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Endocrine and reproductive differences and genetic divergence in two populations of the cockroach *Diploptera punctata*

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ABSTRACT

The viviparous cockroach, *Diploptera punctata*, has been a valuable model organism for studies of the regulation of reproduction by juvenile hormone (JH) in insects. As a result of its truly viviparous mode of reproduction, precise regulation of JH biosynthesis and reproduction is required for production of offspring, providing a model system for the study of the relationship between JH production and oocyte growth and maturation. Most studies to date have focused on individuals isolated from a Hawaiian population of this species. A new population of this cockroach was found in Nakorn Pathom, Thailand, which demonstrated striking differences in cuticle pigmentation and mating behaviours, suggesting possible physiological differences between the two populations. To better characterize these differences, rates of JH release and oocyte growth were measured during the first gonadotrophic cycle. The Thai population was found to show significantly earlier increases in the rate of JH release, and oocyte development as compared with the Hawaiian population. Breeding experiments to determine the degree of interfertility between the two populations demonstrated greatly reduced fertility in crosses between the two populations. Additionally, levels of genetic divergence between the two populations estimated by sequencing a fragment of the mitochondrial 16S rRNA gene were surprisingly high. The significant differences in physiology and mating behaviours, combined with the reduced interfertility and high levels of sequence divergence, suggest that these two populations of *D. punctata* are quite distinct, and may even be in the process of speciation. Moreover, these studies have important implications for the study of JH function in the reproductive cycle of insects, as differences in timing of rates of JH biosynthesis may suggest a process of heterochrony in reproduction between the two populations.

1. Introduction

Diploptera punctata (Dictyoptera: Blattaria: Blaberidae) is the only truly viviparous cockroach known, and only one of a few truly viviparous insects (Roth, 1970, 1999). They are a burrowing species, living in leaf litter, which serves as a moist substrate for breeding, and a protection from predators (Schal et al., 1984). They often eat the bark of selected tree species and are considered pests in the tropics, particularly Hawaii, where they were introduced (Stay, 1999).

Hawaiian *D. punctata* has been a model organism for physiological studies of the regulation of juvenile hormone (JH) biosynthesis, the key hormone in reproduction in insects. As a consequence of its truly viviparous mode of reproduction, JH biosynthesis and oocyte development must be tightly regulated in *D. punctata* (Stay and Tobe, 1978; Stay et al., 1983; Tobe, 1980), since the inappropriate production of JH during the period of gestation (pregnancy) results in abortion of the developing embryos. JH production must therefore remain at low levels to permit a viable pregnancy (Stay and Coop, 1973). In addition, precise control of the timing of JH biosynthesis is not limited to pregnancy, but is required at all stages of reproduction. The mechanical stimulation of mating and spermatophore insertion releases the inhibition on the corpora allata (CA) (possibly mediated by allatostatins), resulting in a rapid increase in JH biosynthesis (Engelmann, 1959; Rankin and Stay, 1987). JH biosynthesis occurs exclusively in the paired CA, endocrine glands associated with the retrocerebral complex; these glands increase in size during the reproductive cycle as a result of cell growth and division. As a consequence of the ease of manipulation of the CA and the high rates of JH biosynthesis in this species, *D. punctata* has proven to be a useful model for the study of development and

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reproduction. As JH is released into the haemolymph at rates equal to biosynthesis, production of vitellogenin is induced, as is its uptake by the basal oocytes in the bilateral ovaries (Stay and Tobe, 1978). Thus, JH biosynthesis in adult female *D. punctata* is closely correlated with the gonadotrophic cycle (Stay and Tobe, 1978; Stay et al., 1983; Tobe, 1980).

A population of *D. punctata* was collected from Nakorn Pathom, Thailand in 2004. Comparison of the laboratory Hawaiian and the Thai populations revealed readily apparent morphological differences as well as behavioural differences in mating. Rates of JH release were therefore measured during the gonadotrophic cycle in the two populations, as was oocyte development, to determine if the Hawaiian and Thai populations differ in these respects. Differences between the two populations were also investigated by performing mating experiments to determine if interpopulation crosses are fertile. Finally, a fragment of the mitochondrial 16S rDNA gene was sequenced in order to assess genetic divergence.

2. Materials and methods

2.1. Animal maintenance

Both Hawaiian and Thai populations of *D. punctata* were reared in an incubator at 27 1C in 12:12 photoperiod. Animals were fed water and Lab Chow (Purina, St. Louis, MO) *ad libitum*. Newly molted females were picked in the morning and maintained in glass containers until dissected. A 1:1 ratio of males and females were placed in each container to ensure all females were mated.

2.2. Radiochemical assay (RCA) for juvenile hormone

The RCA was performed *in vitro* as previously described by Tobe and Pratt (1974) and Pratt and Tobe (1974), and modified by Tobe and Stay (1977) and Tobe and Clarke (1985). Basal oocyte length was measured to ascertain the stage of physiological development of each animal.

2.3. Mating experiments

Reciprocal crosses were performed between Hawaiian and Thai *D. punctata*. Parameters for each cross were assembled by placing males and females of either population together in cages when males were all 7 days old (from adult emergence) and females were fourth instars (approximately 43 days since birth) so that their adult emergence would occur in the presence of the males (Stay, 1999). Parental crosses were performed in large groups of 70–162 individuals (25–72 females), housed in clear acrylic "cages" (of size 13.5 31.5 52.0 cm). They were checked regularly for nymphs throughout the gestation period which is known to be about 70–71 days in Hawaiian *D. punctata* (Stay, 1999). Any nymphs found were then raised in separate glass jars until adults, if male, or fourth instars, if female. Then they were paired with 2–4 members of the opposite sex from either the pure Hawaiian or Thai populations to make the F_1 crosses. The jars were each checked for nymphs during the gestation period.

2.4. DNA extraction, PCR, and DNA sequencing

One head (25 mg) of a pregnant female of each population of *D. punctata* was removed, snap frozen in a mixture of dry ice and ethanol, and ground into a powder using a small mortar and pestle. Total genomic DNA isolation was adapted from the DNeasy protocol for purification of total DNA from animal tissues (Qiagen). Lysis time using Proteinase K was 3 h at 56 1C. To further purify the genomic DNA, a phenol/chloroform extraction, followed by an ethanol precipitation, was performed (Sambrook and Russell, 2001).

PCR amplification of a 415-bp fragment of the mitochondrial 16S rRNA gene was performed using forward (5⁰-TTA CGC TGT TAT CCC TTA-3⁰) and reverse (5⁰-CGC CTG TTT ATC AAA AAC AT-3⁰) primers adapted from Kambhampati and Smith (1995). The resulting amplification product contained about 1/3 of the gene. PCR conditions were a modification of Kambhampati (1995), with an annealing temperature of 50 1C. The amplified product was gel-purified (QIAquick, Qiagen), and then cloned into the TOPO TA cloning vector (Invitrogen). For each individual, at least three clones were sequenced and compared in order to eliminate sequencing and other polymerase artifacts. Sequences were deposited in Genbank under the accession numbers EU580104, EU580105. Voucher specimens of each *Diploptera* population were deposited at the Royal Ontario Museum (Toronto), Ent Spec. No. 102957-102960.

2.5. Sequence analysis

Additional 16S insect sequences were downloaded from the NCBI databases, and aligned with sequences obtained from the two *Diploptera* populations using the program ClustalW (Higgins et al., 1992). This alignment was subsequently modified by hand in order to incorporate information concerning rRNA secondary structure (Buckley et al., 2000). Phylogenetic analyses, including maximum parsimony, neighbour-joining/minimum evolution distance methods, and maximum likelihood methods, were performed on the aligned nucleotide sequences using PAUP* v.4.0b10 (Swofford, 2002). Bootstrap methods were used to assess the degree of confidence of nodes in the phylogeny (Felsenstein, 1985). Corrected pairwise distances were also calculated in PAUP* (Swofford, 2002). The species included in the phylogenetic analyses were as follows: Blaberidae: *Angustonicus amieuensis* (GenBank accession no. AJ870994) *Archimandrita tessellata* (U17761), *Blaberus atropos* (U17763), *Blaberus craniifer* (U17765), *Blaberus discoidalis* (U17767), *Blaberus giganteus* (U17771), *Byrsotria fumigata* (U17769), *Epilampra azteca* (U17783), *Eublaberus posticus* (U17785), *Geoscapheus woodwardi* (AB036178), *Gromphadorhina portentosa* (U17787), *Macropanesthia rhinoceros* (AB036177), *Nauphoeta cinerea* (U17797), *Panchlora nivea* (U17814), *Panesthia angustipennis* (AB036179), *Phoeotalia pallida* (U17816), *Phortioeca phoraspoides* (U17819), *Pycnoscelus surinamensis* (U17821), *Rhyparobia maderae* (U17825), *Salganea esakii* (AB036180), *Salganea taiwanensis* (AB036181), *Schultesia lampyridiformis* (U17827), *Trichoblatta pygmaea* (AB036182); Blattellidae: *Parcoblatta pensylvanica* (U17818); Blattidae: *Blatta orientalis* (U17774); Cryptocercidae: *Cryptocercus darwini* (AF126779), *Cryptocercus garciai* (AF126774), *Cryptocercus kyebangensis* (AF310220), *Cryptocercus matilei* (AJ519677), *Cryptocercus primarius* (AY631406), *Cryptocercus punctulatus* (AF126773), *Cryptocercus relictus* (AY631412), *Cryptocercus wrighti* (AF126772); Acrididae: *Locusta migratoria* (AY856117).

2.6. Statistical analysis

One-way ANOVA was performed to determine if rates of JH release were different between the two populations at oocyte lengths of 0.6–0.72 mm on day 1, and 1.63–1.78 mm on days 6 and 7. For this test, the JH release data were square-root transformed. Two-way ANOVA was performed to determine if the difference between populations in overall rate of development during the gonadotrophic cycle was significant. Both Mann–Whitney and

two-sample *t*-tests were used to test for significance between populations in rates of JH release at each individual day of the gonadotrophic cycle. These data were initially assessed for variance using the *F*-two sample test for variance and were analyzed using either the two-sample *t*-test assuming equal or unequal variances, as appropriate. Data are given as mean7 S.E.M. in all appropriate figures. All statistical analyses were run using GraphPad Prism 4.01 (2004, GraphPad Software).

3. Results

Comparison of the laboratory Hawaiian and the Thai populations revealed readily apparent morphological differences, as well as behavioural differences in the timing of mating. Most obvious is a difference in the colour of the tanned cuticle (the Hawaiian population displays a glossy dark brown cuticle, whereas the Thai population has a matte orange-brown cuticle) (Fig. 1). Another difference between the two populations is in mating behaviour. Hawaiian *D. punctata* females will mate immediately upon emergence, while in Thai, mating can be delayed for up to 16 h in some individuals.

Fig. 1. Day 4 adult female *Diploptera punctata*. The Thai female (left) displays a matte orange cuticle whereas the Hawaiian female (right) displays a glossy brown cuticle.

3.1. JH biosynthesis

As a consequence of our observations that Hawaiian and Thai populations of *D. punctata* mate at different times following emergence, rates of JH release were measured to determine if there were physiological correlates associated with this behavioural difference. The rates of JH release are displayed in Fig. 2 for both Hawaiian and Thai mated females. Since release follows a cycle closely correlated to the growth of basal oocytes, we measured rates of JH release as a function of the gonadotrophic cycle. On day 1, there was a significant increase in JH release by CA of Thai females in comparison to that of Hawaiian females. Rates of JH release in day 0 virgin Thai females were 6.2 pmol h^{-1} per pair CA (S.E. ¼ 1.7; n ¼ 3–5), whereas Hawaiian virgins on day 0 were 3.0 pmol h^{-1} per pair CA (S.E. ¼ 0.83; n ¼ 4) (data not graphed). During pre-vitellogenesis, JH release in both populations increased at similar rates, but the initial rates of JH release were higher for the mated Thai females (one-way ANOVA: P ¼ 0.0120, $F_{1,70}$ ¼ 2.48). Rates of JH release in Thai females, increased at basal oocyte lengths of 0.5 mm, and reached a maximum at 1.3 mm. In comparison, mated Hawaiian females displayed a more discrete peak in JH release at oocyte lengths of 1.3–1.4 mm. At the completion of vitellogenesis and during chorionation, Thai females showed a more rapid reduction (one-way ANOVA: P ¼ 0.0001, $F_{1,59}$ ¼ 24.36) in JH release, declining to levels that were lower than those of the Hawaiian population.

3.2. Oocyte development

Basal oocytes were larger at adult eclosion and at least initially developed at a faster rate in Thai females in comparison to Hawaiian females. A two-way ANOVA showed statistical significance between Thai and Hawaiian females (P ¼ 0.0005, F ¼ 14.0) (Fig. 3). There was a more rapid growth of basal oocytes between the time of mating and day 1 in Thai females, but growth rates on the following days were similar. Oocytes from Thai females attained final mature length (size) more rapidly than those from the Hawaiian population. Thai females oviposited by day 6, whereas Hawaiian females did so 1 day later (data not provided because of small sample size).

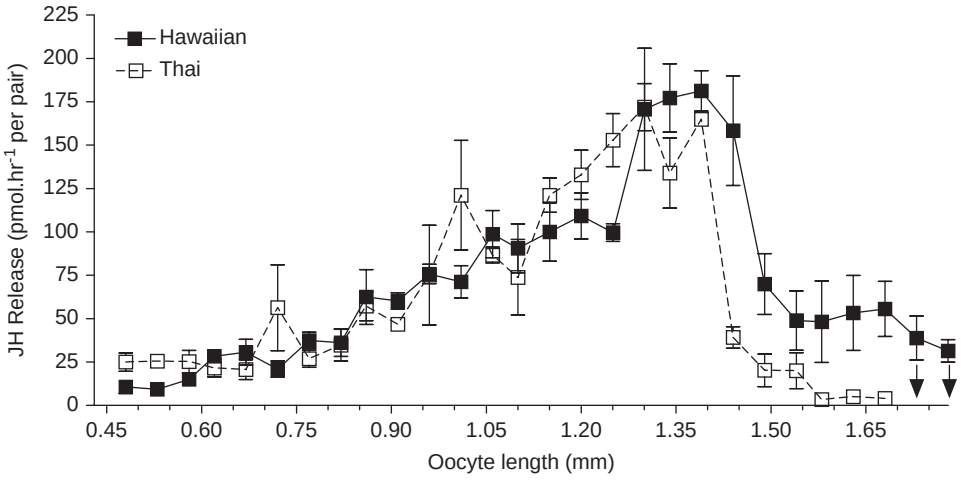

Fig. 2. Rates of JH biosynthesis as a function of basal oocyte length. Radiochemical assays using ^{14}C-methionine were used to measure rates of JH release throughout the gonadotrophic cycle of Thai and Hawaiian mated females. These rates are indicative of the physiological age during the gonadotrophic cycle. Arrows indicate 'missing' JH rates in Thai females as a consequence of earlier oviposition; n ¼ 3–5.

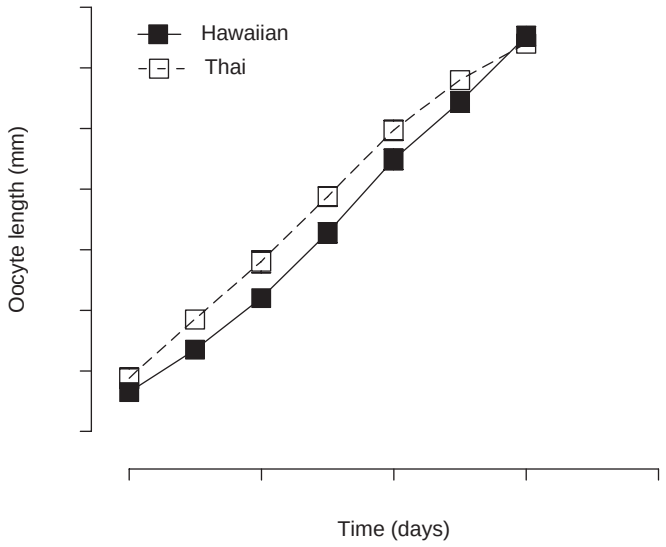

Fig. 3. Rate of oocyte development throughout the gonadotrophic cycle of the two populations. Two-way ANOVA showed a statistical significance (P ¼ 0.0005) between populations as a function of time (Po 0.0001), but the interaction between the two populations was not significant (P ¼ 0.6796). Although development for both populations are indicated until day 6, oocytes of Hawaiian females are known to develop until day 7 or 8 (unpublished observation); n ¼ 3–5.

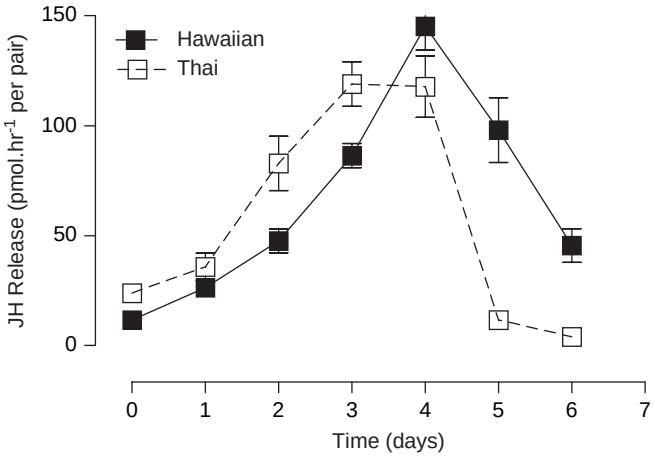

Fig. 4. Rates of JH release of the Hawaiian and Thai populations as a function of age. Mann–Whitney U and two-sample t-tests were performed between the two populations for each day, showing significance for days 0, (Po 0.05); 2, (Po 0.02); 3, (Po 0.01); and 5, (Po 0.001), but not day 1 or 4 (significance considered Po 0.05). There was insufficient data to perform a t-test on day 6. Two-way ANOVA was also performed, showing statistical significance of age on JH release (Po 0.0001, F ¼ 36.71, d.f. ¼ 6) and also the interaction between time and population (Po 0.0001, F ¼ 9.71, d.f. ¼ 6), but not of population alone (P ¼ 0.1547, F ¼ 2.04, d.f. ¼ 1); n ¼ 10–15.

3.3. JH release during development

Plotting of JH release as a function of oocyte length shows punctuated measurements. However, plotting of release as a function of time reveals an overall rate of JH release that is correlated to oocyte development. This suggests that JH release is not wholly dependant on the gonadotrophic cycle, but is controlled by other factors as well (Fig. 4). Rates of JH release differed between Thai and Hawaiian females, and maximal rates were attained earlier in Thai females, as was the decline in rates at the end of the vitellogenic cycle. Thai females displayed a more gradual prolonged increase in JH release, whereas Hawaiian females displayed a sharp peak. Following the peak, Hawaiian females showed a more gradual decline in JH production when compared to the sharp decrease seen in Thai females. Mann–Whitney U and two-sample t-tests were performed to compare JH release of Thai and Hawaiian animals on each day of the gonadotrophic cycle. Significance was observed for days 0, 2, 3, and 5 (level of significance Po 0.05—day 0; Po 0.02—day 2; Po 0.01—day 3; Po 0.001—day 5), but not on days 1 and 4. There were insufficient data for the Thai adult females to perform this analysis for day 6, as the majority had oviposited by this time.

3.4. Mating experiments

In the mating experiments, only 12 matings were observed for parental crosses. Of these, only two were with teneral females (cuticle not yet tanned, indicative of an early day 0 individual). Results of parental and F_1 crosses are shown in Table 1. Two of the parental crosses failed to produce any F_1 offspring, but seven others produced nymphs. Note that each parental cross potentially constituted an entire cage with 25–72 virgin females available for mating with a male of the other population. In normal Hawaiian *D. punctata*, each female usually bears 10–12 nymphs—one for each of her 12 ovarioles (Stay, 1999). Similarly, Thai *Diploptera* have 12 ovarioles, although it is uncertain whether 12 offspring is the norm for these animals. The expected number of nymphs was over 3800 from a total of 321 adult females, assuming that each female bears 12 nymphs, but the total number of F_1 nymphs found was only 88, an average of 3.64 nymphs per female. Because of the long generation time of *D. punctata*, not all F_1 crosses were performed. No nymphs were produced by any F_1 females that matured in the presence of F_1 males.

3.5. DNA sequences of mitochondrial 16S rRNA

The 16S mitochondrial gene fragment isolated from the Thai and Hawaiian *D. punctata* populations was subjected to phylogenetic analysis in order to assess their phylogenetic position as well as level of sequence divergence relative to other insect sequences. A variety of phylogenetic methods, including parsimony, likelihood and distance analyses, all resulted in highly similar topologies. Bootstrap methods were used to assess the robustness of the phylogenetic results (Felsenstein, 1985). The results of the phylogenetic analyses are summarized in Fig. 5. Although some of the basal nodes are not well supported, some of the more recent divergences, such as within Blaberidae, as well as the *Cryptocercus* genus, are fairly well resolved. The two *D. punctata* population samples clearly form a monophyletic group, which is well supported, but the level of divergence between the two populations is more similar to that found between different species within a genus, or even different genera. Using branch lengths as an estimate of sequence divergence, the *D. punctata* populations show divergences similar to that found among different species of the genus *Cryptocercus* (Fig. 5). The divergences are also comparable to some species belonging to different genera within Blaberidae, for example, *G. woodwardi* and *M. rhinoceros*, and are considerably greater than that found among species of the *Blaberus* genus. Simple pairwise maximum likelihood estimated distances show similar patterns of divergence, with distances between the two populations estimated (under the GTR+G model) to be as large as 11.7% (see Appendix A).

4. Discussion

Given the previous data from *D. punctata* on JH biosynthesis, and the observed differences in the timing of mating between the

Table 1
Results of parental and F_1 crosses between Hawaiian and Thai *Diploptera punctata*

Parental cross	F_1	F_1 cross	F_2
Hawaiian female Thai male	7 nymphs	F_1 female 3 Hawaiian males	7 nymphs
		F_1 female 3 Thai males	0 nymphs
		F_1 male 3 Hawaiian females	21 nymphs, 3 failed birth[a]
		F_1 male 3 Hawaiian females	3 nymphs
		F_1 male 3 Thai males	0 nymphs
Hawaiian female Thai male	11 nymphs	None[b]	
Hawaiian female Thai male	31 nymphs	None	
Hawaiian female Thai male	10 nymphs	None	
Hawaiian female Thai male	0 nymphs	None	
Thai female Hawaiian male	9 nymphs	F_1 female F_1 male	0 nymphs
		F_1 female 2 Hawaiian, 2 Thai males	6 nymphs, female abort[c]
		F_1 male 2 Hawaiian, 2 Thai females	9 nymphs
Thai female Hawaiian male	8 nymphs	None	
Thai female Hawaiian male	12 nymphs	None	
Thai female Hawaiian male	0 nymphs	None	

[a] The three nymphs were dead and affixed to each other; one appeared fully tanned and developed, but the others were teneral and had only developed for 47 days (after Stay and Coop, 1973). All had eyespots.
[b] Not all F_1 crosses were performed; those that were not, or parental crosses that did not result in any F_1, are listed as "None".
[c] It is probable that one of the males in the breeding experiment was misidentified and was actually a female, as a full ootheca of 12 oocytes was aborted.

two populations, we have measured both the rates of JH release and oocyte development. Comparisons of rates of JH release showed that the Thai *Diploptera* displayed significantly higher rates during much of the gonotrophic cycle. Taken together with the observed 12–16 h delay in mating, this increase may be attributable to a physiological adaptation in Thai females, to provide a higher titre of JH over a shorter interval. That is, factors that work to control JH biosynthesis may result in a stimulation of biosynthesis to a greater degree (or less inhibition) in Thai females, resulting in an increase in the rate of oocyte development.

During the period of oocyte development and maturation, rates of JH release by Thai and Hawaiian females differ at two important time intervals: prior to vitellogenesis (days 0–2) and during chorionation (days 5 and 6). The possible presence of high JH titre at the end of oocyte development could potentially compromise pregnancy—thus, rates of JH production should be low at this time to permit normal pregnancy and gestation. As such, that these two populations display differences in the timing of JH biosynthesis, and thus oocyte development, indicates that the timing and duration of pregnancy in the Thai population may differ from that of Hawaiian *D. punctata*. Stay and Coop (1973) found that pregnancy lasted approximately 73 days in Hawaiian animals, with associated precise markers of embryonic development. Similar studies must also be performed on Thai *D. punctata* to determine if the rate of embryonic development is similar or identical to the Hawaiian population.

Hawaiian females mate immediately upon emergence, when their cuticles are untanned and not yet sclerotized. It has been postulated that either these animals represent visual cues, which present a stark contrast to older females and leaf litter, or their pheromones are released from the untanned cuticle resulting in mating in these newly molted animals (Sreng, 1993). As Thai animals do not mate immediately after emergence, but rather 12–16 h later, these findings suggest a pheromonal cue. It is also possible that the pheromone cue released from Thai females differs from that of Hawaiian females, as they result in mating at a later time. Once mating has occurred, however, initial rates of JH release are considerably higher in Thai females relative to Hawaiian females (Fig. 2). In view of the delay in mating of newly eclosed Thai females, the initial rapid increase in rates of JH release suggests an acceleration of the gonadotrophic cycle. In contrast, rates of JH release in virgin Thai females are low relative to the rates observed in mated females and comparable to Hawaiian females prior to mating.

Differing rates of JH release between the two populations can be seen during vitellogenesis (particularly for oocyte lengths 1.15–1.4 mm), and again during chorionation (oocyte lengths 1.5–1.8 mm) (Fig. 2). The rates of JH release also differ between the two populations during pre- and post-vitellogenesis, the same stages at which Pratt et al. (1990) and Stay et al. (1991) found Hawaiian females to be most sensitive to inhibition of JH release by allatostatins. It has been postulated that changes in the quantities and affinities of two CA allatostatin receptors cause rates of JH biosynthesis to change during the gonadotrophic cycle (Pratt et al., 1990; Stay et al, 1991). These differences between populations might therefore be explained through differences in receptor abundance or affinity to allatostatins at the level of the CA.

While the rates of JH release between populations parallel each other throughout the gonadotrophic cycle (Fig. 2), Thai females display faster rates of oocyte growth and a more rapid gonadotrophic cycle than Hawaiian females (Fig. 4). Higher rates of JH release in Thai females may be related to the accelerated rates of oocyte development seen in this population.

Differences in developmental timing in these two populations may be controlled by one (or more) mechanisms. It is well established that allatostatins, in part, regulate the production of JH in *D. punctata* (Stay and Woodhead, 1994; Pratt et al., 1990; Stay et al., 1991). The cycle of allatostatin expression in the brain of *D. punctata* has been shown to be the inverse of that of JH release by the CA (Garside et al., 2003). Differences in the level and timing of allatostatin expression between the two populations may account for some of the differences in the profile of JH biosynthesis and oocyte development during the gonadotrophic cycle. For example, allatostatin precursor expression may remain at low levels during vitellogenesis in Thai *Diploptera* for a longer interval, permitting a longer period of uptake of vitellogenin into the oocytes. Alternatively, because initial rates of JH release are higher in Thai than Hawaiian animals, inhibition of JH production may be reduced in Thai *Diploptera* as a consequence of a lower level of allatostatin expression.

Another means by which the timing of JH release could be altered is through internal regulation of the CA themselves.

Fig. 5. Phylogeny of insect 16S mitochondrial sequences. This tree represents the 50% majority rule consensus of 25 shortest trees found in a maximum parsimony heuristic search (50 replicates). The tree was rooted using the locust as the outgroup sequence. Branch lengths were estimated by maximum likelihood under the GTR+G model (shown above branches). Bootstrap percentages from maximum parsimony analyses (100 replicates) are shown below nodes, followed by bootstrap percentages from minimum evolution distance analyses (100 replicates). I ¼ Blaberidae, II ¼ Blattellidae, III ¼ Cryptocercidae, IV ¼ Blattidae, V ¼ Acrididae.

Once inhibition of JH production is 'released' following mating, cells of the CA begin to increase in number, size and volume (Szibbo and Tobe, 1981; Chiang and Schal, 1994; Tobe and Stay, 1977). This activity is necessary to put in place intracellular structures involved in the rise in JH release following mating (Stay et al., 1984). Volumetric changes within the CA are the result of the rapid proliferation of organelles involved in the synthesis of JH (Johnson et al., 1993). Little is known about the mechanisms that regulate CA cytology in *Diploptera*, but it is thought that brain factors do play a part, since neural disconnection causes a mitotic

wave in the CA in virgin females (Chiang et al., 1996, 1999). A difference in timing of expression or potency of these as yet unidentified brain factors may contribute to the overall cycle of JH biosynthesis during oocyte development, and so may also show differences between populations to allow for differences in developmental timing.

The difference in timing of JH biosynthesis for either population of *Diploptera* has implications not only on mating times, oocyte development, and pregnancy, but also on the viability of offspring. The precise inhibition of JH production during pregnancy is essential for successful embryonic development. Thus, differences in the timing and regulation of JH biosynthesis may influence brood number and viability within each population (Stay and Coop, 1973). The time course of embryonic development of Hawaiian *Diploptera* has been documented (Stay, 1999; Stay et al., 2002), whereas nothing is known of the Thai population other than the increased rate of oocyte development and JH release described here. Thus, mating experiments, where populations of *D. punctata* were crossed, reveal that the number of nymphs was significantly reduced, with an average of 3.64 nymphs per female (as opposed to the normal 12). Further analysis must be performed in order to determine this with any certainty.

It would be informative to understand why Hawaiian and Thai populations of *D. punctata* show such low fertility in interbreeding experiments. In this regard, some behavioural differences are noteworthy. In Hawaiian *D. punctata*, males will only mate with females when the females have just emerged as adults and are still teneral (Stay, 1999). In Thai *Diploptera*, males will mate with females after cuticular tanning has begun. Similarly, in the mating experiments, almost all observed matings were with females with tanned or partially tanned cuticles. This difference in the timing of mating which may be related to rates of JH release may be partially responsible for lower hybrid viability. Miyatake and Shimizu (1999) showed that the developmental period and time of mating are genetically correlated in the fly *Bactrocera cucurbitae*, and have led to prezygotic isolation—even when the difference is only 1 h. *Bactrocera tryoni* and *Bactrocera neohumeralis* appear to be unable to hybridize solely because of the difference in time of mating (Lewontin and Birch, 1966). For this reason, it will be important to measure the duration of the gestation period in each *D. punctata* cross, as well as in control crosses of Hawaiian female Hawaiian male and Thai female Thai male. This should provide a better indicator of the gestation period in normal, intrapopulation crosses, to compare to interpopulation crosses. Taken together with the differences in timing of oocyte development, it is possible that the regulation of JH biosynthesis provides a physiological basis for a reproductive barrier.

It is important to note that there is a considerable difference in laboratory generations between these two populations. The Hawaiian population was originally established in our laboratory in 1975, with additional Hawaiian introductions in 1988 and 1994, whereas the Thai population was acquired and established in early 2004. Mating experiments were performed in the summer of 2005, thus allowing about 10 generations in our laboratory, whereas the Hawaiian colony has had many more generations to adjust to a contained colony environment. This no doubt would play a role in the behaviour and physiology of these populations, as a steady food and water supply, close proximity of teneral females with adult males, and a lack of predation are all present in laboratory colonies. Inbreeding and selection for rapid reproduction, as would occur under laboratory conditions, would probably influence the Hawaiian population more than the Thai population at the time of the cross-mating experiments.

Physiological data have shown an increased rate of oocyte development and JH biosynthesis, combined with the reduced viability in hybrid numbers seen in the cross mating experiments. These data suggest a breakdown in physiological compatibility between the Thai and Hawaiian populations of *D. punctata*.

Given these marked physiological and behavioural differences, a portion of the 16S mitochondrial gene was sequenced in order to determine the level of genetic divergence between the two populations, and their phylogenetic position relative to other cockroach sequences. This particular fragment was selected as it has been extensively sequenced in insects, and widely used, well-conserved PCR primers have been developed for this region (Simon et al., 1994, 2006). 16S and other mitochondrial genes have been widely used for phylogenetic studies in general (Simon et al., 1994, 2006), and for cockroaches and related insects in particular (Kambhampati, 1995; Kambhampati et al., 1996; Burnside et al., 1999; Davison et al., 2001; Clark and Khambhampati, 2003). The results of our phylogenetic analysis of cockroach 16S sequences are largely congruent with previous studies of cockroach systematics, with many of the basal nodes which are the subject of current disagreement, such as the position of *Cryptocercus* and possible monophyly of Cryptocercidae and Blattidae (Kambhampati, 1995; Maekawa and Matsumoto, 2000; Inward et al., 2007), as well as the relative phylogenetic positions of subfamilies within Blaberidae (Maekawa et al., 2001), being not particularly well resolved in our study.

The level of genetic divergence in 16S between the two *D. punctata* populations was found to be fairly large (11.7%), and this is also reflected in lengths of the branches separating the two *D. punctata* populations in the 16S phylogeny, as compared with other cockroaches for which 16S has been characterized for closely related species (for example, *Blaberus*, *Salganea* and *Cryptocercus*). Previous studies of 16S in wood-feeding *Cryptocercus* cockroaches found comparable divergences between geographically distinct populations thought to constitute separate species (Kambhampati et al., 1996). These populations were also found to have marked differences in karyotype, and almost complete reproductive isolation in mating experiments (Kambhampati et al., 1996). Moreover, studies of allozymes in these species found no evidence of any hybrids (Aldrich et al., 2004). The greatly reduced fertility and viability of the interbred *D. punctata* populations, combined with the substantial level of divergence in the 16S data, and the marked physiological and behavioural differences is suggestive of reduced gene flow between the two populations and that they may even be in the process of speciating. This possibility would require further investigation, in order to characterize individuals from populations sampled throughout the distribution of *D. punctata*.

5. Conclusion

The differences between Thai and Hawaiian populations of *D. punctata* suggest that the Hawaiian population may not be representative of all *Diploptera* in terms of reproductive physiology. The shift in timing of oocyte development has an impact on all reproductive events in adult females, because the precise control of oocyte development by JH is linked to mating behaviour and pregnancy. This suggests that heterochrony has occurred at the level of JH biosynthesis and reproduction. It follows that there is low interpopulation viability, and this is supported by the species-level molecular divergence between the populations. This evidence means that the Thai population should be considered in future studies of reproduction, as it has all of the advantages of *Diploptera* as a model organism, but may be more similar to ancestral viviparous cockroaches. For example, differences in levels of allatostatin expression or cytology of CA cells may be responsible for the difference in rates of JH biosynthesis.

Further experiments are warranted to distinguish between these possibilities. The Thai population, which may represent a new species (based on hybrid breakdown and molecular divergence data), will be useful in studies of JH production and oocyte growth in the future.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinsphys.2008.02.009.

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