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Lipophorin-facilitated hydrocarbon uptake by oocytes in the German cockroach *Blattella germanica* (L.)

Yongliang Fan[1], Jody Chase[2], Veeresh L. Sevala[1,*] and Coby Schal[1,†]

[1]*Department of Entomology, Box 7613, North Carolina State University, Raleigh, NC 27695-7613, USA* and [2]*Directorate for Education and Human Resources, National Science Foundation, 4201 Wilson Boulevard, Suite 875, Arlington, VA 22230, USA*

*Present address: Paradigm Genetics, Research Triangle Park, NC 27709, USA
†Author for correspondence (e-mail: coby_schal@ncsu.edu)

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Summary

Large amounts of hydrocarbons accumulate during vitellogenesis in the developing basal oocytes of the German cockroach *Blattella germanica* (L.), and all ovarian hydrocarbons are deposited into an egg case (ootheca) during oviposition. Hydrocarbons are not synthesized by the ovaries, but are delivered by hemolymph lipoproteins and accumulate within the basal oocytes. A native *B. germanica* hydrocarbon, [^{3}H]3,11-dimethylnonacosane, injected into adult females of various ages, was taken up by the ovaries in relation to oocyte growth. Ovarian uptake of the hydrocarbon was low in day 0–1 females, increased dramatically between days 3 and 6 and declined sharply through oviposition on day 8–9; ovarian uptake of the hydrocarbon was low during a 21-day pregnancy that followed. [1-^{14}C]Propionate, which becomes incorporated into methyl-branched hydrocarbons, was injected into 5-day-old vitellogenic females to monitor the *de novo* biosynthesis of hydrocarbons and the time course of hydrocarbon deposition in the ovary. Propionate was rapidly incorporated into hydrocarbons within 4 h. Hydrocarbon uptake by the ovaries, however, was three times higher 24 h after injection than 4 h after injection, showing that hydrocarbons are slowly and continuously deposited in oocytes. This result was confirmed with topical application of [^{3}H]3,11-dimethylnonacosane: ovarian uptake was three times higher after 24 h than after 4 h. *In vitro* incubations of sternites, which synthesize hydrocarbons, with [^{14}C]propionate and ovaries, showed that both hemolymph and purified high-density lipophorin facilitated ovarian uptake of newly synthesized hydrocarbons; maximum uptake occurred with 10 % hemolymph or 1 mg ml^{-1} high-density lipophorin. These results were confirmed with sternites treated with [^{3}H]3,11-dimethylnonacosane and co-incubated with ovaries. This is the first report to show that copious amounts of maternal hydrocarbons are provisioned in oocytes and to demonstrate the existence of a hydrocarbon transport pathway involving hemolymph high-density lipophorin.

Key words: *Blattella germanica*, cockroach, lipophorin, ovary, hydrocarbon transport, integument.

Introduction

All insects and plants depend on epicuticular lipids for water balance, protection from pathogens and environmental stresses, and some cuticular lipids are used as intra- and inter-specific cues, as pheromones and allelochemicals, respectively (Howard, 1993; Nelson and Blomquist, 1995). Hydrocarbons (HCs), represented mainly by normal and methyl-branched alkanes and alkenes, are the major cuticular lipids in insects, and their hydrophobic properties contribute significantly to water retention and water balance (Hadley, 1984; Gibbs, 1998).

Studies in different groups of insects implicate the oenocytes, large abdominal cells rich in smooth endoplasmic reticulum and mitochondria, as the site of HC synthesis (Romer, 1991). The tissue localization of oenocytes varies considerably among insects, suggesting that the site of HC synthesis in insects varies with the location of oenocytes. In *Tenebrio molitor*, the oenocytes are grouped along the upper side of the tracheal trunks, separated from the fat body (Romer, 1991), and in larvae of the butterfly *Calpodes ethlius* oenocytes are also found in the hemocoel (Jackson and Locke, 1989). In the locusts *Schistocerca gregaria* and *Locusta migratoria*, the oenocytes are found only in the peripheral fat body, which is situated beneath the abdominal epidermis (Diehl, 1975; Katase and Chino, 1984). The oenocytes of *Drosophila melanogaster* are within the abdominal epidermis, and complete feminization of the HC pheromone mixture produced by males was recently induced by targeted expression of the *transformer* gene in adult oenocytes (Ferveur et al., 1997), in support of the idea that

oenocytes synthesize HCs. In the American and German cockroaches *Periplaneta americana* and *Blattella germanica*, respectively, the oenocytes are also distributed near the epidermal cells of the abdominal tergites and sternites and are separated from the hemocoel by a basal membrane (Kramer and Wigglesworth, 1950; Liang and Schal, 1993).

A large proportion of the HCs are directed to the exterior of the cuticle. Clearly then, when oenocytes are situated in the hemocoel, a hemolymph transport pathway would be required for delivery of HCs to the epidermis and ultimately to the epicuticle. Such a pathway, involving hemolymph high-density lipophorin (HDLp), has now been described in many insects (for a review, see Schal et al., 1998b). Interestingly, however, it is also prominent in insects whose oenocytes are adjacent to epidermal cells in the integument. In all studies that have examined internal lipids, a large but developmentally regulated pool of internal HCs has been found (Dwyer et al., 1986; Guo and Blomquist, 1991; Gu et al., 1995; Young et al., 1999; Sevala et al., 1999, 2000), and in most of these studies, the internal HCs appear to be qualitatively similar to the cuticular HCs (Chino and Downer, 1982; Schal et al., 1994, 1998b; Gu et al., 1995; Sevala et al., 2000; Young et al., 2000). The rationale proposed for internal HCs is that hemolymph HDLp functions primarily to deliver HCs from oenocytes to integumental tissues that do not synthesize HCs, including the head and thorax (including the legs and wings).

However, our studies of adult *B. germanica* females indicate that a major fraction of the internal HCs is localized within oocytes, suggesting that an important function of hemolymph HCs and HDLp is to provision the developing oocytes as well as cuticular surfaces overlying regions that do not synthesize HCs. Because neither the fat body nor the gonads can synthesize HCs, large amounts of HCs must be transported from the oenocytes and deposited in the ovaries prior to ovulation (Gu et al., 1995). Moreover, as large amounts of HCs are found in the egg case (Schal et al., 1994, 1998b; Young et al., 2000), it appears that the female might provision her progeny with maternal HCs and possibly HDLp, the latter as a yolk protein precursor.

In many insects, almost half the yolk mass is composed of lipid imported by vitellogenin and lipophorin. Yet nothing is known about the mechanism by which HCs are deposited in insect oocytes, the role of HCs in embryonic development and HC–lipophorin interactions in the embryo. In this paper, we report the results of *in vivo* and *in vitro* analyses of HC transport and uptake by the ovary and the role of lipophorin in HC transport. We use the German cockroach *B. germanica* as a model system because (i) it is large and therefore readily amenable to physiological and biochemical approaches, (ii) its HCs have been chemically well characterized (Augustynowicz et al., 1987; Carlson and Brenner, 1988; Jurenka et al., 1989), (iii) the biochemical pathways of HC biosynthesis are well understood (Chase et al., 1990, 1992; Blomquist et al., 1993), (iv) the sites and developmental time course of HC synthesis are better known than in any other arthropod (Gu et al., 1995; Schal et al., 1994; Young et al., 1999), (v) HCs constitute the major lipid of lipophorin, representing up to 50 % of the lipids (Sevala et al., 1999), (vi) the ovaries and integument take up massive amounts of HCs (Schal et al., 1998b) and (vii) lipophorin is also taken up by the oocytes and therefore acts as a yolk protein precursor (see below). Thus, we combine the advantages of the well-characterized cockroach HCs and lipophorin system with physiological measurements and immunoassays to describe HC transport to the ovary.

Materials and methods

Chemicals

Sodium [1-^{14}C]propionic acid (2.04 GBq mmol^{-1}) was purchased from NEN Research Products (Boston, MA, USA). [11,12-^{3}H]3,11-Dimethylnonacosane (approximately 1.11 TBq mmol^{-1}) was a gift from Dr Glenn Prestwich (Salt Lake City, UT, USA). TC199 medium was purchased from Specialty Media (Lavallette, NJ, USA), and all the remaining chemicals were purchased from Sigma (St Louis, MO, USA). Organic solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Animals

The German cockroach *Blattella germanica* (L.) colony was maintained in incubators at 27±0.5 °C, approximately 50 % relative humidity and on 12 h:12 h light:dark photoperiod. Newly emerged adult females were separated from the colony on the day of adult eclosion (day 0) and maintained in separate plastic cages. Insects were provided with Purina 5012 Rat Chow (Purina Mills, St Louis, MO, USA) and water *ad libitum*. Females were mated on day 6 and they oviposited 2–3 days later under these conditions. Females were always maintained in groups because solitary females are reproductively repressed (Gadot et al., 1989; Holbrook et al., 2000).

Biosynthesis and transport of hydrocarbons in vivo *and* in vitro

Methylmalonyl CoA, derived from propionate, can serve as a methyl-branch donor in the synthesis of methyl-branched HCs in *B. germanica* (Chase et al., 1990). Since over 80 % of the HCs are methyl-branched (Jurenka et al., 1989), overall HC synthesis can be followed. [1-^{14}C]Propionate (16.65 kBq) in 0.5 µl of TC199 medium was injected into 5-day-old adult females. The females were provisioned with food and water in Petri dishes in an incubator at 27 °C. Ovaries were dissected after 4 or 24 h, rinsed thoroughly at least three times in saline, and HCs were extracted from the ovaries and the rest of the body separately, purified and assayed for radioactivity. *In vivo* HC transport and uptake experiments were confirmed with the native HC 3,11-dimethylnonacosane. Synthetic racemic [11,12-^{3}H]3,11-dimethylnonacosane (21.46 kBq) was either topically applied to the abdominal tergites or injected into the hemocoel in 0.4 µl of hexane. After predetermined incubation times, the ovaries were dissected, rinsed thoroughly, lipids were extracted, HCs purified and the radioactivity in the HC fraction analyzed by liquid scintillation spectrometry.

For *in vitro* studies, abdominal sternites 3 and 4 from 5-day-old virgin females were co-incubated with a pair of ovaries of various ages in 500 µl of TC199 medium [adjusted to 410 mosmol l^{-1} by the addition of 55 mmol l^{-1} NaCl and 40 mmol l^{-1} Hepes, pH 7.4, and sterilized by filtration through a 0.22 µm low protein binding filter (Millipore, Bedford, MA, USA) just prior to use] and 25.9 kBq of [1-^{14}C]propionate. All incubations were at 27 °C with constant shaking on an orbital waving shaker (VWR, Atlanta, GA, USA) to oxygenate the tissues. After 4 or 24 h incubations, the tissues were removed and analyzed for labeled HCs. As required, either purified HDLp or hemolymph was added to the medium. These experiments were repeated with labeled HC. Sternites 3 and 4 from 5-day-old virgin females were dissected and rinsed thoroughly in saline, and [^{3}H]3,11-dimethylnonacosane (21.46 kBq) was topically applied in 0.5 µl of hexane on the cuticular surface of the sternites. The sternites were co-incubated with ovaries in TC199 and processed as above.

Extraction and quantification of hydrocarbons

The surface lipids were extracted from egg cases as described by Young and Schal (1997). Briefly, each egg case was immersed in 2 ml of *n*-hexane containing 15 µg of *n*-hexacosane as internal standard, agitated gently for 5 min, and the solvent was decanted into a clean vial. This procedure was repeated, and the egg case was subjected to a final rinse with 1 ml of hexane. The three hexane extracts were combined and subjected to HC purification and analysis by gas chromatography.

Internal lipids were extracted by a modified Bligh and Dyer (1959) procedure (Gu et al., 1995). For samples to be analyzed by gas chromatography, 30 µg of *n*-hexacosane was added as internal standard. Lipids were extracted from various tissues by homogenization in water for 30 s (Kontes micro ultrasonic cell disruptor, Vineland, NJ, USA), and the homogenate was extracted with hexane:methanol:water (2:1:1). Samples were vortexed vigorously and centrifuged at 2000 ***g*** (IEC-Centra7, International Equipment, Needham Heights, MA, USA) for 10 min. A sample of the hexane phase was loaded onto a Biosil-A (Bio-Rad, Richmond, CA, USA) mini-column (approximately 500 mg of silica gel in a glass-wool-stoppered Pasteur pipette), and the HCs were eluted with 7 ml of hexane. The solvent was reduced with a gentle stream of N_2, and unlabeled HCs were quantified by gas chromatography whereas radioactive HCs were assayed in 3 ml of scintillation fluid (ScintiSafe EconoF, Fisher) by liquid scintillation spectrometry (LS5801, Beckman, Fullerton, PA, USA).

For gas chromatography analysis, the hexane was reduced to 1–2 µl with N_2 and analyzed on an HP 5890II (Hewlett-Packard, Palo Alto, CA, USA) gas chromatograph equipped with a flame-ionization detector and interfaced with an HP 3365II ChemStation. Splitless injection was made into a 25 m×0.32 mm×1 µm HP-1 capillary column operated at 150 °C for 2 min, then increased at 10 °C min^{-1} to 280 °C and held for 10 min. The injector and detector were held at 280 and 300 °C, respectively.

Hemolymph collection and lipophorin isolation and purification

Hemolymph was collected from ice-anesthetized day 2–4 females by severing the cerci and gently pressing the abdomen. Hemolymph was collected into chilled 1.5 ml microcentrifuge tubes containing cold saline and protease inhibitors (0.05 mol l^{-1} sodium phosphate buffer, pH 7.0, containing 0.15 mol l^{-1} NaCl, 10 mmol l^{-1} EDTA, 5 mmol l^{-1} glutathione, 2 mmol l^{-1} phenylmethyl sulfonyl fluoride, 10 µg ml^{-1} leupeptin and 10 µg ml^{-1} pepstatin), centrifuged at 735 ***g*** for 2 min at 4 °C to pellet the hemocytes, and the plasma was stored at –80 °C.

HDLp was purified by KBr density-gradient ultracentrifugation, as described by Shapiro et al. (1984) and previously applied to *B. germanica* by Gu et al. (1995) and Sevala et al. (1997, 1999). Briefly, plasma obtained from virgin females was mixed with 2.58 g of KBr in saline and adjusted to 5.8 ml. The KBr mixture was placed into a Beckman 13.5 ml QuickSeal tube, overlaid with 7.7 ml of freshly prepared 0.9 % NaCl, and subjected to ultracentrifugation with slow acceleration and deceleration at 285 000 ***g*** for 22 h at 4 °C in a Beckman L8-70M ultracentrifuge using a fixed angle rotor (70.1 Ti). Fractions of 400 µl were collected starting from the top of the tube. The purity of lipophorin fractions was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Pure lipophorin fractions were pooled, concentrated and dialyzed against 10 mmol l^{-1} phosphate-buffered saline, pH 7.4, using a Centricon-10 microconcentration tube with a 10×10^{3} molecular mass cut-off membrane (Amicon, Danvers, MA, USA). Protein concentration was measured by the Bradford method (Bradford, 1976) with bovine serum albumin as standard.

Enzyme-linked immunosorbent assay (ELISA)

The lipophorin titer in hemolymph, ovaries and egg cases was determined by indirect ELISA as described by Sevala et al. (1999), with minor modifications. Diluted (1:2000 to 1:8000) hemolymph (100 µl), ovary extract or egg case extract and a series of HDLp standards (1–100 ng ml^{-1}) in coating buffer (50 mmol l^{-1} sodium carbonate/bicarbonate buffer, pH 9.4) were bound to each well of an Immunoware high-binding 96-well ELISA plate (Pierce, Rockford, IL, USA) by incubating overnight at 4 °C. Several blanks were also included in each plate. The plates were rinsed three times with phosphate-buffered saline containing Tween-20 (8 mmol l^{-1} sodium phosphate, 2 mmol l^{-1} potassium phosphate, 140 mmol l^{-1} sodium chloride, 10 mmol l^{-1} potassium chloride and 0.05 % Tween-20, pH 7.4) and blocked for 1 h with 1 % bovine serum albumin at 37 °C. The wells were then filled with 100 µl of diluted HDLp antiserum in phosphate-buffered saline containing Tween-20 (1:500) and normal rabbit serum, and incubated for 1 h. Plates were washed three times and loaded with 100 µl of anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma) diluted 1:10 000 in phosphate-buffered saline containing Tween-20, and incubated for 1 h at 37 °C. Plates were washed again and developed at room

temperature with 100 µl of enzyme substrate, *p*-nitrophenyl phosphate (Pierce), and the reaction was stopped after 30 min by adding 100 µl of 2 mol l^{-1} NaOH to each well. Absorbance was read at 405 nm in a PowerWave200 automated microplate reader (Bio-Tek, Winooski, VT).

Results

Accumulation of hydrocarbons in the ovaries and egg case

The reproductive cycle of female *B. germanica* is depicted in the inset to Fig. 1. The German cockroach has panoistic ovarioles: the basal oocytes mature synchronously between days 0 and 9 in response to juvenile hormone (for a review, see Schal et al., 1997). All females were mated on day 6 and most oviposited on day 9 (Fig. 1). Although *B. germanica* is oviparous, females exhibit functional ovoviviparity: fertilized eggs are oviposited into an egg case which remains attached to the vestibulum of the female between days 9 and 28. During this 'pregnancy', the new basal oocytes in the ovaries remain repressed. After parturition, the female resumes a new vitellogenic cycle and the basal oocytes grow to approximately 2.5 mm in length (not shown).

Corrected for hemolymph volume (see Sevala et al., 1999), total HCs in the hemolymph declined in the young female soon after eclosion (Fig. 1), probably because large amounts of hemolymph HCs were deposited on the teneral epicuticle (Young and Schal, 1997; Young et al., 1999). Subsequently, hemolymph HC levels remained relatively constant at approximately 160 µg through the remainder of the reproductive cycle. In contrast, levels of ovarian HCs changed considerably in relation to oocyte maturation and the reproductive cycle. Levels of ovarian HCs increased during oocyte maturation and declined sharply at oviposition on day 9. Subsequently, the ovaries did not accumulate HCs during 'pregnancy' (days 9–25). While the exterior of the egg case contained only 22.6 µg of HCs, its interior contained 224.6 µg, which approximately equaled the difference between the ovarian HC contents before and after oviposition (231.6 and 8.2 µg, respectively). Thus, copious amounts of HCs accumulate in the ovaries and are then transferred into the oviposited egg case. This pattern is also evident in the second reproductive cycle: day 25 ovaries contained only 0.7 µg of HCs, while the ovaries and egg case accumulated 198.8 µg of HCs by day 34. Newly formed egg cases contained 6.00±0.287 mg lipids (mean ± S.E.M., *N*=6), representing 16.3±0.77 % of the fresh mass of the egg case, while internal HCs represented 4.48±0.58 % of total egg case lipids.

These data, coupled with previous results showing that the ovaries do not synthesize HCs (Gu et al., 1995), demonstrate that HCs must be shuttled to the ovaries through the hemolymph. Moreover, transfer of ovarian HCs to the interior of the egg case suggests that ovarian HCs are deposited into the maturing basal oocytes and not the follicle cells that encase them. We examined the hemolymph–ovary transport pathway both *in vivo* and *in vitro*.

In vivo *biosynthesis and transport of hydrocarbons to the ovaries*

Because HC synthesis is developmentally regulated in adult females (Schal et al., 1994), females of different ages were injected with 21.46 kBq of [^{3}H]3,11-dimethylnonacosane, the major HC in the native HC profile, to control for HC synthesis and therefore examine only its distribution within the female. Females were incubated for 24 h at 27 °C, their epicuticular lipids extracted with hexane, the ovaries were dissected and thoroughly rinsed, and cuticular and ovarian HCs were purified and assayed by liquid scintillation spectrometry. HC uptake by the ovaries was low on days 0–2, increased on days 3–4, peaked on days 5–6 and declined sharply thereafter (Fig. 2A). The ovaries of pregnant females took up no HCs, as was also indicated by the gas chromatography data (Fig. 1), and neither did the egg case on days 16 and 26; the exterior

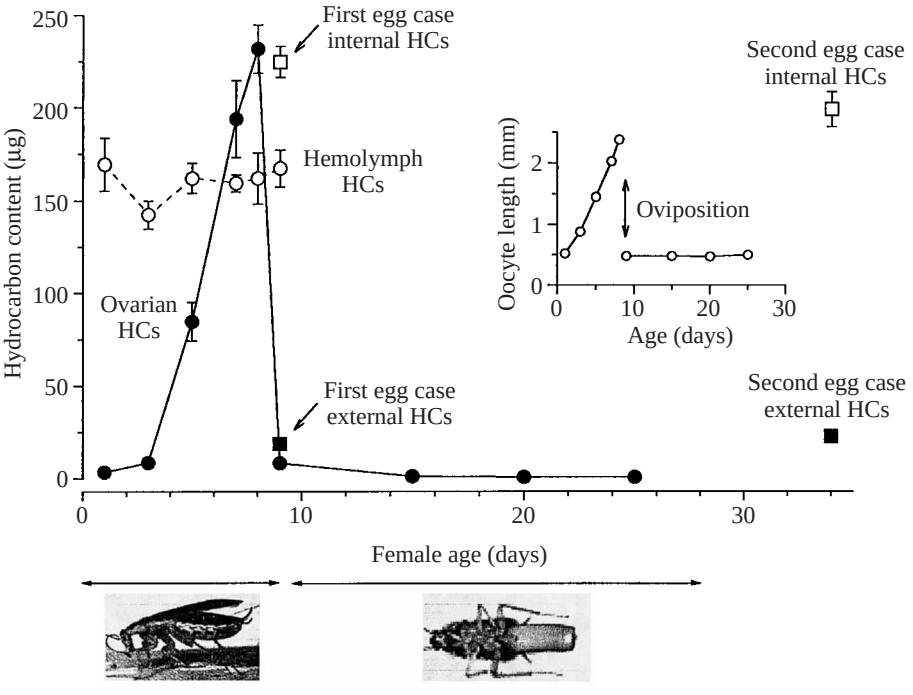

Fig. 1. Hydrocarbon (HC) content of the hemolymph, ovaries and first and second egg cases. Hydrocarbon content was determined by gas chromatography. Inset: time course of the reproductive cycle, represented by oocyte length; oviposition occurs on day 9 and pregnancy follows. The images represent a vitellogenic female (left) between days 0 and 8 and a 'pregnant' female (right) between days 9 and 28. Values are means ±1 S.E.M.; some error bars are obscured by the symbol. *N*=7 for each mean except on day 7, where *N*=5, and day 8, where *N*=9.

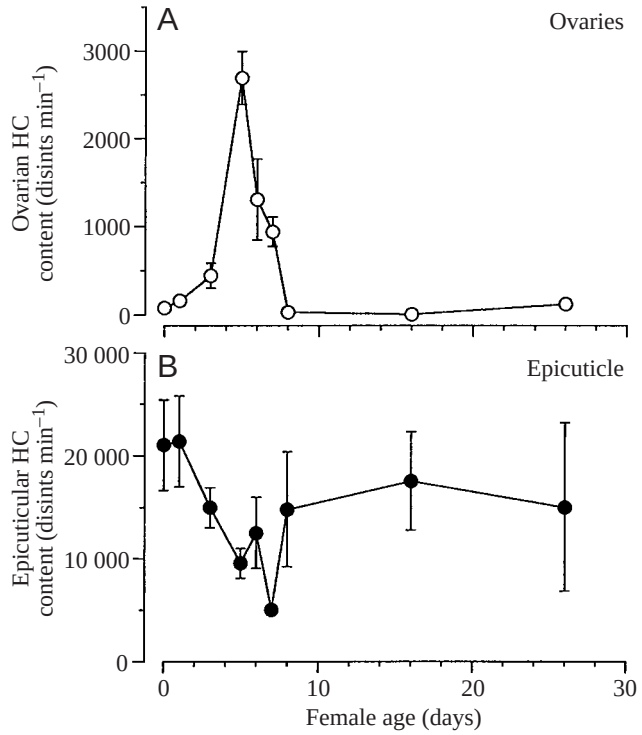

Fig. 2. Age-dependent *in vivo* hydrocarbon (HC) deposition in the ovary and epicuticle in relation to the reproductive cycle. [^{3}H]3,11-Dimethylnonacosane (21.46 kBq) was injected in 0.4 µl of hexane into the hemocoel of females of various ages, and uptake of [^{3}H]3,11-dimethylnonacosane by the ovary (A) and epicuticle (B) was assayed 24 h later. Values are means ±1 S.E.M.; some error bars are obscured by the symbol. *N*=8 for each mean, except on day 0, where *N*=10, day 1, where *N*=13, day 6, where *N*=6, and day 8, where *N*=9.

of these egg cases contained approximately 200 disints min^{-1} of HC (data not shown), probably from contamination from the cuticular surface.

Interestingly, when HCs were not directed to the ovaries, more HCs were deposited on the epicuticular surface (Fig. 2B). As expected, the teneral cuticle received large amounts of HCs (1.7 % of injected HCs). As HC uptake by the ovaries increased, the HCs delivered to the epicuticle declined by 69 % and, subsequently, as the ovaries of pregnant females were refractory to HC deposition, cuticular uptake increased 2.6-fold.

We compared the time course of HC biosynthesis and its deposition in the ovaries by measuring the incorporation of [^{14}C]propionate into HCs in the ovaries and the rest of the body 4 and 24 h after injection. Young and Schal (1997) showed that, after injection of radiolabeled propionate into *B. germanica* nymphs, incorporation into HCs was complete within 2–4 h. The *in vivo* data presented in Table 1 confirm that HC biosynthesis was rapid. Within 4 h, 132×10^3 disints min^{-1} was incorporated into HCs and no further HCs were synthesized by 24 h (116 978 disints min^{-1}; P>0.05; Student's t-test). However, the distribution of the newly biosynthesized methyl-branched HCs changed during the 24 h incubation. While the amount of labeled HCs in the ovaries increased 3.7-fold, the amount of labeled HCs in the rest of the body declined by 23.3 % at 24 h (Table 1).

Table 1. In vivo *hydrocarbon biosynthesis and transport*

	Hydrocarbon synthesis and distribution (disints min^{-1})	
Tissue	4 h	24 h
Ovary pair	5315±695	19 867±2879
Rest of the body	126 651±7803	97 111±6486

Day 4 females were injected with 16.65 kBq of [1-^{14}C]propionate, and the ovaries were dissected 4 h or 24 h after injection. Hydrocarbons were extracted from the ovaries and the rest of the body, and radioactivity was assayed by liquid scintillation spectrometry.

Values are means ±1 S.E.M. (*N*=5).

These results were confirmed with topical application of [^{3}H]3,11-dimethylnonacosane (21.46 kBq) to the tergites of day 5 females. Both ovaries were dissected after 4 or 24 h, and the amount of radioactive HCs was analyzed. After 4 h, the ovaries contained 9574±958 disints min^{-1} of HCs (mean ± S.E.M., *N*=5), representing only 0.75 % of the topically applied HC. However, 24 h after topical application of the labeled HC, 2.7 times more labeled HCs appeared in the ovaries, representing 2 % (25 690±2256 disints min^{-1}) of total labeled HCs.

Involvement of lipophorin in hydrocarbon transport to the ovaries

The hydrophobic nature of HCs suggests that plasma lipoproteins might shuttle them from the integument to storage and deposition sites. Indeed, in a number of insect species, hemolymph HCs associate with an HDLp (Chino, 1985; Schal et al., 1998b; Sevala et al., 2000). We tested the hypothesis that HDLp delivers HCs to oocytes by quantifying HC uptake by the ovary in the presence of either hemolymph or HDLp. Sternites 3 and 4 from 4-day-old virgin females were co-incubated for 24 h with an ovary from day 5 females in the presence of 25.9 kBq of [1-^{14}C]propionate. With TC199 medium alone, large amounts of HCs were biosynthesized (95 918±24 946 disints min^{-1}, mean ± S.E.M., *N*=5), but most of the HCs remained with the sternites and only 78±29 disints min^{-1} was deposited into the ovary; the amount deposited in the ovary was subtracted as background from all other incubations. The amount of labeled HCs in the ovary increased with increasing hemolymph concentrations in TC199, reaching a plateau at 10 % hemolymph (Fig. 3).

The capacity of HDLp to accept HCs from the integument was also tested by incubating sternites 3 and 4 with 0–2.0 mg ml^{-1} HDLp in the presence of labeled propionate. HDLp was isolated from plasma by KBr density-gradient equilibrium ultracentrifugation and its purity confirmed by SDS–PAGE (Fig. 4A). Our results indicated that loading of newly synthesized HCs onto HDLp increased linearly in

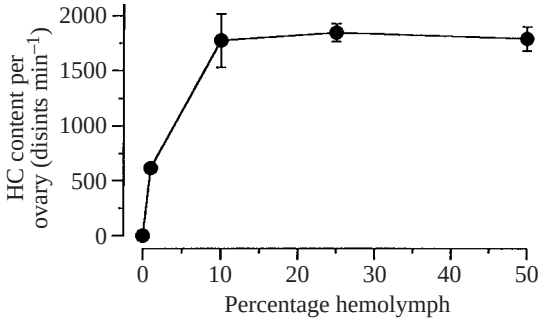

Fig. 3. Accumulation of newly synthesized hydrocarbons (HCs) in the ovary *in vitro*. An ovary from a day 4 female was co-incubated with abdominal sternites 3 and 4 from a day 5 female in 500 µl of TC199 medium containing 25.9 kBq of [1-^{14}C]propionate and 0–50 % *Blattella germanica* plasma. Values are means ±1 S.E.M. (*N*=3–5). Some error bars are obscured by the symbol.

relation to the amount of HDLp in the medium (Fig. 4B). HDLp is thus necessary to effect translocation of HCs from the integument to the hemolymph, and its presence would be absolutely mandatory for transferring newly synthesized or stored HCs to the ovaries.

This was confirmed with sternites and ovary co-incubations. Without HDLp, most of the labeled HCs remained associated with the sternites, and only 137±24.5 disints min^{-1} (mean ± S.E.M., *N*=5) was deposited in the ovary; this amount was subtracted as background from subsequent incubations. Incubations of an ovary with varying concentrations of HDLp yielded similar results to incubations with hemolymph, with maximum deposition of HCs in the ovary occurring at concentrations of HDLp above 1 mg ml^{-1} (Fig. 4C). Interestingly, this represents approximately 11 % hemolymph, because the HDLp concentration in hemolymph is approximately 9 µg µl^{-1} (Sevala et al., 1999). Furthermore, this transfer occurred without facilitation by a lipid transfer particle (LTP).

The relationship between ovarian uptake of HCs and ovarian age was examined *in vitro* using co-incubations of sternites 3 and 4 from day 5 virgin females together with ovaries of various ages and 1 mg ml^{-1} HDLp. [^{3}H]3,11-Dimethylnonacosane was topically loaded onto the sternites so that only transfer of labeled HC to the ovary was considered. As expected from the *in vivo* results (Figs 1, 2), HC uptake increased steadily with growth of the ovary and peaked on day 8, just before ovulation; it declined sharply after oviposition on day 9 (Fig. 5A). To relate ovarian uptake of HCs to their physiological stage, ovaries from day 6–9 females were co-incubated *in vitro* with sternites from day 5 females, [^{14}C]propionate and 10 % hemolymph. Day 7 ovaries took up the largest amount of ^{14}C-labeled HCs (Fig. 5B).

Lipophorin uptake by oocytes

Developmental studies of the lipophorin content of the ovaries and egg case showed that the ovaries sequester lipophorin from the hemolymph (Fig. 6), and the increase in lipophorin content within the ovaries corresponded well with the increase in the HC content of the ovaries (Fig. 1). Moreover, as shown for HCs, almost all ovarian lipophorin is subsequently recovered from the egg case. The trivial amount of lipophorin remaining in the ovaries disappears by day 15 (data not shown), suggesting that one or more oocyte was resorbed during ovulation.

Discussion

Oocyte maturation in oviparous animals is characterized by a vitellogenic period during which all maternally derived yolk protein precursors are sequestered in the oocyte, usually through receptor-mediated endocytosis (Bu and Schwartz, 1994; Sappington and Raikhel, 1995, 1998; Raikhel and Snigirevskaya, 1998; Burmester and Scheller, 1999). Large amounts of maternal lipids are also sequestered (Speake and Thompson, 1999), and lipid accumulation in eggs has been reported in many insects (for reviews, see Engelmann, 1970; Kunkel and Nordin, 1985). Important egg lipids are phospholipids for membrane formation during embryogenesis, and triacylglycerol is used mainly as an energy source.

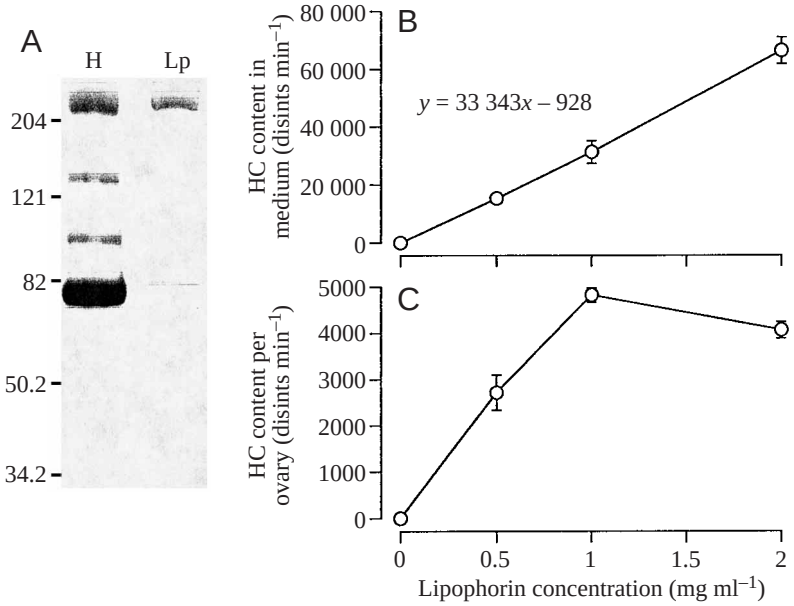

Fig. 4. Accumulation of newly synthesized hydrocarbons (HCs) in the medium and ovary *in vitro*. An ovary from a day 4 female was co-incubated with abdominal sternites 3 and 4 from a day 5 female in 500 µl of TC199 medium containing 25.9 kBq of [1-^{14}C]propionate and various concentrations of purified *Blattella germanica* lipophorin. (A) SDS–PAGE of hemolymph (H) and purified lipophorin (Lp). (B) Accumulation of newly synthesized hydrocarbons in the medium; r^2=0.999, P<0.0001. (C) Accumulation of newly synthesized hydrocarbons in the ovary. Values are means ±1 S.E.M. (*N*=3–5). Some error bars are obscured by the symbol.

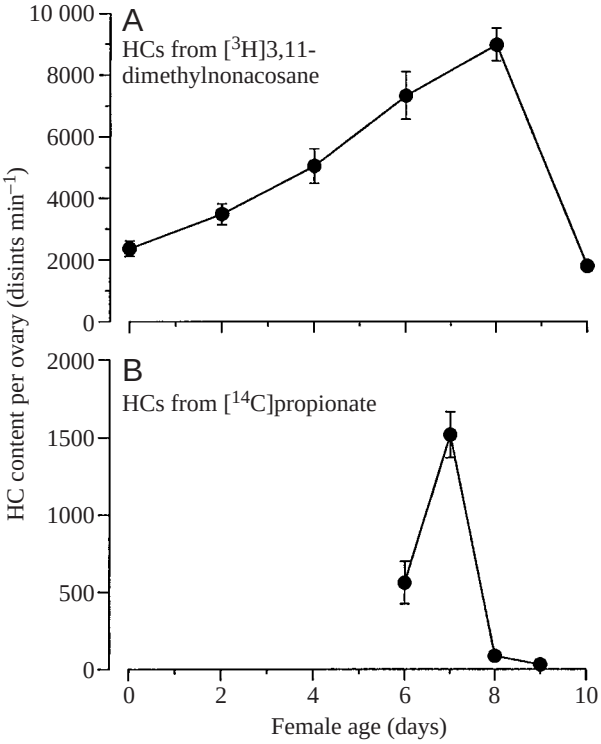

Fig. 5. Ovarian uptake of hydrocarbons (HCs) *in vitro*. (A) [^{3}H]3,11-Dimethylnonacosane (21.46 kBq) was topically applied onto sternites 3 and 4 of a day 5 female, and the sternites were then co-incubated in TC199 with ovaries of different ages in the presence of 1 mg ml^{-1} purified high-density lipophorin (HDLp). [^{3}H]3,11-Dimethylnonacosane levels in the ovary were assayed by liquid scintillation spectrometry. (B) Sternites 3 and 4 from a day 6 female were co-incubated with an ovary from a day 6, 7, 8 or 9 female in 500 µl of TC199 medium containing 10 % hemolymph and [1-^{14}C]propionate (25.9 kBq). Both sets of incubations were at 27 °C for 24 h. Values are means ±1 S.E.M. (*N*=8). Some error bars are obscured by the symbol.

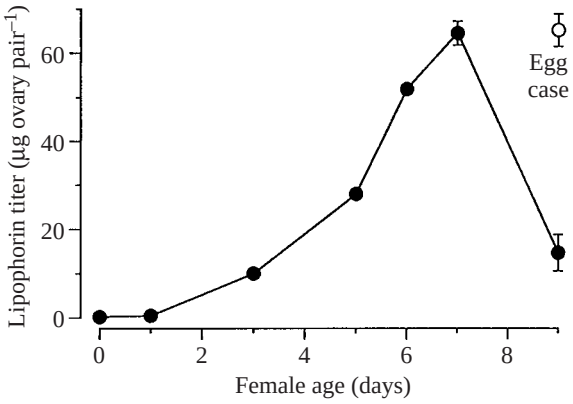

Fig. 6. Accumulation of lipophorin in ovaries *in vivo*. The amount of lipophorin in the ovaries and egg case was determined by ELISA. Values are means ±1 S.E.M. (*N*=9). Some error bars are obscured by the symbol.

Surprisingly though, there are few quantitative studies on apolar lipids in insect eggs, even though both embryos and neonates in most insects require extensive waterproofing. Nelson and Sukkestad (1970) found complex mixtures of branched HCs, including mono-, di- and trimethyl HCs in eggs of the moth *Manduca sexta* and, surprisingly, the HC content of egg lipids is high (9.45 %) (Kawooya and Law, 1988) compared with that of *B. germanica* (4.85 %). Kawooya and Law (1988) calculated that there is twice as much sterol and 10 times more HCs in the eggs of *M. sexta* than can be attributed to the combined total of egg lipophorin and vitellogenin. Although HCs appear to represent a significant lipid class in insect eggs, we are unaware of other quantitative studies of HCs in insect eggs.

Our previous investigations with *B. germanica* revealed more than 400 µg of 'internal' HCs just before ovulation (Schal et al., 1994). This amount declined dramatically after oviposition, suggesting that the eggs were provisioned with HCs and constituted a major fraction of internal HCs in adult females. Herein, we confirmed that 232 µg of these internal HCs is deposited in the ovaries, much more than the less than 160 µg in the hemolymph (Fig. 1) or approximately 150 µg found on the epicuticular surface of the female (Schal et al., 1994; Young et al., 1999). Moreover, we showed that all ovarian HCs were transferred to the egg case, confirming that HCs are localized within the oocytes and not in other ovarian tissues, such as follicle cells. Since the ovaries do not synthesize HCs *de novo* (Gu et al., 1995), a hemolymph transport pathway must be involved, and our results demonstrate that hemolymph is indeed required to effect the transfer of HCs from the sternal integument (probably oenocytes) to the ovary (Fig. 3).

Seminal work by Chino and co-workers showed that in several insect species hemolymph HCs are associated with HDLp, a major hemolymph lipoprotein of 600 kDa. In most insects, HDLp is characterized by two constituent apoproteins, apoLp-I and apoLp-II, with approximate molecular masses of 240 kDa and 80 kDa, respectively (Fig. 4A) (Chino et al., 1981; Chino, 1985; Law and Wells, 1989; Kanost et al., 1990; Law et al., 1992; Van der Horst et al., 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994; Arrese et al., 2000). HDLp serves multiple functions, including that of a juvenile hormone binding protein in beetles, termites, flies, bees and cockroaches (Trowell, 1992; King and Tobe, 1993; Sevala et al., 1997; Engelmann and Mala, 2000). Its most recognized function, however, is in bulk transport of lipids, including phospholipids, diacylglycerol (DAG), carotenoids, hydrocarbons and, in some mosquitoes, triacylglycerol (Ford and Van Heusden, 1994). Lipids may comprise up to 50 % of the mass of lipophorin, but its lipid composition varies greatly among species and probably in relation to physiological stages.

In many insects, HCs constitute the major lipid carried by HDLp, and the dynamic transformations of lipophorin may be more closely related to reproductive physiology in adults and the periodic replenishment of HCs in the new cuticle of immatures than with DAG-fueled flight. In cockroaches, termites, locusts, beetles, flies and moths, HDLp shuttles HCs to the cuticle, and in some insects, to specialized pheromone glands (for reviews, see Schal et al., 1998a,b). The fat body,

too, contains HCs (Gu et al., 1995; Young et al., 1999), and our present results show that the ovaries also serve as an important HC deposition site in females. Because lipophorin is the major HC transporter in the hemolymph, the HC profiles of the hemolymph, lipophorin and cuticle are generally similar (e.g. Chino and Downer, 1982; Schal et al., 1998b; Sevala et al., 2000). In some insects, male and female HDLp carry different HCs, some of which serve as sex pheromones, and the sex-specific composition of lipophorin is determined by the types of HCs biosynthesized by sex-specific oenocytes [for example, cockroach and locust (Katase and Chino, 1984; Gu et al., 1995), *Drosophila melanogaster* (Pho et al., 1996) and moths (Schal et al., 1998a)] and not by features of lipophorin.

In the moth *M. sexta*, a lipid transfer particle (LTP) plays a role in the delivery of lipids to the developing oocytes and the conversion of adult HDLp to egg very high density lipophorin (Liu and Ryan, 1991). In some insects, LTP also carries HCs (Blacklock and Ryan, 1994), and in the American cockroach HCs comprise 40 % of the lipids in LTP, while HDLp carries only 28 % of HCs; however, the latter is much more abundant in the hemolymph (Takeuchi and Chino, 1993). However, our results, together with previous *in vitro* work with purified HDLp (Katase and Chino, 1982, 1984), suggest that LTP is not necessary for exchange of HCs between the integument and HDLp and between HDLp and the ovary. We found maximum HC uptake by the ovary from sternites with either 10 % hemolymph (Fig. 3) or 1 mg ml^{-1} HDLp (Fig. 4C). Since the concentration of HDLp in the hemolymph of the German cockroach is approximately 9 mg ml^{-1} (Sevala et al., 1999), the *in vitro* uptake data agree well with the *in situ* conditions. Nevertheless, it is possible that LTP may be associated with, or even synthesized by, the incubated tissues, and these experiments will have to be repeated with LTP antibodies to block LTP function.

Unlike vertebrate lipoproteins, insect lipophorin is generally considered to be a reusable particle that shuttles lipids among tissues without entering cells (Chino and Kitazawa, 1981; Van Heusden et al., 1991). Chino et al. (1977) suggested that lipophorin must act as a reusable lipid shuttle because insect eggs contain larger amounts of lipid than could be accounted for by ovarian lipophorin and vitellogenin. Indeed, in some insects, no lipophorin can be detected in oocytes or it represents only a minor fraction of total egg protein, for example in *Rhodnius prolixus* (Gondim et al., 1989), suggesting that lipophorin acts primarily as a shuttle. In other insects, however, oocytes sequester lipophorin, and ovarian lipophorin is physically and immunologically identical to hemolymph lipophorin, suggesting a common origin (Thomas and Gilbert, 1969; Chino et al., 1977). Furthermore, immunocytological observations and tracking of [^{3}H]DAG-labeled lipophorin and [^{35}S]apoLp-labeled lipophorin showed that HDLp from adult *M. sexta* hemolymph was sequestered by oocytes without recycling the lipophorin back to the hemolymph (Kawooya and Law, 1988; Kawooya et al., 1988; Van Antwerpen et al., 1993). In the moth *Hyalophora cecropia*, HDLp and vitellogenin enter the oocytes through the same endocytic mechanism, presumably involving receptor-mediated processes; HDLp is then converted into very high density particles within the eggs (Kulakosky and Telfer, 1990; Telfer and Pan, 1988; Telfer et al., 1991). The clearest documentation of lipophorin uptake into oocytes comes from recent studies of the mosquito *Aedes aegypti*. During the first vitellogenic cycle, *Lp* gene expression is upregulated after a blood meal and hemolymph lipophorin is transported into the yolk granules of the developing oocyte (Sun et al., 2000). The lipophorin receptor in the mosquito oocyte is distinct from the vitellogenin receptor (Cheon et al., 2001), unlike in *Hyalophora cecropia* oocytes (Kulakosky and Telfer, 1990).

An early study identified non-vitellogenin proteins with a high lipid content, presumably lipophorin, in ovaries of *B. germanica* (Kunkel and Pan, 1976). Our polyclonal antibodies against hemolymph HDLp recognize ovarian proteins in this species (Fig. 6), suggesting that lipophorin enters the oocytes. Yet, the amount of lipophorin in the ovaries appears to be low relative to the amount of vitellin, and a calculation of the HC:lipophorin ratio suggests that not all HCs enter the oocytes with internalized lipophorin. We recovered 232 μg of HCs from a mature ovary pair containing approximately 40 basal oocytes (Fig. 1), but only 65 μg of lipophorin (Fig. 6). Since all hemolymph HCs are carried by HDLp, and the hemolymph contains approximately 9 μg HDLp μl^{-1} and approximately 10 μg HCs μl^{-1} (Sevala et al., 1999), it follows that 1 μg of HDLp carries approximately 1 μg of HCs. Clearly, HDLp must either unload the bulk of the HCs at the oocyte surface (as a reusable shuttle) or enter the basal oocytes, unload HCs and other lipids and recycle to the hemolymph. Regardless, the age-related uptake of HC and HDLp in the ovaries is identical to vitellogenin uptake, suggesting either that common receptors are used or a coordinated proliferation of independent receptors for vitellogenin and lipophorin. Interestingly, it appears that, in *Manduca sexta*, 90 % of the lipids that accumulate in the egg are delivered by low-density lipophorin that is not internalized (Kawooya and Law, 1988). Cockroaches do not have low-density lipophorin, leaving open the question of how HCs might be deposited in the ovaries.

In the cockroach *Diploptera punctata*, HDLp content increases to approximately 1.5 μg per oocyte early in vitellogenesis, but declines rapidly to non-detectable levels in ovulated eggs (King and Tobe, 1993). This pattern indicates that lipophorin shuttles out of the oocytes, that it is rapidly metabolized within the oocytes or that lipophorin is associated only with follicle cells and not with oocytes. Notably, in this viviparous cockroach, the bulk of maternal lipids is taken up by embryos, not oocytes. In ovoviviparous *Leucophaea maderae*, much smaller amounts of lipophorin than vitellogenin enter the eggs, as in *B. germanica*, but nothing is known about its ovarian HCs. It would be of interest to relate the reproductive mode of cockroaches (oviparity, ovoviviparity, viviparity) to maternal strategies of provisioning HCs into the eggs. The relatively high HC content of *B. germanica* HDLp, compared with other insects, including other cockroaches (e.g. *Periplaneta americana*) (Chino and Kitazawa, 1981), is probably related to

its role in supplying HCs to a large batch of externally incubated oocytes, and it is possible that less HCs are provisioned in cockroaches that internally incubate their embryos.

It follows then, that in *B. germanica*, even though HDLp serves many functions, including that of a juvenile-hormone-binding protein, its titer will fluctuate in relation to shuttling HCs (Sevala et al., 1999). Soon after eclosion, the teneral cuticle has a large demand for HCs (Fig. 2B) (Schal et al., 1994). As the oocytes begin to grow, they too sequester large amounts of HCs, and the female meets these increasing demands for apolar lipids by producing more HCs and HDLp, elevating hemolymph volume (Sevala et al., 1999) and redirecting HC deposition away from the cuticle (Fig. 2B).

The mechanism(s) of HDLp-mediated delivery of HCs to oocytes, the function(s) of lipophorin within the oocyte and the fate of ovarian lipophorin and HCs have not been studied in any insect, and recent investigations of lipid accumulation in the ovaries of other arthropods have yet to identify lipoprotein carriers (Ravid et al., 1999). It is reasonable to hypothesize that maternal HCs serve to provision the embryonic and neonate cuticle with a waterproofing layer. We have shown previously that the female provisions the exterior of her egg case with an HC profile that has a melting temperature 15 °C higher than that of her own cuticular HCs; this would provide the egg case with superior waterproofing (Young et al., 2000). Our preliminary radiotracer experiments show that maternal HCs that are deposited in the egg appear on the neonate epicuticle (C. Schal, unpublished results). Moreover, although the embryo can synthesize HCs *de novo* around dorsal closure (Y. Fan and C. Schal, unpublished results), their contribution to the neonate cuticle is relatively minor. Our working hypothesis, thus, is that maternal lipophorin within the embryo serves to transport maternally sequestered HCs to the embryonic and neonate cuticles.

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