

1 **Negligible effects of ocean acidification on *Eurytemora affinis* (Copepoda)**  
2 **offspring production**

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21

22 **Abstract**

23 Ocean acidification is caused by increasing amounts of carbon dioxide dissolving in the oceans  
24 leading to lower seawater pH. We studied the effects of lowered pH on the calanoid copepod  
25 *Eurytemora affinis* during a mesocosm experiment conducted in a coastal area of the Baltic Sea.  
26 We measured copepod reproductive success as a function of pH, chlorophyll *a* concentration,  
27 diatom and dinoflagellate biomass, carbon to nitrogen (C:N) ratio of suspended particulate  
28 organic matter, as well as copepod fatty acid composition. The laboratory-based experiment  
29 was repeated four times during four consecutive weeks, with water and copepods sampled from

1 pelagic mesocosms enriched with different CO<sub>2</sub> concentrations. In addition, oxygen radical  
2 absorbance capacity (ORAC) of animals from the mesocosms was measured weekly to test  
3 whether the copepod's defence against oxidative stress was affected by pH. We found no effect  
4 of pH on offspring production. Phytoplankton biomass, as indicated by chlorophyll *a*  
5 concentration and dinoflagellate biomass, had a positive effect. The concentration of  
6 polyunsaturated fatty acids in the females were reflected in the eggs and had a positive effect  
7 on offspring production, whereas monounsaturated fatty acids of the females were reflected in  
8 their eggs but had no significant effect. ORAC was not affected by pH. From these experiments  
9 we conclude that *E. affinis* seems robust against direct exposure to ocean acidification on a  
10 physiological level, for the variables covered in the study. *E. affinis* may not have faced acute  
11 pH stress in the treatments as the species naturally face large pH fluctuations.

12

## 13 **1 Introduction**

14 The concentration of carbon dioxide (CO<sub>2</sub>) in the atmosphere is rising at a ten times faster rate  
15 than during the past 55 million years. The oceans absorb CO<sub>2</sub> from the atmosphere leading to  
16 lower seawater pH and reduction in carbonate concentration. Since pre-industrial times the  
17 ocean acidity has increased by 28% (IPCC, 2013). The fast increase in CO<sub>2</sub> and change in  
18 seawater chemistry will have adverse effects on many marine species and ecosystems (Fabry et  
19 al., 2008; Kroeker et al., 2010). Due to lower buffering capacity of brackish water, the Baltic  
20 Sea is especially sensitive to elevated CO<sub>2</sub> (Havenhand, 2012). Modelling suggests a decrease  
21 of 0.26-0.40 pH units for the Baltic Sea by the year 2100 (BACC II, 2015). In addition, high  
22 CO<sub>2</sub> levels interact with other climate change related factors that may have negative effects on  
23 marine organisms (Kroeker et al., 2013; Talmage and Gobler, 2012). Especially the coastal  
24 zones are under heavy pressure from anthropogenically driven ocean acidification due to  
25 eutrophication and oxygen minimum zones (Fabry et al., 2008; Melzner et al., 2013; Wallace  
26 et al., 2014).

27 Copepods are the most abundant zooplankton in the oceans. They constitute major parts  
28 of the diet of juvenile fish, and are hence an important part of the food web. Lowered pH may  
29 disturb the acid-base balance, thereby altering the reproduction, hatching, and development  
30 (Kurihara et al., 2004; Mayor et al., 2007; Weydmann et al., 2012). Besides the direct effects  
31 of acidification, rising CO<sub>2</sub> can adversely affect consumers and food webs due to changed  
32 nutritional value of prey (Rossoll et al., 2012). Polyunsaturated fatty acids (PUFA) are essential

1 metabolites for copepods and need to be obtained from the diet. Certain PUFA have specific  
2 roles in central processes of copepod reproduction including egg production (20:5 $\omega$ 3 EPA), egg  
3 hatching (22:6 $\omega$ 3 DHA), and development (18:3 $\omega$ 3 and 18:5 $\omega$ 3) (Jónasdóttir et al., 2009).  
4 Important  $\omega$ 3 fatty acids decreased significantly in the diatom *Thalassiosira pseudonana* grown  
5 at high CO<sub>2</sub>, with lower levels of PUFA with following decreased egg production in the copepod  
6 *Acartia tonsa* (Rossoll et al., 2012). Further, CO<sub>2</sub>-related changes in the fatty acid composition  
7 and content of several primary producers have been reported (Bermúdez et al., 2016, and  
8 references therein). Furthermore, ocean acidification induced changes in phytoplankton species  
9 composition can have an indirect effect on food quantity and quality for heterotrophic  
10 consumers. Elevated CO<sub>2</sub> levels can increase C:N ratios of primary producers, which alter their  
11 nutritional value and can adversely affect the growth and reproduction of copepods (Schoo et  
12 al., 2013).

13 Ocean acidification can induce oxidative stress in marine organisms (Tomanek et al.,  
14 2011; Kaniewska et al., 2012). Hence, biochemical responses to low pH conditions, such as  
15 changed activity of antioxidants and enzymes may show higher sensitivity than for example  
16 survival and reproduction (Gorokhova et al., 2010; Zhang et al., 2012). An enhanced  
17 antioxidant defence in response to increased reactive oxygen species (ROS) concentration may  
18 occur at the expense of reduced investment in other metabolic processes, such as growth and  
19 reproduction. The defence capacity against oxidative stress can be assessed by measuring the  
20 capacity to quench ROS (see review by Monaghan et al., 2009).

21 *E. affinis* is a common copepod in the Baltic Sea and dominates the zooplankton  
22 community together with *Acartia bifilosa* in the study area during summer. *E. affinis* is an egg-  
23 bearing copepod that produces subitaneous eggs during summer and diapause eggs in autumn.  
24 The copepods recruit from small overwintering populations, and by hatching from the sediment  
25 (Katajisto et al., 1998). Previous studies on the effects of ocean acidification on *A. bifilosa* from  
26 the Baltic Sea have shown adverse effects in combination with warming (Vehmaa et al., 2012a,  
27 2013). The increase in egg production with warmer temperature was lower when copepods were  
28 simultaneously exposed to warmer temperature and lowered pH (Vehmaa et al., 2012a).

29 The main objectives of this study were to examine effects of ocean acidification on  
30 reproductive success and antioxidant defence of the copepod *E. affinis*, as well as measuring  
31 the effects of food quality and quantity on offspring production. We studied how lowered pH,  
32 phytoplankton biomass (indicated as chlorophyll *a*), biomass of diatoms and dinoflagellates and

1 the C:N ratio of particulate organic matter (POM) affect the offspring, i.e., nauplii production  
2 in *E. affinis*. In addition, we looked at the effect of pH on essential fatty acids of incubated egg-  
3 bearing females to reveal indirect effects via the food. We also tested whether the fatty acid  
4 levels of the females were reflected in their eggs under a range of  $f\text{CO}_2$  values representative  
5 for the future ocean (IPCC, 2013).

6

## 7 **2 Material and Methods**

### 8 **2.1 Experimental set-up**

9 The study was conducted using KOSMOS mesocosms (Riebesell et al., 2013) within the  
10 framework of the SOPRAN project (Paul et al., 2015). The mesocosms were located at  
11 Storfjärden, an offshore pelagic area in the vicinity of Tvärminne Zoological Station  
12 (University of Helsinki) Baltic Sea (59°51'20"N, 23°15'42"E) from the beginning of June until  
13 the middle of August, 2012. Storfjärden has a maximum depth of 34 m. The water is brackish  
14 with mean salinity 6. The area receives inflow of freshwater from the river Svartån, and  
15 periodical inflows of cold water from the open Baltic Sea with higher salinity (Niemi, 1976).  
16 Six mesocosms, consisting of 17 m deep bags made of thermoplastic urethane, each enclosing  
17 ~55 m<sup>3</sup>, were moored on site on June 12. The mesocosms were covered by a net (mesh size 3  
18 mm) at the top and the bottom during filling and left open for four days before the net was  
19 removed and the top was pulled up 1.5 m above the water surface and closed at the bottom (see  
20 Riebesell et al., 2013 and Paul et al., 2015 for details on the experimental design) to enclose the  
21 natural plankton community. The water column was mixed at the beginning of the experiment  
22 in order to avoid a salinity stratification. Four of the mesocosms were stepwise manipulated  
23 with CO<sub>2</sub> enriched seawater, during three consecutive days. Two bags were untreated and used  
24 as controls. Due to outgassing, CO<sub>2</sub> was also added on day 15 of the experiment to the upper 7  
25 m of the high CO<sub>2</sub> mesocosms to maintain the treatment levels. No nutrients were added. The  
26 average  $f\text{CO}_2$  levels during the period of our incubation experiments ( $t_1$ - $t_{30}$ ) were 346, 348,  
27 494, 868, 1075 and 1333  $\mu\text{atm}$  (Paul et al., 2015).

### 28 **2.2 Sampling and incubations**

29 Our copepod experiment was conducted during a four-week period with weekly incubations.  
30 We sampled water and copepods from the mesocosms on days  $t_3$ ,  $t_{10}$ ,  $t_{17}$  and  $t_{24}$  ( $t_0$  being the

1 day of first addition of CO<sub>2</sub> into the bags). Zooplankton was sampled with a 300 µm net (Ø 17  
2 cm) from 17 m depth to the surface from all mesocosms and transferred to containers pre-filled  
3 with 4 L of seawater from a depth of 9 m from the respective mesocosm. On the same day,  
4 unfiltered water samples were taken from each mesocosm with depth-integrated water samplers  
5 (IWS, HYDRO-BIOS, Kiel) which take equal amount of seawater from every depth (0-17 m).  
6 In order to minimize handling of the restricted water available, to keep food conditions as  
7 similar to *in situ* conditions as possible, and to avoid gas exchange, the water was directly  
8 transferred into airtight 1.2 L Duran bottles for incubations. Water samples and zooplankton  
9 were transported to a light- and temperature controlled room at Tvärminne Zoological Station.  
10 Egg-bearing females of *E. affinis* (n = 10 per treatment) were incubated in the 1.2 L Duran glass  
11 bottles which contained mesocosm water. Temperature and pH were measured before adding  
12 the copepods to the bottles. Bottles were filled up and sealed without airspace, ensuring no air  
13 bubbles were present, to prevent CO<sub>2</sub>-outgassing. The bottles were slowly inverted after sealing  
14 and incubated in a 16:8 h light-dark cycle at *in situ* temperature, as an attempt to match the  
15 natural environment. A light source was installed above the incubation bottles, yielding 7 µmol  
16 m<sup>-2</sup> s<sup>-1</sup> (LI-COR LI-1000). All pH and temperature measurements were conducted with an  
17 Ecosense pH10 pH/temperature Pen directly from the bottles before closing and directly after  
18 opening (Table 1). The pen was calibrated with standard buffer solutions (Centipur, Titripac  
19 pH 4.00, 7.00 and 10.00) every second day. The bottles were inverted three times a day and  
20 their location on the shelf was randomly changed.

21 Each incubation lasted four days. Copepods and nauplii were gently filtered once daily  
22 onto a 250 µm and 30 µm mesh, respectively. The status of the adult copepods was checked  
23 under a dissecting microscope by submerging the sieve in a petri dish filled with water from  
24 respective mesocosm, before returning the copepods to bottles containing new unfiltered  
25 seawater sampled the same day from respective mesocosm. The nauplii were preserved in acid  
26 Lugol's solution and counted under a dissecting microscope (Nikon SMZ800, 25 ×  
27 magnification). As we could not follow individual copepods, we counted the nauplii produced  
28 daily, and the number of live females in the incubation bottles (survival > 95 %) when filtering  
29 out the nauplii. Only first stage nauplii of *E. affinis* were included in the analyses. The number  
30 of nauplii produced per female was calculated from the daily nauplius count divided by the  
31 number of females in the bottles. The bottles with new water was temperature-adjusted in the  
32 climate chamber before transferring the copepods. When changing the water we checked for

1 oxygen depletion every second day with a hand held oxygen probe (YSI Environmental  
2 ProODO) in the old water used in the incubation bottles.

3 At the end of each weekly incubation (*t*7, *t*14, *t*21, *t*28) the copepods were counted and  
4 checked for eggs and survival. Egg sacs were cut off from incubated egg-bearing females, with  
5 a thin needle and transferred to pre-weighted tin cups. The females were then stored separately.  
6 The samples were frozen in an ultra-freezer (-80 °C) until fatty acids were measured by gas  
7 chromatography as fatty acid methyl esters (FAMES) following instructions in Klein Breteler  
8 et al. (1999). Fatty acids were separated into three groups that were used in the analyses;  
9 polyunsaturated (PUFA), monounsaturated (MUFA) and saturated fatty acids (SAFA) and were  
10 expressed as ng mg dry weight<sup>-1</sup>.

11 With each start of the weekly, sub-experiments, female *E. affinis* with egg sacs were  
12 picked from the mesocosms for analyses of oxygen radical absorbance capacity (ORAC). The  
13 animals ( $n = 30 \pm 2$ ) were carefully moved with tweezers onto a piece of plankton net gauze and  
14 stored in Eppendorf tubes in -80 °C until they were homogenised in 150 µl Tris-EDTA buffer  
15 containing 1% sarcosyl. The antioxidative capacity was assayed as ORAC according to Ou et  
16 al. (2001). As a source of peroxy radicals, we used 2,2-azobis(2-amidinopropane)  
17 dihydrochloride (AAPH) (152.66 mM) and fluorescein was used as a fluorescent probe (106  
18 nM). We used trolox (218 µM, Sigma-Aldrich) as a standard and the assay was performed on  
19 a 96-well microplate and to each well, 20 µL sample, 30 µL AAPH and 150 µL fluorescein  
20 were added. ORAC values were normalized to protein concentration and expressed as mg  
21 Trolox equivalents mg protein<sup>-1</sup>. Protein concentration was measured with NanoOrange® (Life  
22 Technologies).

23 Phytoplankton was sampled every second day, fixed with acidic Lugol's iodine (2% final  
24 concentration) and counted with the inverted microscope method (Utermöhl, 1958). Samples  
25 for chlorophyll *a* (Chl *a*) measurements were collected onto GF/F filters and measured as  
26 described by Welschmeyer (1994).

27 Samples for carbon (C) and nitrogen (N) concentrations were collected as for Chl *a* and  
28 stored in glass petri dishes at -20°C until analyses. For further details on sampling and analyses,  
29 please refer to Paul et al. (2015).

30

## 1 2.3 Statistical analyses

### 2 2.3.1 Nauplii production

3 A linear mixed effects model (LMM) was applied, as we did repeated measures of nauplii  
4 production of the same groups of individuals from the same mesocosms, to test if pH or food  
5 quantity and quality affected the nauplii production of *E. affinis*. Collinearity between all  
6 explanatory variables was checked (Pearson's product-moment correlation). Chl *a*  
7 concentration and the abundance of filamentous cyanobacteria correlated. As these correlating  
8 variables explain partly the same thing, the variable that explained the variation in nauplii  
9 production the best (Chl *a*) was included in the model. In the model the average number of  
10 nauplii produced female<sup>-1</sup> day<sup>-1</sup> (log-transformed) for each treatment was set as response  
11 variable. Incubation pH (calculated as weekly mean values from daily measurements from  
12 incubation bottles), Chl *a* concentration, biomass of diatoms (*Chaetoceros* sp. *Skeletonema*  
13 *marinoi* and pennate diatoms, total µg C L<sup>-1</sup>), C:N <55µm fraction of POM, biomass of  
14 mixotrophic dinoflagellates (*Amylax triacantha*, *Dinophysis* spp., *Heterocapsa triquetra* and  
15 *Micracanthodinium* spp., size range ~10-100 µm, total µg C L<sup>-1</sup>) and incubation temperature  
16 were used as fixed effects (Table 2). We used only the most abundant diatoms as the other  
17 species had a very scarce and inconsistent abundance in the samples. The main groups of  
18 diatoms were present in all mesocosms. The smaller fraction of C:N <55 µm was used instead  
19 of total C:N as the total fraction may have included large zooplankton such as copepods which  
20 could affect the results. The explanatory variables used included data of each mesocosm of the  
21 corresponding day of sampled water used for the incubations. When sampling days were  
22 missing, the average values (of total µg C L<sup>-1</sup> for diatoms and dinoflagellates, and mol:mol of  
23 C:N) for the previous and the next day were used. Day nested within week, nested within  
24 mesocosm, was used as random intercept as nauplii production of the same animals was  
25 measured four times per week and as weekly incubations were dependent on each other, and  
26 they were repeatedly sampled from the same mesocosms. The model simplifications were done  
27 manually in backward stepwise manner by removing the non-significant effects and by using  
28 Akaike's information criterion (AIC) to achieve the minimum adequate model for the data. We  
29 report t-statistics of the retained variables for the LMMs (Table 3).

### 1 2.3.2 Fatty acids

2 Linear mixed effects models were applied to test if pH has a direct effect on the fatty acid  
3 content of female copepods. EPA, DHA, and their precursor 18:3 $\omega$ 3 autocorrelated strongly  
4 with each other, and with total PUFA (Pearson's product-moment correlation); therefore we  
5 decided to use PUFA in the LMM. Separate models were made for each fatty acid group, which  
6 was set as response variable, with pH as fixed effect and mesocosm as random effect. To test  
7 the effects of essential fatty acids on weekly nauplii production, we used separate LMMs, as  
8 PUFA and MUFA autocorrelated. In the models, PUFA, MUFA and SAFA were used as fixed  
9 effects and mesocosm was tested as random factor (Table 2).

10 To test whether female fatty acid content are reflected in the fatty acid content of eggs,  
11 each fatty acid group (PUFA, MUFA and SAFA) was tested separately in a LMM. In the model,  
12 fatty acids of eggs was set as response variable and female fatty acid content as fixed effect;  
13 mesocosm was used as random factor. Not all females had egg sacs left at the end of weeks 3  
14 and 4 and therefore not enough material (egg sacs) was obtained for all treatments. The  
15 variables of corresponding samples that were missing the egg data were therefore removed.

### 16 2.3.3 Antioxidative capacity

17 We tested whether there was an effect of pH on the copepods' antioxidant capacity (ORAC)  
18 with a LMM. ORAC was set as response variable, pH (measured the same day from water  
19 samples taken for incubations) as fixed factor and mesocosm was set as random factor. In  
20 addition, to test for potential correlation between ORAC and nauplii production, a Pearson's  
21 product-moment correlation was performed. In the ORAC data, values for mesocosms 5  
22 (control) and 6 (868  $\mu$ atm) were missing.

23 For all models, model validation was done by plotting the standardised residuals against  
24 the fitted values. All statistical analyses were performed with R 2.15.2 and the nlme-package  
25 (Pinheiro et al., 2012) was used for the LMM analyses (R Development Core Team, 2012).

26

## 27 3 Results

28 The oxygen saturation was continuously high (>93.8%) in all incubations (Table 1).  
29 Temperature in the climate-controlled room followed the *in situ* temperature except during the  
30 fourth weekly incubation (*t*24-*t*28) when the room was not adjusted to the sudden *in situ* drop



1 in temperature that occurred. Temperature in the treatment bottles increased from around 10°C  
2 in the first week to 15°C during the fourth week (Table 1). The pH remained stable in the bottles  
3 (SD < 0.08 within a week based on daily measurements, (Table 1) and matched the *in situ* pH  
4 and CO<sub>2</sub> treatments. Chl *a* concentration was relatively stable at ~2 µg L<sup>-1</sup> in all mesocosms but  
5 then decreased to ~1 µg L<sup>-1</sup> on *t*17. A significant positive effect of CO<sub>2</sub> on Chl *a* was observed  
6 after *t*17 (Paul et al., 2015). Dinoflagellates were on average 4.41±1.39 µg CL<sup>-1</sup> (+SD) (range  
7 0-7.32) and declined rapidly after *t*17. The C:N values included in our analyses (our sampling  
8 days) were on average 7.66±0.42 (range 6.13-8.77). A more comprehensive description of C:N  
9 is found in Paul et al. (2015). The diatoms included in our analyses were on average 0.06±0.10  
10 (range 0-0.53 µg C L<sup>-1</sup>). CO<sub>2</sub> treatment did neither effect dinoflagellates, C:N <55 µm, nor  
11 diatoms.

12 Nauplii production in incubations was highest in water from M3, 1075 µatm (pH 7.6)  
13 with on average 12.6±9.6 nauplii produced per female per day during the whole study period.  
14 For clarity and easier comparison between studies within this mesocosm project, average *f*CO<sub>2</sub>  
15 levels (*t*1-*t*30) are included in Fig 1 to describe the treatments. The effect of pH on nauplii  
16 production was not statistically significant. Particulate matter C:N (< 55µm) had no impact on  
17 nauplii production. Chl *a* concentration, as an indicator of total food availability had a positive  
18 effect (LMM; *t* = 5.440, *p* = < 0.001, Fig. 2a). Dinoflagellate biomass (*t* = 2.731, *p* = 0.008,  
19 Fig. 2c) stimulated nauplii production, whereas diatom biomass (LMM; *t* = -4.231, *p* = <0.001,  
20 Fig. 2b) had an adverse effect. There was a positive relationship between incubation  
21 temperature and nauplii production (*t* = 3.388, *p* = <0.004) (Table 3).

22 The fatty acid contents (ng mg dry weight<sup>-1</sup>) of the females were not affected by pH  
23 (LMM *p* = > 0.5). Female MUFA and PUFA content significantly affected the MUFA and  
24 PUFA content of the eggs (LMM MUFA; *t* = 2.922, *p* = 0.012, LMM PUFA; *t* = 2.864, *p* =  
25 0.013), whereas female SAFA did not (Fig. 3 a-c, LMM; *t* = -1.497, *p* = 0.158). Female PUFA  
26 concentration stimulated nauplii production (LMM; *t* = 3.989, *p* = 0.001), whereas MUFA  
27 (LMM; *t* = 2.031, *p* = 0.058), and SAFA content had no statistically significant effect (LMM; *t*  
28 = 0.644, *p* = 0.528, Fig. 4 a-c, Table 3).

29 ORAC was not affected by pH (LMM; *t* = -0.057, *p* = 0.580) and there was no correlation  
30 between female ORAC and nauplii production (*rho* = 0.297. *p* = 0.180) (Fig. 5).

31

## 1    **4    Discussion**

### 2    **4.1    Effects of lowered pH**

3    Experimental CO<sub>2</sub> concentrations did not affect the nauplii production of *E. affinis* in the current  
4    study. However, nauplii production in our incubations corresponded well with patterns of  
5    nauplii abundance observed in the mesocosm bags. The total number of copepods in the  
6    mesocosms showed no significant relation with CO<sub>2</sub> either (Lischka et al., 2015). This is also  
7    in line with findings of Niehoff et al. (2013), who found no effect of CO<sub>2</sub> on zooplankton  
8    community development or abundance of single taxa in a similar mesocosm study in  
9    Kongsfjorden, Svalbard.

10        The physicochemical conditions in the research area is naturally fluctuating, therefore the  
11    plankton community may be adapted to large variability in CO<sub>2</sub> concentration and pH. In  
12    addition, organisms such as copepods are exposed to daily variation in pH and there is evidence  
13    that species performing vertical migration may be more robust to changes in CO<sub>2</sub> (Lewis et al.,  
14    2013). *E. affinis* undertakes diel vertical migration and particularly ovigerous *E. affinis* females  
15    stay below 20 m depth and experience >0.5 units change (7.51-8.1) in pH on a daily basis  
16    (Almén et al., 2014), in the area where the current study was conducted. Thus, this could  
17    partially explain why *E. affinis* reproduction did not respond to lowered pH. Cripps et al. (2014),  
18    on the other hand, found severely reduced nauplii survival for *Acartia tonsa* kept at a pCO<sub>2</sub> of  
19    1000 µatm, while other life stages were less affected. There appears to be a large variation in  
20    CO<sub>2</sub> sensitivity between species, even for organisms from the same study area. During this  
21    KOSMOS study, Vehmaa et al. (2015) found a negative effect of increased fCO<sub>2</sub> on body size  
22    and development index for *A. bifilosa*, another common copepod in the Baltic Sea. The  
23    increasing hatching rate of *E. affinis* with higher temperature reported by Andersen and Nielsen  
24    (1997) is also reflected in our results with higher incubation temperatures, affecting the nauplii  
25    production positively.

### 26    **4.2    Effects of food**

27    We found that nauplii production was positively affected by food availability (Chl *a*  
28    concentration, Fig. 2a). Our results are in agreement with Zervoudaki et al. (2014) who neither  
29    found discernible effects of lowered pH, whereas both higher temperature and food  
30    concentration (Chl *a*) positively affected egg production in *A. clausi* in a low nutrient

1 Mediterranean system. According to fractionated Chl *a* measurements during the mesocosm  
2 campaign (Paul et al., 2015) >90% of the Chl *a* consisted of nanophytoplankton (<20 µm),  
3 which possibly constituted an important food source for the filter-feeding *E. affinis* (Motwani  
4 and Gorokhova, 2013).

5 Although nauplii production of *E. affinis* was negatively affected by diatoms, no effect of  
6 CO<sub>2</sub> on diatom abundance was found. The abundance of diatoms was high during the first days  
7 but then declined rapidly. Low hatching frequency has, however, previously been observed for  
8 *E. affinis* during the diatom spring bloom in the same area (Ask et al., 2006). Some diatoms  
9 contain inhibitory compounds or lack essential nutrients that may be crucial for copepod  
10 reproduction (Lee et al., 1999). In the current study, diatoms consisted of *Chaetoceros* spp.,  
11 *Skeletonema marinoi* and pennate diatoms. Vehmaa et al. (2012b) reported low egg production  
12 for *E. affinis* on a *S. marinoi* dominated diet in the study area. *Skeletonema* can produce  
13 potentially harmful aldehydes affecting copepod egg production (Ianora and Miralto, 2010).  
14 Significant negative correlation between *Chaetoceros* spp. and *E. affinis* hatching frequency  
15 has also been reported (Ask et al., 2006). However, there could potentially be a non-causal  
16 relationship between low diatom abundance and high nauplii production. It is possible that the  
17 end of the diatom bloom and peak abundance coincided (Ask et al., 2006). Dinoflagellates are  
18 in some cases considered superior food source for copepods, as opposed to diatoms (Ianora et  
19 al., 2004; cf. Vehmaa et al., 2012b). In this study dinoflagellates positively stimulated nauplii  
20 production. Dinoflagellates probably contributed to nutritional quality as they are high in  
21 essential fatty acids (Galloway and Winder, 2015). We do not know to which extent the  
22 copepods fed on the different species; however, *E. affinis* is able to feed on both *H. triquetra*  
23 and *Dinophysis* spp., although the latter has toxic strains (Setälä et al., 2009).

24 We realize that some copepods and nauplii probably were introduced with the unfiltered  
25 water to the incubation bottles. We assume that it did not have a major effect on the results as  
26 the copepod nauplii abundance did not vary between the mesocosms (Lischka et al., 2015), and  
27 only *E. affinis* nauplii were counted. We observed a lot of epibionts (*Vorticella*) attached to  
28 adult copepods during the third week in the mesocosms. This was probably due to ageing  
29 (Jamieson and Santer, 2003), or the lack of predators that would otherwise have removed  
30 infested individuals which are more visible due to epibionts causing impaired escape ability  
31 (Souissi et al., 2013). The age of the *E. affinis* adults incubated in our experiments, was  
32 estimated to 2-3 weeks to >1 month. The higher age structure of *E. affinis* occurring in the

1 mesocosms, as well as the decreasing Chl *a* levels could partly explain the decreased nauplii  
2 production in the third and fourth week of the experiment. Decreasing levels of PUFA in  
3 females towards the fourth week (Bermúdez et al., 2016), could also have affected copepod  
4 nauplii production. In the current study, the natural phytoplankton composition in the  
5 mesocosms did not change significantly due to CO<sub>2</sub> (Bermúdez et al., 2016; Annegret Stuhr,  
6 pers. comm.). Rossoll et al. (2013) and Bermúdez et al. (2016) suggest that a dampening of  
7 CO<sub>2</sub>-effects can be expected for coastal communities adapted to strong natural fluctuations (cf.  
8 Waldbusser and Salisbury, 2014), as also proposed here. Rossoll et al. (2013) found no changes  
9 in phytoplankton community composition and no direct effect of lowered pH or indirect CO<sub>2</sub>  
10 effect, via changed food quality on *A. tonsa* reproduction, exposed to similar treatment levels  
11 as in the present study.

### 12 **4.3 Antioxidative capacity and fatty acids**

13 Our results suggest that the oxidative balance was maintained in the copepods in all treatments  
14 regardless of pH, as we did not observe any change in ORAC. As noted by Vehmaa et al. (2013),  
15 ORAC is affected by lowered pH, rather in combination with warmer temperatures, but not by  
16 moderately lowered pH alone. An oxidative imbalance, favouring ROS production can result  
17 in oxidative stress, as ROS can attack biomolecules, such as lipids, proteins and DNA  
18 (Monaghan et al., 2009). Developmental stage (Fanjul-Moles and Gonsebatt, 2012),  
19 environmental condition (Lushchak, 2011), as well as feeding activity (Furuhagen et al., 2014)  
20 can affect levels of oxidative stress, suggesting the importance of measuring several biomarkers  
21 (Monaghan et al., 2009). We conclude that *E. affinis* did not face pronounced pH stress and  
22 therefore seems fairly robust to future ocean acidification, at least based on results in the present  
23 manuscript.

24 Analyses of fatty acid concentration in *E. affinis* females from our incubations revealed  
25 that PUFA in females was transferred to the eggs and stimulated nauplii production  
26 significantly, whereas no significant effect of pH on FA content in females was revealed.  
27 Despite the fact that Rossoll et al. (2012) found CO<sub>2</sub> induced changes in fatty acid content of  
28 phytoplankton in laboratory-based experiments, no CO<sub>2</sub> induced changes on phytoplankton or  
29 copepod fatty acid composition were found during the current mesocosm study (Bermúdez et  
30 al., 2016). The authors suggest that phosphorus limitation, being homogeneous in all  
31 mesocosms as nutrient addition was not practised, may have a stronger influence on community  
32 composition and their associated fatty acid profile than CO<sub>2</sub>. Isari et al. (2015) found neither

1 direct effects on copepod vital rates, nor indirect effects, via phytoplankton fatty acid  
2 composition, in two copepods *Acartia granii* and *Oithona davisae*. However, most PUFA  
3 showed a positive correlation with pCO<sub>2</sub> during part of a mesocosm study in Svalbard, which  
4 the authors attribute to taxonomical changes due to rising dinoflagellate abundances (Leu et al.,  
5 2013). In the present study female MUFA were reflected in their eggs, whereas SAFA were  
6 not, and none of them had a significant effect on nauplii production. These fatty acids, at least  
7 MUFA, are rather used for metabolism and storage (McMeans et al., 2012).

## 9 **5 Conclusions**

10 From our results we conclude that *E. affinis* is not sensitive to near future levels of ocean  
11 acidification on a physiological level for the variables measured in the study. Offspring  
12 production was not affected after one generation. Food quality, in terms of dinoflagellate  
13 biomass and higher PUFA stimulated nauplii production, but we observed no difference in fatty  
14 acid composition due to pH. We neither observed an effect of pH on ORAC. In the study area  
15 *E. affinis* is probably adapted to high pH variability due to diel vertical migration and may,  
16 therefore, not have faced pronounced pH stress from the treatment levels used in this study. We  
17 found that the effects of food quantity had an impact on nauplii production of *E. affinis*. For the  
18 time we conducted the laboratory based experiments, we, however, did not observe an indirect  
19 CO<sub>2</sub> effect via phytoplankton biomass. Chl *a* concentration correlated positively with CO<sub>2</sub>, but  
20 only clearly discernible for picophytoplankton from *t*25 onwards (Paul et al., 2015) and we  
21 sampled no longer than *t*27. How the indirect effect of CO<sub>2</sub>, (via the food) would affect the  
22 copepods on a longer time scale remains unclear. Future studies should focus on copepod  
23 adaptation in relation to coastal pH variability and tolerance towards extreme events.

## 25 **Author contribution**

26 A-K.A., A.V., A.B. and J.E.-Ö. designed and conducted the laboratory experiment. A-K.A.  
27 counted the nauplii samples, S.L. counted mesozooplankton and ciliates from the mesocosms  
28 and A.S. counted phytoplankton. S.F. analysed ORAC, A.P. analysed C:N samples, J.R.B.  
29 analysed fatty acids and L.B. analysed Chl *a*. A-K.A. and A.V. performed the statistical  
30 analyses and A-K.A. wrote the manuscript with contributions from all co-authors. Project  
31 coordinator: U.R.

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1 **Tables**

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3 Table 1.  $f\text{CO}_2$  values ( $t1-t30$ ), average weekly pH, temperature and dissolved oxygen (DO) and  
 4 saturation in incubation bottles.

$f\text{CO}_2$ treatment ( $\mu\text{atm}$ )	Mesocosm	week	pH	temp. (C°)	DO mg l <sup>-1</sup>	DO%
346	1	1	8.12	11.21	10.61	96.0
	1	2	8.24	14.51	10.30	98.7
	1	3	8.12	15.08	8.71	99.5
	1	4	8.03	15.80	9.42	93.8
348	5	1	8.14	10.00	10.94	96.7
	5	2	8.20	13.37	10.64	98.3
	5	3	8.07	14.99	9.88	99.8
	5	4	8.02	15.10	9.61	98.9
494	7	1	7.93	9.98	10.87	96.2
	7	2	8.02	13.31	10.62	97.7
	7	3	7.90	15.00	9.96	100.6
	7	4	7.91	14.96	9.60	98.7
868	6	1	7.68	10.24	10.83	95.2
	6	2	7.80	13.33	10.56	97.3
	6	3	7.74	15.01	9.85	99.6
	6	4	7.76	15.13	9.65	98.9
1075	3	1	7.59	10.23	10.85	96.4
	3	2	7.72	13.63	10.61	98.3
	3	3	7.67	14.60	10.00	101.4
	3	4	7.71	15.29	9.57	98.5
1333	8	1	7.52	9.96	10.07	96.0
	8	2	7.63	13.35	10.65	98.0
	8	3	7.59	14.76	9.98	100.5
	8	4	7.62	15.14	9.72	99.7

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- 1 Table 2. Variables that were used in the full LMM models (numbers indicate separate models).  
 2 Repeated measures were used as random effects in the models, as samples from the same enclosures  
 3 are dependent on each other.

LMM	Fixed effects	Definition	Response variable
1	pH	The ocean acidification effect	Nauplii production
	Chl <i>a</i>	The food quantity effect	
	Diatoms	The food quality effect	
	C:N<55µm		
	Dinoflagellates		
	Incubation temp.		
2	Incubation pH	The ocean acidification effect	Fatty acids in females:
3			PUFA
4			MUFA
			SAFA
5	Fatty acids in females: PUFA	Relationship between female fatty acids and their eggs	Fatty acids in eggs:
6	MUFA		PUFA
7	SAFA		MUFA
			SAFA
8	Fatty acids in females: PUFA		Nauplii production
9	MUFA		
10	SAFA		
11	pH		ORAC

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1 Table 3. T-statistics of the retained fixed effects in the LMM.

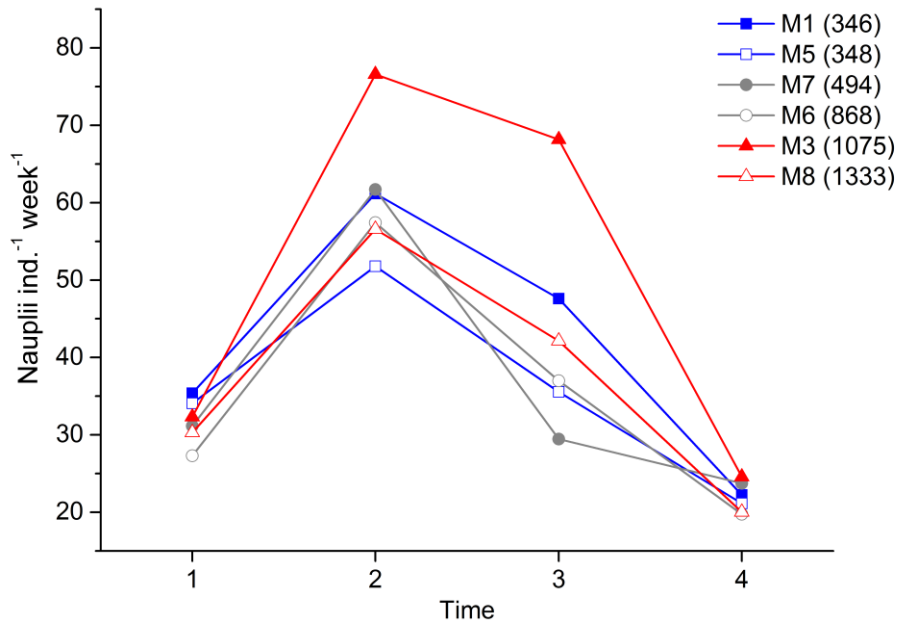
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LMM	Response variable	Variable	value	df	t	p
1	Nauplii production*	Chl <i>a</i>	1.09±0.20	69	5.440	<0.001
		Diatoms	-2.79±0.66	69	-4.231	<0.001
		Dinoflagellates	0.14±0.05	69	2.731	0.008
		Incubation temp.	0.16±0.05	17	3.388	0.004
Fatty acids in females:						
2	PUFA	Incubation pH	75.99±112.80	16	0.673	0.510
3	MUFA		-7.70±34.60	16	-0.223	0.827
4	SAFA		-135.27±325.21	16	-0.416	0.683
Fatty acids in eggs:		Fatty acids in females:				
5	PUFA	PUFA	1.15±0.40	13	2.864	0.013
6	MUFA	MUFA	1.08±0.37	13	2.922	0.012
7	SAFA	SAFA	-2.51±1.68	13	-1.497	0.158
Fatty acids in females:		Fatty acids in females:				
8	Nauplii production	PUFA	0.09±0.02	17	3.989	0.001
9		MUFA	0.185±0.09	17	2.031	0.058
10		SAFA	0.006±0.01	17	0.644	0.528
11	ORAC	Incubation pH	-0.02±0.04	15	0.057	0.580

\*log-transformed

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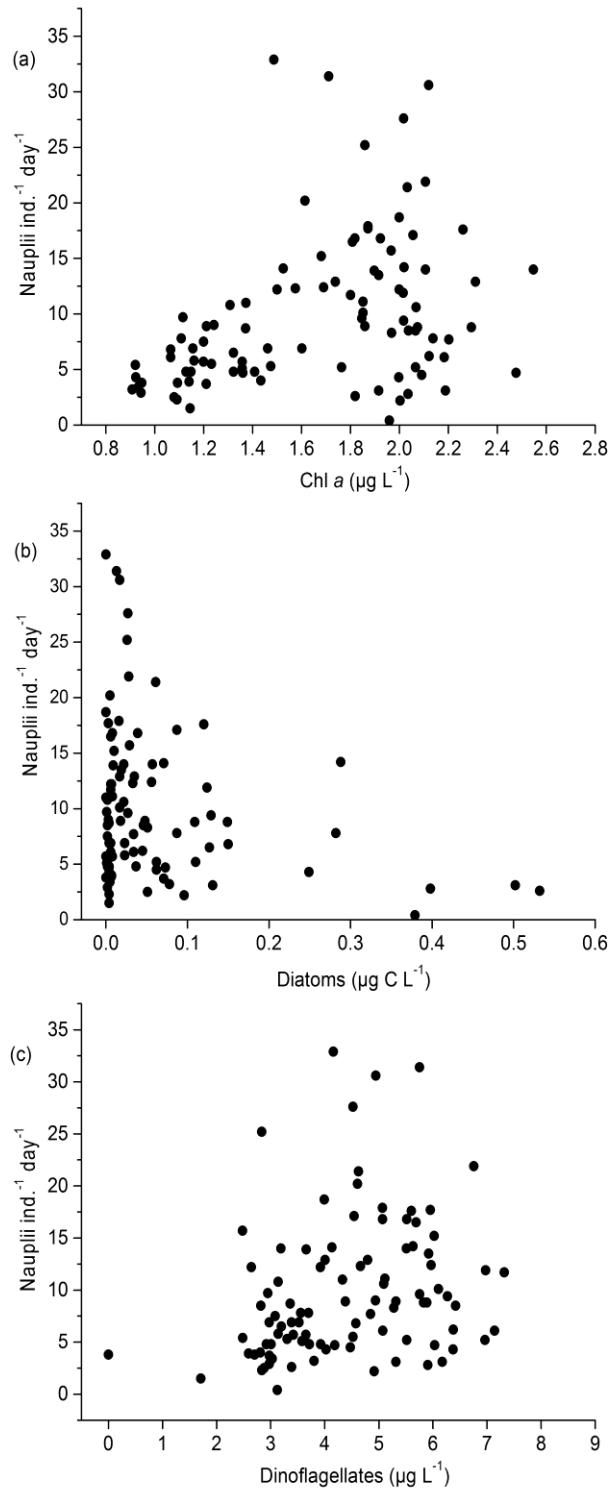
## 1 Figures



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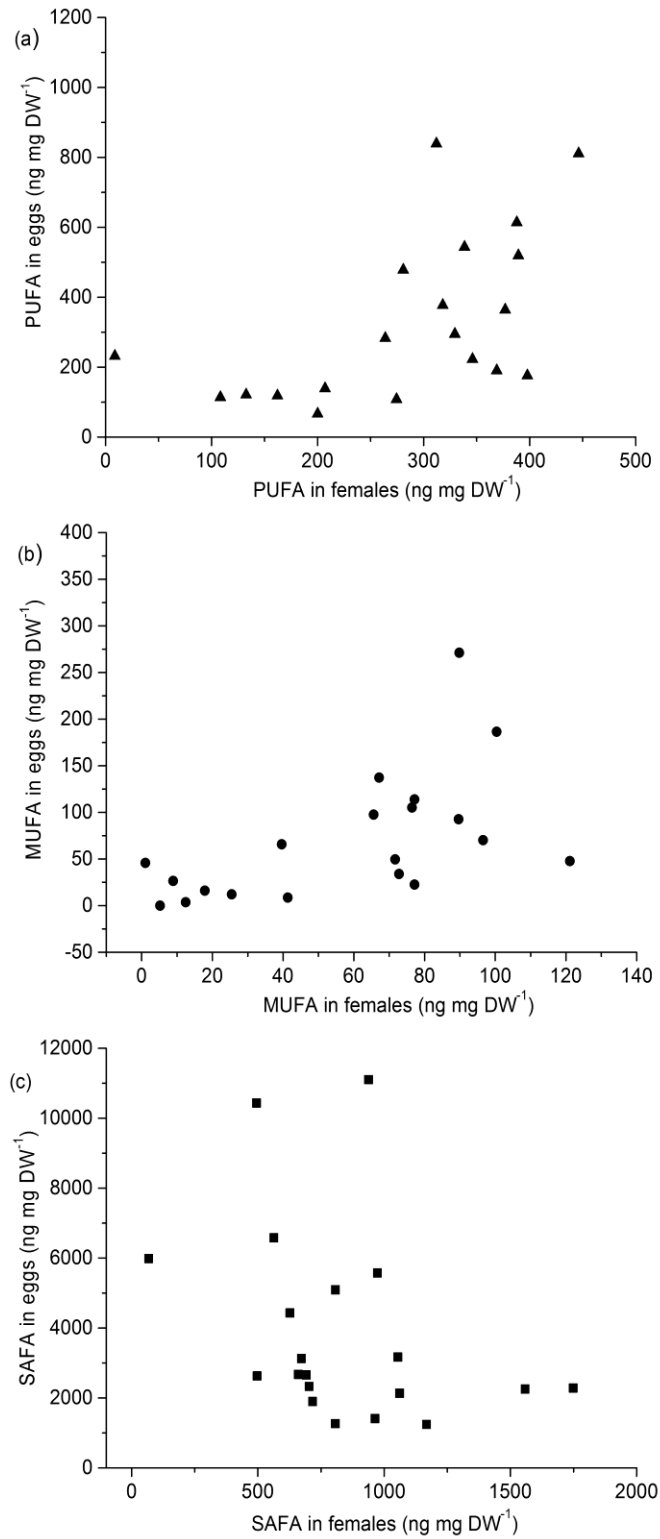
3 Fig. 1. Weekly nauplii production, as averages of 10 females per bottle, for all mesocosms  
4 (treatment target  $fCO_2$  in brackets, as averages of  $t1-t30$ ). Time point 1 is the average weekly  
5 nauplii production  $t3-t7$ , 2 =  $t10-t14$ , 3 =  $t17-t21$ , and 4 =  $t24-t28$ .





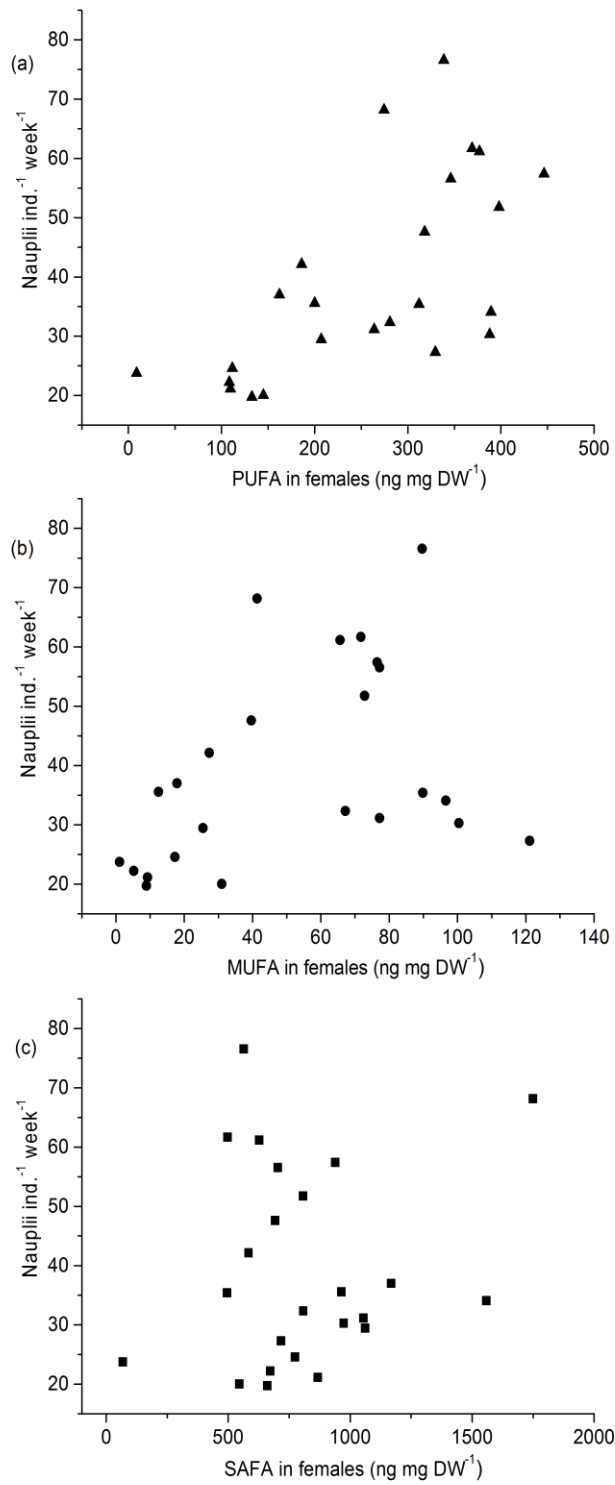
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2 Fig. 2. Daily nauplii production of *E. affinis* as a function of a) Chl *a* concentration, b) diatom  
 3 biomass, and c) dinoflagellate biomass.



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2 Fig. 3. Fatty acids; a) PUFA, b) MUFA and c) SAFA content of females and eggs.

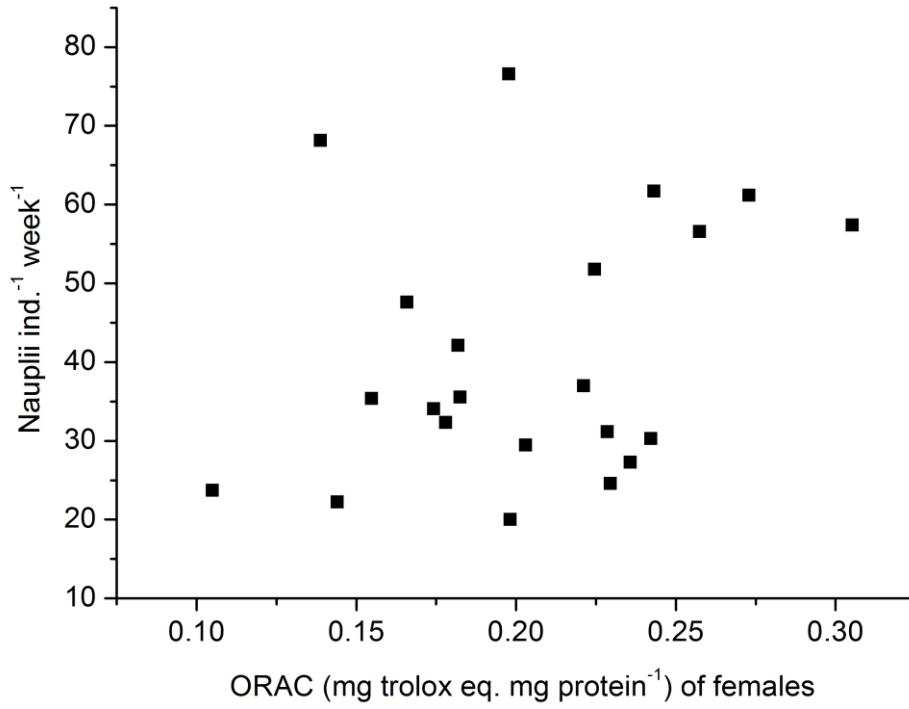


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2 Fig. 4. Relationship between nauplii production and female a) PUFA, b) MUFA and c) SAFA

3 content.

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Fig. 5. Correlation between weekly ORAC of *E. affinis* females and nauplii production (as averages of 10 females).