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

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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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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^{TM} (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).

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

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):

$$D = \frac{v^2\tau}{2} \tag{2}$$

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

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

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 ^{TM} 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