Assessing a species thermal tolerance through a multiparameter approach: the case study of the deep-sea hydrothermal vent shrimp *Rimicaris exoculata*

Juliette Ravaux¹ · Nelly Léger¹ · Gérard Hamel² · Bruce Shillito¹

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Abstract

Assessing species thermal tolerance requires identification of their thermal strategies and evaluation of their ability to cope with temperature fluctuations. The mobilization of the molecular heat stress response (HSR), which is a proxy for the thermal tolerance, would be part of the strategy of species colonizing highly variable thermal environments. We here investigate multiple parameters of the HSR in the deep-sea vent shrimp *Rimicaris exoculata* that colonizes such environments. The set points of the HSR induction, compared to those of the coastal species *Palaemonetes varians*, clearly reflect a high thermotolerance in this species, while the HSR is proved to be rarely mobilized in the *R. exoculata* natural populations. Finally, the compilation of multiple parameters such as the upper thermal limit and several thresholds of the HSR, as well as thermal behavior observations, allows us to provide a more accurate picture of the combination and complementarity of strategies that can account for the overall thermal tolerance of the species.

Keywords Thermotolerance · Thermal stress · Heat shock response · Thermoregulation · Escape response · hsp70 · Pressure experiment

Introduction

Assessing species thermal tolerance is a major challenge in anticipating their physiological responses to ongoing climate change and ultimately their ability to cope with it. The common evaluation of species tolerance to heat comprises the in vivo assessment of the upper thermal limit, as well as of the induction threshold of the molecular heat stress response (HSR) (see for example Somero 2010; Tomanek 2010; Morris

et al. 2013). A review on marine species proposed that their response to thermal stress depends on the thermal heterogeneity of their habitat (Tomanek 2010). Species inhabiting ecosystems with a high thermal heterogeneity would frequently induce the HSR, as part of their strategy to occupy this thermal niche (Tomanek 2010). Based on this assumption, we here address the following questions: Do species colonizing highly variable thermal environments have HSR set points that reflect a higher tolerance to heat? Do they frequently mobilize the HSR in their natural environment?

The onset of the HSR delineates the limits of normal physiological functions, beyond which the fitness of the organism is negatively impacted since the HSR is believed to imply high metabolic costs (Angilletta 2009). The HSR comprises the induction of the stress protein Hsp70 (heat shock protein of 70-kDa molecular weight), an evolutionarily conserved chaperone that is mobilized upon cellular damage, and is therefore extensively utilized as a bioindicator of environmental stress (review in Morris et al. 2013). As previously debated for the determination of another indicator of thermal limit, the critical thermal maximum (CT_{max}, which is a behavioral response) (Chown et al. 2009; Vinagre et al. 2015), the experimental

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✉ Juliette Ravaux
juliette.ravaux@upmc.fr

¹ BOREA Biologie des Organismes et Ecosystèmes Aquatiques, UMR 7208 MNHN CNRS SU UA UCN IRD 207, Equipe Adaptation aux Milieux Extrêmes, Sorbonne Université, Bât. A, 4e étage, 7 Quai St Bernard, 75005 Paris, France

² IMPMC Institut de Minéralogie, de Physique des Matériaux et Cosmochimie, UMR CNRS SU 7590, Sorbonne Université, Paris Cedex 05, France

procedures can affect the HSR set points. For a given temperature of heat shock, the previous acclimation temperature (Barua and Heckathorn 2004; Ravaux et al. 2012, 2016), the heating ramp (Cottin et al. 2010a), the duration of exposure to the maximum temperature (Ravaux et al. 2012), and finally the time of recovery after the heat shock (Cottin et al. 2010a) may all influence the intensity of the HSR, or the temperature at which it is triggered. Therefore, although the set points of the HSR are major indices for the evaluation of thermal tolerance, the diversity of experimental procedures complicates the interspecies comparisons as well as the identification of potentially common patterns between different species. This reflects the limits of assessing the thermal tolerance by using a simple index that would not reflect the complexity of the response in the organism, which depends on multiple parameters.

The present study addresses the thermal tolerance of the deep-sea hydrothermal vent shrimp *Rimicaris exoculata*, an emblematic species of the Mid-Atlantic Ridge vent sites (see Schmidt et al. 2008 for review). This species lives in a thermal environment where the variability is the highest among aquatic habitats (Bates et al. 2010). *R. exoculata* thrives at close proximity of the vent fluid emissions, in the zone of chaotic mixing of hot hydrothermal fluids with cold abyssal seawater, resulting in thermal regimes characterized by variations of high magnitude over short spatial and temporal scales (see for examples Tunnicliffe et al. 1998; Lee 2003; McMullin et al. 2000; Bates et al. 2010). This species must thus have evolved molecular mechanisms and/or behavioral strategies to cope with extreme temperature fluctuations, as well as with constant threat of exposure to deleterious temperatures. However, our knowledge of the thermal tolerance of this species, as well as for other deep-sea vent species, remains relatively limited, due to both the complex access to the vent sites, and the difficulty of sampling and conducting in vivo experiments on deep-sea animals in good physiological conditions (see review in Van Dover and Lutz 2004 and Shillito et al. 2014). In the last two decades, in vivo experiments, using equipment that retain and/or reconstitute the deep-sea hydrostatic pressure, gradually provided the first data on upper thermal limits for several hydrothermal species (see for example Airriess and Childress 1994; Lee 2003; Girguis and Lee 2006; Bates et al. 2010; Shillito et al. 2014). But to date, there is still no data on the set points of the heat stress response in deep-sea hydrothermal species. Previous studies of the broad molecular response of *R. exoculata* to a 1-h exposure at 30 °C allowed the identification of the *hsp70* stress genes, and more specifically those induced by heat stress (Ravaux et al. 2007; Cottin et al. 2010a, b). A parallel evaluation of the level of stress gene expression in specimens freshly collected at the same site, at their natural pressure, strongly suggested that the shrimp did not induce their expression in situ (Ravaux et al. 2009). Here, we assess the thermal tolerance of this species,

through in vivo heat exposure experiments under deep-sea pressure conditions, by defining the thresholds for both the molecular and behavioral stress responses at temperatures ranging from 20 °C to the CTmax (38.5 °C; Shillito et al. 2006), and for durations ranging from several minutes to 1 h. The thermal tolerance of *R. exoculata* is further compared to that of the coastal shrimp *Palaemonetes varians*, which also lives in a thermally variable environment, by comparing several indices obtained with similar experimental procedures: the set points of the HSR, as well as the CTmax. The stress gene expressions obtained for these in vivo experiments are further compared to the natural stress gene expression levels in shrimp collected at the corresponding vent sites (TAG and Snake Pit), in order to compare the in vivo data interpretation to the actual in situ situation. We finally combined all the indices provided here and in our previous studies on *R. exoculata* to propose a review of the thermal tolerance strategies in this deep-sea hydrothermal vent species. Our results, besides providing data about the ecology and adaptation in the deep-sea hydrothermal environment, provide valuable information about the processes and strategies of heat stress responses in thermally variable environments.

Materials and methods

Animal collection

Rimicaris exoculata were collected from the Mid-Atlantic Ridge Snake Pit (23° 23' N, 44° 58' W, about 3500-m depth) and TAG (26° 08' N, 44° 49' W, about 3600-m depth) vent sites, using the suction device of the ROV *Victor 6000* (BICOSE 2014 cruise). Shrimp were collected and recovered at deep-sea pressure by using the PERISCOP system, composed of an in situ sampling cell directly clamped on the nozzle of the submersible's suction device and an isobaric recovery device (Shillito et al. 2008). The PERISCOP system aims at greatly reducing the collection trauma: during initial suction sampling, the suction power may be moderated in situ by the operators, and the shrimp are directly transferred from their natural habitat towards the sampling cell, thereby avoiding several meters of transit at high speed through the submersible's suction tube. The sampling cell is further stored inside the PERISCOP in order to maintain deep-sea pressure during the ascent: pressure generally remained within 70 to 84% of in situ pressure (35–36 MPa), as opposed to full decompression using conventional means. In two cases, however, pressure drops occurred during recovery (see Fig. 1). Finally, the thermal insulation equipment of PERISCOP is sufficient to prevent temperature rising above 15 °C during the whole sampling process (Shillito et al. 2008).

Upon their recovery on the ship deck, the shrimp were either frozen in liquid nitrogen or transferred in a 20-l

Fig. 1 Sample treatment and choice of the control specimens. **a** Samples experimental treatments. This table details, for each heat shock experiment, the temperature and duration of the shock, as well as the shrimp treatments in the pressurized equipment: PERISCOP (recovery pressure upon collection), BALIST (duration and temperature of maintenance), and IPOCAMP (protocol for heat shock experiments). (*) The two heat shocks of 10 min at 30 °C both gave very low levels of stress gene expression (*hsp70-F1*) despite their difference of pressure during the collection process with PERISCOP: 0.144 ± 0.140 ($n = 5$) for the 26.5-MPa batch, and 0.004 ± 0.001 ($n = 5$) for the 0.1-MPa batch. Therefore, only the 0.1-MPa batch was presented in Fig. 4. **b** Inducible stress gene expression (*hsp70-F1*) in the different batches of control

specimens. Stress gene expressions are expressed as means for $n = 5$ individuals ($\pm$ SEM) of relative *hsp70* form1 mRNA amount normalized to *RPL8* abundance (reference gene). For all experimental conditions, the relative level of inducible stress gene expression is very low with a mean relative expression for all the specimens of 0.140 ± 0.052 ($n = 20$), when compared to the levels obtained following a heat shock (see Fig. 4). Since neither the maintenance in BALIST nor the pressure loss in PERISCOP affected the *hsp70* expression levels in the control specimens, the same control batch that corresponds to the duration of the experiment in IPOCAMP (6 h at 11 °C) was chosen for all the heat shock experiments analyses

aquarium (IPOCAMP™, Fig. 2a; Shillito et al. 2014) operated at 30 MPa, in flow-through mode (20 l/h flow rate), in order to achieve several temperature incubations.

In vivo experiments

A total of 9 independent heat shock experiments were conducted in the pressurized tanks in order to determine the thresholds for *hsp70* expression (Fig. 1). For each experiment, a batch of 10 to 20 specimens was placed in a cage, and maintained at about 11 °C during 3 h before the heat shock, and 2 h after the heat shock until the end of the experiment. Heat shocks were obtained by immersing a heat exchanger situated on the water inlet line into a temperature-controlled waterbath which had been pre-set to the desired shock temperature (Fig. 2a). Once inside the pressure vessel, the incoming seawater was guided towards the cage via a PVC tube, thereby producing a steep

temperature increase (see Fig. 2b), and allowing rapid stabilization at the heat shock temperature. Retrieving the heat exchanger from the waterbath at the end of the shock insured a rapid temperature decrease (Fig. 2b). Temperature recordings in the cage were obtained by using two Pt-100 autonomous temperature loggers (S2T6000D, NKE Instruments) placed at the bottom and top of the cage, with both probe tips situated about 10 cm apart (Fig. 2a, and Fig. 4b for temperature profiles). The difference in temperature between these two probes never exceeded 0.5 °C during the stable phases, i.e., before the heat shock, or during the plateau of the heat shock. Possibly, this difference reached 3–4 °C for 1 or 2 min during the initiation of heating or cooling. The survival rate was 100% for all experiments except for the 10-min exposure at a temperature reaching 39 °C (see Fig. 4). When experiments ended, all specimens were dissected to sample the abdominal muscles that were immediately frozen in liquid nitrogen.

Fig. 2 Experimental setup for in vivo experiments at 30-MPa pressure. (a) The pressure vessel IPOCAMP (internal diameter 20 cm, height 60 cm; Shillito et al. 2014) contains a PVC cage closed at the top with a transparent polyethylene lid. Three sapphire windows in the pressure vessel lid allow the insertion of an endoscope (e) and two optical fiber light guides, for the behavioral observation during the experiment. Large arrows indicate the inlet and outlet of circulating sea-water, which is forced through the cage ventilation holes via a PVC tube. Two Pt100 probes are positioned inside the cage in the upstream water flow (T1), and under the lid at the top of the cage (T2). (b) Example of temperature profiles, obtained for a 30 °C heat shock experiment with T1 probe (bottom) and T2 probe (top), showing the homogeneity of the temperature in the cage

In some cases, freshly sampled shrimp were maintained a few hours at 30-MPa pressure and 8–10 °C temperature in another flow-through aquarium of 8-l volume (BALIST, described in Ravaut et al. 2013), prior to the proper heat shocks in IPOCAMP described above. This was the case when either (1) the IPOCAMP aquarium was not available due to other experiments already occurring at the time of sampling, or (2) the heating performances of the IPOCAMP were not needed (control experiments at constant temperature), or (3) the PERISCOP device had failed retaining pressure during recovery, in which case a few hours recovery period was decided to ascertain survival of sampled animals. The details for each experiment, as well as the controls chosen to evaluate the stress gene expression potentially triggered by these modifications, are provided in Fig. 1. The level of inducible stress gene expression was quantified in the different batches of control specimens, and proved to be very low in all experimental conditions (Fig. 1). Since neither the preliminary maintenance in BALIST nor the pressure loss in PERISCOP affected the *hsp70* expression levels in the control specimens, the same control batch that corresponds to the duration of the experiment in IPOCAMP (6 h at 11 °C) was chosen for all the heat shock experiments analyses.

Behavioral analysis

Four experiments were partially recorded by using an endoscope (Fort, Dourdan, France), a CCD camera (JVC, TK-C1380), and a DVD recorder (DVO-1000MD Sony): (1)

control maintenance at 11 °C, (2) heat shock at 20 °C for 1 h, (3) heat shock at 24 °C for 1 h (a preliminary experiment that was not further used for stress gene expression), (4) heat shock at 30 °C for 30 min. For each experiment, the behavior of the shrimp was observed every minute of the video footage when the temperature reached the plateau and remained constant, which corresponds to mean temperatures of 11.5 ± 0.2 °C ($n = 132$), 20.0 ± 0.1 °C ($n = 12$), 23.7 ± 1.1 °C ($n = 44$), and 30.3 ± 0.2 °C ($n = 23$). For each observation point, the shrimp that were swimming either in the swarm or out of the swarm were counted, as well as the total number of shrimp that were forming a swarm (see Fig. 3).

Quantification of *hsp70* expression using real-time quantitative RT-PCR

The total RNA was extracted from ground tissues (abdominal muscles) (MiniLys, Bertin technologies) using Trizol reagent (Invitrogen) in accordance with the manufacturer protocol. The RNA (0.5 µg) was treated to remove DNA contamination by using the Turbo-DNAse kit (Ambion), and then reversely transcribed to cDNA with the oligo(dT)₁₈ primer and Superscript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions.

The expression of *hsp70* gene was assessed by qPCR with specific primers (see Supplementary Table). Each reaction of qPCR was run in triplicate, and corresponded to 5 individuals for each experimental condition. All reactions were performed

Fig. 3 Behavioral observations of the shrimp swarm upon different temperatures during in vivo experiments. The upper graph depicts the proportion of shrimp assembled in swarm (black dots) and the proportion of shrimp that are swimming either in or out of the swarm (white dots), expressed as mean % $\pm$ S.D., for each of the four in vivo experiments. The total number of individuals was $n = 11$ for 11 °C, $n = 20$

for 20 °C, $n = 14$ for 24 °C, and $n = 12$ for 30 °C. The pictures below the graph are views of the cage in the IPOCAMP aquarium (see Fig. 2), which illustrate the different types of swarm observed: steady swarm at 11 °C and 20 °C, dynamic swarm at 24 °C, and unstable swarm at 30 °C. Body length of the observed shrimps is about 4–5 cm

on the LightCycler® 480 II Real-Time PCR Detection System (Roche, France), using Sybr Green I Master (Roche, France). The PCR program consisted of an initial 5 min step at 95 °C, followed by 45 cycles consisting of 30 s of denaturation at 94 °C, 30 s of annealing at the optimal annealing temperature (60 °C), and 30 s at 72 °C. The measurement of fluorescence during the 70 to 95 °C melting curve showed a single and discrete peak for all primers tested. One negative control and one dilution series protocol of pooled cDNA were included in each run. The dilution series were used to construct a relative standard curve to determine the PCR efficiencies and for further quantification analysis. In all experiments, all primers gave amplification efficiencies of 90–100%. Data were analyzed with the LightCycler® 480 software and the *hsp70* expression was normalized to the reference gene *RPL8* (60S ribosomal protein L8, previously chosen as the reference gene to study the *hsp70* expression in *R. exoculata*; Cottin et al. 2010a).

Results

Behavior observations during in vivo experiments

The behavior of the shrimp was observed at 11 °C ($n = 132$; 11.5 ± 0.2 °C), 20 °C ($n = 12$; 20.0 ± 0.1 °C), 24 °C ($n = 44$; 23.7 ± 1.1 °C), and 30 °C ($n = 23$; 30.3 ± 0.2 °C) (Fig. 3). When maintained at constant 11 °C and 20 °C, the shrimp aggregated, with a mean of $88.2 \pm 12.4\%$ of the individuals

in the aggregation at 11 °C, and $93.8 \pm 3.1\%$ at 20 °C. The shrimp were quite motionless, with one or two individual occasionally swimming to reposition itself in the group ($2 \pm 4.8\%$ of shrimp actively swimming at 11 °C, and $3.3 \pm 3.9\%$ at 20 °C), and the aggregation was therefore named “steady swarm.” At 24 °C, the shrimp were also aggregated most of the time with a mean of $65.7 \pm 19.6\%$ of individuals in the group, but they were more active than at 11 °C and 20 °C, with a few individuals actively swimming back and forth from the group ($18 \pm 11.3\%$). The aggregation was consequently named “dynamic swarm.” At 30 °C, the shrimp formed an aggregation that often dispersed, resulting in a percentage of individuals in the aggregation of $44.2 \pm 27.2\%$, and a similar proportion of actively swimming individuals ($48.2 \pm 15.5\%$), which was therefore named “unstable swarm.”

Thresholds of *hsp70* expression induction

Four members of the *hsp70* family were previously identified in *Rimicaris exoculata*, two heat-inducible forms named “*R. exoculata hsp70* form1” and “*R. exoculata hsp70* form2,” and two constitutive forms named “*R. exoculata hsc70* form3” and “*R. exoculata hsc70* form4” (Cottin et al. 2010a). The relative abundance of these four *hsp70* transcripts was quantified following heat shocks at temperatures of 20 °C, 25 °C, and 30 °C (Supplementary Figure). These expression profiles confirmed that both forms 1 and 2 were inducible, whereas *hsp70* forms 3 and 4 were constitutively

expressed and not further induced following heat shocks. However, due to the difficulty of quantifying the basal levels of *hsp70* form2 when it is not induced, the heat shock response was further quantified by using the level of expression of the inducible *hsp70* form1 for all the following experiments of heat exposure.

Temperature threshold for the onset of the *hsp70* expression

The relative abundance of the inducible *hsp70* form1 transcripts following 1-h heat shocks at temperatures of 20 °C, 25 °C, and 30 °C is presented in Fig. 4a (corresponding temperature profiles are presented in Fig. 4b). The levels of *hsp70* form 1 following the 20 °C and 25 °C shocks were comparable to the 11 °C control specimens (0.29), with 0.42

for 20 °C shock shrimp and 0.24 for 25 °C shock shrimp. The 30 °C shock triggered a significant and massive induction of the *hsp70* form1 (Kruskal-Wallis, $p = 0.013$; Mann-Whitney two-sided test $p = 0.008$), with a mean normalized expression level of 6.7, corresponding to 23-fold relatively to the control specimens at 11 °C.

Duration threshold for the onset of the *hsp70* expression

The *hsp70* form1 expression was quantified for heat shocks at 30 °C of either 60 min, 30 min, or 10 min (Fig. 4a and b). A significant induction, relative to the control specimens kept at 11 °C, was only observed for the 60-min-length heat exposure (Kruskal-Wallis, $p = 0.0039$; Mann-Whitney two-sided test $p = 0.008$).

Fig. 4 Thresholds for the heat stress response induction in *Rimicaris exoculata*. Seven independent heat shock experiments, and one control maintenance, are represented on this figure. A series of three 60-min shocks was done at 20.0 ± 0.1 °C, 25.6 ± 0.3 °C, and 30.3 ± 0.2 °C (referred to as 20 °C, 25 °C, and 30 °C shock in the text). Another series of three 10-min shocks was achieved at temperatures increasing up to 29.6 °C, 35.4 °C, and 38.6 °C (referred to as 30 °C, 35 °C, and 39 °C in the text). Finally, one 30-min shock was done at 30.1 ± 0.3 °C (referred to as 30 °C in the text). In both (a) and (b), the 60-min heat shocks are represented by black circles, the 30-min heat shock by crosses, and 10-min heat shocks by white circles. (a) Hsp70 expression levels. The levels of *hsp70* form1 mRNA were measured after each heat exposure

experiment and are expressed as means for 5 individuals ($\pm$ S.E.M.) of relative amount of hsp70-F1 normalized to *RPL8* abundance (reference gene). The mortality of *R. exoculata* specimens was quantified at the end of the 10-min experiments and is indicated on the graph as gray bars, i.e., 0% for 30 °C and 35 °C, and 36.4% for 39 °C experiment ($n = 11$). (b) Temperature profiles of in vivo heat shocks. The heat shock experiments were conducted in IPOCAMP pressurized aquaria, and consisted of 3-h maintenance at 11.5 ± 0.2 °C (referred to as 11 °C in the text), followed by heat exposures and finally 2 h of recovery at 11 °C. The temperature profiles of the maintenance and recovery periods are not represented here, and only the heat exposure data are given

Heat shock response close to the maximal thermal limit

In order to explore the boundaries of the HSR, specimens of *R. exoculata* were exposed to acute heat shocks at temperature ranging from 30 °C, to near their upper thermal limit (38.5 ± 2 °C CTmax; Shillito et al. 2006) (Fig. 4a and b). Heat shocks of 10-min duration at 30 °C (maximum temperature reached of 29.6 °C) and 35 °C (maximum temperature reached of 35.4 °C; 3 min at 35 °C and 7 min above 30 °C) did not trigger any HSR. The relative amount of *hsp70* form 1 mRNA was 0.004 for the 30 °C shock and 0.57 for the 35 °C shock, when compared to the level of 0.29 in the control specimens kept at 11 °C. The shrimp were all alive and at the end of these two experiments, i.e., 2 h after the heat exposure. The 10-min shock at 39 °C (maximum temperature reached of 38.6 °C; 3 min at 38 °C and 7 min above 30 °C) yielded a significant HSR relative to the 11 °C control specimens (Kruskal-Wallis test, $p = 0.0008$; Mann-Whitney two-sided test $p = 0.008$), and a mortality of 36.4% (4 individuals out of 11) was observed at the end of the experiment.

Natural levels of stress gene expression

The level of inducible stress gene expression *hsp70* form 1 was quantified in two batches of shrimp, recovered with the PERISCOP device in order to better preserve their natural physiological state (Fig. 5). In both batches, the majority of the shrimp showed very low levels of stress gene expression, with 90% (TAG) and 85% (Snake Pit) of the individuals having *hsp70* form1 relative levels below 0.5, and a total mean level of expression of 0.31 ± 0.16 (TAG) and 0.39 ± 0.16 (Snake Pit). When compared to the heat-shocked shrimp, either for 10 min at 39 °C or for 1 h at 30 °C, these freshly collected specimens showed very low levels of *hsp70* form 1 expression, i.e., between 11-fold and 22-fold less. For comparison, the data from the Rainbow vent site (from Ravaux et al. 2009) were added to the graph, with a mean level of expression of $5.10^{-3} \pm 1.10^{-3}$, which corresponds to a very low expression of about 900- to 1300-fold less than that of the heat-shocked shrimp.

Discussion

Maintaining deep-sea pressure during recovery and experimentation

The animals studied in this work originate from hydrothermal vent sites (TAG and Snake Pit) situated at 3500–3600-m depth. Specimens collected with a submersible, subjected to pressure and temperature

Fig. 5 Natural levels of inducible stress gene expression in *Rimicaris exoculata* specimens from diverse vent sites. Shrimp were recovered with the PERISCOP device at 3500-m depth on TAG and Snake Pit sites, maintained at 26 to 30.3 MPa and ≤ 15 °C throughout the ascent, and immediately frozen on board, in order to preserve their natural physiological state. The stress gene expressions are expressed as means of *hsp70* form1 mRNA levels normalized to *RPL8* abundance for $n = 21$ (TAG) and $n = 13$ (Snake Pit). Previous results obtained for specimens recovered at Rainbow site at 2300-m depth, and maintained at 23 MPa and ≤ 15 °C throughout the ascent, are presented ($n = 15$; Ravaux et al. 2009). For comparison, the levels of *hsp70* form1 stress gene expression obtained from heat shock experiments are indicated on the graph, i.e., 4.3 ± 1.1 for 10 min at 39 °C and 6.7 ± 1.1 for 1 h at 30 °C (Fig. 4a)

variations during ascent towards the surface, were characterized by a major proportion of motionless individuals for which survival was difficult to ascertain. Using the PERISCOP recovery device greatly reduced such trauma, and provided sampling batches close to 100% survival, with specimens displaying a very active behavioral response upon pressure release of the PERISCOP, a straightforward indication of survival and physiological fitness that allows further in vivo experiments on these specimens using pressure aquaria. Since two batches of shrimp were subjected to decompression during the ascent with the PERISCOP, we further evaluated the potential impact of this decompression on the stress gene expression (Fig. 1). Samples recovered at pressure of 0.1 MPa and 3.2 MPa were alive and showed very low levels of inducible stress gene expression, comparable to the levels measured in the animals recovered at 26.7 MPa. Since the pressure loss during the ascent did not affect the stress gene expression, these batches of animals were further used for heat exposure experiments, but were nevertheless only compared with each other and not with batches recovered at 26 MPa (see Fig. 1 for sample details).

Due to technical limitations inherent to our pressure aquaria, the incubations described in this work were conducted at a pressure of 30 MPa (maximum working pressure), a value exceeding 80% of the 35 to 36 MPa prevailing in situ.

To what extent this pressure shift impairs the physiological functions of the studied specimens remains unknown, and it should be kept in mind that the animals observed in this study were not incubated at their exact natural pressure conditions. However, the 100% survival experienced in our experiments (except for the 39 $^{\circ}C$ exposure) was also observed for another study of *R. exoculata* using 72-h pressure incubations during the same cruise (Auguste et al. 2016), and indicates the satisfactory biological state of the studied specimens. Moreover, all our heat incubations were undertaken at the same pressure of 30 MPa, therefore allowing inter-experiment comparisons of response intensities.

Assessing a species thermal tolerance through HSR set points: comparison of the deep-sea shrimp *R. exoculata* and the coastal shrimp *P. varians*

A species thermal tolerance cannot accurately be evaluated with a single heat stress response set point. The HSR set points can indeed vary according to several parameters, like the temperature and duration of the heat shock, the recovery period after the heat shock, or the prior acclimation temperature. The assessment of a species thermal tolerance, and further comparison to other species, by using a single set point for the HSR could then lead to an incorrect or at least incomplete

◀ **Fig. 6** Assessment of species' thermal tolerance through multiparameter comparison of the heat shock response. **a** Theoretical representation of the stress gene expression pattern as a function of the duration and the temperature of the heat shock. The zone of stress gene expression extends between the optimal temperature (T_{opt}), below which the stress response is not induced whatever the duration of the shock, and the critical thermal maximum (CTmax), above which the survival is limited. When the temperature of the heat shock increases ($t_2 > t_1$), the duration for the onset of the stress gene expression decreases ($d_2 < d_1$). For a given temperature of heat shock, mortality (hatched gray area) occurs when the temperature of the heat shock approaches the CTmax, as well as for long exposures to high temperatures. The intensity of the stress gene expression can vary within the stress gene expression zone and is not depicted here. **b** Theoretical representation of the stress expression zones extrapolated from the experimental data obtained for *Palaemonetes varians* acclimated at 10 °C (species A) or 20 °C (species B), and for *Rimicaris exoculata* (species C). The experimental data for the heat stress response (HSR) induction of these species are represented on the graph panel **c** below. The shift of the stress gene expression zone along the x -axis for species A and B is due to the acclimation, which modifies the upper thermal limit and the HSR set points ("acclimation effect"). Two shifts indicated by white arrows are shown for species C stress gene expression zone when compared to species B, one along the x -axis (higher CTmax) and one along the y -axis (increasing of the duration threshold for inducing the HSR). The two zones however merge on their left part, meaning that species C zone is larger than species B zone. The comparison of these zones leads to a classification of these 3 species according to their thermal tolerance as follows: species A < species B < species C. **c** Representation of the experimental data of the heat stress response in *Rimicaris exoculata* and *Palaemonetes varians*, as a function of the duration and the temperature of the heat shock. The data for *R. exoculata* are those for *hsp70* form 1 from Fig. 4a, and for *P. varians* those for *hsp70* form 1 from Ravaux et al. (2012). Open symbols display the heat shock conditions that did not trigger the HSR, while full symbols represent the conditions that induced the HSR. The CTmax, index of the upper thermal limit, is indicated as a vertical line crossing the x -axis for *R. exoculata* (solid line, 38.5 °C; Shillito et al. 2006) and for *P. varians* acclimated to 10 °C (dotted line, 31 °C; Ravaux et al. 2012) and 20 °C (dashed line, 36 °C; Ravaux et al. 2012). The curves outline for each species the area which includes all the points with an induced HSR, therefore delimiting the stress gene expression zone for the current data

conclusion. We here propose to compare the thermal tolerance of two species, the vent shrimp *R. exoculata* and the coastal shrimp *Palaemonetes varians* (Ravaux et al. 2012), by using multiple parameters of the HSR, i.e., the set points as a function of the temperature of the heat shock and the duration of the heat shock, as well as of the acclimation temperature (10 °C or 20 °C for *P. varians*). The CTmax data were also considered the upper limit for the HSR. The recovery period after the heat shock (2 h) and the temperature profiles of the heat shocks (sudden exposure in minutes range to the shock temperature) were similar for all the experiments, in order to ensure comparable experimental procedures.

A general theoretical representation of the expected pattern of the HSR is proposed in Fig. 6a. A theoretical representation of the stress gene expression of *P. varians* acclimated either at 10 or at 20 °C, as a function of both the temperature and the duration of the heat exposure, is proposed in Fig. 6b (extrapolation of the empirical data from Ravaux et al. 2012,

displayed in Fig. 6c). The acclimation results in a shift of both the CTmax and the stress gene expression set points along the x -axis (indicated as "acclimation effect" on Fig. 6b), resulting in a shift of the stress gene expression zone.

Since the natural environmental temperature (measured within *R. exoculata* aggregations) at the TAG site ranges from 5 to 10 °C (Table 1), we could expect HSR set points comparable to those of *P. varians* specimens acclimated at a similar temperature of 10 °C. However, *R. exoculata* clearly has a higher thermal tolerance than *P. varians* acclimated at 10 °C, with a shift of the CTmax and the stress expression zone to higher temperatures (along the x -axis), as well as a shift of the expression zone to higher duration of heat exposure (along the y -axis) (Fig. 6b and c). *R. exoculata* also surprisingly shows a higher thermal tolerance than *P. varians* acclimated at 20 °C, as shown by a shift of the CTmax to higher temperatures along the x -axis, as well as a shift of the expression zone along the y -axis for high temperatures. This would mean that the threshold duration for stress gene expression is higher in *R. exoculata* and therefore allows short exposure to high temperatures without triggering the HSR. However, the stress gene expression zone of *R. exoculata* does not simply shift to higher temperatures when compared to 20 °C acclimated *P. varians*, since both zones merge for heat exposures of 1 h at about 30 °C. This means that *R. exoculata* has a wider zone of stress gene expression, which extends to temperatures far below the CTmax for sustained exposures of 1 h.

A comparison of a single HSR set point, like the temperature threshold that triggers the HSR, would have led to the conclusion of a similar thermal tolerance between *R. exoculata* and *P. varians* (20 °C acclimated), since this threshold is about 30 °C for *R. exoculata* and 31 °C for *P. varians* (1-h heat shock, 2 h of recovery post-shock). With this multiparameter approach, *R. exoculata* clearly appears more thermotolerant than *P. varians*, regarding both the CTmax and the duration of the heat stress that induce the onset of the HSR. This result has significant ecological implications, since this would mean that *R. exoculata* is not affected by short temperature variations at high temperatures in its natural environment, which is an advantage in a thermal environment that is highly variable on short time scales.

Assessment of the thermal tolerance in *R. exoculata* through the behavioral heat stress response threshold (thermoregulation and escape response)

In addition to the determination of the molecular HSR thresholds, we analyzed the behavior of the shrimp during exposure to diverse temperatures, in order to determine the threshold of a common thermal behavior upon heat stress, i.e., the escape response (see Fig. 3). This allowed us to propose the existence of another thermal behavior in *R. exoculata*, i.e., a

thermoregulation behavior, which relies on the occurrence of active and social aggregations of individuals.

This species is indeed known to occur in aggregations of thousands of individuals, with densities estimated to be in the 1500 to 3000 individuals per square meter range (Van Dover et al. 1988, Copley et al. 1997, Gebruk et al. 2000). The term “swarm” was previously used by several authors to describe *R. exoculata* aggregations, but more in a descriptive sense, by analogy with bee swarms, since very little information is available about the ethology of *Rimicaris* (Segonzac et al. 1993). This species is believed to form aggregations as a result of restricted availability of substratum exposed to hydrothermal fluids, which fuel the chemoautotrophic metabolism of its bacterial epibiosis that fulfill most of its nutritional needs (Polz et al. 1998; Gebruk et al. 2000; Ponsard et al. 2013). The aggregations could also form as a consequence of the selection of a thermal niche on the chimney walls at close proximity of the source (Ravaux et al. 2009). In both cases, the aggregations would form in response simply to the distribution of resources and/or temperature of the habitat, which define these groups as *active and non-social aggregations* (review in Ritz et al. 2011). In our in vivo experiments, the shrimp aggregations occurred in an homogeneous environment and in the absence of environmental cues, therefore suggesting they form not only as a result of independent attraction of individuals to a resource, but also as a result of social behavior. We indeed observed aggregations including up to 90% of the total shrimp in experimental environments where the water temperature was homogeneous and stable (either 11 °C, 20 °C, 25 °C, or 30 °C; see Fig. 3). This here precludes the involvement of a thermal stimulus, such as a warm source or a temperature gradient that would induce a choice, in the formation of these aggregations. Since the water circulating in these experimental environments was surface filtered seawater, the shrimp were exposed to neither any food nor chemical stimulus related to the hydrothermal fluids. This leads us to the conclusion that *R. exoculata* form *active and social aggregations*, which refers to groups of individuals that

“self-organize” and maintain cohesion because of the many derived benefits (review in Ritz et al. 2011).

The activity in the swarms increased with the experimental temperature, and the aggregations were therefore named “steady swarm” at 11 °C and 20 °C, “dynamic swarm” at 24 °C, and “unstable swarm” at 30 °C (Fig. 3). While the steady swarms would reflect optimal temperature conditions, the enhancement of the level of active moving, and especially swimming, at higher temperatures could be interpreted as an avoidance of non-optimal temperatures. The onset of an escape response was previously proposed for *R. exoculata* for temperatures above 24 °C $\pm$ 2 °C (Ravaux et al. 2003). However, while the escape behavior would ultimately cause dispersal of the aggregation, the shrimp aggregations were maintained at 24 °C despite the enhanced movement level. This increased activity, if not solely explained by an escape response, may therefore be part of a thermoregulation behavior. The shrimp may indeed actively move their swimming appendages, while staying close to the swarm, in order to mix the water streams from hot hydrothermal fluid and cold abyssal water, and consequently maintain an optimal temperature in the swarm. Such behavioral responses to high temperatures have already been extensively described in social insects, like bees and wasps, that use active thermoregulation through wing fanning to cool the nest temperature (see review in Jones and Oldroyd 2007). The in situ temperature measurements in the shrimp swarm would support this hypothesis of a thermoregulation process since, from datasets of several hundred of measurements, the maximum temperature reported did not exceed 25 °C and the mean temperature did not exceed 16 °C (Table 1). The very low level of natural stress gene expression in the shrimp, directly sampled in the swarms at three different vent sites (see Fig. 6), also strongly suggest for the occurrence of a thermoregulation process that would prevent the shrimp from being exposed to high deleterious temperatures. The onset of the thermoregulation behavior would then occur between 20 °C and 24 °C.

Table 1 Habitat temperature of *Rimicaris exoculata* aggregations. This table only reports studies that provide data of direct in situ measurements of temperatures within shrimp aggregations. The temperature values given by the authors as personal communications were not included in

the table, as for the 20–30 °C range (G. Thompson in Van Dover et al. 1988) and the 10–40 °C range (Gebruk et al. 2000). S.D.: standard deviation, *N*: number of temperature measurements

<i>T</i> mean $\pm$ S.D. (°C)	<i>T</i> min (°C)	<i>T</i> max (°C)	<i>N</i>	Site	Reference
–	10	15	2	Snake Pit	Segonzac et al. 1993
13.2 $\pm$ 5.5	–	–	9	Rainbow	Desbruyères et al. 2001
11	4.7	25.0	12–15	Rainbow	Geret et al. 2002
8.7 $\pm$ 2.3	3.8	14.7	327	Rainbow	Zbinden et al. 2004
6.2 $\pm$ 0.4 to 16 $\pm$ 1.3	5.3	18.2	274	Rainbow	Schmidt et al. 2008
4.6 $\pm$ 0.4 to 10.2 $\pm$ 3.2	2.8	17.4	935	TAG	Schmidt et al. 2008

When exposed to a constant temperature of 30 °C, the shrimp swarm became unstable, dissociating and re-organizing itself very often, with about half of the shrimp actively swimming in the cage (Fig. 3). This behavior could therefore be interpreted as an escape response, and its onset coincides with the molecular stress response threshold for prolonged exposure to 30 °C (Fig. 4). Escape responses, known as a primary defense used by ectotherms to prevent exposure to temperature extremes, were previously shown to be conspicuous among mobile vent species (Bates et al. 2010).

Multiparameter assessment of the thermal tolerance in *R. exoculata*

A synthetic scheme of the thermal tolerance strategies in *R. exoculata*, which compiles data from this study as well as from our previous studies, is proposed in Fig. 7. This compilation of multiple parameters (thermal limits, thresholds of molecular and thermal behaviors) gives a more accurate picture of the combination and complementarity of strategies that can account for the overall thermal tolerance of the species.

The thermal limits of this species, from below 2 °C to about 38 °C, define *R. exoculata* as an eurythermal species. It is indeed neither a warm specialist nor a thermophilic species like the vent annelid *Alvinella pompejana* that colonizes the smoker walls of the Pacific Ridge sites, and would have an optimal thermal range expanding from above 20 °C to beyond 40 °C (Ravaux et al. 2013). The *R. exoculata* upper thermal

limit is far above the temperatures reported from in situ measurements in the shrimp swarms, which do not exceed 25 °C (Table 1). This is consistent with the observations of other mobile vent species that seek relatively cool habitat temperatures when compared to their heat tolerance, in order to avoid exposures to deleterious temperatures in their unpredictably variable environment (Bates et al. 2010).

In the temperature range from 16 °C (boundary of the mean in situ temperatures in the swarms) and 38 °C (average CTmax), a combination of behavioral and molecular strategies allows the shrimp to cope with its peculiar thermal environment. The thermoregulation behavior of the swarm, occurring beyond 20 °C, would help to decrease the temperature back to optimal conditions. If the water temperature increases above 30 °C, the thermoregulation behavior is accompanied by a capacity to endure exposures to temperatures ranging up to the upper thermal limit without triggering the heat stress response, provided that it is limited to durations in the 10 min to 1 h range. For prolonged exposures to temperatures above 30 °C, these processes are relayed by the onset of both the escape response and the molecular HSR (stress gene induction).

Conclusions

This study clearly showed that the set points of the HSR induction reflect a high thermotolerance in the deep-sea shrimp

Fig. 7 Review of the strategies accounting for the thermal tolerance of *Rimicaris exoculata*. Diagram summarizing data and subsequent hypotheses on *R. exoculata* thermal tolerance, from the present study as well as from our previous studies (Ravaux et al. 2003; Shillito et al. 2006; Ravaux et al. 2009; Cottin et al. 2010a). The mean habitat temperature range of the shrimp aggregations is indicated on the diagram as a black rectangle on the temperature scale bar (4.6 °C to 16 °C), and is a compilation of available data obtained from direct in situ measurements in the

shrimp swarms (see Table 1). Above the temperature bar are the behavioral and molecular strategies implied in the thermal tolerance of *R. exoculata*, and below the temperature bar is a scheme of the corresponding behavior observed in vivo. See the “Discussion/Review of the thermal tolerance strategies in *R. exoculata*” section for further explanations. CTmin, critical thermal minimum; CTmax, critical thermal maximum

R. exoculata. In response to the second question raised in the introduction of this work, i.e., do species colonizing highly variable thermal environments mobilize frequently the HSR in their natural environment, our results provided evidence that it is not the case for *R. exoculata*. The HSR was only rarely triggered in the natural sampled specimens, which would be due to a thermoregulation behavior. Such thermal behaviors (thermoregulation, escape response) are very difficult to identify in natural swarms of *R. exoculata*, where in situ behaviors most probably result from various constraints such as hydrodynamics, access to the fluid emissions, and availability of substrate, as well as social interactions between individuals. It therefore makes it difficult to distinguish the involvement of each of these latter factors in the structure and function of *R. exoculata* swarms, and to further determine whether the distribution of the individuals presents a pattern in relation to environmental, physiological, or ecological factors.

By studying diverse aspects of the heat stress response, we demonstrated that this species has evolved both molecular mechanisms and behavioral strategies to cope with extreme temperature fluctuations, as well as with constant threat of exposure to deleterious temperatures. The compilation of multiple parameters, used in this study to evaluate and compare species thermal tolerance, highlights the complementarity of the strategies involved in a species response to heat stress at scales ranging from the molecular to the population levels.

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