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Journal of Insect Physiology 51 (2005) 803–809
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Cuticular hydrocarbon synthesis and its maternal provisioning to embryos in the viviparous cockroach *Diploptera punctata*

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Received 15 December 2004; received in revised form 18 March 2005; accepted 22 March 2005

Abstract

Embryos of the viviparous cockroach *Diploptera punctata* accumulate large amounts of hydrocarbon (HC) of either maternal or embryonic origin. HC synthesis and its accumulation in maternal and embryonic tissues were measured over the course of gestation. Female abdominal integument was the only tissue that synthesized appreciable amounts of HC in vitro, and did so at an increasing rate from the time of mating to mid-pregnancy, when rates of synthesis declined. The embryos synthesized HC at rates $<1\%$ those of the female, showing that the majority of HC detected in and on embryos was of maternal origin. The brood sac that houses the developing embryos did not synthesize HC in vitro, indicating that HC must be transported from the female abdominal integument to the embryos. The mass of female epicuticular HC was constant at $\sim$183 μg, while her internal HC increased fourfold from mating to mid-pregnancy, then declined until parturition. The decline in internal HC reflected both declining HC synthesis in the female and greater export to the embryos, as embryonic internal HC increased 250-fold prior to parturition. An external HC coating over the oothecal covering and chorion of the embryos increased to mid-pregnancy, then declined. Unlike oviparous cockroaches, *D. punctata* females fed throughout the reproductive cycle, reflecting the nutritional demands of continuously provisioning the developing embryos.

Keywords: *Diploptera punctata*; Hydrocarbons; Viviparity; Maternal investment; Feeding

1. Introduction

Insects display a range of maternal contributions to offspring development. Most are oviparous, investing resources in eggs only prior to ovulation. Some are ovoviviparous, retaining eggs internally and providing water and protection, but not nutrition, until embryogenesis is complete. Viviparity, though uncommon, is widespread among insects and has evolved at least once, sometimes repeatedly, in each of 11 orders (Hagan, 1951). Viviparous females both retain and nourish developing embryos until they reach an advanced state of development. Regardless of reproductive strategy, all females must meet certain requirements to produce viable offspring: embryos must receive sufficient nutrients to support development, and must be protected against desiccation and pathogens. It is of interest to relate the timing, magnitude, and mechanisms of these essential investments to the reproductive mode (i.e., oviparity, ovoviviparity, viviparity) of the female.

The cockroaches (Dictyoptera, Blattaria) offer an excellent opportunity to investigate the evolution of maternal provisioning strategies. Among over 4000 described species of cockroaches, there are many oviparous and ovoviviparous representatives. True viviparity, however, has been found in only one cockroach species, the Pacific beetle cockroach *Diploptera punctata*. In this species, females retain developing embryos in a glandular brood sac during a 2-month gestation. When embryos complete dorsal closure and

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0022-1910/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2005.03.008

can drink, the brood sac begins to secrete protein-rich milk that supports embryonic growth, such that first instar nymphs at birth have 50 times the dry weight of eggs at oviposition (Stay and Coop, 1973). In 50-day old females (~65% through embryogenesis), milk collected from the brood sac consists of about 46% protein, 5% amino acid, 25% carbohydrate, and 20% lipids—of which half are nutritive substances, and the other half wax or hydrocarbons (Ingram et al., 1977). This milk is undoubtedly responsible for embryo dry weight gain during gestation, and the glycoprotein fraction is secreted by the brood sac itself (Stay and Coop, 1974; Ingram et al., 1977; Williford et al., 2004). The sources of the hydrocarbon components are unclear.

D. punctata embryos also bear a conspicuous waxy coating from the day they are oviposited into the brood sac, but the coating is most abundant during the middle 50% of gestation. This coating consists of 89% hydrocarbons, 6% long chain alcohols, 4% wax esters, and 1% aldehydes, and likely represents another aspect of maternal investment in *D. punctata* (Nelson et al., 2004). All insects, at all developmental stages, bear cuticular lipids that are crucial to water balance and pathogen exclusion (Nelson and Blomquist, 1995). Hydrocarbons (HC) are an important maternal investment in the oviparous cockroach *Blattella germanica*, in which HC provisioned to developing oocytes are not metabolized by embryos, but are retained in the hemocoel and cuticle of the first two nymphal instars (Fan et al., 2002, unpublished data).

Here we investigate the source of embryonic HC in *D. punctata*. Studies of the cockroaches *Periplaneta americana* and *B. germanica* have implied or demonstrated that HC synthesis is restricted to oenocytes, large cells rich in smooth endoplasmic reticulum and mitochondria, associated with abdominal epidermis in these species (Fan et al., 2003; reviewed in Schal et al., 2003). We can conceive of three plausible sources of the HC layer found on *D. punctata* embryos. First, it may be synthesized either by the glandular cells of the brood sac and provided to the embryos via brood sac milk, or by the subcuticular cells and passed through the brood sac cuticle. In addition to secretory cells and subcuticular cells, the brood sac epithelium, a derivative of abdominal epidermis, contains duct cells. However neither oenocytes nor morphological evidence for the synthesis of HC has been described for the brood sac (Stay and Coop, 1974). Second, HC may be synthesized elsewhere in the female and transported to the brood sac and embryos. Or, third, HC may be synthesized by the embryos themselves. The latter is least likely, as the HC layer is already present on embryos no more than a day old, and HC within these embryos would represent HC provided in yolk. This study distinguishes between these three possible scenarios, and relates patterns of HC synthesis in females and embryos to stages of embryonic development and the temporal pattern of female feeding.

2. Materials and methods

2.1. Insects

Cockroaches were maintained at $27 \pm 0.3\,^{\circ}C$ under a 12:12 h light:dark cycle, and were provided water and Purina rat chow ad libitum. Females mate upon adult ecdysis (day 0), and newly mated females were removed from the colony daily and maintained in separate plastic cages. Under these conditions, oviposition occurs on about day 8 after adult ecdysis, embryonic dorsal closure on day 20, and parturition about days 70–73 (Stay and Coop, 1973; Holbrook et al., 1998).

2.2. In vitro hydrocarbon synthesis

We tested various female tissues (tergites, sternites, thorax, wings, digestive tract, and brood sac) and embryos for in vitro synthesis of HC by incubation with [1-^{14}C]propionate, which serves as a traceable carbon donor in the synthesis of methyl-branched HC, allowing comparison of rates of HC synthesis in various tissues (Chase et al., 1990). This is likely to be a good estimator of total HC synthesis in *D. punctata*, in which both embryonic and maternal external HC consisted of >97% methyl-branched HC (Nelson et al., 2004). Each tissue was incubated in a 4 ml glass vial with 500 µl cockroach saline (Kurtti and Brooks, 1976), adjusted to 360 mOsm L^{-1}, containing 0.0185 MBq (0.5 µCi) [1-^{14}C]propionate (2035 MBq $mmol^{-1}$; New England Nuclear Research Products, Boston, MA) for 3 h at 27 °C with continuous gentle shaking.

To determine which tissue synthesizes HC, three females (days 23 and 30) were dissected and the head, dorsal thorax, ventral thorax, wings, legs, digestive tract, brood sac, and abdominal sternites and tergites were incubated. Sternites were freed of fat body and incubated, with internal surface contacting the medium, in three batches, with sternites 1–2 together, 3–4 together, and 5 to posterior together. Tergites were similarly incubated in three batches, consisting of tergites 1–3, 4–6, and 7 to posterior.

Changes in HC biosynthesis throughout the course of the reproductive cycle were investigated by incubating sternites three and four of the mother, one clutch of 12 embryos (cut into anterior and posterior halves with a sharp razor blade with the internal surfaces contacting the medium) and one brood sac (with hemolymph surface contacting the medium). After 3 h, 0.5 ml methanol was added and the tissue disrupted with a sonicator probe (Kontes, Vineland, NJ) for 30 s. The probe was rinsed into the vial with 1 ml hexane.

Following vigorous vortexing and 10 min centrifugation at 1500*g*, a 0.5 ml aliquot of the hexane was subjected to flash column chromatography on silica gel (100–200 mesh, ~500 mg) in a Pasteur pipette minicolumn, and HC eluted with 6 ml hexane, the solvent was removed under N_2, replaced with liquid scintillation cocktail, and the HC fraction quantified by liquid scintillation spectrometry with a counting efficiency of 97%.

2.3. Hydrocarbon extraction and quantification

Lipids were extracted from the female brood sac, from the exterior and interior of the embryos, and the exterior and interior of the female. Females were anesthetized with CO_2, and the brood sac everted by gentle pressure to the abdomen. To extract an individual brood sac, a female was held for 30 s with the everted brood sac exposed to 7 ml *n*-hexane containing 15 μg *n*-hexacosane as an internal standard. This process was repeated with clean hexane excluding the standard, and the two extracts were combined. The brood sac was restored to its internal orientation with a blunt probe, and cuticular HC were extracted from individual females using three 2 ml hexane soaks, each 1 min in duration, and the first of these contained 15 μg *n*-hexacosane. External HC of embryos were extracted by the same method, with one clutch of 12 embryos per extract.

Females and embryos previously extracted for external lipids were disrupted with sharp forceps, then a sonicator probe for 30 s, and their internal lipids were extracted by a modification (Young and Schal, 1997) of the procedure of Bligh and Dyer (1959). *n*-Hexacosane (15 μg) was included in each sample as an internal standard. The chloroform phase was reduced to dryness with N_2 and the lipid extract resuspended in hexane. In the case of embryos, the internal extract likely included both embryonic cuticle and developing cuticle of the first instar nymph, since these were internal to the chorion.

HC were purified by flash chromatography as already described. All samples were reduced under N_2 and analyzed by gas-liquid chromatography (GC, HP5890II) with a FID and interfaced with HP ChemStation (Rev. A.09.03). Splitless injection was made into a 30 m × 0.32 mm ID × 1 μm film thickness HP-5 capillary column operated at 150 °C for 1 min, ramped at 10 °C min^{-1} to 320 °C, and held for 20 min. The injector and detector were held at 300 °C and 310 °C, respectively. Helium was used as carrier gas at an average linear velocity of 35 cm s^{-1}.

2.4. Feeding

To measure food intake in females over the course of gestation, we separated females of known ages into 150 × 25 mm petri dishes, with three mated females of the same age per dish. Each dish was provided with a paper shelter and water ad libitum. One pellet of Purina rat chow was provided per dish, with the chow placed in a vial cap in the center of a 60 mm petri dish. The pellet was weighed daily. Five control pellets were set up under identical conditions without cockroaches to account for fluctuations due to changes in humidity. Two batches of mated females were used, one initiated with day 0 females and a second with day 32 females.

3. Results

3.1. Hydrocarbon synthesis

HC synthesis in adult females was localized in the abdomen, with the sternites and tergites together accounting for 99.6±0.4% (mean±SE) of the total in vitro HC synthesis accomplished by female tissues (Fig. 1). Sternites 3–4 alone accounted for 33.6±4.7% of total HC synthesis. The brood sac of day 30 females did not produce detectable amounts of methyl-branched HC.

The rate of female HC synthesis, as measured in vitro in sternites 3–4, varied over the course of the reproductive cycle, increasing to day 30 and declining thereafter (Fig. 2A). Embryonic HC biosynthesis (Fig. 3A) was first measured on day 20, 12 days after oviposition, and followed a pattern similar to that of the female, increasing sharply to day 30 and then declining to parturition. However, embryonic HC biosynthesis was always 30- to 900-fold less than that of female sternites 3–4 (i.e., ~90–2700-fold less than in whole females). Embryonic HC synthesis was greatest relative to female synthesis on day 60, when HC biosynthesized by embryos was 3.3±0.5% of HC synthesized by sternites 3–4. Since sternites 3–4 account for about a third of female HC synthesis, embryos synthesized HC at a maximum of ~1% the rate of the female. Of the cumulative HC synthesis measured over the course of

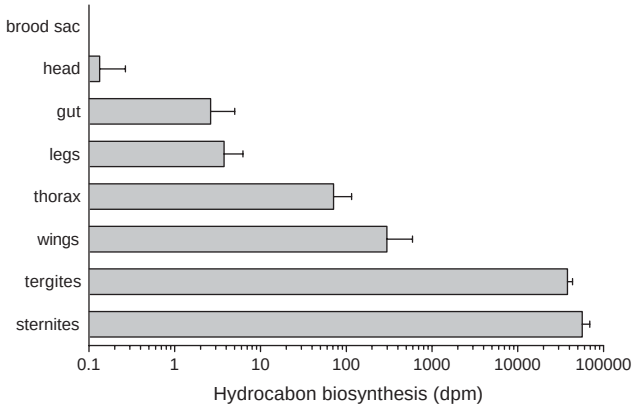

Fig. 1. In vitro HC biosynthesis by tissues of female *D. punctata* ($n = 3$ for each mean, one d-23 female and two d-30 females). Data are mean + SEM.

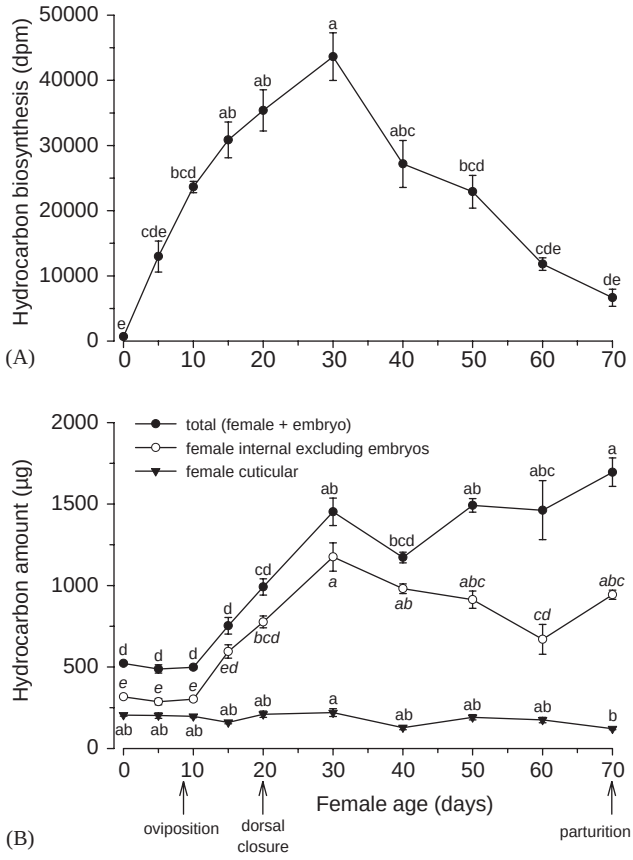

Fig. 2. Changes in (A) biosynthesis by sternites 3 and 4 and (B) accumulation of HC in female *D. punctata* over the course of the first reproductive cycle. In (B), total HC is the sum of HC measured from internal and external female, internal and external embryos, and brood sac. Arrows on the abscissa in (B) refer to reproductive landmarks: oviposition on day 8, dorsal closure on day 20, and parturition on day 70. Data are mean ± SEM ($n = 3$–11 per mean). Means with any letter designation in common within each variable are not significantly different at the $\alpha = 0.05$ level (ANOVA; Scheffe test).

gestation, embryos accounted for 0.36%. Brood sacs did not synthesize a measurable quantity of methyl-branched HC at any point during gestation (Fig. 3A).

3.2. Hydrocarbon accumulation

Female cuticular HC remained relatively constant at 182.8 ± 6.1 µg per female over the entire reproductive cycle (Fig. 2B). On the other hand, female internal HC content (Fig. 2B) roughly reflected the rates of HC biosynthesis, increasing nearly fourfold from day 0 to a peak of 1174.5 ± 86.6 µg on day 30. Female HC stores then declined by about 40% to day 60, apparently reflecting the combined effects of decreasing synthesis and increasing export of HC to embryos.

The external HC content of a clutch of 12 embryos increased rapidly from nearly zero on day 15 to a peak of 9.9 ± 0.6 µg per clutch on day 30, then declined by 63% to 3.7 ± 1.4 µg on day 70 (Fig. 3C). Embryonic

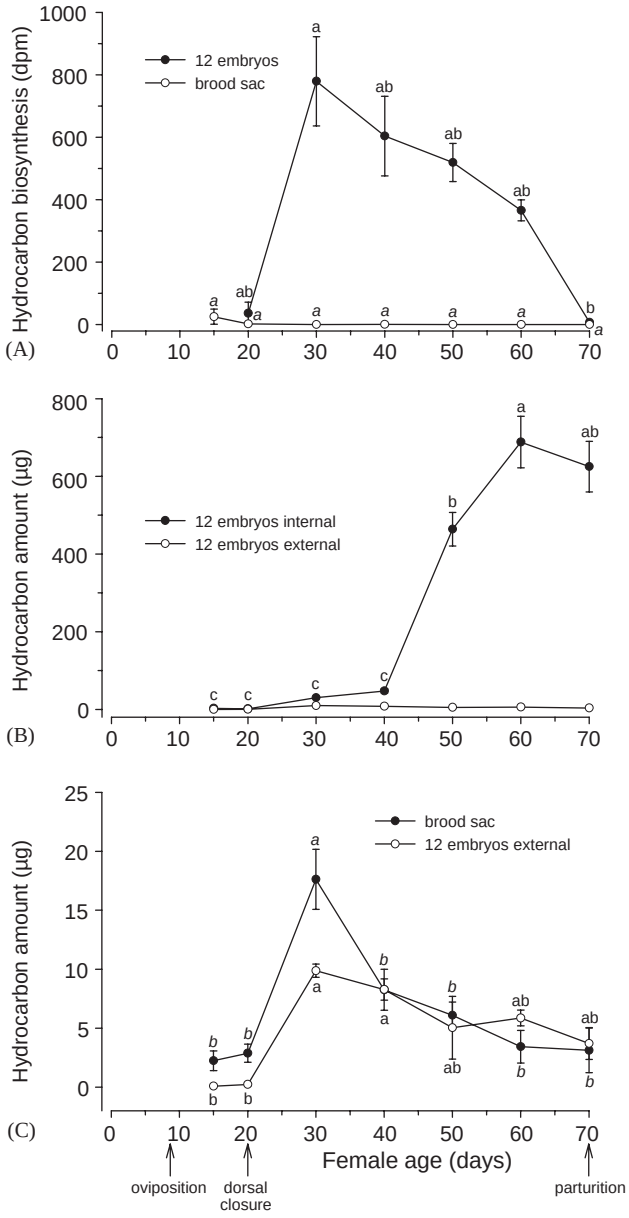

Fig. 3. Changes in (A) biosynthesis and (B and C) accumulation of HC in embryos and the brood sac over the course of the reproductive cycle. Data for the external surface of the embryos in (B) are duplicated in (C) on a more suitable scale. Reproductive landmarks are as in Fig. 2. Data are mean ± SEM ($n = 3$–6 per mean). Means with any letter designation in common are not significantly different at the $\alpha = 0.05$ level (ANOVA; Scheffe test).

internal HC content was also low (2.7 ± 0.1 µg per clutch) on day 15, but continued to increase beyond day 30 (Fig. 3B), when external HC began to decline (Fig. 3C). Internal HC increased about 250-fold to a peak of 688.2 ± 66.3 µg per 12 embryos on day 60, then declined by about 10% to day 70. The amount of internal HC lost by females between days 30 and 60 was 505.4 ± 125.9 µg, slightly less than the 653.9 ± 66.8 µg gained by embryos (internal and external) during the same time. This difference was not statistically

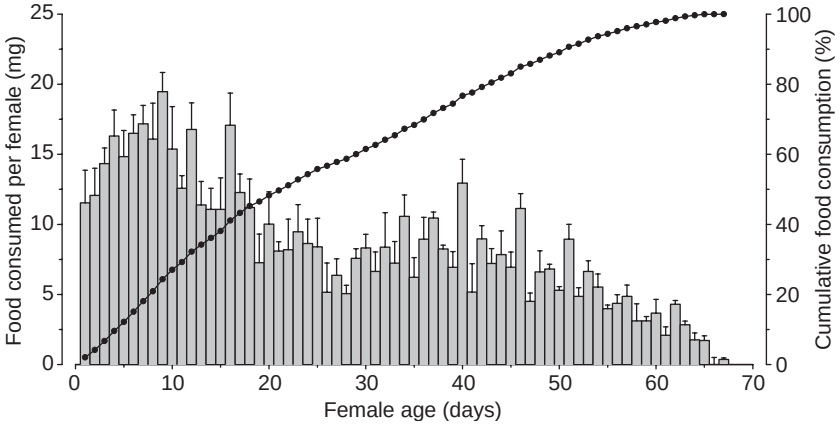

Fig. 4. Daily and cumulative food consumption by female *D. punctata* over the course of the reproductive cycle. Data are mean + SEM ($n = 5$ per mean).

significant, but the trend that embryos gained more than females lost probably reflects the joint contributions of both female storage and de novo biosynthesis to embryo HC content.

HC on the surface of the brood sac followed a pattern similar to that on the external surface of the embryo, increasing 8-fold between days 15 and 30 to a peak of $17.6 \pm 2.6\,\mu g$, then declining by 84% to day 70 (Fig. 3C). However, brood sac HC always represented a small fraction ($<2.7\%$) of total embryonic HC.

Total HC content of the female-embryo system (Fig. 2B) increased over the course of gestation, confirming that continued synthesis by the female (Fig. 2A), and to some extent embryos (Fig. 3A), result in total system HC gains. A simple linear regression of total-system HC content, as measured by GC, on cumulative biosynthesis, as measured by in vitro incubation of tissues, is highly significant ($R^2 = 0.813$, $P < 0.001$).

3.3. Feeding

Females ate throughout the reproductive cycle, with an early broad peak before oviposition on day 8, a minimum around day 27, and a secondary broad peak around day 40 (Fig. 4). After day 40, feeding gradually declined to undetectable levels just prior to parturition. Half of the cumulative food consumption took place in the first third of the reproductive cycle, and low levels of feeding occurred around the time of maximum HC biosynthesis.

4. Discussion

4.1. Patterns of HC biosynthesis and feeding related to reproductive mode

In contrast to oviparous and ovoviviparous cockroaches, which partition all nutrient investment to the developing oocytes over a short period of time, the viviparous *D. punctata* provisions its oocytes with yolk before oviposition and then continues to provision the developing embryos with protein and carbohydrate over the course of a 60-day gestation. The present results show that *D. punctata* sustains a similar continuous investment in embryonic HC. This is in contrast to the well-documented developmental course of *B. germanica*, an oviparous cockroach that forms a sclerotized ootheca and retains it externally until the nymphs hatch. *B. germanica* females exhibit high levels of food consumption and HC synthesis prior to oviposition (Cochran, 1983; Schal et al., 1994). During this time, HC are extensively transported to the ovaries, and female internal HC content drops by at least 50% upon oviposition (Schal et al., 1994, Fan et al., 2002). After oviposition, HC synthesis and feeding also decline, and "pregnant" females may fast for days at a time (Cochran, 1983; Schal et al., 1994).

Patterns of feeding and HC biosynthesis in female *D. punctata* reflect this contrast in reproductive strategy. Whereas in *B. germanica* HC synthesis peaks prior to oviposition, HC synthesis in *D. punctata* increases more than threefold from oviposition to mid-pregnancy, corresponding to greater maternal investment in embryos than in oocytes. Similarly, female internal HC content does not plunge at the time of oviposition, but declines gradually only after day 30, apparently due to increased transfer of HC to embryos. Furthermore, *D. punctata* females continue feeding measurably throughout pregnancy—this feeding is, in fact, necessary to sustain normal embryonic development, and females starved after oviposition are unable to sustain viable embryos (Stay and Coop, 1973).

We were unable to establish a close relationship between rates of feeding and HC synthesis as in *B. germanica*. *D. punctata* feeding peaked roughly ten days prior to peak HC synthesis, but the actual time lag

could not be assessed since feeding was measured daily, and HC synthesis every 10 days. Furthermore, the feeding study was conducted with two cohorts of females, one batch initiated at day 0 and the second batch initiated with day 32 females. Therefore, low levels of feeding around day 30, when HC synthesis was greatest, may have been caused by artificially depressed feeding in recently handled females. Nonetheless, the fact that all *D. punctata* females fed measurably throughout the reproductive cycle clearly distinguishes their nutritional strategy from that of oviparous and ovoviviparous cockroaches.

A causal link has been established between feeding and HC synthesis in *B. germanica*, in which starvation causes a subsequent cessation of HC production in both nymphs and adults (Schal et al., 1994; Young and Schal, 1997). The coincidence of feeding and HC synthesis in *D. punctata* indicates a similar relationship in this species, suggesting that HC biosynthesis is an expensive process whose sustenance requires extensive accumulation and processing of nutrients.

4.2. Patterns of embryonic HC accumulation

This study was preceded by the observation that *D. punctata* embryos are coated in waxy HC throughout gestation. Apparently of maternal origin, this coating occurs outside the chorion, and is most abundant between days 23 and 53 (Nelson et al., 2004). Nelson et al. (2004) collected this material from embryos of day 48 females; they recovered 6.4 μg of lipid per clutch, and found it to consist of 89% HC. The nearest time point in the present study was day 50, when 5.04 μg external HC was detected per clutch. If this quantity represents 89% of the total lipids coating the embryos, then each clutch would have had about 5.7 μg total lipids, a value that agrees well with that reported by Nelson et al. This study also corroborates the previous observation that the waxes were most abundant during the middle 50% of gestation; we found that embryo external HC increased markedly after day 20, and declined between days 30 and 70.

HC extracted from the everted brood sac followed a pattern similar to that of the embryos, but their relationship to the embryonic wax coating is unclear. The brood sac HC could be simply the brood sac's own cuticle, or a combination of brood sac cuticle, HC in transit to the embryos and HC of the wax coating the embryos. Finally, internal extracts from embryos included not only their internal stores, but also the developing embryonic and nymphal cuticles. As expected, embryonic internal HC content increased greatly after mid-pregnancy, representing the preparation of the nymphal cuticle for unprotected existence outside the female.

4.3. Source of embryonic HC

The source of embryonic HC, both internal and external, was almost exclusively maternal. Given that total HC accumulation in the female-embryo system on day 70 was 1695 μg, and that embryos accounted for only 0.36% of total synthesis, embryos could have been responsible for about 6 μg of this total. This amounts to 1% of the embryonic HC content of 628.9 μg on day 70. Thus the female must have contributed about 99% of the HC found in and on the embryos. This is in contrast to *B. germanica*, in which embryos, physiologically isolated from the female, synthesize about 19% of their HC content prior to hatching (Fan et al., unpublished data). Maternal contribution of HC to embryos in *D. punctata* is further evidenced by the simultaneous decrease in female HC and increase in embryonic HC after day 30. In *B. germanica*, maternal HC provisioned to the eggs are retained in the first and the second instar nymphs (Fan et al., unpublished data); it is possible that the even greater maternal contribution of HC in *D. punctata* represents a similarly long-term investment in the waterproofing of offspring. In this regard, *D. punctata* is particularly interesting because, unlike other cockroach species, development from neonates to adults may be completed in as few as three molt cycles (Holbrook and Schal, 2004).

4.4. Anatomical sites of HC biosynthesis

The tissue responsible for female HC synthesis was the abdominal integument, with sternites synthesizing more than tergites, as previously shown for *B. germanica* (Gu et al., 1995; Young et al., 1999). The specialized brood sac of *D. punctata* is derived from abdominal integument, and Nelson et al. (2004) suggested that the brood sac itself might synthesize the HC that are provisioned to the embryos, particularly those found on the exterior of the chorion. However, such synthesis was not found in this study. This result is consistent with an emerging body of evidence that insect HC synthesis occurs in oenocytes of both holometabolous and hemimetabolous species. In the cockroaches *P. americana* and *B. germanica*, oenocytes are associated with the abdominal integument, and, as in *D. punctata*, only preparations of the abdominal integument biosynthesize HC in vitro (reviewed in Schal et al., 2003). Thus, while temporal patterns of HC synthesis in *D. punctata* have diverged in association with its viviparous reproductive mode, the site of HC synthesis does not differ from that reported from other cockroaches.

Given that the brood sac does not synthesize HC, yet HC are transferred from mother to embryos, we suggest that HC are transported by lipophorin from oenocytes associated with the abdominal integument to the sites of deposition on and within embryos. Brood sac milk proteins are structurally related to lipocalins, a family of

proteins sometimes involved in transport of hydrophobic molecules (Williford et al., 2004). It is conceivable that milk proteins, in addition to their nutritive function, may serve as carrier proteins for HC that have already been transported from oenocytes to brood sac. However, they cannot serve in this capacity throughout the reproductive cycle, since eggs and embryos are already coated in HC prior to the production of brood sac milk (Nelson et al., 2004). Thus the wax coating over the embryos must pass across the brood sac by some other route.

Acknowledgements

This research was supported in part by the Blanton J. Whitmire Endowment at North Carolina State University, and grants from the NSF (IBN-9817075), and USDA-NRICGP (2002-35302-12488 and 2004-35302-14880). We thank R. Santangelo and J. Sun for technical help with rearing cockroaches.

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