulates the secretion of PTTH, although the mechanism by which these two events are coordinated remains unknown (Nijhout, 1994).

In addition to the effects JH has on development and metamorphosis of insects, JH has been shown to influence social interactions between insects, including aggressive behavior in the honey bee, *Apis mellifera* (Pearce et. al, 2001), and territorial and parental behavior in the burying beetle, *Nicrophorus orbicollis* (Scott, 2006a). Juvenile hormone may mediate territorial behavior as a form of courtship, especially if an encounter between males is witnessed by a female. This behavior may then reflect the fitness of a male as a mate, especially if we consider that the juvenile hormone transferred to the female at mating may give her offspring a greater

chance at survival. In some lepidopterans, such as the moth, *Heliothis virescens*, JH is a controlling factor in the development of reproductive organs, and it is also a component of the sperm package transferred to the female during mating (Wedell, 2005). This nuptial gift contains JH, along with nutrients, and in some cases a mating plug or chemical signal to prevent or discourage other males from mating with that female. These spermatophores can be as much as 6 to 10% of the male's body weight, thus males must insure their investment (Rutowski, 1998). Females can benefit greatly from the transfer of this gift. The transfer of JH caused a marked increase in oocyte development in female *Heliothis virescens* (Pszczolkowski et al., 2006) and *Chorthippus brunneus* (Butlin et al., 1986). Treatment with JH alone produced the same results in *Manduca sexta* (Sasaki and Riddiford, 1984), resulting in greater fecundity in all cases. Futhermore, a study by Doussourd et al. (1988) demonstrated that the spermatophore contained an alkaloid compound that actually provide protection for the eggs against predators.

If territorial and aggressive behaviors are correlated with JH in male insects, these behaviors may in fact reflect the ability of a male butterfly to provide a superior spermatophore to the female at mating, resulting in greater fecundity for that female. Thus, for species in which females can choose whether to mate or not, females may use the outcome of territorial battles between males to determine the most attractive mate, and one that will provide the greatest reproductive success. Because females of *P. polyxenes* have been shown to come to leks not to judge courtship displays, but rather to judge quality of male territory (Lederhouse, 1978), it is very likely that they would use outcome of territorial battles to determine who is the resident of a desirable territory and therefore the superior male.

In the first part of this study I tested the hypothesis that the level of aggression and territorial behavior in butterflies is influenced by variation in the amount of juvenile hormone in

the hemolymph prior to metamorphosis. I manipulated JH levels using the analogue methoprene. The inhibitory action of JH on metamorphosis and the fact that JH is only produced in insects and arthropods alone has led to recent study of JH and JH mimics in insecticides. As a pesticide, JH and its mimics bring with them the promise of few toxic effects on non-target species outside the arthropods (Minakuchi and Riddiford, 2006), and great effort has gone into developing stable JH analogues, including methoprene, pyriproxyfen, fenoxycarb, and juvabione, to be used as insect growth regulators. The JH mimic methoprene is used in lieu of juvenile hormone in this study because of its increased stability in the hemolymph, increased length of action, and the consistent ability to mimic the physiological actions of JH (Nijhout, 1994; Riddiford and Truman, 1993). After butterflies emerged, I staged male-male encounters and observed all behaviors and winners. In the second part of the study, I tested the hypothesis that such aggression, and perhaps the increased JH level, is reflected in reproductive success in terms of mate-choice by females that witnessed the encounter and chose a mate based on the outcome of the encounter.

I used two species, *Papilio polyxenes* and *Vanessa cardui*, to test the hypothesis that JH mediates territorial and aggressive behaviors. *Papilio polyxenes*, the Eastern Black Swallowtail, is a medium-sized butterfly in the family Papilionidae. It frequents meadows, fields and open forests from southern Canada throughout the eastern United States, south into Columbia, Ecuador and northern Peru. There are four subspecies of *P. polyxenes*, *P. asterius* being that most common throughout North America. *Larval P. polyxenes* larvae feed on many plants, including parsnip (*Pastinaca sativa*), hemlock (*Conium maculatum*), dill (*Anethum graveolens*), and parsley (*Petroselinum crispum*). Male *P. polyxenes* males exhibit territorial behaviors (Lederhouse, 1978). In addition to the unique lek behavior described above, wing-span and body

weight are not significant factors in determining male-male encounter outcome (Lederhouse, 1978). Thus, there is increased likelihood that hormones such as JH can have a significant influence on territorial outcome and mate choice, making *P. polyxenes* an ideal model for the study.

Vanesssa cardui, the Painted Lady, is a small butterfly in the family Nymphalidae. It is the most widely distributed butterfly in the world (Klein), found everywhere in the world except South America, Australia, and the Arctic. *Vanessa cardui* tend to inhabit bright, open environments, preferring clover fields, meadows, and hilly country, in addition to marshes and dunes (Harris, 1999). Larvae of *V. cardui* feed on many host-plants, including thistles (*Asteraceae spp.*), hollyhocks and mallows (Malvaceae *spp.*), and various legumes (Fabaceae *spp.*), preferring thistles as adults (Klein). Like *P. polyxenes*, *V. cardui* males display territorial behaviors, and most males win exclusive use of their defended areas through territorial battles (Harris, 1999). These traits make the species another ideal model for this study.

Materials and Methods

1. Experimental Animals

Larvae of the Eastern Black Swallowtail, *P. polyxenes*, were obtained from Shady Oak Butterfly Farm in Brooker, Florida. As recommended by Chris Hebdon (personal comm.), larvae were reared individually in plastic cups fitted with paper-cloth covers. Each cup contained a parsley stem as food for the larva. The paper-cloth cover was moistened each day to prevent desiccation of parsley and of larvae. New parsley was added as needed. Larvae of the painted lady, *Vanessa cardui*, were purchased in a culture set, along with food, from Carolina Biological Supply. They were reared individually in small cups with a nutrient mash until pupation.

Sex of larvae was determined according to their translucent pits, visible under a dissecting microscope, as described by Schaefer and Taylor, 2006. Females possess longitudinally-elongated, translucent spots on the underside of the eighth and ninth abdominal segments, which are absent in males.

A 12-hour light period beginning at 6:00 A.M. followed by a 12-hour dark period at 6:00 P.M. was used until pupation. At that point, the light cycle was switched to a 15-hour light cycle beginning at 6:00 A.M. to mimic the changing of seasons which brings about eclosion. Agro-Lite plant light bulbs (Phillips™, 60W) were used in the lamps to provide full spectrum lighting.

Upon pupation, chrysalises were removed from individual cups and pinned to the side of a mesh enclosure, which was divided to allow for identification of individuals upon eclosion. Pupae were lightly sprayed with water frequently to prevent desiccation. Larvae of the Painted Lady pupated in approximately 2 weeks, and eclosed after 1-2 weeks of pupation, while larvae of the Eastern Black Swallowtail pupated in approximately 1 month, and eclosed approximately 1 month after pupation.

Upon eclosion, a distinctive mark was made on the base of the underside of the forewing with a colored Sharpie marker to allow for individual identification. Males of *P. polyxenes* were identified based on color dimorphism, while in *V. cardui* males were identified based on narrower abdomen size.

Male and female adults were housed separately in 65-gallon mesh reptile/insect cages (Carolina Biological Supply). Encounters were staged in a 78x96x96" Butterfly Habitat (Educational Science Online Science and Nature Store).

2. Methoprene Treatments

Methoprene acid (#M6682, Sigma) was applied to *P. polyxenes* individuals on the second day of the last instar in dosages according to Fry (2006). Methoprene was applied to *V. cardui* individuals on the first day of the last instar. Treatments were given on the dorsal surface of the caterpillars. Large caterpillars (mass ≥1.00 g) received a dose of 1 μl of 0.298 mM (0.0926 μg/larva) in acetone, and small caterpillars (mass <0.99 g) received a dose of 1 μl of 0.0298 mM (0.00926 μg/larva) dissolved in acetone. Control individuals received 1μl of acetone.

3. Hemolymph Sampling

Hemolymph was drawn from *P. polyxenes* on the 5th day of the last instar, and from *V. cardui* on the 2nd day of the last instar. The posterior end of the caterpillar was restrained using a glass microscope slide, and a 30-gauge needle was used to prick the the dorsal epidermis of the 3rd segment from the anterior end of the caterpillar. A 5 μl capillary tube was used to draw 5μl of the hemolymph from the bubble formed on the surface of the caterpillar. Hemolymph was immediately mixed with 2% NaCl (5μl NaCl /μl hemolymph), and acetonitrile (5μl acetonitrile /μl hemolymph). Samples were frozen at -20°C until needed for JH assay.

4. Male-male Encounters

Male-male encounters were staged in a 75-gallon mesh enclosure containing a food source. A smaller mesh enclosure containing a female was set up within the larger enclosure to allow for female observation of the encounter. The winner of the encounter was determined to be the male that chased the other away in the horizontal flights characterized by Kemp and Wicklund (2001). If no clear winner was determined after 15 minutes, the encounter was declared a tie.

After the winner of the encounter was determined, or a tie was declared, the female was released from the small enclosure. She was allowed to choose a male, and both the male she went to first as well as the male she spent more of the next 15 minutes nearest was recorded. If the female displayed no interest in either male, she was considered as having no preference.

5. Hemolymph analysis

Hemolymph was analyzed as in Shu et. al (1997). Each sample, plus 0.0926 μg methoprene acid as an internal standard, was extracted three times with 500 μl hexane, shaken and centrifuged at 7000 rpm for 5 minutes for each extraction. The extracts were dried in a SpeedVac. Residues were re-suspended in 200 μl hexane. This solution for four individuals of the same treatment was pooled, and, along with two rinses of each vial (200 μl hexane each), were eluted through a 1μl void volume neutral aluminum oxide column with hexane, 10% diethyl ether in hexane (E/H) and 30% E/H. The 30% E/H fraction was dried in a SpeedVac. 75 μl d_4-methanol was then added to each sample pool followed by addition of 75μl 5% trifluoroacetic acid (TFA) in d_4-methanol (very slowly while shaking in a 60˚C water bath). The reaction mixture was then dried in a SpeedVac. Residues were re-suspended in 2μl hexane and subjected to GC-MS analysis. JH-II was monitored at mass to charge ratio (*m/z*) 90 and *m/z* 225.

Results

Due to high mortality in *Papilio polyxenes,* I was unable to conduct male encounter trials. In the one trial that was able to be completed, the high-dosed male won the encounter over the male given a low dose.

Male butterflies of *V. cardui* treated with high doses of methoprene were more aggressive and active than those that were not treated or those given a lower dose. Treated males were more

likely to display the behaviors associated with increased aggression, in particular beating wings against, walking on, and hopping towards the other male. Males given a lower dose of methoprene and untreated males were more likely to display social behaviors that were less aggressive, such as touching or 'exploring' the other male with antennae. Encounters rarely resulted in chasing, which is considered the most aggressive behavior of those observed during this study (Kemp and Wicklund, 2001) (Table 1). In addition, treated males were less timid in the enclosures and much more difficult to catch.

Male *V. cardui* treated with methoprene were significantly more likely to win encounters over males that were not treated (Binomial test, z-ratio=+1.55, p=0.042, Figure 1). Males that were treated with high doses of methoprene won more encounters over males given a low dose (Figure 2), although there was no significance (Binomial test, p=0.156)

Females exhibited no preference for either treated or untreated males, and showed no interest in males at all during the experiment. They usually remained motionless, went directly to the food source and began to feed, or flew in a direction that avoided either male.

Hemolymph analysis for juvenile hormone by gas chromatography was not able to be completed at this time due to equipment failure and time constraints.

Methoprene Treatment group	Average Behaviors per Male per Treatment				
	Beating wings	Walk over	Touch with antennae	'Hop' towards	Chase
High dose n=5	2.40	0.80	0.00	0.60	0.00
Low dose n=14	1.14	0.36	0.24	0.43	0.07
Untreated n=10	0.60	0.20	0.10	0.30	0.00

Table 1. Behaviors exhibited by different treatment groups of *V. cardui* butterflies during male-male encounters.
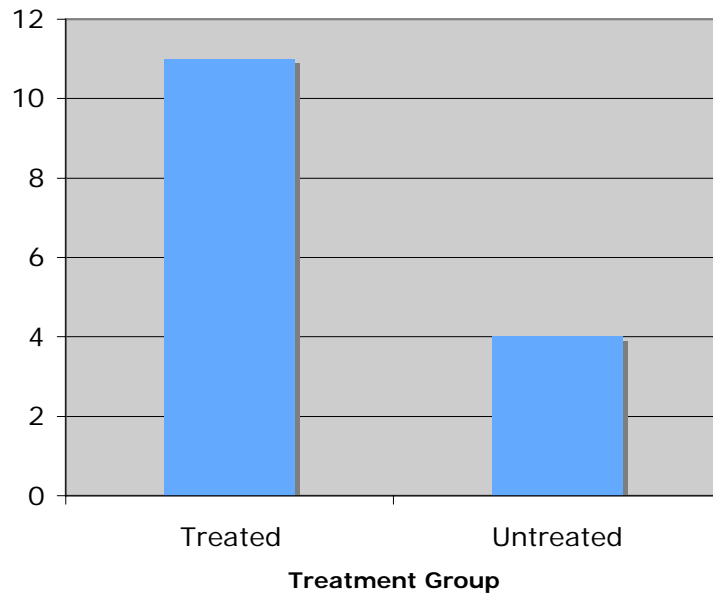
Figure 1. Outcome of male-male contests between male *V. cardui* treated with methoprene acid and untreated individuals, n=15.
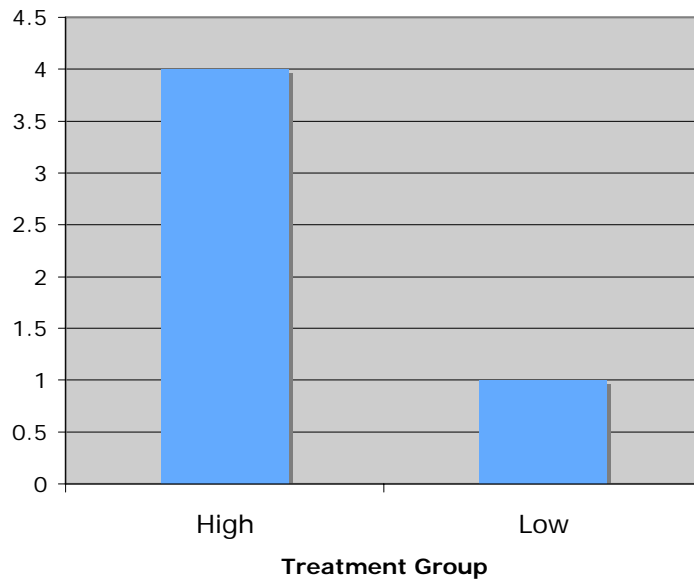

Figure 2. Outcome of male-male contests between individuals given high dose (0.0926µg/µl) and low dose (0.00926 µg/µl) of methoprene acid, n=5.

Discussion/Conclusions

The results clearly demonstrate that juvenile hormone is a chemical mediator of territorial and aggressive behavior in male butterflies, much in the same way that JH influences the aggressive and social behavior of the ant, *Myrmicaria eumenoides* (Lengyel et al., 2007) and aggressive and parental behavior in the burying beetle (Scott, 2006). Males were treated with two different doses of methoprene acid, a JH mimic. Treated males with methoprene were more likely to display increased aggressive behavior and won significantly more encounters over males that were not treated. Males that were treated with the high dose of methoprene were more likely to win encounters over males given the low dose, but a small sample size influenced the statistical difference of the latter. The outcomes of encounters clearly showed that males treated with higher doses of methoprene were dominant over those males given a low dose. This result supports the hypothesis that juvenile hormone acts as a chemical mediator of aggression in male *Vanessa cardui*.

It is interesting that even treated males rarely displayed the behaviors considered more aggressive, such as the elaborate aerial maneuvers described by Kemp and Wicklund (2001), or the active forcing of the subordinate male off of the territory in dispute. Rather, conflicts were often resolved with limited physical interaction between males. Males treated with higher doses were more likely to display more aggressive behaviors like batting wings, and males treated with a lower dose or untreated males were more likely to display more social and less aggressive behaviors such as touching the other butterfly with antennae. Butterflies represent an excellent model with which to observe war-of-attrition encounters like these. Their inability to inflict physical damage on one another without harming themselves has led to this graded aggression, which allows males to settle disputes with a minimum of physical costs.

The outcome of territorial battles is important in female mate choice in many animal species. Testosterone in many vertebrate animals increases aggressive behavior, which leads to increased frequency of mating behavior and greater mating success. For example, male Dark-eyed Juncos, *Junco hyemalis*, injected with testosterone, display more aggressive behaviors and more frequent and elaborate mating displays. Females preferred these males, and interestingly, mating success increased testosterone levels in males as a positive feedback (Enstrom et. al, 1997). Similarly, JH levels in Caribbean fruit flies (*Anastrepha suspensa*) are higher in males who have mated, and have been shown to increase sexual behavior and sex pheromone production and mating success, as these flies are also preferred by females (Teal et. al, 2000). However, the link between hormones, aggression, and mating has not been solidified in invertebrates.

Female butterflies in this study showed no interest in interacting with the males so I am unable to determine if females respond differently to males with different levels of JH. In the natural setting, females of *P. polyxenes* and *V. cardui* may not actively choose mates but rather make the choice of whether to mate with a male that approaches her. Perching males, like *Vanessa cardui,* wait for a female to enter his territory and fly out to her, while patrolling males, like *Papilio polyxenes,* actively search for mates throughout their adult lives (Lederhouse, 1978). Thus it makes sense that a female might only respond if mating is first initiated by the male. Since the males in this study did not approach any of the females, there were no opportunities for female choice. It is difficult to encourage experimental animals to mate with each other, so perhaps a better way to carry out this portion of the study would be to house a portion of treated and untreated males with females in the same enclosure and analyze the hemolymph of males

which are mated and rejected on their own. In this way, the study would perhaps more accurately reflect the dynamics of a natural mating situation in which mating behavior is not forced.

Future studies with larger sample sizes and a greater variety of dosage levels of methoprene as well as a wider range of species could perhaps give more definitive results as to the affect of JH on aggressive behavior in insects and allow the generalization of results to other species within the lepidopteran genera. In addition, a radioimmunoassay of the JH content in the hemolymph would provide a more reliable method of analyzing the hormone levels.

In conclusion, the support for the hypothesis that juvenile hormone influences aggressive and territorial behavior adds to the growing knowledge base of the mediation of behavior by hormones. While the female mate-choice hypothesis was not supported in this study, the potential for association between juvenile hormone and greater mating success suggests that juvenile hormone could be the insect analogue to testosterone, and warrants further study.

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