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Sexual dimorphism in Grp78 and Hsp90A heat shock protein expression in the estuarine copepod *Eurytemora affinis*

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Abstract Aquatic organisms are constantly exposed to both natural and anthropogenic stressors. Under stress conditions, they elicit a cellular stress response, involving heat shock proteins (HSPs). HSPs are essential to protect proteins against aggregation and to help in the folding of native proteins or refolding of damaged ones. Because of their conservation among taxons and their inducibility after environmental/chemical stress, HSPs are commonly used as ecological and ecotoxicological biomarkers. However, the appropriate use of such molecular tools requires the investigation of the influence of biotic factors on their basal levels. As a first step in biomarker characterization, the present study aims to evaluate the impact of the reproductive cycle on the expression of the two major HSPs, *Grp78* and *Hsp90A* in the estuarine copepod *Eurytemora affinis*. The constitutive expression of both genes in males was weak when compared to female levels suggesting gender-specific stress tolerance. Transcript levels gradually increased during oogenesis and maximal levels were recorded in ovigerous females. The present data support the view that the reproductive condition of individuals has to be considered as a confounding factor in stress evaluation by HSP quantification.

Keywords Biomarker · Confounding factor · Crustacean · GRP · HSP · Reproduction

Introduction

Estuarine health has become a major research concern in recent years, as reported by an increasing number of

publications (Sun et al. 2012). Estuaries provide essential habitats for many species including plants, invertebrates (e.g. crustaceans, worms) and vertebrates (e.g. birds, fish), sustaining the vital biodiversity of coastal regions. They also represent hotspots for settlement of human populations due to their strategic geographical location. Estuaries are thus highly dynamic ecosystems subjected to both natural pressure—such as variations of salinity, turbidity, oxygenation, and temperature—and a wide range of toxic anthropogenic effluents from conurbations, industry, and agriculture. Estuarine aquatic organisms are thus constantly exposed to both environmental and chemical stressors. The Seine estuary situated in the English Channel is one of the most important estuaries of the European Northwest continental shelf. This estuary is characterized by a low zooplanktonic biodiversity predominated by the calanoid copepod *Eurytemora affinis* which can represent 90 to 99 % of zooplankton (Mouny and Dauvin 2002). Over the years, *E. affinis* has become a relevant ecological and ecotoxicological bioindicator (Forget-Leray et al. 2009). Indeed, this euryhaline copepod represents an essential basal organism in the trophic web of estuaries (Mauchline 1998; Winkler and Greve 2004) but also of salt marshes and brackish waters all over the Northern Hemisphere from America to Asia and Europe. It has been shown to have remarkable adaptive capabilities to high variations of salinity and temperature, helping it to maintain its position in these specific environments (Bradley et al. 1988; Devreker et al. 2008; Ketzner and Bradley 1982). Moreover, *E. affinis* has shown the capacity to accumulate environmental contaminants in both field and laboratory studies suggesting its role in biogeochemical cycles of various pollutants such as hydrophobic organic compounds or synthetic steroids (Cailleaud et al. 2007a, b, 2009, 2011a). In recent years, research studies have focused on developing tools to evaluate the effects of such environmental contaminants on *E. affinis* larval development, mortality, swimming behavior, or enzymatic

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activities (Cailleaud et al. 2011b; Forget et al. 2002; Forget-Leray et al. 2005; Lesueur et al. 2013). The *E. affinis* copepods are appropriate test organisms because of their small size, sexual dimorphism (male geniculate antenna), short generation time, and ease of culturing in the laboratory (Cailleaud et al. 2011b; Lesueur et al. 2013; Michalec et al. 2013).

Over the past decades, heat shock proteins (HSPs) have become common ecological and ecotoxicological biomarkers due to (1) their key role in the cellular protective response, (2) their substantial conservation among taxons, and (3) their inducibility after environmental stress (Bierkens 2000). HSPs, also known as stress proteins or chaperones, are highly conserved proteins of varying molecular weight (10–150 kDa) and are constitutively expressed in all organisms under non-stress conditions (Feder and Hofmann 1999). They are essential for protecting proteins against aggregation and help in the folding of native proteins or refolding of damaged ones. Stress conditions suddenly increase the synthesis of HSPs, contributing to maintaining cellular homeostasis (Mallouk et al. 1999).

In order to expand the panel of biomarkers for the evaluation of the impact of stressors on *E. affinis*, we recently identified two HSP cDNAs in *E. affinis*, i.e., *Grp78* (78-kDa glucose-regulated protein) and heat shock protein 90A (*Hsp90A*; Xuereb et al. 2012). *Grp78* and *Hsp90A* were shown to be induced in *E. affinis* after salinity and temperature shocks in both laboratory and field populations (Xuereb et al. 2012), suggesting their possible use as general stress biomarkers in this species. GRP78, also referred to as the immunoglobulin heavy-chain-binding protein BiP is the endoplasmic reticulum paralog of HSP70. GRP78 is evolutionarily conserved from yeast to human (Lee 2001 for review). In vertebrates, it is thought to be involved in unfolded protein response, oxidative stress, calcium depletion, anti-apoptosis signaling, and defense system (Coe and Michalak 2009; Lee et al. 1999; Liu et al. 1997; Rao et al. 2002). In crustaceans, GRP78 may be implicated in protein folding and immune function (Luan et al. 2009). HSP90A is the cytosolic inducible form of HSP90. HSP90 plays crucial roles in both vertebrates and invertebrates in the folding of various proteins (Picard 2002), in the regulation of the activity of numerous factors involved in apoptosis and immunity (Joly et al. 2010), and in supporting various components of the steroid hormone receptors (Echeverria and Picard 2010). The main limitation to the reliable use of such general stress biomarkers in monitoring studies lies in the influence of biotic factors on their basal levels. Such confounding factors could lead to misinterpretation of marker responses. It has been shown—especially in fish and crustaceans—that the HSP responses vary according to tissue, organism, developmental stage, or reproduction (Cui et al. 2010; Iwama et al. 2004; Zhang et al. 2009; Zhao et al. 2011). In this context, we investigated the influence of both gender and reproductive stage on the *E. affinis* *Grp78* and *Hsp90A* expression by culturing copepods under constant optimal conditions.

Material and methods

Eurytemora affinis stabulation

Copepods were collected into the oligo-mesohaline zone (salinity 4.8) of the Seine estuary (longitude 0°15'52"E, latitude 49°29'19"N; Haute-Normandie, France) using a horizontal plankton net (200- μ m mesh size) in spring 2010 (16.6 °C). Immediately after sampling, the copepods were transferred into insulated containers and were quickly brought back to the laboratory. Copepods were reared in 40-L aquariums filled with artificial brackish water (a mixture of UV-treated filtered (1 μ m) sea water and deionized water) in optimal culture conditions, i.e., allowing high reproduction and developmental rates (Devreker et al. 2009). Briefly, copepods were kept at 15 \pm 1 °C and salinity 15 under constant aeration. The photoperiod was maintained at 12:12-h light/dark. The copepods were fed every 2 days with a mixture of *Rhodomonas marina* and *Isochrysis galbana* receiving a total of 20,000 cells mL⁻¹. Algae cultures were grown at 20 °C in 10-L tanks under 24-h fluorescent illumination and constant aeration in Conway medium.

After an acclimation period of 3 weeks, males and females were sorted using a stereomicroscope (Leica Wild M3B) according to their sexual stage. Samples of 50 individuals (three replicates per stage, i.e., 750 individuals for all five stages) were quickly rinsed with deionized RNase-free water before being frozen in liquid nitrogen and stored at -80 °C until further investigation.

Total RNA isolation and reverse transcription

Samples were ground using micro-pestles (Eppendorf, Le Pecq, France) during 3 freeze/thaw cycles in liquid nitrogen, and were then homogenized in 250 μ L of nuclease-free ultrapure water (Sigma-Aldrich, Saint Quentin Fallavier, France). Total RNAs were extracted using Tri-Reagent LS (Euromedex, Mundolsheim, France). Genomic DNA digestion and RNA purification were conducted with TURBO DNA-free® kit (Ambion Applied Biosystems, Courtaboeuf, France) according to the manufacturer's recommendations. RNA integrity was checked by electrophoresis on a 2 % agarose gel. Quantification and purity were evaluated using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA). RNA samples were stored at -80 °C until used for further experiments.

Reverse transcription was conducted from 1 μ g of total RNA with M-MLV reverse transcriptase RNase H minus (Promega, Charbonnières, France) using oligo(dT)₂₀ in the presence of Recombinant RNasin® Ribonuclease Inhibitor (Promega). Finally, complementary first-strand DNA

(cDNA, 40 μ L) were diluted in 60 μ L of ultra-pure water and stored in 5- μ L aliquots at -20°C .

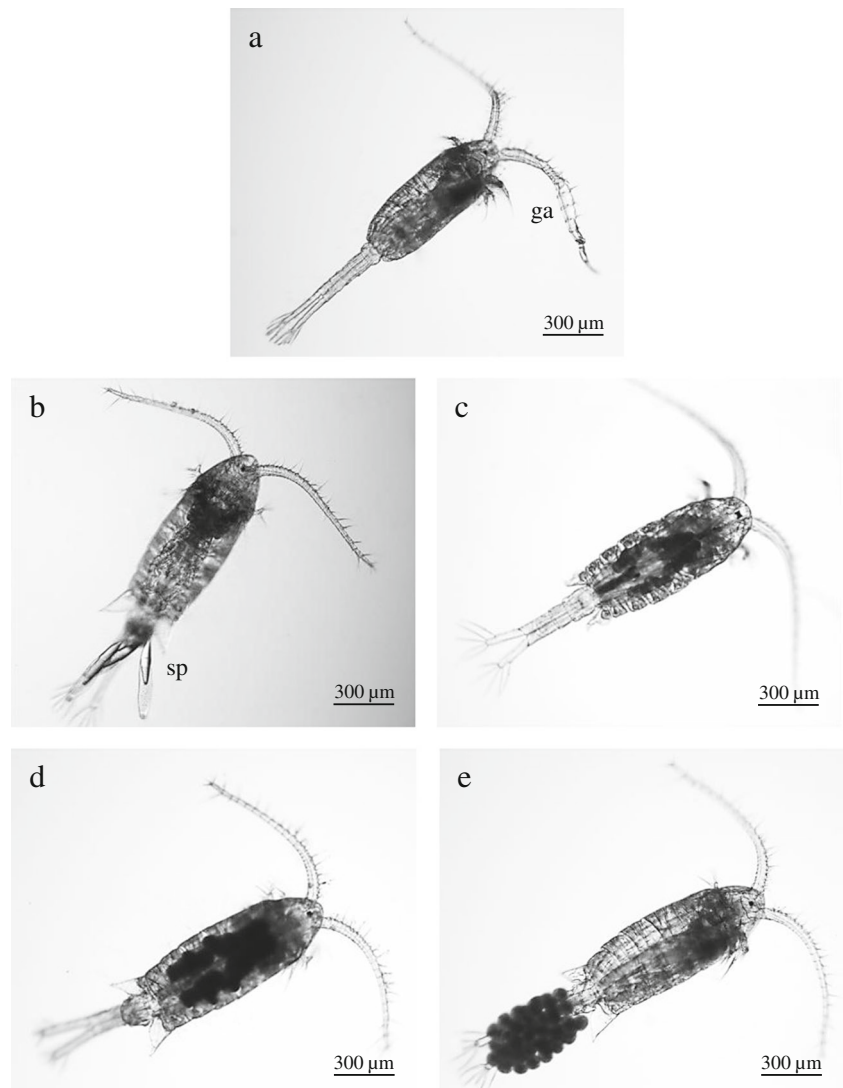
Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) reactions were conducted as previously described using qPCR-*grp78*.F/qPCR-*grp78*.R and qPCR-*hsp90A*.F/qPCR-*hsp90A*.R primer sets (Xuereb et al. 2012). Briefly, amplifications were carried out in duplicate on the Rotor-Gene Q 2-plex HRM (QIAGEN, Courtaboeuf, France) from 5 μ L of cDNA using the QuantiTect[®] SYBR[®] Green Master Mix (1X, QIAGEN) and forward/reverse primers (0.5 μ M each). Non template controls were systematically performed to check the absence of DNA contamination. After an initial denaturation step at $95^{\circ}\text{C}/15$ min, cDNA were amplified for 45 cycles of $94^{\circ}\text{C}/15$ s, $59^{\circ}\text{C}/20$ s, and $72^{\circ}\text{C}/15$ s. After thermocycling, cDNA were denatured by a rapid increase to

95°C and hybridized again for 20 s at 68°C . The melting curve was finally determined during a slow temperature elevation from 55 to 99°C ($0.2^{\circ}\text{C s}^{-1}$). The specificity of PCR products and the absence of primer dimers were checked for all amplifications using the dissociation curve.

The expression levels of target genes were calculated according to the absolute quantification method as previously described (Xuereb et al. 2011). Briefly, the quantification cycle values (C_q ; cycle number from which fluorescence is detected above the noise threshold) were collected with the Rotor-Gene Q series software (QIAGEN) using the comparative quantification method. To convert C_q values into cDNA copy number, a specific standard curve was established for each primer pair from 10-fold serial dilutions of purified PCR products (from 10^9 to 10^1 cDNA copies, in triplicate). For that, *grp78* and *hsp90A* were amplified from a cDNA sample with HotStar HiFidelity DNA Polymerase (QIAGEN) according to the manufacturer's manual and purified with QIAquick

Fig. 1 Reproductive stages of *E. affinis*. **a** Male (ventral view; *ga* geniculate antenna). **b** Female stage 1, immature (dorsal view). Any oocyte can be observed in the oviducts (*sp* spermatophore). **c** Female stage 2A, initiating maturation (dorsal view). The female presents small early vitellogenic oocytes in thin oviducts. **d** Female stage 2B, advanced maturation (dorsal view). The female presents enlarged oviducts full of late-vitellogenic oocytes. **e** Female stage 3, ovigerous (dorsal view). The female—without any oocyte in the oviducts—carries a cluster of eggs



PCR® Purification Kit (QIAGEN). cDNA concentration was determined with a NanoDrop® ND-1000 spectrophotometer and dilution series were performed in ultra-pure water. Dilutions of medium range (corresponding to 10^5 and 10^4 cDNA copies) called “standard points,” were distributed in 5- μ L aliquots and stored at -20°C . These standard points were systematically amplified during the qPCR runs to confirm the reliability of amplification reading and to correct the Y-intercept of standard curve equation. The correlation coefficient of standard curves was 0.999, whereas *grp78* and *hsp90A* PCR efficiencies were, respectively, 1.771 and 1.775.

Statistics

Statistical procedures were carried out with the SigmaStat 3.5 software (Systat Software, Chicago, USA). Normality and equality of variances were checked using, respectively, the Kolmogorov–Smirnov ($p=0.365$ and $p=0.398$ for *Grp78* and *Hsp90A*, respectively) and Levene median ($p=0.679$ and $p=0.481$ for *Grp78* and *Hsp90A*, respectively) tests. Statistical differences between samples were assessed using the one way ANOVA coupled with the Student–Newman–Keuls test as post hoc analysis ($p<0.05$).

Results

Identification of *Eurytemora affinis* reproductive stages

Reproductive stages identified in *E. affinis* are illustrated in Fig. 1. Only one stage was highlighted in males (Fig. 1a). Four stages were determined in females according to their oviduct appearance. The immature stage 1 (Fig. 1b) was characterized by the absence of any visible oocytes in the oviducts whereas the presence of vitellogenic oocytes was typical of stage 2 (Fig. 1c, d). This stage was subdivided into two groups according to the maturing phase of oogenesis, i.e., stage 2A (small early vitellogenic oocytes in narrow oviducts), and 2B (enlarged oviducts full of late-vitellogenic oocytes). Finally, stage 3 females were ovigerous without visible oocytes (Fig. 1e).

Grp78 and *Hsp90A* expression during *Eurytemora affinis* reproductive cycle

Overall, *Grp78* transcript levels were ten times higher than *Hsp90A* ones (Fig. 2). The lowest levels were detected in males for both genes, i.e., 1.7×10^7 and 8×10^5 copies for *Grp78* (Fig. 2a) and *Hsp90A* (Fig. 2b), respectively. In females, transcript amounts gradually increased during oogenesis from stages 1 to 3. Thus, levels varied from 2.6×10^7 to 6.2×10^7 for *Grp78* (Fig. 2a) and 2×10^6 to 5.2×10^6 for *Hsp90A* (Fig. 2b).

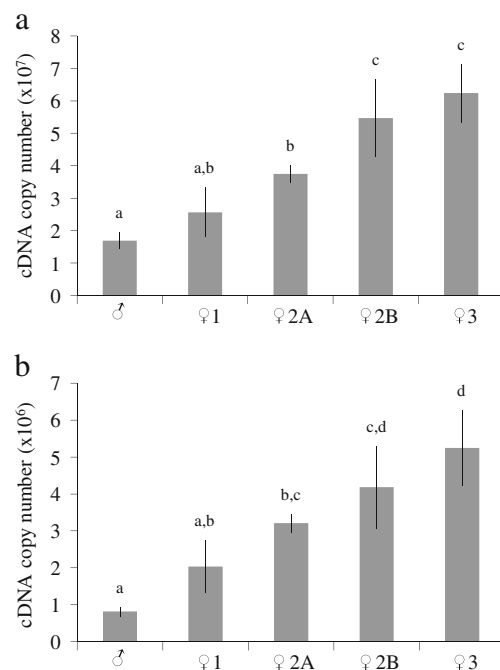


Fig. 2 *Grp78* and *Hsp90A* expression during the reproductive cycle of *E. affinis*. *Grp78* (a) and *Hsp90A* (b) expression was studied by quantitative PCR according to sexual differentiation (♂, male; ♀, female) and oogenesis stage (stages 1 to 3). Each bar represents the mean of cDNA copy number \pm standard deviation of independent biological samples. Significant differences are indicated by different letters ($p<0.005$)

Discussion

While the *E. affinis* developmental cycle and copulation were described as early as the 1970s (Katona 1971, 1975), few data are available regarding their reproductive stages. Recently, Dur et al. chose to describe the reproductive cycle of *E. affinis* according to spawning and hatching delay criteria in order to model the population dynamics of egg bearing copepods (Dur et al. 2009). In the present study, we opted to characterize reproductive stages according to morphological criterions. Mature males were distinguished through their geniculate antennae. However, they were not categorized due to the absence of other distinctive morphological feature allowing the identification of various testicular stages. In females, the classification was based—as previously described in calanoids—on the observation of sexual maturation and gravidity (Tourangeau and Runge 1991). We thus determined four stages from initiating vitellogenesis to egg production.

In order to evaluate the influence of sex and reproductive stage on *Grp78* and *Hsp90A* expression in *E. affinis*, we opted to quantify transcript levels by real-time PCR. This approach—which benefits from specificity and sensitivity—is particularly adapted to study gene expression in microcrustaceans since only a small amount of biological material is needed. The lack of *E. affinis* transcriptome data

led us to quantify the target cDNAs in males and females using the absolute method (Xuereb et al. 2012). In agreement with previous data, the basal transcript levels of *Grp78* were higher than *Hsp90A* ones, irrespective of sex or reproductive stage (Xuereb et al. 2012). Our results highlighted a lower expression of both genes in males than in females. This molecular sexual dimorphism may underlie gender-specific stress tolerance. In particular, the substantial basal levels of HSPs may give female higher tolerance to stress conditions. Indeed, previous studies have demonstrated in fish that high levels of HSP70 were correlated with an ability to cope with environmental changes (Nakano and Iwama 2002). Interestingly, *E. affinis* females—as shown in others copepod species—present a higher survival rate than males after salinity stress (Beyrend-Dur et al. 2009; Cervetto et al. 1999; Chen et al. 2006). Moreover, Mikulski et al. showed that the weak HSP constitutive expression in male *Daphnia magna* was correlated with the active selection of a relatively stable environment whereas females—who present high constitutive HSP levels—select habitats that offer optimal conditions for growth and offspring, even if exposed to variable environmental conditions (Mikulski et al. 2011). Interestingly, male *E. affinis* were shown to be particularly abundant in bottom waters, where energetic cost for organisms is thought to be reduced (Devreker et al. 2008). In the same way, male *E. affinis* present higher locomotion capabilities than females in osmotic stress, in order to maintain themselves in optimal salinity condition (Michalec et al. 2010).

For both genes, we found evidence of an increase of expression during oogenesis. Accordingly, *Grp78* and *Hsp90* were shown to be strongly expressed in crustacean ovaries (Jiang et al. 2009; Li et al. 2009, 2012; Luan et al. 2009; Zhang et al. 2009). Whereas the present study is, to our knowledge, the first focusing on *Grp78* expression during the arthropod reproductive cycle, several studies have shown that the *Hsp90* expression tended to be enhanced during oocyte maturation (Wu and Chu 2008; Zhao et al. 2011). Although the role of HSP90 in the ovary physiology is not well known, literature data suggest that HSP90 could be involved in the regulation of vitellogenin synthesis and in the activation of the EcR pathway in crustaceans and insects (Arbeitman and Hogness 2000; Wu and Chu 2008). The increase of *Hsp* expression during oocyte maturation and maximal levels recorded in ovigerous females might help to provide efficient anti-stress machinery ensuring optimal offspring production.

Nowadays, the quantification of HSPs is commonly used as an indicator of general stress. With the aim of developing such biomarkers in the relevant ecotoxicological species *E. affinis*, we previously showed that *Grp78* and *Hsp90A* were induced after salinity and temperature shocks in laboratory and in field populations, underlying the interest of evaluating the role of HSPs in the ecology of *E. affinis* during long-term monitoring of natural populations. However, in this paper, we

have highlighted that the constitutive *Grp78* and *Hsp90A* expression varied according to the sex and reproductive stage in *E. affinis*, and may be responsible for differential stress tolerance. Our results underline that the reproductive condition of individuals has to be considered as a confounding factor in *E. affinis*, requiring the composition of the population to be taken into account in HSP assays. By helping to define baseline values, this work is a first step to provide a framework for the routine use of such general biomarkers.

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