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Characterization of termite lipophorin and its involvement in hydrocarbon transport

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Abstract

The transport of lipids constitutes a vital function in insects and requires the plasma lipoprotein lipophorin. In all insects examined to date, cuticular hydrocarbons are also transported through the hemolymph by lipophorin, and in social insects they play important roles not only in water proofing the cuticle but also in nestmate recognition. High-density lipophorin (HDLp), isolated from *Reticulitermes flavipes* plasma by KBr gradient ultracentrifugation, contains 66.2% protein and 33.8% lipids; hydrocarbons constitute its major neutral lipid (20.4% of total lipids). Anti-lipophorin serum was generated in rabbit and its specific association with lipophorin, and not with any other plasma proteins, was verified with Western blotting. Immunoprecipitation also confirmed that this antibody specifically recognizes lipophorin, because all hemolymph hydrocarbons of the termites *R. flavipes* and *R. lucifugus* and the cockroach *Supella longipalpa*, which associate only with lipophorin, were recovered in the immunoprecipitated protein. Cross-reactivity of the antiserum with lipophorin from related species was investigated by double immunodiffusion with 10 termite species in the genera *Reticulitermes*, *Coptotermes*, *Zootermopsis*, and *Kalotermes*, and with five cockroach species. Involvement of lipophorin in hydrocarbon transport was shown by injecting HDLp antiserum into *Zootermopsis nevadensis* and then monitoring the de novo biosynthesis of hydrocarbons and their transport to the cuticular surface; the antiserum significantly disrupted hydrocarbon transport. ELISA revealed a gradual increase in the lipophorin titer in successively larger *R. flavipes* workers, and differences among castes in lipophorin titers were highest between nymphs and first instar larvae.

Keywords: *Reticulitermes flavipes*; ELISA; Hydrocarbon; Lipophorin; Termite

1. Introduction

Lipids serve a variety of important structural and functional roles in all organisms. Hydrocarbons that coat the outer surface of the cuticle constitute a lipid class of vital importance to arthropods because they serve multiple functions in development and reproduction (Howard, 1993; Blomquist et al., 1998). In social insects, hydrocarbons not only maintain water balance, but the species-, colony-, and caste-specific epicuticular hydrocarbon profiles serve as recognition cues (Bagnères et al., 1991; Lorenzi et al., 1997; Singer, 1998; Vander Meer and Morel, 1998). Thus, social insects must closely regulate their epicuticular and exocrine secretions (Vauchot et al., 1996; Blomquist et al., 1998). Because termites are blind (except primary reproductives) and live inside galleries, sensory recognition via chemical communication is essential to this mode of life and it is mediated by cuticular lipids acting as pheromones (Bagnères et al., 1998).

Hemolymph transport of lipids in insects is facilitated by plasma lipoproteins (Chino, 1985), which are synthesized by fat body cells and secreted to the hemolymph, where relatively large quantities can be found (Van der Horst et al., 1993; Soulages and Wells, 1994). The predominant lipoprotein in resting insects is lipophorin, a high-density lipoprotein (HDLp, density ~1.12 g ml^{-1}) that transports a variety of lipophilic molecules (Van der Horst et al., 1993; Canavoso et al.,

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2001; Sevala et al., 1997). In general, lipophorin contains diacylglycerol, phospholipids, cholesterol, carotenoids, and hydrocarbons (reviews: Chino, 1985; Canavoso et al., 2001). In cockroaches, large amounts of hydrocarbons associate with HDLp, and in the adult female German cockroach, *Blattella germanica*, HDLp delivers hydrocarbons not only to the epicuticle, but also to vitellogenic oocytes (Schal et al., 1998; Fan et al., 2002). Recently, we showed for the first time in a social insect that HDLp transports hydrocarbons through the hemolymph in the dampwood termite *Zootermopsis nevadensis* (Hagen), and suggested that it might play an important role in regulation of the externalization and internalization of hydrocarbons (Sevala et al., 2000). This process may help to explain how the cuticular profile is modulated in various physiological stages (Mpuru et al., 2001). Lipophorin might also be essential in modulating intraspecific chemical profiles that are especially important in social insects (Bagnères et al., 1991, 1996).

In the Eastern subterranean termite, *Reticulitermes flavipes* (Kollar), hemolymph HDLp was identified as a high affinity carrier of juvenile hormone (Okot-Kotber and Prestwich, 1991a). It was characterized as a 700 kDa protein and under denaturing conditions it dissociated into two apoproteins with estimated molecular sizes of 230 and 76 kDa. Only the 230 kDa apoprotein interacted with a specific photoaffinity label for the juvenile hormone III binding site (Okot-Kotber and Prestwich, 1991a). Lipophorins of similar characteristics were detected as juvenile hormone binding proteins in other termite species, including *Z. nevadensis* and the Formosan termite, *Coptotermes formosanus* (Shiraki) (Okot-Kotber and Prestwich, 1991b).

To investigate the multifunctional roles of HDLp in termite physiology and behavior, it is necessary to develop tools to quantify its developmental profile in the hemolymph. We undertook the present study to purify a termite HDLp, raise antibodies against it, and develop and validate a sensitive enzyme-linked immunosorbent assay (ELISA) to quantify lipophorin titers in various termite castes. Because the antiserum raised against lipophorin recognizes lipophorin of other termite species, it offers a general tool for studies on lipophorin. The newly developed ELISA should facilitate studies of lipid mobilization and chemical communication in termites.

2. Materials and methods

2.1. Insects

R. flavipes individuals were from a laboratory colony recently collected near Raleigh, NC (USA) and maintained at North Carolina State University. Other subterranean species were collected in Europe, including *Reticulitermes grassei* (collected in forêt de la Coubre, Charente-Maritime, France), *R. banyulensis* (Bézier, France), *R. balkanensis* (Marathon, Greece), *R. lucifugus* (Marseille, France) and it subspecies *R. (lucifugus) corsicus* (Sartène, Corsica, France), *R. santonensis* (île d'Oleron, Charente-Maritime, France), and a newly described species (Bagnères et al., 2003; Uva et al., 2004), *R. urbis* (from Domène, Isère, France). *C. formosanus*, the Formosan subterranean termite, was from the University of Hawaii, Manoa, HI, USA), *Z. nevadensis*, a dampwood species, was collected in the Toiyabe National Forest (Sierra County, CA, USA), and *Kalotermes flavicollis*, a drywood species, was collected near Marseille, France. Five cockroach species, *Cryptocercus punctulatus*, *Periplaneta americana*, *Supella longipalpa*, *Blaberus cranifer*, and *Diploptera punctata*, were obtained from laboratory colonies at North Carolina State University.

2.2. Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA), except the following: immunodiffusion discs and Pansorbin cells were from ICN (Costa Mesa, CA, USA), nitrocellulose (0.45 μm), 4–15% SDS-PAGE pre-cast ready gels, and broad range molecular weight markers were from Bio-Rad (Hercules, CA, USA), *p*-nitrophenyl phosphate (pNPP), 10% bovine serum albumin (BSA), and 96 well ELISA plates were from Pierce (Rockford, IL, USA), and [1-^{14}C]propionate (specific activity 56.7 mCi/mmol) was from ARC (St. Louis, MO, USA). Organic solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.3. Hemolymph collection

Hemolymph was collected under a microscope from ice-anesthetized termite workers by severing the antennae and gently pressing the body. Calibrated capillaries pre-loaded with 2 μl bleeding buffer (0.05 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl, 10 mM ethylenediamide tetra acetic acid, 5 mM glutathione, 2 mM phenylmethyl sulfonyl fluoride, 10 μg ml^{-1} leupeptin, and 10 μg ml^{-1} pepstatin), were used to collect hemolymph. The buffer/hemolymph solution was transferred into chilled, 1.5 ml microcentrifuge tubes and centrifuged at 400×*g* for 2 min at 4 °C to remove the hemocytes. Plasma was stored at −80 °C.

2.4. Isolation of lipophorin from *R. flavipes*

Hemolymph was collected from the homogenate of approximately 1600 *R. flavipes* workers of various ages. The heads were severed and the bodies and heads were gently homogenized on ice with a few strokes of a

pestle in an Eppendorf tube containing cold phosphate buffered saline (PBS) fortified with protease inhibitors. HDLp was purified by KBr equilibrium density-gradient ultracentrifugation as described by Shapiro et al. (1984) and previously applied to *Z. nevadensis* by Sevala et al. (2000). Briefly, hemolymph was mixed with 2.58 g KBr in PBS and adjusted to 5.8 ml. The KBr mixture was placed into a Beckman 13.5 ml QuickSeal tube (Fullerton, CA, USA), overlaid with 7.7 ml freshly prepared 0.9% NaCl, and subjected to ultracentrifugation with slow acceleration and deceleration at $285,000\times g$ for 22 h at 4 °C in a Beckman L8-70M ultracentrifuge using a fixed angle 70.1Ti rotor. Fractions of 400 μl were collected from the top of the tube and characterized on 4–15% SDS-PAGE. Molecular sizes of proteins were computed from log plots of standard molecular mass markers versus their R_f values. The following molecular mass markers were used: myosin (205 kDa), β-galactosidase (119 kDa), BSA (98 kDa), ovalbumin (52.3 kDa), carbonic anhydrase (36.8 kDa), soybean trypsin inhibitor (30.1 kDa), lysozyme (22 kDa), and aprotinin (7.6 kDa).

Pure lipophorin fractions were pooled, concentrated, and dialyzed against 10 mM PBS (pH 7.4) using a Centricon-10 microconcentration tube with a 10,000 MW cut-off membrane (Amicon, Danvers, MA, USA). Protein concentration was measured by the Bradford method (Bradford, 1976) with BSA as standard.

2.5. Chemical composition of lipophorin

2.5.1. Separation of lipids by thin layer chromatography (TLC)

Total lipids were extracted from KBr-purified HDLp according to Bligh and Dyer (1959). Lipid classes were separated by TLC according to Bjostad and Roelofs (1984). The TLC plates (Silica gel 60-F_{254}, Merck, Damstadt, Germany) were developed twice in 200:250:10:1 ether/benzene/ethanol/acetic acid to 8 cm beyond the origin, and then twice with 80:20:2 hexane/ether/acetic acid to 18 cm above the origin. Lipids were visualized by exposure to iodine vapor. Lipid bands corresponding to authentic standards were scraped into vials, extracted 3× with 2 ml 50:50 chloroform/methanol (phospholipids and monoacylglycerol were eluted from silica with 100% methanol). Nonadecanoic acid (100 μg) was added as an internal standard to each lipid class, except hydrocarbons, which received 100 μg of *n*-octacosane. The lipid classes to be trans-esterified were evaporated to dryness under a N_2 stream, 1 ml of BF_3/methanol was added, and the vials were heated at 100 °C for 1 h. Fatty acid methyl esters were extracted by adding 1 ml water and 0.5 ml hexane, the vials were shaken vigorously and the hexane phase was analyzed by gas chromatography (GC). A non-polar HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) was used in a HP5890 GC (Agilent, Avondale, PA, USA). The splitless injector and FID were set at 280 and 300 °C, respectively. Column temperature was at 150 °C for 2 min, then increased by 5 °C per min to 260 °C, and held for 10 min.

2.5.2. Radio-TLC

To measure de novo lipid biosynthesis, 100 *R. flavipes* workers were fed a small piece of filter paper (0.25 mm^3) loaded with 50 μCi of [1-^{14}C]sodium acetate. Twelve hours later, hemolymph was collected, lipophorin was immunoprecipitated, lipophorin-bound lipids extracted, lipids were separated by TLC and radioactivity was monitored using a Bioscan system 200 image scanner (Washington, DC, USA).

2.6. Preparation of lipophorin antiserum

A small amount of pre-immune blood was collected from each of two New Zealand white rabbits. Then, 250 μg HDLp in 10 mM PBS, pH 7.4, was emulsified thoroughly in Freund's complete adjuvant and injected subcutaneously at multiple sites into each of the two rabbits. Four booster injections of 100 μg in Freund's incomplete adjuvant were given at 3-week intervals; rabbits were bled 1 week after the final injection. The blood was clotted uncovered for 1 h at 37 °C, then overnight at 4 °C, and centrifuged at $10,000\times g$ for 10 min to remove coagulated blood cells. The antiserum was stored in aliquots at −80 °C.

2.7. Immunological techniques

To test for cross-reactivity of the antiserum with hemolymph of other termite species and cockroaches, double radial immunodiffusion was conducted using 0.9% agarose gels as described by Ouchterlony (1949). The procedures of Sevala et al. (1999) were followed. Briefly, hemolymph was mixed with PBS at the ratio of 1:30 (v/v), and loaded into the peripheral wells of the immunodiffusion discs. The center well of each disc contained 1:2 PBS dilution of the antiserum generated against *R. flavipes* lipophorin. The immunodiffusion discs were held in the dark at 4 °C and precipitin lines were checked 48 h later.

Cross-reactivity of the antiserum was confirmed, and its specificity for lipophorin was checked by immunoblotting (Western). Briefly, hemolymph proteins were separated on 4–15% SDS-PAGE. Gels were rinsed in transfer buffer (3.02 g Tris base, 14.4 g glycine and 200 ml methanol to make 1 l buffer) for 15 min. Proteins were electroblotted for 2 h onto a nitrocellulose transfer membrane (0.45 μm) pre-wetted with transfer buffer in a Bio-Rad mini-gel transfer apparatus. The membrane was blocked with 5% non-fat dry milk in PBS-T (8 mM sodium phosphate, 2 mM potassium phosphate,

140 mM sodium chloride, 10 mM potassium chloride, 0.05% Tween-20, pH 7.4) overnight at 4 °C. The membrane was then probed with lipophorin antiserum followed by a secondary antiserum conjugated to alkaline phosphatase. The antigen–antibody complex was visualized using pNPP as an enzyme substrate.

Because all hemolymph hydrocarbons associate with lipophorin, specific immunoprecipitation of lipophorin should remove all hydrocarbons from a hemolymph sample. Three microliters of insect blood collected from severed antennae, 97 μl distilled H_2O, 100 μl termite lipophorin antiserum, and 200 μl-40 mM Tris buffer were mixed and incubated overnight at 4 °C with shaking. Meanwhile, 60 μl Pansorbin cells were centrifuged for 1 min at 8000×*g* to remove the storage buffer. Cells were re-suspended and washed three times with immunoprecipitation buffer. The lipophorin–antibody complex was added to the pre-washed Pansorbin cells, vortexed to re-suspend the cells, and incubated at room temperature for 1 h. The mixture was centrifuged for 10 min at 8000×*g*, the supernatant was carefully decanted, and lipids were extracted from both supernatant and pellet using the Bligh and Dyer (1959) method. Hydrocarbons were purified from a silica gel column by elution with hexane, and analyzed by GC following the methods of Fan et al. (2002).

2.8. ELISA of HDLp

An indirect ELISA was developed to quantify the lipophorin titers in different stages and castes of the subterranean termite *R. flavipes*. To determine the optimum primary antibody dilution, 100 μl of diluted (1:200,000) extracts of termite workers, or 100 μl of a series of HDLp standards (1–100 ng ml^{-1}) in coating buffer (50 mM sodium carbonate–bicarbonate buffer, pH 9.4), was bound to each well of Immunoware high-binding 96-well ELISA plates by incubating overnight at 4 °C. Several blanks were also included in each plate. The plates were rinsed 3× with PBS-T, and blocked for 1 h with 1% BSA at 37 °C. Each well was then loaded with 100 μl of diluted lipophorin antiserum (dilutions of 1:250, 1:500, 1:1000, 1:2000, 1:4000) or 100 μl of 1:100 diluted pre-immune serum in PBS-T, containing 1% BSA, and incubated at 37 °C for 1 h. Plates were washed 3× and loaded with 100 μl goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase diluted 1:10,000 in PBS-T, and incubated for 1 h at 37 °C. Plates were washed 3× again and developed at room temperature with 100 μl per well of enzyme substrate pNPP; the reaction was stopped after 30 min by adding 50 μl of 2 N NaOH to each well. Absorbance was read at 405 nm in a PowerWave-X automated microtiter plate reader (Bio-Tek, Winooski, VT, USA).

To determine the optimum secondary antibody dilution 100 μl of diluted HDLp (10 ng ml^{-1}) in coating buffer was bound to each well of 96-well ELISA plates by incubating overnight at 4 °C. After washing and blocking, the plates were incubated at 37 °C for 1 h with 100 μl diluted lipophorin antiserum in PBS-T (various dilutions from 1:100 to 1:10,000) containing 1% BSA. Plates were washed 3×, as before, and loaded with 100 μl of the secondary antibody diluted 1:500, 1:10,000, 1:20,000, 1:30,000, and 1:60,000. The rest of the procedure was as before.

The ELISA calibration curve was generated with 0–200 ng ml^{-1} HDLp, primary antibody dilution of 1:1000 and secondary antibody dilution of 1:10,000. One hundred microliters of diluted (1:200,000) extracts of various termite castes, or 100 μl of a series of HDLp standards in coating buffer, was bound to each well of 96-well ELISA plates by incubating overnight at 4 °C. The plates were rinsed, blocked, loaded with 100 μl of diluted (1:1000) lipophorin antiserum or 100 μl of PBS-T containing 1% BSA, and incubated at 37 °C for 1 h, as before. The rest of the procedure was as before.

2.9. Lipophorin titers in termites

A termite colony consists of larvae, workers (several stages), soldiers, nymphs (several stages), reproductives, and eggs. Different *R. flavipes* instars and castes were collected: first larval instar, workers of various stages (various sizes), first nymphs, nymphs with short- and long-wing pads, soldiers, and primary and secondary reproductives. All were homogenized in PBS and centrifuged at 8000×*g* for 10 min. The supernatants were assayed with ELISA and lipophorin titer was calculated as ng lipophorin mg^{-1} body mass.

3. Results

3.1. Purification and chemical composition of lipophorin

Lipophorin of *R. flavipes* was reported as a high mass (ca. 700 kDa) glycosylated lipoprotein (Okot-Kotber and Prestwich, 1991a). To obtain pure lipophorin for preparation of antiserum, termite worker plasma was subjected to KBr gradient ultracentrifugation and aliquots of fractions were evaluated on SDS-PAGE. HDLp dissociated into two polypeptides with molecular weights of 210 and 85 kDa (Fig. 1). The lipophorin containing fractions 11–19 were combined, dialyzed and concentrated, and used to generate antibodies in rabbits. Fraction 15, recovered at a density of 1.10 g ml^{-1}, represents HDLp and is shown in Fig. 1.

HDLp was purified by KBr gradient ultracentrifugation from two groups of 30 worker termites. The two HDLp fractions (each ~90 μg) contained 66.2% protein

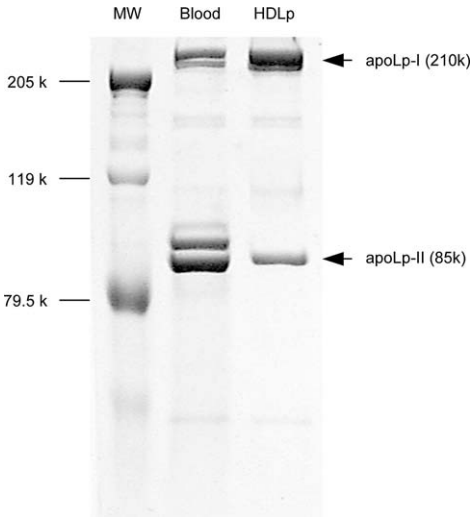

Fig. 1. SDS-PAGE (4–15%) analysis of hemolymph proteins and purified HDLp from *R. flavipes* workers. MW, molecular weight markers; Blood, hemolymph of *R. flavipes* workers; HDLp, high-density lipophorin purified by KBr gradient ultracentrifugation.

and 33.8% lipids (Table 1). Phospholipids constituted the most abundant lipid class, followed by hydrocarbons, monoacylglycerol, free fatty acids, diacylglycerol, and trace amounts of triacylglycerol; cholesterol was not detected.

Although the two termite species *R. flavipes* and *R. lucifugus* have remarkably different hydrocarbon profiles, the respective epicuticular hydrocarbons and HDLp-associated hydrocarbons of each species are similar (Fig. 2).

[1-^{14}C]acetate fed to termite workers was incorporated almost exclusively into newly biosynthesized hydrocarbons, with only minor amounts incorporated into phospholipids and diacylglycerol (Fig. 3). Thus, hydrocarbons are the major lipid class biosynthesized and mobilized by HDLp in termite workers.

Table 1
Composition of high-density lipophorin isolated from plasma of *R. flavipes*

	Mass (%)
Protein	66.2
Total lipids	33.8
Hydrocarbons	20.4
Triacylglycerol	0.9
Free fatty acids	6.1
Diacylglycerol	2.8
Cholesterol	Not detected
Monoacylglycerol	13.7
Phospholipids	56.1

Lipid percentages are expressed as a percentage of total lipids ($N = 2$ groups, each of 30 termites, equivalent to ~90 μg HDLp), as determined by GC.

3.2. Characterization of lipophorin antiserum

Species cross-reactivity of the lipophorin antiserum was investigated with Ouchterlony immunodiffusion on 10 termite species and five cockroach species. The antiserum produced a single precipitin line with hemolymph of *R. flavipes*, *R. grassei*, *R. banyulensis*, *R. balkanensis*, *C. formosanus*, *R. (lucifugus) corsicus*, *R. lucifugus*, *R. urbis*, and *R. santonensis* (Fig. 4). A weak precipitin line formed with hemolymph of *Z. nevadensis* and a nearly invisible line against *K. flavicollis* hemolymph. Cross-reactivity with hemolymph of the cockroaches *C. punctulatus*, *P. americana*, *S. longipalpa*, *B.*

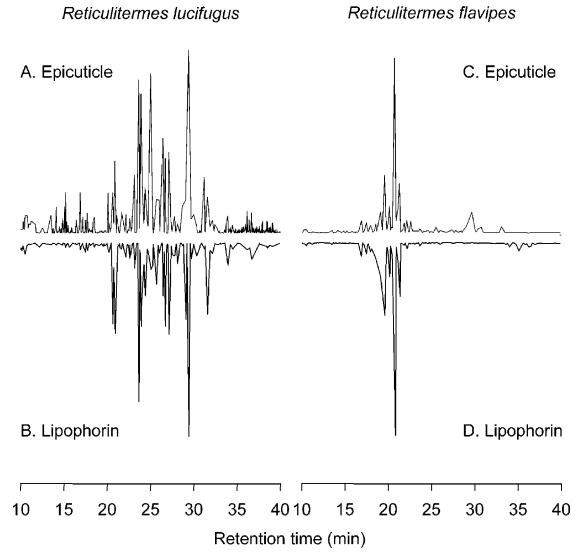

Fig. 2. Gas chromatograms of hydrocarbons extracted from the cuticular surface and from lipophorin of *R. flavipes* and *R. lucifugus*.

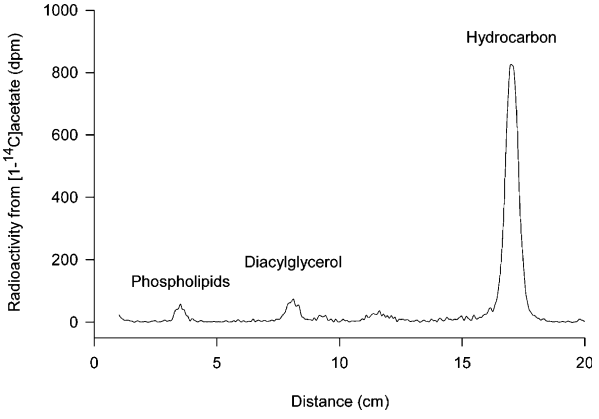

Fig. 3. Newly synthesized radiolabeled lipids associated with lipophorin of *R. flavipes*. About 100 workers were fed 50 μCi [1-^{14}C]sodium acetate on a 0.25 mm^2 filter paper. After 12 h hemolymph was collected, lipophorin was isolated by immunoprecipitation and lipids extracted and separated by TLC.

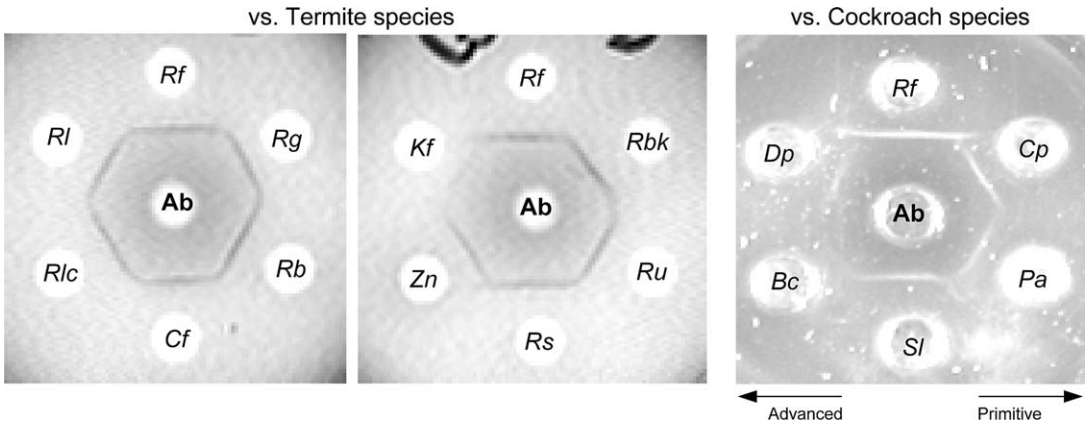

Fig. 4. Ouchterlony immunodiffusion showing cross-reactivity of *R. flavipes* lipophorin antiserum with hemolymph of other termite species and cockroaches. The center wells (Ab) contained antiserum diluted 1:2 in PBS. The peripheral wells contained 1 μl hemolymph from the indicated species in 30 μl PBS. The immunodiffusion discs were developed in the dark at 4 °C, and checked for precipitin lines 48 h later. Rf, *Reticulitermes flavipes*; Rg, *R. grassei*; Rb, *R. banyulensis*; Cf, *Coptotermes formosanus*; Rlc, *R. (lucifugus) corsicus*; Rl, *R. lucifugus*; Rbk, *R. balkanensis*; Ru, *R. urbis*; Rs, *R. santonensis*; Zn, *Zootermopsis nevadensis*; Kf, *Kalotermes flavicollis*; Cp, *Cryptocercus punctulatus*; Pa, *Periplaneta americana*; Sl, *Supella longipalpa*; Bc, *Blaberus craniifer*; Dp, *Diploptera punctata*.

cranifer, and *D. punctata* was much weaker compared with *R. flavipes* (Fig. 4). With the pre-immune rabbit serum in the center well neither HDLp nor hemolymph of *R. flavipes* showed precipitin lines (data not shown).

Cross-reactivity of the antiserum with hemolymph of various termite and cockroach species was also examined with Western blotting. Cross-reactivity was found in all termite species assayed. The polyclonal lipophorin antiserum bound specifically to lipophorin, but both subunits of lipophorin were recognized, except in *Z. nevadensis* where only apolipophorin-I was detected with immunostaining (Fig. 5). An independent Western blot with fresh *Z. nevadensis* blood confirmed that only apolipophorin-I interacted with the antiserum. Hemolymph of two cockroach species was also examined by Western blotting, which showed that both apolipophorin-I and apolipophorin-II were detected (data not shown).

3.3. Hydrocarbons associated with lipophorin: immunoprecipitation assays

The hemolymph of two termite species, *R. flavipes* and *R. lucifugus*, and one cockroach species, *S. longipalpa*, was assayed by immunoprecipitation. The hydrocarbon profiles of the supernatants and immunoprecipitated pellets, assayed by GC, are shown in Fig. 6. With a 1:30 ratio of insect blood/antiserum, hydrocarbons were found only in the immunoprecipitates of the two termite species, but not in the supernatants (Fig. 6A,B). However, with the same blood/antiserum ratio a small amount of hydrocarbon was found in the

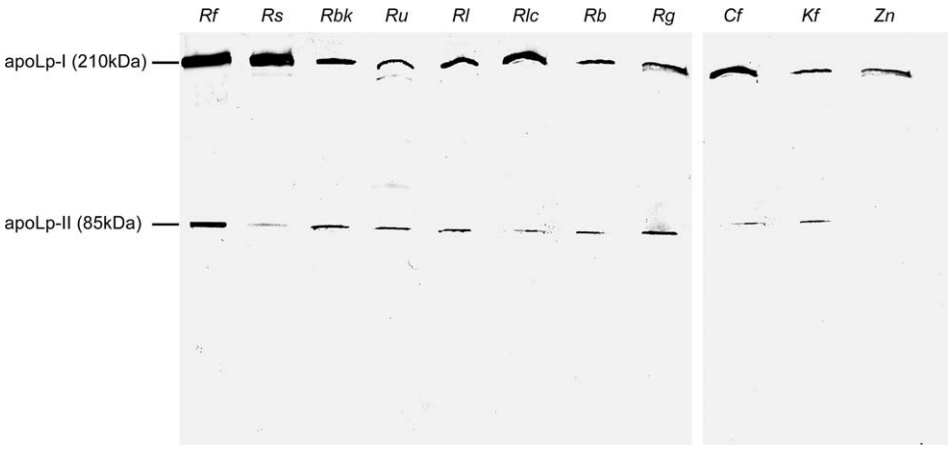

Fig. 5. Immunoblot of termite and cockroach hemolymph. Ten microliters of diluted hemolymph (1:1000) were separated by SDS-PAGE, immunoblotted onto nitrocellulose membrane, and probed with antiserum against *R. flavipes* HDLp, followed by a secondary antiserum conjugated to alkaline phosphatase. The antigen–antibody complex was visualized with pNPP. Species abbreviations as in Fig. 4.

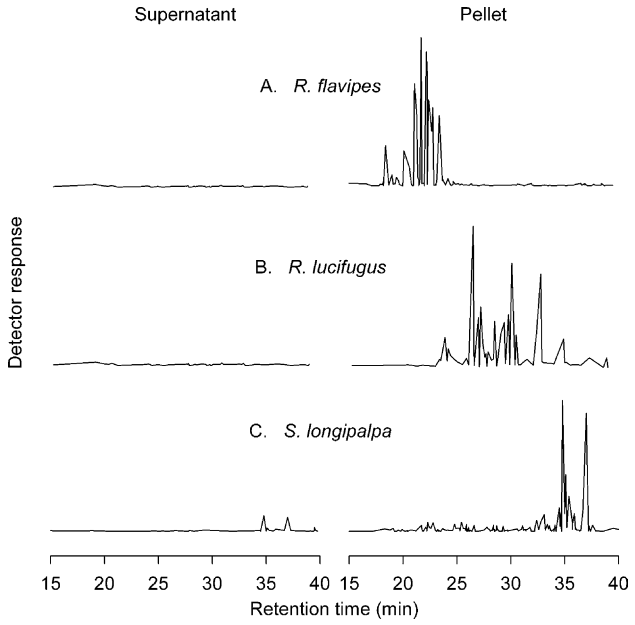

Fig. 6. Gas chromatograms of hydrocarbons extracted from the supernatant and pellet obtained from specific immunoprecipitation of hemolymph of *R. flavipes*, *R. lucifugus*, and *S. longipalpa* with antiserum against *R. flavipes* lipophorin. The pellets and supernatants of immunoprecipitated hemolymph of the three species were solvent-extracted and analyzed by GC.

supernatant of *S. longipalpa* hemolymph (Fig. 6C); nevertheless, most hydrocarbons were found in the immunoprecipitated pellet.

3.4. Disruption of hydrocarbon transport

Z. nevadensis workers were injected with 0.5 μCi of [1-^{14}C]propionate in 0.5 μl PBS to monitor de novo biosynthesis of methyl-branched hydrocarbons and their transport to the cuticular surface. A time course of cuticular and internal hydrocarbons was conducted 0.5, 1, 2, 4, 6, and 8 h after injection. Hydrocarbon biosynthesis peaked between 1 and 2 h after injection, and subsequently no more hydrocarbons were synthesized (ANOVA, $F_{5,\,33} = 7.431$, $P < 0.0001$) (Fig. 7A). Externalization of newly synthesized hydrocarbons, on the other hand, was low in the first 2 h after injection, but it increased gradually between 2 and 6 h (ANOVA, $F_{5,\,33} = 5.462$, df = 38, 6, $P = 0.0009$) (Fig. 7A).

Because hydrocarbons are associated with HDLp injecting lipophorin antibody should impede hydrocarbon transport from the internal hydrocarbon pools to the external cuticular surface. To interfere with hydrocarbon transport, 5 μl of lipophorin antiserum or pre-immune serum was injected 2 h after [1-^{14}C]propionate injection. Two hours later (i.e., 4 h after [1-^{14}C]propionate injection), when we expected no more biosynthesis of hydrocarbons but extensive transport to the cuticular surface, cuticular and internal

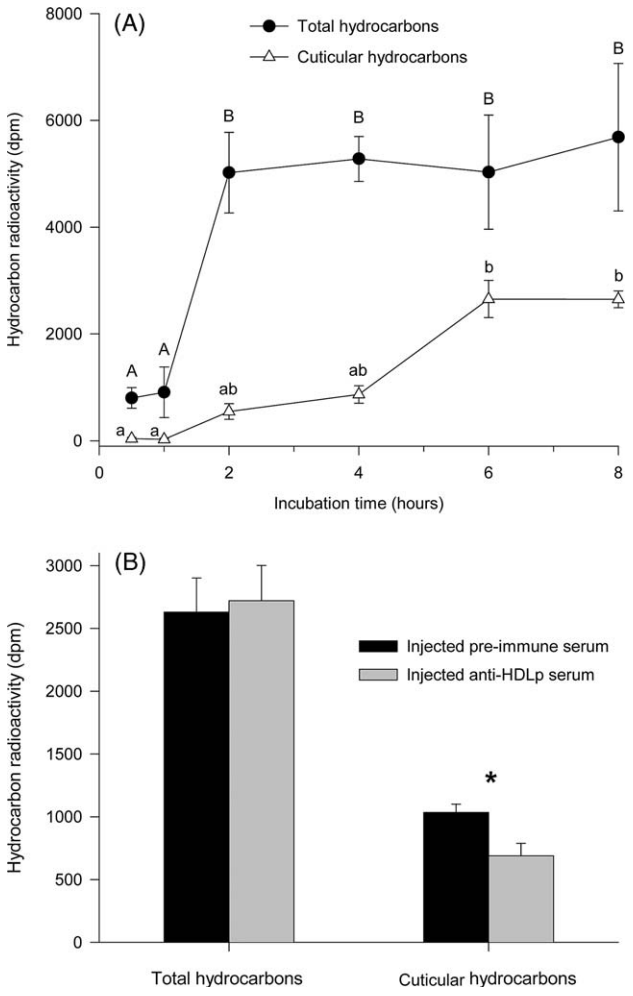

Fig. 7. Time-course of de novo hydrocarbon biosynthesis by *Z. nevadensis* workers and disruption of its transport to the cuticle. (A) Each worker termite was injected 0.5 μCi of [1-^{14}C]propionate and both cuticular and internal lipids were extracted at the times indicated. Hydrocarbons were fractionated on silica gel columns and quantified by LSC. Data are mean ± SEM (N is 7 for each mean, except for 1 h, where $N = 6$). Means with different letters are significantly different (ANOVA, Tukey-Kramer, $P < 0.05$). (B) Each termite was injected 0.5 μCi of [1-^{14}C]propionate, as above, but 2 h later each termite was injected 5 μl of either pre-immune serum (control) or lipophorin antiserum to block the transport of newly synthesized hydrocarbons. Two hours later the cuticular and internal hydrocarbons were extracted, fractionated on silica gel columns, and quantified by LSC. Data are mean ± SEM (N is 8 for control and 10 for antiserum injected). Significant differences (t-test, $P < 0.05$) are indicated by *.

lipids were extracted and hydrocarbons were purified. Equal amounts of radiolabeled hydrocarbons were biosynthesized during the 4 h assay in termites injected either lipophorin antiserum or pre-immune serum (t-test, $t_{16} = 0.224$, $P = 0.826$) (Fig. 7B). However, the antiserum significantly suppressed externalization of newly synthesized hydrocarbons to the cuticular surface (t-test, $t_{16} = 2.729$, $P < 0.015$). These data support

the model that lipophorin delivers newly synthesized hydrocarbons to the cuticular surface.

3.5. ELISA optimization

Standard concentrations of purified HDLp were used to develop an ELISA. To determine the appropriate concentration of the primary lipophorin antiserum, it was diluted to different concentrations and incubated with various concentrations of lipophorin. Pre-immune serum was used as a negative control. The pre-immune serum did not react even with 100 ng ml^{-1} of lipophorin (Fig. 8A). The absorbance increased linearly with all antiserum dilutions, and its high sensitivity in the ELISA is demonstrated by linearity at dilutions of 1:1000 or 1:2000 (Fig. 8A).

Several dilutions were used to determine the optimum concentration of the secondary antibody. The secondary antibody, diluted 1:5000, generated an optical density at 405 nm above 2.00 when the primary antibody dilution was between 1:100 and 1:10,000 at HDLp concentration of 10 ng ml^{-1} (data not shown). Dilution of the secondary antibody greater than 1:30,000 yielded absorbance values below 1.00, whereas a dilution of 1:10,000 resulted in absorbance values between 1.00 and 2.00. When the microplate wells were coated with 100 μl of 10 ng ml^{-1} HDLp, the concentration of primary antibody (dilutions from 1:100 to 1:10,000) did not alter the final absorbance values (data not shown).

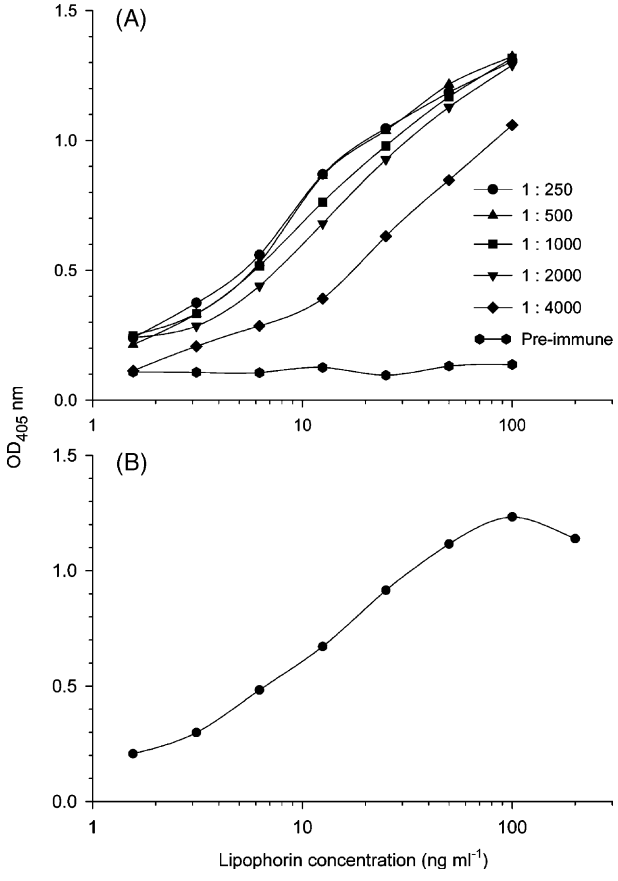

Fig. 8. ELISA optimization and standard curve of lipophorin from *R. flavipes*. (A) Various concentrations of lipophorin were loaded into the ELISA microplate and subsequently probed with various concentrations of primary antibody. Secondary antibody (1:10,000 dilution) of goat anti-rabbit was used. (B) Standard curve using the lipophorin antiserum for *R. flavipes*, at a concentration of 1:1000, and secondary antibody at a concentration of 1:10,000.

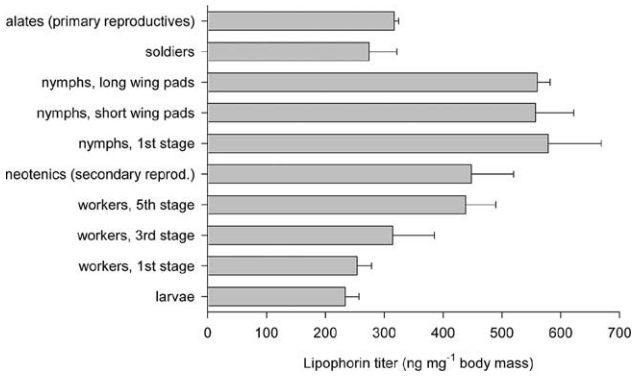

Fig. 9. ELISA quantification of lipophorin in various castes of *R. flavipes*. Three replicates per mean. See text for description of stages and castes.

An ELISA calibration curve was generated with 0–200 ng ml^{-1} HDLp, primary antibody dilution of 1:1000 and secondary antibody dilution of 1:10,000 (Fig. 8B). The absorbance increased linearly ($r = 0.971$) between 3.125 and 100 ng HDLp (Fig. 8B). The absorbance declined at 200 ng lipophorin when compared to the absorbance at 100 ng lipophorin.

3.6. Lipophorin titers in different termite stages and castes

As a foundation for later studies on the role of lipophorin in caste differentiation, we quantified the lipophorin titers in different stages and castes and calculated the lipophorin titer as a function of fresh body mass. The lipophorin titer increased steadily, from 228 to 459 ng mg^{-1} body mass, as termite workers grew in mass (Fig. 9). Soldiers and primary reproductives contained significantly less lipophorin in relation to their large size, probably because of much greater allocation of body mass to cuticular structures (e.g., soldier mandibles). Maximal lipophorin titers, up to 600 ng mg^{-1} body mass, were measured in nymphs.

4. Discussion

A requirement for studies of the structure and function of lipophorin in insects is to develop sensitive

and specific antibodies that can be used to immunolocalize lipophorin, quantify its titer, and for studies of lipophorin transport function. In the present paper, a polyclonal antiserum was grown against termite HDLp, its specificity against various termites and cockroaches was characterized, a highly sensitive quantitative ELISA was developed, and the antiserum was used to disrupt hydrocarbon transport by HDLp.

The characteristics of the HDLp purified from *R. flavipes* generally agree with previous reports on termite lipophorins. Our molecular weight estimates for apoLp-I (210 kDa) and apoLp-II (85 kDa) generally agree with those of Okot-Kotber and Prestwich (1991a) for the same species (230 and 76 kDa, respectively) and with estimates of 220 and 82 kDa for HDLp of *Z. nevadensis* (Sevala et al., 2000). This, together with the immunoblotting experiment and previous research (Okot-Kotber and Prestwich, 1991b), suggest that all termites across a wide spectrum of species possess analogous HDLp that share common general properties.

Termites are closely related to wood-eating cockroaches. Considering common characteristics, it has been suggested that within the Dictyoptera, Blattodea (cockroaches) are a sister group of Mantodea (mantids), while Isoptera (termites) is a sister group of this complex (e.g., Wheeler et al., 2001). We compared the cross-reactivity of antiserum against *R. flavipes* lipophorin with lipophorins from other termite species as well as several representative species of cockroaches. Interestingly, hemolymph from all the termite species we examined cross-reacted with antiserum prepared against *R. flavipes* (Rhinotermitidae) lipophorin. Although radial immunodiffusion experiments showed no cross-reactivity with hemolymph of *K. flavicollis* (Kalotermitidae) (a drywood species that is relatively distantly related to subterranean species) and low reactivity with *Z. nevadensis* (Termopsidae) (more closely related to subterranean species), Western blots confirmed cross-reactivity in these evolutionarily distant relatives of the subterranean termites (see Kambhampati and Eggleton, 2000 for taxonomic relationships of termites). Surprisingly, the hemolymph of several species cross-reacted only with apoLp-I in western blots. ApoLp-II of *Z. nevadensis* was not recognized by antibodies against native lipophorin of *R. flavipes*, and apoLp-II of *R. santonensis*, a very closely related species to *R. flavipes* (Bagnères et al., 1990), was only weakly recognized by the antiserum.

Cross-reactivity of the antiserum with hemolymph of cockroaches was much weaker than with hemolymph of termites. Nevertheless, a clear phylogenetic pattern emerged, showing greater reactivity with the more primitive cockroaches in the Cryptocercidae (*Cryptocercus*), Blattidae (*Periplaneta*) and Blattellidae (*Supella*), and essentially no cross-reactivity with the hemolymph of the advanced Blaberidae (*Blaberus* and *Diploptera*), in support of the wood-feeding cockroaches (*Cryptocercus*) as closely related to Isoptera (see Kambhampati, 1995; Lo et al., 2000). Using antiserum against *Blattella* lipophorin, Sevala et al. (1999) showed the presence of an immunologically cross-reactive protein in hemolymph of *Supella* (both in the Blattellidae), and no cross-reactivity with the hemolymph of *Periplaneta* and *Diploptera*. These findings are therefore in agreement with others showing antigenic cross-reactivity of lipophorin among phylogenetically related species, but less cross-reactivity with species in more distant taxa (Chino and Kitazawa, 1981; Ryan et al., 1984; Schulz et al., 1987; King and Tobe, 1993).

With the exception of *Z. nevadensis*, the antiserum against *R. flavipes* lipophorin antiserum recognized both subunits of lipophorin of all other termite species. Conversely, our antibody against *Blattella* lipophorin revealed strong immunoreactivity with the apoLp-I subunit and little or no cross-reactivity with apoLp-II (Sevala et al., 1999). We had suggested that the latter could be explained based on the native structure of HDLp, because apoLp-I constitutes the outer portion of the lipophorin particle, with apoLp-II forming its inner core (Kanost et al., 1990; Soulages and Wells, 1994). Lack of interaction of the antiserum with apoLp-II could then be due to the fact that antibodies were generated against the native particle. If so, then in termites, it appears that the lipophorin particle dissociated upon injection into rabbit and the antibodies generated therefore recognize epitopes that are associated with both apolipophorins.

Lipophorin contains about 35–50% lipid and its primary function in insects is to transport lipids through the hemolymph (Chino, 1985; Kanost et al., 1990; Van der Horst et al., 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994; Sevala et al., 1999; Ryan and van der Horst, 2000; Canavoso et al., 2001). All lipophorins characterized thus far contain a significant amount of phospholipid that forms the particle surface, and a hydrophobic neutral lipid core. The type and amount of neutral lipids vary according to the physiological stage and across insect species. In most flight-capable insect species, including adult *Manduca sexta* and adult *Locusta migratoria*, the neutral lipid is predominantly *sn*-1,2-diacylglycerol. Nevertheless, even in larvae, as in *Drosophila melanogaster*, diacylglycerol may constitute the major neutral lipid (Canavoso et al., 2001). Triacylglycerol-rich lipophorins have been reported in the dipteran infraorder Culicomorpha (*Aedes aegypti*) and the suborder Nematocera (Pennington and Wells, 2002). Lipophorins of the cockroaches *B. germanica* and *P. americana* and termites (this paper), on the other hand, contain hydrocarbons as the major class of neutral lipids.

Although experimental evidence for involvement of lipophorin in hydrocarbon transport is meager, several studies have documented both the uploading of hydrocarbons into lipophorin and their downloading to the epicuticle and ovaries (reviews: Chino, 1985; Schal et al., 1998, 2003; Fan et al., 2002). In this paper, for the first time in a social insect, we provide experimental support for the model that hydrocarbons are delivered to the cuticle by lipophorin. Injection of lipophorin-specific antiserum into termites significantly disrupted the transfer of newly synthesized hydrocarbons to the cuticular surface. Obviously, careful experiments are needed to confirm that this was not an indirect effect of disrupting other physiological processes, such as juvenile hormone transport, which is also served by lipophorin. Nevertheless, together with other experimental results from cockroaches (Gu et al., 1995; Fan et al., 2002), and genetic manipulations in *Drosophila* (Ferveur et al., 1997), the evidence is quite compelling that most, if not all, cuticular and ovarian hydrocarbons are delivered to these tissues by lipophorin.

A unique system of post-embryonic development has evolved in termites, in which eggs hatch into first instar larvae that subsequently undergo differentiation into various castes or morphs. Some larvae develop through nymphal stages (long wing pads) into primary reproductives, while other nymphs, with short wing pads, develop into neotenics (Buchli, 1958). Other larvae develop into workers, which in turn can transform into secondary reproductives and soldiers. The reproductive and soldier castes are the only castes considered terminal stages in subterranean species.

The lipophorin titer in *R. flavipes* workers increased steadily as termite workers grew in mass (Fig. 9). Maximal lipophorin titers, up to 600 ng mg^{-1} body mass, were measured in nymphs. Soldiers contained significantly less lipophorin in relation to their large size, probably caused by the large mass of their mandibles and head, which contains more muscle and an enlarged frontal gland used in defense. The extra weight of the thoracic musculature in primary reproductives (wings were shed before the assays) may also lead to a lower ratio between lipophorin titer and body mass in this stage (Fig. 9). It is also possible, however, that in these terminal stages hydrocarbon transport to the cuticle is minimal, and therefore less lipophorin is needed in the hemolymph. In contrast, during each successive molt the cuticular hydrocarbons are lost, requiring that earlier larval stages mobilize hydrocarbons to the newly formed cuticle. It is not surprising therefore that early larval stages have high titers of lipophorin.

Nevertheless, lipophorin carries other lipids whose titers change in relation to development (Okot-Kotber et al., 1993) and caste differentiation. For example, juvenile hormone, which appears to play a central role in caste differentiation, is also carried by lipophorin. As pointed out by Sevala et al. (1999), the various lipids carried by lipophorin vary both temporally and quantitatively, suggesting that a clear relationship is unlikely between the titer of lipophorin and that of any one of its ligands. Even a single class of lipid—for instance, hydrocarbons—varies greatly during development. All castes of termites contain the same hydrocarbons, but in different proportions, presumably as mediators of species- and caste-recognition (Blomquist et al., 1979; Bagnères et al., 1991; Clément and Bagnères, 1998). Lipophorin appears to be involved in effecting these changes (Sevala et al., 2000), further suggesting that variation in lipophorin titer may coincide with changes in caste-specific lipids.

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