Biological Invasions

Present and future invasion perspectives of an alien shrimp in South Atlantic coastal waters - an experimental assessment of functional biomarkers and thermal tolerance

--Manuscript Draft--

Manuscript Number:	BINV-D-17-00556R2
Full Title:	Present and future invasion perspectives of an alien shrimp in South Atlantic coastal waters - an experimental assessment of functional biomarkers and thermal tolerance
Article Type:	Research paper
Keywords:	tropical shrimp; invasive species; warming oceans; rocky reef; thermal biology; stress physiology
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Funding Information:		
	Fundação para a Ciência e a Tecnologia (SFRH/BD/92975/2013)	Ms Carolina Madeira
	Fundação para a Ciência e a Tecnologia (SFRH/BD/109618/2015)	Ms Vanessa Mendonça
	Fundação para a Ciência e a Tecnologia (SFRH/BPD/115298/2016)	Dr Miguel C. Leal
	Fundação para a Ciência e a Tecnologia (PTDC/MAR-EST/2141/2012)	Not applicable
	Fundação para a Ciência e a Tecnologia (UID/MAR/04292/2013)	Not applicable
	Fundação para a Ciência e a Tecnologia (PT) (UID/Multi/04378/2013)	Not applicable

Abstract: Climate change, particularly ocean warming, is thought to benefit the spread of invasive species due to their increased tolerance to temperature fluctuations as compared to native species. The physiological tolerance of invasive species as a potential mechanism driving invasion success is therefore a subject that merits further study. Specifically, we need to adequately evaluate the potential of species invasions under changing environmental conditions, so that adequate preventive measures can be taken to minimize any impacts on coastal ecosystems. Here, we experimentally evaluated the physiological responses of a recent invader in the Southern Atlantic, the shrimp Lysmata lipkei, under a warming ocean scenario. Adult shrimps were collected

	from rocky shores in southeastern Brazil and subjected to experimental trials under a control and a +3˚C scenario. Molecular biomarkers (in gills and muscle), upper thermal limits, acclimation response ratios, thermal safety margins, mortality rates, estimates of body condition and energy reserves were measured over one month. Results suggest that higher temperatures elicit physiological adjustments at the molecular level, underpinning a high thermal tolerance. In addition, results indicated substantial acclimation capacity, with no evidence of decreased performance under an ocean-warming scenario. Thermal safety margins were low for shrimp from intertidal rock pools but high for shrimp from subtidal habitats. We conclude that the thermal tolerance of this shrimp species may favor its ongoing invasion along the Southwestern Atlantic Ocean, mainly in subtidal habitats, both under present and future thermal conditions.
Response to Reviewers:	RESPONSE TO REVIEWERS: Reviewer #1: Comment #1: The authors should update the reader about the new predictions of magnitude and duration of heat waves with climate change (see Perkins-Kirkpatrick, S. E., & Gibson, P. B. (2017). Changes in regional heatwave characteristics as a function of increasing global temperature. Nature Publishing Group, 7(1), 12256.) Response #1: We understand the reviewer's comment, but this study only focuses on the effects of gradual warming on the shrimp's physiology. To avoid any misunderstanding, we decided to delete any references to extreme events or heat waves. In this sense, we only added another few sentences that update the reader on gradual warming predictions: "The world's oceans have been warming at alarming rates over the past four decades (up to 0.5ºC.decade-1 in certain oceanic locations; Varela et al. 2018) and driving major shifts in species distributions (Loarie et al., 2009; Hoefnagel and Verberk, 2016)." (page 4, lines 77-79); and "By the year 2100, models predict that water temperatures in the Southern Atlantic will increase by 3˚C (IPCC 2013), resulting in a summer average of 27˚C to 29˚C in subtidal areas and an average temperature of 32˚C in the intertidal area of São Sebastião" (page 19, lines 464-467). Comment #2: It is not clear to me what the authors are seeking? To assess the response when the climate will be warmer or to see how the species is dealing with heat waves. If it's to assess the warming climate option (from what I understood), then the increase of LPO (and the absence of any defense system markers) at the end of the 28 days is an indicator of oxidative stress, meaning that the species is not coping well. Therefore I would not say that the species is dealing well and have the potential to invade more. The fact that no mortality was observed and no effect was shown at the organismal-level is surely a matter of time. Therefore, I do not agree with the conclusion of the study. Response #2: As we mentioned in the response to comment #1, we removed from the manuscript any reference to extreme events or heat waves in order to make it clearer to the reader that this study focuses on gradual ocean warming predictions. We also rephrased/added information in objectives section to address the reviewer's comment: "Here, we investigated the physiological responses of the invasive shrimp Lysmata lipkei in present-day conditions as these may already favor the establishment of heat tolerant species in subtropical areas, as well as tested the responses of this shrimp to chronic heat stress, adjusted to the gradual warming projected for Southeastern Brazil by the end of this century" (pages 5-6, lines 124-128). We also understand the reviewer's concern about the conclusion of the study. However, there is a significant amount of literature that suggests that lipid peroxidation may not always indicate deleterious effects, but may just be a consequence of increased respiration rates and consequent higher flux of ROS (see list of references bellow), and may even in some cases induce an adaptive response. In respect to this, there should be a threshold level below which LPO levels are important for physiological functions in biomembranes (e.g. protective function as a signalling molecule stimulating gene expression and cell survival), and above which LPO levels are undoubtedly deleterious leading to membrane damage and pathological conditions (cytotoxic role, inhibiting gene expression and promoting cell death). In order to be sure whether the LPO levels in this study are being deleterious to the organ/whole organism, apoptosis or necrosis levels would need to have been measured or observed (which is no longer possible as we do not have samples prepared for specific apoptosis assays or histology techniques). As a consequence, and by interpreting biomarker results of our study,

both hypothesis (the increase in LPO being a reflection of increased metabolism or having deleterious consequences) are plausible but whole body results suggest no performance decrements. So we further developed an explanation for our hypothesis as well as added the reviewer's hypothesis to the text as an alternative explanation, as it is still possible that it is only a matter of time before changes in condition indicators are observed, and only longer experimental trials would be able to detect a significant shift in growth or energy balance (page 18, lines 427-443): "LPO results combined with an absence of changes in growth, lipid reserves or mortality after chronic warming suggest that LPO is likely functioning as a cell signaling mechanism in L. lipkei, which may in the long-term induce an adaptation process (Niki 2012; Ayala et al. 2014). Alternatively, longer experimental trials are needed to detect deleterious effects of LPO in condition indicators. Current studies on the dynamics of shifts in the lipid composition of cell membranes suggest that temperature increase affects the balance between long-chain polyunsaturated fatty acids (PUFAs) and small-chain saturated fatty acids (SFA) (Imbs and Yakovleva 2012). Thermal stress may induce a reduction in multiple PUFAs over time, leading to structural modifications in bio-membranes and causing leakiness (Hillyer et al. 2017). Additionally, long-term changes in overall respiration or mitochondrial membrane composition/density can affect LPO, which might explain the LPO increase solely after 28 days of exposure to higher temperature. Future studies should combine histopathological analyses with LPO biochemical measurements in order to observe the damage extension of cell membranes in organs, and confirm whether those alterations are within threshold ranges of normal metabolic processes or, alternatively, if they indicate apoptosis and/or necrosis (Madeira et al. 2014)."
References:
• Ayala A, Muñoz MF, Argüelles S (2014) Lipid peroxidation: production, metabolism and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative Medicine and Cellular Longevity 2014, Article ID 360438, 31 pages
• Kagan VE (2018) Lipid peroxidation in biomembranes. Chapter 4: The role of LPO in normal physiological processes. CRC Press, 189 pp
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Comment #3: Some of the information given in the methods and results should be given in the introduction section (Lines 153-159; 338-355: all literature data are not results, results are the temperature data from the tide pool sensors and from the satellite).
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Comment #4: Line 240: anti-oxidant should be replace by antioxidant in all the text
Response #4: This was corrected throughout the manuscript (page 4, line 95; page 9, line 219; page 17, line 414; page 19, line 447; page 29, line 698; page 29, line713).

Comment #5: Lines 293: Only one species was studied here, please correct the sentence.
Response #5: The sentence was corrected (page 11, line 269).

Comment #6: No discussion is given about the increase of AChE.
Response #6: A few sentences were added to the discussion to explain the significant interaction between temperature and time in AChE, as follows: "Also noteworthy, the significant interaction between temperature and time for AChE in muscle tissue (which mostly occurs at neuromuscular junctions (Valenzuela and Akaaboune 2007)) suggests a higher rate of neurotransmission and muscle activity (contraction and relaxation). Klose and Robertson (2004) suggest that high temperatures can induce synaptic thermoprotection and increase the release of neurotransmitters in animals. In addition, this is also an indicator of higher metabolism, which may enhance performance, foraging efficiency and predator avoidance, but it may also result in more energy expenditure." (pages 17-18, lines 420-427).

Comment #7: Figure 2 should be replaced by ESM2.
Response #7: Figure 2 was removed from the main manuscript as suggested and placed in the electronic supplementary material (new Fig. ESM3). We maintained Fig. ESM2 in the supplementary material after considering comments from all reviewers, in an attempt to simplify the manuscript and reduce its length. We leave the final decision to the editor on whether to include fig. ESM2 in the manuscript, but we don't think it is essential as that information is also described in the methods section and we don't want to duplicate it in a figure.

Comment #8: Figure 5 should replace Figure 7.
Response #8: We thank the reviewer's comment. However, figure 5 is limited to biomarker results, and the study included many more physiological parameters than biomarkers alone. Instead, we introduced some changes to figure 7 in order to include more details, and uploaded it as a graphical abstract. Figure 5 was removed from the manuscript and is now in the electronic supplementary material as fig. ESM7.

Comment #9: Figures ESM5 and ESM6 should be in the text to replace the figure 5.
Response #9: Corrected as suggested (new figure 4A-D, see caption in page 38-39, lines 935-941): "Fig. 4 Biomarker behavior (mean+SD) along time (one month) under two temperature treatments (29ºC and 32ºC): A) Hsp70 (heat shock protein 70 kDa) and Ub (ubiquitin); B) CAT (catalase) and LPO (lipid peroxides), C) GST (glutathione-S-transferase) and SOD (superoxide dismutase), D) AChE (acetylcholinesterase – only measured in muscle). Numbers next to letters stand for (1) gills and (2) muscle. Significant differences (p-value ≤ 0.05) from the control group are presented with colored asterisks (*)."

Reviewer #3

Comment #1: First, I request including the original data of the biochemical indicators, e.g., bar graphs for all indicators and all time points for both gill and muscle tissue. While I enjoy the cartoons, an original research paper should include the graphs of the original data (they are exceptions but not in this case).
Response #1: The original biomarker data (bar graphs with +SD and asterisks for significant differences) are now included in the main manuscript (new figure 4A-D, see caption in page 38-39, lines 935-941; see also response to comment #9 of reviewer 1).

Comment #2: Second, several comparative studies have shown that more heat tolerant species, among them intertidal species, generally show less plasticity in their acclimation response than species from less extreme thermal environments, such as the subtidal, for both crustaceans and gastropods. This would mean that a subtropical to tropical intertidal species like L. lipkei should be less capable of tolerating fluctuating thermal conditions that trigger thermal acclimation than a similar species that is limited to the subtidal. The results of this study suggest that this may not hold true for L. lipkei. It may be important to refer to these studies and discuss them in the context of the study's results to highlight the novelty of their findings.
Response #2: We thank the reviewer for the suggestion and we added a few sentences to the discussion focusing on this aspect of the results (page 19-20, lines 464-475): "By the year 2100, models predict that water temperatures in the Southern Atlantic will increase by 3˚C (IPCC 2013), resulting in a summer average of 27˚C to 29˚C in subtidal areas and an average temperature of 32˚C in the intertidal area of São Sebastião, which is still well below the CTMax estimate for this species. The high acclimation capacity of L. lipkei to increased temperature over long exposure times indicates that not only is this species capable to accommodate future temperature change, but also that thermal history may push upper thermal limits beyond, as already documented for other crustaceans (Ober et al. 2016). According to Ravaux et al. (2016), the ARR value of 0.6 obtained for L. lipkei does represent a genuine acclimatory capacity. Our results thus challenge previous findings suggesting a poor acclimation capacity in thermal tolerant species, such as intertidal ones (Stillman 2004; Ravaux et al. 2016; Madeira et al. 2018b)."

Comment #3: The authors did not adequately discuss why the acclimation response is different at day 7 and 28 IMHO. Why is there a second phase of the response on day 28 that is limited to lipid peroxidation? How does this correlate with the temporal

dynamics of shifts in the lipid composition of cell membranes? Or is it more likely that longer-term changes in overall respiration or mitochondrial membrane composition or density cause this indicator to go up after 28 but not 7 days? It would be interesting to discuss the possible biochemical causes.
Response #3: We added a few sentences to the discussion explaining these results as suggested by the reviewer (page 18, lines 432-439): “Current studies on the dynamics of shifts in the lipid composition of cell membranes suggest that temperature increase affects the balance between long-chain polyunsaturated fatty acids (PUFAs) and small-chain saturated fatty acids (SFA) (Imbs and Yakovleva 2012). Thermal stress may induce a reduction in multiple PUFAs over time, leading to structural modifications in bio-membranes and causing leakiness (Hillyer et al. 2017). Additionally, long-term changes in overall respiration or mitochondrial membrane composition/density can affect LPO, which might explain the LPO increase solely after 28 days of exposure to higher temperature.”.

Comment #4: The same is the case for the differences between tissues. Why are the indicators of oxidative stress up-regulated in gill but not in muscle tissue? Is the antioxidant capacity of muscle tissue generally greater? Or is the biochemistry of gill cells more likely to produce higher levels of reactive oxygen species early on during acclimation?
Response #4: We thank the reviewer for this suggestion, and included a few sentences in the discussion about the significant differences in responses for the two tissue types (page 18-19, lines 443-448): “Finally, the different biomarker responses observed for muscle and gill tissues suggest tissue-specific bio-chemistry and function. Gill metabolism is highly aerobic and has a high rate of O2 diffusion and mitochondrial activity, which may lead to a higher level of ROS accumulation and induce a proportionally higher response of antioxidants during acclimation (Verlecar et al. 2008; Madeira et al. 2016).”

Comment #5: I assume that the authors collected the samples at a similar time during the day? Many of the indicators of oxidative and heat stress undergo endogenous (mainly circadian) rhythms and thus may differ simply because of differences in sampling time during the day of collection. The authors should confirm that they sampled during a similar time of the day on each collection day.
Response #5: Yes, samples were always collected at the same time (~9 am). This information was included in the manuscript (page 8, line 189).

Comment #6: The study could be improved if the authors also would have collected respiration rates but this may be another study.
Response #6: We agree with the reviewer’s comment, however, the budget for this project did not include the acquisition of respirometers, and so we could not measure respiration or metabolic rates. However, we are now planning a future project where we will start measuring respiration and metabolic rates in addition to all other physiological measurements we already perform at our lab, so it is definitely in our future research plans. Thank you for the nice suggestion.

Comments #7: The were some concerns about the English before and there is still room for improvement by someone with a good command of English.
Response #7: The paper was sent for an English revision.

Reviewer #4:

Comment #1: Overall, this manuscript presents useful information on the thermal tolerance of a marine invader that may be predicted to do well as temperatures rise. However, I did not feel that these data were presented as clearly as they should be. The text is too long, and the data are presented in an unnecessarily complicated way.
Response #1: The figures were changed in order to make the presentation of the data clearer (as also suggested by the other reviewers, in summary: figures 2 and 5 in the first version were sent to ESM, figure 7 was changed and uploaded as a graphical abstract, new figure 4 in the revised manuscript with original biomarker data). The manuscript was shortened (some parts of the text were rearranged, or deleted) and thoroughly revised to improve the reading flow.

Comment #2: Some of the introduction/abstract struck me as boilerplate or

unsupported - e.g. "However, the physiological tolerance of invasive species as a potential mechanism driving invasion success has been overlooked." I do not agree with this statement at all; while I do believe it's a subject that merits further study, I would like to see the manuscript more carefully represent the novelty and interest of this work.
Response #2: A significant part of the text was rearranged/changed in order to improve the introduction and discussion sections. In particular, we changed that sentence to “The physiological tolerance of invasive species as a potential mechanism driving invasion success is therefore a subject that merits further study. Specifically, we need to adequately evaluate the potential of species invasions under changing environmental conditions, so that adequate preventive measures can be taken to minimize any impacts on coastal ecosystems.” (page 3, lines 54-58). See also other examples: “Here, we investigated the physiological responses of the invasive shrimp Lysmata lipkei in present-day conditions as these may already favor the establishment of heat tolerant species in subtropical areas, as well as tested the responses of this shrimp to chronic heat stress, adjusted to the gradual warming projected for Southeastern Brazil by the end of this century.” (page 5-6, lines 124-128); “Our results thus challenge previous findings suggesting a poor acclimation capacity in thermal tolerant species, such as intertidal ones (Stillman 2004; Ravaux et al. 2016; Madeira et al. 2018b)” (page 20, lines 473-475) ; “Interestingly, climate change predictions for the study area project an increase in precipitation (Marengo 2007; PBMC, 2014) and food availability in coastal areas through runoff of terrestrial inputs, which could potentially fuel benthic energetic pathways (Mendonça et al. 2018). This is expected to favor epibenthic feeders, which include some important consumers in intertidal habitats, such as Lysmata lipkei, and many non-native species which are usually capable to exploit and often monopolize surplus trophic resources (Sorte et al. 2013).” (pages 20-21, lines 491-497); “Biomarker responses are therefore often suitable indicators to support management policies by prioritizing areas and species that need local or regional intervention. However, physiological biomarkers should ideally be used in combination with both traditional (e.g. biodiversity metrics, water-quality analyses) and recent methodological approaches (e.g. molecular markers, metabolomics and proteomics) to integrate lines of evidence determining the invasion potential.” (page 21, lines 503-508), etc…

Comment #3: There are also several studies I would have expected to see referenced, particularly Sorte et al. 2013, which presents a meta-analysis comparing native and invasive species' physiological responses to changing environmental conditions.
Response #3: We thank the reviewer for the suggestion. We now include a discussion of our results in light of the findings of Sorte et al. 2013 (page 16, lines 382-386): “This main finding is aligned to the meta-analysis results reported by Sorte et al. (2013) which analyzed performance of 150 non-native species under different environmental conditions and concluded that these organisms seem to be well-adapted and capable of thriving in warmed aquatic ecosystems.”

Comment #4: Along similar lines, I did not think the data were represented well in the figures. Many figures had an infographic-like feel (especially Fig 7), and presented surprisingly little of the actual study data. The authors have carried out a number of assays, and the figures should display far more of the data in a clear and straightforward fashion. Most of the current figures look like presentation slides, which makes it very hard for the reader to interpret since conclusions are primarily represented instead of actual data. (For instance, Figure 5 is a summary, with no error bars, etc on any numbers.) Figure 6 is good, and a good template for how the biomarker and body composition data should be presented. I think a process figure like Figure 2 would be useful, but Figure 2 as presented (output from a program?) is unnecessarily complex and difficult to follow.
Response #4: We changed several figures to comply with all of the reviewers´ suggestions. We performed some changes on fig.7 and although Biological Invasions does not have a template for graphical abstracts online or printed, we will upload it as such and leave the final decision to the editor whether to include it as a graphical abstract or as a final summary image in the main text or not include it at all. Figure 5 was sent to ESM and a new figure 4 was constructed (with bar graphs and SD for all biomarkers, temperatures, sampling times and tissues). Figure 2 (your guess is correct, it is the output of an online software suggested by FELASA for planning, constructing, and visualizing experimental design – additionally, the program performs

	several analyses on the experimental design until it is correctly validated by the software) was sent to ESM as well. Comment #5: One more specific comment I have is on the use of temperature. I appreciate that the authors are using multiple measures of environmental temperature, but I think some of the temperature data available are perhaps not accurate enough to really conduct the sort of TSM analyses the authors are attempting. (I also, frankly, found the TSM text to be difficult to follow and a detraction from the very nice thermal tolerance data presented in the manuscript.) In particular, the use of satellite data as a proxy for a subtidal temperature in TSM analyses strikes me as questionable, and I would suggest dropping this. Response #5: We appreciate the reviewer's comment. The temperature data used to calculate the maximal habitat temperature in the subtidal was actually retrieved from field probes and not from satellite data. This mistake in the manuscript text was corrected (page 11, lines 268-269; see also new Fig. ESM6 in the supplementary material with field probe temperature data for air, tide pools and subtidal). Comment #6: The manuscript needs considerable rewriting / reorganization to be suitable for publication. However, if it is shortened and the data presented in a more straightforward fashion (in both text and figures), and the introduction and discussion likewise clarified with a more careful consideration of previous work and the limits of inference for these data, I think it could be a valuable addition to the marine invasion literature. Response #6: The manuscript text was simplified, clarified; some parts were rearranged/rewritten or deleted, throughout the entire manuscript. The figures were changed according to suggestions from all reviewers. Finally, the manuscript was sent for an English revision. Please re-read the entire manuscript for an overview of all the changes made (the changes that are addressing specific scientific questions of reviewers are highlighted in yellow, however, changes in the writing style or English are not highlighted, so please re-read). Check also changes made to the electronic supplementary material.

Lisbon, 20nd of November 2018

Dear Editor,

I hereby send you the revised manuscript “Present and future invasion perspectives of an alien shrimp in South Atlantic coastal waters – an experimental assessment of functional biomarkers and thermal tolerance” to be submitted for publication in Biological Invasions.

We hope this version addresses all the issues raised by the reviewers and that you find it suitable for publication.

We thank you in advance for your attention. We’re looking forward to hearing from you.

Kind regards,

Carolina Madeira (on behalf of all authors)

RESPONSE TO REVIEWERS:

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Comment #1: The authors should update the reader about the new predictions of magnitude and duration of heat waves with climate change (see Perkins-Kirkpatrick, S. E., & Gibson, P. B. (2017). Changes in regional heatwave characteristics as a function of increasing global temperature. Nature Publishing Group, 7(1), 12256.)

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Response #2: As we mentioned in the response to comment #1, we removed from the manuscript any reference to extreme events or heat waves in order to make it clearer to the reader that this study focuses on gradual ocean warming predictions. We also rephrased/added information in objectives section to address the reviewer's comment: "Here, we investigated the physiological responses of the invasive shrimp *Lysmata lipkei* in present-day conditions as these may already favor the establishment of heat tolerant species in

subtropical areas, as well as tested the responses of this shrimp to chronic heat stress, adjusted to the gradual warming projected for Southeastern Brazil by the end of this century" (pages 5-6, lines 124-128). We also understand the reviewer's concern about the conclusion of the study. However, there is a significant amount of literature that suggests that lipid peroxidation may not always indicate deleterious effects, but may just be a consequence of increased respiration rates and consequent higher flux of ROS (see list of references bellow), and may even in some cases induce an adaptive response. In respect to this, there should be a threshold level below which LPO levels are important for physiological functions in biomembranes (e.g. protective function as a signalling molecule stimulating gene expression and cell survival), and above which LPO levels are undoubtedly deleterious leading to membrane damage and pathological conditions (cytotoxic role, inhibiting gene expression and promoting cell death). In order to be sure whether the LPO levels in this study are being deleterious to the organ/whole organism, apoptosis or necrosis levels would need to have been measured or observed (which is no longer possible as we do not have samples prepared for specific apoptosis assays or histology techniques). As a consequence, and by interpreting biomarker results of our study, both hypothesis (the increase in LPO being a reflection of increased metabolism or having deleterious consequences) are plausible but whole body results suggest no performance decrements. So we further developed an explanation for our hypothesis as well as added the reviewer's hypothesis to the text as an alternative explanation, as it is still possible that it is only a matter of time before changes in condition indicators are observed, and only longer experimental trials would be able to detect a significant shift in growth or energy balance (page 18, lines 427-443): "LPO results combined with an absence of changes in growth, lipid reserves or mortality after chronic warming suggest that LPO is likely functioning as a cell signaling mechanism in *L. lipkei*, which may in the long-term induce an adaptation process (Niki 2012; Ayala et al. 2014). Alternatively, longer experimental trials are needed to detect deleterious effects of LPO in condition indicators. Current studies on the dynamics of shifts in the lipid composition of cell membranes suggest that temperature increase affects the balance between long-chain polyunsaturated fatty acids (PUFAs) and small-chain saturated fatty acids (SFA) (Imbs and Yakovleva 2012). Thermal stress may induce a reduction in multiple PUFAs over time, leading to structural modifications in bio-membranes and causing leakiness (Hillyer et al. 2017). Additionally, long-term changes in overall respiration or mitochondrial membrane composition/density can affect LPO, which might explain the LPO increase solely after 28 days of exposure to higher temperature. Future studies should combine histopathological analyses with LPO biochemical measurements in order to observe the damage extension of cell membranes in organs, and confirm whether those

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References:

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Response #8: We thank the reviewer's comment. However, figure 5 is limited to biomarker results, and the study included many more physiological parameters than biomarkers alone. Instead, we introduced some changes to figure 7 in order to include more details, and uploaded it as a graphical abstract. Figure 5 was removed from the manuscript and is now in the electronic supplementary material as fig. ESM7.

Comment #9: Figures ESM5 and ESM6 should be in the text to replace the figure 5.

Response #9: Corrected as suggested (new figure 4A-D, see caption in page 38-39, lines 935-941): "Fig. 4 Biomarker behavior (mean+SD) along time (one month) under two temperature treatments (29ºC and 32ºC): A) Hsp70 (heat shock protein 70 kDa) and Ub (ubiquitin); B) CAT (catalase) and LPO (lipid peroxides), C) GST (glutathione-S-transferase) and SOD (superoxide dismutase), D) AChE (acetylcholinesterase – only measured in muscle). Numbers next to letters stand for (1) gills and (2) muscle. Significant differences (p-value ≤ 0.05) from the control group are presented with colored asterisks (*)."

Reviewer #3

Comment #1: First, I request including the original data of the biochemical indicators, e.g., bar graphs for all indicators and all time points for both gill and muscle tissue. While I enjoy the

cartoons, an original research paper should include the graphs of the original data (they are exceptions but not in this case).

Response #1: The original biomarker data (bar graphs with +SD and asterisks for significant differences) are now included in the main manuscript (new figure 4A-D, see caption in page 38-39, lines 935-941; see also response to comment #9 of reviewer 1).

Comment #2: Second, several comparative studies have shown that more heat tolerant species, among them intertidal species, generally show less plasticity in their acclimation response than species from less extreme thermal environments, such as the subtidal, for both crustaceans and gastropods. This would mean that a subtropical to tropical intertidal species like *L. lipkei* should be less capable of tolerating fluctuating thermal conditions that trigger thermal acclimation than a similar species that is limited to the subtidal. The results of this study suggest that this may not hold true for *L. lipkei*. It may be important to refer to these studies and discuss them in the context of the study's results to highlight the novelty of their findings.

Response #2: We thank the reviewer for the suggestion and we added a few sentences to the discussion focusing on this aspect of the results (page 19-20, lines 464-475): "By the year 2100, models predict that water temperatures in the Southern Atlantic will increase by 3˚C (IPCC 2013), resulting in a summer average of 27˚C to 29˚C in subtidal areas and an average temperature of 32˚C in the intertidal area of São Sebastião, which is still well below the CTMax estimate for this species. The high acclimation capacity of *L. lipkei* to increased temperature over long exposure times indicates that not only is this species capable to accommodate future temperature change, but also that thermal history may push upper thermal limits beyond, as already documented for other crustaceans (Ober et al. 2016). According to Ravaux et al. (2016), the ARR value of 0.6 obtained for *L. lipkei* does represent a genuine acclimatory capacity. Our results thus challenge previous findings suggesting a poor acclimation capacity in thermal tolerant species, such as intertidal ones (Stillman 2004; Ravaux et al. 2016; Madeira et al. 2018b)."

Comment #3: The authors did not adequately discuss why the acclimation response is different at day 7 and 28 IMHO. Why is there a second phase of the response on day 28 that is limited to lipid peroxidation? How does this correlate with the temporal dynamics of shifts in the lipid composition of cell membranes? Or is it more likely that longer-term changes in overall respiration or mitochondrial membrane composition or density cause this indicator to

go up after 28 but not 7 days? It would be interesting to discuss the possible biochemical causes.

Response #3: We added a few sentences to the discussion explaining these results as suggested by the reviewer (page 18, lines 432-439): "Current studies on the dynamics of shifts in the lipid composition of cell membranes suggest that temperature increase affects the balance between long-chain polyunsaturated fatty acids (PUFAs) and small-chain saturated fatty acids (SFA) (Imbs and Yakovleva 2012). Thermal stress may induce a reduction in multiple PUFAs over time, leading to structural modifications in bio-membranes and causing leakiness (Hillyer et al. 2017). Additionally, long-term changes in overall respiration or mitochondrial membrane composition/density can affect LPO, which might explain the LPO increase solely after 28 days of exposure to higher temperature.".

Comment #4: The same is the case for the differences between tissues. Why are the indicators of oxidative stress up-regulated in gill but not in muscle tissue? Is the antioxidant capacity of muscle tissue generally greater? Or is the biochemistry of gill cells more likely to produce higher levels of reactive oxygen species early on during acclimation?

Response #4: We thank the reviewer for this suggestion, and included a few sentences in the discussion about the significant differences in responses for the two tissue types (page 18-19, lines 443-448): "Finally, the different biomarker responses observed for muscle and gill tissues suggest tissue-specific bio-chemistry and function. Gill metabolism is highly aerobic and has a high rate of O_2 diffusion and mitochondrial activity, which may lead to a higher level of ROS accumulation and induce a proportionally higher response of antioxidants during acclimation (Verlecar et al. 2008; Madeira et al. 2016)."

Comment #5: I assume that the authors collected the samples at a similar time during the day? Many of the indicators of oxidative and heat stress undergo endogenous (mainly circadian) rhythms and thus may differ simply because of differences in sampling time during the day of collection. The authors should confirm that they sampled during a similar time of the day on each collection day.

Response #5: Yes, samples were always collected at the same time (~9 am). This information was included in the manuscript (page 8, line 189).

Comment #6: The study could be improved if the authors also would have collected respiration rates but this may be another study.

Response #6: We agree with the reviewer's comment, however, the budget for this project did not include the acquisition of respirometers, and so we could not measure respiration or metabolic rates. However, we are now planning a future project where we will start measuring respiration and metabolic rates in addition to all other physiological measurements we already perform at our lab, so it is definitely in our future research plans. Thank you for the nice suggestion.

Comments #7: The were some concerns about the English before and there is still room for improvement by someone with a good command of English.

Response #7: The paper was sent for an English revision.

Reviewer #4:

Comment #1: Overall, this manuscript presents useful information on the thermal tolerance of a marine invader that may be predicted to do well as temperatures rise. However, I did not feel that these data were presented as clearly as they should be. The text is too long, and the data are presented in an unnecessarily complicated way.

Response #1: The figures were changed in order to make the presentation of the data clearer (as also suggested by the other reviewers, in summary: figures 2 and 5 in the first version were sent to ESM, figure 7 was changed and uploaded as a graphical abstract, new figure 4 in the revised manuscript with original biomarker data). The manuscript was shortened (some parts of the text were rearranged, or deleted) and thoroughly revised to improve the reading flow.

Comment #2: Some of the introduction/abstract struck me as boilerplate or unsupported - e.g. "However, the physiological tolerance of invasive species as a potential mechanism driving invasion success has been overlooked." I do not agree with this statement at all; while I do believe it's a subject that merits further study, I would like to see the manuscript more carefully represent the novelty and interest of this work.

Response #2: A significant part of the text was rearranged/changed in order to improve the introduction and discussion sections. In particular, we changed that sentence to "The physiological tolerance of invasive species as a potential mechanism driving invasion success is therefore a subject that merits further study. Specifically, we need to adequately evaluate the potential of species invasions under changing environmental conditions, so that adequate

preventive measures can be taken to minimize any impacts on coastal ecosystems." (page 3, lines 54-58). See also other examples: "Here, we investigated the physiological responses of the invasive shrimp *Lysmata lipkei* in present-day conditions as these may already favor the establishment of heat tolerant species in subtropical areas, as well as tested the responses of this shrimp to chronic heat stress, adjusted to the gradual warming projected for Southeastern Brazil by the end of this century." (page 5-6, lines 124-128); "Our results thus challenge previous findings suggesting a poor acclimation capacity in thermal tolerant species, such as intertidal ones (Stillman 2004; Ravaux et al. 2016; Madeira et al. 2018b)" (page 20, lines 473-475) ; "Interestingly, climate change predictions for the study area project an increase in precipitation (Marengo 2007; PBMC, 2014) and food availability in coastal areas through runoff of terrestrial inputs, which could potentially fuel benthic energetic pathways (Mendonça et al. 2018). This is expected to favor epibenthic feeders, which include some important consumers in intertidal habitats, such as *Lysmata lipkei*, and many non-native species which are usually capable to exploit and often monopolize surplus trophic resources (Sorte et al. 2013)." (pages 20-21, lines 491-497); "Biomarker responses are therefore often suitable indicators to support management policies by prioritizing areas and species that need local or regional intervention. However, physiological biomarkers should ideally be used in combination with both traditional (e.g. biodiversity metrics, water-quality analyses) and recent methodological approaches (e.g. molecular markers, metabolomics and proteomics) to integrate lines of evidence determining the invasion potential." (page 21, lines 503-508), etc…

Comment #3: There are also several studies I would have expected to see referenced, particularly Sorte et al. 2013, which presents a meta-analysis comparing native and invasive species' physiological responses to changing environmental conditions.

Response #3: We thank the reviewer for the suggestion. We now include a discussion of our results in light of the findings of Sorte et al. 2013 (page 16, lines 382-386): "This main finding is aligned to the meta-analysis results reported by Sorte et al. (2013) which analyzed performance of 150 non-native species under different environmental conditions and

concluded that these organisms seem to be well-adapted and capable of thriving in warmed aquatic ecosystems."

Comment #4: Along similar lines, I did not think the data were represented well in the figures. Many figures had an infographic-like feel (especially Fig 7), and presented surprisingly little of the actual study data. The authors have carried out a number of assays, and the figures should display far more of the data in a clear and straightforward fashion. Most of the current figures look like presentation slides, which makes it very hard for the reader to interpret since conclusions are primarily represented instead of actual data. (For instance, Figure 5 is a summary, with no error bars, etc on any numbers.) Figure 6 is good, and a good template for how the biomarker and body composition data should be presented. I think a process figure like Figure 2 would be useful, but Figure 2 as presented (output from a program?) is unnecessarily complex and difficult to follow.

Response #4: We changed several figures to comply with all of the reviewers´ suggestions. We performed some changes on fig.7 and although Biological Invasions does not have a template for graphical abstracts online or printed, we will upload it as such and leave the final decision to the editor whether to include it as a graphical abstract or as a final summary image in the main text or not include it at all. Figure 5 was sent to ESM and a new figure 4 was constructed (with bar graphs and SD for all biomarkers, temperatures, sampling times and tissues). Figure 2 (your guess is correct, it is the output of an online software suggested by FELASA for planning, constructing, and visualizing experimental design – additionally, the program performs several analyses on the experimental design until it is correctly validated by the software) was sent to ESM as well.

Comment #5: One more specific comment I have is on the use of temperature. I appreciate that the authors are using multiple measures of environmental temperature, but I think some of the temperature data available are perhaps not accurate enough to really conduct the sort of TSM analyses the authors are attempting. (I also, frankly, found the TSM text to be difficult to follow and a detraction from the very nice thermal tolerance data presented in the manuscript.) In particular, the use of satellite data as a proxy for a subtidal temperature in TSM analyses strikes me as questionable, and I would suggest dropping this.

Response #5: We appreciate the reviewer's comment. The temperature data used to calculate the maximal habitat temperature in the subtidal was actually retrieved from field probes and not from satellite data. This mistake in the manuscript text was corrected (page 11, lines 268-

269; see also new Fig. ESM6 in the supplementary material with field probe temperature data for air, tide pools and subtidal).

Comment #6: The manuscript needs considerable rewriting / reorganization to be suitable for publication. However, if it is shortened and the data presented in a more straightforward fashion (in both text and figures), and the introduction and discussion likewise clarified with a more careful consideration of previous work and the limits of inference for these data, I think it could be a valuable addition to the marine invasion literature.

Response #6: The manuscript text was simplified, clarified; some parts were rearranged/rewritten or deleted, throughout the entire manuscript. The figures were changed according to suggestions from all reviewers. Finally, the manuscript was sent for an English revision. Please re-read the entire manuscript for an overview of all the changes made (the changes that are addressing specific scientific questions of reviewers are highlighted in yellow, however, changes in the writing style or English are not highlighted, so please re-read). Check also changes made to the electronic supplementary material.

Present and future invasion perspectives of an alien shrimp in South Atlantic coastal waters – an experimental assessment of functional biomarkers and thermal tolerance

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Running title: Effects of increased temperature on tropical alien shrimp

Acknowledgements

The present research was financially supported by the Portuguese Foundation for Science and Technology (FCT) through the WarmingWebs project (PTDC/MAR-

EST/2141/2012), the “Investigador FCT” position granted to C.V. and the strategic projects UID/MAR/04292/2013 granted to MARE and UID/Multi/04378/2013 granted to UCIBIO. Authors C.M. and V.M. acknowledge PhD grants (SFRH/BD/92975/2013 and SFRH/BD/109618/2015, respectively), and M.C.L. acknowledges a post-doc grant (SFRH/BPD/115298/2016), all granted by FCT. The funding institution had no influence in the study design, collection and analysis of datasets or in the decision to publish the work. Authors would like to thank everyone who helped during field work and experimental trials, and to a Megan Walters for proofreading.

Abstract

Climate change, particularly ocean warming, is thought to benefit the spread of invasive species due to their increased tolerance to temperature fluctuations as compared to native species. The physiological tolerance of invasive species as a potential mechanism driving invasion success is therefore a subject that merits further study. Specifically, we need to adequately evaluate the potential of species invasions under changing environmental conditions, so that adequate preventive measures can be taken to minimize any impacts on coastal ecosystems. Here, we experimentally evaluated the physiological responses of a recent invader in the Southern Atlantic, the shrimp *Lysmata lipkei*, under a warming ocean scenario. Adult shrimps were collected from rocky shores in southeastern Brazil and subjected to experimental trials under a control and a +3˚C scenario. Molecular biomarkers (in gills and muscle), upper thermal limits, acclimation response ratios, thermal safety margins, mortality rates, estimates of body condition and energy reserves were measured over one month. Results suggest that higher temperatures elicit physiological adjustments at the molecular level, underpinning a high thermal tolerance. In addition, results indicated substantial acclimation capacity, with no evidence of decreased performance under an ocean-warming scenario. Thermal safety margins were low for shrimp from intertidal rock pools but high for shrimp from subtidal habitats. We conclude that the thermal tolerance of this shrimp species may favor its ongoing invasion along the Southwestern Atlantic Ocean, mainly in subtidal habitats, both under present and future thermal conditions.

Keywords tropical shrimp, invasive species, warming oceans, rocky reefs, thermal biology

Introduction

The world's oceans have been warming at alarming rates over the past four decades (up to 0.5ºC.decade^{-1} in certain oceanic locations; Varela et al. 2018) and driving major shifts in species distributions (Loarie et al., 2009; Hoefnagel and Verberk, 2016). As a consequence, distribution gaps are being created in wide geographic areas, which have been invaded by opportunistic species (Burgiel and Muir 2010). The study of the thermal biology of invasive species is expected to elucidate the physiological mechanisms driving the establishment of non-native species in new locations as global warming continues (Kelley 2014). The physiological tolerance hypothesis (Zerebecki and Sorte 2011) predicts that the latitudinal range occupied by a given species is a surrogate of its ability to acclimate to varying thermal regimes, which may ultimately increase its potential to invade thermally vacant niche space across biogeographic regions. Different thermal environments experienced by a species along its invasion process may affect biochemical interactions, cellular metabolism (Tomanek and Somero 2002; Angilletta et al. 2006) and, organism performance (Pörtner and Farrell 2008). However, how physiology shapes biogeographical distributions of invasive species is still overlooked (Somero 2002; Cortes et al. 2016). Previous studies on marine species have shown significant effects of water temperature on heat shock protein expression (Hofmann and Somero 1995; Tomanek and Somero 2002; Shabtay and Arad 2005; Clark et al. 2008; Madeira et al. 2015b), immune and antioxidant functions (Pannunzio and Storey 1998; Pérez-Casanova et al. 2008; Madeira et al. 2013), including scavenging capacity (Yuan et al. 2013), metabolite synthesis (Barton 2002; Viant et al. 2003), metabolic performance (Magozzi and Calosi 2015; Payne et al. 2016), physio-ecological short-term processes (e.g. rates of ingestion, defecation, respiration and excretion) (Yuan et al. 2009, 2013), and long-term processes, such as growth (Angilletta

2004; Rushworth et al. 2011; Ye et al. 2011). As such, the successful establishment of non-indigenous species will require the tolerance of a possibly different range of temperatures (and varying rates of temperature change) in the new environment through physiological adjustments (Kelley 2014). In other words, high acclimation and adaption abilities are required for a species to be able to invade a new habitat or geographic region. In particular, Kelley (2014) and Lejeusne et al. (2014) proposed that, compared to native species, invasive species already distributed over a large latitudinal range are expected to (i) be more resistant to acute thermal stress owing to previous adaptation to broad geographic thermal widths over evolutionary or historical time, (ii) show lower rates of oxygen consumption and consequent greater resistance to environmental stresses, and (iii) have higher survival rates under chronic stress due to higher acclimation capacity. The identification of physiological phenotypes of successful invaders can contribute to recognize key features that predispose certain species to become established in new habitats (Kolar and Lodge 2001; Kelley 2014).

The caridean shrimp *Lysmata lipkei* (as described by Okuno and Fiedler, 2010) is native to the West Pacific region and has recently invaded the tropical and subtropical Western Atlantic (Pachelle et al. 2016). Specifically, it is considered an exotic species along the Eastern coast of Brazil where it can be found in tide pools or shallow waters (~15m depth) (Pachelle et al. 2016). Since this species is fairly recent to science, it is useful to gather specific information on its thermal biology and physiology to monitor the expansion of its invasive populations in the wild. Additionally, experimental trials are needed to understand the ability of *Lysmata* shrimps to become invasive under present and future thermal conditions predicted under consensual climate change scenarios.

Here, we investigated the physiological responses of the invasive shrimp *Lysmata lipkei* in present-day conditions as these may already favor the establishment of heat tolerant

species in subtropical areas, as well as tested the responses of this shrimp to chronic heat stress, adjusted to the gradual warming projected for Southeastern Brazil by the end of this century. In this research, we followed a multi-parameter approach (molecular, cellular and whole-body) to detect metabolic changes and focused on alterations from control (current summer SST) to future temperature, derived from gradual warming by 2100 (+3ºC SST anomaly). We maintained individuals under experimental temperature conditions and (i) compared selected biomarkers that modulate stress responses in different tissues (gills and muscle) over exposure time in both temperature settings, and (ii) compared upper thermal limits, acclimation ability and thermal safety margins, as well as (iii) performance parameters growth, body condition, accumulation of energy reserves and mortality rates at the end of exposure time in both thermal treatments.

Materials and methods

Ethical guidelines

The experiments here performed were approved by the competent authorities both in Portugal and Brazil (Direcção Geral de Alimentação e Veterinária and Comissão de Ética no Uso de Animais, respectively, authorization documents 0421/000/000/2013 and 13.1.981.53.7). The experimental design followed ARRIVE guidelines as well as directions by the Federation of European Laboratory Animal Science Associations (see Festing and Altman, 2002; Hau and Schapiro, 2010).

Animal collection and field temperature measurements

The biological model used in this study was the shrimp species *Lysmata lipkei*. Wild specimens (n = 120, mean ± SD total length = 4.04 cm ± 1.06 cm, mean ± SD total

151 weight = 1.01 g ± 0.83 g) were collected with hand nets in shallow waters (<50 cm) of
152 intertidal rocky reefs in Southeastern Brazil (23˚49'42''S, 45˚26'29''W, Barequeçaba
153 Beach, São Sebastião, São Paulo, Fig. 1B). Animals were transported to the marine field
154 station (CEBIMar - Universidade de São Paulo, S. Sebastião) in plastic containers with
155 aerated sea water where they were allowed to acclimate for two weeks at local seawater
156 temperature (29.0˚C ± 0.5). Shrimp welfare was assessed daily (e.g. parasites, lesions,
157 feeding behavior) and animals were fed frozen food daily *ad libitum*.
158 The thermal environment of this species was assessed at the initial introduction
159 locations (northeastern Brazil – Ceará and Rio Grande do Norte, Pachelle et al. 2016,
160 Fig. 1B) and at the study location (S. Sebastião, Brazil, Fig. 1B). The temperature at this
161 species' original geographic occurrence (Japan) was also analyzed (Fig. 1A). Data were
162 collected from several sources, including: i) sea temperature database (SST data
163 available from www.seatemperature.org); and ii) Hobo V2 probes placed at the study
164 site (S. Sebastião), glued to rocks of 8 replicate tide pools during low tide, using epoxy
165 polyamide cement Tubolit®. *In situ* temperature data was recorded continuously during
166 the warm season months (from December to February, 56 days) in 2015/16.
167 Measurements were taken at intervals of 2 hours, 24/7.
168
169 Experimental layout
170 The experimental trials in captivity were performed in two separate semi-open aquaria
171 systems of 200 L each under natural light conditions (14L:10D) but no direct sunlight.
172 Each system consisted of 2 replicate glass tanks (25 × 25 × 25 cm), one seawater
173 deposit, one sump, and a UV filter (see Fig. ESM1). After acclimation, *L. lipkei*
174 specimens were randomly allocated into experimental tanks (n = 30 individuals.tank^{-1}),
175 which were then randomly attributed to either the control or the +3ºC treatment (2

176 replicate tanks per temperature). Temperature was steadily increased (0.10˚C.hr^{-1}) to
177 treatment conditions: a) control (29.0˚C ± 0.5˚C), reflecting current mean summer water
178 temperature at the collection site, and b) thermal challenge (+3ºC; 32.0˚C ± 0.5˚C),
179 corresponding to projected mean summer water temperature for the tropical/subtropical
180 Atlantic by 2100 (gradual warming scenario RCP8.5, IPCC 2013). Tanks were supplied
181 with filtered seawater (35) and aeration allowing oxygen saturation (95 to 100%).
182 Environmental enrichment was achieved by adding small cobbles to tanks. Thermostats
183 were used to hold temperature constant for 28 days (Eheim® Jager Heater 150W,
184 Germany). Sampling was performed at several timepoints (see Fig. ESM2-ESM3 in the
185 electronic supplementary material) by euthanizing the individuals through longitudinal
186 transection. Sample size varied within the ranges reported in previous studies (Madeira
187 et al. 2012b, 2016, Vinagre et al. 2013):
188 i) For molecular biomarker analyses, animals were sampled once a week, at day 0, 7,
189 14, 21 and 28 days, always at the same period of the day (~ 9 am). Five individuals
190 were randomly sampled from the two replicate tanks for each temperature and each time
191 point (5 individuals × 5 timepoints × 2 temperatures, total n = 50 individuals). Gill and
192 muscle tissues were frozen at -80˚C. This timeline follows OECD recommendations for
193 repeated exposure toxicity studies, and was adapted to aquatic animals.
194 ii) For the determination of upper thermal limits, a subset of individuals was randomly
195 sampled from each temperature treatment after 7 and 28 days of exposure, and
196 subsequently subjected to Critical Thermal Maximum (CTMax) trials (13-14
197 individuals × 2 timepoints × 2 temperatures, total n = 54). CTMax is a dynamic method
198 widely used for the determination of thermal limits in ectothermic animals (Kaspari et
199 al. 2015). Individuals were maintained in a thermostatic bath with a steady heating of
200 1˚C each 15 min (Fig. ESM4 in the electronic supplementary material). This heating

rate is consistent to observations in tide pools during summer days, and follows the recommendations of Vinagre et al. (2015) for the use of ecologically realistic warming ramps. All individuals subjected to one CTMax trial were then removed from the experiment.

iii) For the assessment of body condition, morphometric measurements of the animals (total length and weight, 26 individuals × 2 timepoints × 2 temperatures, total n = 104) were taken using an icthyometer and a scale, respectively. The determination of energy reserves was performed using the same muscle samples referred in point i). Sampling times for whole-body assessments were performed at the beginning (0 days) and at the end (28 days) of the experiment. Mortality was monitored throughout trials.

Laboratory analyses

Molecular biomarkers

Molecular biomarkers were chosen based on their role on the cellular stress response (CSR): i) heat shock protein 70 kDa (Hsp70) is a chaperone with an adaptive value, repairing damaged proteins upon thermal stress (Feder and Hofmann 1999; Coles and Brown 2003; Hofmann 2005; Madeira et al. 2012a); ii) ubiquitin (Ub) targets irreversibly damaged proteins for proteasome degradation (Logan and Somero 2011; Tang et al. 2014); iii) antioxidant enzymes (catalase – CAT, glutathione-S-transferase – GST, superoxide dismutase – SOD) neutralize reactive oxygen species (ROS) and oxidation products (e.g. lipid peroxides) that arise at high temperature (Lushchak 2011; Vinagre et al. 2012), iv) lipid peroxides (LPO) are markers of damage to cell membranes (Pannunzio and Storey 1998; Logan and Somero 2011); v) acetylcholinesterase (AChE) is a neurotoxicity marker, it ends synaptic transmission at cholinergic synapses by catalyzing the breakdown of acetylcholine. When its activity is

disrupted or inhibited, it can lead to paralysis and possible cardio-vascular failure (Assis et al. 2012; Singh et al. 2013).

Samples of gills and muscle tissues (ca. 200-250 mg) were stored in 1.5 mL microtubes, and homogenated in a phosphate buffered saline solution (1 mL PBS, 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to extract the most soluble cytosolic proteins, following the methods previously described by Madeira et al. (2018a). Protein quantification and kinetic assays (Table 1) were undertaken using the colorimetric methods described and adapted for 96-well microplates (Madeira et al. 2015a) (Table ESM1 shows a detailed description of each method). Total protein was quantified using the Bradford method (Bradford 1976) to standardize all biomarker results.

Energy reserves

C:N ratios indicate transitions linked to changes in main organic components, such as proteins (N rich) and lipids (C rich) (Vollenweider 1985). Therefore, the C:N ratio was used as a proxy of energy metabolism/reserves, and % C used as a proxy of lipid reserves (following Sterner and Elser 2002). Muscle samples were lyophilized (by freeze-drying in vacuum) and grounded to a fine homogeneous powder. Samples of ~0.5 mg were loaded into tin cups and analyzed using an Elementar Isoprime continuous-flow mass spectrometer (GV Instruments) coupled to a vario PYRO cube elemental analyser (Elementar, Hanau, Germany). Reference materials (acetanilide; Stable Isotope Research Facility, Indiana University, USA) were assayed at the beginning of each run and after every 10 samples. Additionally, total protein was also analyzed as a proxy of energy reserves, since proteins can serve as respiratory substrates

250 when other energy sources are depleted (for instance due to stress; Alberts et al. 2002).
251 Total protein was extracted as described in the previous section (Bradford 1976).
252
253 *Data analyses*
254 Some calculations were performed previously to statistical analyses as follows:
255 i) Biomarker results were all standardized by the total protein in each sample.
256 ii) Thermal limits (CTMax) were calculated for each species as:

257 $$(1)\ \mathrm{CTMax}_{(\mathrm{species})} = \sum \frac{(T_{end-point})}{n}$$

258 where $T_{end\text{-}point}$ stands for the temperature at which an end-point was observed for any
259 given individual and n is sample size. Coefficients of variation (% CV) were calculated
260 to estimate intraspecific CTMax variation as follows:

261 $$(2)\ \%\mathrm{CV} = \left(\frac{\mathrm{SD}}{\mathrm{Mean}}\right) \times 100$$

262 The acclimation capacity was determined by: i) comparing mean CTMax values after
263 short term and longer term exposure to both temperature conditions (CTMax_{T28} –
264 CTMax_{T7} at each temperature and $\mathrm{CTMax}_{32^{\circ}C}$ – $\mathrm{CTMax}_{29^{\circ}C}$ at 7 and 28 days); ii)
265 acclimation response ratio (ARR, (Claussen 1977)) as ΔCTMax / Δtemperature.
266 Thermal safety margin (TSM, i.e. warming tolerance) was calculated for the intertidal
267 and subtidal environments as the difference between mean CTMax for this species and
268 Maximum Habitat Temperature (MHT) determined through field measurements at
269 intertidal and subtidal habitats. This difference indicates how close the test species is to
270 its upper thermal limit.
271 iii) Body condition was calculated by Fulton's K index, from morphometric data, as
272 follows:

273 $$(3)\ \mathrm{K} = 100\mathrm{M_t}/\mathrm{L_t^3}$$

274 where M_t is the total wet mass (mg) and L_t is the total length (mm) (Ricker 1975).

To calculate the C:N ratio of each muscle sample, %C and %N were first divided by the molar weight of each element (12.0107 $g.mol^{-1}$ for carbon and 14.0067 $g.mol^{-1}$ for nitrogen), and then the molar ratio was calculated. Additionally, mortality records were calculated as percentages (%) and compared across treatments.

Exploratory analyses were performed on the datasets to identify outliers (using the 1.5 coefficient), and to test normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test). When, at least, one of the assumptions of a specific test (e.g. normality or homoscedasticity) were not met, either a data transformation (e.g. logarithmic) was performed or a non-parametric test was used (this applies to all the statistical analyses performed). The principal objective of statistical analyses was to evaluate the main temperature effects (present day vs predicted from warming modeling) and/or acclimation capacity (as time-dependent temperature effects) on the several physiological parameters measured (from molecular to cellular to individual level).

Factorial ANOVAs were carried out to detect: a) which biomarkers contribute to differentiate between temperature treatments (29˚C vs 32˚C), exposure times (T0, T7, T14, T21, T28), and tissue types (gills and muscle; following the statistical approaches by Whitehead and Crawford 2005, and Madeira et al. 2016); b) differences between biomarker levels throughout the experiment at different temperatures (through post-hoc Tukey tests). For simplicity, the results only show significant differences from control (29˚C) at each time-point. A Principal Component Analysis (PCA) was carried out for each tissue to detect biomarkers that correlate with temperature and time and that contribute to explain the variance in the dataset. Upper thermal limit data were analyzed with a factorial ANOVA (temperature – 29˚C vs 32˚C, and time – T7 vs T28, as categorical predictors, and CTMax as dependent variable). Tukey's post-hoc tests for unequal N were carried out to detect differences between groups. Finally, Fulton's K

condition index, total protein, % C, and C:N ratio were also analyzed with factorial ANOVAs (temperature - 29˚C vs 32˚C, and time – T0 vs T28, as factors). Statistical analyses were performed in Statistica (version 13.0, Statsoft Inc. USA) and the *p*-value of 0.05 was used to decide on statistical significance.

Results

Characterization of the thermal environments of rocky reefs at the original and newly colonized areas

Temperature satellite data from the past 5 years showed that the average SST (Fig. 2A) in several coastal cities of Japan (from the Boso Peninsula to the Ryukyu Islands) ranges from 19.9˚C (February) to 28.1˚C (August), with maximum coastal water temperatures reaching 30˚C during summer. Average sea surface temperatures along the northeastern Brazilian coast vary from 26.6˚C to 28.9˚C throughout the year, reaching maximum temperatures of 29.7˚C (Fig. 2A). Finally, at our study location, S. Sebastião (Fig. 2B), satellite data showed that temperature ranges approximately from 24˚C to 26˚C during the warm months (from December to March) with maximum water temperatures of 29˚C during March.

Temperature data from field probes in S. Sebastião (Southeastern Brazil) showed a mean daily minimum and maximum temperatures ranging from 18.43˚C to 37.60˚C (Fig. ESM5, ESM6, and Table ESM2). There was an absolute maximum record of 41.50˚C (MHT) in three of the tide pools sampled. Mean monthly temperatures varied between 25.72˚C to 29.32˚C, and February was the warmest month recorded in the data loggers. Monthly temperature variation (average maximum – average minimum) was 16.60˚C ± 2.74˚C, while average daily temperature variation was 4.78˚C ± 1.06˚C.

Temperature effects on *Lysmata lipkei*

Stress assessment at lower levels of biological organization

Overall, *Lysmata lipkei* showed physiological changes in response to increased temperature during the experimental period. Significant effects were observed for biomarkers Hsp70, CAT, GST and SOD (Table 2, see also Table ESM3 for basal biomarker levels). Significant differences in biomarker levels between tissues were also detected (Table 2), as well as significant interaction between factors for some of the biomarkers (Table 2). In particular, significant temperature *vs*. time interactions were recorded for all biomarkers. The temperature-tissue-time interaction was also significant for Ub and SOD. Based on the PCA (Fig. 3, see table ESM4 for factor loadings), Hsp70 and SOD were the gill biomarkers that correlated the most to temperature, while no particular biomarker associated with time. In the muscle tissue, Ub and CAT were correlated with temperature, and AChE and GST were the biomarkers most correlated to time. Additionally, the variance explained by PC1 and PC2 in the gills (60.02% and 19.30%, for PC1 and PC2, respectively) was higher than in the muscle (31.85% and 23.48% for PC1 and PC2, respectively).

Significant biomarker changes under increased temperature for each sampling time and tissue are presented in Fig. 4. Shrimp biomarkers were more responsive to temperature increase after seven days of exposure (all biomarkers in gills, and CAT and AChE in muscle). After the first week of exposure, biomarker levels returned to control conditions, with no differences between temperature treatments at day 14 and 21. Nevertheless, a significant change was observed again after 28 days, coherently in both tissues and characterized by an increase in LPO levels. A common pattern was conspicuous for both tissues: biomarkers that significantly responded to the temperature challenge showed the same pattern/direction of response (increase) and similar response

timings. Nevertheless, biomarker response in the gills showed slightly higher fold-changes than in the muscle tissue (see Fig. ESM7 for a summary of fold-change results).

The most responsive biomarkers in both tissues were CAT and LPO. Hsp70, Ub, GST and SOD only showed changes in gill tissue. Almost all biomarkers showed a rapid response to an increased thermal load, except for LPO, which took 28 days to show a significant change.

Stress assessment at higher levels of biological organization

Lysmata lipkei showed mean CTMax values ranging from 36.6˚C to 38.4˚C at control and increased temperature, respectively, with significantly higher CTMax at 32˚C than at control temperature ($F = 49.60$, p-value <0.01). An interactive effect of acclimation time *vs.* temperature was observed for CTMax ($F = 11.68$, p-value <0.01), suggesting that acclimation time affects thermal tolerance plasticity (Fig. 5). Particularly, significant differences were found for CTMax between 29˚C and 32˚C, after acclimating for 7 and 28 days, but the degree of plasticity (i.e., environmentally induced phenotypic change) was higher after longer acclimation times. An acclimation capacity of 0.87˚C was recorded after 7 days and 2.51˚C after 28 days, with an overall ARR of 0.6. A negative TSM of -3.99˚C was recorded in the intertidal environment and a positive TSM of 9.45˚C was recorded in the subtidal environment.

The condition indicators analyzed for *L. lipkei* exposed to different temperature conditions only showed significant differences for total protein (Table 3), with lower protein content at 32˚C when compared to the 29˚C treatment. A significant temperature-time interaction was also solely observed for total protein, particularly at T28 (p-value = 0.031). All other condition indicators (Fulton's K, % C, and C:N) were

similar between experimental treatments and throughout the experiment. No shrimp mortality was recorded throughout the experiments.

Discussion

Understanding the panoply of responses and adaptations of invasive species to changes in environmental temperature can provide notable insights about the patterns and processes of species invasions under climate change scenarios (Zerebecki and Sorte 2011). In this study, we showed that the recently invasive shrimp species *Lysmata lipkei* in the Southwestern Atlantic is capable of substantial acclimation to chronic elevated temperature, with no mortality observed over experimental trials. This main finding is aligned to the meta-analysis results reported by Sorte et al. (2013) which analyzed performance of 150 non-native species under different environmental conditions and concluded that these organisms seem to be well-adapted and capable of thriving in warmed aquatic ecosystems. Overall, our results show that (i) this shrimp species is sensitive enough to elicit a detectable response to high temperature (in accordance with other studies with caridean shrimps, e.g. Allan et al. 2006; González-Ortegón et al. 2013; Reiser et al. 2014; Magozzi and Calosi 2015); (ii) the intensity of stress and duration of exposure are key features determining the onset of biochemical and physiological adjustments (as also observed by, e.g., Buckley et al. 2004; Schulte et al. 2011; Madeira et al. 2016 for several ectotherms); and that iii) no variations were detected in condition indices and lipid reserves between treatments, suggesting that shrimps maintained their weight and condition.

It is important to consider the similarity between temperature regimes along the Southern Atlantic Ocean and at the shrimps' original geographic distribution in Japan. All temperature datasets showed that the average yearly temperature range in the

400 invaded areas in NE and SE Brazil (about 22˚C – 28˚C) is very similar to that of their
401 native range in Japan (about 20˚C – 28˚C), with mean maximal temperatures
402 approaching 30˚C. *In situ* data also showed that these shrimps experience wide
403 temperature variations in the invaded area. Considering that *L. lipkei* inhabit the
404 intertidal and shallow subtidal habitats in their original geographical area (Okuno and
405 Fiedler 2010), which are naturally prone to abiotic variation, these shrimps are likely
406 equipped with adaptive mechanisms that allow them to withstand extremely variable
407 environments (Ravaux et al. 2016). In fact, tolerance to temperature fluctuations has
408 already been proposed as a relevant physiological trait that might underlie successful
409 species invasions (McMahon 2002; Zerebecki and Sorte 2011; Kelley 2014).
410 Molecular mechanisms underpinning *L. lipkei* eurythermality varied with thermal load
411 and time, as observed for other decapod crustaceans (e.g. Tavares-Sánchez et al. 2004;
412 Qian et al. 2012; Vinagre et al. 2014; Madeira et al. 2015b; Rodríguez-Fuentes et al.
413 2017). Biomarkers Hsp70 and SOD in the gills, and Ub and CAT in the muscle were
414 mostly associated with temperature, while damage to lipids (LPO) was mostly
415 associated with the duration of exposure. These results suggest that chaperone and
416 antioxidant defenses can play an important role during thermal stress by stabilizing
417 damaged proteins (Tomanek 2010) and protecting cells from the toxic effects of ROS
418 (Lesser 2006), thereby allowing the proper management of cellular functioning and the
419 maintenance of homeostasis across environmental temperature changes (Farcy et al.
420 2009). This seems to be enough to promote survival and avoid general metabolic failure
421 (Halpin et al. 2002; Lesser 2006; Yamashita et al. 2010; Madeira et al. 2013) in this
422 shrimp species. Also noteworthy, the significant interaction between temperature and
423 time for AChE in muscle tissue (which mostly occurs at neuromuscular junctions
424 (Valenzuela and Akaaboune 2007)) suggests a higher rate of neurotransmission and

425 muscle activity (contraction and relaxation). Klose and Robertson (2004) suggest that
426 high temperatures can induce synaptic thermoprotection and increase the release of
427 neurotransmitters in animals. In addition, this is also an indicator of higher metabolism,
428 which may enhance performance, foraging efficiency and predator avoidance, but it
429 may also result in more energy expenditure. LPO results combined with an absence of
430 changes in growth, lipid reserves or mortality after chronic warming suggest that LPO is
431 likely functioning as a cell signaling mechanism in *L. lipkei*, which may in the long-
432 term induce an adaptation process (Niki 2012; Ayala et al. 2014). Alternatively, longer
433 experimental trials are needed to detect deleterious effects of LPO in condition
434 indicators. Current studies on the dynamics of shifts in the lipid composition of cell
435 membranes suggest that temperature increase affects the balance between long-chain
436 polyunsaturated fatty acids (PUFAs) and small-chain saturated fatty acids (SFA) (Imbs
437 and Yakovleva 2012). Thermal stress may induce a reduction in multiple PUFAs over
438 time, leading to structural modifications in bio-membranes and causing leakiness
439 (Hillyer et al. 2017). Additionally, long-term changes in overall respiration or
440 mitochondrial membrane composition/density can affect LPO, which might explain the
441 LPO increase solely after 28 days of exposure to higher temperature. Future studies
442 should combine histopathological analyses with LPO biochemical measurements in
443 order to observe the damage extension of cell membranes in organs, and confirm
444 whether those alterations are within threshold ranges of normal metabolic processes or,
445 alternatively, if they indicate apoptosis and/or necrosis (Madeira et al. 2014). Finally,
446 the different biomarker responses observed for muscle and gill tissues suggest tissue-
447 specific bio-chemistry and function. Gill metabolism is highly aerobic and has a high
448 rate of O_2 diffusion and mitochondrial activity, which may lead to a higher level of ROS

accumulation and induce a proportionally higher response of antioxidants during acclimation (Verlecar et al. 2008; Madeira et al. 2016).

The activation of those cellular stress responses has been previously linked to shifts in organismal thermal tolerance (Feder and Hofmann 1999; Whitley et al. 1999; Madeira et al. 2015b; Rahlff et al. 2017). Our study corroborates these findings since CTMax of *L. lipkei* were considerably high (between 36.6˚C to 38.4˚C) among ectothermic animals, and are in line with upper thermal limits recorded for other tropical shrimps (e.g., 39.7˚C for *Palaemon northropi*, 36˚C for *Litopenaeus vannamei*, 34.7˚C for *Hippolyte obliquimanus,* (Ravaux et al. 2016) and 39ºC for *Pachygrapsus transversus* (Vinagre et al. 2016)), as well as for other invasive crustaceans (e.g. 33˚C for *Palaemon macrodactylus,* Lejeusne et al. 2014; 35˚C for *Carcinus maenas,* Cuculescu et al. 1998). In fact, an integrative study of the physiology of invasive vs native species found that the mean upper thermal limits for invasive decapods was around 35˚C (Kelley 2014), which suggests that the CTMax of *L. lipkei* is higher than average. This ultimately suggests that their high thermal tolerance may have been crucial in the invasion of Southern Atlantic coastal habitats. The TSM values here calculated for intertidal and subtidal habitats (-3.99˚C and 9.45˚C, respectively) suggest that *L. lipkei* may expand further south and more into subtidal habitats along the Brazilian coast. By the year 2100, models predict that water temperatures in the Southern Atlantic will increase by 3˚C (IPCC 2013), resulting in a summer average of 27˚C to 29˚C in subtidal areas and an average temperature of 32˚C in the intertidal area of São Sebastião, which is still well below the CTMax estimate for this species. The high acclimation capacity of *L. lipkei* to increased temperature over long exposure times indicates that not only is this species capable to accommodate future temperature change, but also that thermal history may push upper thermal limits beyond, as already documented for other crustaceans (Ober et

al. 2016). According to Ravaux et al. (2016), the ARR value of 0.6 obtained for *L. lipkei* does represent a genuine acclimatory capacity. Our results thus challenge previous findings suggesting a poor acclimation capacity in thermal tolerant species, such as intertidal ones (Stillman 2004; Ravaux et al. 2016; Madeira et al. 2018b).

Acclimation is often thought to impose considerable metabolic costs to organisms (Angilletta et al. 2004), as it relies on energetic reserves otherwise used for different functions, but the evidence so far is weak because such costs are difficult to quantify (Tomanek and Somero 2002). Our study agrees with Clark et al. (2013) in that acclimation in aquatic ectotherms typically occurs within 1-3 weeks, not imposing burdening energy costs for this invasive shrimp. No differences were detected either in mortality and growth rates, physiological condition or the quantity of lipid reserves after experimental trials. This indicates that adults of *L. lipkei* were able to maintain homeostasis, and to keep energy balance and oxygen consumption within narrow ranges as observed for other invertebrate species (see Kühnhold et al. 2016). Verberk et al. (2016) explained this balance based on the fact that organisms depend on a constant and sufficient flux of O_2 from their environment to their metabolizing tissues in order to ensure an adequate ATP supply to cover all physiological demands and still maintain the energy status (Rodríguez-Fuentes et al. 2017). It should be stressed that shrimps were fed *ad libitum* in this study and could have met any energy demands by increasing food intake. Interestingly, climate change predictions for the study area project an increase in precipitation (Marengo 2007; PBMC, 2014) and food availability in coastal areas through runoff of terrestrial inputs, which could potentially fuel benthic energetic pathways (Mendonça et al. 2018). This is expected to favor epibenthic feeders, which include some important consumers in intertidal habitats, such as *Lysmata lipkei*, and many non-native species which are usually capable to exploit and often monopolize

surplus trophic resources (Sorte et al. 2013). Nevertheless, it is worth noting that organismal-level responses, such as metabolic rates and long term processes, such as growth and reproduction (Pörtner and Knust 2007), may not have the same time-course as cellular-level responses (Rahlff et al. 2017). Longer experimental trials, including all life-cycle stages, may be necessary to detect changes at higher levels of biological complexity.

Biomarker responses are therefore often suitable indicators to support management policies by prioritizing areas and species that need local or regional intervention. However, physiological biomarkers should ideally be used in combination with both traditional (e.g. biodiversity metrics, water-quality analyses) and recent methodological approaches (e.g. molecular markers, metabolomics and proteomics) to integrate lines of evidence determining the invasion potential.

Conclusions

The main conclusion is that *Lysmata lipkei* can easily acclimate to increased water temperature. This capacity may enable this species to continue its invasion to tropical and subtropical areas along the Southwestern Atlantic coast. Results further suggest that *L. lipkei*, similarly to other non-indigenous species, shows specific physiological traits that make them successful invaders, namely (i) tolerance to upper thermal limits, and (ii) strong ability to acclimate and make physiological adjustments while maintaining performance. The recognition of such phenotypic traits can help scientists and managers to identify species that have the physiological ability to tolerate both present and future abiotic environments in specific geographic regions. This should be an important tool to predict which areas are more susceptible for invasions, or range expansions, and

523 develop preventive measures to avoid the introduction of non-native species or allow
524 better management in locations where invaders have already settled.
525
526 **Electronic supplementary material** The online version of this article contains
527 supplementary material, which is available to authorized users.
528
529 **Compliance with ethical standards**
530 *Research involving animals:* the authors declare that animal experiments followed legal
531 guidelines for laboratory animal science and were authorized by competent authorities,
532 as stated in the materials and methods subsection "Ethical guidelines".
533 *Declaration of interest:* the authors declare that they have no conflicts of interest.
534
535 **References**
536 Alberts B, Johnson A, Lewis J, Al. E (2002) How cells obtain energy from food. In:
537 Molecular Biology of the Cell, 4th editio. Garland Science, New York, NY, USA,
538 Allan EL, Froneman PW, Hodgson AN (2006) Effects of temperature and salinity on
539 the standard metabolic rate (SMR) of the caridean shrimp *Palaemon peringueyi*. J
540 Exp Mar Bio Ecol 337:103–108. doi: 10.1016/j.jembe.2006.06.006
541 Angilletta MJ, Bennett AF, Guderley H, et al (2006) Coadaptation: A unifying principle
542 in evolutionary thermal biology. Physiol Biochem Zool 79:282–294. doi:
543 10.1086/499990
544 Angilletta MJ, Steury TD, Sears MW (2004) Temperature, Growth Rate, and Body Size
545 in Ectotherms: Fitting Pieces of a Life-History Puzzle. Integr Comp Biol 44:498–
546 509. doi: 10.1093/icb/44.6.498
547 Assis CRD, Linhares AG, Oliveira VM, et al (2012) Comparative effect of pesticides on

548 brain acetylcholinesterase in tropical fish. Sci Total Environ 441:141–150. doi:
549 10.1016/j.scitotenv.2012.09.058
550 Ayala A, Muñoz MF, Argüelles S (2014) Lipid peroxidation: production, metabolism
551 and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid
552 Med Cell Longev 2014:Article ID 360438, 31 pages. doi:
553 10.1093/europace/eux022
554 Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to
555 changes in circulating corticosteroids. Integr Comp Biol 42:517–525. doi:
556 10.1093/icb/42.3.517
557 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram
558 quantities of protein utilizing the principle of protein-dye binding. Anal Biochem
559 72:248–254. doi: 10.1016/0003-2697(76)90527-3
560 Buckley B a, Place SP, Hofmann GE (2004) Regulation of heat shock genes in isolated
561 hepatocytes from an Antarctic fish, *Trematomus bernacchii*. J Exp Biol 207:3649–
562 3656. doi: 10.1242/jeb.01219
563 Burgiel SW, Muir A a. (2010) Invasive Species , Climate Change and Ecosystem-Based
564 Adaptation : Addressing Multiple Drivers of Global Change. Global Invasive
565 Species Programme (GISP), Washington, DC, US and Nairobi, Kenya
566 Clark MS, Fraser KPP, Burns G, Peck LS (2008) The HSP70 heat shock response in the
567 Antarctic fish *Harpagifer antarcticus*. Polar Biol 31:171–180. doi:
568 10.1007/s00300-007-0344-5
569 Clark TD, Sandblom E, Jutfelt F (2013) Aerobic scope measurements of fishes in an era
570 of climate change: respirometry, relevance and recommendations. J Exp Biol
571 216:2771–2782. doi: Doi 10.1242/Jeb.084251
572 Claussen D (1977) Thermal acclimation in ambystomatid salamanders. Comp Biochem

573 Physiol - A Mol Integr Physiol 58:333–340.
574 Coles SL, Brown BE (2003) Coral bleaching - Capacity for acclimatization and
575 adaptation. Adv Mar Biol 46:183–223. doi: 10.1016/S0065-2881(03)46004-5
576 Cortes PA, Puschel H, Acuña P, et al (2016) Thermal ecological physiology of native
577 and invasive frog species: do invaders perform better? Conserv Physiol 4:cow056.
578 doi: 10.1093/conphys/cow056
579 Cuculescu M, Hyde D, Bowler K (1998) Thermal tolerance of two species of marine
580 crab, *Cancer pagurus* and *Carcinus maenas*. J Therm Biol 23:107–110.
581 Ellman GL, Courtney KD, Andres Jr V, Featherstone RM (1961) A new and rapid
582 colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol
583 7:88-90,IN1-IN2,91-95. doi: 10.1016/0006-2952(61)90145-9
584 Farcy É, Voiseux C, Lebel JM, Fiévet B (2009) Transcriptional expression levels of cell
585 stress marker genes in the pacific oyster *Crassostrea gigas* exposed to acute
586 thermal stress. Cell Stress Chaperones 14:371–380. doi: 10.1007/s12192-008-
587 0091-8
588 Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the
589 stress response: evolutionary and ecological physiology. Annu Rev Physiol
590 61:243–282. doi: 10.1146/annurev.physiol.61.1.243
591 Festing MFW, Altman DG (2002) Guidelines for the design and statistical analysis of
592 experiments using laboratory animals. ILAR J 43:244–258. doi:
593 10.1093/ilar.43.4.244
594 González-Ortegón E, Pascual E, Drake P (2013) Respiratory responses to salinity,
595 temperature and hypoxia of six caridean shrimps from different aquatic habitats. J
596 Exp Mar Bio Ecol 445:108–115. doi: 10.1016/j.jembe.2013.04.006
597 Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S transferases. The first

598 enzymatic step in mercapturic acid formation. Biol Chem 7130–7139.
599 Halpin PM, Sorte CJ, Hofmann GE, Menge B a (2002) Patterns of Variation in Levels
600 of Hsp70 in Natural Rocky Shore Populations from Microscales to Mesoscales.
601 Integr Comp Biol 42:815–824. doi: 10.1093/icb/42.4.815
602 Hau J, Schapiro SJ (2010) Handbook of Laboratory Animal Science: Essential
603 Principles and Practices - Volume I, 3rd edn. CRC Press, Taylor and Francis
604 Group, Boca Raton, FL, USA
605 Hillyer KE, Dias DA, Lutz A, et al (2017) Metabolite profiling of symbiont and host
606 during thermal stress and bleaching in the coral *Acropora aspera*. Coral Reefs
607 36:105–118. doi: 10.1007/s00338-016-1508-y
608 Hoefnagel KN, Verberk WCEP (2016) Long-term and acute effects of temperature and
609 oxygen on metabolism, food intake, growth and heat tolerance in a freshwater
610 gastropod. J Therm Biol 68:1–12. doi: 10.1016/j.jtherbio.2016.11.017
611 Hofmann GE (2005) Patterns of Hsp gene expression in ectothermic marine organisms
612 on small to large biogeographic scales. Integr Comp Biol 45:247–255. doi:
613 10.1093/icb/45.2.247
614 Hofmann GE, Somero GN (1995) Evidence for protein damage at environmental
615 temperatures : Seasonal changes in levels of ubiquitin conjugates and hsp70 in the
616 intertidal mussel *Mytilus trossulus*. J Exp Biol 198:1509–1518.
617 Imbs AB, Yakovleva IM (2012) Dynamics of lipid and fatty acid composition of
618 shallow-water corals under thermal stress: An experimental approach. Coral Reefs
619 31:41–53. doi: 10.1007/s00338-011-0817-4
620 IPCC (2013) Atlas of Global and Regional Climate Projections. Clim Chang 2013 Phys
621 Sci Basis Contrib Work Gr I to Fifth Assess Rep Intergov Panel Clim Chang
622 Annexe I:1311–1394. doi: 10.1017/CBO9781107415324.029

623 Johansson LH, Borg LA (1988) A spectrophotometric method for determination of
624 catalase activity in small tissue samples. Anal Biochem 174:331–336. doi:
625 10.1016/0003-2697(88)90554-4
626 Kaspari M, Clay NA, Lucas J, et al (2015) Thermal adaptation generates a diversity of
627 thermal limits in a rainforest ant community. Glob Chang Biol 21:1092–1102. doi:
628 10.1111/gcb.12750
629 Kelley AL (2014) The role thermal physiology plays in species invasion. Conserv
630 Physiol 2:1–14. doi: 10.1093/conphys/cou045
631 Klose MK, Robertson RM (2004) Stress-induced thermoprotection of neuromuscular
632 transmission. Integr Comp Biol 44:14–20. doi: 10.1093/icb/44.1.14
633 Kolar CS, Lodge DM (2001) Progress in invasion biology: Predicting invaders. Trends
634 Ecol Evol 16:199–204. doi: 10.1016/S0169-5347(01)02101-2
635 Kühnhold H, Kamyab E, Novais S, et al (2016) Thermal stress effects on energy
636 resource allocation and oxygen consumption rate in the juvenile sea cucumber,
637 *Holothuria scabra* (Jaeger, 1833). Aquaculture 467:1–9. doi:
638 10.1016/j.aquaculture.2016.03.018
639 Lejeusne C, Latchere O, Petit N, et al (2014) Do invaders always perform better?
640 Comparing the response of native and invasive shrimps to temperature and salinity
641 gradients in south-west Spain. Estuar Coast Shelf Sci 136:102–111. doi:
642 10.1016/j.ecss.2013.11.014
643 Lesser MP (2006) Oxidative Stress in Marine Environments: Biochemistry and
644 Physiological Ecology. Annu Rev Physiol 68:253–278. doi:
645 10.1146/annurev.physiol.68.040104.110001
646 Loarie SR, Duffy PB, Hamilton H, et al (2009) The velocity of climate change. Nature
647 462:1052–1055. doi: 10.1038/nature08649

Logan C a, Somero GN (2011) Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper). Am J Physiol Regul Integr Comp Physiol 300:R1373-83. doi: 10.1152/ajpregu.00689.2010

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101:13–30. doi: 10.1016/j.aquatox.2010.10.006

Madeira C, Leal MC, Diniz MS, et al (2018a) Thermal stress and energy metabolism in two circumtropical decapod crustaceans: Responses to acute temperature events. Mar Environ Res. doi: 10.1016/j.marenvres.2018.08.015

Madeira C, Madeira D, Diniz MS, et al (2016) Thermal acclimation in clownfish: An integrated biomarker response and multi-tissue experimental approach. Ecol Indic. doi: 10.1016/j.ecolind.2016.07.009

Madeira C, Madeira D, Diniz MS, et al (2017) Comparing biomarker responses during thermal acclimation: A lethal vs non-lethal approach in a tropical reef clownfish. Comp Biochem Physiol Part A Mol Integr Physiol 204:104–112. doi: 10.1016/j.cbpa.2016.11.018

Madeira C, Madeira D, Vinagre C, Diniz M (2015a) Octocorals in a changing environment: Seasonal response of stress biomarkers in natural populations of Veretillum cynomorium. J Sea Res 103:120–128.

Madeira C, Mendonça V, Flores AAV, et al (2018b) High thermal tolerance does not protect from chronic warming – A multiple end-point approach using a tropical gastropod, Stramonita haemastoma. Ecol Indic. doi: 10.1016/j.ecolind.2018.04.044

Madeira D, Mendonça V, Dias M, et al (2015b) Physiological, cellular and biochemical thermal stress response of intertidal shrimps with different vertical distributions: Palaemon elegans and Palaemon serratus. Comp Biochem Physiol - Part A Mol

673 Integr Physiol 183:107–115. doi: 10.1016/j.cbpa.2014.12.039
674 Madeira D, Narciso L, Cabral HN, et al (2012a) HSP70 production patterns in coastal
675 and estuarine organisms facing increasing temperatures. J Sea Res 73:137–147.
676 doi: 10.1016/j.seares.2012.07.003
677 Madeira D, Narciso L, Cabral HN, et al (2013) Influence of temperature in thermal and
678 oxidative stress responses in estuarine fish. Comp Biochem Physiol Part A Mol
679 Integr Physiol 166:237–243. doi: 10.1016/j.cbpa.2013.06.008
680 Madeira D, Narciso L, Cabral HN, Vinagre C (2012b) Thermal tolerance and potential
681 impacts of climate change on coastal and estuarine organisms. J Sea Res 70:32–41.
682 doi: 10.1016/j.seares.2012.03.002
683 Madeira D, Vinagre C, Costa PM, Diniz MS (2014) Histopathological alterations,
684 physiological limits, and molecular changes of juvenile Sparus aurata in response
685 to thermal stress. Mar Ecol Prog Ser 505:253–266. doi: 10.3354/meps10794
686 Magozzi S, Calosi P (2015) Integrating metabolic performance, thermal tolerance, and
687 plasticity enables for more accurate predictions on species vulnerability to acute
688 and chronic effects of global warming. Glob Chang Biol 21:181–194. doi:
689 10.1111/gcb.12695
690 Marengo JA (2007) Mudanças Climáticas Globais e seus Efeitos sobre a
691 Biodiversidade: Caracterização do Clima Atual e Definição das Alterações
692 Climáticas para o Território Brasileiro ao Longo do Século XXI. Série
693 Biodiversidade 26:212. doi: 10.1017/CBO9781107415324.004
694 McMahon RF (2002) Evolutionary and physiological adaptations of aquatic invasive
695 animals: r selection versus resistance. Can J Fish Aquat Sci 59:1235–1244. doi:
696 10.1139/f02-105
697 Mendonça V, Madeira C, Dias M, et al (2018) What's in a tide pool? Just as much food

web network complexity as in large open ecosystems. PLoS One. doi: 10.1371/journal.pone.0200066

Niki E (2012) Do antioxidants impair signaling by reactive oxygen species and lipid oxidation products? FEBS Lett 586:3767–3770. doi: 10.1016/j.febslet.2012.09.025

Ober GT, Thornber C, Grear J, Kolbe JJ (2016) Ecological differences influence the thermal sensitivity of swimming performance in two co-occurring mysid shrimp species with climate change implications. J Therm Biol 64:26–34. doi: 10.1016/j.jtherbio.2016.11.012

Okuno J, Fiedler C (2010) Lysmata lipkei: A new species of Peppermint Shrimp (Decapoda, Hippolytidae) from warm temperate and subtropical waters of Japan. In: Fransen C, de Grave S, Ng P (eds) Studies of Malacostraca: Lipke Bijdeley Holthuis Memorial Volume - Crustaceana Monographs. Koninklijke Brill, NV. Leiden, Netherlands., pp 597–610

Pachelle PPG, Anker A, Mendes CB, Bezerra LEA (2016) Decapod crustaceans from the state of Ceará, northeastern Brazil: an updated checklist of marine and estuarine species, with 23 new records. Zootaxa 4131:1–63. doi: 10.11646/zootaxa.4131.1.1

Pannunzio TM, Storey KB (1998) Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. J Exp Mar Bio Ecol 221:277–292. doi: 10.1016/S0022-0981(97)00132-9

Payne NL, Smith JA, van der Meulen DE, et al (2016) Temperature dependence of fish performance in the wild: Links with species biogeography and physiological thermal tolerance. Funct Ecol 903–912. doi: 10.1111/1365-2435.12618

PBMC, 2014: Base científica das mudanças climáticas. Contribuição do Grupo de Trabalho 1 do Painel Brasileiro de Mudanças Climáticas ao Primeiro Relatório da

723 Avaliação Nacional sobre Mudanças Climáticas [Ambrizzi, T., Araujo, M. (eds.)].
724 COPPE. Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, 464
725 pp
726 Pérez-Casanova JC, Rise ML, Dixon B, et al (2008) The immune and stress responses
727 of Atlantic cod to long-term increases in water temperature. Fish Shellfish
728 Immunol 24:600–609. doi: 10.1016/j.fsi.2008.01.012
729 Pörtner HO, Farrell A (2008) Physiology and climate change. Science 322:690–692.
730 doi: 10.1126/science.1163156
731 Pörtner HO, Knust R (2007) Climate change affects marine fishes through the oxygen
732 limitation of thermal tolerance. Science 315:95–97. doi: 10.1126/science.1135471
733 Qian Z, Liu X, Wang L, et al (2012) Gene expression profiles of four heat shock
734 proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*.
735 Comp Biochem Physiol C Toxicol Pharmacol 156:211–20. doi:
736 10.1016/j.cbpc.2012.06.001
737 Rahlff J, Peters J, Moyano M, et al (2017) Short-term molecular and physiological
738 responses to heat stress in neritic copepods *Acartia tonsa* and *Eurytemora affinis*.
739 Comp Biochem Physiol Part A Mol Integr Physiol 203:348–358. doi:
740 10.1016/j.cbpa.2016.11.001
741 Ravaux J, Léger N, Rabet N, et al (2016) Plasticity and acquisition of the thermal
742 tolerance (upper thermal limit and heat shock response) in the intertidal species
743 *Palaemon elegans*. J Exp Mar Bio Ecol 484:39–45. doi:
744 10.1016/j.jembe.2016.07.003
745 Reiser S, Herrmann JP, Temming A (2014) Thermal preference of the common brown
746 shrimp (*Crangon crangon*, L.) determined by the acute and gravitational method. J
747 Exp Mar Bio Ecol 461:250–256. doi: 10.1016/j.jembe.2014.08.018

748 Ricker WE (1975) Computation and interpretation of biological statistics of fish
749 populations. Bull Fish Res Board Can 191:1–382.
750 Rodríguez-Fuentes G, Murúa-Castillo M, Díaz F, et al (2017) Ecophysiological
751 biomarkers defining the thermal biology of the Caribbean lobster *Panulirus argus*.
752 Ecol Indic 78:192–204. doi: 10.1016/j.ecolind.2017.03.011
753 Rushworth KJW, Smith SDA, Cowden KL, Purcell SW (2011) Optimal temperature for
754 growth and condition of an endemic subtropical anemonefish. Aquaculture
755 318:479–482. doi: 10.1016/j.aquaculture.2011.06.004
756 Schulte PM, Healy TM, Fangue NA (2011) Thermal performance curves, phenotypic
757 plasticity, and the time scales of temperature exposure. Integr Comp Biol 51:691–
758 702. doi: 10.1093/icb/icr097
759 Shabtay A, Arad Z (2005) Ectothermy and endothermy: evolutionary perspectives of
760 thermoprotection by HSPs. J Exp Biol 208:2773–81. doi: 10.1242/jeb.01705
761 Singh A, Jaiswal SK, Sharma B (2013) Effect of low temperature stress on
762 acetylcholinesterase activity and its kinetics in 5th instar larvae of Philosamia
763 ricini. J Biochem Res 1:17–25.
764 Somero GN (2002) Thermal physiology and vertical zonation of intertidal animals:
765 optima, limits and costs of living. Integr Comp Biol 42:780–789. doi: doi:
766 10.1093/icb/42.4.780
767 Sorte CJB, Ibáñez I, Blumenthal DM, et al (2013) Poised to prosper? A cross-system
768 comparison of climate change effects on native and non-native species
769 performance. Ecol Lett 16:261–270. doi: 10.1111/ele.12017
770 Sterner RW, Elser JJ (2002) Ecological Stoichiometry: The biology of elements from
771 molecules to the biosphere. Princeton University Press, NJ, USA
772 Stillman JH (2004) A comparative analysis of plasticity of thermal limits in porcelain

773 crabs across latitudinal and intertidal zone clines. Int Congr Ser 1275:267–274.
774 doi: 10.1016/j.ics.2004.09.034
775 Sun Y, Oberley LW, Li Y (1988) A simple method for clinical assay of superoxide
776 dismutase. Clin Chem 34:497–500.
777 Tang C-H, Leu M-Y, Shao K, et al (2014) Short-term effects of thermal stress on the
778 responses of branchial protein quality control and osmoregulation in a reef-
779 associated fish, Chromis viridis. Zool Stud 53:21. doi: 10.1186/s40555-014-0021-7
780 Tavares-Sánchez OL, Gómez-Anduro G a, Felipe-Ortega X, et al (2004) Catalase from
781 the white shrimp *Penaeus (Litopenaeus) vannamei:* molecular cloning and protein
782 detection. Comp Biochem Physiol B Biochem Mol Biol 138:331–7. doi:
783 10.1016/j.cbpc.2004.03.005
784 Tomanek L (2010) Variation in the heat shock response and its implication for
785 predicting the effect of global climate change on species' biogeographical
786 distribution ranges and metabolic costs. J Exp Biol 213:971–979. doi:
787 10.1242/jeb.038034
788 Tomanek L, Somero GN (2002) Interspecific- and acclimation-induced variation in
789 levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock
790 transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*):
791 implications for regulation of hsp gene expression. J Exp Biol 205:677–685.
792 Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by
793 thiobarbituric acid test. Anal Biochem 86:271–278. doi: 10.1016/0003-
794 2697(78)90342-1
795 Valenzuela IMP, Akaaboune M (2007) Acetylcholinesterase mobility and stability at
796 the neuromuscular junction of living mice. Mol Biol Cell 18:2904–2911. doi:
797 10.1091/mbc.E07

798 Varela R, Lima FP, Seabra R, et al (2018) Coastal warming and wind-driven upwelling:
799 A global analysis. Sci Total Environ 639:1501–1511. doi:
800 10.1016/j.scitotenv.2018.05.273
801 Verberk WCEP, Overgaard J, Ern R, et al (2016) Does oxygen limit thermal tolerance
802 in arthropods? A critical review of current evidence. Comp Biochem Physiol -Part
803 A Mol Integr Physiol 192:64–78. doi: 10.1016/j.cbpa.2015.10.020
804 Verlecar XN, Jena KB, Chainy GBN (2008) Seasonal variation of oxidative biomarkers
805 in gills and digestive gland of green-lipped mussel *Perna viridis* from Arabian Sea.
806 Estuar Coast Shelf Sci 76:745–752. doi: 10.1016/j.ecss.2007.08.002
807 Viant MR, Werner I, Rosenblum ES, et al (2003) Correlation between heat-shock
808 protein induction and reduced metabolic condition in juvenile steelhead trout
809 (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. Fish Physiol
810 Biochem 29:159–171. doi: 10.1023/B:FISH.0000035938.92027.81
811 Vinagre C, Dias M, Roma J, et al (2013) Critical thermal maxima of common rocky
812 intertidal fish and shrimps - A preliminary assessment. J Sea Res 81:10–12. doi:
813 10.1016/j.seares.2013.03.011
814 Vinagre C, Leal I, Mendonça V, et al (2016) Vulnerability to climate warming and
815 acclimation capacity of tropical and temperate coastal organisms. Ecol Indic
816 62:317–327. doi: 10.1016/j.ecolind.2015.11.010
817 Vinagre C, Leal I, Mendonça V, Flores AA V (2015) Effect of warming rate on the
818 critical thermal maxima of crabs, shrimp and fish. J Therm Biol 47:19–25. doi:
819 10.1016/j.jtherbio.2014.10.012
820 Vinagre C, Madeira D, Mendonça V, et al (2014) Effect of temperature in multiple
821 biomarkers of oxidative stress in coastal shrimp. J Therm Biol 41:38–42. doi:
822 10.1016/j.jtherbio.2014.02.005

823 Vinagre C, Madeira D, Narciso L, et al (2012) Impact of climate change on coastal
824 versus estuarine nursery areas: Cellular and whole-animal indicators in juvenile
825 seabass *Dicentrarchus labrax*. Mar Ecol Prog Ser 464:237–243. doi:
826 10.3354/meps09885
827 Vollenweider R (1985) Elemental and biochemical composition of plankton biomass:
828 some comments and explorations. Arch fur Hydrobiol 105:11–29.
829 Whitehead A, Crawford DL (2005) Variation in tissue-specific gene expression among
830 natural populations. Genome Biol 6:R13. doi: 10.1186/gb-2005-6-2-r13
831 Whitley D, Goldberg SP, Jordan WD (1999) Heat shock proteins: A review of the
832 molecular chaperones. J Vasc Surg 29:748–751. doi: 10.1016/S0741-
833 5214(99)70329-0
834 Yamashita M, Yabu T, Ojima N (2010) Stress Protein HSP70 in Fish. Aqua-BioScience
835 Monogr 3:111–141. doi: 10.5047/absm.2010.00304.0111
836 Ye L, Yang S-Y, Zhu X-M, et al (2011) Effects of temperature on survival,
837 development, growth and feeding of larvae of Yellowtail clownfish *Amphiprion*
838 *clarkii* (Pisces: Perciformes). Acta Ecol Sin 31:241–245. doi:
839 10.1016/j.chnaes.2011.06.003
840 Yuan X, Yang H, Meng L, et al (2013) Impacts of temperature on the scavenging
841 efficiency by the deposit-feeding holothurian *Apostichopus japonicus* on a
842 simulated organic pollutant in the bivalve-macroalage polyculture from the
843 perspective of nutrient budgets. Aquaculture 406–407:97–104. doi:
844 10.1016/j.aquaculture.2013.05.009
845 Yuan X, Yang H, Wang L, et al (2009) Bioenergetic responses of sub-adult sea
846 cucumber *Apostichopus japonicus* (Selenka) (Echinodermata: Holothuroidea) to
847 temperature with special discussion regarding its southernmost distribution limit in

China. J Therm Biol 34:315–319. doi: 10.1016/j.jtherbio.2009.05.001

Zerebecki RA, Sorte CJB (2011) Temperature tolerance and stress proteins as mechanisms of invasive species success. PLoS One. doi: 10.1371/journal.pone.0014806

873 **Tables**

874

875 **Table 1** Methods used for biomarker analyses. Abb: Hsp70 – heat shock protein 70; Ub
876 – total ubiquitin; CAT – catalase; LPO – lipid peroxides; GST – glutathione-S-
877 transferase; SOD – superoxide dismutase; AChE – acetylcholinesterase; ELISA –
878 Enzyme Linked Immunosorbent Assay; EC – Enzyme Comission number.

Biomarker	Type of analysis	EC	References
Hsp70	protein quantification, indirect ELISA	-	Madeira et al. 2017
Ub	protein quantification, direct ELISA	-	Madeira et al. 2017
CAT	enzymatic/kinetic assay (activity)	1.11.1.6	Johansson and Borg 1988
LPO	quantification of malondialdehyde	-	Uchiyama and Mihara 1978
GST	enzymatic/kinetic assay (activity)	2.5.1.18	Habig et al. 1974
SOD	enzymatic/kinetic assay (activity)	1.15.1.1	Sun et al. 1988
AChE	enzymatic/kinetic assay (activity)	3.1.1.7	Ellman et al. 1961

879

880 **Table 2** Statistical analyses (factorial ANOVAs) showing main effects of temperature
881 (29˚C, 32˚C) and tissue (gills, muscle) and their interactive effects, as well as
882 interactions with time (0, 7, 14, 21 and 28 days) on selected molecular biomarkers from
883 the species *L. lipkei.* Significant results (p-value ≤0.05) are indicated (*). Abb: Hsp70 –
884 heat shock protein 70kDa; Ub – ubiquitin; CAT – catalase; LPO – lipid peroxides; GST
885 – glutathione-S-transferase; SOD – superoxide dismutase; AChE – acetylcholinesterase;
886 Temp – Temperature; T*T*T – Temperature × Time × Tissue; NA – not available.

	Hsp70		**Ub**		**CAT**		**LPO**		**GST**		**SOD**		**AChE**	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Temperature	21.9	***	0.9	ns	16.2	***	0.8	ns	10.0	**	7.9	**	3.544	ns
Tissue	181.2	***	82.6	***	218.7	***	21.1	***	104.4	***	169.8	***	NA	NA
Temp*Time	4.3	**	3.1	*	4.5	**	10.9	***	2.7	*	3.0	*	2.676	*
Temp*Tissue	3.1	ns	1.1	ns	0.7	ns	0.6	ns	0.4	ns	0.0	ns	NA	NA
T*T*T	1.6	ns	4.6	**	2.0	ns	1.3	ns	1.4	ns	2.3	*	NA	NA

Levels of significance: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, ns: non-significant ($p > 0.05$).

892

893 **Table 3** Factorial ANOVAs showing the main effects of temperature (29˚C vs 32˚C)
894 and interactive effects with time (T0 vs T28) on condition indicators for *L. lipkei*.
895 Significant results (p-value ≤ 0.05) are presented in bold. Abb: Temp – Temperature.

	Fulton's K		**Total protein**		**%C**		**C:N molar ratio**	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Temperature	0.069	0.793	**5.639**	**0.022**	1.240	0.272	0.608	0.440
Temp x Time	0.720	0.408	**4.784**	**0.043**	0.034	0.854	0.072	0.792

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914 **Figure captions**

915

916 **Fig. 1** (a) Original geographical distribution range of the caridean shrimp *Lysmata lipkei*
917 according to Okuno and Fiedler (2010). The locations shown are the sampling sites
918 from which specimens were collected and first taxonomically identified in 2010. (b) *L.*
919 *lipkei* known occurrences in Brazilian coastal waters: NE Brazil (in the states of Ceará –
920 Camocim and Icapuí, and Rio Grande do Norte – Areia Branca), where the first
921 introductions of this species were observed (Pachelle et al. 2016), and SE Brazil where
922 shrimps were collected for this study (São Sebastião, São Paulo State). This image was
923 prepared using GoogleEarth® images

924

925 **Fig. 2** a) Present SST temperatures (˚C) in *L. lipkei* original distribution range (Japan,
926 data from the locations where these shrimps were first caught and described: Okinawa,
927 Boso Peninsula, Ryukyu Islands) and at the first introduction locations in Brazil (Ceará
928 and Rio Grande do Norte states, in the northeastern coast); b) Present and projected
929 temperatures for 2100 (+3˚C) in São Sebastião (SP, Brazil) coastal waters – new
930 colonized environment. Note: present monthly average sea surface temperatures were
931 calculated from satellite data for the past 5 years

932

933 **Fig. 3** Ordination diagrams of the first two axes of the PCA carried out for each
934 sampled tissue of *Lysmata likpei* exposed to 29˚C and 32˚C considering all sampling
935 times (0, 7, 14, 21 and 28 days of exposure): a) gills and b) muscle

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937 **Fig. 4** Biomarker behavior (mean+SD) along time (one month) under two temperature
938 treatments (29ºC and 32ºC): A) Hsp70 (heat shock protein 70 kDa) and Ub (ubiquitin);

939 B) CAT (catalase) and LPO (lipid peroxides), C) GST (glutathione-S-transferase) and
940 SOD (superoxide dismutase), D) AChE (acetylcholinesterase – only measured in
941 muscle). Numbers next to letters stand for (1) gills and (2) muscle. Significant
942 differences (p-value ≤ 0.05) from the control group are presented with colored asterisks
943 (*).
944
945 **Fig. 5** Critical Thermal Maxima (CTMax) measured in *Lysmata lipkei* after 7 days and
946 28 days at two different acclimating conditions predicting exposure to present average
947 summer temperatures (29˚C, control) and future average summer temperatures (32˚C) in
948 intertidal rocky reefs of Southeastern Brazil. Significant differences (p-value ≤ 0.05) are
949 indicated with an asterisk (*)
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Present and future invasion perspectives of an alien shrimp in South Atlantic coastal waters – an experimental assessment of functional biomarkers and thermal tolerance

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Running title: Effects of increased temperature on tropical alien shrimp

Acknowledgements

The present research was financially supported by the Portuguese Foundation for Science and Technology (FCT) through the WarmingWebs project (PTDC/MAR-

EST/2141/2012), the “Investigador FCT” position granted to C.V. and the strategic projects UID/MAR/04292/2013 granted to MARE and UID/Multi/04378/2013 granted to UCIBIO. Authors C.M. and V.M. acknowledge PhD grants (SFRH/BD/92975/2013 and SFRH/BD/109618/2015, respectively), and M.C.L. acknowledges a post-doc grant (SFRH/BPD/115298/2016), all granted by FCT. The funding institution had no influence in the study design, collection and analysis of datasets or in the decision to publish the work. Authors would like to thank everyone who helped during field work and experimental trials, and to a Megan Walters for proofreading.

Abstract

Climate change, particularly ocean warming, is thought to benefit the spread of invasive species due to their increased tolerance to temperature fluctuations as compared to native species. The physiological tolerance of invasive species as a potential mechanism driving invasion success is therefore a subject that merits further study. Specifically, we need to adequately evaluate the potential of species invasions under changing environmental conditions, so that adequate preventive measures can be taken to minimize any impacts on coastal ecosystems. Here, we experimentally evaluated the physiological responses of a recent invader in the Southern Atlantic, the shrimp *Lysmata lipkei*, under a warming ocean scenario. Adult shrimps were collected from rocky shores in southeastern Brazil and subjected to experimental trials under a control and a +3˚C scenario. Molecular biomarkers (in gills and muscle), upper thermal limits, acclimation response ratios, thermal safety margins, mortality rates, estimates of body condition and energy reserves were measured over one month. Results suggest that higher temperatures elicit physiological adjustments at the molecular level, underpinning a high thermal tolerance. In addition, results indicated substantial acclimation capacity, with no evidence of decreased performance under an ocean-warming scenario. Thermal safety margins were low for shrimp from intertidal rock pools but high for shrimp from subtidal habitats. We conclude that the thermal tolerance of this shrimp species may favor its ongoing invasion along the Southwestern Atlantic Ocean, mainly in subtidal habitats, both under present and future thermal conditions.

Keywords tropical shrimp, invasive species, warming oceans, rocky reefs, thermal biology, stress physiology

Introduction

The world's oceans have been warming at alarming rates over the past four decades (up to 0.5ºC.decade^{-1} in certain oceanic locations; Varela et al. 2018) and driving major shifts in species distributions (Loarie et al., 2009; Hoefnagel and Verberk, 2016). As a consequence, distribution gaps are being created in wide geographic areas, which have been invaded by opportunistic species (Burgiel and Muir 2010). The study of the thermal biology of invasive species is expected to elucidate the physiological mechanisms driving the establishment of non-native species in new locations as global warming continues (Kelley 2014). The physiological tolerance hypothesis (Zerebecki and Sorte 2011) predicts that the latitudinal range occupied by a given species is a surrogate of its ability to acclimate to varying thermal regimes, which may ultimately increase its potential to invade thermally vacant niche space across biogeographic regions. Different thermal environments experienced by a species along its invasion process may affect biochemical interactions, cellular metabolism (Tomanek and Somero 2002; Angilletta et al. 2006) and, organism performance (Pörtner and Farrell 2008). However, how physiology shapes biogeographical distributions of invasive species is still overlooked (Somero 2002; Cortes et al. 2016). Previous studies on marine species have shown significant effects of water temperature on heat shock protein expression (Hofmann and Somero 1995; Tomanek and Somero 2002; Shabtay and Arad 2005; Clark et al. 2008; Madeira et al. 2015b), immune and antioxidant functions (Pannunzio and Storey 1998; Pérez-Casanova et al. 2008; Madeira et al. 2013), including scavenging capacity (Yuan et al. 2013), metabolite synthesis (Barton 2002; Viant et al. 2003), metabolic performance (Magozzi and Calosi 2015; Payne et al. 2016), physio-ecological short-term processes (e.g. rates of ingestion, defecation, respiration and excretion) (Yuan et al. 2009, 2013), and long-term processes, such as growth (Angilletta

2004; Rushworth et al. 2011; Ye et al. 2011). As such, the successful establishment of non-indigenous species will require the tolerance of a possibly different range of temperatures (and varying rates of temperature change) in the new environment through physiological adjustments (Kelley 2014). In other words, high acclimation and adaption abilities are required for a species to be able to invade a new habitat or geographic region. In particular, Kelley (2014) and Lejeusne et al. (2014) proposed that, compared to native species, invasive species already distributed over a large latitudinal range are expected to (i) be more resistant to acute thermal stress owing to previous adaptation to broad geographic thermal widths over evolutionary or historical time, (ii) show lower rates of oxygen consumption and consequent greater resistance to environmental stresses, and (iii) have higher survival rates under chronic stress due to higher acclimation capacity. The identification of physiological phenotypes of successful invaders can contribute to recognize key features that predispose certain species to become established in new habitats (Kolar and Lodge 2001; Kelley 2014).

The caridean shrimp *Lysmata lipkei* (as described by Okuno and Fiedler, 2010) is native to the West Pacific region and has recently invaded the tropical and subtropical Western Atlantic (Pachelle et al. 2016). Specifically, it is considered an exotic species along the Eastern coast of Brazil where it can be found in tide pools or shallow waters (~15m depth) (Pachelle et al. 2016). Since this species is fairly recent to science, it is useful to gather specific information on its thermal biology and physiology to monitor the expansion of its invasive populations in the wild. Additionally, experimental trials are needed to understand the ability of *Lysmata* shrimps to become invasive under present and future thermal conditions predicted under consensual climate change scenarios.

Here, we investigated the physiological responses of the invasive shrimp *Lysmata lipkei* in present-day conditions as these may already favor the establishment of heat tolerant

species in subtropical areas, as well as tested the responses of this shrimp to chronic heat stress, adjusted to the gradual warming projected for Southeastern Brazil by the end of this century. In this research, we followed a multi-parameter approach (molecular, cellular and whole-body) to detect metabolic changes and focused on alterations from control (current summer SST) to future temperature, derived from gradual warming by 2100 (+3ºC SST anomaly). We maintained individuals under experimental temperature conditions and (i) compared selected biomarkers that modulate stress responses in different tissues (gills and muscle) over exposure time in both temperature settings, and (ii) compared upper thermal limits, acclimation ability and thermal safety margins, as well as (iii) performance parameters growth, body condition, accumulation of energy reserves and mortality rates at the end of exposure time in both thermal treatments.

Materials and methods

Ethical guidelines

The experiments here performed were approved by the competent authorities both in Portugal and Brazil (Direcção Geral de Alimentação e Veterinária and Comissão de Ética no Uso de Animais, respectively, authorization documents 0421/000/000/2013 and 13.1.981.53.7). The experimental design followed ARRIVE guidelines as well as directions by the Federation of European Laboratory Animal Science Associations (see Festing and Altman, 2002; Hau and Schapiro, 2010).

Animal collection and field temperature measurements

The biological model used in this study was the shrimp species *Lysmata lipkei*. Wild specimens (n = 120, mean ± SD total length = 4.04 cm ± 1.06 cm, mean ± SD total

weight = 1.01 g ± 0.83 g) were collected with hand nets in shallow waters (<50 cm) of intertidal rocky reefs in Southeastern Brazil (23˚49'42''S, 45˚26'29''W, Barequeçaba Beach, São Sebastião, São Paulo, Fig. 1B). Animals were transported to the marine field station (CEBIMar - Universidade de São Paulo, S. Sebastião) in plastic containers with aerated sea water where they were allowed to acclimate for two weeks at local seawater temperature (29.0˚C ± 0.5). Shrimp welfare was assessed daily (e.g. parasites, lesions, feeding behavior) and animals were fed frozen food daily *ad libitum*.

The thermal environment of this species was assessed at the initial introduction locations (northeastern Brazil – Ceará and Rio Grande do Norte, Pachelle et al. 2016, Fig. 1B) and at the study location (S. Sebastião, Brazil, Fig. 1B). The temperature at this species' original geographic occurrence (Japan) was also analyzed (Fig. 1A). Data were collected from several sources, including: i) sea temperature database (SST data available from www.seatemperature.org); and ii) Hobo V2 probes placed at the study site (S. Sebastião), glued to rocks of 8 replicate tide pools during low tide, using epoxy polyamide cement Tubolit®. *In situ* temperature data was recorded continuously during the warm season months (from December to February, 56 days) in 2015/16. Measurements were taken at intervals of 2 hours, 24/7.

Experimental layout

The experimental trials in captivity were performed in two separate semi-open aquaria systems of 200 L each under natural light conditions (14L:10D) but no direct sunlight. Each system consisted of 2 replicate glass tanks (25 × 25 × 25 cm), one seawater deposit, one sump, and a UV filter (see Fig. ESM1). After acclimation, *L. lipkei* specimens were randomly allocated into experimental tanks (n = 30 individuals.tank^{-1}), which were then randomly attributed to either the control or the +3ºC treatment (2

replicate tanks per temperature). Temperature was steadily increased ($0.10^{\circ}C.hr^{-1}$) to treatment conditions: a) control ($29.0^{\circ}C \pm 0.5^{\circ}C$), reflecting current mean summer water temperature at the collection site, and b) thermal challenge (+3ºC; $32.0^{\circ}C \pm 0.5^{\circ}C$), corresponding to projected mean summer water temperature for the tropical/subtropical Atlantic by 2100 (gradual warming scenario RCP8.5, IPCC 2013). Tanks were supplied with filtered seawater (35) and aeration allowing oxygen saturation (95 to 100%). Environmental enrichment was achieved by adding small cobbles to tanks. Thermostats were used to hold temperature constant for 28 days (Eheim® Jager Heater 150W, Germany). Sampling was performed at several timepoints (see Fig. ESM2-ESM3 in the electronic supplementary material) by euthanizing the individuals through longitudinal transection. Sample size varied within the ranges reported in previous studies (Madeira et al. 2012b, 2016, Vinagre et al. 2013):

i) For molecular biomarker analyses, animals were sampled once a week, at day 0, 7, 14, 21 and 28 days, always at the same period of the day (~ 9 am). Five individuals were randomly sampled from the two replicate tanks for each temperature and each time point (5 individuals × 5 timepoints × 2 temperatures, total n = 50 individuals). Gill and muscle tissues were frozen at -80˚C. This timeline follows OECD recommendations for repeated exposure toxicity studies, and was adapted to aquatic animals.

ii) For the determination of upper thermal limits, a subset of individuals was randomly sampled from each temperature treatment after 7 and 28 days of exposure, and subsequently subjected to Critical Thermal Maximum (CTMax) trials (13-14 individuals × 2 timepoints × 2 temperatures, total n = 54). CTMax is a dynamic method widely used for the determination of thermal limits in ectothermic animals (Kaspari et al. 2015). Individuals were maintained in a thermostatic bath with a steady heating of 1˚C each 15 min (Fig. ESM4 in the electronic supplementary material). This heating

rate is consistent to observations in tide pools during summer days, and follows the recommendations of Vinagre et al. (2015) for the use of ecologically realistic warming ramps. All individuals subjected to one CTMax trial were then removed from the experiment.

iii) For the assessment of body condition, morphometric measurements of the animals (total length and weight, 26 individuals × 2 timepoints × 2 temperatures, total n = 104) were taken using an icthyometer and a scale, respectively. The determination of energy reserves was performed using the same muscle samples referred in point i). Sampling times for whole-body assessments were performed at the beginning (0 days) and at the end (28 days) of the experiment. Mortality was monitored throughout trials.

Laboratory analyses

Molecular biomarkers

Molecular biomarkers were chosen based on their role on the cellular stress response (CSR): i) heat shock protein 70 kDa (Hsp70) is a chaperone with an adaptive value, repairing damaged proteins upon thermal stress (Feder and Hofmann 1999; Coles and Brown 2003; Hofmann 2005; Madeira et al. 2012a); ii) ubiquitin (Ub) targets irreversibly damaged proteins for proteasome degradation (Logan and Somero 2011; Tang et al. 2014); iii) antioxidant enzymes (catalase – CAT, glutathione-S-transferase – GST, superoxide dismutase – SOD) neutralize reactive oxygen species (ROS) and oxidation products (e.g. lipid peroxides) that arise at high temperature (Lushchak 2011; Vinagre et al. 2012), iv) lipid peroxides (LPO) are markers of damage to cell membranes (Pannunzio and Storey 1998; Logan and Somero 2011); v) acetylcholinesterase (AChE) is a neurotoxicity marker, it ends synaptic transmission at cholinergic synapses by catalyzing the breakdown of acetylcholine. When its activity is

disrupted or inhibited, it can lead to paralysis and possible cardio-vascular failure (Assis et al. 2012; Singh et al. 2013).

Samples of gills and muscle tissues (ca. 200-250 mg) were stored in 1.5 mL microtubes, and homogenated in a phosphate buffered saline solution (1 mL PBS, 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to extract the most soluble cytosolic proteins, following the methods previously described by Madeira et al. (2018a). Protein quantification and kinetic assays (Table 1) were undertaken using the colorimetric methods described and adapted for 96-well microplates (Madeira et al. 2015a) (Table ESM1 shows a detailed description of each method). Total protein was quantified using the Bradford method (Bradford 1976) to standardize all biomarker results.

Energy reserves

C:N ratios indicate transitions linked to changes in main organic components, such as proteins (N rich) and lipids (C rich) (Vollenweider 1985). Therefore, the C:N ratio was used as a proxy of energy metabolism/reserves, and % C used as a proxy of lipid reserves (following Sterner and Elser 2002). Muscle samples were lyophilized (by freeze-drying in vacuum) and grounded to a fine homogeneous powder. Samples of ~0.5 mg were loaded into tin cups and analyzed using an Elementar Isoprime continuous-flow mass spectrometer (GV Instruments) coupled to a vario PYRO cube elemental analyser (Elementar, Hanau, Germany). Reference materials (acetanilide; Stable Isotope Research Facility, Indiana University, USA) were assayed at the beginning of each run and after every 10 samples. Additionally, total protein was also analyzed as a proxy of energy reserves, since proteins can serve as respiratory substrates

when other energy sources are depleted (for instance due to stress; Alberts et al. 2002). Total protein was extracted as described in the previous section (Bradford 1976).

Data analyses

Some calculations were performed previously to statistical analyses as follows:

i) Biomarker results were all standardized by the total protein in each sample.

ii) Thermal limits (CTMax) were calculated for each species as:

$$(1)\ \mathrm{CTMax}_{(\mathrm{species})} = \sum \frac{(T_{end-point})}{n}$$

where $T_{end\text{-}point}$ stands for the temperature at which an end-point was observed for any given individual and n is sample size. Coefficients of variation (% CV) were calculated to estimate intraspecific CTMax variation as follows:

$$(2)\ \%\mathrm{CV} = \left(\frac{\mathrm{SD}}{\mathrm{Mean}}\right) \times 100$$

The acclimation capacity was determined by: i) comparing mean CTMax values after short term and longer term exposure to both temperature conditions ($CTMax_{T28}$ – $CTMax_{T7}$ at each temperature and $CTMax_{32°C}$ – $CTMax_{29°C}$ at 7 and 28 days); ii) acclimation response ratio (ARR, (Claussen 1977)) as ΔCTMax / Δtemperature. Thermal safety margin (TSM, i.e. warming tolerance) was calculated for the intertidal and subtidal environments as the difference between mean CTMax for this species and Maximum Habitat Temperature (MHT) determined through field measurements at intertidal and subtidal habitats. This difference indicates how close the test species is to its upper thermal limit.

iii) Body condition was calculated by Fulton's K index, from morphometric data, as follows:

$$(3)\ \mathrm{K} = 100\mathrm{M_t}/\mathrm{L_t^3}$$

where M_t is the total wet mass (mg) and L_t is the total length (mm) (Ricker 1975).

To calculate the C:N ratio of each muscle sample, %C and %N were first divided by the molar weight of each element (12.0107 $g.mol^{-1}$ for carbon and 14.0067 $g.mol^{-1}$ for nitrogen), and then the molar ratio was calculated. Additionally, mortality records were calculated as percentages (%) and compared across treatments.

Exploratory analyses were performed on the datasets to identify outliers (using the 1.5 coefficient), and to test normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test). When, at least, one of the assumptions of a specific test (e.g. normality or homoscedasticity) were not met, either a data transformation (e.g. logarithmic) was performed or a non-parametric test was used (this applies to all the statistical analyses performed). The principal objective of statistical analyses was to evaluate the main temperature effects (present day vs predicted from warming modeling) and/or acclimation capacity (as time-dependent temperature effects) on the several physiological parameters measured (from molecular to cellular to individual level).

Factorial ANOVAs were carried out to detect: a) which biomarkers contribute to differentiate between temperature treatments (29˚C vs 32˚C), exposure times (T0, T7, T14, T21, T28), and tissue types (gills and muscle; following the statistical approaches by Whitehead and Crawford 2005, and Madeira et al. 2016); b) differences between biomarker levels throughout the experiment at different temperatures (through post-hoc Tukey tests). For simplicity, the results only show significant differences from control (29˚C) at each time-point. A Principal Component Analysis (PCA) was carried out for each tissue to detect biomarkers that correlate with temperature and time and that contribute to explain the variance in the dataset. Upper thermal limit data were analyzed with a factorial ANOVA (temperature – 29˚C vs 32˚C, and time – T7 vs T28, as categorical predictors, and CTMax as dependent variable). Tukey's post-hoc tests for unequal N were carried out to detect differences between groups. Finally, Fulton's K

condition index, total protein, % C, and C:N ratio were also analyzed with factorial ANOVAs (temperature - 29˚C vs 32˚C, and time – T0 vs T28, as factors). Statistical analyses were performed in Statistica (version 13.0, Statsoft Inc. USA) and the *p*-value of 0.05 was used to decide on statistical significance.

Results

Characterization of the thermal environments of rocky reefs at the original and newly colonized areas

Temperature satellite data from the past 5 years showed that the average SST (Fig. 2A) in several coastal cities of Japan (from the Boso Peninsula to the Ryukyu Islands) ranges from 19.9˚C (February) to 28.1˚C (August), with maximum coastal water temperatures reaching 30˚C during summer. Average sea surface temperatures along the northeastern Brazilian coast vary from 26.6˚C to 28.9˚C throughout the year, reaching maximum temperatures of 29.7˚C (Fig. 2A). Finally, at our study location, S. Sebastião (Fig. 2B), satellite data showed that temperature ranges approximately from 24˚C to 26˚C during the warm months (from December to March) with maximum water temperatures of 29˚C during March.

Temperature data from field probes in S. Sebastião (Southeastern Brazil) showed a mean daily minimum and maximum temperatures ranging from 18.43˚C to 37.60˚C (Fig. ESM5, ESM6, and Table ESM2). There was an absolute maximum record of 41.50˚C (MHT) in three of the tide pools sampled. Mean monthly temperatures varied between 25.72˚C to 29.32˚C, and February was the warmest month recorded in the data loggers. Monthly temperature variation (average maximum – average minimum) was 16.60˚C ± 2.74˚C, while average daily temperature variation was 4.78˚C ± 1.06˚C.

Temperature effects on *Lysmata lipkei*

Stress assessment at lower levels of biological organization

Overall, *Lysmata lipkei* showed physiological changes in response to increased temperature during the experimental period. Significant effects were observed for biomarkers Hsp70, CAT, GST and SOD (Table 2, see also Table ESM3 for basal biomarker levels). Significant differences in biomarker levels between tissues were also detected (Table 2), as well as significant interaction between factors for some of the biomarkers (Table 2). In particular, significant temperature *vs*. time interactions were recorded for all biomarkers. The temperature-tissue-time interaction was also significant for Ub and SOD. Based on the PCA (Fig. 3, see table ESM4 for factor loadings), Hsp70 and SOD were the gill biomarkers that correlated the most to temperature, while no particular biomarker associated with time. In the muscle tissue, Ub and CAT were correlated with temperature, and AChE and GST were the biomarkers most correlated to time. Additionally, the variance explained by PC1 and PC2 in the gills (60.02% and 19.30%, for PC1 and PC2, respectively) was higher than in the muscle (31.85% and 23.48% for PC1 and PC2, respectively).

Significant biomarker changes under increased temperature for each sampling time and tissue are presented in Fig. 4. Shrimp biomarkers were more responsive to temperature increase after seven days of exposure (all biomarkers in gills, and CAT and AChE in muscle). After the first week of exposure, biomarker levels returned to control conditions, with no differences between temperature treatments at day 14 and 21. Nevertheless, a significant change was observed again after 28 days, coherently in both tissues and characterized by an increase in LPO levels. A common pattern was conspicuous for both tissues: biomarkers that significantly responded to the temperature challenge showed the same pattern/direction of response (increase) and similar response

timings. Nevertheless, biomarker response in the gills showed slightly higher fold-changes than in the muscle tissue (see Fig. ESM7 for a summary of fold-change results).

The most responsive biomarkers in both tissues were CAT and LPO. Hsp70, Ub, GST and SOD only showed changes in gill tissue. Almost all biomarkers showed a rapid response to an increased thermal load, except for LPO, which took 28 days to show a significant change.

Stress assessment at higher levels of biological organization

Lysmata lipkei showed mean CTMax values ranging from 36.6˚C to 38.4˚C at control and increased temperature, respectively, with significantly higher CTMax at 32˚C than at control temperature ($F = 49.60$, p-value <0.01). An interactive effect of acclimation time *vs.* temperature was observed for CTMax ($F = 11.68$, p-value <0.01), suggesting that acclimation time affects thermal tolerance plasticity (Fig. 5). Particularly, significant differences were found for CTMax between 29˚C and 32˚C, after acclimating for 7 and 28 days, but the degree of plasticity (i.e., environmentally induced phenotypic change) was higher after longer acclimation times. An acclimation capacity of 0.87˚C was recorded after 7 days and 2.51˚C after 28 days, with an overall ARR of 0.6. A negative TSM of -3.99˚C was recorded in the intertidal environment and a positive TSM of 9.45˚C was recorded in the subtidal environment.

The condition indicators analyzed for *L. lipkei* exposed to different temperature conditions only showed significant differences for total protein (Table 3), with lower protein content at 32˚C when compared to the 29˚C treatment. A significant temperature-time interaction was also solely observed for total protein, particularly at T28 (p-value = 0.031). All other condition indicators (Fulton's K, % C, and C:N) were

similar between experimental treatments and throughout the experiment. No shrimp mortality was recorded throughout the experiments.

Discussion

Understanding the panoply of responses and adaptations of invasive species to changes in environmental temperature can provide notable insights about the patterns and processes of species invasions under climate change scenarios (Zerebecki and Sorte 2011). In this study, we showed that the recently invasive shrimp species *Lysmata lipkei* in the Southwestern Atlantic is capable of substantial acclimation to chronic elevated temperature, with no mortality observed over experimental trials. This main finding is aligned to the meta-analysis results reported by Sorte et al. (2013) which analyzed performance of 150 non-native species under different environmental conditions and concluded that these organisms seem to be well-adapted and capable of thriving in warmed aquatic ecosystems. Overall, our results show that (i) this shrimp species is sensitive enough to elicit a detectable response to high temperature (in accordance with other studies with caridean shrimps, e.g. Allan et al. 2006; González-Ortegón et al. 2013; Reiser et al. 2014; Magozzi and Calosi 2015); (ii) the intensity of stress and duration of exposure are key features determining the onset of biochemical and physiological adjustments (as also observed by, e.g., Buckley et al. 2004; Schulte et al. 2011; Madeira et al. 2016 for several ectotherms); and that iii) no variations were detected in condition indices and lipid reserves between treatments, suggesting that shrimps maintained their weight and condition.

It is important to consider the similarity between temperature regimes along the Southern Atlantic Ocean and at the shrimps' original geographic distribution in Japan. All temperature datasets showed that the average yearly temperature range in the

invaded areas in NE and SE Brazil (about 22˚C – 28˚C) is very similar to that of their native range in Japan (about 20˚C – 28˚C), with mean maximal temperatures approaching 30˚C. *In situ* data also showed that these shrimps experience wide temperature variations in the invaded area. Considering that *L. lipkei* inhabit the intertidal and shallow subtidal habitats in their original geographical area (Okuno and Fiedler 2010), which are naturally prone to abiotic variation, these shrimps are likely equipped with adaptive mechanisms that allow them to withstand extremely variable environments (Ravaux et al. 2016). In fact, tolerance to temperature fluctuations has already been proposed as a relevant physiological trait that might underlie successful species invasions (McMahon 2002; Zerebecki and Sorte 2011; Kelley 2014).

Molecular mechanisms underpinning *L. lipkei* eurythermality varied with thermal load and time, as observed for other decapod crustaceans (e.g. Tavares-Sánchez et al. 2004; Qian et al. 2012; Vinagre et al. 2014; Madeira et al. 2015b; Rodríguez-Fuentes et al. 2017). Biomarkers Hsp70 and SOD in the gills, and Ub and CAT in the muscle were mostly associated with temperature, while damage to lipids (LPO) was mostly associated with the duration of exposure. These results suggest that chaperone and antioxidant defenses can play an important role during thermal stress by stabilizing damaged proteins (Tomanek 2010) and protecting cells from the toxic effects of ROS (Lesser 2006), thereby allowing the proper management of cellular functioning and the maintenance of homeostasis across environmental temperature changes (Farcy et al. 2009). This seems to be enough to promote survival and avoid general metabolic failure (Halpin et al. 2002; Lesser 2006; Yamashita et al. 2010; Madeira et al. 2013) in this shrimp species. Also noteworthy, the significant interaction between temperature and time for AChE in muscle tissue (which mostly occurs at neuromuscular junctions (Valenzuela and Akaaboune 2007)) suggests a higher rate of neurotransmission and

muscle activity (contraction and relaxation). Klose and Robertson (2004) suggest that high temperatures can induce synaptic thermoprotection and increase the release of neurotransmitters in animals. In addition, this is also an indicator of higher metabolism, which may enhance performance, foraging efficiency and predator avoidance, but it may also result in more energy expenditure. LPO results combined with an absence of changes in growth, lipid reserves or mortality after chronic warming suggest that LPO is likely functioning as a cell signaling mechanism in *L. lipkei*, which may in the long-term induce an adaptation process (Niki 2012; Ayala et al. 2014). Alternatively, longer experimental trials are needed to detect deleterious effects of LPO in condition indicators. Current studies on the dynamics of shifts in the lipid composition of cell membranes suggest that temperature increase affects the balance between long-chain polyunsaturated fatty acids (PUFAs) and small-chain saturated fatty acids (SFA) (Imbs and Yakovleva 2012). Thermal stress may induce a reduction in multiple PUFAs over time, leading to structural modifications in bio-membranes and causing leakiness (Hillyer et al. 2017). Additionally, long-term changes in overall respiration or mitochondrial membrane composition/density can affect LPO, which might explain the LPO increase solely after 28 days of exposure to higher temperature. Future studies should combine histopathological analyses with LPO biochemical measurements in order to observe the damage extension of cell membranes in organs, and confirm whether those alterations are within threshold ranges of normal metabolic processes or, alternatively, if they indicate apoptosis and/or necrosis (Madeira et al. 2014). Finally, the different biomarker responses observed for muscle and gill tissues suggest tissue-specific bio-chemistry and function. Gill metabolism is highly aerobic and has a high rate of O_2 diffusion and mitochondrial activity, which may lead to a higher level of ROS

accumulation and induce a proportionally higher response of antioxidants during acclimation (Verlecar et al. 2008; Madeira et al. 2016).

The activation of those cellular stress responses has been previously linked to shifts in organismal thermal tolerance (Feder and Hofmann 1999; Whitley et al. 1999; Madeira et al. 2015b; Rahlff et al. 2017). Our study corroborates these findings since CTMax of *L. lipkei* were considerably high (between 36.6˚C to 38.4˚C) among ectothermic animals, and are in line with upper thermal limits recorded for other tropical shrimps (e.g., 39.7˚C for *Palaemon northropi*, 36˚C for *Litopenaeus vannamei*, 34.7˚C for *Hippolyte obliquimanus,* (Ravaux et al. 2016) and 39ºC for *Pachygrapsus transversus* (Vinagre et al. 2016)), as well as for other invasive crustaceans (e.g. 33˚C for *Palaemon macrodactylus,* Lejeusne et al. 2014; 35˚C for *Carcinus maenas,* Cuculescu et al. 1998). In fact, an integrative study of the physiology of invasive vs native species found that the mean upper thermal limits for invasive decapods was around 35˚C (Kelley 2014), which suggests that the CTMax of *L. lipkei* is higher than average. This ultimately suggests that their high thermal tolerance may have been crucial in the invasion of Southern Atlantic coastal habitats. The TSM values here calculated for intertidal and subtidal habitats (-3.99˚C and 9.45˚C, respectively) suggest that *L. lipkei* may expand further south and more into subtidal habitats along the Brazilian coast. By the year 2100, models predict that water temperatures in the Southern Atlantic will increase by 3˚C (IPCC 2013), resulting in a summer average of 27˚C to 29˚C in subtidal areas and an average temperature of 32˚C in the intertidal area of São Sebastião, which is still well below the CTMax estimate for this species. The high acclimation capacity of *L. lipkei* to increased temperature over long exposure times indicates that not only is this species capable to accommodate future temperature change, but also that thermal history may push upper thermal limits beyond, as already documented for other crustaceans (Ober et

al. 2016). According to Ravaux et al. (2016), the ARR value of 0.6 obtained for *L. lipkei* does represent a genuine acclimatory capacity. Our results thus challenge previous findings suggesting a poor acclimation capacity in thermal tolerant species, such as intertidal ones (Stillman 2004; Ravaux et al. 2016; Madeira et al. 2018b).

Acclimation is often thought to impose considerable metabolic costs to organisms (Angilletta et al. 2004), as it relies on energetic reserves otherwise used for different functions, but the evidence so far is weak because such costs are difficult to quantify (Tomanek and Somero 2002). Our study agrees with Clark et al. (2013) in that acclimation in aquatic ectotherms typically occurs within 1-3 weeks, not imposing burdening energy costs for this invasive shrimp. No differences were detected either in mortality and growth rates, physiological condition or the quantity of lipid reserves after experimental trials. This indicates that adults of *L. lipkei* were able to maintain homeostasis, and to keep energy balance and oxygen consumption within narrow ranges as observed for other invertebrate species (see Kühnhold et al. 2016). Verberk et al. (2016) explained this balance based on the fact that organisms depend on a constant and sufficient flux of O_2 from their environment to their metabolizing tissues in order to ensure an adequate ATP supply to cover all physiological demands and still maintain the energy status (Rodríguez-Fuentes et al. 2017). It should be stressed that shrimps were fed *ad libitum* in this study and could have met any energy demands by increasing food intake. Interestingly, climate change predictions for the study area project an increase in precipitation (Marengo 2007; PBMC, 2014) and food availability in coastal areas through runoff of terrestrial inputs, which could potentially fuel benthic energetic pathways (Mendonça et al. 2018). This is expected to favor epibenthic feeders, which include some important consumers in intertidal habitats, such as *Lysmata lipkei*, and many non-native species which are usually capable to exploit and often monopolize

surplus trophic resources (Sorte et al. 2013). Nevertheless, it is worth noting that organismal-level responses, such as metabolic rates and long term processes, such as growth and reproduction (Pörtner and Knust 2007), may not have the same time-course as cellular-level responses (Rahlff et al. 2017). Longer experimental trials, including all life-cycle stages, may be necessary to detect changes at higher levels of biological complexity.

Biomarker responses are therefore often suitable indicators to support management policies by prioritizing areas and species that need local or regional intervention. However, physiological biomarkers should ideally be used in combination with both traditional (e.g. biodiversity metrics, water-quality analyses) and recent methodological approaches (e.g. molecular markers, metabolomics and proteomics) to integrate lines of evidence determining the invasion potential.

Conclusions

The main conclusion is that *Lysmata lipkei* can easily acclimate to increased water temperature. This capacity may enable this species to continue its invasion to tropical and subtropical areas along the Southwestern Atlantic coast. Results further suggest that *L. lipkei*, similarly to other non-indigenous species, shows specific physiological traits that make them successful invaders, namely (i) tolerance to upper thermal limits, and (ii) strong ability to acclimate and make physiological adjustments while maintaining performance. The recognition of such phenotypic traits can help scientists and managers to identify species that have the physiological ability to tolerate both present and future abiotic environments in specific geographic regions. This should be an important tool to predict which areas are more susceptible for invasions, or range expansions, and

develop preventive measures to avoid the introduction of non-native species or allow better management in locations where invaders have already settled.

Electronic supplementary material The online version of this article contains supplementary material, which is available to authorized users.

Compliance with ethical standards

Research involving animals: the authors declare that animal experiments followed legal guidelines for laboratory animal science and were authorized by competent authorities, as stated in the materials and methods subsection "Ethical guidelines".

Declaration of interest: the authors declare that they have no conflicts of interest.

References

Alberts B, Johnson A, Lewis J, Al. E (2002) How cells obtain energy from food. In: Molecular Biology of the Cell, 4th editio. Garland Science, New York, NY, USA,

Allan EL, Froneman PW, Hodgson AN (2006) Effects of temperature and salinity on the standard metabolic rate (SMR) of the caridean shrimp *Palaemon peringueyi*. J Exp Mar Bio Ecol 337:103–108. doi: 10.1016/j.jembe.2006.06.006

Angilletta MJ, Bennett AF, Guderley H, et al (2006) Coadaptation: A unifying principle in evolutionary thermal biology. Physiol Biochem Zool 79:282–294. doi: 10.1086/499990

Angilletta MJ, Steury TD, Sears MW (2004) Temperature, Growth Rate, and Body Size in Ectotherms: Fitting Pieces of a Life-History Puzzle. Integr Comp Biol 44:498–509. doi: 10.1093/icb/44.6.498

Assis CRD, Linhares AG, Oliveira VM, et al (2012) Comparative effect of pesticides on

brain acetylcholinesterase in tropical fish. Sci Total Environ 441:141–150. doi: 10.1016/j.scitotenv.2012.09.058

Ayala A, Muñoz MF, Argüelles S (2014) Lipid peroxidation: production, metabolism and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014:Article ID 360438, 31 pages. doi: 10.1093/europace/eux022

Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 42:517–525. doi: 10.1093/icb/42.3.517

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi: 10.1016/0003-2697(76)90527-3

Buckley B a, Place SP, Hofmann GE (2004) Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*. J Exp Biol 207:3649–3656. doi: 10.1242/jeb.01219

Burgiel SW, Muir A a. (2010) Invasive Species , Climate Change and Ecosystem-Based Adaptation : Addressing Multiple Drivers of Global Change. Global Invasive Species Programme (GISP), Washington, DC, US and Nairobi, Kenya

Clark MS, Fraser KPP, Burns G, Peck LS (2008) The HSP70 heat shock response in the Antarctic fish *Harpagifer antarcticus*. Polar Biol 31:171–180. doi: 10.1007/s00300-007-0344-5

Clark TD, Sandblom E, Jutfelt F (2013) Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J Exp Biol 216:2771–2782. doi: Doi 10.1242/Jeb.084251

Claussen D (1977) Thermal acclimation in ambystomatid salamanders. Comp Biochem

Physiol - A Mol Integr Physiol 58:333–340.

Coles SL, Brown BE (2003) Coral bleaching - Capacity for acclimatization and adaptation. Adv Mar Biol 46:183–223. doi: 10.1016/S0065-2881(03)46004-5

Cortes PA, Puschel H, Acuña P, et al (2016) Thermal ecological physiology of native and invasive frog species: do invaders perform better? Conserv Physiol 4:cow056. doi: 10.1093/conphys/cow056

Cuculescu M, Hyde D, Bowler K (1998) Thermal tolerance of two species of marine crab, *Cancer pagurus* and *Carcinus maenas*. J Therm Biol 23:107–110.

Ellman GL, Courtney KD, Andres Jr V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88-90,IN1-IN2,91-95. doi: 10.1016/0006-2952(61)90145-9

Farcy É, Voiseux C, Lebel JM, Fiévet B (2009) Transcriptional expression levels of cell stress marker genes in the pacific oyster *Crassostrea gigas* exposed to acute thermal stress. Cell Stress Chaperones 14:371–380. doi: 10.1007/s12192-008-0091-8

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282. doi: 10.1146/annurev.physiol.61.1.243

Festing MFW, Altman DG (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J 43:244–258. doi: 10.1093/ilar.43.4.244

González-Ortegón E, Pascual E, Drake P (2013) Respiratory responses to salinity, temperature and hypoxia of six caridean shrimps from different aquatic habitats. J Exp Mar Bio Ecol 445:108–115. doi: 10.1016/j.jembe.2013.04.006

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S transferases. The first

enzymatic step in mercapturic acid formation. Biol Chem 7130–7139.

Halpin PM, Sorte CJ, Hofmann GE, Menge B a (2002) Patterns of Variation in Levels of Hsp70 in Natural Rocky Shore Populations from Microscales to Mesoscales. Integr Comp Biol 42:815–824. doi: 10.1093/icb/42.4.815

Hau J, Schapiro SJ (2010) Handbook of Laboratory Animal Science: Essential Principles and Practices - Volume I, 3rd edn. CRC Press, Taylor and Francis Group, Boca Raton, FL, USA

Hillyer KE, Dias DA, Lutz A, et al (2017) Metabolite profiling of symbiont and host during thermal stress and bleaching in the coral *Acropora aspera*. Coral Reefs 36:105–118. doi: 10.1007/s00338-016-1508-y

Hoefnagel KN, Verberk WCEP (2016) Long-term and acute effects of temperature and oxygen on metabolism, food intake, growth and heat tolerance in a freshwater gastropod. J Therm Biol 68:1–12. doi: 10.1016/j.jtherbio.2016.11.017

Hofmann GE (2005) Patterns of Hsp gene expression in ectothermic marine organisms on small to large biogeographic scales. Integr Comp Biol 45:247–255. doi: 10.1093/icb/45.2.247

Hofmann GE, Somero GN (1995) Evidence for protein damage at environmental temperatures : Seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. J Exp Biol 198:1509–1518.

Imbs AB, Yakovleva IM (2012) Dynamics of lipid and fatty acid composition of shallow-water corals under thermal stress: An experimental approach. Coral Reefs 31:41–53. doi: 10.1007/s00338-011-0817-4

IPCC (2013) Atlas of Global and Regional Climate Projections. Clim Chang 2013 Phys Sci Basis Contrib Work Gr I to Fifth Assess Rep Intergov Panel Clim Chang Annexe I:1311–1394. doi: 10.1017/CBO9781107415324.029

Johansson LH, Borg LA (1988) A spectrophotometric method for determination of catalase activity in small tissue samples. Anal Biochem 174:331–336. doi: 10.1016/0003-2697(88)90554-4

Kaspari M, Clay NA, Lucas J, et al (2015) Thermal adaptation generates a diversity of thermal limits in a rainforest ant community. Glob Chang Biol 21:1092–1102. doi: 10.1111/gcb.12750

Kelley AL (2014) The role thermal physiology plays in species invasion. Conserv Physiol 2:1–14. doi: 10.1093/conphys/cou045

Klose MK, Robertson RM (2004) Stress-induced thermoprotection of neuromuscular transmission. Integr Comp Biol 44:14–20. doi: 10.1093/icb/44.1.14

Kolar CS, Lodge DM (2001) Progress in invasion biology: Predicting invaders. Trends Ecol Evol 16:199–204. doi: 10.1016/S0169-5347(01)02101-2

Kühnhold H, Kamyab E, Novais S, et al (2016) Thermal stress effects on energy resource allocation and oxygen consumption rate in the juvenile sea cucumber, *Holothuria scabra* (Jaeger, 1833). Aquaculture 467:1–9. doi: 10.1016/j.aquaculture.2016.03.018

Lejeusne C, Latchere O, Petit N, et al (2014) Do invaders always perform better? Comparing the response of native and invasive shrimps to temperature and salinity gradients in south-west Spain. Estuar Coast Shelf Sci 136:102–111. doi: 10.1016/j.ecss.2013.11.014

Lesser MP (2006) Oxidative Stress in Marine Environments: Biochemistry and Physiological Ecology. Annu Rev Physiol 68:253–278. doi: 10.1146/annurev.physiol.68.040104.110001

Loarie SR, Duffy PB, Hamilton H, et al (2009) The velocity of climate change. Nature 462:1052–1055. doi: 10.1038/nature08649

Logan C a, Somero GN (2011) Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper). Am J Physiol Regul Integr Comp Physiol 300:R1373-83. doi: 10.1152/ajpregu.00689.2010

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101:13–30. doi: 10.1016/j.aquatox.2010.10.006

Madeira C, Leal MC, Diniz MS, et al (2018a) Thermal stress and energy metabolism in two circumtropical decapod crustaceans: Responses to acute temperature events. Mar Environ Res. doi: 10.1016/j.marenvres.2018.08.015

Madeira C, Madeira D, Diniz MS, et al (2016) Thermal acclimation in clownfish: An integrated biomarker response and multi-tissue experimental approach. Ecol Indic. doi: 10.1016/j.ecolind.2016.07.009

Madeira C, Madeira D, Diniz MS, et al (2017) Comparing biomarker responses during thermal acclimation: A lethal vs non-lethal approach in a tropical reef clownfish. Comp Biochem Physiol Part A Mol Integr Physiol 204:104–112. doi: 10.1016/j.cbpa.2016.11.018

Madeira C, Madeira D, Vinagre C, Diniz M (2015a) Octocorals in a changing environment: Seasonal response of stress biomarkers in natural populations of Veretillum cynomorium. J Sea Res 103:120–128.

Madeira C, Mendonça V, Flores AAV, et al (2018b) High thermal tolerance does not protect from chronic warming – A multiple end-point approach using a tropical gastropod, Stramonita haemastoma. Ecol Indic. doi: 10.1016/j.ecolind.2018.04.044

Madeira D, Mendonça V, Dias M, et al (2015b) Physiological, cellular and biochemical thermal stress response of intertidal shrimps with different vertical distributions: Palaemon elegans and Palaemon serratus. Comp Biochem Physiol - Part A Mol

Integr Physiol 183:107–115. doi: 10.1016/j.cbpa.2014.12.039

Madeira D, Narciso L, Cabral HN, et al (2012a) HSP70 production patterns in coastal and estuarine organisms facing increasing temperatures. J Sea Res 73:137–147. doi: 10.1016/j.seares.2012.07.003

Madeira D, Narciso L, Cabral HN, et al (2013) Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comp Biochem Physiol Part A Mol Integr Physiol 166:237–243. doi: 10.1016/j.cbpa.2013.06.008

Madeira D, Narciso L, Cabral HN, Vinagre C (2012b) Thermal tolerance and potential impacts of climate change on coastal and estuarine organisms. J Sea Res 70:32–41. doi: 10.1016/j.seares.2012.03.002

Madeira D, Vinagre C, Costa PM, Diniz MS (2014) Histopathological alterations, physiological limits, and molecular changes of juvenile Sparus aurata in response to thermal stress. Mar Ecol Prog Ser 505:253–266. doi: 10.3354/meps10794

Magozzi S, Calosi P (2015) Integrating metabolic performance, thermal tolerance, and plasticity enables for more accurate predictions on species vulnerability to acute and chronic effects of global warming. Glob Chang Biol 21:181–194. doi: 10.1111/gcb.12695

Marengo JA (2007) Mudanças Climáticas Globais e seus Efeitos sobre a Biodiversidade: Caracterização do Clima Atual e Definição das Alterações Climáticas para o Território Brasileiro ao Longo do Século XXI. Série Biodiversidade 26:212. doi: 10.1017/CBO9781107415324.004

McMahon RF (2002) Evolutionary and physiological adaptations of aquatic invasive animals: r selection versus resistance. Can J Fish Aquat Sci 59:1235–1244. doi: 10.1139/f02-105

Mendonça V, Madeira C, Dias M, et al (2018) What's in a tide pool? Just as much food

web network complexity as in large open ecosystems. PLoS One. doi: 10.1371/journal.pone.0200066

Niki E (2012) Do antioxidants impair signaling by reactive oxygen species and lipid oxidation products? FEBS Lett 586:3767–3770. doi: 10.1016/j.febslet.2012.09.025

Ober GT, Thornber C, Grear J, Kolbe JJ (2016) Ecological differences influence the thermal sensitivity of swimming performance in two co-occurring mysid shrimp species with climate change implications. J Therm Biol 64:26–34. doi: 10.1016/j.jtherbio.2016.11.012

Okuno J, Fiedler C (2010) Lysmata lipkei: A new species of Peppermint Shrimp (Decapoda, Hippolytidae) from warm temperate and subtropical waters of Japan. In: Fransen C, de Grave S, Ng P (eds) Studies of Malacostraca: Lipke Bijdeley Holthuis Memorial Volume - Crustaceana Monographs. Koninklijke Brill, NV. Leiden, Netherlands., pp 597–610

Pachelle PPG, Anker A, Mendes CB, Bezerra LEA (2016) Decapod crustaceans from the state of Ceará, northeastern Brazil: an updated checklist of marine and estuarine species, with 23 new records. Zootaxa 4131:1–63. doi: 10.11646/zootaxa.4131.1.1

Pannunzio TM, Storey KB (1998) Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. J Exp Mar Bio Ecol 221:277–292. doi: 10.1016/S0022-0981(97)00132-9

Payne NL, Smith JA, van der Meulen DE, et al (2016) Temperature dependence of fish performance in the wild: Links with species biogeography and physiological thermal tolerance. Funct Ecol 903–912. doi: 10.1111/1365-2435.12618

PBMC, 2014: Base científica das mudanças climáticas. Contribuição do Grupo de Trabalho 1 do Painel Brasileiro de Mudanças Climáticas ao Primeiro Relatório da

Avaliação Nacional sobre Mudanças Climáticas [Ambrizzi, T., Araujo, M. (eds.)]. COPPE. Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil, 464 pp

Pérez-Casanova JC, Rise ML, Dixon B, et al (2008) The immune and stress responses of Atlantic cod to long-term increases in water temperature. Fish Shellfish Immunol 24:600–609. doi: 10.1016/j.fsi.2008.01.012

Pörtner HO, Farrell A (2008) Physiology and climate change. Science 322:690–692. doi: 10.1126/science.1163156

Pörtner HO, Knust R (2007) Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315:95–97. doi: 10.1126/science.1135471

Qian Z, Liu X, Wang L, et al (2012) Gene expression profiles of four heat shock proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*. Comp Biochem Physiol C Toxicol Pharmacol 156:211–20. doi: 10.1016/j.cbpc.2012.06.001

Rahlff J, Peters J, Moyano M, et al (2017) Short-term molecular and physiological responses to heat stress in neritic copepods *Acartia tonsa* and *Eurytemora affinis*. Comp Biochem Physiol Part A Mol Integr Physiol 203:348–358. doi: 10.1016/j.cbpa.2016.11.001

Ravaux J, Léger N, Rabet N, et al (2016) Plasticity and acquisition of the thermal tolerance (upper thermal limit and heat shock response) in the intertidal species *Palaemon elegans*. J Exp Mar Bio Ecol 484:39–45. doi: 10.1016/j.jembe.2016.07.003

Reiser S, Herrmann JP, Temming A (2014) Thermal preference of the common brown shrimp (*Crangon crangon*, L.) determined by the acute and gravitational method. J Exp Mar Bio Ecol 461:250–256. doi: 10.1016/j.jembe.2014.08.018

Ricker WE (1975) Computation and interpretation of biological statistics of fish populations. Bull Fish Res Board Can 191:1–382.

Rodríguez-Fuentes G, Murúa-Castillo M, Díaz F, et al (2017) Ecophysiological biomarkers defining the thermal biology of the Caribbean lobster *Panulirus argus*. Ecol Indic 78:192–204. doi: 10.1016/j.ecolind.2017.03.011

Rushworth KJW, Smith SDA, Cowden KL, Purcell SW (2011) Optimal temperature for growth and condition of an endemic subtropical anemonefish. Aquaculture 318:479–482. doi: 10.1016/j.aquaculture.2011.06.004

Schulte PM, Healy TM, Fangue NA (2011) Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. Integr Comp Biol 51:691–702. doi: 10.1093/icb/icr097

Shabtay A, Arad Z (2005) Ectothermy and endothermy: evolutionary perspectives of thermoprotection by HSPs. J Exp Biol 208:2773–81. doi: 10.1242/jeb.01705

Singh A, Jaiswal SK, Sharma B (2013) Effect of low temperature stress on acetylcholinesterase activity and its kinetics in 5th instar larvae of Philosamia ricini. J Biochem Res 1:17–25.

Somero GN (2002) Thermal physiology and vertical zonation of intertidal animals: optima, limits and costs of living. Integr Comp Biol 42:780–789. doi: doi: 10.1093/icb/42.4.780

Sorte CJB, Ibáñez I, Blumenthal DM, et al (2013) Poised to prosper? A cross-system comparison of climate change effects on native and non-native species performance. Ecol Lett 16:261–270. doi: 10.1111/ele.12017

Sterner RW, Elser JJ (2002) Ecological Stoichiometry: The biology of elements from molecules to the biosphere. Princeton University Press, NJ, USA

Stillman JH (2004) A comparative analysis of plasticity of thermal limits in porcelain

crabs across latitudinal and intertidal zone clines. Int Congr Ser 1275:267–274. doi: 10.1016/j.ics.2004.09.034

Sun Y, Oberley LW, Li Y (1988) A simple method for clinical assay of superoxide dismutase. Clin Chem 34:497–500.

Tang C-H, Leu M-Y, Shao K, et al (2014) Short-term effects of thermal stress on the responses of branchial protein quality control and osmoregulation in a reef-associated fish, Chromis viridis. Zool Stud 53:21. doi: 10.1186/s40555-014-0021-7

Tavares-Sánchez OL, Gómez-Anduro G a, Felipe-Ortega X, et al (2004) Catalase from the white shrimp *Penaeus (Litopenaeus) vannamei:* molecular cloning and protein detection. Comp Biochem Physiol B Biochem Mol Biol 138:331–7. doi: 10.1016/j.cbpc.2004.03.005

Tomanek L (2010) Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J Exp Biol 213:971–979. doi: 10.1242/jeb.038034

Tomanek L, Somero GN (2002) Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of hsp gene expression. J Exp Biol 205:677–685.

Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 86:271–278. doi: 10.1016/0003-2697(78)90342-1

Valenzuela IMP, Akaaboune M (2007) Acetylcholinesterase mobility and stability at the neuromuscular junction of living mice. Mol Biol Cell 18:2904–2911. doi: 10.1091/mbc.E07

Varela R, Lima FP, Seabra R, et al (2018) Coastal warming and wind-driven upwelling: A global analysis. Sci Total Environ 639:1501–1511. doi: 10.1016/j.scitotenv.2018.05.273

Verberk WCEP, Overgaard J, Ern R, et al (2016) Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. Comp Biochem Physiol -Part A Mol Integr Physiol 192:64–78. doi: 10.1016/j.cbpa.2015.10.020

Verlecar XN, Jena KB, Chainy GBN (2008) Seasonal variation of oxidative biomarkers in gills and digestive gland of green-lipped mussel *Perna viridis* from Arabian Sea. Estuar Coast Shelf Sci 76:745–752. doi: 10.1016/j.ecss.2007.08.002

Viant MR, Werner I, Rosenblum ES, et al (2003) Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead trout (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. Fish Physiol Biochem 29:159–171. doi: 10.1023/B:FISH.0000035938.92027.81

Vinagre C, Dias M, Roma J, et al (2013) Critical thermal maxima of common rocky intertidal fish and shrimps - A preliminary assessment. J Sea Res 81:10–12. doi: 10.1016/j.seares.2013.03.011

Vinagre C, Leal I, Mendonça V, et al (2016) Vulnerability to climate warming and acclimation capacity of tropical and temperate coastal organisms. Ecol Indic 62:317–327. doi: 10.1016/j.ecolind.2015.11.010

Vinagre C, Leal I, Mendonça V, Flores AA V (2015) Effect of warming rate on the critical thermal maxima of crabs, shrimp and fish. J Therm Biol 47:19–25. doi: 10.1016/j.jtherbio.2014.10.012

Vinagre C, Madeira D, Mendonça V, et al (2014) Effect of temperature in multiple biomarkers of oxidative stress in coastal shrimp. J Therm Biol 41:38–42. doi: 10.1016/j.jtherbio.2014.02.005

Vinagre C, Madeira D, Narciso L, et al (2012) Impact of climate change on coastal versus estuarine nursery areas: Cellular and whole-animal indicators in juvenile seabass *Dicentrarchus labrax*. Mar Ecol Prog Ser 464:237–243. doi: 10.3354/meps09885

Vollenweider R (1985) Elemental and biochemical composition of plankton biomass: some comments and explorations. Arch fur Hydrobiol 105:11–29.

Whitehead A, Crawford DL (2005) Variation in tissue-specific gene expression among natural populations. Genome Biol 6:R13. doi: 10.1186/gb-2005-6-2-r13

Whitley D, Goldberg SP, Jordan WD (1999) Heat shock proteins: A review of the molecular chaperones. J Vasc Surg 29:748–751. doi: 10.1016/S0741-5214(99)70329-0

Yamashita M, Yabu T, Ojima N (2010) Stress Protein HSP70 in Fish. Aqua-BioScience Monogr 3:111–141. doi: 10.5047/absm.2010.00304.0111

Ye L, Yang S-Y, Zhu X-M, et al (2011) Effects of temperature on survival, development, growth and feeding of larvae of Yellowtail clownfish *Amphiprion clarkii* (Pisces: Perciformes). Acta Ecol Sin 31:241–245. doi: 10.1016/j.chnaes.2011.06.003

Yuan X, Yang H, Meng L, et al (2013) Impacts of temperature on the scavenging efficiency by the deposit-feeding holothurian *Apostichopus japonicus* on a simulated organic pollutant in the bivalve-macroalage polyculture from the perspective of nutrient budgets. Aquaculture 406–407:97–104. doi: 10.1016/j.aquaculture.2013.05.009

Yuan X, Yang H, Wang L, et al (2009) Bioenergetic responses of sub-adult sea cucumber *Apostichopus japonicus* (Selenka) (Echinodermata: Holothuroidea) to temperature with special discussion regarding its southernmost distribution limit in

China. J Therm Biol 34:315–319. doi: 10.1016/j.jtherbio.2009.05.001

Zerebecki RA, Sorte CJB (2011) Temperature tolerance and stress proteins as mechanisms of invasive species success. PLoS One. doi: 10.1371/journal.pone.0014806

Tables

Table 1 Methods used for biomarker analyses. Abb: Hsp70 – heat shock protein 70; Ub – total ubiquitin; CAT – catalase; LPO – lipid peroxides; GST – glutathione-S-transferase; SOD – superoxide dismutase; AChE – acetylcholinesterase; ELISA – Enzyme Linked Immunosorbent Assay; EC – Enzyme Comission number.

Biomarker	Type of analysis	EC	References
Hsp70	protein quantification, indirect ELISA	-	Madeira et al. 2017
Ub	protein quantification, direct ELISA	-	Madeira et al. 2017
CAT	enzymatic/kinetic assay (activity)	1.11.1.6	Johansson and Borg 1988
LPO	quantification of malondialdehyde	-	Uchiyama and Mihara 1978
GST	enzymatic/kinetic assay (activity)	2.5.1.18	Habig et al. 1974
SOD	enzymatic/kinetic assay (activity)	1.15.1.1	Sun et al. 1988
AChE	enzymatic/kinetic assay (activity)	3.1.1.7	Ellman et al. 1961

Table 2 Statistical analyses (factorial ANOVAs) showing main effects of temperature (29˚C, 32˚C) and tissue (gills, muscle) and their interactive effects, as well as interactions with time (0, 7, 14, 21 and 28 days) on selected molecular biomarkers from the species *L. lipkei.* Significant results (p-value ≤0.05) are indicated (*). Abb: Hsp70 – heat shock protein 70kDa; Ub – ubiquitin; CAT – catalase; LPO – lipid peroxides; GST – glutathione-S-transferase; SOD – superoxide dismutase; AChE – acetylcholinesterase; Temp – Temperature; T*T*T – Temperature × Time × Tissue; NA – not available.

	Hsp70		**Ub**		**CAT**		**LPO**		**GST**		**SOD**		**AChE**	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Temperature	21.9	***	0.9	ns	16.2	***	0.8	ns	10.0	**	7.9	**	3.544	ns
Tissue	181.2	***	82.6	***	218.7	***	21.1	***	104.4	***	169.8	***	NA	NA
Temp*Time	4.3	**	3.1	*	4.5	**	10.9	***	2.7	*	3.0	*	2.676	*
Temp*Tissue	3.1	ns	1.1	ns	0.7	ns	0.6	ns	0.4	ns	0.0	ns	NA	NA
T*T*T	1.6	ns	4.6	**	2.0	ns	1.3	ns	1.4	ns	2.3	*	NA	NA

Levels of significance: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, ns: non-significant ($p > 0.05$).

Table 3 Factorial ANOVAs showing the main effects of temperature (29˚C vs 32˚C) and interactive effects with time (T0 vs T28) on condition indicators for *L. lipkei*. Significant results (p-value ≤ 0.05) are presented in bold. Abb: Temp – Temperature.

	Fulton's K		**Total protein**		**%C**		**C:N molar ratio**	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Temperature	0.069	0.793	**5.639**	**0.022**	1.240	0.272	0.608	0.440
Temp x Time	0.720	0.408	**4.784**	**0.043**	0.034	0.854	0.072	0.792

Figure captions

Fig. 1 (a) Original geographical distribution range of the caridean shrimp *Lysmata lipkei* according to Okuno and Fiedler (2010). The locations shown are the sampling sites from which specimens were collected and first taxonomically identified in 2010. (b) *L. lipkei* known occurrences in Brazilian coastal waters: NE Brazil (in the states of Ceará – Camocim and Icapuí, and Rio Grande do Norte – Areia Branca), where the first introductions of this species were observed (Pachelle et al. 2016), and SE Brazil where shrimps were collected for this study (São Sebastião, São Paulo State). This image was prepared using GoogleEarth® images

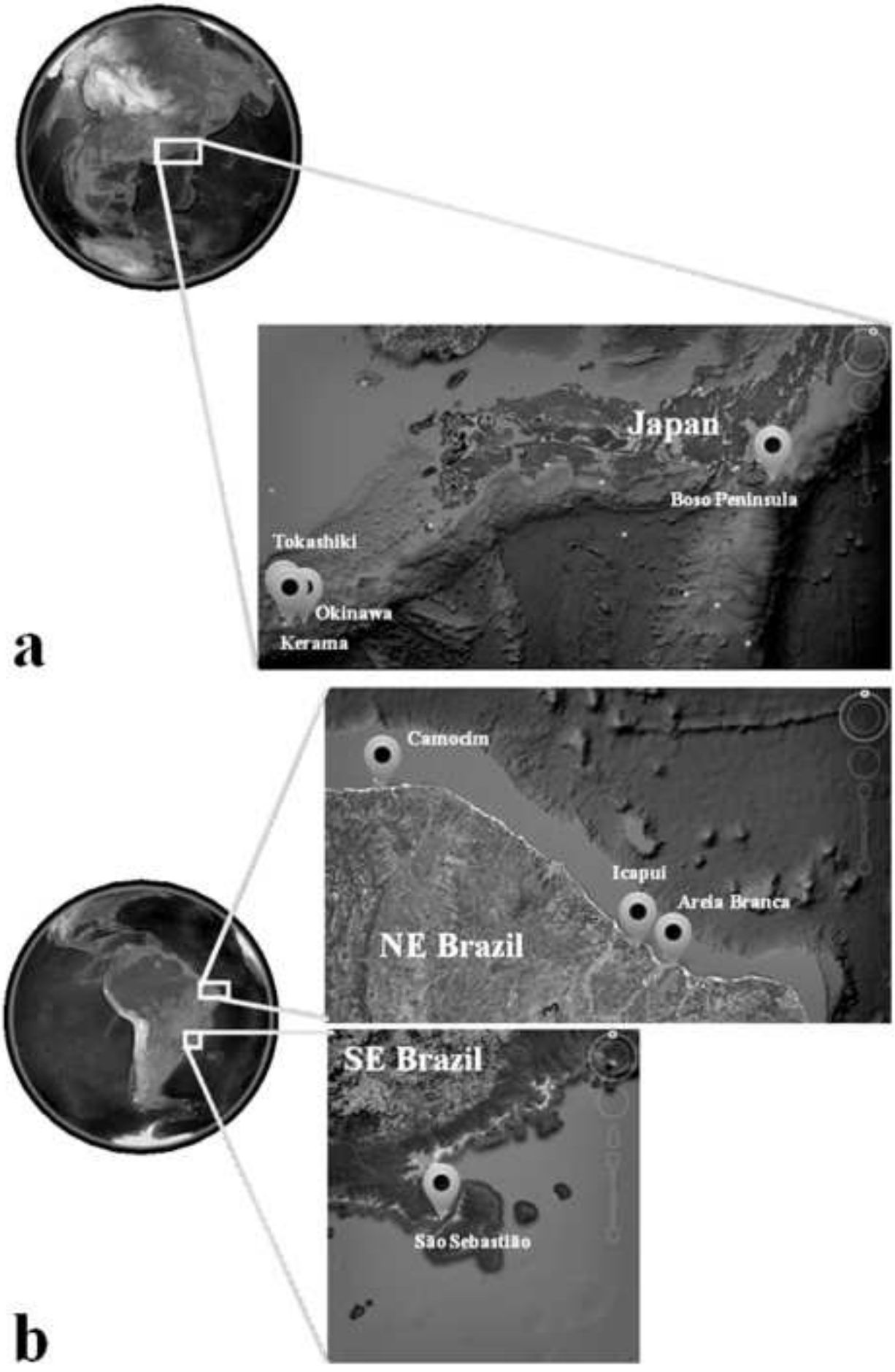
Fig. 2 a) Present SST temperatures (˚C) in *L. lipkei* original distribution range (Japan, data from the locations where these shrimps were first caught and described: Okinawa, Boso Peninsula, Ryukyu Islands) and at the first introduction locations in Brazil (Ceará and Rio Grande do Norte states, in the northeastern coast); b) Present and projected temperatures for 2100 (+3˚C) in São Sebastião (SP, Brazil) coastal waters – new colonized environment. Note: present monthly average sea surface temperatures were calculated from satellite data for the past 5 years

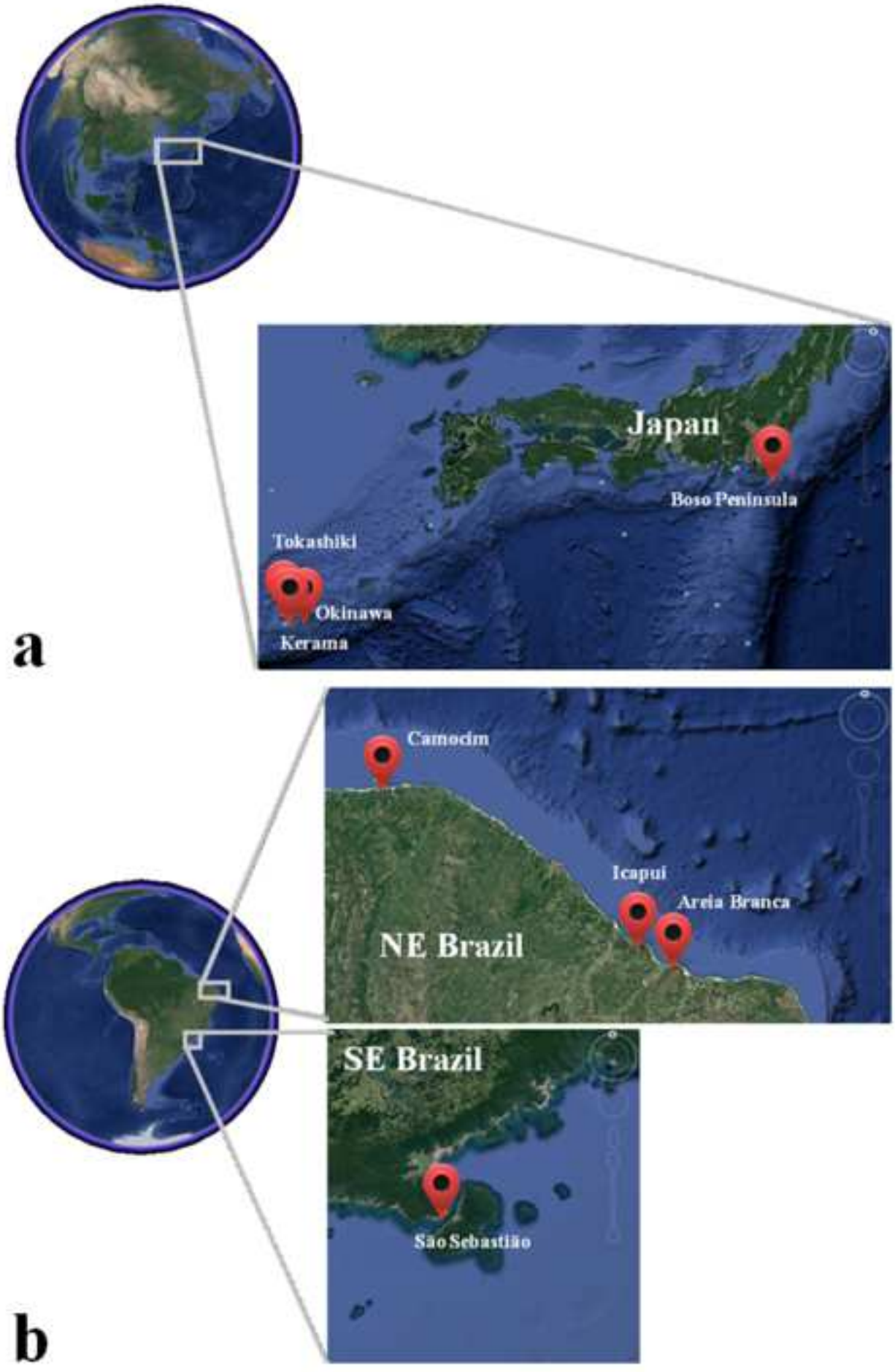
Fig. 3 Ordination diagrams of the first two axes of the PCA carried out for each sampled tissue of *Lysmata likpei* exposed to 29˚C and 32˚C considering all sampling times (0, 7, 14, 21 and 28 days of exposure): a) gills and b) muscle

Fig. 4 Biomarker behavior (mean+SD) along time (one month) under two temperature treatments (29ºC and 32ºC): A) Hsp70 (heat shock protein 70 kDa) and Ub (ubiquitin);

B) CAT (catalase) and LPO (lipid peroxides), C) GST (glutathione-S-transferase) and SOD (superoxide dismutase), D) AChE (acetylcholinesterase – only measured in muscle). Numbers next to letters stand for (1) gills and (2) muscle. Significant differences (p-value ≤ 0.05) from the control group are presented with colored asterisks (*).

Fig. 5 Critical Thermal Maxima (CTMax) measured in *Lysmata lipkei* after 7 days and 28 days at two different acclimating conditions predicting exposure to present average summer temperatures (29˚C, control) and future average summer temperatures (32˚C) in intertidal rocky reefs of Southeastern Brazil. Significant differences (p-value ≤ 0.05) are indicated with an asterisk (*)





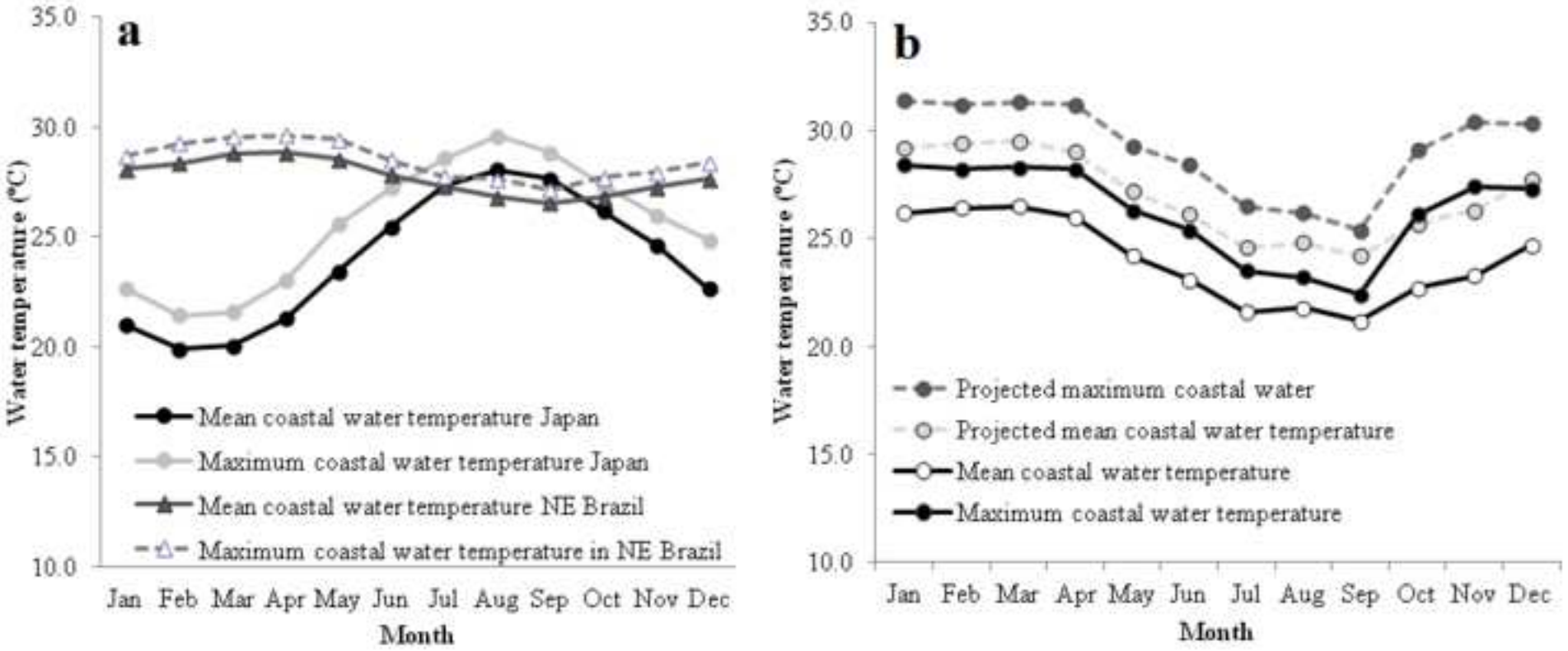


Fig 2 color

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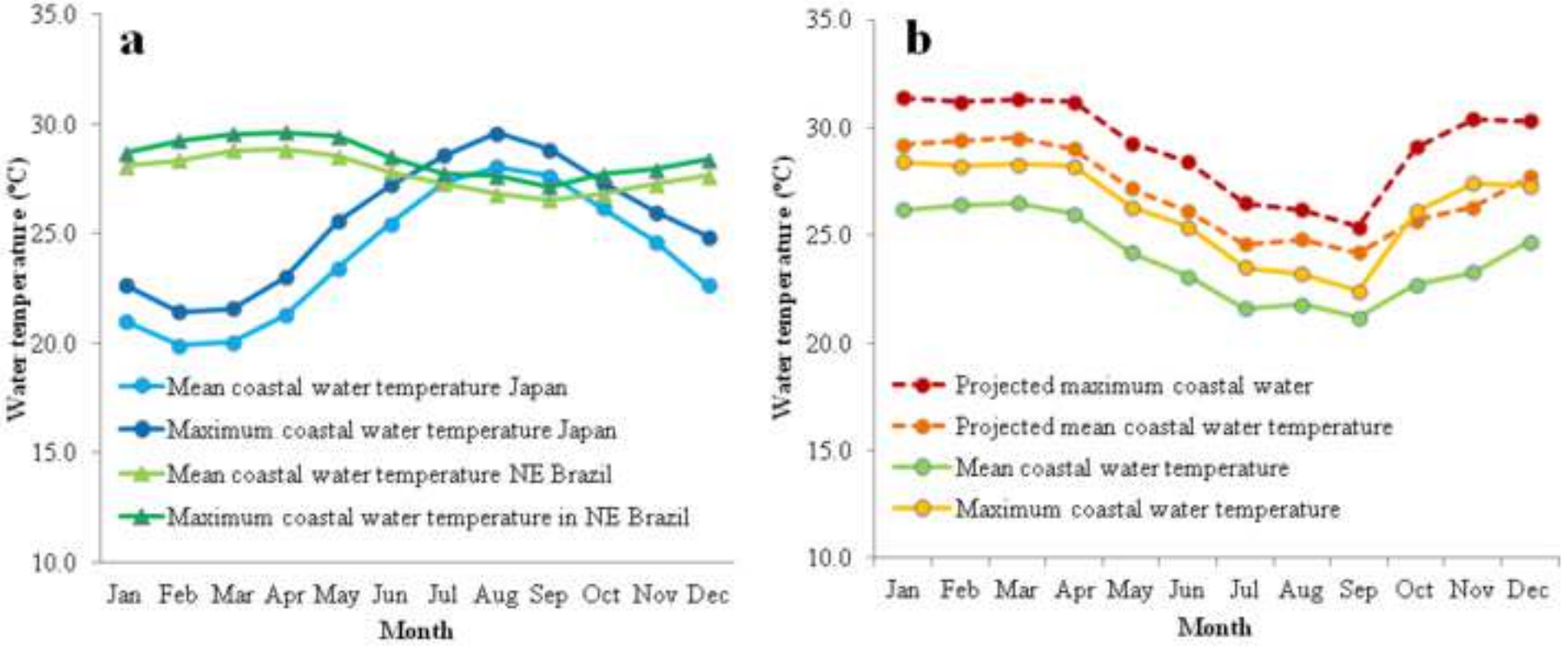

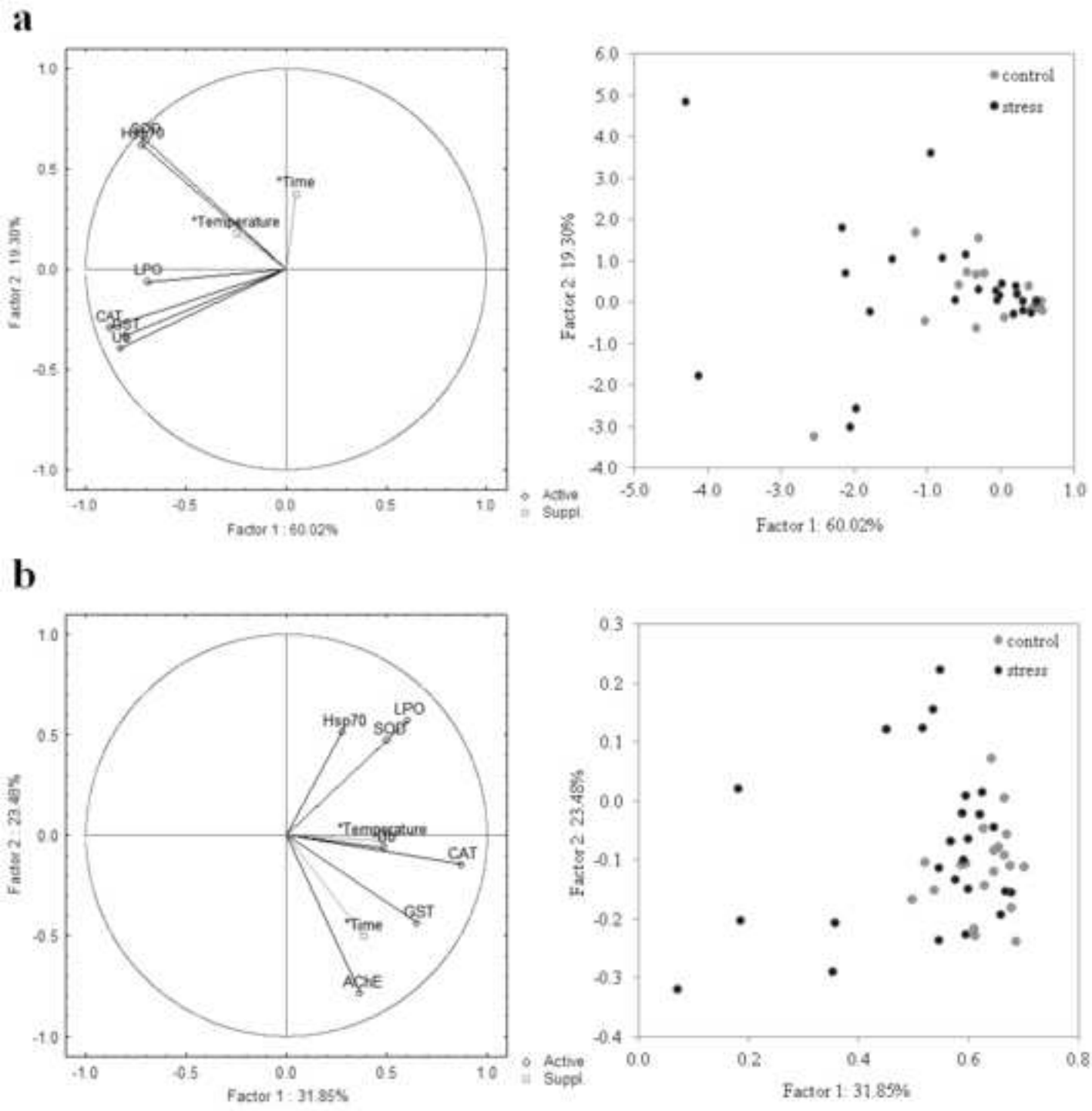

Fig 3 color

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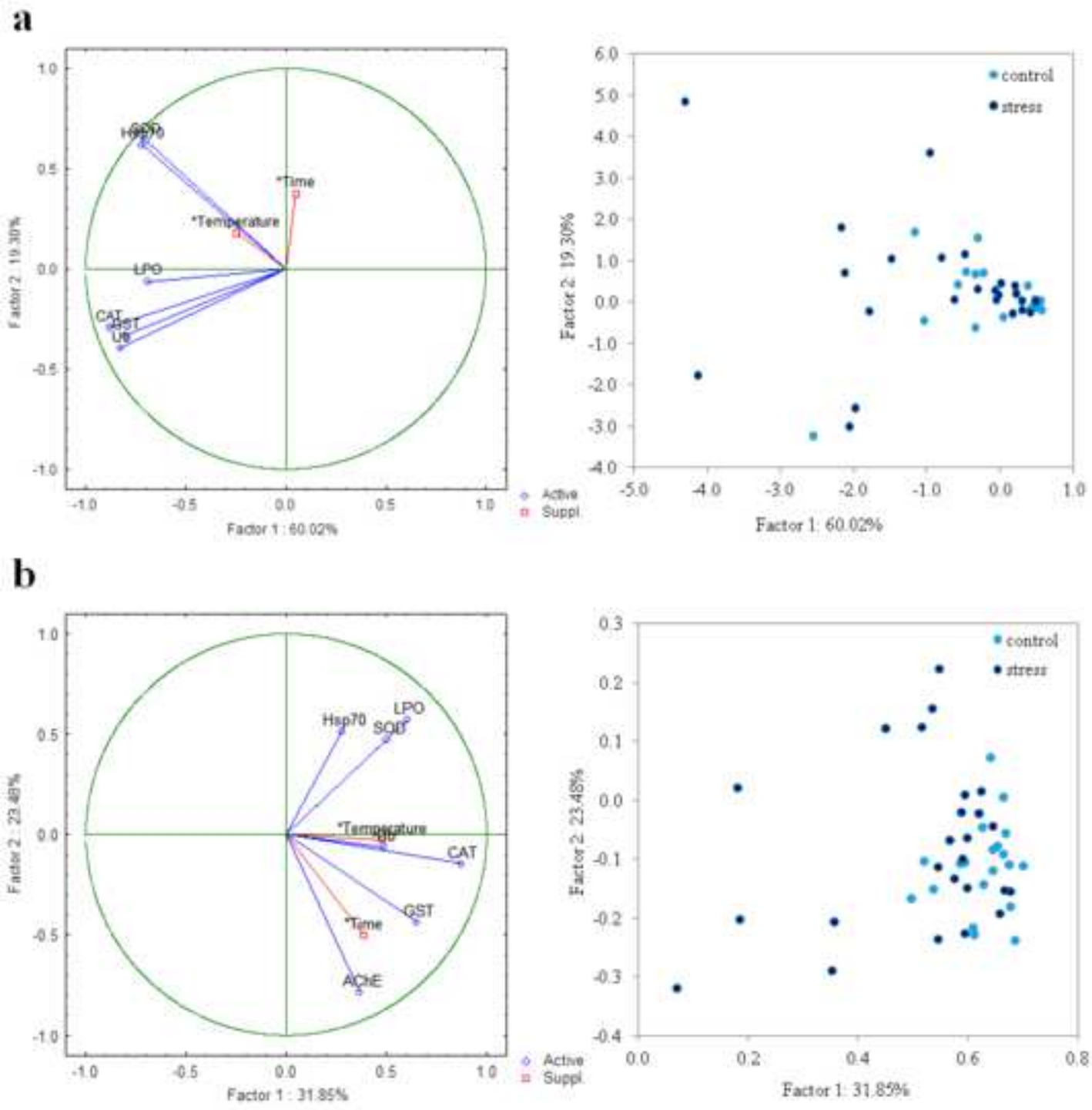

Fig 4a B&W

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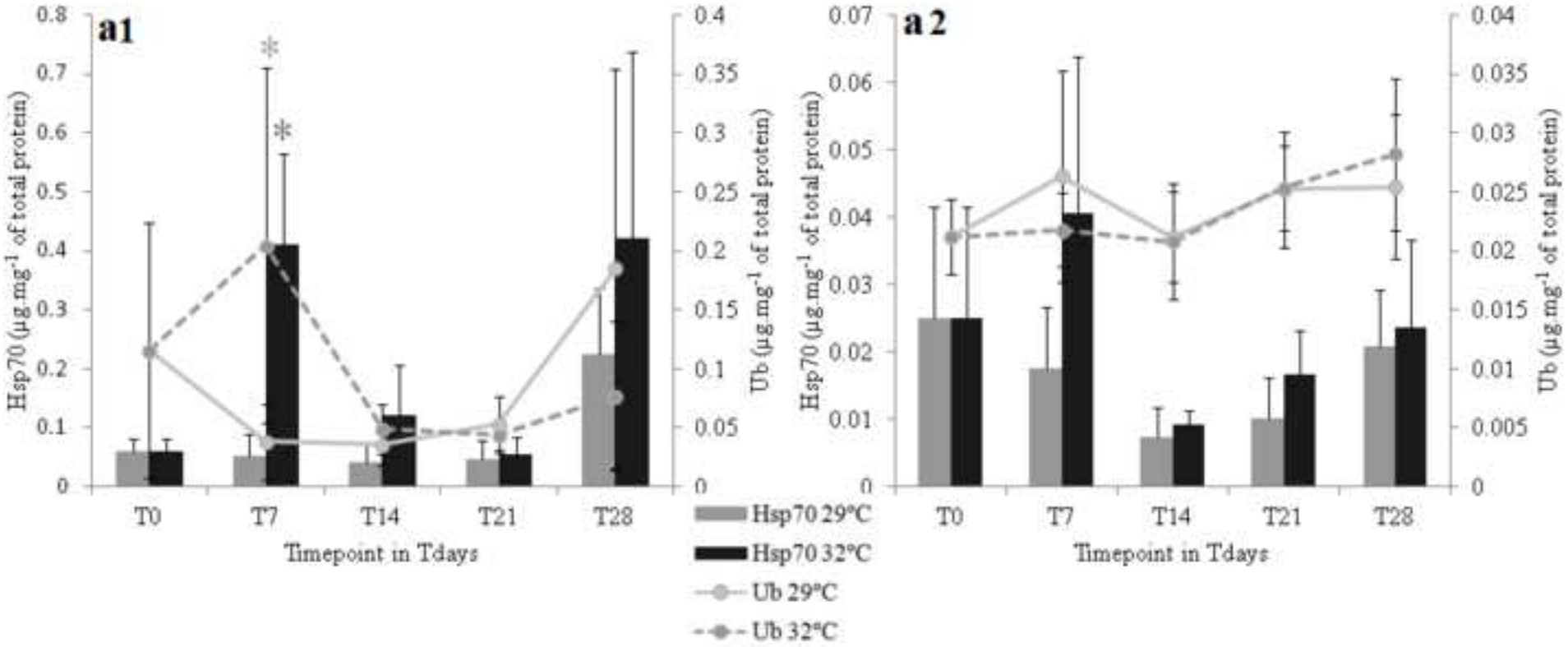

Fig 4a color

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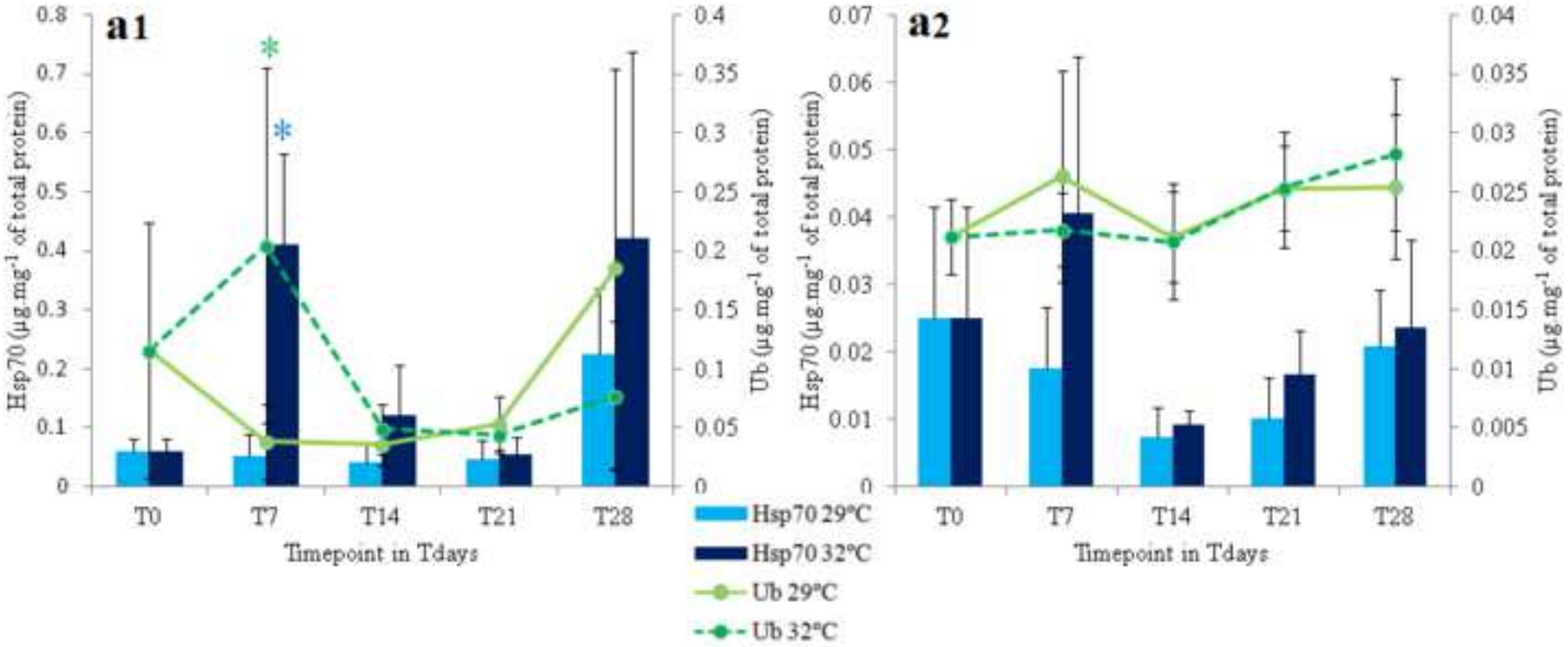

Fig 4b B&W

Click here to access/download;Figure;Fig4B_B&W.tif

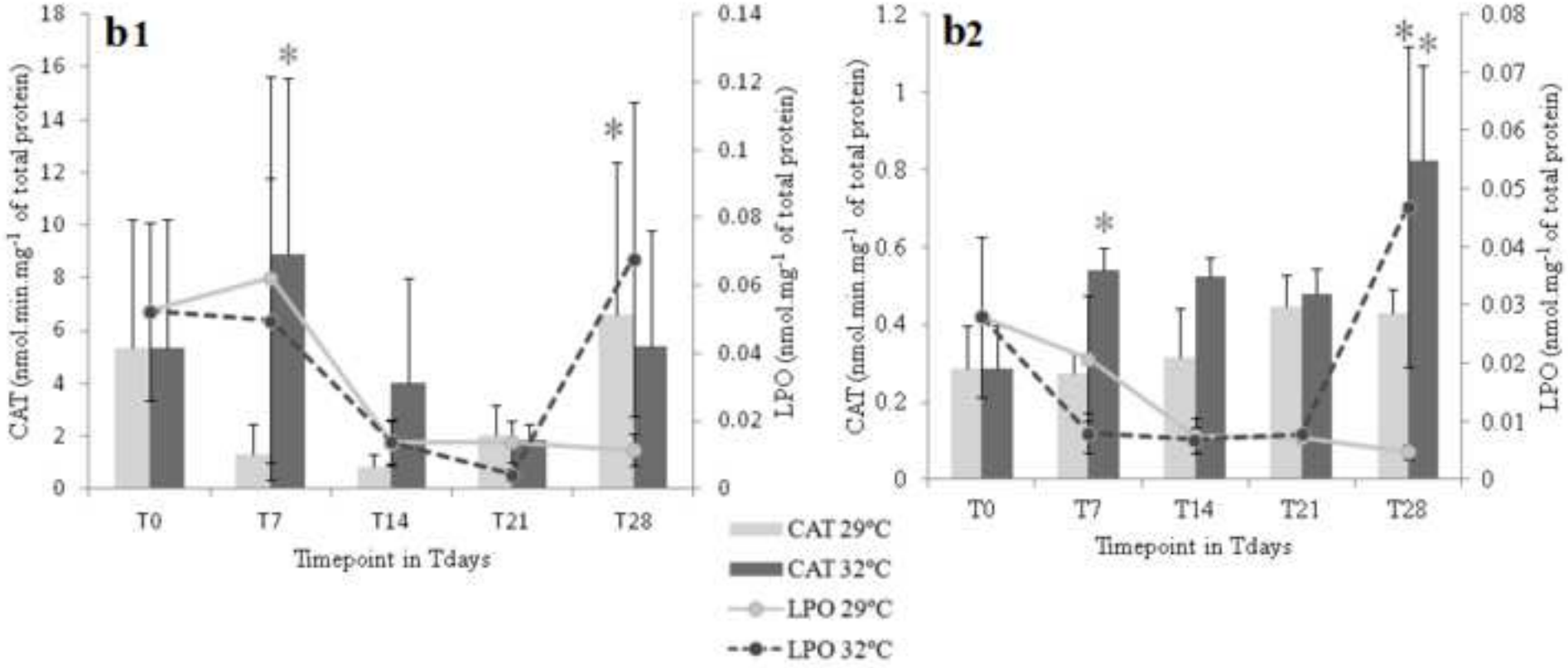

Fig 4b color

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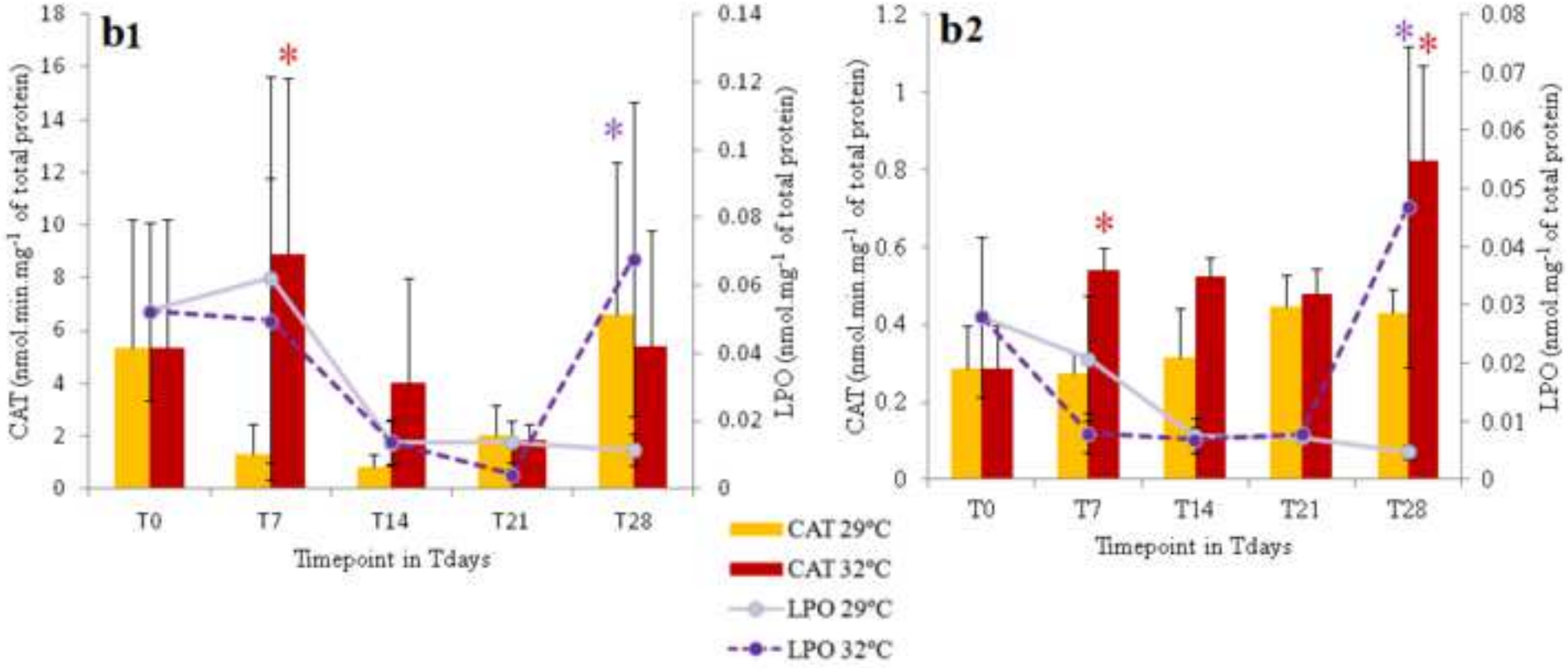

Fig 4c B&W

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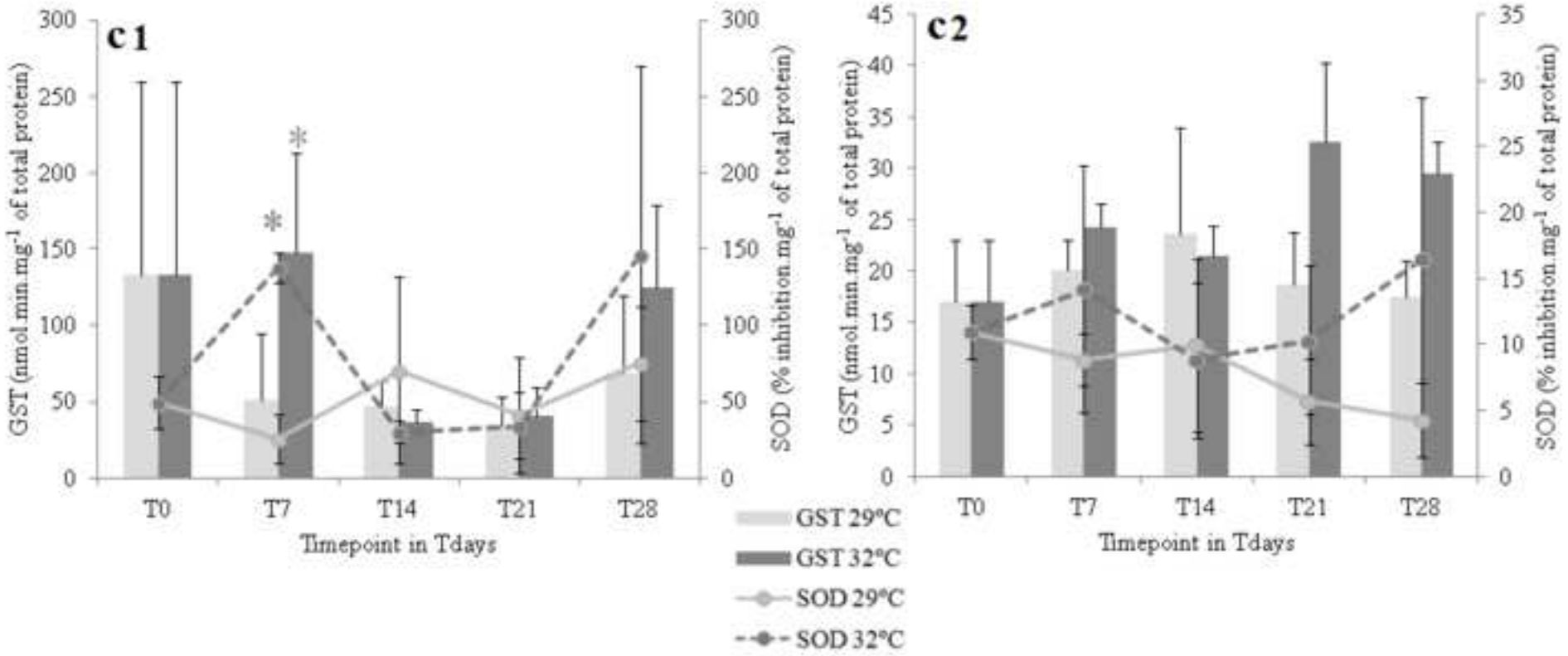

Fig 4c color

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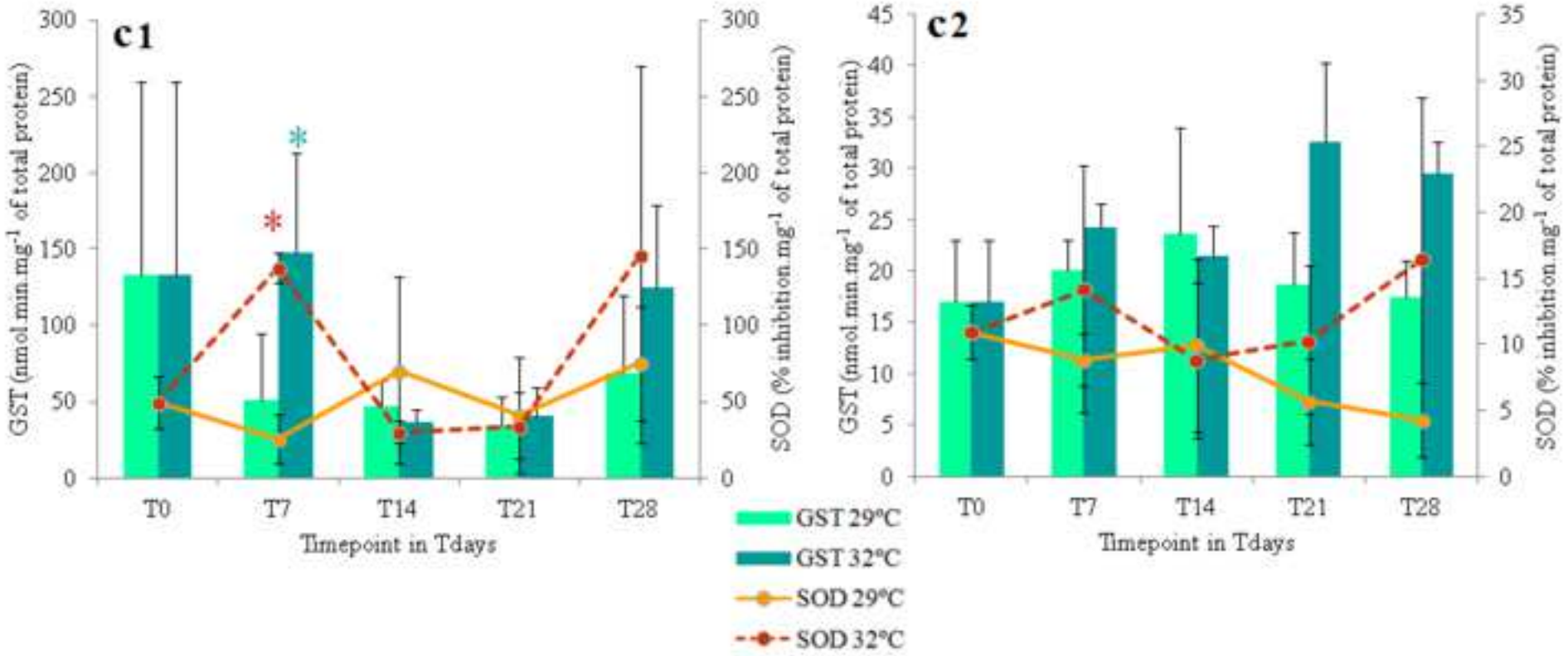

Fig 4d B&W

Click here to access/download;Figure;Fig4D_B&W.tif

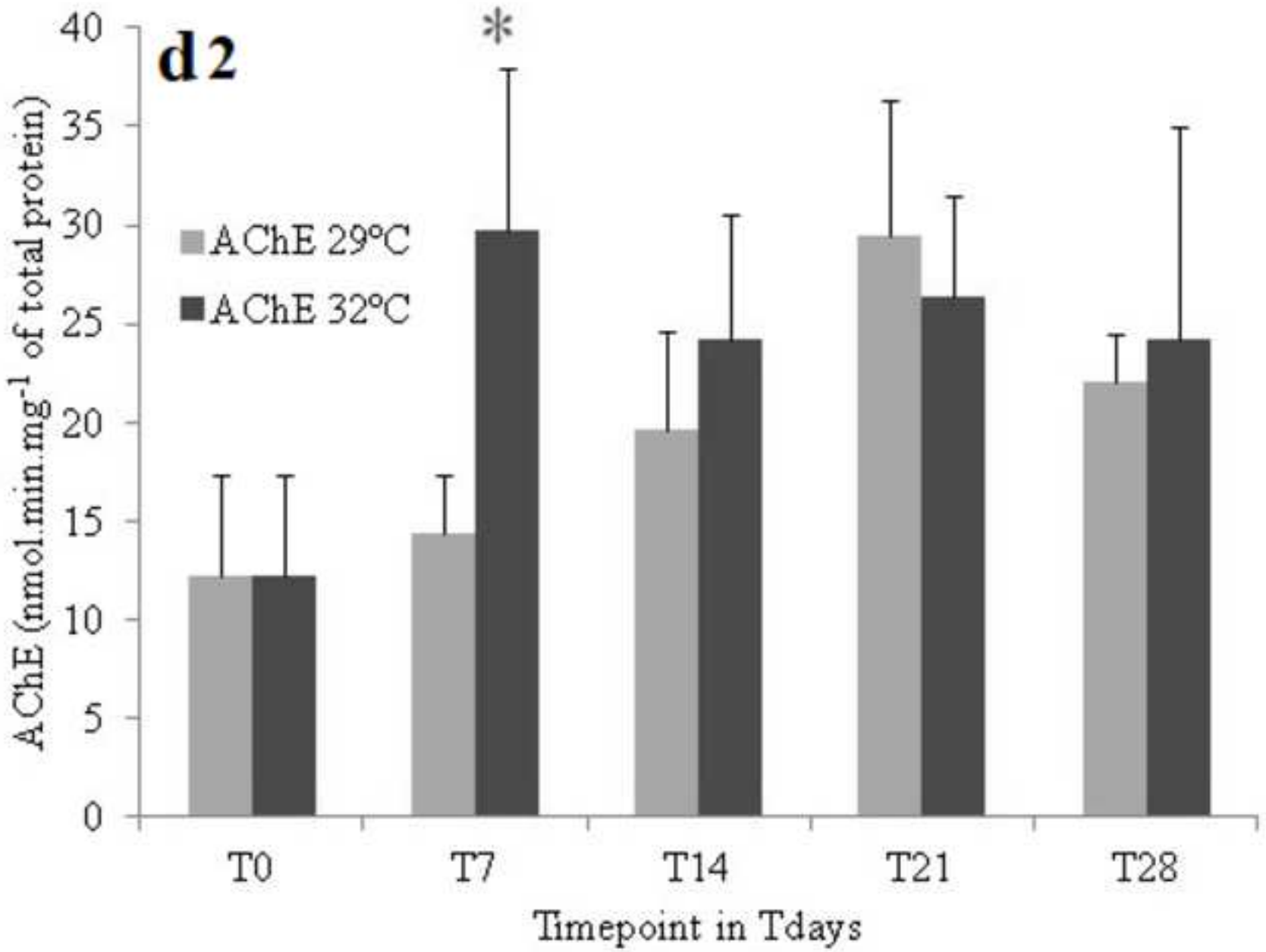

Fig 4d color

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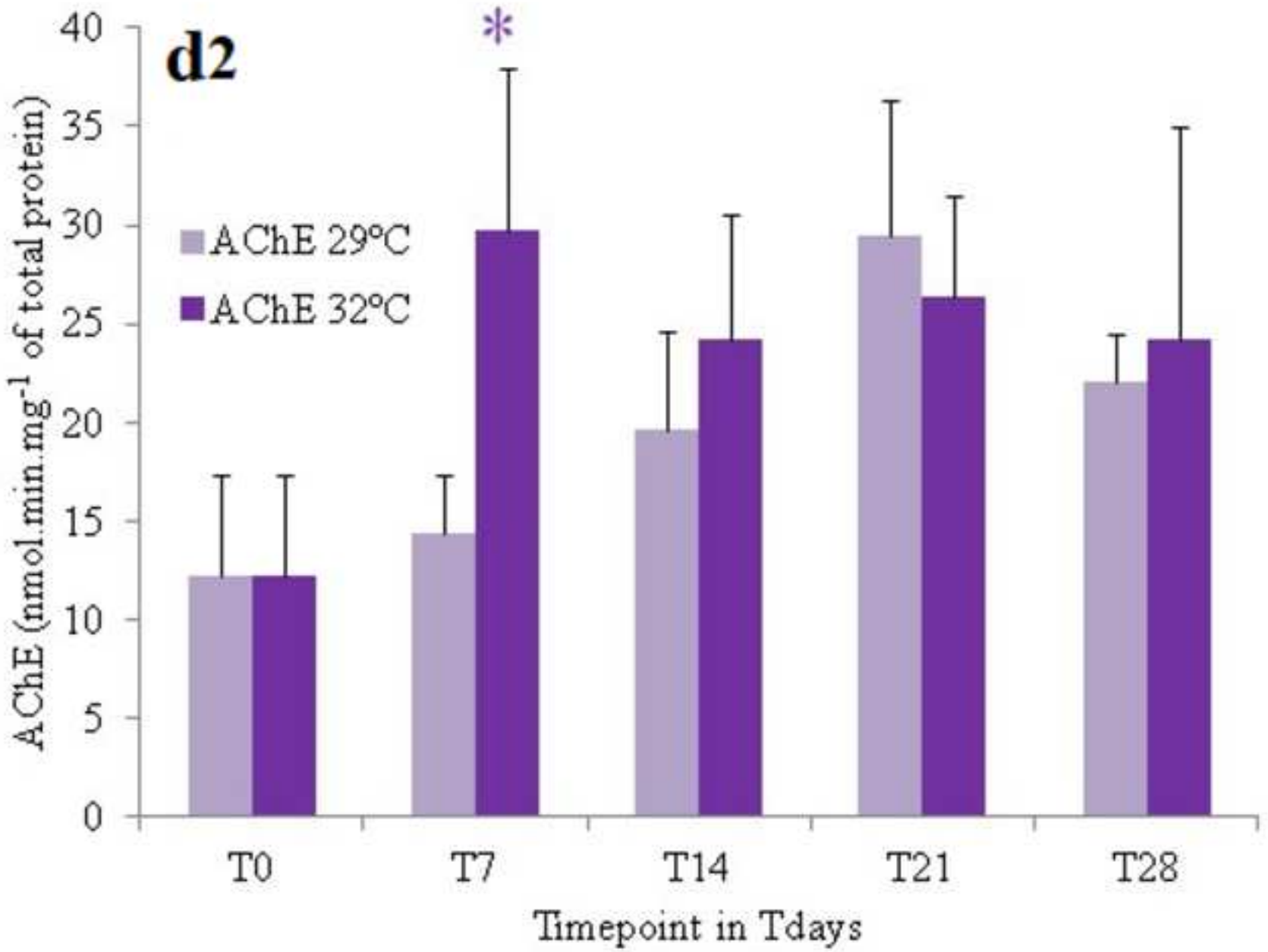

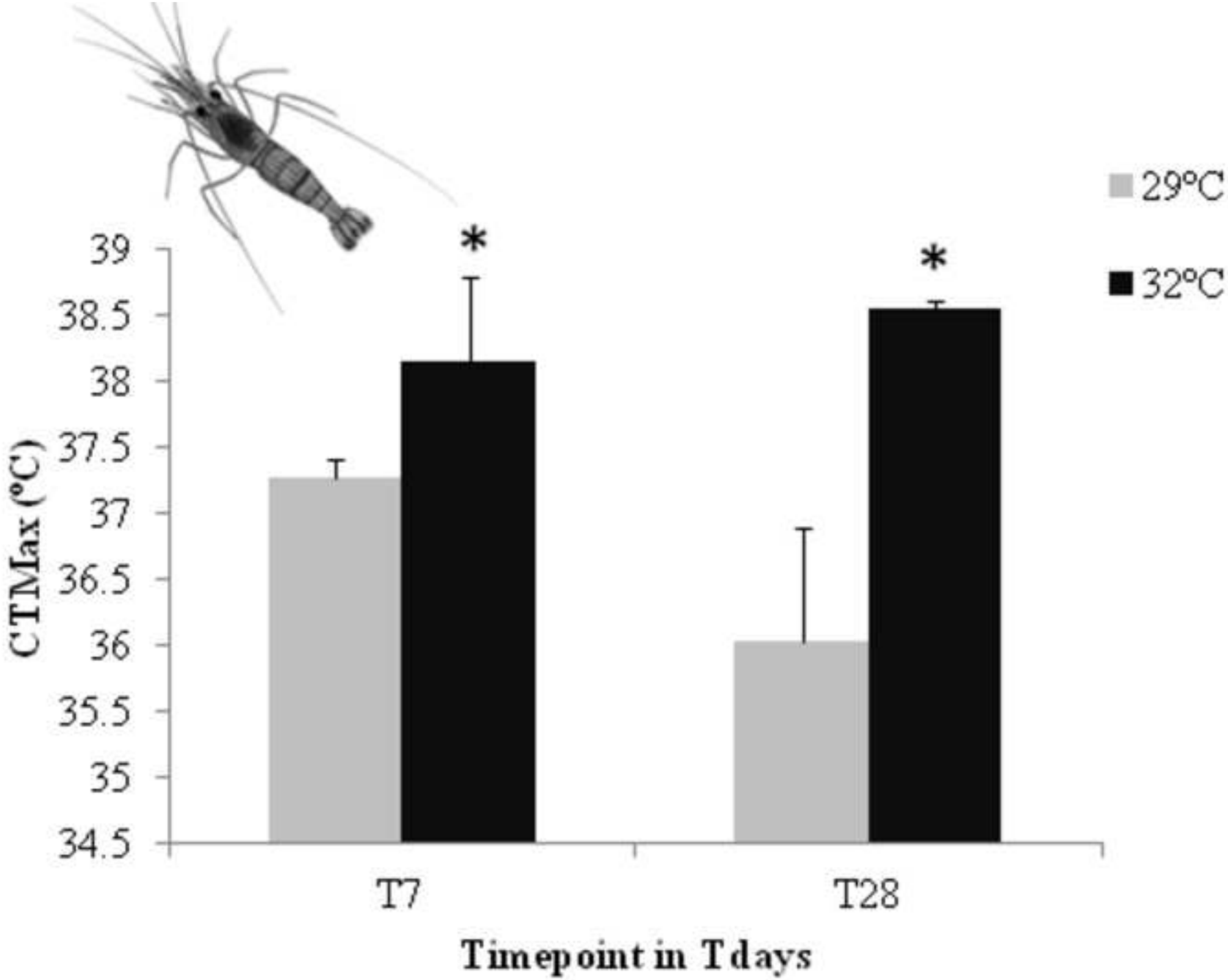

Fig 5 color

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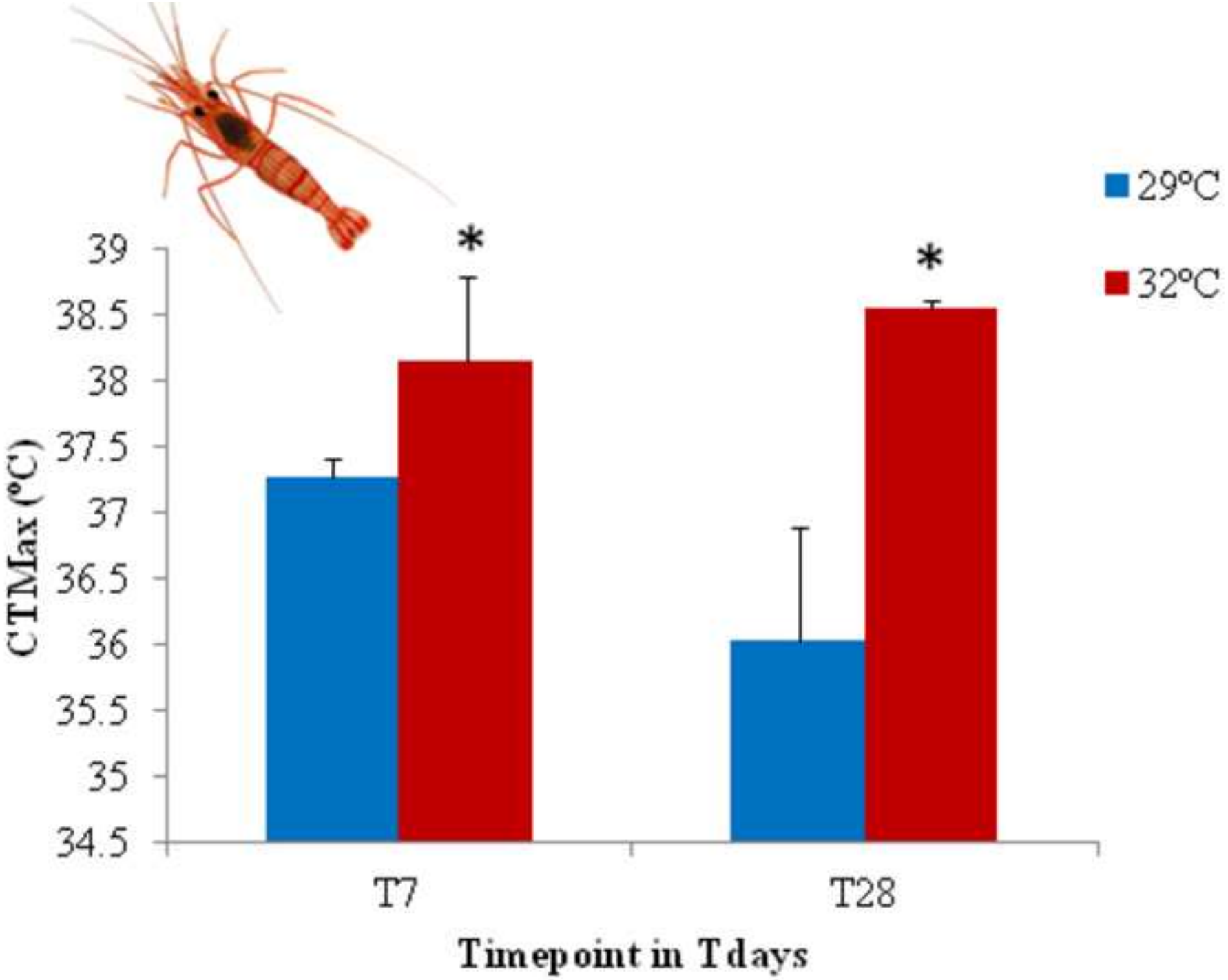
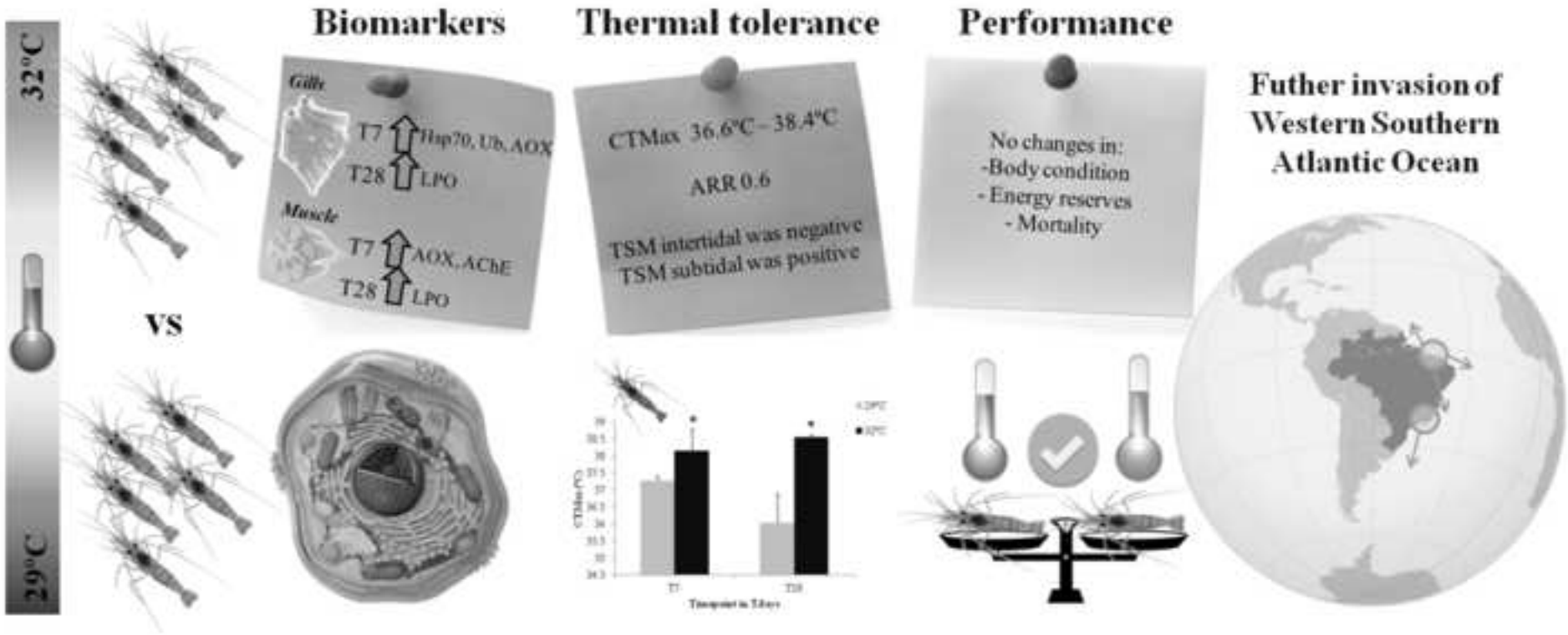


graphical abstract B&W



graphical abstract color

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