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RESEARCH ARTICLE

The importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster

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ABSTRACT

Although thermal performance is widely recognised to be pivotal in determining species' distributions, assessment of this performance is often based on laboratory-acclimated individuals, neglecting their proximate thermal history. The thermal history of a species sums the evolutionary history and, importantly, the thermal events recently experienced by individuals, including short-term acclimation to environmental variations. Thermal history is perhaps of greatest importance for species inhabiting thermally challenging environments and therefore assumed to be living close to their thermal limits, such as in the tropics. To test the importance of thermal history, the responses of the tropical oyster *Isognomon nucleus* to short-term differences in thermal environments were investigated. Critical and lethal temperatures and oxygen consumption were improved in oysters that previously experienced elevated air temperatures, and were associated with an enhanced heat shock response, indicating that recent thermal history affects physiological performance as well as inducing short-term acclimation to acute conditions. These responses were, however, associated with trade-offs in feeding activity, with oysters that experienced elevated temperatures showing reduced energy gain. Recent thermal history, therefore, seems to rapidly invoke physiological mechanisms that enhance survival of short-term thermal challenge but also longer term climatic changes and consequently needs to be incorporated into assessments of species' thermal performances.

KEY WORDS: Ecological relevance, Energy absorption efficiency, Heat-shock response, Microclimate, Thermal response, Trade-offs

INTRODUCTION

Understanding the thermal responses of ectothermic species is of increasing importance in the light of global warming, especially when considering the effect of climate shifts and instability on the realised thermal niche of species (Etterson and Shaw, 2001; Helmuth et al., 2002; Pörtner, 2002; Pörtner and Knust, 2007; Chown et al., 2010; Bozinovic et al., 2011; Chapperon and Seuront, 2011; Rezende et al., 2011; Anttila et al., 2014; Palumbi et al., 2014; Verberk et al., 2015). The suite of thermal responses utilised by a species, which can entail complex, integrated processes at whole-organism, cellular and molecular levels, determines their sensitivity to climatic influences and represents the central mechanism for species' thermal adaptation (Pörtner, 2002, 2010; Kassahn et al., 2009; Marshall et al., 2010; Bartolini et al., 2013). In addition, thermal responses can occur at different time scales ranging from rapid compensatory adjustments and phenotypic trait plasticity that drive thermal acclimatisation at the individual level, up to evolutionary changes and thermal adaptation, which occur over multiple generations (Burggren and McMahon, 1981; Pörtner, 2010; Somero, 2010; Palumbi et al., 2014). The prediction of species' responses to thermal challenges are therefore complex, especially given the differential effects of acute versus chronic thermal stress and the fact that our understanding of acclimation and adaptation processes is limited by the relatively short timescales usually adopted to study them (Stillman, 2002, 2003; Bozinovic et al., 2011; Rezende et al., 2011, 2014; Magozzi and Calosi, 2015). Indeed, the role of previous climatic conditions experienced by individuals constitutes an often neglected aspect when species' actual thermal acclimation and adaptation capacities are investigated. To address this knowledge gap, this paper explores the influence of proximate thermal history (i.e. the recent thermal events experienced by an individual) in shaping species' thermal responses and climate sensitivity, using rocky shore species as a model system.

Intertidal communities constitute one of the most widely distributed and tractable systems to understand how species can acclimate or adapt to extremely dynamic environments (Helmuth, 1998; Helmuth and Hofmann, 2001; Helmuth et al., 2002, 2006; Chapperon and Seuront, 2011; Giomi et al., 2014; Fusi et al., 2016). In recent years, the interaction of major environmental drivers, combined with the biotic components of the intertidal habitat, has received great attention as a model system for determining the suite of responses species can exhibit to environmental challenges (Barry et al., 1995; Helmuth, 1998; Helmuth and Hofmann, 2001; Helmuth et al., 2002, 2006; Harley and Helmuth, 2003; Burrows et al., 2008; Mislan et al., 2009; Chapperon and Seuront, 2011; Sokolova et al., 2012; Mislan et al., 2014). In particular, many studies have investigated species' tolerance thresholds to forecast their vulnerability to climate change and to formulate projections that range from individual to community levels (e.g. Helmuth and Hofmann, 2001; Stillman, 2002, 2003; Bartolini et al., 2013; Magozzi and Calosi, 2015; Verberk et al., 2015). Thus, it is not surprising that the recurrent terms adopted to describe the survival expectancy of intertidal organisms facing climate change are 'stress' and 'risk' (see Helmuth and Hofmann, 2001; Helmuth et al., 2002; Mislan et al., 2009; Denny et al., 2011). This approach, however, appears incomplete, as the information required to accurately formulate reliable predictions about the acclimation and adaptive potentials of species requires the incorporation of an individual's

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thermal history. The proximate thermal history of a species can impact an organism's contemporary performance at a variety of levels, from short-term, molecular responses in terms of induction of heat shock proteins and subsequent energy allocation, to longer term selection of resistant genotypes. The influence of the thermal experience of a species will be strongly shaped by environmental conditions experienced in the natural habitat of an individual. Currently, it is debated whether species in the tropics are living closer to their thermal limits and, therefore, have less capacity for thermal acclimation than their temperate contemporaries (Tewksbury et al., 2008; Dillon et al., 2010; Huey et al., 2012; Sunday et al., 2012, 2014; Fusi et al., 2015; Seebacher et al., 2015). However, as the influence of thermal history will be strongly shaped by the environmental conditions experienced by species in their habitat, it seems conceivable that tropical species, which frequently face extremely harsh climates, may be adapted to manifest a more efficient thermal response to climatic anomalies than their temperate counterparts. The aim of the present study was, first, to provide evidence of the adaptive value derived from the colonisation of habitats that experience recurrent severe heat stress by estimating the costs and the benefits associated with a species' thermal response in the high intertidal zone in the tropics. Second, as laboratory acclimation generally results in artificial conditioning and often masks innate responses generated by environmental conditions experienced in the natural habitat (Terblanche et al., 2007; Fangue et al., 2011), we investigated species responses to environmentally realistic conditions. In this case, because environmental extremes occur when individuals are emersed by the falling tide, aerial conditions pose the greatest challenge to intertidal species, as air temperatures, and more importantly substrate temperatures (Marshall et al., 2010), rapidly increase upon emersion compared with the more thermally stable seawater environment. The present paper demonstrates the need to incorporate the ecologically relevant thermal history of individuals when attempting to measure realistic thermal responses of organisms.

MATERIALS AND METHODS

Collection sites and animal maintenance

The oyster *Isognomon nucleus* (Lamarck 1819) (Pteriidae, Bivalvia) is widespread within tropical latitudes in the Indo-Pacific region where it inhabits rocky shores. In the Gulf of Thailand, typically, *I. nucleus* is abundant (~4200 m^{-2}) and shows a clear vertical distribution between mid- to high-shore (~2.5–3.25 m above mean sea level, tidal range ~3.5 m; see Samakraman et al., 2010). As such, *I. nucleus* inhabits a physically stressful environment, being emersed for long periods (~8 h per tidal cycle) and naturally facing daily air temperature variation between ~18 and 40°C (M.G., unpublished data; Samakraman et al., 2010; Dong et al., 2015).

More than 2000 individuals of *I. nucleus* were collected from the mid-shore along the rocky shores of Sichang Island (Gulf of Thailand: 13°08′52″N, 100°48′11″E), during early spring 2013. Animals were subsequently transferred to the University of Palermo, Italy, within 32 h in moist tissues. Animals were maintained in atidal conditions for more than 1 month in 20 l aquaria in aerated, filtered (10 µm) seawater, with constant water flow maintained using immersion pumps. During this period, oysters were acclimated at 26±1°C and 28.5±0.5‰, mirroring the seawater conditions in Sichang Island (T. Yeemin, personal communication), fed with the microalgae *Isochrysis galbana* (25,000 cells ml^{-1}), and maintained under a constant dark:light regime (12 h:12 h) (Ezgeta-Balić et al., 2011). Animals were maintained in these conditions to attempt to remove the possible influence of any environmental cues (e.g. tidal cycle, thermal variation, air exposure) or mid-term (few weeks) thermal history on their contemporary thermal responses. Separate groups of animals were then exposed to single events of different thermal challenges during emersion (see below for details) and, as a result, it was considered that any difference these groups of animals exhibited in their thermal responses would be a direct result of their recent, short-term, thermal history.

General experimental procedures

Laboratory procedures were designed to evaluate the physiological and functional effects of acute heat exposure that *I. nucleus* could experience during daytime emersion periods. Two different temperatures were applied during 4 h of air exposure; 26°C as a control temperature (coincident with the water temperature in the acclimatisation tanks), and 40°C, which represents the mean of the maximal air temperatures recorded at substrate level along the high-shore in Sichang Island (C. Meepoka and M.G., personal communication). Unfed (~24 h) oysters (valve length 18.3± 0.1 mm, range 14.0–24.0 mm) were emersed by removing the water from the aquaria with an immersion pump to minimise disturbance. During emersion, to accurately regulate air temperature close to the substratum, three natural daylight UVA heat lamps (75 W; Model Repti Zoo, Italy) were placed at different heights above the animals. This setup allowed the oysters to reach the experimental temperatures required in less than 5 min, and maintained stable (±1°C) thermal conditions for the duration of air exposure. A mercury thermometer (±0.1°C) was placed in contact with the experimental individuals to continuously monitor the ambient temperature around the shells, and micro-T-type thermocouples (La Termotecnica s.n.c., Bruino, Italy) were inserted within the valves of individuals to measure the correspondence between the oysters' body and ambient temperatures (differences between the two measurements were <0.5°C). Immediately after the end of the 4 h emersion, oysters from the two treatments were re-immersed in seawater by inverting the flow direction of the immersion pump to minimise disturbance. Two different water temperatures were adopted: 26°C (equal to the acclimatisation condition) and 35°C (mimicking the temperature that oysters encounter when the water of the incoming tide heats up on the sun-exposed rocks and first washes the oysters; M.G., personal observation) to give a two-factorial crossed experimental design (four treatments: 26°C_A–26°C_W, i.e. from 26°C during air exposure to 26°C when re-immersed; 26°C_A–35°C_W; 40°C_A–26°C_W and 40°C_A–35°C_W). These four treatments were used for all the experiments with the exception of the analysis of the lethal temperature and the first experiment of oxygen consumption rate (see below). In these two experiments, an increasing temperature ramp of 2±0.2°C h^{-1}, starting from 26°C, was applied immediately after re-immersion to approach the warming of seawater occurring when the incoming tide washes over the sun-exposed rocks (M.G., personal observation). Consequently, because animals were re-immersed in 26°C water and gradually heated, the treatments 26°C_A–35°C_W and 40°C_A–35°C_W, which represent rapid, acute increases in temperature on immersion, were omitted. Despite the fact that the ramping procedure did not accurately simulate the realistic temperature profiles that the oyster would experience during incoming tides, these assays were performed to generate thermal tolerance estimations suitable for comparative purposes (Overgaard et al., 2012; Nguyen et al., 2014). Consequently, the tolerance thresholds achieved with the ramping procedures should not be assumed as general thermal limits of *I. nucleus*.

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Mortality

Temperature-induced mortality rate and 50% lethal temperature (LT_{50}) were measured in groups of 50 oysters subjected to three different treatments: animals permanently submerged at 26°C (i.e. without simulated emersion: 26°C_W) and animals that were previously emersed for 4 h in air at 26°C or 40°C (i.e. 26°C_A–26°C_W and 40°C_A–26°C_W). When re-immersed, each group of animals was subjected to the temperature ramp starting at 26°C as described above and mortality (individuals with open valves and non-reactive to a tactile stimulus) was recorded at 1°C incremental increases in temperature (i.e. every 30 min). Dead individuals were immediately removed from the experimental tank and the experiments were terminated when all oysters had died.

Respirometric measurements

Oysters, whose shells were rubbed with 95% ethanol to remove epizoic organisms, were placed individually into respiration chambers. Respirometric measurements were carried out using the electrode micro-respiration System MRS-8 (Unisense, Aarhus, Denmark), in eight micro-respiration glass chambers containing ~4 ml of filtered (<0.45 µm) and sterilised (autoclaved) seawater. To ensure the constant mixing of the water, each chamber was stirred with a glass-embedded micro-magnet and an individual stirring device. Rates of oxygen consumption ($\dot{M}_{O_2}$) were measured with an O_2 Clark-type electrode and continuously monitored with the specific MicOx software (both Unisense). Both the oxygen consumption of the electrode and the signal drift were minimal and their interference with readings was considered insignificant (Revsbech, 1989). Calibration of the O_2 electrodes was performed at each experimental temperature in a N_2-saturated solution (0% air saturation) and aerated seawater (100% saturation). The eight respiratory chambers were placed in a common rack and immersed in a temperature-controlled water bath (type RTE 101, Neslab, USA).

Two types of $\dot{M}_{O_2}$ experiments were performed. The first was designed to evaluate the thermal tolerance of *I. nucleus* under a classical heating ramp procedure. Two groups of animals were exposed to 26 or 40°C for 4 h in air (using the heat lamps described above) and, after re-immersion, the temperature ramp was applied (see 'General experimental procedures', above). $\dot{M}_{O_2}$ was measured at every 2°C interval (i.e. every hour) until a discernible deviation from the temperature-induced exponential increase was observed. The second experiment was designed to realistically simulate the effect of different temperatures during emersion on the $\dot{M}_{O_2}$ of *I. nucleus* measured immediately after re-immersion at 26 or 35°C (i.e. an acute difference in emersion temperature, without performing a gradual thermal ramp). All four crossed treatments were performed on 16 animals (i.e. 26°C_A–26°C_W, 26°C_A–35°C_W, 40°C_A–26°C_W and 40°C_A–35°C_W, $\sum N$=2 emersion temperatures×2 re-immersion temperatures×16=64). While the first experiment provides valuable indications of the oyster's thermal tolerance for comparative purposes, the second experiment was aimed at realistically approximating the temperature fluctuations that could be experienced by *I. nucleus* in its highly variable habitat during natural tidal cycles. At the end of both $\dot{M}_{O_2}$ experiments, the flesh of individuals was excised from the valves, dried at 105°C for 24 h and reweighed. Respiration rates were reported as oxygen consumption per unit mass and expressed as µmol O_2 h^{-1} g^{-1} dry tissue.

Feeding and assimilation responses

Clearance rates of oysters were quantified by measuring the removal of algal cells (*Isochrysis galbana*) added into pre-filtered seawater (<0.45 µm, Whatmann GF/C filters, salinity 28–29‰) at a starting concentration of 25,000 cells ml^{-1}, which was the concentration at which pseudo-faeces formation and the inhibition of feeding processes were avoided (Sarà et al., 2008, 2013; Ezgeta-Balić et al., 2011). Feeding and assimilation responses were determined for all four crossed treatments (i.e. 26°C_A–26°C_W, 26°C_A–35°C_W, 40°C_A–26°C_W and 40°C_A–35°C_W) and each treatment was repeated twice using 8 animals per repetition ($\sum N$=2 emersion temperatures×2 re-immersion temperatures×2 repetitions×8=64). Eight unfed (~24 h) oysters were individually placed into separate beakers containing 500 ml of filtered seawater and a magnetic stirring bar. To assess the possible effect of sedimentation of algal cells, a beaker containing only seawater was used as a control in each treatment replicate. After ~1 h following re-immersion, the algal culture was added to each beaker. After 5 min, a 20 ml subsample was removed with a syringe from the centre of each beaker, and cell density was measured using a Coulter Counter (100 µm of aperture size) (Z2 Beckman Coulter, Inc., USA). Samples were then collected every 30 min for 2 h; cell concentrations were calculated as the mean of 3 subsample counts, and clearance rate (in l h^{-1}) was calculated as:

$$\text{Clearance rate} = V \times (c_1 - c_2)/\text{time}, \quad (1)$$

where V is the volume of water, and c_1 and c_2 are the mean cell concentrations at the beginning and end of each time increment (h).

The efficiency with which organic material is absorbed from the ingested food material (assimilation efficiency, AE) was calculated by the ratio method of Conover (1966):

$$\text{AE} = (F - E)/[(1 - E)F], \quad (2)$$

where F is the ash-free dry mass:dry mass ratio of the food ingested and E is the ash-free dry mass:dry mass ratio of the faeces.

Faeces accumulated in the beakers were collected 24 h after the start of every experimental session by filtering the water from 5 replicates (pooled in order to obtain a measurable quantity) with GF/C filters that had been washed, ashed and preweighed, and the salts washed out with 0.5 mol l^{-1} ammonium formate. Similarly, the remaining algal mass was determined from 3 replicates with the same type of filters. Filters were dried at 105°C for 24 h, weighed, and then ashed at 450°C for 4 h before being weighed a second time to calculate the mass of organic material combusted. Before each weighing, filters were cooled in desiccators to avoid changes in mass resulting from ambient humidity levels. The absorbed energy rate (J g^{-1} h^{-1}) was obtained by multiplying the maximum clearance rate by the assimilation efficiency and food concentration (mg l^{-1}).

hsp70 mRNA expression

A total of eight groups of *I. nucleus* were employed to quantify *hsp70* mRNA expression. In addition to the four treatment groups described above (26°C_A–26°C_W, 26°C_A–35°C_W, 40°C_A–26°C_W and 40°C_A–35°C_W, N=8), four further treatments were applied, with individuals (N=8) kept for 4 h in air at 26 and 40°C, to check for the activation of *hsp70* expression following exposure to air. A further two samples acted as controls and were taken from the remaining oysters left in seawater at 26°C and collected at the beginning (N=4) and at the end (N=8) of the experiment to check for possible effects of manipulation or any temporal variation in *hsp70* expression.

For all specimens, the entire flesh of individuals was immediately excised from valves manually opened with a scalpel, and then immersed in 2 ml RNA*later* (Qiagen, Austin, TX, USA). All

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samples were left at 4°C for 24 h and subsequently stored at −80°C until analysis. Total RNA was isolated from ∼30 mg of tissue using Eastep Universal RNA Extraction Kit (Promega, USA) and quantified using NanoDrop ND-1000 (Thermo, USA); 1 µg of total RNA was used as the template for synthesis of the first strand of cDNA using PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). Four degenerate primer pairs [Table S1; SeaActinF and SeaActinR (Clark et al., 2008), CAL5 and CAL7 (Duda and Palumbi, 1999), BtubF1 and ABtub2r (Einax and Voigt, 2003), 18SF and 18SR] were used to amplify the *β-actin*, *calmodulin*, *β-tubulin* and *18S* rRNA gene; and one degenerate primer pair (dAIHSP70F and dAIHSP70R) was used to amplify the *hsp70* gene (Song et al., 2006; Table S1). A 486 bp partial *β-actin* gene (GenBank accession no. KJ888939), a 333 bp *calmodulin* gene (GenBank accession no. KJ88894), a 1017 bp *β-tubulin* gene (GenBank accession no. KJ888941), a 869 bp *18S* rRNA gene (GenBank accession no. KJ888943) and a 627 bp partial *hsp70* gene (GenBank accession no. KJ888940) were amplified from *I. nucleus*. The real-time PCR primers (Table S1) were designed based on these sequences using Beacon Designer 7 software (Premier Biosoft International, USA). Real-time PCR was carried out on a CFX96 Real-Time PCR System (Bio-Rad, USA) in a 20 µl reaction volume containing 10 µl of master mix (DyNAmo Flash SYBR Green qPCR Kit, Thermo Scientific, USA), 0.8 µl of each primer (10 nmol µl^{-1}), 1 µl of cDNA template and 7.4 µl of RNase-free water. PCR conditions were: 95°C, 7 min; 40 cycles of 95°C, 20 s; 60°C, 1 min, with a final dissociation curve step.

All samples were measured in triplicate. The relative expression of *hsp70* was analysed using Bio-Rad CFX Manager 3.1 software (Bio-Rad, USA), with *β-actin*, *β-tubulin*, *calmodulin* and *18S* rRNA selected as the reference genes.

Data analysis

Statistical analyses and exponential or sigmoidal regression fits were performed with Sigmastat 3.1 and SigmaPlot 11.0, respectively (SSPS Inc., Point Richmond, CA, USA). Non-linear regressions, calculated using a 3-parameter sigmoidal equation [$y=a/(1+e^{-(x-x_0)/b})$], and exponential growth function ($y=y_0+ae^{bx}$) were used to fit the temperature-dependent mortality and temperature-dependent $\dot{M}_{O_2}$, respectively. Critical temperatures were ascribed to the last temperatures above which $\dot{M}_{O_2}$ significantly deviated from the exponential increase (Sokolova and Pörtner, 2003; Giomi and Pörtner, 2013). For analyses of $\dot{M}_{O_2}$, feeding process traits and *hsp70* mRNA expression, data were compared using one-way ANOVA with treatment as a fixed factor and Tukey's *post hoc* tests.

RESULTS

Mortality rate and lethal temperature

All *I. nucleus* survived water temperatures from 26 to 41°C. As temperatures increased further, mortality increased, showing different rates among the three experimental treatments (Fig. 1). The onset of mortality and the LT_{50} in the permanently submerged samples were 42 and 48°C, respectively. In the sample emersed in air at 26°C, the onset of mortality remained at 42°C, while the LT_{50} extended up to 49.5°C. These thresholds further rose in the sample emersed in air at 40°C, which showed onset of mortality at 47°C and a LT_{50} of 53°C. The shape of the curves also varied greatly among experimental treatments, with an acute, steep curve in the samples emersed at 26°C, gradually becoming less acute for the samples emersed at 26°C and becoming even more gentle for the samples emersed at 40°C.

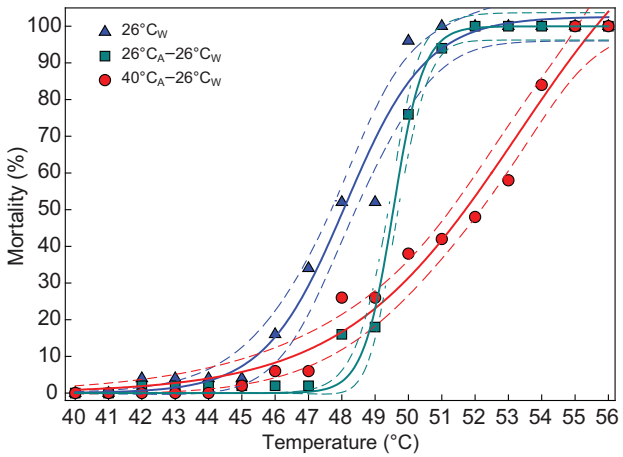

Fig. 1. Temperature-dependent mortality of *Isognomon nucleus* measured along a 2°C h^{-1} thermal ramp. The lethal temperature threshold (LT_{50}) for individuals continuously immersed in water at 26°C is 48°C (blue triangles). In contrast, following 4 h emersion at 26°C before re-immersion, the LT_{50} rises to 50°C (green squares) and further increases to 53°C (red circles) when oysters undergo emersion at 40°C. Each curve was extrapolated using 50 individuals. Regressions were performed with 3-parameter sigmoidal equations and the dashed lines represent the 95% confidence intervals for each regression.

Temperature-dependent rate of oxygen consumption

Oysters that underwent an acute thermal stress during emersion displayed an enhanced thermal tolerance following re-immersion (Fig. 2). Respiration rate ($\dot{M}_{O_2}$) showed a temperature-induced exponential increase within the range 27–39°C in animals emersed at 26°C ($y=-8.6907+3.2707^{(0.0621x)}$, $r^2=0.9988$, $P<0.001$) while this range was extended to 41°C in animals emersed at 40°C

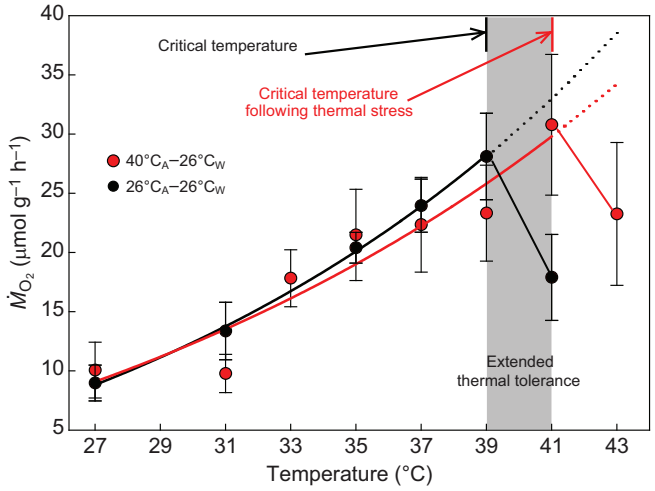

Fig. 2. Temperature-dependent rate of oxygen consumption ($\dot{M}_{O_2}$) of *I. nucleus* measured along a 2°C h^{-1} thermal ramp. The metabolic response of oysters initially emersed in air at 26°C rises exponentially during the progressive increase of temperature on re-immersion and is sustained up 39°C, where the $\dot{M}_{O_2}$ rapidly decreases [$y=-8.6907+3.2707^{(0.0621x)}$, $r^2=0.9988$, $P<0.001$]. This threshold represents the critical temperature and indicates the failure of compensatory mechanisms supporting the thermal response. In contrast, when exposed to warmer air (40°C), animals exhibit a higher critical temperature, indicating an increase of thermal tolerance up to 41°C [$y=-9.5423+4.4081^{(0.0534x)}$, $r^2=0.9085$, $P<0.01$] (means±s.e.m., $N=16$).

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(y=−9.5423+4.4081$^{(0.0534x)}$, r^2=0.9085, P<0.01). A further increase of temperature resulted in a marked reduction of $\dot{M}_{O_2}$ in both groups, indicating that the critical thermal threshold for aerobic metabolism had been reached.

$\dot{M}_{O_2}$ was, however, similar in all groups of animals when oysters were not subjected to an extended thermal ramp, with the exception of those re-immersed at 35°C after exposure in air at 26°C (Tukey's tests following one-way ANOVA, $F_{7,127}$=31.8, P<0.001; Fig. 3). Indeed, this treatment showed a pronounced increase of $\dot{M}_{O_2}$, indicating that the largest thermal response occurs when immersion at high temperature is not preceded by acute heat exposure during emersion in air. In contrast, the lowest $\dot{M}_{O_2}$ values were recorded in oysters that had previously experienced emersion at 40°C, whether they were re-immersed at 35°C or at 26°C.

Feeding and assimilation responses

Clearance rate

The clearance rate measured in animals that experienced emersion at 26°C showed a similar trend, independent from the temperature of re-immersion (Fig. 4A). In these two treatments, clearance rates remained low initially, with an evident increase in the last 30 min, but with much higher values compared with those of animals previously emersed at 40°C. After exposure to 40°C air, animals showed very low (re-immersed at 35°C) or even negligible (re-immersed at 26°C) clearance rates during the first 90 min, followed by a progressive recovery after 120 min (Fig. 4A).

Assimilation efficiency

AE was higher in oysters previously emersed at 40°C than at 26°C. The absence of pseudo-faeces during the experiment indicated that the concentration of microalgae was below the threshold of satiation (Fig. 4B).

Absorbed energy rate

Oysters emersed in air at a lower temperature (26°C) optimised the energy absorbed in comparison with those exposed to higher temperatures (40°C; Fig. 4C).

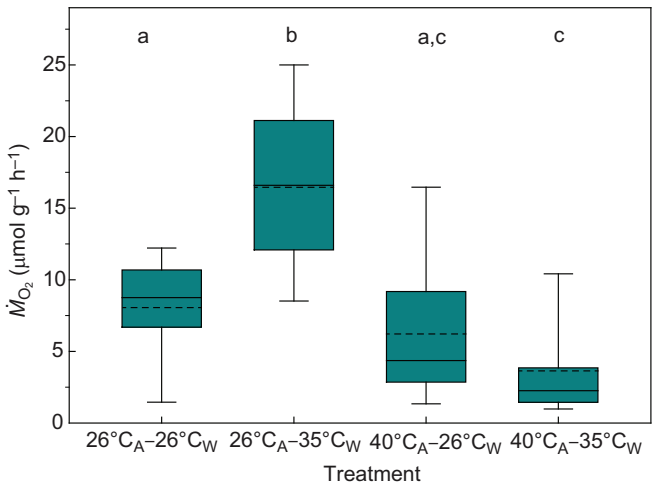

Fig. 3. $\dot{M}_{O_2}$ of *I. nucleus* re-immersed in water at 26 or 35°C after 4 h of exposure in air at 26 or 40°C. The boxes enclose the first and third quartiles; the ends of the whiskers represent the 5th/95th percentiles, the solid lines the median and the dashed lines the mean. Different letters indicate statistically significant differences between treatments (Tukey's test following one-way ANOVA, N=16, P<0.05).

hsp70 mRNA expression

Isognomon nucleus showed different expression patterns of inducible *hsp70* mRNA following exposure to different treatments and temperature regimes (Fig. 5). Both control

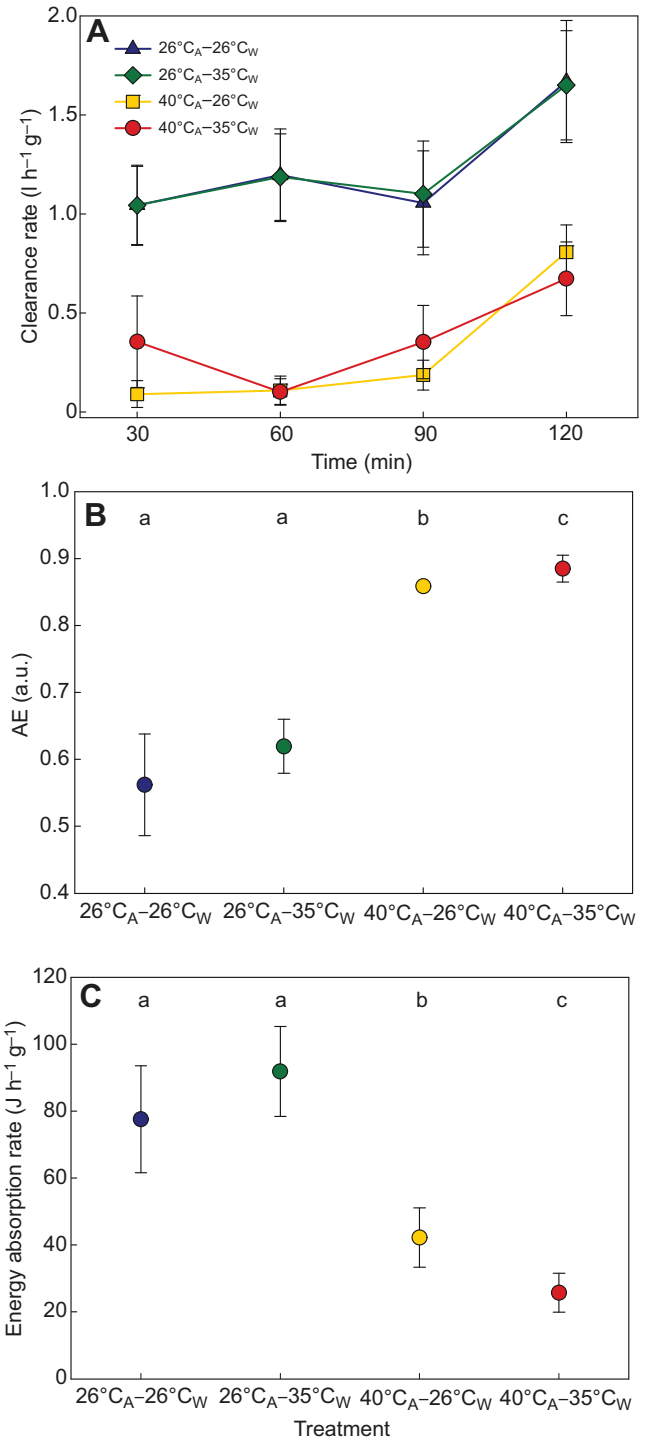

Fig. 4. Feeding responses of *I. nucleus* at 26 or 35°C after 4 h of exposure in air at 26 or 40°C. (A) Clearance rate. (B) Assimilation efficiency (AE). (C) Combining the clearance rate with the assimilation efficiency. The calculated energy absorption rate shows that oysters that do not undergo severe thermal stress perform better and enhance their energy intake from feeding. Means±s.e.m., N=16. In B and C, different letters indicate statistically significant differences between treatments (Tukey's test following one-way ANOVA, P<0.05).

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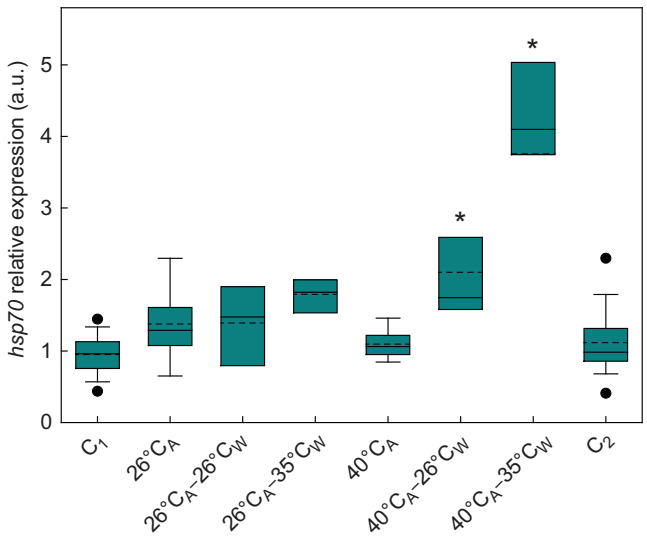

Fig. 5. Relative expression of *hsp70* RNA in *I. nucleus* following exposure to different treatments and temperature regimes. C_1 and C_2 are control animals continuously immersed at 26°C and collected at the beginning and end of the experimental trial, respectively; 26°C_A and 40°C_A are animals emersed for 4 h at 26 and 40°C, whereas 26°C_A–26°C_W, 26°C_A–35°C_W, 40°C_A–26°C_W and 40°C_A–35°C_W are the four experimental groups, emersed and re-immersed in different temperature combinations (see Materials and methods for details). The boxes enclose the first and third quartiles; the ends of the whiskers represent the 5th/95th percentiles, the solid lines the median and the dashed lines the mean. Asterisks indicate groups that are significantly different from both C_1 and C_2 controls (Tukey's test following one-way ANOVA, $P<0.05$, $N=8–32$).

groups sampled at the beginning and at the end of the experiment (26 and 40°C air-emersed groups) and the groups exposed to 26°C in air and re-immersed at 26 or 35°C showed similar low levels of *hsp70* mRNA. In contrast, when oysters were emersed at 40°C and then re-immersed at either 26 or 35°C, they showed a pronounced increase in *hsp70* expression with respect to both control groups (one-way ANOVA, $F_{7,127}=31.8$, $P<0.05$; Fig. 5).

DISCUSSION

The present study revealed unexpected responses of *I. nucleus* when exposed to acute heat stress. Variation in animal body temperatures during emersion produced a direct effect on subsequent physiological thermal responses in *I. nucleus*. The lethal temperature threshold (LT_{50}), thermal tolerance (critical temperature) and oxygen consumption rates ($\dot{M}_{O_2}$) were clearly improved in animals that previously experienced more severe heat stress. This suggests that the physiological responses activated to endure high temperatures experienced in air remain functional when animals are re-immersed. Thus, this mechanism is not only responsible for limiting and repairing heat-induced damage but also has a direct and positive effect on proximate physiological performance. Individuals emersed at 40°C also showed a higher *hsp70* expression response compared with the other treatments. The induction set-point for *hsp70* synthesis is known not only to be temperature specific but also to vary with the thermal history of the organism (Feder and Hofmann, 1999; Buckley et al., 2001; Fangue et al., 2011; Dong et al., 2014). For *I. nucleus*, it is evident that the heat-shock response (at the transcriptional level) was only activated in those individuals emersed in air at 40°C. The positive effect of this *hsp* mRNA production can be seen in oysters re-immersed at 26°C, which displayed a 2°C extension of thermal tolerance and more than a 3°C extension of lethal temperature compared with those emersed in air at 26°C, clearly illustrating the importance of recent thermal history. The heat-shock response was further magnified when oysters were re-immersed at the higher water temperature of 35°C, suggesting that the induction of *hsp70* expression increased in warmer water.

The animals emersed at 26°C and then immersed at 35°C showed a dramatic rise of $\dot{M}_{O_2}$, which probably reflects the energetic requirement to sustain the temperature-increased metabolic demand when emersed. In contrast, the $\dot{M}_{O_2}$ for animals emersed at 40°C and re-immersed at 35°C was unaffected by re-immersion in hot water. These findings suggest a possible adaptive benefit derived from the colonisation of upper shore levels and physically harsh environments. The occurrence of acute heat exposure during emersion (the 40°C treatment) appears to provide the necessary physiological pre-requisites to activate the molecular responses that are able to mitigate any heat-induced denaturation, and therefore enhances temperature-related performance when animals are re-immersed by the tide, even in warm water.

The evident benefit for thermal tolerance and performance derived from the exposure to acute heat during emersion can be related to associated costs in terms of the feeding processes and absorbed energy. It has already been established that the cascade of molecular responses activated by heat stress entails energetic costs due, for example, to the expression of repairing chaperones, *de novo* synthesis of proteins or adjustment of cellular membrane fluidity (Somero, 2002). The costs associated with the thermal response and the extended heat tolerance in *I. nucleus* show a similar pattern at a functional level. Oysters previously emersed at 26°C displayed a much higher clearance rate with respect to those emersed at 40°C, regardless of the temperature of the water that they were re-immersed in. Importantly, oysters emersed at 40°C were able to partially compensate for their low clearance rate by optimising their assimilation efficiency. The combination of these two functional parameters, however, shows that the oysters emersed at lower temperatures are able to maximise their energy absorption as compared with those emersed at 40°C, indicating a clear trade-off between enhanced thermal tolerance, driven by the energetically costly *hsp* response, and the capacity to optimise energy intake and assimilation following heat stress. Increasing water temperatures have been demonstrated to enhance predation activity and feeding rates of mobile intertidal gastropods (Yamane and Gilman, 2009). In contrast, within each air exposure treatment of the present study, feeding performance of the oyster did not differ between the two temperatures, indicating that these traits may be less susceptible to temperature change in sessile molluscs.

The evaluation of key functional responses of a tropical, intertidal oyster exposed to different thermal challenges during emersion showed evidence of costs and benefits linked to the colonisation of such an inhospitable environment. Importantly, these results illustrate that the environmental conditions previously experienced by organisms (e.g. their proximate thermal history), which are often suppressed during laboratory conditioning, have a great impact on an animal's overall performance. The importance of recent thermal history, therefore, needs to be considered when trying to reproduce ecologically relevant thermal responses in the laboratory, especially in terms of the methodologies used, as different studies have shown that measures of thermal tolerance (e.g. thermal tolerance window and the critical thermal limits) are dependent on the experimental procedures adopted to quantify them (Chown et al., 2009; Terblanche et al., 2007, 2011; Mitchell and Hoffmann, 2010;

Santos et al., 2011; Schulte et al., 2011; Overgaard et al., 2012; Ribeiro et al., 2012; Nguyen et al., 2014).

Local and regional climatic features, as well as latitudinal thermal clines, drive intertidal species distribution patterns and subsequent community structure (Helmuth et al., 2002). Indeed, species replacement along a geographic gradient often reflects differences in the local climatic conditions (Barry et al., 1995; Somero, 2002, 2010). At a smaller scale, different vulnerabilities to heat stress are associated with the relative distribution patterns along the vertical gradient of the shore, where species distributed at higher tidal heights exhibit pronounced physiological thermal adaptations (Somero, 2002; Dong et al., 2008). Such inter-specific differences in thermal tolerance highlight how high-shore species are generally able to maintain higher performance during acute warming as compared with congeneric species that populate the lower shore (Tomanek and Somero, 1999; Stillman and Somero, 2000; Harley and Helmuth, 2003; Seabra et al., 2011). These patterns of different thermal tolerance also occur at the intra-specific level and are responsible for disjunct distributions and local population extinctions in response to climatic anomalies and shifts of species ranges (Helmuth and Hofmann, 2001; Helmuth et al., 2011; Somero, 2010; Sorte et al., 2010). The present results further downscale, within the same population, the variability of organismal thermal responses as a reflection of different individual thermal histories.

In conclusion, the present study illustrates the necessity of investigating the realistic thermal history of organisms to accurately interpret their responses to climatic patterns and subsequent consequences. Firstly, contrary to what has been proposed for algae (Limberger et al., 2014), thermal history shapes the heat response of ectotherms and drives trade-offs between costs and benefits of acute heat exposure. Secondly, colonisation of harsh environments appears to infer an adaptive value with a magnified resilience to climatic variability. Natural habitats, such as intertidal coasts, are not homogeneous in terms of temperature profiles, and thermal niches of different breadth and variance co-occur at small spatial scales (Williams and Morritt, 1995; Helmuth, 1998; Helmuth et al., 2006; Chapperon and Seuront, 2011; Fusi et al., 2016). Consequently, individuals co-habiting the same general environment but exposed to such spatial and temporal complexity at different scales may well experience very different microclimatic conditions (Denny et al., 2011). The ability within a population of individuals to tolerate such harsh conditions, through adequate systemic or molecular responses, will provide these individuals with a competitive benefit in terms of tolerance and resilience, which may become fixed within the population. Thirdly, the importance of proximate thermal history in terms of functional traits (e.g. metabolic costs and feeding performances) needs to be integrated into modern bioenergetics frameworks, especially those based on functional trait mechanistic models (e.g. dynamic energy budget models; Kooijman, 2010; Sarà et al., 2014). These models integrate characteristics of the species' niche (Kearney and Porter, 2009; Sarà et al., 2013) with life history traits such as fecundity, body size and growth rate, and can be used to generate predictions about the potential distribution and physiological responses of a species in the light of climate change. The incorporation of information of a species' recent thermal history would, therefore, improve the accuracy of estimates of an individual's thermal responses and hence increase the reliability of predictions generated by mechanistic dynamic energy budget-based species distribution models (Sarà et al., 2013). Fourthly, to accurately forecast species resilience and changes in distribution, the present results showcase the need to downscale our perspective from large scale macro-physiological patterns to local microclimatic levels, generated at the scale appropriate to each species and community (Helmuth et al., 2006, 2014; Harley, 2008; Seabra et al., 2011; Potter et al., 2013). Specifically, in the case of intertidal habitats, substratum slope, orientation and topography operate to create a complex thermal landscape and determine the degree of thermal exposure of intertidal species (Williams and Morritt, 1995; Burrows et al., 2008; Harley, 2008; Helmuth et al., 2011; Seabra et al., 2011; Stafford et al., 2015; Fusi et al., 2016). Individuals that have settled on sun-exposed or shaded surfaces are, for example, subjected to different thermal gradients over a very small scale, even down to centimetres (Williams and Morritt, 1995; Helmuth and Hofmann, 2001; Chapperon and Seuront, 2011; Seabra et al., 2011) and this may reflect differential mortality of individuals when anomalous or extreme climatic events occur (Chan et al., 2006; Ngan, 2006; Harley, 2008; Schneider et al., 2010; Williams et al., 2011; Cartwright and Williams, 2014).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
F.G. conceived the study and drafted the paper. M.G. collected the animals and F.G., C.M. and G.-D.H. performed the experiments. F.G., Y.-W.D., G.A.W. and G.S. contributed substantially to the analyses and interpretation of the data and to the critical revisions of the intellectual content of the paper. All authors contributed to the writing of the final version the manuscript.

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Table S1. primers designed for gene clone and real-time PCR

Primer name	Primer Sequences (5' - 3')	Amplicon size (bp)	PCR efficiency	Source
Gene clone				
SeaActinF	ACCGACTACYTSAKKAAGATCCT	486		Clark et al. 2008
SeaActinR	GAVGCVAGGATGGAGCCRCC			
dAIHSP70F	CAGGAATTCAARCGYAAACAC	627		Song et al. 2006
dAIHSP70R	TTGGTCATKGCTCGYTCTCC			
CAL5	TTYGACAAGGAYGGHGATGG	333		Duda and Palumbi 1999
CAL7	TCDGCYTCNCKRATCATYTCRTC			
BtubF1	CAGGCYGGNCAGTGYGGHAACCAGATTGG	1017		Einax and Voigt 2003
ABtub2r	GTTGTTNGGGATCCAYTCSACGAA			
18SF	TCTWTCAARTGTCTGCCCTAT	869		Self designed
18SR	CTTCCGTCAATTCCTTTAAGTT			
Real-time PCR				
qACTIN-F	AGAGACATCAAGGAGAAG	193	1.983, R^2=0.998	Self designed
qACTIN-R	TACCAGCAGATTCCATAC			
qHSP-F	GACAAGTCAGAGGAGGTT	151	1.945, R^2=0.999	Self designed
qHSP-R	CAGAGTATGTGGTGAAGGT			
qCAL-F	GACTTCCCTGAGTTTCTGACCAT	106	2.003, R^2=0.980	Self designed
qCAL-R	AGCCATCGCCATCCTTGT			
qBTUB-F	TACAATGAAGCCACAGGAG	210	1.959, R^2=0.999	Self designed
qBTUB-R	AACAACATCTAATACGGAGTCT			
q18S-F	CGCTTCGCCTGAATAATG	211	1.905, R^2=0.999	Self designed
q18S-R	CCTCTGACTATCGTTCTTGA			