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Lipophorin of female *Blattella germanica* (L.): characterization and relation to hemolymph titers of juvenile hormone and hydrocarbons

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Abstract

High density lipophorin (HDLp) from the hemolymph of the German cockroach, *Blattella germanica* (L.) (Family Blattellidae), has an apparent molecular weight of 670 kDa, with an isoelectric point of 7.0 and a density of 1.109 g/ml. It is composed of two subunits, apolipoprotein-I (212 kDa) and apolipoprotein-II (80 kDa), and consists of 51.4% lipid, 46.2% protein and 2.4% carbohydrate. Hydrocarbons constitute 42.2% of the total lipids which also contain diacylglycerol, cholesterol and phospholipid. Lipophorin is rich in the amino acids glutamic acid, aspartic acid, lysine, valine, and leucine. Specificity of a polyclonal antibody was demonstrated by Western blotting and Ouchterlony immunodiffusion: the antiserum recognized native HDLp and apolipoprotein-I, but not apolipoprotein-II, purified vitellin, or other hemolymph proteins. It also recognized a protein in the hemolymph of *Supella longipalpa* (Blattellidae) but did not cross-react with hemolymph proteins from *Periplaneta americana* (Blattidae) or *Diploptera punctata* (Blaberidae). An enzyme-linked immunosorbent assay was developed to measure the HDLp titer in the hemolymph of adult females. The titer of HDLp, a juvenile hormone binding protein, exhibited no clear relationship to the changing titer of juvenile hormone in hemolymph. The hemolymph titer of hydrocarbon, which is also carried by HDLp, showed some functional relation to the concentration of HDLp in the hemolymph. Because it concurrently serves multiple functions in insect development and reproduction, lipophorin titer might covary with the titers of lipid ligands that occur at high concentrations and require extensive shuttling through the hemolymph.

Keywords: *Blattella germanica*; Lipophorin; Juvenile hormone; Hydrocarbon; Hemolymph volume; ELISA

1. Introduction

Lipophorin (Lp) is a ubiquitous hemolymph lipoprotein that is widely distributed among insects. It has been purified and characterized from the hemolymph of insect species in the Diptera (*Sarcophaga bullata*, Van Mellaert et al., 1985; *Aedes aegypti*, Van Heusden et al., 1997; *Drosophila melanogaster*, Fernando-Warnakulasuriya and Wells, 1988), Hymenoptera (*Apis mellifera*, de Kort and Koopmanschap, 1986), Coleoptera (*Leptinotarsa decemlineata*, de Kort and Koopmanschap, 1987), Lepidoptera (several species, see review: Soulages and Wells, 1994), Hemiptera (*Triatoma*, Ganzalez et al., 1991; *Rhodnius*, Coelho et al., 1997), Orthoptera (*Locusta migratoria*, Chino and Kitazawa, 1981), Isoptera (several species, Okot-Kotber and Prestwich, 1991), and Dictyoptera (*Periplaneta americana*, de Kort and Koopmanschap, 1986; *Leucophaea maderae*, Rayne and Koeppe, 1988; *Nauphoeta cinerea*, Kindle et al., 1989; *Diploptera punctata*, King and Tobe, 1988; *Blattella germanica*, Gu et al., 1995). Structural studies indicate that Lp is a glycolipoprotein with a density of 1.020 to 1.270 g/ml (Beenakkers et al., 1988). High density Lp (HDLp; 1.063 to 1.210 g/ml) contains up to 50% lipids and the protein moiety consists of two apoproteins: apolipoprotein-I (apoLp-I; 220–250 kDa) and apolipoprotein-II (apoLp-II; 70–80 kDa) (for reviews, see Chino, 1985; Kanost et al., 1990; Law et al., 1992; Van der Horst et al., 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994).

Lipophorins serve multiple functions, including as plasma coagulagens that mediate insect immune responses (Bohn, 1986) and as hemostatic agents (Coodin and Caveney, 1992). As a carrier of lipids,

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HDLp facilitates absorption of neutral and polar lipids from the gut, and it transports diacylglycerols from the fat body to muscle to fuel flight (Chino, 1985; Van der Horst et al., 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994). Lipophorin also delivers pheromones to the cuticle (Gu et al., 1995; Pho et al., 1996) and specialized pheromone glands (Schal et al., 1998a), hydrocarbons to the epicuticle, fat body, and ovaries (Chino et al., 1977; Chino, 1985; Katagiri and de Kort, 1991; Gu et al., 1995; Schal et al., 1998b), and retinoids to yet undetermined locations (Kutty et al., 1996). It has specific, high-affinity binding sites for juvenile hormone (JH) III in species in the Coleoptera, Isoptera, Diptera, Hymenoptera and Dictyoptera (Trowell, 1992; Sevala et al., 1997) and, in this context, the primary functions of HDLp are thought to be facilitation of transport of the hydrophobic hormone from the site of synthesis to target tissues (Whitmore and Gilbert, 1972) and protection of the hormone from enzymatic degradation by hemolymph esterases and epoxide hydrolases (Sanburg et al., 1975a, b; Hammock et al., 1975; Goodman, 1990; Lanzrein et al., 1993; Touhara et al., 1996). Thus, HDLp has been implicated in the regulation of hemolymph hormone titers during critical stages of development and reproduction. Lipophorin also binds carotenoids and hydrophobic xenobiotics, but the degree of selectivity and function(s) of these interactions remain to be elucidated.

The density of Lp and its lipid composition vary widely among species and developmental and physiological stages, probably reflecting its immediate function. High density Lp of *Blattella germanica* binds JH III with high affinity and high specificity (Sevala et al., 1997) and it carries hydrocarbon to the epicuticle and ovary (Gu et al., 1995; Schal et al., 1998b). We undertook the present study to examine the biochemical and immunological properties of HDLp and to determine whether alterations of hemolymph JH and hydrocarbon titers during the reproductive cycle of female *B. germanica* are accompanied by changing titers of HDLp.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma (St Louis, MO), except the following: immunodiffusion discs were from ICN (Costa Mesa, CA), nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP) from Promega (Madison, WI), nitrocellulose (0.2 μm) from Bio-Rad (Hercules, CA), 96 well ELISA plates and 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonic acid (ABTS) were from Pierce (Rockford, IL), and d_4-methanol from Aldrich (Milwaukee, WI).

2.2. Insects

The *B. germanica* colony was maintained in an incubator at 27 ± 0.3°C under a 12:12 h light:dark cycle and fed Purina rat chow (#5012) and water ad libitum. Adult male and female insects were separated from the colony within several hours after adult eclosion and were maintained in separate groups. Only adult females were used in experiments. Females were mated on day 6 and they oviposited 3 days later. Females of *B. germanica* incubate their maturing embryos in an external ootheca that remains attached at the female's vestibulum (see Schal et al., 1997). Under our laboratory conditions oothecae hatched on day 28, 19 days after oviposition.

2.3. Oocyte maturation and hemolymph volume

The basal oocytes of dissected ovaries were measured with an ocular micrometer in a dissecting microscope. We followed the procedure described and validated by Gu et al. (1995) for hemolymph volume determinations. Chilled females were injected 26 000 dpm [carboxyl-^{14}C]inulin (1.7 mCi/g; NEN Research Products, DuPont Co., Boston, MA) and maintained at 27°C for 2 h. Hemolymph samples were collected in 5-μl capillary tubes from a clipped cercus and subjected to liquid scintillation spectrometery.

2.4. Isolation of lipophorin by KBr density gradient ultracentrifugation

Lipophorin was isolated by KBr density-gradient ultracentrifugation using the method of Shapiro et al. (1984) as modified by Gu et al. (1995). Hemolymph (400 μl) was collected from 4-day-old virgin females, and the plasma was subjected to ultracentrifugation 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 were collected from the top of the gradient and the purity of each fraction was assessed by native and SDS polyacrylamide gel electrophoresis (PAGE) using 4–15% gradient slab gels, according to the procedure of Laemmli (1970). Gels were stained with Coomassie blue to visualize proteins. Protein-bound carbohydrates were stained by periodic acid-Schiff reagent as described by Chippendale and Beck (1966). The HDLp-containing fractions were pooled, concentrated and dialysed against 10 mM phosphate buffered saline, pH 7.4, using an Amicon Ultrafree-15 centrifugal filter device with a 10 000 MW cut-off membrane.

2.5. Production of polyclonal antibody

Lipophorin was purified by KBr density-gradient ultracentrifugation and dialyzed. A 500-μl aliquot containing 350 μg of HDLp in 10 mM sodium phosphate

buffer, pH 7.4, containing 0.9% NaCl was emulsified thoroughly in an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites into each of two New Zealand White rabbits. Three booster injections were given at 2-week intervals and the rabbits were bled 1 week after the final injection. The blood was incubated for 1 h at room temperature, overnight at 4°C, and centrifuged to remove coagulated blood cells. The serum was stored in aliquots at − 78°C.

2.6. Ouchterlony immunodiffusion and western blotting

To test the specificity of the antiserum, double radial immunodiffusion was conducted using 1% agarose gels as described by Ouchterlony (1949). Hemolymph proteins were separated by gel electrophoresis and transferred onto nitrocellulose membrane (0.2 μm) following the method of Towbin et al. (1979). The HDLp band was identified by incubating the membrane with HDLp antiserum followed by a secondary antiserum conjugated to alkaline phosphatase. The antigen–antibody complex was visualized by using NBT and BCIP as a substrate. Control experiments were performed to check the specificity of the antiserum as described by Sevala et al. (1992), and no cross reactive bands were found on the membrane.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The lipophorin titer in hemolymph of adult females was determined by indirect ELISA as described by Popham and Chippendale (1993). Two hundred microliters of diluted hemolymph and a series of HDLp standards in coating buffer (50 mM sodium carbonate–bicarbonate buffer, pH 9.4) were bound to each well of an Immunoware-96 well ELISA plate (high binding) by incubating overnight at 4°C. Several blanks were also included in each plate. The plates were rinsed three times with phosphate buffered saline with Tween-20 (PBST; 8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride and 0.05% Tween-20, pH 7.4) and blocked for 1.5 h with 2% normal rabbit serum at 27°C. Then wells were filled with 200 μl of diluted HDLp-antiserum in PBST (1:500) containing normal rabbit serum, and incubated for 2 h. Plates were washed three times and loaded with 200 μl anti-rabbit immunoglobulin conjugated to horseradish peroxidase diluted in PBST, and incubated for 2 h at 27°C. Plates were washed again and developed at room temperature with 200 μl of enzyme substrate ABTS and the reaction was stopped after 30 min by adding 100 μl of 1% sodium dodecyl sulfate to each well. Absorbance was read at 405 nm in a Bio-Tek automated microplate reader. To demonstrate specificity and validity of the assay, serially diluted hemolymph, purified vitellin from *Blattella*, and bovine serum albumin were included in the assay.

2.8. Protein, amino acid, carbohydrate, and lipid analyses

Total protein concentration in hemolymph and HDLp samples was determined using the Bio-Rad protein assay (Bradford, 1976) with bovine serum albumin as standard. Amino acid analysis of purified HDLp was performed by phenyl isothiocyanate chemistry using an Applied Biosystems 420 amino acid analyzer.

The presence of covalently bound carbohydrates was determined by staining native- and SDS-PAGE with the glycoprotein stain periodic acid-Schiff reagent. The amount of total sugars associated with HDLp was determined by the anthrone assay using mannose as a standard (Roe and Dailey, 1966).

Lipids were extracted from purified HDLp by the Bligh and Dyer (1959) procedure. The chloroform phase was reduced to dryness with N_2 and total lipids were determined colorimetrically using the vanillin reagent (Goldsworthy et al., 1972) with diolein as standard.

2.9. Thin layer chromatography of lipids

Radiolabeled lipids were generated by injecting females with sodium [1-^{14}C]acetic acid (specific activity 51.7 mCi/mmol, Sigma). Hemolymph was collected 8 h later and HDLp was purified by KBr gradient ultracentrifugation. The fractions containing HDLp were pooled and lipids were extracted (Bligh and Dyer, 1959) and analyzed by thin layer chromatography (TLC) using hexane, ethyl ether, and acetic acid (80:20:2) as developing solvents. The TLC plates were scanned for radioactivity using a Bioscan system 200 image scanner.

2.10. Gas chromatography–mass spectrometric determination of hemolymph JH titer

Hemolymph was collected from CO_2-anesthetized adult females as described previously (Sevala et al., 1997) and centrifuged at 6000***g*** at 4°C for 8 min to remove hemocytes. Hemolymph samples were processed for JH titer determination as described by Bergot et al. (1981) and Baker et al. (1984), except that the final clean-up was by open column chromatography (Shu et al., 1997). An aliquot of hemolymph (50 μl) was transferred to a chilled vial containing 250 μl of acetonitrile, 250 μl of 2% NaCl, and 200 pg JH III-ethyl ester as an internal standard. The samples were stored at − 78°C. The acetonitrile-brine mixture was extracted with three 500 μl volumes of hexane which were combined and concentrated in a SpeedVac concentrator (ISS 100, Savant Instruments, Inc., Farmingdale, NY). The extract was resuspended in hexane and eluted through an Al_2O_3 (6%

water added) column (1 ml void volume) with hexane, 10% ether–hexane, and 30% ether–hexane. The JH-containing 30% ether–hexane fraction was dried in the SpeedVac concentrator for d_3-methoxyhydrin derivatization. Seventy five microliters d_4-methanol was slowly added to the residue, followed by 75 μl 5% trifluoroacetic acid in d_4-methanol, while shaking. After derivatization in an oven at 60°C for 15 min, the extracts were dried in the SpeedVac concentrator. The residue was dissolved in hexane and eluted through an Al_2O_3 column with hexane, 30% ether–hexane to remove any underivatized compounds, and ethyl acetate–hexane (1:1) to obtain d_3-methoxyhydrins. The ethyl acetate–hexane fraction was dried in the SpeedVac concentrator. The residue was dissolved in 2 μl hexane and injected on a 30 m Alltech EconoCap Carbowax capillary column (0.25 mm ID, 0.25 μm film thickness) for analysis in an HP-5890 gas chromatograph (Hewlett-Packard, Avondale, PA) coupled to an HP-5972 mass selective detector. The d_3-methoxyhydrin derivative of JH III was monitored for fragments at *m*/*z* 76 and 225; d_3-methoxyhydrin derivatives of JH II and JH I were monitored for fragments at *m*/*z* 90 and 225 and *m*/*z* 90 and 239, respectively. The internal standard, JH III-ethyl, was monitored at *m*/*z* 76 and 239 for its d_3-methoxyhydrin derivative. Total abundance of specific JHs was quantified against that for the internal standard using calibration curves obtained with authentic standards of JH III, JH III-ethyl ester, JH II, and JH I. Our limit of detection for synthetic JH I, II, and III is 10 pg/sample, with a recovery rate of ca. 80% for the three synthetic JHs.

2.11. Extraction and quantification of hydrocarbon from hemolymph and HDLp

Hemolymph (> 1 μl) was collected into chilled calibrated capillary tubes. Lipids were extracted from hemolymph or purified HDLp by the Bligh and Dyer (1959) procedure with *n*-hexacosane (1 μg) as an internal standard. The chloroform phase was reduced to dryness with N_2, replaced with hexane, and the samples loaded onto silicic acid mini-columns (BioSil-A, Bio-Rad Labs, Richmond, CA). Hydrocarbons were eluted with 7 ml hexane, the solvent reduced with N_2, and the samples were analyzed on a HP 5890II GC equipped with a flame-ionization detector and interfaced with a 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 per minute to 280°C and held for 10 min. The injector and detector were held at 280 and 300°C, respectively.

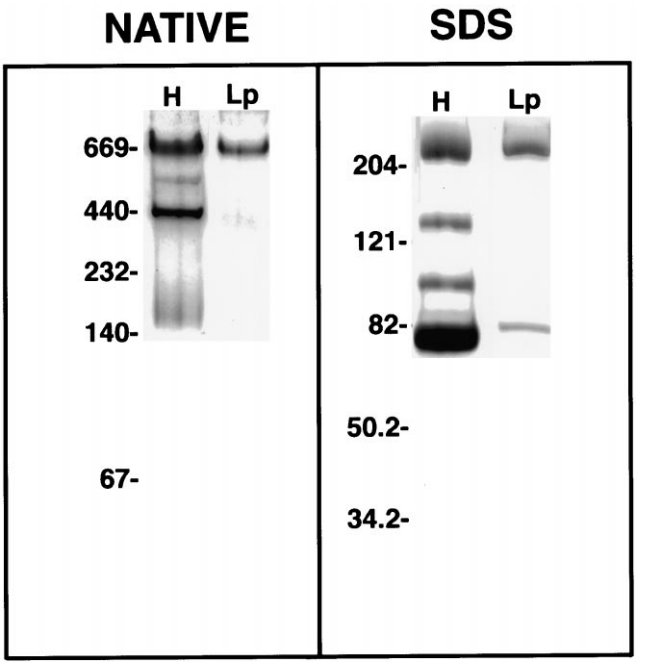

Fig. 1. Polyacrylamide gel electrophoretic analysis of purified lipophorin from the hemolymph of *B. germanica*. Left panel, native-PAGE and right panel, SDS-PAGE. Hemolymph = lane H; Lipophorin = lane Lp.

3. Results

3.1. Chemical composition of lipophorin

Lipophorin was purified from the hemolymph of *B. germanica* by KBr gradient ultracentrifugation. The density of HDLp was found to be 1.109 g/ml, as previously shown (Gu et al., 1995). The purity of the fractions containing HDLp was confirmed by native and SDS-PAGE. The apparent molecular weight of HDLp as determined by native-PAGE was found to be 670 kDa (Fig. 1, left panel), and SDS-PAGE indicated that HDLp consists of two apoproteins, apoLp-I and apoLp-II with apparent molecular weights of 212 and 80 kDa, respectively (Fig. 1, right panel). The isoelectric point (pI) of the native protein was found to be 7.0.

HDLp contains 46.2% protein (Table 1). We found that the vanillin reagent-based assay for total lipids sig-

Table 1
Composition of native lipophorin from *B. germanica* hemolymph

Component	Weight (%) (n = 3–8)
Protein	46.2
Total lipids	51.4
Hydrocarbon[a]	(42.2)
Carbohydrate	2.4

[a]Hydrocarbons are expressed as a percentage of the total lipids, as determined by GLC.

nificantly underestimated the lipid content of HDLp as compared with a gravimetric assay, which yielded 51.4% lipids. This was due to the unusually high hydrocarbon content of the lipid fraction (42.2%), and we determined that even 100 μg of hydrocarbons gave little absorbance with the vanillin test. This is partly due to the fact that vanillin reacts with unsaturated lipids, and in female *Blattella*, cuticular hydrocarbons mainly consist of *n*-alkanes and mono- and di-methyl alkanes (Augustynowicz et al., 1987; Jurenka et al., 1989). The hydrocarbons carried by HDLp are identical to those found on the epicuticle, as previously shown (Gu et al., 1995). TLC analysis of the lipids indicated a high hydrocarbon content, diacylglycerol, cholesterol and phospholipid, and trace amounts of carotenoids. No triacylglycerols were detected in association with HDLp. [1-^{14}C]Acetic acid, injected into adult females, was primarily incorporated into hydrocarbon, with only minor amounts incorporated into phospholipid and diacylglycerol (Fig. 2). The holoprotein stained positively with the glycoprotein stain periodic acid-Schiff reagent (not shown) and carbohydrates accounted for 2.4% of the HDLp particle, suggesting that HDLp is a glycolipoprotein. The amino acid composition presented in Table 2 indicates that HDLp consists of a high proportion of the amino acids aspartic acid, glutamic acid, valine, leucine, and lysine.

3.2. Characterization of lipophorin antiserum

The specificity of the polyclonal antiserum raised against HDLp was determined by three different approaches, namely, western blotting, immunodiffusion and ELISA. Western blotting and immunostaining of HDLp after SDS-PAGE showed that the HDLp antiserum reacted with apoLp-I (Fig. 3). Furthermore, in immunodiffusion assays the antiserum produced a single precipitin arc with purified HDLp (Fig. 4). When ELISA was used to further confirm the specificity, the wells containing HDLp gave significant absorbance readings and none of the control wells and pure vitellin showed any cross-reactivity with the antiserum.

Hemolymph obtained from adults of different species of cockroaches and also from various stages of *B. germ-*

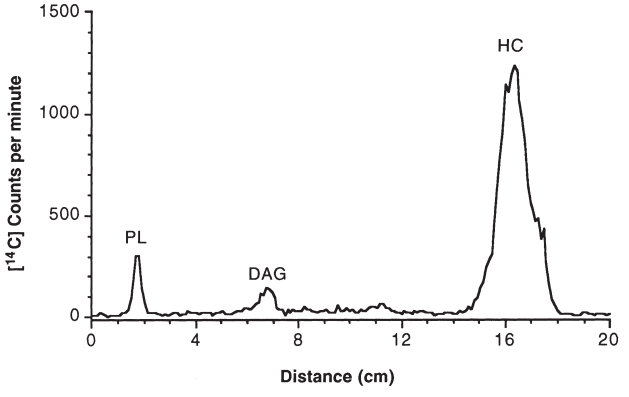

Fig. 2. Radio thin layer chromatogram of lipids associated with *B. germanica* lipophorin. Five day old virgin females were injected with 0.5 μCi sodium [1-^{14}C]acetic acid (specific activity 51.7 mCi/mmol) each in 1 μl saline. After 8 h hemolymph was collected. Lipophorin was isolated by density gradient ultracentrifugation and lipids were separated on TLC. PL: phospholipid, DAG: diacylglycerol, and HC: hydrocarbon.

Table 2
Amino acid composition of native lipophorin from *B. germanica* hemolymph

Amino acid	Mean mol (%) (n = 2)
Asparagine/aspartic acid	10.99
Threonine	4.59
Serine	5.08
Glutamine/glutamic acid	11.03
Proline	3.80
Glycine	7.39
Alanine	8.01
Valine	9.47
Isoleucine	2.41
Leucine	12.28
Tyrosine	3.38
Phenylalanine	5.40
Histidine	3.37
Lysine	9.89
Arginine	2.87

Cysteine, tryptophan and methionine not determined.

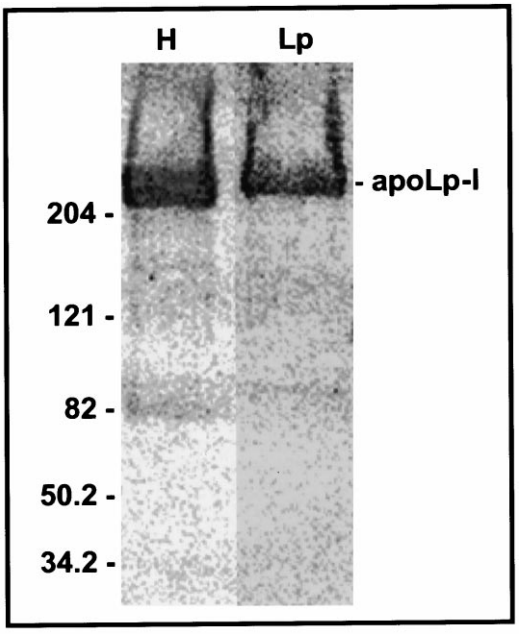

Fig. 3. Western blot of lipophorin and hemolymph of *B. germanica*. The hemolymph (lane H) and lipophorin (lane Lp) samples were analyzed by 4–15% SDS-PAGE gradient gels. Proteins were transferred to nitrocellulose and the blots were immunologically stained with HDLp antiserum.

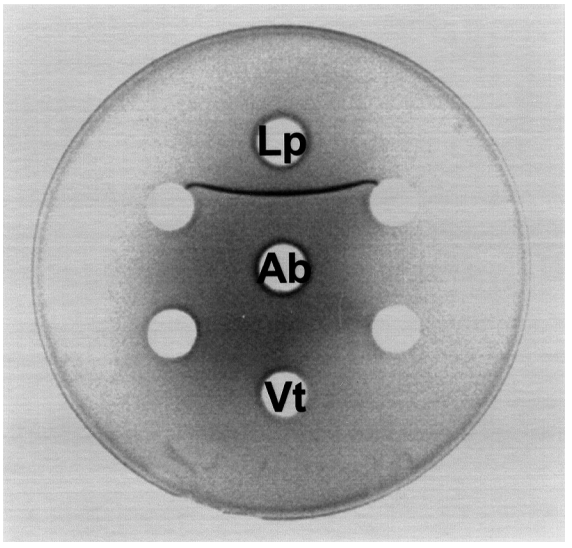

Fig. 4. Ouchterlony immunodiffusion showing cross-reactivity of lipophorin antiserum with purified lipophorin. Center well (Ab) contained lipophorin antiserum. The wells Lp and Vt contained purified lipophorin and vitellin, respectively.

anica was tested for cross-reactivity using double radial immunodiffusion. Precipitin arcs were observed with all tissues from *B. germanica* and with *Supella longipalpa* hemolymph indicating the presence of immunologically cross-reactive lipophorins in these two species of cockroaches. The *B. germanica* HDLp antiserum failed to recognize proteins in the hemolymph of *Periplaneta americana* and *Diploptera punctata*, two cockroaches representing the families Blattidae and Blaberidae (data not shown).

3.3. Hemolymph titers and contents of lipophorin, juvenile hormone and hydrocarbons

The reproductive cycle of female *B. germanica* is described in Fig. 5(A). The basal oocytes synchronously matured between days 0 and 9. All females were mated on day 6 and they oviposited on day 9. While the ootheca was attached to the vestibulum of the female, between days 9 and 28, the new basal oocytes in the ovaries grew little.

To permit calculations of total hemolymph proteins, JH, and hydrocarbons, we determined hemolymph volume throughout the reproductive cycle. The volume of the hemolymph changed in relation to the reproductive cycle (Fig. 5(B)). It increased during oocyte maturation (days 0 to 7) and sharply declined just before oviposition. Hemolymph volume remained low throughout pregnancy.

The profiles of hemolymph protein and HDLp titers appear to follow similar patterns in the adult female during the reproductive cycle (Fig. 6(A)). The concentration of HDLp in the hemolymph increased progressively from day 1 (3.21 μg/μl), peaked on day 5 (9.2 μg/μl),

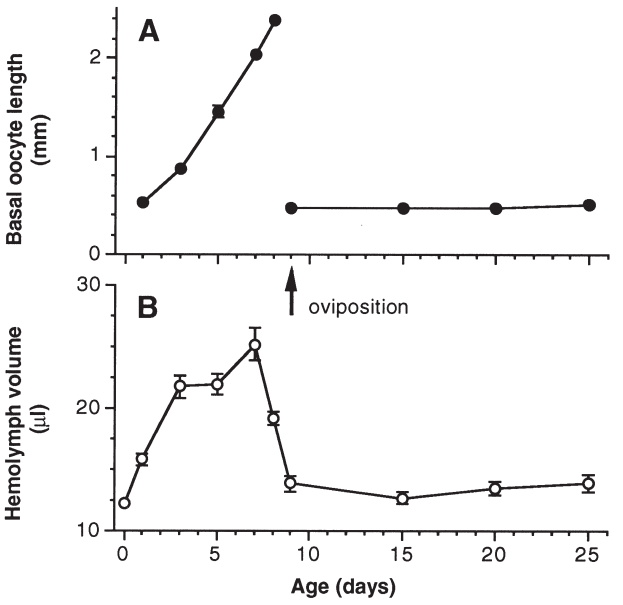

Fig. 5. Oocyte maturation in female *B. germanica* (A). Oocyte length was measured with a dissecting microscope. Oviposition occurred on day 9 (arrow). Hemolymph volume (B) was determined with the [^{14}C]inulin dilution method. Each point represents the mean of 8–10 individual determinations. Vertical bars indicate the standard error of the mean and some are obscured by the symbol.

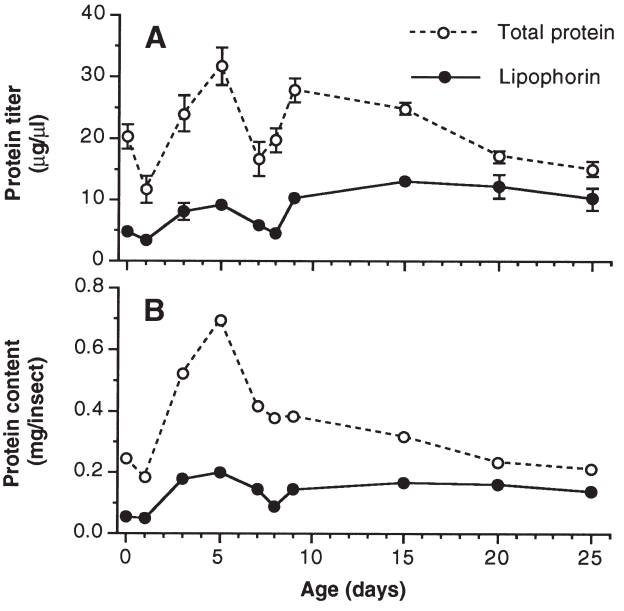

Fig. 6. Titers of lipophorin and protein concentration in the hemolymph of adult female *B. germanica* (A). Each point represents the mean of 5–8 replicates. Vertical bars indicate the standard error of the mean. Hemolymph protein and lipophorin contents (B) were calculated by multiplying the respective protein titer by hemolymph volume.

and sharply declined on day 8 (4.50 μg/μl), the day before oviposition. The HDLp titer began to rise again at the time of oviposition (day 9) and remained relatively constant at about 10.25 μg/μl until day 25, while the female carried an ootheca. Total protein concentration

followed a similar pattern except that the titer declined gradually from days 10 to 25 after adult emergence.

Corrected for hemolymph volume, the quantity of hemolymph HDLp appeared much more constant throughout the reproductive cycle after an initial rise to day 3 (Fig. 6(B)). Other hemolymph proteins exhibited a dramatic rise during vitellogenesis and a decline that started before ovulation and continued until near the end of gestation.

The data presented in Fig. 7(A) show that the hemolymph JH concentration rose from 7.04 pg/μl on day 1 to a maximum concentration of 173.4 pg/μl by day 7. The titer sharply declined to 6.82 pg/μl on day 8 and became undetectable shortly after oviposition (day 9). Juvenile hormone begins to reappear in the hemolymph on day 25 (11.02 pg/μl), 3–4 days before the embryos hatch from the attached ootheca. The changes in JH titer were remarkably positively correlated with changes in hemolymph volume. Hence, the cycle of total circulating JH (JH titer multiplied by hemolymph volume) was identical to changes in JH titer (Fig. 7(A)).

Hemolymph hydrocarbons are destined to the cuticle and ovaries (Gu et al., 1995; Schal et al., 1998b). Early in the adult stage the female experienced a sharp decline in hemolymph hydrocarbons (Fig. 7(B)), probably in relation to uptake by the new epicuticle (Young and Schal, 1997). During vitellogenesis (days 3 to 7) the concentration of hydrocarbons in the hemolymph reached a low of 6.32 μg/μl, in relation to extensive uptake by the ovaries (V.S., unpublished data). Just before ovulation, when the oocytes no longer accepted hemolymph proteins, the concentration of hydrocarbons dramatically

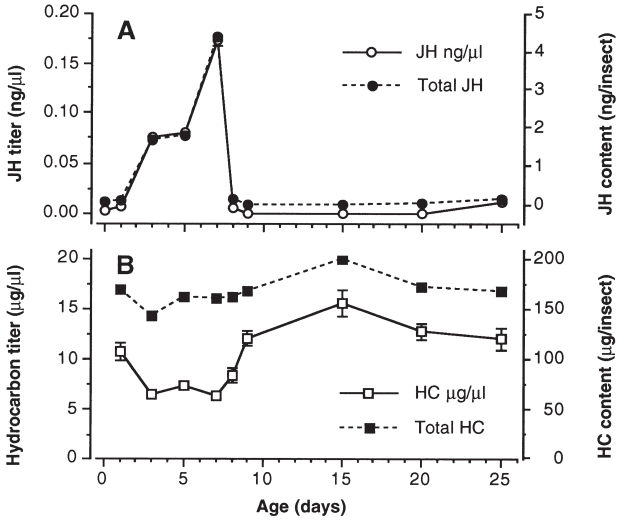

Fig. 7. Hemolymph titers and contents of juvenile hormone (A) and hydrocarbons (B) in adult female *B. germanica*. Hemolymph JH and hydrocarbon content were calculated by multiplying the respective titer by hemolymph volume. Each point in (A) represents the mean of three replicates of pooled 50 μl of hemolymph (from 20–30 females) and in (B) the average of 10 determinations. Vertical bars indicate the standard error of the mean.

increased in the hemolymph and reached a peak of more than 15 μg/μl near mid-pregnancy. While 225 μg of hydrocarbons shuttled through the hemolymph and into the developing oocytes (Schal et al., 1998b), total hydrocarbons in the hemolymph remained remarkably constant at around 160 μg (Fig. 7(B)).

4. Discussion

4.1. Composition of lipophorin

Insect lipophorins characterized to date have a density of 1.063–1.210 g/ml (Ryan et al., 1984; Beenakkers et al., 1988). The density of *Blattella* Lp falls within this range and it can be considered a high density Lp. It has an apparent molecular weight of 670 kDa and it is composed of two subunits, one large apolipoprotein (212 kDa) and a small apolipoprotein (80 kDa). Other studies on the structure of Lp from different species of insects have also indicated that HDLp is composed of two subunits, but the molecular weight of the large subunit generally ranges from 220 to 250 kDa (275 kDa in larval *Drosophila melanogaster*; Fernando-Warnakulasuriya and Wells, 1988) and the small subunit from 70 to 85 kDa (Chino, 1985; Kanost et al., 1990; Soulages and Wells, 1994). The *Blattella* HDLp has a pI of 7.0 and stains positively with the periodic acid-Schiff reagent for the presence of carbohydrates, confirming the glycolipoprotein nature of *B. germanica* HDLp, like other lipophorins.

Lipophorin's major function is to transport lipids through the hemolymph and it contains about 35 to 50% lipid (Chino, 1985; Kanost et al., 1990; Van der Horst et al., 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994). The HDLp of cockroaches is especially rich in lipids, containing 50% of its mass in lipids in *P. americana* (Chino and Kitazawa, 1981) and 51.4% in *B. germanica* (Table 1). Interestingly, our study indicates that hydrocarbons constitute the major component (42.2%) of the total lipids associated with HDLp, supporting the view that hydrocarbon is transported by hemolymph HDLp from the sites of synthesis, presumably the oenocytes which are associated with the integument, to sites of deposition, including the epicuticle, fat body and ovaries (Chino, 1985). Indeed, the hydrocarbon chromatographic profile of these tissues are very similar in *B. germanica* (see Schal et al., 1998b), in support of this model. Moreover, the vast majority of newly synthesized lipid in adult females was hydrocarbon, with much lower amounts of diacylglycerol and phospholipid (Fig. 2).

Two major groups of insects have emerged based on the lipids carried by Lp. The Lp of flight-capable insects is loaded with a large complement of diacylglycerol, which is used to fuel flight (Chino, 1985; Van der Horst

et al., 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994). Conversely, non-flying insects and those that use amino acids or carbohydrates as fuel substrates during flight (e.g., Katagiri and de Kort, 1991) carry much less diacylglycerol. Within this group, insects that deposit large amounts of hydrocarbons on their epicuticle carry a large proportion of these lipids in their HDLp. *Blattella germanica* fits this pattern. In these insects caution must be exercised when lipid determinations are performed using colorimetric tests that fail to detect hydrocarbons and saturated fatty acids (e.g., the vanillin test appears to detect only double bonds in lipids).

The amino acid composition of *B. germanica* HDLp shows considerable similarity to lipophorins from other species, such as, *D. punctata* (King and Tobe, 1992), *Philosamia cynthia* (Chino et al., 1977), *Aedes aegypti* (Ford and Van Heusden, 1994), and others. Indeed, an earlier principal component analysis of amino acid compositions concluded that lipophorins from various insects formed a distinct group, separate from vitellogenins (Kunkel and Nordin, 1985). However, *Blattella* HDLp has a lower proportion of aspartic acid and threonine and higher amounts of valine, leucine and lysine compared with several other lipophorins. It is important to note that the acid hydrolysis prevented separation of asparagine from aspartate, and glutamine from glutamate.

We examined the immunological similarity of *B. germanica* HDLp to lipophorins from other cockroach species by using the rabbit polyclonal antibodies generated against HDLp. The data indicate the presence of an immunologically cross-reactive protein in hemolymph of *Supella* which belongs to the same family (Blattellidae) as *B. germanica* and no cross-reaction was observed with the hemolymph of *Periplaneta* (Blattidae) and *Diploptera* (Blaberidae) (data not shown). These findings are in agreement with others showing antigenic cross-reactivity within stages and sexes of the same species and even among phylogenetically related species, but no cross-reactivity among species in different orders (Chino and Kitazawa, 1981; Ryan et al., 1984; Schulz et al., 1987; King and Tobe, 1992). Interestingly, apoLp-II polypeptides from several insect orders exhibit extensive cross-reactivity to apoLp-II antibodies, suggesting that apoLp-I is much less conserved (Ryan et al., 1984) and that our antibodies primarily recognized apoLp-I. The immunological observations appear to be supported by partial amino acid sequence data, showing that HDLp of *Drosophila* shares high sequence similarity with HDLp of the blowfly (both Diptera), but little similarity to HDLp of a cockroach or locust (Kutty et al., 1996). These studies suggest that HDLp is not evolutionarily conserved among insects. Interestingly, however, despite their immunological differences, insect lipophorins exhibit little functional species-specificity (Chino, 1985).

Immunoblotting of *Blattella HDLp* and hemolymph revealed strong immunoreactivity with the apoLp-I subunit and little or no cross reaction to apoLp-II. The lack of interaction of the antiserum with apoLp-II is partly due to the fact that antibodies in our study were generated to native HDLp and it has been well established that apoLp-I constitutes the outer portion of the Lp particle, with apoLp-II forming its inner core (Kanost et al., 1990; Soulages and Wells, 1994). Thus, the antibodies generated would recognize the epitopes that are associated with the more exposed region of apoLp-I.

4.2. Titers of lipophorin and JH

We examined changes in hemolymph HDLp titers of adult females in relation to concurrent changes in the titers of hydrocarbons and JH, which HDLp carries. Lipophorin titers were determined by indirect ELISA because this method was shown to be more sensitive than a direct method (Popham and Chippendale, 1993). The concentration of HDLp in hemolymph increased from day 1 after emergence to a peak on day 5, followed by a gradual decline to just before oviposition on day 9. The HDLp titers remained high (10.25 μg/μl) during gestation between days 9 and 25 (Fig. 6(A)). Hemolymph proteins followed a similar pattern to HDLp throughout the first reproductive cycle, with HDLp comprising a lower fraction of total proteins during vitellogenesis and a much higher fraction (up to 66%) in late pregnancy.

The titers of HDLp have been measured in several other insect groups and the titers seem to vary widely among life stages and species. For example, adult females of the silkworm, *Philosamia cynthia*, have 10 μg HDLp per μl of hemolymph (Chino et al., 1977), adults of *Triatoma* have 20–60 μg/μl (Ganzalez et al., 1991), pharate adults of *Hyalophora cecropia* have 8–27 μg/μl (Telfer et al., 1991), adults of *Rhodnius prolixus* 40 μg/μl (Gondim et al., 1989), and adult female *D. punctata* contained 4–12 μg HDLp per μl of hemolymph (King and Tobe, 1993). Lipophorin concentration in hemolymph of *B. germanica* appears to be related to two major reproductive events. Until day 5, females ingest large amounts of food (Hamilton and Schal, 1988; Schal et al., 1994) which they convert to vitellogenin for oocyte maturation. The relationship between elevated HDLp concentrations and active feeding fits well with the conjecture that feeding stimulates HDLp synthesis (Shelby and Chippendale, 1996; Van Heusden et al., 1997). Between days 3 and 7 the oocytes maximally grow (Fig. 5(A)) in relation to elevated feeding and drinking and increasing hemolymph volume (Fig. 5(B)) and JH titer (Fig. 7(A)). Between days 9 (oviposition) and 28 (hatch) gravid females feed little and only sporadically. Hemolymph proteins continue to decline, but HDLp titer remains constant during this period (Fig. 6). This suggests that HDLp is not sequestered by the

oocytes (unlike vitellogenin), but remains within a significantly reduced hemolymph volume.

Lipophorin functions as a high affinity JH binding protein in the hemolymph of *Blattella* (Sevala et al., 1997) implying a possible relationship between titers of HDLp and JH in hemolymph. We tested this hypothesis by measuring JH titer during the first reproductive cycle. As expected, JH titer rose from day 1 to day 7 (Fig. 7(A)). The JH concentration in the hemolymph declined sharply before oviposition and remained low or undetectable (< 0.2 pg/μl) until 3–4 days before hatching of the first ootheca. Clearly, the HDLp concentration in hemolymph (Fig. 6) was unrelated to JH titer, in agreement with previous reports with other insects (for reviews, see Goodman and Chang, 1985; Trowell, 1992). The lack of a correlation between JH titer and the titer of its binding protein is especially notable in the last instar of *B. germanica*. JH titer declines early and remains low throughout the rest of this stadium, whereas the HDLp titer peaks in mid-stadium and declines subsequently (V.S., unpublished results).

Our results on the temporal changes in JH titer in relation to the reproductive cycle in *B. germanica* agree well with the expected cycle based on work with ovoviviparous and viviparous cockroaches (see Tobe and Stay, 1985; Feyereisen, 1985). They also concur with the cycle of JH biosynthesis by the corpora allata and the pattern of oocyte maturation (Gadot et al., 1989; Fig. 5(A)). However, the JH titer differs appreciably from a previous report with the same species. Camps et al. (1987) noted a peak of 11 pg JH III/μl hemolymph in day 5 females (basal oocyte length 1.43 mm), reared at 26°C. Our peak JH titer was 173.4 pg/μl in day 7 females (basal oocyte length 2.03 ± 0.04 mm), reared at 27°C (Fig. 5(B)). While a fraction of this 15-fold difference may be attributable to different rates of JH biosynthesis in the two laboratories (see Bellés et al., 1987; Gadot et al., 1989), and possible strain differences, other methodological differences should be considered. Nonetheless, we conclude that *B. germanica* exhibits a typical dictyopteran reproductive pattern, manifested by a relatively high JH titer during vitellogenesis and a clear relationship between JH synthesis, JH titer, and oocyte maturation.

4.3. Titers of lipophorin and hydrocarbons

Lipophorin also shuttles hydrocarbons from the integument to the epicuticle, fat body, and ovaries (Chino, 1985; Gu et al., 1995). The relatively high hydrocarbon content of *B. germanica* HDLp, as compared with other insects, including other cockroaches (e.g., *P. americana*; Chino and Kitazawa, 1981), is likely related to its role in supplying hydrocarbons to the epicuticle and developing oocytes. The data indicate some functional congruence between the HDLp and hydrocarbon profiles. Soon after eclosion the teneral cuticle has a large demand for hydrocarbons (Schal et al., 1994). As the oocytes begin to grow, they too sequester large amounts of hydrocarbons (up to 6.0 μg per each of 40 eggs). The female meets these increasing demands for apolar lipids by producing more HDLp (Fig. 6(A)) and elevating hemolymph volume (Fig. 5(B)). This results in minor fluctuations in the hemolymph hydrocarbon titer and a nearly constant level of total hydrocarbons in the hemolymph.

It thus appears that although HDLp performs multiple functions, including as a JH binding protein and in transport of lipids, HC and pheromones (Trowell, 1992; Katagiri and de Kort, 1991; Dillwith et al., 1986; Gu et al., 1995), the bulk of lipid transport in adult females occurs in relation to oocyte maturation. Because various lipids are concurrently transported to the basal oocytes, it is likely that a clearer relationship will emerge between HDLp titer and the varying concentrations of transported lipids. Even at its lowest hemolymph concentration, HDLp has ample capacity to carry JH (Sevala et al., 1997), suggesting that it need not covary with JH titer. In support of this idea, Van Heusden et al. (1997) showed that in the mosquito *Aedes aegypti*, whose flight is fueled by carbohydrates, HDLp increased 3- to 4-fold after blood feeding, presumably in response to a need for lipid transport to the oocytes.

An alternative model to HDLp titer modulation is that HDLp's multiple functions are accomplished by modification of its density. Lipophorin is known to function as a dynamic particle, ranging from a low-density particle that results from an exchangeable association of apoLp-III ($M_r \sim 17$–20 kDa) and diacylglycerol with HDLp, to a very high-density complex that contains little lipids. Recently, Engler et al. (1996) have shown that even HDLp changes appreciably in late larvae and early pupae of *Manduca sexta*. Similar changes occur in *R. prolixus* during egg maturation (Coelho et al., 1997). An early decline in Lp density is probably related to higher rates of uptake of lipids from the midgut than the rates of lipid delivery to the ovary. A subsequent rise in Lp density coincides with rapid lipid uptake by the ovary and onset of oviposition (Coelho et al., 1997). It remains to be determined whether different density lipophorins, which co-exist in the hemolymph at the same time, represent instantaneous snapshots of the lipid loading and unloading process or if they perform different lipid transport functions.

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