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*Published in:*  
Limnology and Oceanography

*DOI:*  
[10.1002/lno.11077](https://doi.org/10.1002/lno.11077)

*Publication date:*  
2019

*Document version*  
Publisher's PDF, also known as Version of record

*Document license:*  
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*Citation for published version (APA):*  
Grebner, W., Berglund, E. C., Berggren, F., Eklund, J., Haroadottir, S., Andersson, M. X., & Selander, E. (2019). Induction of defensive traits in marine plankton-new copepodamide structures. *Limnology and Oceanography*, 64(2), 820-831. <https://doi.org/10.1002/lno.11077>

## Induction of defensive traits in marine plankton—new copepodamide structures

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### Abstract

Marine zooplankton release chemical cues, which trigger defenses in unicellular phytoplankton, such as increased toxin production and changes of colony sizes. Here, we identify the structure of two novel alarm cues belonging to the group of copepodamides. Similar to the known copepodamides, one of the compounds described is shown to trigger both amnesic and paralytic shellfish toxin production and chain length shortening in *Skeletonema marinoi*. In addition, we report the putative structures of another 21 copepodamides, which constitute 28% of the total copepodamides extractable from whole animals, suggesting that the copepodamide concentrations have been underestimated in earlier studies. We introduce a structure-based nomenclature to handle the increasing number of copepodamides. Analysis of 12 copepod species showed that marine calanoid and freshwater cyclopoid copepods contain copepodamides. The only harpacticoid included in the analysis, *Tigriopus californicus*, did not appear to produce detectable amounts of copepodamides. Feeding experiments revealed that copepodamide compositions depend on both diet and species-specific properties. Copepodamides induce both morphological and biochemical defensive traits in phytoplankton and may drive large-scale trait-mediated effects in marine food webs. The more comprehensive list of copepodamides reported here makes it possible to explore the role of the copepodamide signaling system in the pelagic ecosystem in greater detail.

The free water mass of the oceans, the pelagial, is Earth's largest ecosystem, and its primary producers, phytoplankton, account for half of the planet's primary production (Field et al. 1998). The zooplankton community of the upper part of the pelagial is dominated by copepods. In productive areas, they occasionally reach densities of hundreds of individuals per liter (Hamner and Carleton 1979; Ambler et al. 1991) and constitute up to 90% of the zooplankton biomass (Froneman 2001; Pane et al. 2004). Copepods play a key role in the food web as the main vector from the primary producers to higher

trophic levels (Hansen et al. 1994; Verity and Smetacek 1996; Stibor et al. 2004).

Copepods exude unique chemical cues into surrounding waters (Selander et al. 2016). Among the exuded compounds are intraspecific and interspecific cues. This includes species, sex, and even life-stage-specific cues (Heuschele and Selander 2014). Exuded compounds also serve as early warnings of predator presence for prey organisms. Prey responses to predator presence include decreased colony size, induction of toxin production, increased bioluminescence, and increased cell wall silicification (Long et al. 2007; Pondaven et al. 2007; Bergkvist et al. 2012; Selander et al. 2015; Lindström et al. 2017). One group of such predator cues is taurine conjugated lipids, copepodamides. Copepodamides trigger paralytic shellfish and amnesic shellfish toxin production in dinoflagellates and diatoms (Selander et al. 2015; E. Selander et al. unpubl.), increased bioluminescence in *Alexandrium tamarensense* and *Lingulodinium polyedra* (Lindström et al. 2017), and chain length shortening in chain forming diatoms (E. Selander et al. unpubl.).

Copepodamides are characterized by the scaffold and the fatty acid attached to it. The known copepodamides contain common marine fatty acids such as docosahexaenoic (C22:6),

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eicosapentaenoic (C20:5), or stearidonic acid (C18:4). Copepods, however, contain many more fatty acids, and it is possible that more copepodamides exist in addition to the ones currently known. Further, copepodamides have only been measured in three species of calanoid copepods, *Centropages typicus*, *Calanus* sp., and *Pseudocalanus* sp. (Selander et al. 2015). Here, we report the structure of two novel copepodamides and suggest putative structures for an additional 21 copepodamides. The bioactivity and ecological relevance of one of the newly identified compounds is tested in dose response experiments monitoring morphological and biochemical defensive traits of representatives from the two major marine phytoplankton groups, dinoflagellates and diatoms. Additionally, we report the copepodamide composition of 10 common marine copepods in the NE Atlantic surface waters as well as one harpacticoid and one freshwater species and explore the effect of diets with different fatty acid composition on the copepodamide composition.

## Materials and methods

### Copepodamide extraction and purification

Copepodamides from freeze-dried *Calanus finmarchicus* (11.32 g) (*Calanus* AS) were extracted with 200 mL methanol at  $-20^{\circ}\text{C}$  for 48 h. The extract was mixed in a blender and sonicated at  $40^{\circ}\text{C}$  and  $> 20$  kHz, for 30 min, and centrifuged for 5 min at 1800 rpm. The supernatant was collected and the procedure repeated with 150 mL methanol. Copepodamides were separated from less polar compounds by liquid-liquid partitioning (Löfgren et al. 2012). The methanol extract was supplemented with 1% aqueous  $\text{NH}_3^+$ , to a volume of 300 mL and mixed with 300 mL of heptane/methanol (98 : 2). The mixture was incubated on a shaking table for 30 min, transferred to a separation funnel, and 150 mL heptane/methanol (98 : 2) was added. The funnel was shaken vigorously for 3 min. The heptane phase was discarded and the procedure repeated twice with 300 mL portions of heptane/methanol (98 : 2). The methanol phase, containing the copepodamides, was evaporated using a rotary evaporator (Rotavapor R II, Büchi) at  $40^{\circ}\text{C}$ . The extract was resolved in methanol, diluted with milliQ water to 25% methanol (aq), and loaded onto an solid phase extraction (SPE) column (ENVI-18, 19 g, 60 mL; Sigma-Aldrich). Impurities were removed with 70% (v/v) methanol, and the copepodamides eluted in 100% methanol. The copepodamides were further purified by gradient elution on a high-performance liquid chromatography (HPLC; Hitachi ELITE LaChrom L-7100) system with a reversed phase column (KR100, C18,  $5\ \mu\text{m}$ ,  $4.6 \times 150$  mm; Hichrom) The gradient went from 100% A (methanol : acetonitrile : water; 35 : 35 : 35) to 100% B (2-propanol) over 25 min, 100% B was maintained for 5 min before re-equilibrating in 100% A for 7 min before the next injection. Both eluents were supplemented with 0.2% (v/v) formic acid, the flow rate was  $1\ \text{mL}\ \text{min}^{-1}$ . Fractions were collected (Waters Fraction Collector III)

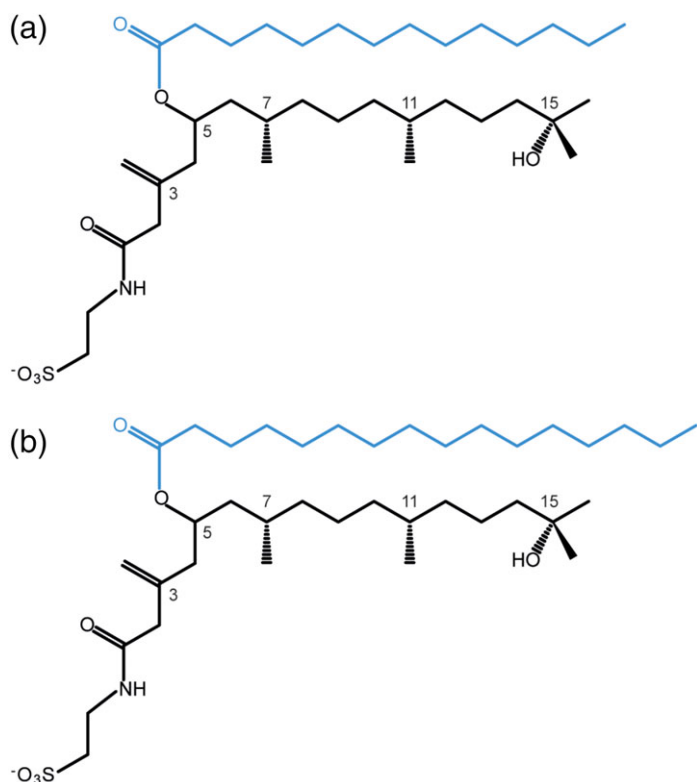
at 15 s intervals. Fractions were pooled based on copepodamide content. A final isocratic HPLC clean up step with methanol : acetonitrile : water : isopropanol, 9 : 8 : 3 : 80, with 0.2% (v/v) formic acid was used. Fractions containing significant amounts of pure copepodamides were pooled and stored at  $-20^{\circ}\text{C}$ .

### Copepodamide analysis

Copepodamides were analyzed on an Agilent 1200 LC system, coupled to an Agilent 6410 triple quadrupole detector with electrospray interface (Agilent Technologies). We used a Prevail column (C18,  $3\ \mu\text{m}$ ,  $2.1 \times 150$  mm, Hichrom), at  $50^{\circ}\text{C}$ , and a gradient from A methanol : acetonitrile : water (35 : 35 : 30) to B 2-propanol, at a flow rate of  $0.2\ \text{mL}\ \text{min}^{-1}$ . Both solvents were supplemented with 0.2% (v/v) formic acid. The gradient started with 5% B, 1 min, followed by a linear increase over 14 min to 82% B, which was maintained for 1 min before re-equilibrating for 8 min with 5% B. The ion source was operated in negative mode, at  $300^{\circ}\text{C}$  and 4.5 kV, at  $7\ \text{L}\ \text{min}^{-1}$  nitrogen flow at 35 Psi. Copepodamides with known flat structure were identified using the following diagnostic fragments: product ion  $m/z$  432.2 for 22:6-, 20:5-, and 18:4-dihydrocopepodamides with precursor ions  $m/z$  708.5, 734.5, and 760.5; product ion  $m/z$  430.2 for 14:0-, 16:0-, 22:6-, 20:5-, and 18:4-copepodamides with precursor ions 658.5, 686.5, 706.5, 732.5, and 758.5; fragmentor voltage 250 V; and collision energy 44 eV. 22:6-Copepodamide was used as external standard for quantification. For the precursor ion scan for additional copepodamides from *C. finmarchicus*, the precursor ions were scanned from  $m/z$  400 to 1000 for product ions  $m/z$  430.2 and 432.2, respective.

### Structure verification of novel copepodamides

To separate and identify the hydrolysable fatty acid side chain in position C5 (Fig. 1) from the copepodamide, we used the alkaline transmethylation modified after Christie (1976). Purified copepodamides were dissolved in 0.2 mL heptane, and 0.5 mL  $0.5\ \text{mol}\ \text{L}^{-1}$  sodium methoxide in dry methanol was added before incubation in a sonication bath for 30 min at room temperature. After neutralization with 50  $\mu\text{L}$  acetic acid and 0.8 mL deionized water, the heptane phase was transferred into a new glass vial. Extraction was repeated once with 0.5 mL heptane, and the combined heptane phases were evaporated under  $\text{N}_2$  flow at room temperature. The fatty acid methyl esters were dissolved in heptane and analyzed by gas chromatography-mass spectrometry (GC-MS) as described in Selander et al. (2015). To verify the identity of the lysocopepodamide scaffold, we extracted the remaining aqueous phase with chloroform. The chloroform was evaporated and the sample resolved in methanol before analysis on HPLC-MS/MS as described above. The sample was compared to nuclear magnetic resonance (NMR)-verified standards of lysocopepodamide, both individually and coinjected (Supporting Information Fig. S1).



**Fig. 1.** Structures of the two novel copepodamides. The black structures show the identified scaffold and the blue the saturated lipids attached to it. **(a)** For 14:0-copepodamide ( $C_{36}H_{68}NO_7S^-$ , molecular weight [MW] 659), tetradecanoic acid (14:0, myristic acid) is attached to the scaffold at position C5. **(b)** For 16:0-copepodamide ( $C_{38}H_{72}NO_7S^-$ , MW 687), hexadecanoic acid (16:0, palmitic acid) is attached to the scaffold at position C5.

### Species-specific copepodamide composition

The marine copepod species *Acartia clausi*, *Centropages hamatus*, *Centropages typicus*, *Metridia longa*, *Oithona* sp., *Pseudocalanus* sp., and *Temora longicornis* were collected outside the Sven Lovén Center for Marine Sciences, Kristineberg, Sweden. *C. finmarchicus*, *Calanus glacialis*, and *Calanus hyperboreus* were collected from water at Svalbard, Norway, 2014. The intertidal harpacticoid *Tigriopus californicus* was purchased from Reefphyto, and the freshwater specie *Cyclops* sp. was collected in a pond in the Gothenburg botanical garden, Sweden. Three to seven copepods were extracted in 1 mL methanol at  $-20^\circ\text{C}$  for at least 24 h. The methanol was evaporated at  $40^\circ\text{C}$  under  $N_2$  flow and the sample resolved in 40–80  $\mu\text{L}$  methanol before analysis by HPLC–MS/MS as described above. The relative proportions (in %) of the different copepodamides analyzed in each sample were compared by principal component analysis (PCA) using SIMCA (Version 14.1 Umetrics).

### Diet-specific copepodamide composition

Adult females of a *T. longicornis* culture were used for feeding experiments. Four to six copepods were placed in 5 mL filtered sea water (salinity 26) and fed with *Rhodomonas* sp. ( $6.5 \times 10^5$  cells  $\text{mL}^{-1}$ ), *Nannochloropsis oculata* ( $3.7 \times 10^5$  cells

$\text{mL}^{-1}$ ), *Dunaliella tertiolecta* ( $5 \times 10^5$  cells  $\text{mL}^{-1}$ ), or *Isochrysis galbana* ( $4.6 \times 10^5$  cells  $\text{mL}^{-1}$ ) for 2 d at  $16^\circ\text{C}$ , photoperiod 12 : 12 (light : dark), light intensity  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  ( $N = 4$  biological replicates). All algae strains were obtained from the GUMACC algae bank (University of Gothenburg, Sweden). Extraction and HPLC–MS/MS analysis of copepodamides were done as described earlier.

### Phytoplankton culturing

*Skeletonema marinoi* strain GF 04-7D (isolated from Gullmarsfjord, Sweden, in 2009) and *Alexandrium minutum* strain GUMACC 83 (isolated from Ria de Vigo, Spain, 1987) were obtained from the GUMACC algae bank (University of Gothenburg, Sweden). *Pseudo-nitzschia seriata* (isolated from Disko Bay, West Greenland, 2013) was obtained from Nina Lundholm, Natural History Museum, University of Copenhagen, Denmark. The algae were cultured in silicate-enriched (*S. marinoi*) or selenium-enriched (*A. minutum*) f/2-medium (Guillard 1975) or L medium (*P. seriata*). Incubation temperature was  $16^\circ\text{C}$ , photoperiod 12 : 12 (light : dark), light intensity  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and salinity 26 for *S. marinoi* and *A. minutum*. *P. seriata* was incubated at  $4^\circ\text{C}$ , photoperiod 22 : 2 (light : dark),  $60 \mu\text{mol photons m}^{-2}$ , and salinity 30.

### Copepodamide dose response experiments

Exponential growing cultures of *S. marinoi*, *P. seriata*, and *A. minutum* were exposed to 16:0- and 18:4-copepodamide (0, 1, 5, 10, 50, 100, and 500  $\text{pmol L}^{-1}$ ,  $n = 4$ , for *S. marinoi*; 0, 1, 25, 50, 100, 150, 250, and 500  $\text{pmol L}^{-1}$ ,  $n = 3$ , for *P. seriata*; and 0, 2, 4, 8, 16, and 24  $\text{nmol L}^{-1}$ ,  $n = 4$  for *A. minutum*). Copepodamides were coated onto the culture vessel walls (8.7 mL scintillation vials for *S. marinoi* and *P. seriata*, 1.5 mL glass tubes for *A. minutum*) by adding them in methanol and evaporating the solvent under  $N_2$  flow,  $30^\circ\text{C}$ . Controls were treated with methanol without copepodamides. Starting cell concentrations were  $\approx 5000$  cells  $\text{mL}^{-1}$  (*S. marinoi*),  $\approx 6500$  cells  $\text{mL}^{-1}$  (*P. seriata*), and  $\approx 10,000$  cells  $\text{mL}^{-1}$  (*A. minutum*). *S. marinoi* and *P. seriata* were incubated on a revolving plankton wheel at 0.5 rpm, while the motile *A. minutum* cells were incubated standing. Incubation conditions were the same as described for cultures stock above. Incubations lasted 3 d for *S. marinoi* and *P. seriata* and 2 d for *A. minutum*. After incubation, the numbers of cells in chains were counted for at least 50 chains from each replicate under an inverted microscope (*S. marinoi*) or the cells harvested for toxin analysis (*P. seriata* and *A. minutum*).

### Toxin analysis

#### Domoic acid analysis

The culture samples consisting of cells suspended in media were acidified with 0.2% (v/v) formic acid and loaded onto SPE columns (Bond Elute C18 LRC 10 mL, 200 mg,  $40 \mu\text{m}$ ; Agilent) to collect total (intracellular and extracellular) domoic acid. Columns were desalted with 5 mL milliQ water with

0.2% (v/v) formic acid. About 750  $\mu\text{L}$  aqueous methanol (50%, v/v) was added to the column and left for 1 h, to extract cells. The samples were eluted into HPLC vials and analyzed on an Agilent 1200 LC system coupled to an Agilent 6410 triple quadrupole detector with electrospray interface, injection volume was 10  $\mu\text{L}$ . We used a reversed phase column (Accucore 2.1  $\times$  150 mm, 2.6  $\mu\text{m}$ , Thermo Fisher Scientific) at 45°C and isocratic elution with 25% acetonitrile (aq) with 0.1% (v/v) formic acid. The flow rate was 0.2 mL  $\text{min}^{-1}$ . The ion source operated in negative mode at 300°C and 4500 V with a nitrogen gas flow of 5.8 L  $\text{min}^{-1}$  at 20 psi. Fragmentor voltage was set at 100 V and collision energy 14 eV. Domoic acid was identified by comparing retention time and diagnostic fragments, precursor ion  $m/z$  312 and product ion  $m/z$  266.2 (Tammilehto et al. 2012), with domoic acid standard solution (National Research Council, Certified Reference Materials Program, Canada).

### Gonyautoxin analysis

The samples were transferred to a 1.5 mL reaction tube and centrifuged for 5 min at 16°C, 12,000  $\times g$ . The supernatant was discarded and the cell pellets lyophilized. Toxins were extracted in 200  $\mu\text{L}$  acetic acid (0.05 mol  $\text{L}^{-1}$ ) by three freeze–thaw cycles. The lysate was centrifuged for 5 min at 12,000  $\times g$ , the supernatant transferred into HPLC vials and analyzed on an Agilent 1200 LC system coupled to an Agilent 6410 triple quadrupole detector with electrospray interface, injection volume was 1  $\mu\text{L}$ . We used a reversed phase chromatography (Accucore 2.1  $\times$  150 mm, 2.6  $\mu\text{m}$ ; Thermo Fisher Scientific) and isocratic elution with 50% acetonitrile (aq) with 0.1% (v/v) acetic acid, flow rate 0.2 mL  $\text{min}^{-1}$ . The electrospray ionization source was operated in positive ionization mode at 4.5 kV and 300°C with a nitrogen gas flow of 10 L  $\text{min}^{-1}$  at 35 psi. Fragmentor voltage was set at 104 V and collision energy 23 eV (gonyautoxin 1, 4) or 19 eV (gonyautoxin 2, 3). Gonyautoxins were identified from comparison of retention time and diagnostic fragments, precursor ion  $m/z$  412.0 and product ion  $m/z$  314.1 for gonyautoxin 1 and 4 and precursor ion 396.0 and product ion 298.1 for gonyautoxin 2 and 3 (Dell'aversano et al. 2005) provided by analysis of standard solutions of gonyautoxin 1,4 and 2,3 (National Research Council, Certified Reference Materials Program, Canada).

### Nonlinear regression fits

We performed nonlinear regression fits according to the Michaelis–Menten equation (GraphPad Prism 7, GraphPad Software) with the obtained phytoplankton response variables in relation to the copepodamide concentrations to get information about the maximum induced response:

$$\text{Phytoplankton response} = \frac{R_{\text{max}} \times C}{K_{\text{m}} + C}$$

where phytoplankton response refers to toxicity or chain length reduction relative to controls,  $C$  denotes copepodamide

concentration,  $R_{\text{max}}$  the maximum induced response, and  $K_{\text{m}}$  the half saturation constant, that is, the concentration inducing half the maximum induction.

## Results

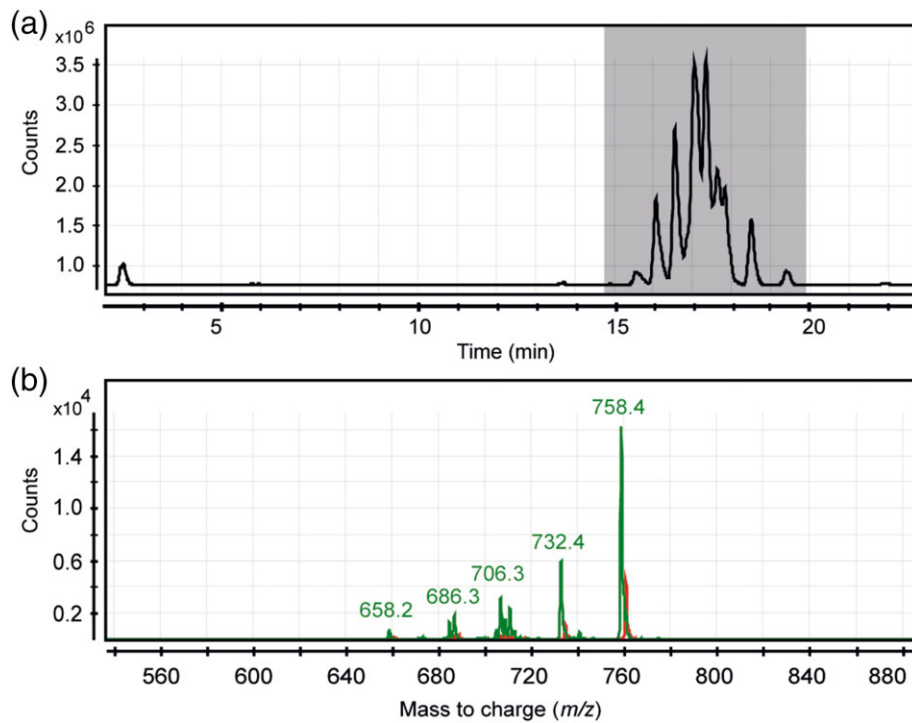
### Structure elucidation of novel copepodamides

To search for additional copepodamides, we performed a precursor scan of a polar lipid extract from *C. finmarchicus* (Fig. 2a). The precursor scan targeting compounds with the diagnostic fragment  $m/z$  430 revealed two prominent peaks with precursor ions (negative mode)  $m/z$  658 and 686 in addition to more than 20 minor constituents (Fig. 2b). GC–MS analysis of fatty acid methyl esters from isolated copepodamides 658 and 686 revealed the fatty acid side chain to be myristic acid (C 14:0) for copepodamide  $m/z$  658 and palmitic acid (C 16:0) for copepodamide  $m/z$  686. MS/MS experiments confirmed that the scaffold part of both compounds is identical to NMR-validated lyso-copepodamide (Selander et al. 2015; Supporting Information Fig. S1). This confirms the structure of 14:0-copepodamide ( $\text{C}_{36}\text{H}_{68}\text{NO}_7\text{S}^-$ ; Fig. 1a) and 16:0-copepodamide ( $\text{C}_{38}\text{H}_{72}\text{NO}_7\text{S}^-$ ; Fig. 1b). Accurate mass of the compounds is consistent with the proposed structures (Supporting information 2).

A targeted search for additional copepodamides- and dihydrocopepodamides-containing fatty acids commonly found in copepods (Tiselius et al. 2012) revealed another 21 putative structures of copepodamides listed in Table 1, although we were unable to purify sufficient amounts for full structural elucidation of these. Together with 14:0- and 16:0-copepodamide, the novel copepodamides contribute 28% of total copepodamides in *C. finmarchicus* extract. We propose a simple structure-oriented nomenclature (Table 1) to name the growing number of copepodamides. The described copepodamides all fall into two groups with either a methyl ( $-\text{CH}_3$ ) or a methylene ( $=\text{CH}_2$ ) group at position C3 (Fig. 1). We refer to these groups as dihydrocopepodamides (former copepodamides A–C) and copepodamides (former copepodamides D–F). Each group is further characterized by different fatty acids attached to position C5 which is indicated by the prefix (e.g., 22:6-copepodamide, a copepodamide with a methylene group at C3 and docosahexaenoic acid [C 22:6] at C5). The deacylated species are in analogy with other acyl lipids referred to as lyso-dihydrocopepodamide (former G) and lyso-copepodamide (former H). The entire group of compounds can collectively be referred to as “copepodamides.”

### Effects of 16:0-copepodamide on phytoplankton

We were able to purify sufficient amounts of 16:0-copepodamide and performed dose response experiments with three microalgae species to confirm its biological activity. We used established copepod-induced systems in diatoms and dinoflagellates. *A. minutum* and *P. seriata* both increase production of toxic secondary metabolites (gonyautoxins and



**Fig. 2.** Total ion chromatogram of an extract from freeze-dried *C. finmarchicus*. **(a)** Counts vs. acquisition time; total ion count precursor ion plot. The area between 15 and 20 min (gray) was extracted and the corresponding spectrums are displayed in **(b)**. **(b)** The green spectrum shows the precursor ions with the taurine fragment of 430.2, and the red spectrum displays the precursor ions with the taurine fragment of 432.2. The precursor ions of 758.4, 732.4, and 706.3 have been characterized earlier, but two previously unknown masses, containing the 430.2 fragment, 658.2 and 686.3, formed prominent peaks in the precursor scan. The analysis was made in negative mode.

domoic acid, respectively) in response to copepod presence (Selander et al. 2015; Tammilehto et al. 2015). *Skeletonema* changes morphology to single celled or shorter chain phenotype when exposed to copepods (Bergkvist et al. 2012). Increased resistance to grazers has been established for defended phenotypes of *Alexandrium* and *Skeletonema*, but the grazer deterrent role of domoic acid is still debated (Bergkvist et al. 2012; Selander et al. 2006; E. Selander et al. unpubl.). 18:4-Copepodamide (copepodamide D) was included as a positive control. It has the same molecular backbone as 16:0-copepodamide and induces toxin formation in *A. minutum* (Selander et al. 2015). 16:0-Copepodamide triggered the grazer-induced phenotypes in all three systems (Fig. 3). *P. seriata* and *S. marinoi* were sensitive to low amounts of copepodamides and signal perception saturated at 50 pmol L<sup>-1</sup> (*S. marinoi*) and 150 pmol L<sup>-1</sup> (*P. seriata*). In contrast, *A. minutum* showed no saturation even at high concentrations (up to 24 nmol L<sup>-1</sup>) and is previously shown to do so up to 28 nmol L<sup>-1</sup> (Selander et al. 2015). However, for the diatoms, the potency of 16:0-copepodamide was lower than that of 18:4-copepodamide as reflected by 63% lower maximum domoic acid increase in *P. seriata* (Fig. 3b) and 19% lower maximum chain length shortening in *S. marinoi* (Fig. 3c). For *A. minutum*, no maximum induced response could be estimated as the copepodamide concentrations tested did not lead

to a saturation of the increased toxin production (Fig. 3a), but for the tested concentrations, no difference in potency of 16:0- and 18:4-copepodamide could be observed.

### Copepodamide composition

The composition of the 10 copepodamides with known structure (lyso-,14:0-, 16:0-, 18:4-, 20:5-, 22:6-copepodamide and lyso-,18:4-, 20:5-, 22:6-dihydrocopepodamide) shows species-specific patterns in 11 tested copepod species (marine species: *Acartia clausi*, *C. finmarchicus*, *C. glacialis*, *C. hyperboreus*, *Centropages hamatus*, *Centropages typicus*, *Metridia longa*, *Oithona* sp., *Pseudocalanus* sp., and *T. longicornis* and the freshwater species: *Cyclops* sp.; Fig. 4a). A single harpacticoid species (*Tigriopus californicus*) was included and did not contain detectable levels of copepodamides.

Some species of the same genus have similar copepodamide composition, such as *Calanus* spp., whereas others like *Centropages typicus* and *Centropages hamatus* cluster far apart (Fig. 4a). The freshwater specie *Cyclops* sp. is not distinctly different from the saltwater species, and has a similar copepodamide signature as *Centropages hamatus*. The presence of copepodamides in the freshwater copepod suggests that copepodamide signaling may be operating in limnic systems too. Copepodamide composition in *Centropages hamatus* and *C. finmarchicus* illustrates the differences in copepodamide profiles from opposite corners of the PCA. *Centropages hamatus*, is dominated by

**Table 1.** Composition of copepodamides in a polar lipid extract of *C. finmarchicus* analyzed by HPLC-MS/MS. The structures of copepodamide A-F are published in Selander et al. (2015), the structures of 14:0- and 16:0-copepodamide are presented in this study, and the remaining copepodamides are not structural elucidated. Copepodamides are now named by the fatty acid residue followed by a suffix reflecting an unsaturated (copepodamide) or saturated (dihydrocopepodamide) scaffold (e.g., 22:6-copepodamide for the former copepodamide D and 22:6-dihydrocopepodamide for the former copepodamide A). Fatty acids marked with \* were also identified in taurine-containing lipids in *Calanus* spp. stage five copepodites in a study of Mayor et al. (2015).

Fatty acid	Scaffold	m/z precursor ion	m/z product ion	Percentage of all listed compounds	Former annotation	
22:6*	Copepodamide	758	430	50.3	D	
22:5		760		9.1		
20:5*		732		9.1		E
16:0*		686		6.6		
18:4*		706		2.2		F
18:1*		712		1.8		
18:3*		708		1.7		
20:4		734		1.6		
14:0*		658		1.5		
16:1*		684		0.6		
18:2*		710		0.5		
18:5		704		0.3		
20:1*		740		0.2		
22:4		762		0.2		
18:0		714		0.1		
16:4		678		0.1		
20:2		738		0.1		
22:6		Dihydrocopepodamide		760		432
16:0	688		1.6			
20:5	734		1.4	B		
22:5	762		1.4			
18:4	708		0.2	C		
20:4	736		0.2			
18:3	710		0.2			
18:1	714		0.1			
18:5	706		0.1			
18:2	712		0.1			
16:1	686		0.1			
14:0	660		0.1			

dihydrocopepodamides (95% of total copepodamides), whereas *C. finmarchicus* contains only 14% dihydrocopepodamides (Fig. 4b).

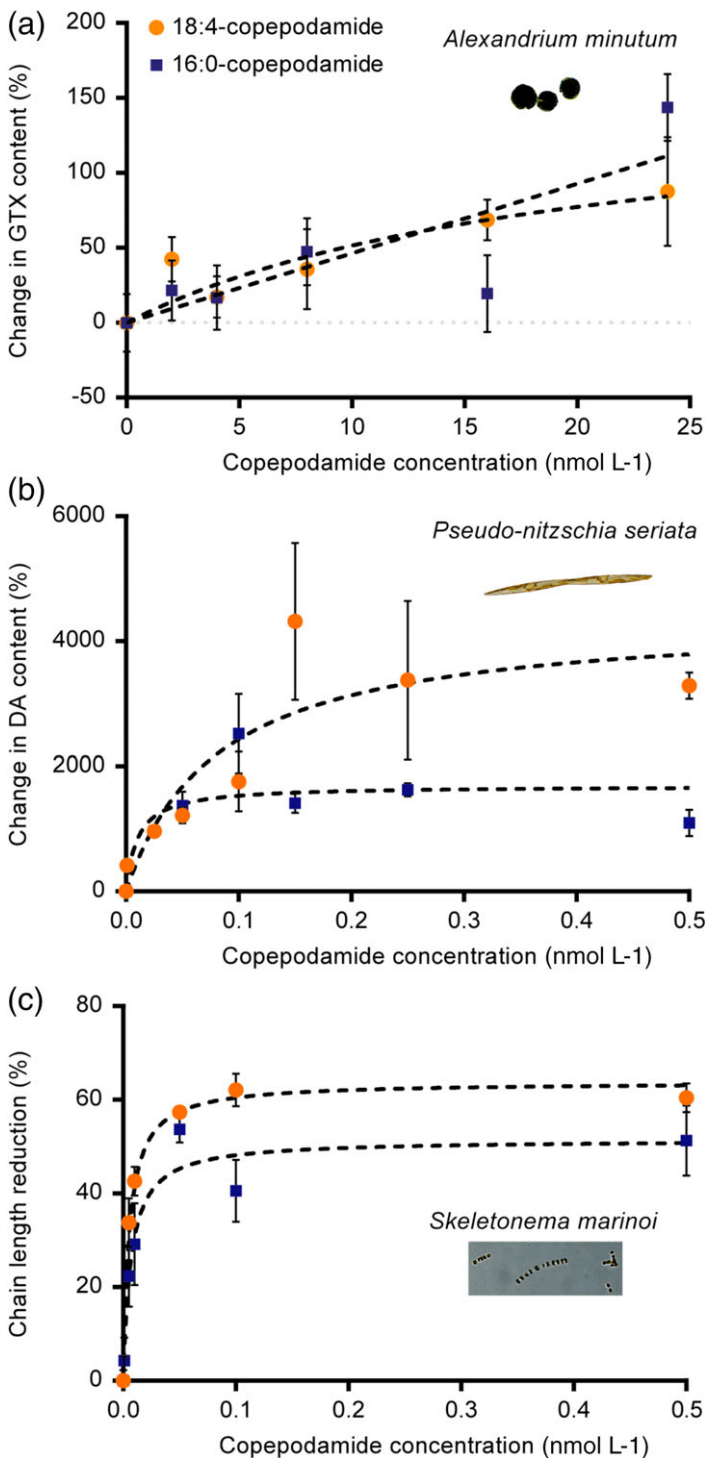
Feeding of *T. longicornis* with different microalgae resulted in a changed composition of copepodamides (Fig. 4c) mirroring the fatty acid content of the algae. Feeding on *D. tertiolecta*, *Rhodomonas* sp., and *I. galbana* resulted in high C18:2-dihydrocopepodamide levels, whereas copepods feeding on *N. oculata* mainly formed C20:5-dihydrocopepodamide. *N. oculata* is rich in C20:5 (Roncarati et al. 2004; Ohse et al. 2015) while *Rhodomonas* algae have a high content of C18:4 and *I. galbana* is rich in C18:4 and C22:6 (Patil et al. 2007; Dahl et al. 2009; Parrish et al. 2012). The fatty acid composition of *D. tertiolecta* is typical for chlorophytes, deficient

in C20:5 and C22:6 but contain some C18:4 (Veloza et al. 2006; Parrish et al. 2012). The food composition had no effect on which type of copepodamide scaffold was synthesized as both *T. longicornis* caught in field and the cultured ones only produced dihydrocopepodamides but not copepodamides.

## Discussion

We report the structure of two novel copepodamides. Both share the identical molecular scaffold of the known copepodamides but are unique in containing saturated acyl groups. In addition, we identify 21 putative copepodamide structures with acyl groups commonly found in copepod organisms. The new compounds constitute a significant portion of the total

copepodamides, which have consequently been underestimated in earlier studies. Ten of the putative structures are likely to be identical to a group of taurine-containing lipids identified in a study of the metabolic response of *Calanus* spp. to environmental warming and ocean acidification (Mayor et al. 2015) further supporting their presence in calanoid copepods.

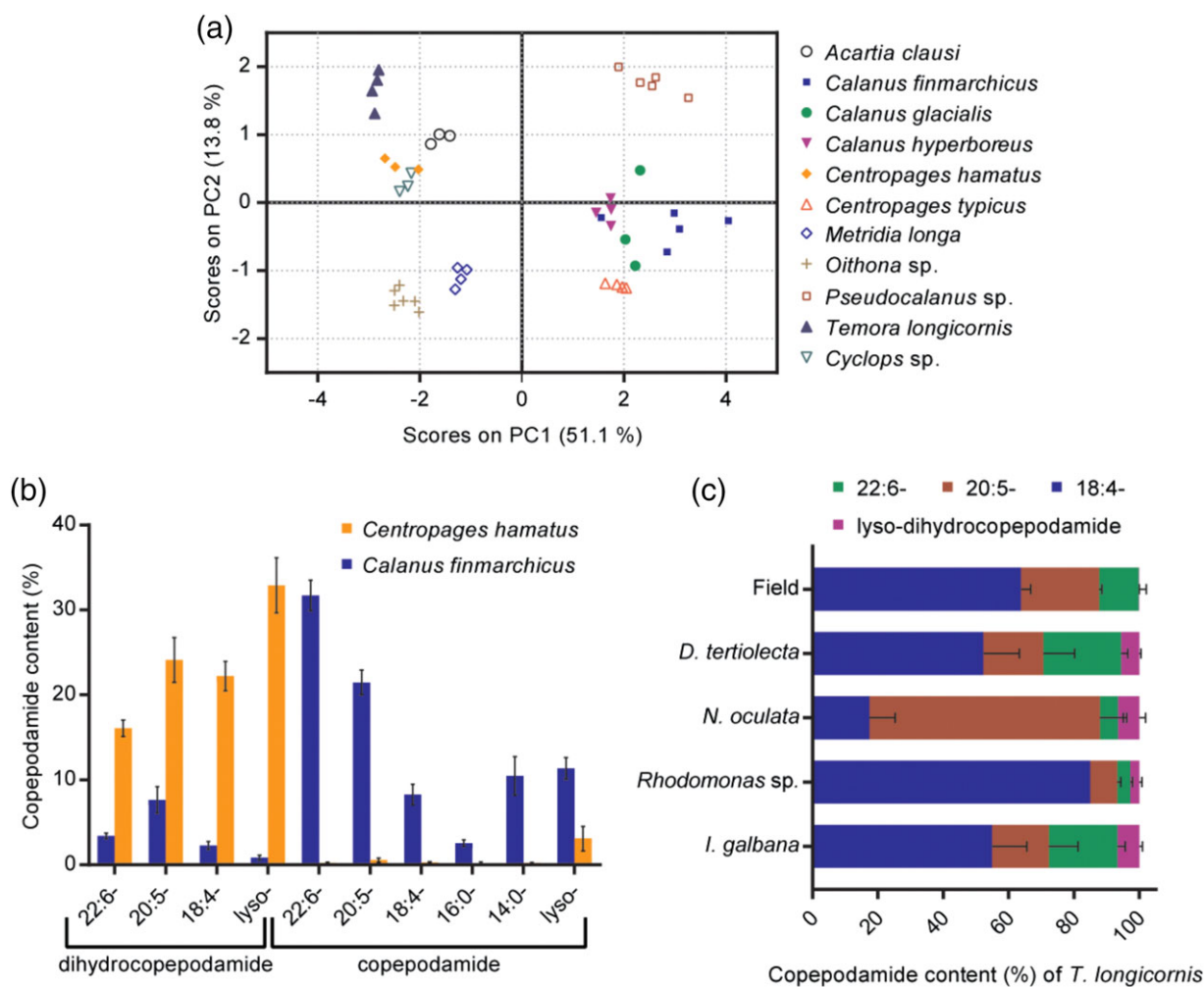


The inducing effect of copepodamides on domoic acid and gonyautoxin production can facilitate harmful algal bloom formation of both amnesic and paralytic shellfish toxin-producing algae. The grazer deterrent role of harmful algal toxins is debated. Evidence is mainly correlative, where algae producing toxins trigger a wide range of responses in grazers. Some grazers are resistant to toxins and feed seemingly unharmed on harmful algae. Others show decreased feeding rates and rejection of toxic prey (Guisande et al. 2002; Xu et al. 2018) or reduced reproductive success (Dutz 1998; Guisande et al. 2002), altogether leading to less grazing pressure for the algae. Several algal toxins like microcystins, paralytic shellfish toxins, and amnesic shellfish toxins are induced by grazer presence, which supports that they serve as grazer deterrents (Selander et al. 2006; Takabayashi et al. 2006; Jang et al. 2007). Both, domoic acid and paralytic shellfish poison accumulate within the tissue of copepods which play a crucial role by transfer of the toxins to distant trophic levels (Tester et al. 2000; Lincoln et al. 2001). As a consequence, harmful algal blooms sometimes have detrimental effects on, for example, fish and mammals (Todd 1993; Landsberg 2002).

Beside increased toxicity, copepod cues also trigger chain length shortening in *Skeletonema*, together with a massive transcriptional response (Bergkvist et al. 2012; Amato et al. 2018; E. Selander et al. unpubl.). Copepods have an one order of magnitude lower grazing rate on single cells compared to longer chains, whereas microzooplankton, like ciliates, prefer the smaller units (Bjærke et al. 2015). By grazing on microzooplankton and large phytoplankton, copepods may act as a switch between two alternate trophic cascades of different food chain lengths and with opposite selective pressure on phytoplankton size distribution (Stibor et al. 2004). Measurements of chain length over time confirm that chain length is negatively correlated to copepod abundance in nature too (Bjærke et al. 2015). Based on the results reported here and earlier findings—a previous study shows that copepodamides induce bioluminescence in *L. polyedra* and *Alexandrium tamarense* (Lindström et al. 2017)—we conclude that copepodamide presence induces a broad range of defensive

**Fig. 3.** Dose response experiments of *A. minutum*, *P. seriata*, and *S. marinoi* treated with copepodamides. Symbols display average toxin induction or colony size reduction normalized to control  $\pm$  SEM after 18:4-copepodamide (circle) or 16:0-copepodamide (square) treatment. Dashed, black lines represent nonlinear least square fits according to the Michaelis–Menten equation. **(a)** Changes in total gonyautoxin 1–4 (GTX) content in *A. minutum* cells in percentage after exposure to increasing concentrations of 18:4-copepodamide and 16:0-copepodamide for 48 h ( $N = 4$  biological replicates). **(b)** Changes in domoic acid (DA) contents in *P. seriata* cells in percentage after exposure to increasing concentrations of 18:4-copepodamide and 16:0-copepodamide for 60 h ( $N = 3$  biological replicates). **(c)** Chain length reduction of *S. marinoi* expressed as percentage of chain length in controls after exposure to increasing concentrations of 18:4-copepodamide and 16:0-copepodamide for 66 h ( $N = 4$  biological replicates, average length of 50 cell chains per replicate). Note the different scales on the axes.





**Fig. 4.** Effects of species affiliation and diet on copepodamide composition. **(a)** PCA on the relative copepodamide composition of 11 different copepod species ( $N = 3$ –6 biological replicates of three to seven individuals per sample). **(b)** Example of the copepodamide profiles of *Centropages hamatus* and *C. finmarchicus* ( $N = 3$  [*Centropages hamatus*];  $N = 5$  [*C. finmarchicus*] biological replicates of five individuals per sample, average  $\pm$  SEM). **(c)** Relative content of copepodamides in percentage of cultured *T. longicornis* exclusively fed with *Rhodomonas* sp., *Nannochloropsis oculata*, *D. tertiolecta*, or *I. galbana* ( $N = 4$  biological replicates of five to six individuals per sample, average  $\pm$  SEM) and *T. longicornis* caught in the field ( $N = 4$  biological replicates of three to five individuals per sample, average  $\pm$  SEM).

traits in phylogenetically distant groups which may cause indirect cascading effects in plankton food webs. In particular, copepods and copepodamides will both favor smaller and/or more defended phytoplankton phenotypes.

A full characterization of copepodamides makes it possible to pursue the molecular and ecological mechanisms of indirect effects of copepods in the pelagic ecosystem. For instance, the signal transduction pathway and downstream molecular mechanisms leading to the expression of defensive phenotypes can be studied in controlled laboratory experiments, and plankton communities can be exposed to copepodamides to separate the indirect, copepodamide-driven effects from direct grazing effects.

Dose response experiments comparing copepodamides containing a saturated or unsaturated fatty acid support the

importance of the presence of a fatty acyl group at position C3 (Selander et al. 2015). Notwithstanding, for the diatoms tested, the potency of the saturated 16:0-coepodamide is lower than for the unsaturated 18:4-coepodamide. This suggests that the identity of the fatty acid side chain is also of some importance for the structure activity relationship and that especially the level of saturation may be important for the activity of the copepodamides. The binding of molecules to proteins is partly driven by entropic terms which mean that molecules with a lower conformational flexibility and less conformational penalty toward the bioactive conformation can bind better than highly flexible molecules (Bostrom et al. 1998; Hoffmann 2000). In comparison to saturated fatty acids, the conformational space of unsaturated fatty acids is smaller, as the double bond introduces a rotational barrier.

Assuming that copepodamides are sensed by microalgae via receptor proteins, copepodamides with a higher conformational stability may lead to better binding to the protein and thus stronger biological activity.

However, the response of *A. minutum* to copepodamides was less sensitive than for the diatoms and there was no difference in the potency of 18:4- and 16:0-copepodamide in toxin induction. Dinoflagellates and diatoms may have evolved different perception or response mechanisms to copepodamides. This is not surprising as dinoflagellates and diatoms are represented in two distinct groups in the eukaryotic tree of life (Burki and Keeling 2014; Worden et al. 2015) and may indicate convergent evolution of copepodamide receptor proteins.

A study monitoring copepodamide concentrations in the field reveals that the average natural concentration of copepodamides in seawater is 0.4 to 2 pmol L<sup>-1</sup>. The same study shows that the effective concentration averages < 0.5% of the nominal concentration when administered by coating copepodamides onto culture vessel walls (E. Selander et al. unpubl.). In the present study, saturation in the dose response curves for the diatoms was reached at nominal concentrations of 0.1–0.5 nmol L<sup>-1</sup>, resulting in 0.5–2.5 pmol L<sup>-1</sup> effective concentrations. Thus, both diatom species respond to copepodamide cues in ecologically relevant concentrations. The lower sensitivity of the dinoflagellate to copepodamides is puzzling, but copepods occasionally reach densities of hundreds per liter (Hamner and Carleton 1979; Ambler et al. 1991) and a single large copepod can exude 120 pmol d<sup>-1</sup> (Selander et al. 2015). This indicates that even these high concentrations are in a possible range of natural occurring copepodamides. Indeed, copepods do induce toxin production in *A. minutum* in laboratory experiments, but the lower sensitivity also suggest that there may be additional cues to the copepodamides involved or alternatively that the standing incubations used for *A. minutum* result in lower effective concentrations than incubation on a rotating plankton wheel used for the diatoms in this study.

Our results indicate that copepods have a specific copepodamide composition, which is linked to the diet and controlled by the amount of available dietary fatty acids. Copepods, although generally omnivorous, are selective feeders on microalgae depending on, for example, size, abundance, or toxicity (Frost 1972; Schultz and Kjørboe 2009; Ray et al. 2016; Xu et al. 2017) and microalgae vary in their fatty acid composition (Roncarati et al. 2004; Patil et al. 2007; Ohse et al. 2015). Additionally, copepods graze on microzooplankton, further differentiating the fatty acid composition of their diet (Tiselius 1989; Calbet and Saiz 2005; Tiselius et al. 2012). Hence, it is conceivable that feeding preferences between different species are mirrored in the copepodamide composition. The specific composition of copepodamides is further influenced by the proportions of dihydrocopepodamides and copepodamides, which seem not to be affected by the diet. It may reflect evolutionary differences in copepodamide biosynthesis among the

copepod species tested. This is obviously also the case for the harpacticoid *Tigriopus californicus*, which does not produce detectable amounts of copepodamides at all. In accordance to the biosynthetic pathway of a group of related compounds in *Tetrahymena* (Kaya and Sano 1991), it is possible that the two copepodamide scaffolds are synthesized prior to the introduction of the fatty acid residue, resulting in a species-specific distribution of dihydrocopepodamides and copepodamides with a diet-dependent variation of the fatty acid side chain.

As the composition of exuded copepodamides of *C. finmarchicus* (Fig. 4b) is similar to the composition of extractable copepodamides (Selander et al. 2015), the specific composition of dihydrocopepodamides and copepodamides observed within different copepod species can have ecological consequences. Dihydrocopepodamides are approximately 10 times more potent inducers of paralytic shellfish toxin production in dinoflagellates than copepodamides (Selander et al. 2015). Thus, formation of harmful algae blooms may be more promoted by copepods rich in dihydrocopepodamides such as *Acartia clausi*, *Centropages hamatus*, and *T. longicornis*.

Further, this study represents the first record of copepodamides in freshwater copepods and suggests that copepodamides may act as cue compounds in limnic systems too.

It is currently not known why copepods synthesize copepodamides and why the compounds are released into the water, but kairomones, chemical cues benefitting the receiving organism, are often released involuntarily where the compounds original purpose, for example, metabolism, improves overall fitness (Pohnert et al. 2007). One possible hypothesis is that copepodamides serve a function as emulsifiers in the digestive tract of copepods in analogy to taurine-containing lipids found in the gastric juice of crabs and bile salts in other animals (Van Den Oord et al. 1965; Maldonado-Valderrama et al. 2011). Alternatively, Mayor et al. (2015) discuss a speculative role of taurine lipids found in *Calanus* in the transportation and protection of metabolic substrates from their storage location to the point of oxidative catabolism within mitochondria. Beside this, a variety of possible functions of copepodamides is conceivable and further studies, for example, regarding the localization and biosynthesis of copepodamides are needed to learn more about the function of copepodamides within the copepods.

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#### Acknowledgments

This work was supported by a fellowship from the German Research Foundation GR-4716/1-1 (to W.G.), the Olle Engkvist Byggmästare

Foundation (to E.S.), the Danish Research Council, grant DFF-1323-00258 (to S.H.), and the Swedish Research Council VR No. 2015-05491 (to E.S.).

*Submitted 19 December 2017*

*Revised 29 June 2018*

*Accepted 11 October 2018*

**Conflict of Interest**

None declared.

*Associate editor: Peter Hernes*