Aquaculture Reports 6 (2017) 35–42

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Copepod swimming behavior, respiration, and expression of stress-related genes in response to high stocking densities

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ARTICLE INFO

Article history:
Received 16 November 2016
Received in revised form 2 March 2017
Accepted 2 March 2017
Available online 9 March 2017

Keywords:
Copepods
Swimming-behavior
Respiration
Gene-expression
Stress

ABSTRACT

Using copepod nauplii as live feed in aquaculture hatcheries could solve high mortality rates of first-feeding fish larvae due to malnutrition. However, implementing the use of copepod nauplii on an intensive production scale requires a stable production at preferably high densities, which is problematic for calanoid copepod species like *Acartia tonsa*. In the present study, we evaluated the response of copepods experiencing stress under high-density conditions by assessing the acute stress level of *A. tonsa*. Control density was at 100 ind. L^{-1} while the treatments were increased stepwise up to 10,000 ind. L^{-1}. Three biological/physiological end-points were studied: swimming behavior, respiration rate and expression level of stress-related genes.

None of the elevated densities caused any significant change in swimming behavior, respiration rate or gene expression level. This study suggests that adults of *A. tonsa* do not exhibit any measurable acute stress response when exposed to high culture densities for 12 h.

1. Introduction

One major challenge in fish production is the high mortality rates of first-feeding fish larvae caused by malnutrition (reviewed in Hamre et al., 2013). Feeding copepod nauplii to fish larvae have shown to decrease the effect of malnutrition by improving survival, fitness, growth and skin pigmentation compared to traditional live feed like brine shrimps and rotifers (e.g. Øie et al., 2015). These benefits have resulted in a growing interest in implementing copepods as live feed on an intensive scale (Abate et al., 2015; Payne and Rippingale, 2001). In order to support the production of fish larvae, a stable copepod production at preferable high densities is required. For instance, a prototype of an intensive recirculation aquaculture system for production of *Acartia tonsa* is currently present at Roskilde University (Denmark) (described in Abate et al., 2015). Similar cultivation systems are available for other calanoid copepod species (e.g. Carotenuto et al., 2012). High copepod densities are, however, considered a challenge when implementing copepods as a live feed on an intensive scale (Ajiboye et al., 2011; Drillet et al., 2011).

High copepod densities can result in different types of stressors including limited food resources, oxygen depletion, accumulation of metabolic products and the physical interaction with other individuals (Jepsen et al., 2015; Ozaki et al., 2010; Støttrup and Norsker, 1997). The negative effect of the individual and multiple stressors in high-density conditions can explain why copepods are difficult to raise in dense cultures (e.g. Støttrup and McEvoy 2003; Jepsen et al., 2007). How these stressors are inter-related and how they affect each other is not well understood. Nevertheless, an optimal density at which a copepod population has its optimal "output" has been demonstrated as proposed and modeled by Drillet and Lombard (2013) and Drillet et al. (2014a,b).

Culture densities ranging from 50 to 600 mature *A. tonsa* L^{-1} have been reported without having any general negative effects on the copepods (Jepsen et al., 2007; Ogle, 1979; Peck and Holste, 2006; Støttrup et al., 1986). For adult densities ranging from 100–250 ind. L^{-1}, *A. tonsa* have been reported to have an optimal egg production of 25–39 eggs female^{-1} day^{-1} (e.g. Franco et al., 2017). High-density studies of adult *A. tonsa* up to 6000 ind. L^{-1} have been reported with negative responses on adult survival,

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http://dx.doi.org/10.1016/j.aqrep.2017.03.001

fecundity and developmental time as well as cannibalism of eggs and nauplii (e.g. Drillet et al., 2014a,b; Franco et al., 2017). Despite of copepods potential economic value in aquaculture industries, knowledge about their biology and physiology in relation to high densities is, however, limited (Abate et al., 2015; Drillet et al., 2011).

So far, the mechanisms underlying the difficulties of rearing high-density copepod cultures are still unknown. In the present study, we are challenging the perception of adult copepods being stressed at high-density conditions up to 10,000 ind. L^{-1} by examining three physiological end-points: swimming behavior, respiration rate and gene expression analysis of stress-related genes (*ferritin*, *hsp70*, *hsp90*).

We want to examine the following:

- If high-density conditions up to 10,000 ind. L^{-1} are stressful for individuals of *A. tonsa*, will we find a change in swimming behavior compared to lower densities (100 ind. L^{-1})?
- Will there be changes in respiration rates over densities indicating a stress response?
- Will three chosen transcriptional stress biomarkers (*ferritin*, *hsp70*, *hsp90*) exhibit changes in gene expression between high – and low-density conditions?

2. Materials and methods

2.1. Copepod cultures

The culture strain of *A. tonsa* originated from Øresund (N 56°/E 12°; Denmark) where the animals were collected by the National Institute of Aquatic Resources, Danish Technical University (Denmark) in 1981 (identity code: DFH.AT1) (Støttrup et al., 1986). The strain has been cultivated for 36 years under constant salinity, temperature and light conditions (0.2 μm filtered seawater, salinity 30–32 psu, 17 °C, oxygen >60%, dim light). The copepods have been kept at the same conditions for 25 years in 60 L polyethene tanks at Roskilde University (Denmark) and fed the mono-algae, *Rhodomonas salina,* in excess (>800 μg C L^{-1}; Berggreen et al., 1988). *R. salina* was cultivated in 2 L round-bottom flasks diluted daily with Guillard's (F/2) medium (Guillard and Ryther, 1962). Algae-cultivation took place under a stable temperature (17 °C) with constant CO_2 supply and light (PAR ~80 μE m^{-2} s^{-1}).

For the three experiments conducted, a mixture of CIV-CV copepodites and mature individuals of *A. tonsa*, measured under a dissection microscope (Olympus SZ 40, Olympus opticals (Europa) GmBH, Hamburg, Germany) at 40× magnification (prosome length: 780 ± 70 μm/675 ± 85 μm, n = 250, female:male ratio: ~1:1, referred to as adults) from 24 to 26 days old stocks (grown from cold-stored eggs), were being used. The stock densities ranged from ~ 500–1000 ind. L^{-1}. The density of 100 adults L^{-1} was used to represent very low-density conditions, while 5000 and 10,000 ind L^{-1} represented high culture densities. In addition to these densities, we included densities of 1250 ind L^{-1} for the swimming behavior and 500, 1250 and 2500 adults L^{-1} in the respiration experiment to ensure that, despite technical limitations, we could monitor response for lower densities. Incubation periods of 1 h, 8 h and 12 h were applied for all three experiments.

2.2. Swimming behavior

Besides densities of 100, 5000 and 10,000 ind. L^{-1} an additional density of 1250 ind. L^{-1} were used as a low-density treatment. In preliminary studies (not shown here), we found that 100 ind. L^{-1} in some cases is difficult to record since there are not enough animals for capture. To avoid this, we chose 1250 ind. L^{-1} as the lowest possible density at which we could get sufficient recordings of the animals for analysis. After being transferred gently with a 400 μm mesh to triplicate 250 mL tissue-culture flasks, the copepods were left to rest in complete darkness for 15 min in order to calm the convective water movement. Each replicate was recorded at 25 fps by a monochrome USB3 digital camera (model DMK23UM021; The Imaging Source Europe GmbH, Bremen, Germany) after 1 h, 8 h and 12 h. The camera was mounted with a 105 mm Nikkon lens in a setup described in Hansen et al. (2010b). In brief, light was provided by an infrared diode collimated by a Fresnel lens directing the light beams directly into the camera, which gave very high contrast and allowed optimal apparatus setting of the lens. With this setup, the entire depth of the tissue flask was visible. The videos were stored directly on a PC as Quicktime movies, which subsequently were analyzed using the motion analysis software Labtrack 4™ (BioRAS, Kvistgaard, Denmark).

The motion analysis extracted the calibrated vertical and horizontal positions of the copepods from the Quicktime movie while keeping track of the time of multiple copepods simultaneously. The motion analyses were conducted at time steps corresponding roughly to the time step where the copepod had moved at least one body length in the Quicktime movie.

The digitalized motility patterns were subsequently analyzed for the characteristic motility descriptors following Visser and Kiørboe (2006). An idealized swim path is shown on Fig. 1A. Visser and Kiørboe (2006) suggested that the net displacement l traveled by a copepod can be described by the diffusive random walk model of Taylor (1921).

$$l = \sqrt{2v^2\tau\left(t - \tau\left(1 - e^{-t/\tau}\right)\right)} \quad (1)$$

Eq. (1) estimates the characteristic parameters of random walk behavior. The random walk model describes a particle (here a copepod) as moving in an initially ballistic path at the velocity v over time t. As the copepod shift directions over time, the motility becomes more convoluted and diffusive (Fig. 1A). τ is equal to the time point where the motility changes from ballistic to diffusive and displays similar characteristics as the tumble frequency.

The net displacement l (cm) for each time step was determined as the root mean squared (RMS) for each time step for all individuals recorded for each video recording. In an observational system with two dimensions (horizontal and vertical planes), diffusion rate (D) is given by Eq. (2) (Berg, 1983):

$$D = \frac{v^2\tau}{2} \quad (2)$$

Thus Eq. (2) can be substituted into Eq. (1):

$$l = \sqrt{4D\left(t - \tau\left(1 - e^{-t/\tau}\right)\right)} \quad (3)$$

We then fitted the net displacement by time to Eq. (3) for each treatment (See example in Fig. 1B) by non-linear regression (SAS 9.4 ™ NLIN library), to estimate D (diffusion rate) and τ (tumbling frequency, s^{-1}) of the densities of 100, 1250, 5000 and 10,000 copepods L^{-1}.

2.3. Respiration

Individuals of *A. tonsa* were initially incubated at densities 100, 5000 and 10,000 ind. L^{-1}, but since the average O_2 decline at the lowest density of 100 ind. L^{-1} was not statistically significant ($t = -2.47$, $df = 5$, $p > 0.05$), we repeated the setup with additional densities of 500, 1250, and 2500 ind. L^{-1} to ensure that significant O_2 declines could also be measured at low-density conditions. The copepods were kept in 25 mL gas-tight glass bottles containing seawater (0.2 μm filtered, 30–32 psu, air-equilibrated). Five

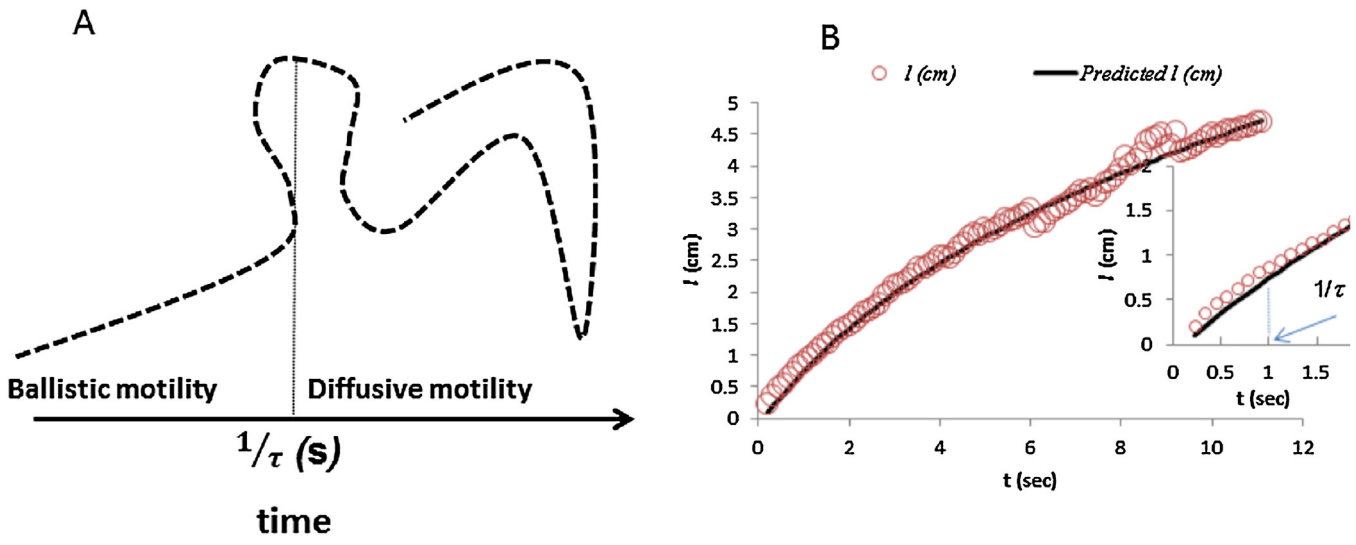

Fig. 1. Panel A shows the conceptual swimming path of *Acartia tonsa*. The animal is initially moving ballistically in a straight path (left hand side of figure). As time passes (marked by arrow shaped x-axis), the behavior changes from ballistic to diffusive. The dotted vertical line denote the time (1/τ), where the *A. tonsa* jumps, and the swimming path becomes convoluted, and the motility changes to diffusive (right hand side of the dotted line on Fig. 1A). Panel B show an example of a treatment where *l* (O) is calculated and fitted to Eq. (3) (full black curve line). The small insert shows where the statistical software (SAS 9.4) estimated the transition from ballistic to diffusive.

replicate bottles were prepared for each animal density and for control incubations without animals. The bottles were mounted on a plankton wheel that was submerged in a temperature-controlled water bath (17 ± 0.5 °C) and rotated at 10 rpm. Oxygen concentration was monitored every 60–120 min using O_2-sensitive planar optodes (SensorSpot, Pyroscience, Germany) glued to the inside of the bottles and interrogated with an optical O_2 meter (FireSting, Pyroscience, Germany). Before and after the experiment, each SensorSpot was individually calibrated in seawater (30–32 psu, temperature 17 °C) at O_2 concentrations corresponding to 100 and 0% air saturation (i.e., 251.5 and 0 μmol O_2 L^{-1}). Individual-specific respiration rates were calculated from the linear decrease in O_2 concentration during the first 6–8.5 h of the experimental incubation, corrected for the minute changes in O_2 concentration observed in the control bottles. Biomass-specific respiration rates were calculated from individual-specific respiration rates by using the relationship between prosome length *L* (μm) and organic carbon content *B* (ng C) in *A. tonsa* determined by Berggreen et al. (1988): $B = 1.11 \times 10^{-5} L^{2.92}$.

2.4. Gene expression

Quadruplicate flasks with densities of 100, 5000 and 10,000 ind. L^{-1} were prepared for each of the following periods: 1, 8 and 12 h of incubation. From each incubation flask, 25 copepods were transferred to 1 mL RNAlater® (Sigma–Aldrich, USA). This resulted in 4 biological replicates for each treatment and incubation period, consisting each of 25 animals. The samples were stored at −80 °C until RNA extraction.

RNA extraction was performed with the RNeasy Mini kit (Qiagen, Germany). The frozen samples were thawed on ice and excess RNAlater® (Sigma–Aldrich, USA) was removed. The animals were homogenized using a disposable micro-pestle in lysis-buffer from the RNeasy Mini kit (Qiagen, Germany). The following RNA extraction and purification were performed according to the manufacturer's protocol. The RNA samples were treated with DNAse I (Fermentas, USA) to remove genomic DNA.

RNA concentrations and purity were measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., USA). RNA integrity was checked, on a denaturing 1% agarose gel stained with ethidium bromide. An aliquot of 100 ng of total RNA from each sample was reverse transcribed to complementary first-strand DNA (cDNA) with TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, USA) using oligo$(dT)_{16}$. The reverse transcription polymerase chain reactions (PCR) were performed as described in the manufacturer's protocol.

Real-time quantitative PCR was done in 12.5 μL reactions with 6.25 μL Brilliant® II Master Mix (Sigma-Aldrich, USA), 0.5 μL 1.0 mM forward primer, 0.5 μL 1.0 mM reverse primer, 2.75 μL of RNase/DNase free water and 2.25 μL of the first strand cDNA template. Primers for *ferritin*, *hsp70* and *β-actin* were used as described in Nilsson et al. (2013) and primers for *hsp90* and *efa-1α* as described in Petkeviciute et al. (2015). The reactions were run on Stratagene Mx3005P (AH Diagnostics, Aarhus V, Denmark) real-time thermal 40 cycler as follows: 95 °C/15 min, 40 cycles [95 °C/30 s], 60 °C/30 s. Each of the biological replicates was run as technical triplicates during the analysis. PCR amplification efficiencies were 95–97%.

Gene expression levels were normalized by using *β-actin* as reference gene and the $2^{-\Delta\Delta Ct}$ method to estimate relative mRNA levels (Livak and Schmittgen, 2001). Two reference genes, *β-actin,* and *EFA-1a*, were initially used but only β-actin exhibited stable expression during the course of the experiment and was therefore chosen for normalization.

2.5. Statistical analysis

The estimated diffusion rates and tumbling frequencies were compared by density by an unbalanced ANOVA using a GLM model (SAS™ software GLM library), to determine whether diffusion rate or tumbling frequency were significantly different between density and time. We used density (treatment) and time as class variable in the statistical model. Time was used as a class variable in the model because we have no reason to hypothesize any linear response in the tested variable to time. The triplicate observations on the 100 ind. L^{-1} were pooled due to very few observations at the low concentration. To that end, we were unable to record any animals in the 100 ind. L^{-1} treatments. We did not do any time step recordings at 1250 ind. L^{-1}. We used an unbalanced ANOVA, to circumvent the different number of replicates and missing time steps among treatments. The ANOVA was followed by a pairwise post hoc test

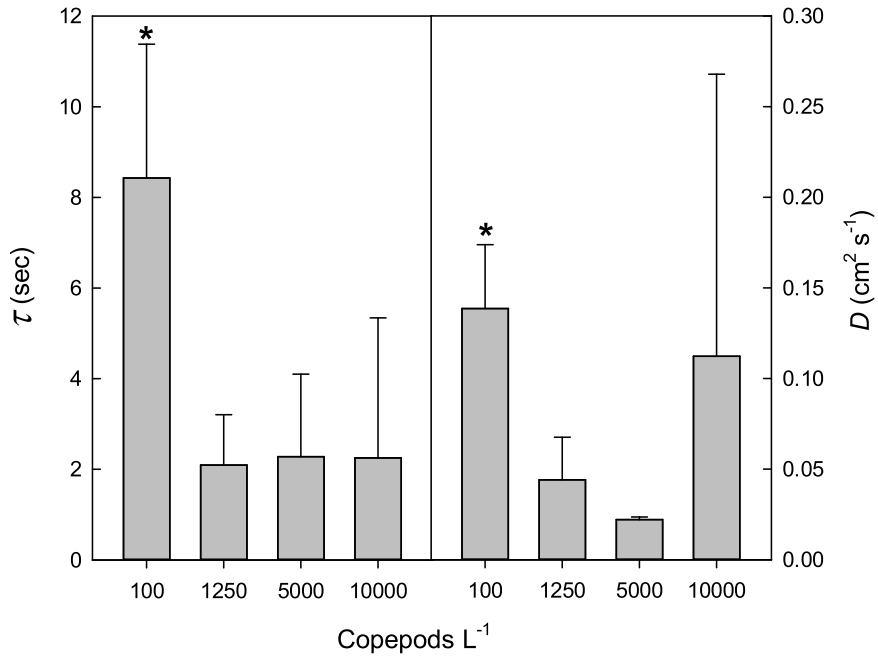

Fig. 2. The characteristic swimming path parameters by the calanoid copepod *Acartia tonsa* (tumbling frequency, τ and the diffusion rate, *D*) derived by equation 3 after one-hour incubation for the four treatments. Error bars are the standard deviation and statistical difference is marked by an asterisk (*).

Table 1
Fit parameters of equation 3 for calculating the net displacement, *l* (cm): $l = \sqrt{4D\left(t - \tau\left(1 - e^{-t/\tau}\right)\right)}$ whereas *D*, is the diffusion rate ($cm^2\ s.^{-1}$), t is the time (s) and τ is tumbling frequency (s^{-1}). Data are fit values ± standard deviations obtained by the non-linear fitting of equation 3 for each of the three replicates for each density and incubation time. Note that due to the low number of observations in the 100 *Acartia tonsa* copepods L^{-1}, the standard deviation has been replaced by standard errors derived from the non-linear fit (marked by an *).

Copepods L^{-1}	time (h)	D ($cm^2\ s^{-1}$)	τ (s)
100	1	0.140 ± 0.035*	8.43 ± 2.95*
100	8	0.049 ± 0.002	5.19 ± 0.42
1250	1	0.044 ± 0.023	2.09 ± 1.11
5000	1	0.022 ± 0.001	2.28 ± 1.82
5000	8	0.017 ± 0.001	1.76 ± 1.44
5000	12	0.027 ± 0.001	1.95 ± 0.61
10000	1	0.110 ± 0.155	2.25 ± 3.10
10000	8	0.024 ± 0.002	1.36 ± 0.24
10000	12	0.019 ± 0.005	0.73 ± 0.45

(Tukey's test) to identify statistical differences among concentrations.

A one-way ANOVA was used to test for significant differences in respiration among the densities.

Since the number of biological replicates (N = 4) was limited in the gene expression analysis, the distribution of the data could not be estimated. Furthermore, low replicate numbers usually result in that the variance among groups that are not well represented. Therefore a multiple *t*-test without assuming equal standard deviation was used to determine if treatments were significantly different with Holm-Šidák step down comparisons (alpha of 5%) (Ludbrook, 1998).

3. Results

3.1. Swimming behavior

Table 1 represents the parameters of equation 3 containing the densities (100, 1250, 5000 and 10,000 ind. L^{-1}), the incubation times (1, 8 and 12 h), the estimated diffusion rates, D, and tumbling frequencies, τ. The diffusion rate is in general terms the volume that the copepods "explore" per second. The apparent decrease in diffusion rate with increasing densities means that the copepods appear to cover a smaller volume of water. The tumbling frequency (τ.) also appears to decrease over time, meaning that the copepods jump more over time at all densities. The ANOVA followed by the Tukey test showed that the diffusion rate and the tumbling frequency of 100 copepods L^{-1} were significantly different ($p < 0.05$) than for the densities of 1250, 5000 and 10,000 copepods L^{-1} after one hour (Fig. 1B, Table 1 and Fig. 2). The three other treatments, excluding the 100 copepods L^{-1}, of 1200, 5000 and 10,000 copepods L^{-1}, however, showed no statistical significant difference in diffusion rate over time (mean ± SD: 0.038 $cm^2\ s^{-1}$ ± 0.027 $cm^2\ s^{-1}$) or in tumbling frequency (mean ± SD: 1.78 s^{-1} ± 1.25 s^{-1}, Table 1).

3.2. Respiration

Biomass-specific respiration rates (nmol O_2 μmol $C^{-1}\ h^{-1}$) versus densities (500, 1250, 2500, 5000 and 10,000 ind. L^{-1}) are shown in Fig. 3. The average respiration rate appears to slightly increase with increasing copepod density, which is then followed by a slight decrease at even higher densities. One-way ANOVA revealed, however, that the respiration rate did not vary significantly with copepod density (df = 4.20; F = 1.066; p = 0.40).

3.3. Gene expression

Relative mRNA levels for copepod densities of 100, 5000 and 10,000 ind. L^{-1} at 1, 8 and 12 h of incubation are given in Fig. 4. Comparison of the relative gene expression levels of *ferritin*, *hsp70* and *hsp90* between densities of 100 ind. L^{-1} did not vary over time (Holm-Šidák method, $p > 0.05$). These densities represent low culture density and were therefore used as controls for statistical comparisons. None of the higher densities exhibited any significant changes in relative mRNA levels of *ferritin*, *hsp70* or *hsp90* for any of the incubation times (Holm-Šidák method, $p > 0.05$, Fig. 4).

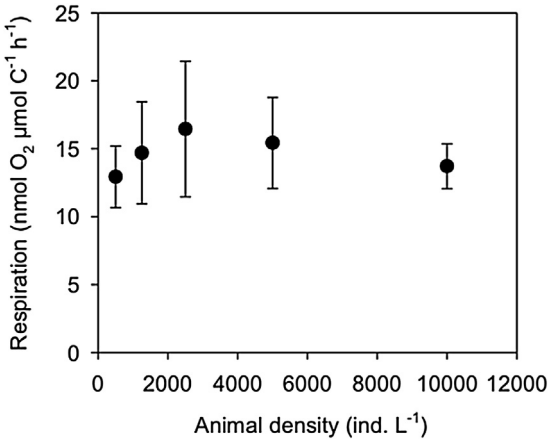

Fig. 3. Biomass-specific respiration of the calanoid copepod *Acartia tonsa* plotted against density (ind. L^{-1}). There was no significant change in respiration rate over densities (one-way ANOVA, df = 4.20; F = 1.066; p = 0.40). Error bars represents standard deviation (n = 5).

4. Discussion

Calanoid copepods, including *A. tonsa*, are suspension feeders and require large volumes of water. Maximum culture densities of *A. tonsa* usually range from 100 to 2,000 ind. L^{-1} (Støttrup, 2006 and references herin). Actual culture-wide densities can, however, be difficult to estimate and only few studies are available on *A. tonsa* densities from eggs to adults (e.g. Franco et al., 2017).

We found that individuals of *A. tonsa* did not exhibit stress response at the behavioral, respiratory or at gene expression level in relation to high-density conditions (10,000 ind. L^{-1}) during 12 h. Furthermore, mortality at densities of up to 20,000 ind. L^{-1} has been shown to be below 5% at incubation times of 48 h for the same copepod stocks as used in the present study (personal communication, Mads Kærhus Olufsen, Roskilde University). This corresponds to other studies with constant daily mortality rates of 5 – 9.75% for densities up to 5000 ind. L^{-1} (Drillet et al., 2014b; Medina and Barata, 2004; Støttrup et al., 1986). A recent study by Franco et al. (2017), using the same strain of *A. tonsa* as in the present study, did however observe a decrease in survival (2-fold) for densities above 1000 ind. L^{-1}.

The >30 year old copepod strain (Støttrup et al., 1986) is well described in the literature. It is an advantage that the same culture baseline applies for many studies. Furthermore, copepods for use as live feed in aquaculture hatcheries would probably end up being cultivated for multiple generations. When starting copepod cultures, high densities are obtained the first few days followed by a decline and stabilization at a lower level (Støttrup et al., 1986; and references herein). So far, there are no clear answers on why *A. tonsa* is difficult to rear at high densities long-term. The present study focused on acute stress responses up to 12 h as a result of high-density conditions and did not investigate the long term responses.

Planktonic organisms cover a wider behavioral spectrum. *A. tonsa* "drift" motionless while searching for food. The drifting is frequently interrupted by swift darts-like motion, where the copepod reorients itself followed by resumed drifting (e.g. Tiselius and Jonsson, 1990). This "run-tumble" behavior was mathematically described by following a non-linear diffusive function by Visser and Kiørboe (2006). At a limited time scale, the drifting motility of the copepod seems linear – and the distance moved over time will increase linearly. This behavior is termed ballistic swimming (Visser and Kiørboe, 2006). Over time, as the copepod reorients itself (tumbles), the swimming path appears increasing more convoluted as the copepod change swimming direction.

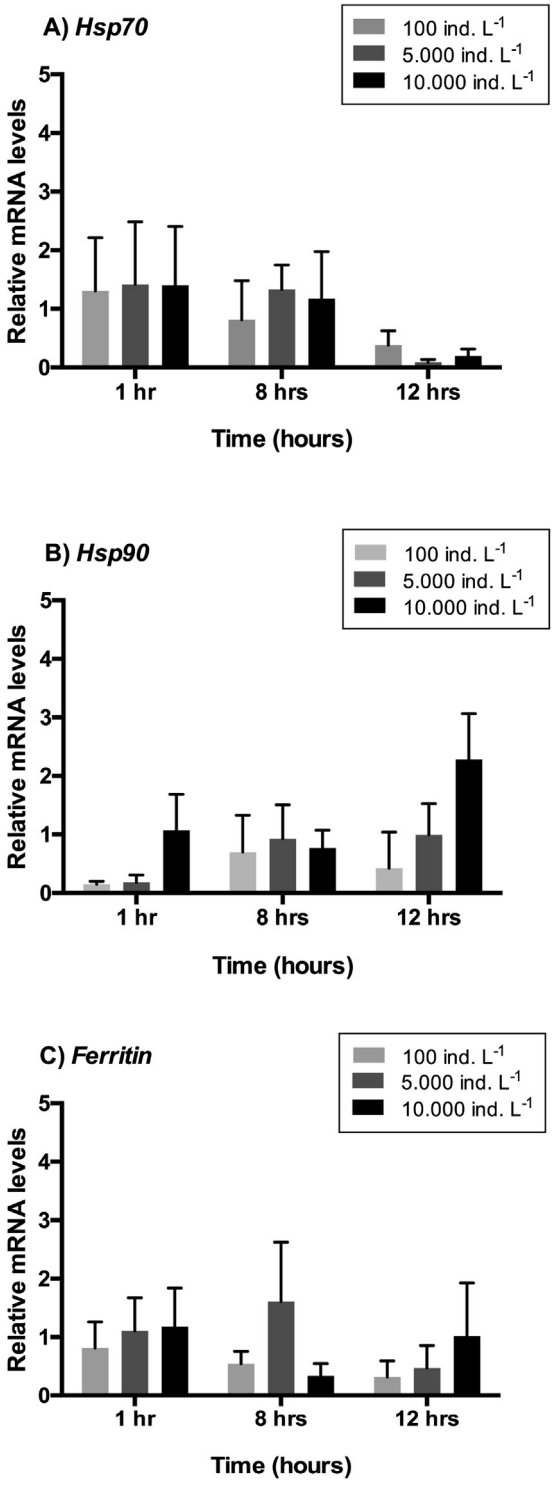

Fig. 4. Real-time quantitative PCR analysis of *hsp70*, *hsp90* and *ferritin* of the calanoid copepod *Acartia tonsa*. Densities of 100 (control, light grey), 5000 (grey) and 10,000 (black) ind. L^{-1} were used to examine gene expression levels of *ferritin* (A), *hsp70* (B) and *hsp90* (C). For each of the three densities, 4 biological replicates were collected after 1, 8 and 12 h of incubation (3 densities, 3 incubation-periods, 4 replicates = 36 samples in total). Each replicate consisted of 25 copepods. For real-time quantitative PCR, each biological replicate was performed with 3 technical replicates. Expression levels were normalized by *β- actin*. Error bars represent standard deviation (N = 4). None of the genes exhibited statistically significant changes in relative mRNA levels (Holm-Šidák, p > 0.05).

This swimming is termed diffusive (Berg and Brown, 1972). The swimming behavior of copepods exposed to various types of environmental stress is not well studied, and there are to our knowledge no reports in the literature on how the balance between ballistic and diffuse swimming is affected by stress.

We examined whether or not adults of *A. tonsa* exhibited changes in swimming behavior up to 10,000 ind. L^{-1}. We expected that the copepods at higher densities would bump into each other and thereby generate more convoluted swimming paths with increased diffusion rate and tumbling frequency. We did, however, not find the expected changes in swimming behavior except for the significantly different 100 ind. L^{-1} treatment. Here, all the copepods behaved within literature values from experiments conducted at much lower copepod density in terms of diffusion rates D (0.038 versus 0.036) and tumbling frequencies τ (1.78 versus 1.7) (Visser and Kiørboe, 2006).

Respiration rate is an integrative indicator of the overall metabolic activity, including motion, feeding and basal requirements (Kiørboe et al., 1985). Organisms have the ability to respond to stress, by increasing their energy demand, which can be reflected by an increased respiration rate. Since the high-densities we are using are much higher than the previously described *A. tonsa* densities (up to 6000 ind. L^{-1}), we expected several fold higher respiration rates in comparison to the low-density conditions used in the present study (e.g. Drillet et al., 2014b; Franco et al., 2017). However, biomass-specific respiration rates did not vary significantly with copepod density (Fig. 3).

The available studies regarding respiration rates in *A. tonsa* are primarily focusing on simultaneous feeding, swimming, fecundity and egg production aspects (e.g. Kiørboe, 2008; Kiørboe et al., 1985). Studies exploring the effect of stress on respiration rates of copepods are limited. Thor (2003) found that the respiration rate of *A. tonsa* (females) were reduced ~ 60% during starvation stress (8.5 nmol O_2 μmol C^{-1} h^{-1},17 °C) compared to non-starved copepods (of 20.0 nmol O_2 μmol C^{-1} h^{-1}, 17 °C). When food was reintroduced, the respiration rate increased within 8 h by ~40% compared to non-starved animals (32 nmol O_2 μmol C^{-1} h^{-1}, 17 °C) (Thor, 2003). The marine isopod, *Idotea balthica,* responded to an abrupt salinity decrease (30–10 psu) with an elevated respiration rate (~40% increase) within the first four hours after exposure. The elevated rate was sustained for 20 h, where after it decreased to the same level as prior the exposure after 30 h (Bulnheim, 1974). Since different stressors were shown to result in changes in respiration for *A. tonsa* and other crustaceans, we expected that the same would apply for density-related stress. Bulnheim (1974), and others, have shown that changes in respiration by crustaceans will occur within minutes to a few hours after stress exposure (e.g. Schapker et al., 2002; Tedengren et al., 1988). This suggests that the 12 h of incubation applied in the present study is considered sufficient to monitor changes in respiration.

Behavior and respiration are usually interrelated. If the behavior were changing toward more movements caused by high density, this would result in increased activity level and thereby be reflected in elevated respiration. Since there were no significant changes in either of the two physiological end-points, analysis of gene expression is a parameter that could give a more direct determination on whether the animals experience stress or not. The expression of stress related genes would change before the stress manifests as end-points like survival, fecundity and growth rate.

Dynamic transcription of genes allows rapid adaptation to external, environmental or physiological changes that affect the organisms. Gene expression analysis of selected genes can be used to monitor the physiological state under stressful conditions and give an early detection of when a stressor is affecting the organism in a negative manner. Several studies, using transcriptional biomarkers for stress, are available for copepods (reviewed in Lauritano et al., 2012). Genes encoding for chaperone molecules are usually up-regulated during stress conditions in order to preserve macromolecules in cells, and the gene expression patterns of chaperone genes could provide valuable information on the underlying mechanisms of copepod stress (reviewed in Lauritano et al., 2012). The genes for heat shock proteins, *hsp70* and *hsp90*, are widely up-regulated in response to a wide range of stressors and represent commonly used transcriptional stress biomarkers in copepods (e.g. Petkeviciute et al., 2015 and references herein). Ferritin is an iron-storage protein that protects macromolecules from damage by reactive oxygen species (Arosio and Levi, 2002; Hintze and Theil, 2006). Up-regulation of *ferritin* has been observed in response to nickel exposure, xenobiotics, and the resting states quiescence and diapause in copepods (Hansen et al., 2010a; Jiang et al., 2013; Nilsson et al., 2013; Tarrant et al., 2008). If adult individuals of *A. tonsa* experienced high-density conditions as being stressful, we expected a change in gene expression for the three chosen transcriptional biomarkers.

Comparison of the relative gene expression levels of *ferritin*, *hsp70* and *hsp90* between densities of 100 ind. L^{-1} did not vary over time (Holm-Šidák step down comparisons, $p > 0.05$). These densities represent low culture density and were therefore used as controls for statistical comparisons. None of the higher densities exhibited any significant changes in relative mRNA levels of *ferritin*, *hsp70* or *hsp90* for any of the incubation times ($p > 0.05$, Fig. 4). Compared to the positive stress responses for *A. tonsa* described in Petkeviciute et al. (2015) and Rahlff et al. (2017), with a fold increase of 63.8 and 185 in *hsp70* at heat shocks up to 30 °C, the observed fold changes in the present study (ranging from 0.028 to 3.074) cannot be considered a true stress response. Changes in gene expression of the heat shock proteins (*hsp70* and *hsp90*) as well as *ferritin* have been reported to happen within an hour to a few hours after exposure to different stressors (e.g. Duan et al., 2016; Petkeviciute et al., 2015). The incubation of up to 12 h is therefore sufficient to see a response on gene expression level.

Treatments at densities of 10,000 ind. L^{-1} during up to 12 h seems not to have a negative effect on adults of *A. tonsa* or induce a stress response that can be detected by behavior, respiration and gene expression. Long-term (>12 h) incubations at densities up to 6000 ind. L^{-1} have been shown to affect egg production and growth (Drillet et al., 2014b; Franco et al., 2017; Jepsen et al., 2007; Medina and Barata, 2004). The difficulties in rearing *A. tonsa* at high-densities might be caused by longer-term factors like oxygen and food depletion as well as accumulation of metabolic products (Jepsen et al., 2015; Ozaki et al., 2010; Støttrup and Norsker, 1997). High densities have, furthermore, been reported to increase egg cannibalism (Ban and Minoda 1994; Camus and Zeng 2009; Drillet et al., 2014a,b). However, *A. tonsa* egg cannibalism has been shown recently to be of minor importance when at high densities. In fact, just a few eggs ingested per copepod every 24 h was demonstrated when excess micro-algal food was present (personal communication, Minh Vu Thi Thuy, Roskilde University).

Other studies concerning the high-density effect on *A. tonsa* have focus on end-points like mortality, growth-rate, and egg production (Drillet et al., 2014b; Franco et al., 2017; Jepsen et al., 2007; Medina and Barata, 2004). The present study was planned to focus on somewhat alternative end-points (behavior, respiration, gene-expression) to act as a relatively fast and comprehensive set of parameters to discover the underlying causes for why *A. tonsa* is difficult to rear in high densities.

5. Conclusion

We found that adult individuals of the calanoid copepod, *A. tonsa*, after >30 years in culture do not exhibit signs on any stress

response at densities of up to 10,000 ind. L^{-1} when assessing swimming behavior, respiration rate, gene expression or mortality as physiological end points within a 12 h time frame. Limitations in copepod densities must be due to other parameters than acute stress.

Acknowledgements

We would like to thank Egle Petkeviciute, Mouloud Rais, Stéphane Gasparini and Fabien Lombard for helping with the preliminary studies. We would also like to thank Thomas A. Rayner for proofreading the manuscript. The Villum foundation project # VKR022608, supported HHJ financially the procurements of equipment used in the video recordings. This work was supported by the Villum Foundation; Project AMPHICOP no. 8960, to B.W.H.

References

Abate, T.G., Nielsen, R., Nielsen, M., Drillet, G., Jepsen, P.M., Hansen, B.W., 2015. Economic feasibility of copepod production for commercial use: result from a prototype production facility. Aquaculture 436, 72–79, http://dx.doi.org/10.1016/j.aquaculture.2014.10.012.

Ajiboye, O.O., Yakubu, A.F., Adams, T.E., Olaji, E.D., Nwogu, N.A., 2011. A review of the use of copepods in marine fish larviculture. Rev. Fish Biol. Fish. 21, 225–246, http://dx.doi.org/10.1007/s11160-010-9169-3.

Arosio, P., Levi, S., 2002. Ferritin, iron homeostasis, and oxidative damage. Free Radic. Biol. Med. 33, 457–463, http://dx.doi.org/10.1016/S0891-5849(02)00842-0.

Ban, S., Minoda, T., 1994. Induction of diapause egg production in Eurytemora affinis by their own metabolites. Hydrobiologia 292/293, 185–189, http://dx.doi.org/10.1007/bf00229940.

Berg, H.C., Brown, D.A., 1972. Chemotaxis in *Escherichia coli* analysed by three-dimensional tracking. Nature 239, 500–504, http://dx.doi.org/10.1038/239500a0.

Berggreen, U., Hansen, B., Kiørboe, T., 1988. Food size spectra, ingestion and growth of the copepod *Acartia tonsa* during development: implications for determination of copepod production. Mar. Biol. 99, 341–352, http://dx.doi.org/10.1007/BF02112126.

Berg, H.C., 1983. Random Walks in Biology. In: Burdige, D.J. (Ed.), Geochemistry of Marine Sediments. Princeton University Press, p. 630.

Bulnheim, H.P., 1974. Respiratory metabolism of *Idotea balthica* (Crustacea, Isopoda) in relation to environmental variables, acclimation processes and moulting. Helgoländer Wissenschaftliche Meeresuntersuchungen 26, 464–480, http://dx.doi.org/10.1007/BF01627627.

Camus, T., Zeng, C., 2009. The effects of stocking density on egg production and hatching success, cannibalism rate, sex ratio and population growth of the tropical calanoid copepod *Acartia sinjiensis*. Aquaculture 287, 145–151, http://dx.doi.org/10.1016/j.aquaculture.2008.10.005.

Carotenuto, Y., Esposito, F., Pisano, F., Lauritano, C., Perna, M., Miralto, A., Ianora, A., 2012. Multi-generation cultivation of the copepod *Calanus helgolandicus* in a re-circulating system. J. Exp. Mar. Bio. Ecol. 418–419, 46–58, http://dx.doi.org/10.1016/j.jembe.2012.03.014.

Drillet, G., Lombard, F., 2013. A first step towards improving copepod cultivation using modelling: the effects of density, crowding, cannibalism, tank design and strain selection on copepod egg production yields. Aquac. Res. 1–10, http://dx.doi.org/10.1111/are.12317.

Drillet, G., Frouël, S., Sichlau, M.H., Jepsen, P.M., Højgaard, J.K., Joardeer, A.K., Hansen, B.W., 2011. Status and recommendations on marine copepod cultivation for use as live feed. Aquaculture 315, 155–166, http://dx.doi.org/10.1016/j.aquaculture.2011.02.027.

Drillet, G., Maguet, R., Mahjoub, M.-S., Roullier, F., Fielding, M.J., 2014a. Egg cannibalism in *Acartia tonsa*: effects of stocking density, algal concentration, and egg availability. Aquac. Int. 22, 1295–1306, http://dx.doi.org/10.1007/s10499-013-9747-1.

Drillet, G., Rais, M., Novac, A., Jepsen, P.M., Mahjoub, M.-S., Hansen, B.W., 2014b. Total egg harvest by the calanoid copepod *Acartia tonsa* (Dana) in intensive culture—effects of high stocking densities on daily egg harvest and egg quality. Aquac. Res. 46, 3028–3039, http://dx.doi.org/10.1111/are.12459.

Duan, Y., Zhang, Y., Dong, H., Zhang, J., 2016. Effect of desiccation on oxidative stress and antioxidant response of the black tiger shrimp Penaeus monodon. Fish Shellfish Immunol. 58, 10–17, http://dx.doi.org/10.1016/j.fsi.2016.09.004.

Franco, S.C., Augustin, C.B., Geffen, A.J., Dinis, M.T., 2017. Growth, egg production and hatching success of *Acartia tonsa* cultured at high densities. Aquaculture 468, 569–578, http://dx.doi.org/10.1016/j.aquaculture.2016.10.044.

Guillard, R.R., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt, and Detonula confervacea (cleve) Gran. Can. J. Microbiol. 8, 229–239, http://dx.doi.org/10.1139/m62-029.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquac. 5, S26–S58, http://dx.doi.org/10.1111/j.1753-5131.2012.01086.x.

Hansen, B.H., Altin, D., Booth, A., Vang, S.H., Frenzel, M., Sørheim, K.R., Brakstad, O.G., Størseth, T.R., 2010a. Molecular effects of diethanolamine exposure on *Calanus finmarchicus* (Crustacea: Copepoda). Aquat. Toxicol. 99, 212–222, http://dx.doi.org/10.1016/j.aquatox.2010.04.018.

Hansen, B.W., Jakobsen, H.H., Andersen, A., Almeda, R., Pedersen, T.M., Christensen, A.M., Nilsson, B., 2010b. Swimming behavior and prey retention of the polychaete larvae *Polydora ciliata* (Johnston). J. Exp. Biol. 213, 3237–3246, http://dx.doi.org/10.1242/jeb.038810.

Hintze, K.J., Theil, E.C., 2006. Cellular regulation and molecular interactions of the ferritins. Cell. Mol. Life Sci. 63, 591–600, http://dx.doi.org/10.1007/s00018-005-5285-y.

Jepsen, P.M., Andersen, N., Holm, T., Jørgensen, A.T., Højgaard, J.K., Hansen, B.W., 2007. Effects of adult stocking density on egg production and viability in cultures of the calanoid copepod *Acartia tonsa* (Dana). Aquac. Res. 38, 764–772, http://dx.doi.org/10.1111/j.1365-2109.2007.01730.x.

Jepsen, P.M., Andersen, C.V.B., Schjelde, J., Hansen, B.W., 2015. Tolerance of un-ionized ammonia in live feed cultures of the calanoid copepod *Acartia tonsa* Dana. Aquac. Res. 46, 420–431, http://dx.doi.org/10.1111/are.12190.

Jiang, J.L., Wang, G.Z., Mao, M.G., Wang, K.J., Li, S.J., Zeng, C.S., 2013. Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to nickel exposure. Comp. Biochem. Physiol. – C Toxicol. Pharmacol. 157, 203–211, http://dx.doi.org/10.1016/j.cbpc.2012.11.001.

Kiørboe, T., Møhlenberg, F., Hamburger, K., 1985. Bioenergetics of the planktonic copepod Acartia tonsa: relation between feeding, egg production and respiration, and composition of specific dynamic action. Mar. Ecol. Prog. Ser. 26, 85–97, http://dx.doi.org/10.3354/meps026085.

Kiørboe, T., 2008. Optimal swimming strategies in mate-searching pelagic copepods. Oecologia 155, 179–192, http://dx.doi.org/10.1007/s00442-007-0893-x.

Lauritano, C., Procaccini, G., Ianora, A., 2012. Gene expression patterns and stress response in marine copepods. Mar. Environ. Res. 76, 22–31, http://dx.doi.org/10.1016/j.marenvres.2011.09.015.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. Methods 25, 402–408, http://dx.doi.org/10.1006/meth.2001.1262.

Ludbrook, J., 1998. Multiple comparison procedures updated. Clin. Exp. Pharmacol. Physiol. 25, 1032–1037, http://dx.doi.org/10.1111/j.1440-1681.1998. tb02179.x.

Medina, M., Barata, C., 2004. Static-renewal culture of *Acartia tonsa* (Copepoda: Calanoida) for ecotoxicological testing. Aquaculture 229, 203–213, http://dx.doi.org/10.1016/S0044-8486(03)00389-2.

Nilsson, B., Jepsen, P.M., Rewitz, K., Hansen, B.W., 2013. Expression of *hsp70* and *ferritin* in embryos of the copepod *Acartia tonsa* (Dana) during transition between subitaneous and quiescent state. J. Plankton Res. 36, 513–522, http://dx.doi.org/10.1093/plankt/fbt099.

Ogle, J., 1979. Adaptation of a brown water culture technique to the mass culture of the copepod *Acartia tonsa*. Gulf Res. Rep. 6, 291–292, http://dx.doi.org/10.18785/grr.0603.10.

Ozaki, Y., Kaneko, G., Yanagawa, Y., Watabe, S., 2010. Calorie restriction in the rotifer *Brachionus plicatilis* enhances hypoxia tolerance in association with the increased mRNA levels of glycolytic enzymes. Hydrobiologia 649, 267–277, http://dx.doi.org/10.1007/s10750-010-0269-9.

Payne, M.F., Rippingale, R.J., 2001. Intensive cultivation of the calanoid copepod *Gladioferens imparipes*. Aquaculture 201, 329–342, http://dx.doi.org/10.1016/S0044-8486(01)00608-1.

Peck, M.A., Holste, L., 2006. Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in Acartia tonsa (Calanoida: Copepoda): optimizing intensive cultures. Aquaculture 255, 341–350, http://dx.doi.org/10.1016/j.aquaculture.2005.11.055.

Petkeviciute, E., Kania, P.W., Skovgaard, A., 2015. Genetic responses of the marine copepod *Acartia tonsa* (Dana) to heat shock and epibiont infestation. Aquac. Rep. 2, 10–16, http://dx.doi.org/10.1016/j.aqrep.2015.04.001.

Rahlff, J., Peters, J., Moyano, M., Pless, O., Claussen, C., Peck, M.A., 2017. Short-term molecular and physiological responses to heat stress in neritic copepods *Acartia tonsa* and *Eurytemora affinis*. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 203, 348–358, http://dx.doi.org/10.1016/j.cbpa.2016.11.001.

Schapker, H., Breithaupt, T., Shuranova, Z., Burmistrov, Y., Cooper, R.L., 2002. Heart and ventilatory measures in crayfish during environmental disturbances and social interactions. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 131, 397–407, http://dx.doi.org/10.1016/S1095-6433(01)00492-5.

Støttrup, J.G., McEvoy, L., 2003. Live Feeds in Marine Aquaculture, 1st ed. Blackwell Science.

Støttrup, J.G., Norsker, N.H., 1997. Production and use of copepods in marine fish larviculture. Aquaculture 155, 231–247, http://dx.doi.org/10.1016/S0044-8486(97)00120-8.

Støttrup, J.G., Richardson, K., Kirkegaard, E., Pihl, N.J., 1986. The cultivation of *Acartia tonsa* Dana for use as a live food source for marine fish larvae. Aquaculture 52, 87–96, http://dx.doi.org/10.1016/0044-8486(86)90028-1.

Støttrup, J.G., 2006. A review on the status and progress in rearing copepods for marine larviculture. Advantages and disadvantages. Among Calanoid, Harpacticoid and Cyclopoid copepods. Av. Nutr. Acuícola VIII, 62–83.

Tarrant, A.M., Baumgartner, M.F., Verslycke, T., Johnson, C.L., 2008. Differential gene expression in diapausing and active *Calanus finmarchicus* (Copepoda). Mar. Ecol. Prog. Ser. 355, 193–207, http://dx.doi.org/10.3354/meps07207.

Taylor, G.I., 1921. Diffusion by continuous movements. Proc. Lond. Math. Soc. 2–20, 196–212, http://dx.doi.org/10.1112/plms/s2-20.1.196.
Tiselius, P., Jonsson, P.R., 1990. Foraging behavior of six calanoid copepods: observations and hydrodynamic analysis. Mar. Ecol. Prog. Ser. 66, 23–33.
Tedengren, M., Arner, M., Kautsky, N., 1988. Ecophysiology and stress response of marine and brackish water *Gammarus species* (Crustacea, Amphipoda) to changes in salinity and exposure to cadmium and diesel-oil. Mar. Ecol. Prog. Ser. 47, 107–116, http://dx.doi.org/10.3354/meps047107.
Thor, P., 2003. Elevated respiration rates of the neritic copepod *Acartia tonsa* during recovery from starvation. J. Exp. Mar. Bio. Ecol. 283, 133–143, http://dx.doi.org/10.1016/S0022-0981(02)00473-2.
Visser, A.W., Kiørboe, T., 2006. Plankton motility patterns and encounter rates. Oecologia 148, 538–546, http://dx.doi.org/10.1007/s00442-006-0385-4.
Øie, G., Galloway, T., Sørøy, M., Holmvaag Hansen, M., Norheim, I.A., Halseth, C.K., Almli, M., Berg, M., Gagnat, M.R., Wold, P.A., Attramadal, K., Hagemann, A., Evjemo, J.O., Kjørsvik, E., 2015. Effect of cultivated copepods (Acartia tonsa) in first-feeding of Atlantic cod (Gadus morhua) and ballan wrasse (Labrus bergylta) larvae. Aquac. Nutr., http://dx.doi.org/10.1111/anu.12352.