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Short-term molecular and physiological responses to heat stress in neritic copepods *Acartia tonsa* and *Eurytemora affinis*

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ABSTRACT

Invertebrates inhabiting shallow water habitats represent particularly appropriate organisms for studying the acclimation potential to environmental stress, since they naturally experience large fluctuations in key abiotic factors such as temperature and salinity. We quantified the biochemical- (mRNA transcripts of 78-kDa glucose-regulated protein (*grp78*), 70-kDa heat shock protein (*hsp70*), 90-kDa heat shock protein (*hsp90*), protein synthesis of HSP70) and organismal- (oxygen consumption rates) level responses to acute heat stress on two neritic copepods (*Acartia tonsa* and *Eurytemora affinis*) with special emphasis on the role of short-term acclimation. Transcripts of *hsp* increased with increasing acute temperature exposure and protein quantities (HSP70) were detectable for 30 h. In *A. tonsa*, HSP70 synthesis was also associated with handling stress. In *E. affinis*, heat-dependent responses were detected in *hsp90*, *grp78* (mRNA) and HSP70 (protein) expression. Acclimation to a warmer temperature significantly decreased the heat stress response in both species. In *A. tonsa*, short-term acclimation to heat was not detected at the organismal level via metabolic rate. This study reveals interspecific differences in both the gene expression of stress molecules (e.g. *hsp90*) as well as the stress factors needed to evoke a stress response (heat vs. handling). We demonstrate that cellular stress markers can be useful measures of short-term thermal acclimation in copepods, which may remain undetected by organismal-level measures.

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1. Introduction

Species inhabiting neritic aquatic habitats are often physiologically well-adapted to cope with the natural fluctuations they experience in environmental conditions. Conditions perceived as stressful may elicit responses at the cellular level, on gene expression and protein synthesis, which help restore an organism's homeostasis. Poikilothermic animals need a strong ability to make physiological adjustments in order to cope with thermal fluctuations and extremes. Thermal acclimation cannot only shift the optimum temperature (T_{opt}) and pejus (pejus = getting worse) temperature (T_p) (Pörtner 2001), but also the thermal tolerance of an organism (Lagerspetz and Vainio 2006) such as increases in the critical thermal maximum (T_c). In order to understand the potential for a species to acclimate to a warmer environment, it is helpful to assess its physiological performance at thermal extremes.

The calanoid copepods *Eurytemora affinis* (Poppe 1880) and *Acartia tonsa* (Dana 1849) are key members of aquatic food webs and are commonly found in dynamic environments, where they face strong spatial gradients and temporal variability (daily to seasonally) in abiotic factors such as salinity, temperature, oxygen, pH and turbulence (Dam 2013; Diekmann et al. 2012). *Eurytemora affinis* typically occurs in lakes and other freshwater habitats, generally avoids salinities >6.5 psu (Viitasalo et al. 1994) but can cope with fluctuations in salinity naturally occurring in some estuaries and salt marsh habitats (Lee 1999b; Lee and Petersen 2003; Peitsch et al. 2000; Xuereb et al. 2012). The acclimation capacity of *E. affinis* to changes in temperature and salinity has been extensively studied (Bradley 1978; Gonzalez and Bradley 1994; Lee and Petersen 2003). Based on changes in egg production rate (*EPR*), the T_{opt} for *E. affinis* was estimated to be ~12 °C (Diekmann et al. 2012). Bradley (1975) demonstrated that individuals from Chesapeake Bay could survive between 0 and 30 °C, at least for short periods of time. Similarly, *Acartia tonsa* is a eurythermal species, which displays short-term tolerance to temperatures from −1 to 32 °C (Gonzalez 1974). While tropical populations occur year-round, in temperate and boreal Atlantic estuaries as well as in the brackish waters of the Baltic Sea, the species only occurs during warmer months and populations persist through the winter as resting eggs (Gonzalez 1974). Based on *EPR*, the optimum salinity for *A. tonsa* was reported to be between 15 and 22 psu (Cervetto et al. 1999; Peck and Holste 2006) and the T_{opt} and the upper T_p of a southwestern Baltic population was

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estimated to be ~23 °C and ~28 °C, respectively (Diekmann et al. 2012), whereas 100% mortality occurred at 34 °C (Holste and Peck 2005).

In addition to organismal-level endpoints such as survival or reproductive rate, which integrate various physiological responses, molecular biomarkers involved in cellular homeostatic mechanisms are potentially more informative indicators of stress. For example, expression profiles of heat-shock proteins (HSP) have been applied as markers for tracking an organism's thermal history (Karouna-Renier and Zehr 1999). The major role of HSP is to assist proper folding of newly formed proteins, repair denatured proteins and aid degradation of proteins after the cell has experienced severe stress (Sørensen et al. 2003). Within a species, we assume that temperatures between T_p and maximum T_c are appropriate for triggering a heat-shock response (HSR). The HSR could be delayed when individuals experience warmer (subsequently lethal) temperatures (DiDomenico et al. 1982). In marine organisms, stressors inducing HSP include changes in salinity, pH, soluble oxygen, desiccation, and pressure (Sørensen et al. 2003). Furthermore, ultraviolet radiation (Tartarotti and Torres 2009; Won et al. 2015), infection (Zhenyu et al. 2004), endocrine disrupting chemicals and heavy metals (Lauritano et al. 2012; Rhee et al. 2009) can also enhance HSP production. Stress-induced HSP occur in different parts of a cell, e.g. members of the HSP70 family are in the cytosol or nucleus, while a different isoform termed 78-kDa glucose-regulated protein (GRP78), which is also referred to as HSP70-5 or immunoglobulin binding protein (BiP), is located in the endoplasmic reticulum (ER). Transcription of the *hsp* gene is self-regulating by locking the heat-shock (transcription) factor-1 (HSF1) into the multi-chaperone-complex when cellular HSP levels reach a certain threshold (Tomanek and Somero 2002). This regulatory mechanism helps avoid detrimental effects caused by constitutive high levels of HSP (Feder 1999; Feder and Hofmann 1999; Krebs and Feder 1997).

HSP (proteins) and *hsp* (genes) have frequently been used to evaluate stress responses in a wide range of marine invertebrates (Clark and Peck 2009a; Greene et al. 2011; Lauritano et al. 2012; Madeira et al. 2012) including stress-mediated HSP70 induction in marine snails, copepods and clams (Barreto et al. 2015; Tomanek and Somero 2002; Voznesensky et al. 2004; Werner 2004). Previous work on the shrimp *Fenneropenaeus chinensis* suggested that ER-HSP GRP78 played a role in immune function and protein folding (Luan et al. 2009). Elevated *hsp70* gene expression during recovery from the quiescent to subitaneous egg stage has been reported for *A.tonsa* (Nilsson et al. 2013), as well as elevated levels of *hsp70* and *hsp90* after heat-shock in acclimated individuals (Petkeviciute et al. 2015). Working with *E. affinis*, Xuereb et al. (2012) reported elevated *grp78* and *hsp90A* mRNA expression levels in response to thermal shocks and also demonstrated an impact of salinity on the quantities of *hsp90A* transcripts.

In the present study, we made use of the HSR to study the potential for short-term heat acclimation of *A. tonsa* and *E. affinis* on a transcriptomic level while also considering the time course of heat shock protein synthesis. For comparative purposes we also determined the oxygen consumption rate (*A. tonsa* only) using oxygen microoptodes after application of a similar heat-acclimation period (24 h). We hypothesized that interspecific differences in the upper T_p and maximum T_c would be reflected in interspecific differences in the HSR. We also expected lower *hsp* transcript quantities in previously acclimated copepods, which were primed for moderate heat stress. Within *A. tonsa*, we hypothesized that acclimation responses would be more easily discernable at the molecular- as opposed to organismal-levels.

2. Materials and methods

2.1. Copepod cultures

Cultures of *A. tonsa* were maintained at a temperature (T) of 20.5 ± 0.5 °C, a salinity (S) of 18.5 ± 0.5 psu and a light regime of 16:8 L:D. Cultures of *E. affinis* were maintained at 10.5 ± 0.5 °C, an S of 4.5 ± 0.5 psu and a light regime of 12:12 L:D. *Acartia tonsa* was cultured for >5 years (~2- to 3-week generation time) whereas *E. affinis* was cultured for ~6 months (~4-week generation time). *Eurytemora affinis* was collected from the Kiel Canal (54°20′N, 9°57′E) with an S of 4–10 psu while *A. tonsa* was collected in Kiel Fjord (54°20′N, 10°09′E) with an S of 12 to 16 psu (Diekmann et al. 2012). Rearing T and S were chosen according to optimum levels for *EPR* (Holste and Peck 2005) or according to conditions copepods experienced at field sites (Diekmann et al. 2012). Copepods were reared in 300-L, semi-static (20-L exchange day^{-1}) tanks at a density of 500 copepods L^{-1} and were provided daily ad libitum portions of the cryptophyte *Rhodomonas baltica* (50,000 cells mL^{-1} = 2700 μg C L^{-1}, (Illing et al. 2015)). Copepods provided this daily ration of this algae display unlimited growth and egg production (Kiørboe et al. 1985; Støttrup and Jensen 1990).

2.2. Experimental handling of copepods

Acartia tonsa used in the experiments were hatched from eggs and reared for approximately three weeks until they reached the adult stage. *Eurytemora affinis* used in experiments were obtained from mixed-stage cultures, which were gently sieved (475 μm) so that only adults or stage V copepodites were present. These late-stage copepods were transferred to a new tank one day prior to the experiment. During experiments, copepods (n ≈ 100) were carefully transferred into 1-L beakers containing 1 L of seawater at the culture salinity and a specific test temperature. This concentration of 100 copepods L^{-1} was chosen to avoid stress reported to occur at higher concentrations that could influence *hsp* expression. Since Lee et al. (2012) provided evidence that 1000 individuals per L of the cyclopoid copepod *Paracyclopina nana* did not induce *hsp70* or *hsp90* expression, we assumed a copepod density of 100 L^{-1} was low enough not to enhance *hsp* expression. The transfer of copepods was done using a sieve that was submersed in water at all times. The beakers with copepods were transferred to incubators (model BK800, Thermo Scientific) where copepods were maintained at the test temperature for 1.5 h (except for the time course of the experiment) with ad libitum food (*R. baltica*), gentle aeration and dim light (~5 μE). Afterwards copepods were used in one of the following experiments: 1) peak induction of *hsp* gene transcription, 2) heat challenge, 3) acclimation, 4) protein time course 5) oxygen consumption (Supplementary files 1&2). Copepods were gently transferred to a sieve submerged in shallow water within a petri dish and then moved to a counting chamber. Any dead individuals were counted and removed. Mortality (in %) was calculated by dividing the number of dead individuals by the total number of individuals and multiplying by 100. All surviving copepods were pooled and rapidly frozen on dry ice and transferred to −80 °C. This sorting process lasted <10 min.

2.3. Exp. 1: peak induction of hsp gene transcription

This experiment was designed to determine the time course of mRNA transcripts for *hsp70* and *hsp90* in *A. tonsa* and for *grp78* and *hsp90* in *E. affinis* after a heat stress. Rhee et al. (2009) determined peak induction for *hsp70* transcripts to occur after 1.5 h, and this experiment was designed to confirm this. For the time series, roughly 100 copepods ($n = 1$) were frozen after 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 h of incubation at the respective temperatures. *Acartia tonsa* was exposed to a mean heat-shock of 28 °C while *E. affinis* was exposed to a temperature of 25 °C (measured temperatures were rounded to the nearest 1 °C). Temperatures are mean values from measurements made in the beakers at the beginning and at the end of the incubation (measured values are given in Supplementary files 1&2). The heat-shock temperatures for the copepods were chosen according their upper T_p as previously mentioned. Since the results for *A. tonsa* indicated an early induction of *hsps*, a higher temporal resolution for the subsequent experiment with *E. affinis* was used. Exposure to the heat stress temperature in this species lasted for ca. 0.25, 0.5, 0.75,

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