Lipid composition of tropical and subtropical copepod species of the genus *Rhincalanus* (Copepoda: Eucalanidae): a novel fatty acid and alcohol signature

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ABSTRACT: *Rhincalanus nasutus*, *R. rostrifrons*, and *R. cornutus* are primarily found in subtropical and tropical waters and accumulate large lipid stores, an uncommon feature in low-latitude copepods. While *R. nasutus* fatty acid and alcohol profiles have been examined previously, little is known about lipids in *R. rostrifrons* or *R. cornutus*. Lipid profiles for wax ester, triacylglycerol, phospholipid, free fatty acid, sterol, and free fatty alcohol fractions were determined for these 3 species collected from the eastern tropical north Pacific, Gulf of California, and Gulf of Mexico. Storage lipids, primarily wax esters, were the dominant component (>88%) of total lipid in all 3 *Rhincalanus* species. *R. nasutus*, however, had distinctly different storage lipid fatty acid and alcohol profiles, with primarily 16:1(n-7)/18:1(n-9) fatty acids and 14:0/16:0 fatty alcohols, while *R. cornutus* and *R. rostrifrons* accumulated 14:0/16:0 fatty acids and 16:1/18:1 fatty alcohols. Species differences also were observed in sterol profiles, with *R. rostrifrons* having a cholesterol content of 75 to 76% versus 85 to 90% in *R. nasutus* and *R. cornutus*. The remainder of the sterol fraction was largely composed of 22-dehydrocholesterol in all species. In all 3 species, phospholipid fractions were dominated by 22:6(n-3), 16:0, 18:0, 20:5(n-3), and 18:1(n-9) fatty acids. The results of the present study suggest that genetic predisposition significantly governs lipid profiles, particularly storage lipids, within this genus. The storage lipid profiles observed in *R. cornutus* and *R. rostrifrons* were unique from their congeners and have not been reported previously for other copepod species.

KEY WORDS: *Rhincalanus* spp. · Copepod · Lipid · Fatty acid · Fatty alcohol · Sterol

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INTRODUCTION

Rhincalanus (family Eucalanidae) is a small genus of copepods, consisting of only 4 species: *R. nasutus*, *R. cornutus*, *R. rostrifrons*, and *R. gigas*. *R. gigas* is an Antarctic, circumpolar species, found only south of 50° S (Lang 1965, Bradford-Grieve et al. 1999). This species is primarily found in the upper 1000 m of the water column, although its distribution varies sub-stantially with location and season (Spiridonov & Kosobokova 1997, Ward et al. 1997). In contrast, *R. nasutus* is most common in tropical and subtropical environments throughout the Indian, Atlantic, and Pacific oceans (Grice 1962, Lang 1965, Bradford-Grieve et al. 1999, Goetze 2003). *R. rostrifrons* and *R. cornutus* also are common in tropical and subtropical environments, but do not have overlapping ranges (Lang 1965, Bradford-Grieve et al. 1999).

R. rostrifrons inhabits the Pacific and Indian oceans, while *R. cornutus* is restricted to the Atlantic Ocean. The subtropical *Rhincalanus* species inhabit the epi- and mesopelagic zones of the water column (Lang 1965), with peaks in abundance often found between 200 and 600 m (Morris & Hopkins 1983, Chen 1986, Sameoto 1986, Ohman et al. 1998, Vinogradov et al. 2004, Schnack-Schiel et al. 2008).

Rhincalanus rostrifrons and *R. cornutus* are sometimes considered to be 2 subspecies of *R. cornutus*: *R. c. rostrifrons* and *R. c. cornutus* (Lang 1965). As there does not seem to be a consensus on the preferred nomenclature in the literature (e.g. Longhurst 1985, Arcos & Fleminger 1986, Chen 1986, Sameoto 1986, Vicencio Aguilar & Fernández-Álamo 1996, Saltzman & Wishner 1997, Fernández-Álamo et al. 2000), we recognize *R. rostrifrons* and *R. cornutus* as separate species, following the most current molecular-based phylogenies of the Eucalanidae family (Goetze 2003, 2010).

All 4 *Rhincalanus* species accumulate wax esters (WEs) as the primary storage lipid (61 to 92% of total body lipid in adults), which are often present in a large oil sac (Lee et al. 1971a, 2006, Lee & Hirota 1973, Graeve et al. 1994, Kattner et al. 1994, Kattner & Hagen 1995, Sommer et al. 2002, Schnack-Schiel et al. 2008). Overall lipid content is high, with reported values ranging from 16 to 69% of dry weight (DW) (Lee & Hirota 1973, Morris & Hopkins 1983, Kattner et al. 1994, Sommer et al. 2002, Schnack-Schiel et al. 2008). While this is not surprising for the high-latitude *R. gigas*, it is uncommon for tropical and subtropical species to accumulate large amounts of lipids, and for the accumulated lipids to be comprised of WEs (Lee & Hirota 1973).

Thus far, only the lipid profiles (fatty acids and fatty alcohol components) of *Rhincalanus gigas* and *R. nasutus* have been reported. Both accumulate 18:1(n-9) and 16:1(n-7) fatty acids and 14:0 and 16:0 fatty alcohols in their storage lipids (Lee et al. 1971a, Graeve et al. 1994, Kattner & Hagen 1995, Schnack-Schiel et al. 2008). This pattern is unique from many other well-studied polar copepods, and a specific fatty acid and alcohol synthesis pathway has been proposed for *R. gigas* (Kattner & Hagen 1995), which *R. nasutus* likely shares, given its similar WE composition. Genetic work indicates that *R. nasutus* and *R. gigas* appear to be more closely related to each other, while *R. rostrifrons* and *R. cornutus* form their own monophyletic group (Goetze 2003, 2010).

The goal of the present study was to investigate the lipid profiles of *Rhincalanus cornutus* and *R. rostrifrons* in order to evaluate their similarity to each other and to their congeners. Lipid profiles for WE, triacylglycerol (TAG), phospholipid, free fatty acid (FFA), sterol, and free fatty alcohol fractions were determined for individuals collected from the eastern tropical north Pacific (ETNP), Gulf of California (GOC), and Gulf of Mexico (GOM). Obtaining this detailed lipid composition data will aid in evaluating the extent of the genetic component in accumulation patterns of storage lipid fatty acids and alcohols in this genus. Additional detailed lipid profiles of *R. nasutus* also were generated, extending the knowledge of lipid composition and partitioning in this species.

MATERIALS AND METHODS

Adult female *Rhincalanus* spp. copepods were collected in the upper 200 to 300 m of the water column during 4 cruises at 3 different locations. Both *R. rostrifrons* and *R. nasutus* were obtained during 2 cruises in the ETNP to the Costa Rica Dome region (9°N, 90°W) during October–November 2007 and December 2008–January 2009, aboard the RVs 'Seward Johnson' and 'Knorr', respectively. Individuals were collected with a 3 m^2 Tucker trawl, paired bongo net system, and a 1 m^2 multiple opening/closing net and environmental sampling system (MOCNESS). *R. cornutus* adult females were obtained during a cruise in the eastern GOM (27°N, 86°W) offshore of Florida during June 25–29, 2007 aboard the RV 'Suncoaster'. Additional *R. nasutus* individuals were collected from the Guaymas Basin, GOC (approximate location: 27°N, 111°W) during June 4 to 12, 2007, aboard the RV 'New Horizon'. Copepods on the GOM and GOC cruises were collected using a 3 m^2 Tucker trawl. Immediately after capture, adult females were sorted and individuals of each species were separated into small containers containing 0.2 µm-filtered seawater at *in situ* temperature. Copepods were kept at *in situ* temperatures for approximately 3 to 12 h to allow them to empty their guts. All individuals were frozen in cryovials at −80°C or below until analyses could be performed back in the lab. For ETNP and GOC cruises, a −80°C ship-board freezer was utilized. For the GOM cruise, copepods were flash-frozen in liquid nitrogen for the duration of the cruise, and transferred to a −80°C freezer upon return to the lab.

Prior to lipid extraction, individuals were thawed and total length and prosome length were measured to the nearest mm. Wet weights (WW, in mg) were estimated using length–weight equations derived

from measurements on additional copepods from these same locations (Cass 2011). *Rhincalanus nasutus* weights were calculated using:

$$\text{Log(WW)} = 3.22 \times \text{Log(TL)} - 1.97 \qquad (1)$$

where TL (mm) was total length ($R^2 = 0.97$, $p < 0.001$, $n = 30$). Weights for *R. cornutus* and *R. rostrifrons* were determined using:

$$\text{Log(WW)} = 3.16 \times \text{Log(PL)} - 1.66 \qquad (2)$$

where PL (mm) was prosome length ($R^2 = 0.95$, $p < 0.001$, $n = 48$). DWs were estimated by conversion from WWs, based on the average percent water of each species at each location (Cass 2011). Copepods were re-grouped into batches of 24 to 46 individuals (each batch comprised a single sample) and refrozen at −80°C until lipid extraction occurred. Five samples in total were analyzed: (1) *R. cornutus* from the GOM cruise; (2) *R. rostrifrons* from the 2007 ETNP cruise; (3) *R. rostrifrons* from the 2008–2009 ETNP cruise; (4) *R. nasutus* from the GOC cruise; and (5) *R. nasutus* from the 2007 ETNP cruise.

Lipid was extracted by homogenizing copepods in 2:1 dichloromethane (DCM):methanol (MeOH) using a tissue grinder. The liquid was then transferred to a capped centrifuge tube containing a few ml of salt water (5%) and shaken. Subsequently, the DCM layer was removed, more DCM was added to the centrifuge tube, and the process was repeated several times. The extracted DCM was dried using anhydrous sodium sulfate and evaporated using a rotary evaporator setup, yielding the total lipid extract.

Separation of the lipid classes was attained with silica columns using 5% deactivated silica gel (Merck silica gel 60, 70–230 mesh; Wakeham & Volkman 1991). Five of the resulting fractions were utilized: WEs, TAGs, free fatty alcohols and sterols, FFAs, and phospholipids. The WE, TAG, FFA, and phospholipid fractions were saponified by heating the sample to 100°C for 2 h with 0.5 N KOH in MeOH. Nonsaponifiable (neutral) fractions (fatty alcohols and sterols) were removed first with hexane. The remaining solution was then acidified (pH < 2) and hexane was used again to recover the acidic fraction (fatty acids). The fatty acids were converted to fatty acid methyl esters (FAMEs) by addition of diazomethane. The neutral WE fraction

and the free fatty alcohol plus sterol fraction were converted to trimethylsilyl (TMS) ethers using N,O-bis(trimethylsilyl)trifluoro-acetamide and pyridine.

Samples were run on a GC (Agilent 6890 gas chromatograph with a flame ionization detector [FID]) or GC-MS (Agilent 6890 gas chromatograph coupled to an Agilent 5793 mass spectrometer). FAME fractions were run on a Restek RTX-WAX column, while TMS ethers utilized a J&W DB-XLB column. Internal standards of methylnonadecanoate for FAMEs and 5α(H)-cholestane for the TMS ethers were added to each sample prior to injection on the GC or GC-MS. Identification of compounds was based on the mass spectra and retention times. The mass of each compound was converted to moles, and molar percentages are reported unless otherwise noted. Total lipid mass and percent mass of each lipid class was calculated by summing identified lipid compounds in all or relevant fractions.

Cluster analyses (using PRIMER 6 software) were used to examine relative similarity between samples. Resemblance matrices for fatty acid, alcohol, or sterol profiles of samples were generated using an S17 Bray-Curtis similarity test (Bray & Curtis 1957).

RESULTS

Lipid classes and weights

Rhincalanus nasutus had total lipid content ranging from 1.2 to 1.3% of WW and 8.8 to 9.4% of DW in both the GOC and the ETNP (Table 1). *R. cornutus* lipid content was 1.0% of WW or 5.2% of DW in the GOM. *R. rostrifrons* from the ETNP showed a higher

Table 1. *Rhincalanus cornutus*, *R. rostrifrons*, and *R. nasutus*. Percent total lipid mass. Percent weights are in mass lipid per mass of copepod. Average wet (WW) and dry weights (DW) are in mg per individual. Average (minimum–maximum in parentheses) prosome length (PL) is noted. N denotes the number of copepods used for lipid samples. *R. rostrifrons* samples are separated by collection year and *R. nasutus* by collection site (GOC: Gulf of California; ETNP: eastern tropical north Pacific). *R. cornutus* were collected in the Gulf of Mexico and *R. rostrifrons* from the ETNP

	% WW	% DW	WW (mg)	DW (mg)	% Water	PL (mm)	N
R. cornutus	1.0	5.2	0.68	0.13	80.6	2.9 (2.6–3.1)	24
R. rostrifrons							
2007	2.3	17.6	0.65	0.09	86.7	2.7 (2.4–3.0)	46
2008	3.1	23.3	0.64	0.09	86.7	2.8 (2.7–3.1)	30
R. nasutus							
GOC	1.3	9.4	1.17	0.16	85.9	3.4 (3.1–3.6)	28
ETNP	1.2	8.8	1.21	0.17	85.9	3.4 (3.1–3.7)	33

lipid content, comprising 2.3 to 3.1% of WW and 17.6 to 23.3% of DW.

Lipid classes in all *Rhincalanus* species were dominated by storage lipids, primarily WEs (Table 2). Total storage lipid (WE + TAG) was ≥88% of the mass

of total lipid. WEs alone accounted for >85% of total lipid and >94% of storage lipid in *R. rostrifrons* and *R. nasutus*. While *R. cornutus* accumulated WEs as the primary storage lipid, WEs only made up 54% of the total lipid and 62% of total storage lipid. *R. cornutus* TAG comprised 34% of total lipid, whereas TAG made up ≤5% of total lipid in the other species. For all species, FFAs were the next highest lipid class (1.5 to 8.2%) after storage lipids, followed by sterols (0.6 to 4.7%), phospholipids (0.4 to 1.8%), and finally free fatty alcohols (0.1 to 1.0%).

Table 2. *Rhincalanus cornutus*, *R. rostrifrons*, and *R. nasutus*. Percentage of total lipid mass of each lipid class by species. *R. rostrifrons* samples are separated by collection year and *R. nasutus* by collection site (GOC: Gulf of California; ETNP: eastern tropical north Pacific). WE: wax esters; TAG: triacylglycerols; FFAlc: free fatty alcohols; FFA: free fatty acids; P-lip: phospholipids

	WE	TAG	FFAlc	Sterol	FFA	P-lip
R. cornutus	54.1	33.9	0.6	1.9	8.2	1.4
R. rostrifrons						
2007	85.6	5.2	1.0	4.7	2.6	0.9
2008	96.5	0.5	0.1	0.6	1.8	0.4
R. nasutus						
GOC	87.1	4.2	0.4	1.2	5.5	1.5
ETNP	91.6	2.6	0.1	2.4	1.5	1.8

Storage lipid fatty acids

Rhincalanus rostrifrons and *R. cornutus* both showed the same trend in storage lipid fatty acid profiles (Table 3). Storage lipid fatty acids (hereinafter meaning the weighted average of WE and TAG fatty acids) primarily were composed of 16:0 and 14:0 fatty acids (67.1 to 84.0%). Other noteworthy components included fatty acids 16:1(n-7) (2.1 to 5.5%), 18:1(n-9)

Table 3. *Rhincalanus cornutus* and *R. rostrifrons*. Fatty acid profiles. WE: wax esters; TAG: triacylglycerols; TSL: total storage lipid (a weighted average of WE and TAG); P-lip: phospholipids; Total FA: total fatty acids. Values are in percent of total moles. *R. rostrifrons* samples are separated by collection year

| | R. cornutus | | | | | R. rostrifrons | | | | | | | | | |
| | | | | | | 2007 | | | | | 2008 | | | | |
	WE	TAG	TSL	P-lip	Total FA	WE	TAG	TSL	P-lip	Total FA	WE	TAG	TSL	P-lip	Total FA
12:0	0.2	0.2	0.2	1.6	0.3	0.1	0.2	0.1	0.4	0.1	0.1	0.2	0.1	1.5	0.2
13:0	0.1	0.1	0.1	0.4	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.0	0.2
14:0	34.3	30.8	32.0	6.7	28.3	42.1	25.2	40.5	3.0	38.3	47.7	37.5	47.5	4.8	45.4
14:1	0.0	0.5	0.3	0.2	0.3	0.3	0.1	0.3	0.0	0.3	0.3	0.2	0.3	0.0	0.3
15+17 branched	0.3	0.3	0.3	0.6	0.3	1.0	0.8	1.0	0.9	1.0	1.4	1.3	1.4	0.4	1.3
15:0	1.0	0.8	0.9	0.7	1.0	1.2	0.9	1.2	0.6	1.4	1.4	1.2	1.4	0.7	1.9
16:0	45.1	30.1	35.1	28.7	34.4	39.2	35.7	38.7	20.8	37.6	36.5	33.2	36.5	28.3	35.9
16:1(n-5)	0.2	0.4	0.4	0.2	0.3	0.2	0.3	0.2	0.1	0.2	0.2	0.3	0.2	0.0	0.2
16:1(n-7)	2.8	6.9	5.5	2.1	5.0	2.2	2.7	2.3	2.2	2.3	2.1	2.5	2.1	1.3	2.1
16:2	0.0	1.6	1.1	0.2	0.9	0.5	0.9	0.6	0.0	0.5	0.0	0.7	0.0	0.0	0.0
16:3(n-4)	0.0	2.1	1.4	0.3	1.2	0.4	1.5	0.6	0.0	0.5	0.0	0.1	0.0	0.0	0.0
16:4(n-1)	0.0	2.3	1.5	0.4	1.3	0.7	2.1	0.8	0.0	0.8	0.4	3.0	0.4	0.0	0.4
Phytanic acid	2.7	0.3	1.1	0.0	1.0	1.8	2.2	1.8	0.3	1.7	2.1	1.0	2.1	0.0	2.0
17:0	1.0	0.1	0.4	0.9	0.9	0.0	0.0	0.0	1.1	0.0	0.0	0.4	0.0	1.7	0.7
17:1	0.0	0.0	0.0	0.3	0.0	0.1	0.1	0.1	0.0	0.1	0.2	0.8	0.2	0.0	0.2
18:0	5.7	2.3	3.4	15.7	5.8	4.8	8.2	5.1	23.6	5.6	2.7	2.7	2.7	17.6	3.1
18:1(n-7)	0.6	0.9	0.8	2.0	1.4	0.6	1.1	0.6	1.6	0.7	0.4	0.4	0.4	1.6	0.5
18:1(n-9)	3.8	6.4	5.5	7.8	7.1	1.4	4.2	1.7	9.2	2.3	1.0	1.9	1.0	5.3	1.2
18:2	0.0	0.5	0.3	0.7	0.3	0.2	1.1	0.3	0.8	0.3	0.2	0.4	0.2	0.6	0.2
18:4(n-3)	0.0	1.3	0.9	0.0	0.8	0.2	0.7	0.2	0.0	0.2	0.2	1.3	0.2	0.0	0.2
20:0	0.2	0.1	0.2	0.3	0.2	0.4	0.7	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.3
20:1	0.0	0.0	0.0	0.7	0.0	0.0	0.2	0.0	0.4	0.0	0.0	0.2	0.0	0.5	0.0
20:4(n-6)	0.0	1.3	0.8	1.7	0.9	0.0	1.2	0.1	3.3	0.4	0.0	0.5	0.0	2.0	0.1
20:5(n-3)	0.0	7.5	5.0	5.9	4.8	0.8	4.4	1.2	6.6	1.6	0.6	2.8	0.6	5.2	1.1
22:6(n-3)	1.8	1.9	1.9	17.4	2.5	0.7	2.8	1.0	20.5	2.1	1.3	4.1	1.4	23.3	1.5
24:1	0.0	0.0	0.0	3.5	0.1	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	3.2	0.0
Other	0.0	1.1	0.7	1.8	0.8	1.0	2.6	1.2	2.8	1.3	0.8	2.8	0.8	1.8	1.0

(1.0 to 5.5%), 18:0 (2.7 to 5.1%), and 20:5(n-3) (0.6 to 6.6%). Resemblance matrices indicated that WE and TAG fatty acid profiles were 75 to 85% similar within species. Generally, TAG fractions had higher percentages of 16:1(n-7), 18:1(n-9), 20:5(n-3), and 22:6(n-3), while WEs had higher amounts of 14:0 and 16:0 fatty acids.

Rhincalanus nasutus showed a very different storage lipid fatty acid profile than *R. cornutus* and *R. rostrifrons* (Table 4). Monounsaturated fatty acids 16:1(n-7) and 18:1(n-9) dominated the profiles, comprising 64.8 to 69.5% of the total fatty acids. Other important fatty acids included phytanic acid (3.6 to 12.1%), 14:0 (2.4 to 4.4%), 16:2 (1.3 to 3.6%), 20:5 (n-3) (0.1 to 5.9%), and 18:2 (1.2 to 2.5%). WE and TAG fatty acid profiles showed 69 to 75% similarity within samples. Variation often occurred in the amount of 16:1(n-7), 18:1(n-9), 16:0, 18:0, 20:5(n-3), and 22:6(n-3), although the types of differences seen between WE and TAG fatty acids were not consistent between the 2 *R. nasutus* samples collected at different locations.

Cluster analyses indicated that storage lipid fatty acid accumulation patterns showed 2 main groupings: *Rhincalanus cornutus/R. rostrifrons* and *R. nasutus*. Between groups, there was only 20% similarity, but within groups all samples were >80% similar (Fig. 1A).

Phospholipid fatty acids

Phospholipid fractions for all species were dominated by 22:6(n-3) (14.7 to 36.9%), 16:0 (18.1 to 28.7%), 18:0 (11.2 to 23.6%), 18:1(n-9) (5.3 to 14.9%), and 20:5(n-3) (5.2 to 9.8%) fatty acids. Total fatty acid profiles were similar to the storage lipid profiles, as the majority of the fatty acids were from WEs and TAGs. Cluster analyses indicated that phospholipid profiles did not show interspecific differences, with all samples showing >70% similarity and no distinct sectioning observed between species, location, or year (Fig. 1B).

Table 4. *Rhincalanus nasutus.* Fatty acid profiles for the Gulf of California (GOC) and eastern tropical north Pacific (ETNP) samples. Values are in percent of total moles. See Table 3 for other abbreviations

|	GOC					ETNP				
	WE	TAG	TSL	P-lip	Total FA	WE	TAG	TSL	P-lip	Total FA
12:0	0.4	0.3	0.4	0.7	0.5	0.1	0.4	0.1	0.2	0.1
13:0	0.0	0.1	0.0	0.4	0.2	0.0	0.0	0.0	0.0	0.0
14:0	4.8	3.0	4.4	3.2	4.3	2.2	3.8	2.4	1.0	2.4
14:1	0.3	0.7	0.4	0.0	0.3	0.4	0.2	0.5	0.0	0.4
15+17 branched	0.1	0.0	0.1	1.1	0.4	0.0	0.5	0.1	0.2	0.1
15:0	0.2	0.0	0.2	0.4	0.3	0.0	0.2	0.0	0.2	0.1
16:0	3.6	0.7	3.0	25.8	7.8	0.7	11.2	1.3	18.1	2.5
16:1(n-5)	0.8	0.8	0.8	0.2	0.7	0.4	0.2	0.5	0.0	0.4
16:1(n-7)	29.6	47.3	33.5	3.0	26.0	35.1	18.0	34.3	2.0	32.5
16:2	0.5	3.9	1.3	0.2	1.0	3.6	1.1	3.6	0.0	3.4
16:3(n-4)	0.0	0.6	0.1	0.1	0.1	2.7	1.3	2.8	0.0	2.6
16:4(n-1)	0.0	0.0	0.0	0.0	0.0	1.7	1.3	1.8	0.0	1.7
Phytanic acid	15.0	1.3	12.1	0.0	8.9	3.6	6.2	3.6	0.2	3.4
17:0	0.0	0.0	0.0	1.1	0.3	0.0	0.0	0.0	0.7	0.0
17:1	0.4	0.3	0.4	0.6	0.4	0.3	0.3	0.3	0.0	0.3
18:0	2.1	0.3	1.7	14.7	4.1	0.5	10.3	1.0	11.2	1.5
18:1(n-7)	1.7	1.4	1.6	5.0	3.2	1.4	1.8	1.4	2.1	1.4
18:1(n-9)	36.9	34.3	36.0	14.9	34.1	30.8	24.2	30.5	11.1	29.5
18:2	0.9	2.3	1.2	1.0	1.2	2.4	1.8	2.5	0.7	2.4
18:4(n-3)	0.0	0.0	0.0	0.0	0.0	0.8	0.7	0.8	0.0	0.8
20:0	0.1	0.0	0.1	0.2	0.2	0.0	0.3	0.0	0.0	0.0
20:1	0.8	0.5	0.7	0.6	0.6	0.7	0.9	0.6	0.5	0.6
20:4(n-6)	0.0	0.5	0.1	1.6	0.2	1.6	1.7	1.6	2.5	1.7
20:5(n-3)	0.0	0.7	0.1	6.3	1.4	5.6	8.1	5.9	9.8	6.1
22:6(n-3)	1.5	0.0	1.2	14.7	3.0	1.6	3.4	1.8	36.9	3.3
24:1	0.0	0.0	0.0	2.0	0.1	0.0	0.0	0.0	0.0	0.0
Other	0.3	1.1	0.5	2.0	0.8	2.4	2.0	2.6	3.6	2.6
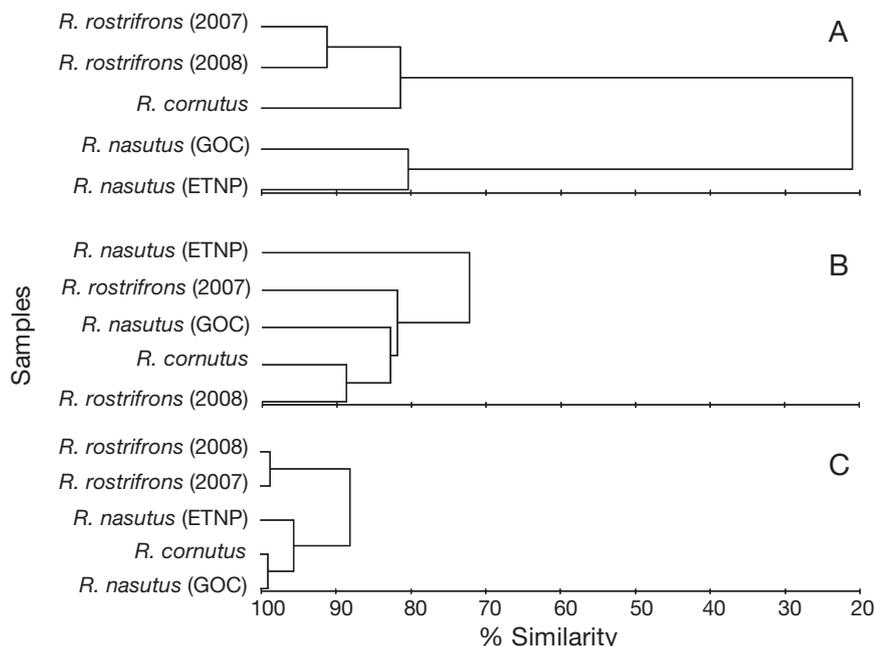
Fig. 1. *Rhincalanus cornutus*, *R. rostrifrons*, and *R. nasutus*. Percent similarity for different lipid fractions: (A) total storage lipid fatty acid profiles, (B) phospholipid fatty acids, and (C) sterol profiles. *R. rostrifrons* samples are separated by collection year and *R. nasutus* by collection site (GOC: Gulf of California; ETNP: eastern tropical north Pacific)

Fatty alcohols

Similar to fatty acids, fatty alcohol profiles were very different between *Rhincalanus cornutus/R. rostrifrons* and *R. nasutus* (Table 5). *R. cornutus* and *R. rostrifrons* WE fatty alcohol profiles almost exclusively consisted of 18:1 (62.6 to 68.8%) and 16:1 (26.4 to 33.7%). In total, these 2 alcohols comprised >95% of WE fatty alcohols. Unfortunately, double-bond position could not be determined for these alcohols, but the presence of single 16:1 and 18:1 peaks indicated that only 1 isomer was present for each alcohol. Other minor alcohol components included 16:0 (2.0 to 4.9%), 14:0 (0.0 to 1.4%), and 18:0 (0.0 to 0.3%). Conversely, *R. nasutus* WE fatty alcohols were primarily 16:0 (58.4 to 66.4%) and 14:0 (24.3 to 34.0%), together making up 91 to 93% of the total fatty alcohols. Also, 18:0 (6.7 to 9.2%) was often the only other fatty alcohol found. Cluster analyses indicated that there was <5% similarity between *R.*

cornutus/R. rostrifrons and *R. nasutus* WE alcohol profiles. Within species as well as between *R. cornutus* and *R. rostrifrons*, similarity was >90%, and *R. rostrifrons* profiles from the 2 sampled locations (ETNP and GOC) were almost identical (99% similarity). The total fatty alcohol profiles were very similar to the WE fatty alcohol profiles, as free fatty alcohols were only a minor lipid component.

Sterols

The primary sterol found in *Rhincalanus cornutus*, *R. rostrifrons*, and *R. nasutus* was cholest-5-en-3β-ol, commonly called cholesterol (Table 6). This comprised around 75 to 76% of total sterols in *R. rostrifrons* and 85.6 to 89.2% of total sterols in *R. cornutus* and *R. nasutus.* The remaining sterols included cholesta-5,22E-dien-3β-ol (22-dehydro-cholesterol) (23.4 to 24.7% in *R. rostrifrons*, 9.5 to 14.4% in *R. cornutus* and *R. nasutus*), and 5α(H)-cholestan-3β-ol (cholestanol) (<2%). Cluster analyses revealed that sterol data formed 2 groups of high similarity: *R. rostrifrons* and *R. nasutus/R. cornutus*. Although sterol profiles showed >85% similarity between groups, within each group, samples were >95% similar to each other (Fig. 1C).

Table 5. *Rhincalanus cornutus*, *R. rostrifrons*, and *R. nasutus*. Fatty alcohol profiles. Fatty alcohols found in the wax ester (WE) fraction are marked separately from total fatty alcohols, which include WE fatty alcohols and free fatty alcohols. Values are in percent (molar) of total fatty alcohols. *R. rostrifrons* samples are separated by collection year and *R. nasutus* by collection site (GOC: Gulf of California; ETNP: eastern tropical north Pacific)

| | *R. cornutus* | | *R. rostrifrons* | | | | *R. nasutus* | | | |
| | | | 2007 | | 2008 | | GOC | | ETNP | |
	WE	Total	WE	Total	WE	Total	WE	Total	WE	Total
12:0	0.0	0.3	0.0	0.0	0.0	0.1	0.0	1.5	0.0	0.0
14:0	1.4	1.7	0.1	0.1	0.0	0.1	24.3	20.9	34.0	33.7
15:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9
16:0	2.0	2.8	3.9	4.5	4.9	5.8	66.4	66.6	58.4	58.4
16:1	33.7	32.5	26.9	25.5	26.4	26.0	0.0	0.0	0.0	0.0
17:0	0.0	1.5	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
17:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
18:0	0.3	0.8	0.3	0.6	0.0	0.3	9.2	11.0	6.7	6.8
18:1	62.6	60.5	68.8	69.3	68.7	67.6	0.0	0.0	0.0	0.0

Table 6. *Rhincalanus cornutus*, *R. rostrifrons*, and *R. nasutus*. Sterols. Values are percent (molar) of total sterols. *R. rostrifrons* samples are separated by collection year and *R. nasutus* by collection site (GOC: Gulf of California; ETNP: eastern tropical north Pacific)

	R. cornutus	*R. rostrifrons* 2007	2008	*R. nasutus* GOC	ETNP
Cholesta-5,22E-dien-3β-ol	14.4	23.4	24.7	13.4	9.5
Cholest-5-en-3β-ol	85.6	75.6	75.3	86.6	89.2
5α(H)-cholestan-3β-ol	0.0	1.0	0.0	0.0	1.3

DISCUSSION

The percent lipid per WW and DW determined during the present study was less than other reported values for *Rhincalanus* spp. Total lipid content for *R. cornutus* collected during the summer from the GOM was reported to be 48.8% of DW (Morris & Hopkins 1983), compared to our values of 5% of DW. Lipid content of *R. nasutus* from various locations ranged from 22 to 42% of DW (Lee et al. 1971a, Schnack-Schiel et al. 2008), also higher than the 8 to 10% of DW reported in the present study. Thus, our total lipid values are likely underestimates of actual lipid content, as estimations of storage lipid made by measuring the volume of lipid sacs of these species during our study (Cass 2011) suggested storage lipid content to be between 30 and 70% of DW. Our low values could be attributed to 2 major factors. The first factor is related to the method for determining lipid mass, which in the present study was determined by summing the lipid peaks found in each fraction. Ohman (1997) points out that the method for determining the lipid mass makes a large difference in the end result, and the method employed in the present study would likely provide lower estimates compared to the more commonly used gravimetric method. When enough sample material is available, lipid mass determination by TLC-FID (thin-layer chromatography with FID) analyses would be a preferable method for bulk lipid content determinations (Ohman 1997). Another factor contributing towards potential underestimation of lipid content was lipid leakage during the body measurement step, when copepods were briefly thawed.

FFA classes (1.5 to 8.2% total lipid) in the present study were slightly higher, on average, than expected. FFAs are generally a minor component in copepods, often not even reported separately. These lipids usually comprise less than 3 to 4% of the total lipids examined (Lee et al. 1971b, Sargent & Falk-Petersen 1988, Ohman 1996, Schnack-Schiel et al.

2008). This discrepancy also may be attributed to the length-measuring step, where copepods were briefly thawed, and it is likely that lipases became active and broke down the other lipid components (particularly phospholipids) into FFAs (Sasaki & Capuzzo 1984, Ohman 1996). It is unlikely though that such degradation would have preferentially mobilized specific fatty acids, and therefore the reported fatty acid relative abundances should be representative of initial conditions prior to any degradation (Sasaki & Capuzzo 1984). As with the lipid leakage, this problem would be remedied by pre-measuring the copepods before they were initially frozen.

The fatty acid profiles observed for adult female *Rhincalanus nasutus* in the ETNP and GOC during the present study were similar to previously determined lipid profiles. Total fatty acid or WE profiles from studies in the Red Sea and off the coast of southern California also showed an abundance of 16:1 and 18:1 fatty acids (Lee et al. 1971a, Sommer et al. 2002, Schnack-Schiel et al. 2008). The main fatty acid contributors to the phospholipid fraction of *R. nasutus* collected off southern California included 20:5 (28.9%), 16:0 (19.4%), 22:6 (17.2%), 18:1 (11.5%), and 18:0 (7.4%) (Lee et al. 1971a), which were the 5 major contributors in the present study as well. Total lipids for *R. nasutus* from the San Lorenzo Channel (near the GOC) found slightly different values for *R. nasutus* (Lavaniegos & López-Cortés 1997), although such differences might be due to seasonal effects or variation in diet (e.g. Dalsgaard et al. 2003, Loh et al. 2008). Total fatty acid profiles for the closely related species *R. gigas* in the Southern Ocean indicated that this species had a similar tendency to accumulate 18:1(n-9) and 16:1(n-7), but their profiles also contained 18:4(n-3), 20:5(n-3), and 22:6(n-6) in large amounts, which has been attributed to dietary sources (Graeve et al. 1994, Kattner et al. 1994, Kattner & Hagen 1995).

Fatty alcohol profiles reported for *Rhincalanus nasutus* in Lee et al. (1971a), Sommer et al. (2002), and Schnack-Schiel et al. (2008) were highly similar to those observed in the present study. Hexadecanol (16:0; 52 to 74% mass), 14:0 (15 to 19%), and octadecanol (18:0; 11 to 16%) made up almost the entire fatty alcohol fraction in those studies. This also was comparable to the fatty alcohol profiles of *R. gigas*, in which 14:0 and 16:0 often made up >90% of the total fatty alcohols (Graeve et al. 1994, Kattner et al. 1994, Kattner & Hagen 1995). These findings are almost

identical to those observed in the present study for *R. nasutus.*

In contrast, total storage lipid fatty acid and WE alcohol profiles observed in adult female *Rhincalanus cornutus* collected from the GOM during early summer and *R. rostrifrons* collected from the ETNP during fall and early winter were not similar to profiles published for any other marine copepods, and the profiles were distinctly different from those of its congeners *R. nasutus* and *R. gigas.* Instead of storage lipids dominated by 16:1(n-7)/18:1(n-9) fatty acids and 14:0/16:0 fatty alcohols, *R. cornutus* and *R. rostrifrons* accumulated 14:0/16:0 fatty acids and 16:1/18:1 fatty alcohols.

It is likely that *Rhincalanus nasutus* and *R. gigas* employ the same strategy for synthesis of WE compo-

nents. As *R. gigas* has a lipid profile that is unique from many other polar species, it has already been suggested that it has a different method of fatty alcohol/acid biosynthesis (Kattner & Hagen 1995). Most copepods have the ability to synthesize 14:0, 16:0, 18:0, and 20:0 fatty acids using Type I fatty acid synthetase (summarized in Sargent & Henderson 1986). From there, subsequent desaturation using a Δ^9 fatty acid desaturase, elongation, and reduction can be achieved, often forming 20:1 and 22:1 fatty acids and alcohols. *R. gigas* is thought to only be able to carry out fatty acid synthesis to the 18:0 point, after which desaturation then yields 18:1(n-9). Palmitic acid (16:0) can also be desaturated to form 16:1(n-7). Major alcohol components are formed by reduction of 16:0 and 14:0 acids to 16:0 and 14:0 fatty alcohols (Kattner & Hagen 1995). *R. nasutus* probably shares this same pathway, as the major components for storage lipids are the same. *R. cornutus* and *R. rostrifrons*, on the other hand, appear to employ a modified version of this pathway (Fig. 2). The 14:0 and some 16:0 fatty acids are left intact to be utilized in WEs, while 16:1(n-7) is reduced to the corresponding alcohol. As we were not able to determine the double-bond position of the alcohols with absolute certainty, the 18:1 alcohol synthesis has 2 pathway options, one of which is employed by these species. The first would be reduction of the 18:1(n-9) fatty acid to the corresponding alcohol. The second option includes elongation of the 16:1(n-7) fatty acid to the 18:1(n-7) form, and then subsequent reduction to the 18:1(n-7) fatty alcohol.

Benefits gained by employing one lipid synthesis pathway over another are uncertain. Energetically, switching the acid and alcohol components yields approximately equivalent amounts of storage lipid energy, as fatty alcohols and acids are incorporated into WEs in a 1:1 ratio, and energetic content is largely based on chain length (Snider et al. 2006). This suggests that no direct energetic advantage is gained by either of the accumulation patterns. Unfortunately, the effects of switching the alcohol and acid components of WEs have not been well studied in relation to the physical properties of WEs. One study performed on a series of synthetic WEs found that addition of a double bond in the alcohol component of a WE decreased the melting point by a larger margin than the addition of an equivalent double bond in the acid component (Patel et al. 2001). This suggests that potential benefits could be gained by employing one strategy over the other, but further study is needed on the physical properties of WEs to determine what such benefits might entail.

Fig. 2. *Rhincalanus nasutus*, *R. gigas*, *R. cornutus*, and *R. rostrifrons*. Proposed pathways for wax ester fatty acid and alcohol biosynthesis. Major steps in general copepod fatty acid and alcohol synthesis pathways are marked. Reactions are labeled as either chain elongation (E), desaturation (D) by Δ^9 desaturase, or reduction (R) to alcohol. Boxes denote major end products. (A) Major pathways for *R. gigas* and *R. nasutus* (modified from Kattner & Hagen 1995). (B) Alternate pathways likely utilized by *R. cornutus* and *R. rostrifrons.*
*: Two different options for 18:1 fatty alcohol production

The types of storage lipids accumulated and their compositions have typically been attributed to a combination of factors, including whether or not diapause is utilized, habitat depth or latitude, and feeding strategy (e.g. Lee et al. 1971a, 2006, Graeve & Kattner 1992, Kattner & Hagen 1995, Albers et al. 1996). In the ETNP, where *Rhincalanus rostrifrons* and *R. nasutus* co-occur, few differences have been observed between the species that would help explain why these closely related species employ 2 different lipid-synthesis pathways. Previously reported abundance data showed similar vertical distributions (peak abundances between 200 and 600 m) for the 2 species (Chen 1986, Sameoto 1986). During our study, all *Rhincalanus* spp. were collected in the upper 200 to 300 m. A comparison of body-composition parameters, including carbon, nitrogen, hydrogen, phosphorus, protein, and lipid content, indicated that few differences existed among species (Cass 2011). To our knowledge, no study has determined whether either *R. rostrifrons* or *R. nasutus* undergoes diapause in this region, although *R. nasutus* has been found to sometimes employ diapause in more temperate regions (Ohman et al. 1998, Schnack-Schiel et al. 2008). Furthermore, no studies have looked at diapause strategies of either *R. cornutus* or *R. rostrifrons* in any location.

Despite similarities in fatty acid and alcohol composition, variation was observed in the type of storage lipids accumulated by *Rhincalanus cornutus* and *R. rostrifrons*. While total amounts of storage lipid were similar between both species (88 to 97% of total lipid), *R. rostrifrons* had higher amounts of WEs (94 to 99%) as a percentage of storage lipid than *R. cornutus* (62%). Higher amounts of TAG in *R. cornutus* in the GOM could be due to several factors, including increased recent feeding (Hakanson 1984, Jónasdóttir 1999), gonad development (Miller et al. 1998), or genetic predisposition (Lee et al. 1972, Hagen et al. 1995, Williams & Biesiot 2004). Results of the present study may support differences based on recent feeding, as protein contents were higher in *R. cornutus* from the GOM (35.5% of DW) than in *R. rostrifrons* from the ETNP (24.3% of DW), based on measurements of individuals collected during the same cruises as in the present study (Cass 2011). Higher protein content suggests that *R. cornutus* were more active than *R. rostrifrons*, potentially supporting an increased feeding effort. Difference in gonad development is an unlikely option, as visual inspection of the copepods used in the present study did not suggest strong variation between the species in ovary or egg development. Genetic predisposition is a possi-

bility, although additional sampling of *R. cornutus* in the GOM and other areas within its range would be needed to allow further certainty on this point.

Although the type and components of storage lipids accumulated in copepods have typically been attributed to a combination of factors, including diapause, habitat depth or latitude, and feeding strategy (e.g. Lee et al. 1971a, 2006, Graeve & Kattner 1992, Kattner & Hagen 1995, Albers et al. 1996), a few studies have noted the possibility that genetic predisposition may play a significant role within some taxa (Lee et al. 1972, Hagen et al. 1995, Williams & Biesiot 2004). The results of our study indicate that genetic predisposition is likely a significant component determining the composition of storage lipids in the *Rhincalanus* group. This is supported by the similarity between storage lipids accumulated within the 2 monophyletic groups formed in the *Rhincalanus* genus: *R. gigas/R. nasutus* and *R. cornutus/R. rostrifrons* (Goetze 2003). This occurs despite the fact that *R. nasutus* and *R. gigas* inhabit different ocean biomes, while the range of *R. nasutus* overlaps with *R. rostrifrons* and *R. cornutus* (Lang 1965, Bradford-Grieve et al. 1999). These unusual storage lipid profiles may be due to different pathways involved in lipid synthesis between the 2 groups (Fig. 2). Further support for a genetic component in storage lipid synthesis patterns is that the WE fatty acid signature of *Pareucalanus attenuatus*, a member of the genus likely most closely related to *Rhincalanus* (Goetze 2003), had a WE fatty acid signature which was >75% similar to *R. nasutus* when both species were collected in the ETNP (Cass 2011). As the present study found, copepods of the same species collected in different locations showed about 80% similarity, suggesting that a value of 75% similarity between *R. nasutus* and *P. attenuatus* indicates a close match. Based on these genetic linkages, it is possible that the WE synthesis pathway observed in *R. nasutus* and *R. gigas* evolved before the *Rhincalanus* lineage split from the *Pareucalanus* lineage. After this point, the ancestor of *R. cornutus* and *R. rostrifrons* split from *R. nasutus* and *R. gigas*, and a new synthesis pathway appeared prior to the evolution of *R. cornutus* and *R. rostrifrons* as separate species. Further work with other members of the *Pareucalanus* genus could help determine how widespread genetically these WE synthesis pathways are within the Eucalanidae family.

Not surprisingly, the phospholipid profiles for the copepods in the present study were very similar. Unlike storage lipids, which often are more reflective of diet (Lee et al. 2006), phospholipid composition is

usually highly regulated to maintain membrane fluidity and function (Devlin 2006). The major fatty acids found in these *Rhincalanus* spp. (22:6(n-3), 16:0, 18:0, 20:5(n-3), and 18:1(n-9)) have been observed in other copepod phospholipid profiles as well, including those at high latitudes (e.g. Lee et al. 1971a, Lee 1974, Albers et al. 1996, Scott et al. 2002), suggesting that most copepods may share a strategy for membrane structure and function.

Furthermore, sterol profiles also were similar within and among species. This is likely due to the fact that cholesterol has many important functions in cellular membranes, including stabilizing membrane structure, affecting membrane permeability, and altering the activity of membrane proteins (Crockett 1998). Such specific sterol compositions are probably attained through preferential retention of dietary cholesterol and other dietary phytosterols, e.g. brassicasterol (24-methylcholesta-5, 22E-dien-3β-ol) and 24-methylenecholesterol (24-methylcholesta-5, 24(28)-dien-3β-ol), that can be easily dealkylated to cholesterol, and subsequent conversion of assimilated sterols to the needed forms (Teshima 1971, Goad 1978, Harvey et al. 1989). Sterol profiles are not well studied in copepods, but one major difference did occur between profiles from our study and previous work. Desmosterol (cholesta-5, 24-dien-3β-ol) was notably absent in the copepods in the present study, a compound which was often the second most abundant sterol found in zooplankton (Harvey et al. 1987, Serrazanetti et al. 1992, 1994, Mühlebach et al. 1999). Desmosterol is usually thought to occur because it is an intermediate in the conversion of dietary phytosterols to cholesterol (Goad 1978). The lack of desmosterol in these subtropical *Rhincalanus* spp. may have to do with the relatively high abundance of heterotrophic versus autotrophic prey in these tropical-subtropical open-ocean systems (M. B. Olson pers. comm.). It also is possible that these copepods have a more efficient or more rapid conversion of ingested phytosterols to cholesterol, such that desmosterol does not accumulate in the body. The minor variation in sterol profiles between the 2 groups (cholesterol content of 75 to 76% in *R. rostrifrons* versus 85 to 90% in *R. cornutus* and *R. nasutus*) may be due to differences in cellular processes related to the amount of cholesterol needed for membrane protein function (Crockett 1998).

In conclusion, the present study indicates that genetic predisposition may play a larger role than previously thought on fatty acid and alcohol accumulation patterns in copepods, particularly *Rhincalanus* spp. Novel studies of storage lipids in *R. cornutus* and *R. rostrifrons* suggest that these 2 species utilize a separate pathway for storage lipid fatty acid and alcohol synthesis than their congeners *R. nasutus* and *R. gigas* (Fig. 2). Further analyses of *Rhincalanus* spp. and the closely related *Pareucalanus* spp. throughout their ranges and in all seasons would help to discern the extent of purely genetic control as well as other factors on lipid profiles.

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