Identifying the gene(s) that allow Tigriopus californicus to survive under thermal stress

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The copepod species, Tigriopus californicus, is one of the most thermally adaptable species in the biosphere. They have been found in tide pools from the coast of Alaska down to the coast of Southern California. However, as all other organisms have limits, the T. californicus are only able to tolerate temperatures up to 34ºC. By comparing gene expression between specimens exposed to high temperatures and those expressed to optimal temperatures, we identified the genes responsible for conferring tolerance to increasing temperatures. After testing gene expression between copepods at 20ºC and at 34ºC, we have determined that both the hsp70 gene and the toll-like receptors of the T. californicus play a role in tolerance to temperature variance.

L'espèce copépode, Tigriopus californicus, est une des espèces les plus thermiquement adaptables de la biosphère. On les trouve dans les bâches depuis la côte de l'Alaska jusqu'à la côte sud de la Californie.

Toutefois, comme tous les autres organismes, les Tigriopus californicus ont des limites et ne peuvent pas tolérer des températures supérieures à 34ºC. En comparant l'expression génétique entre les spécimens exposés à des températures élevées et ceux exposés à des températures optimales, nous avons identifié les gènes responsables de la tolérance à des températures en hausse. Après avoir testé l'expression génétique entre les copépodes à 20ºC et à 34ºC, nous avons déterminé que le gène hsp70 et les récepteurs de type Toll du T. californicus jouent tous deux un rôle dans la tolérance aux variations de température.

INTRODUCTION

Climate change is becoming an ever-increasingly important issue [1]. As habitats and ecosystems begin to change with the emission of greenhouse gases, animal populations must adapt or face potential extinction. All species possess certain abiotic conditions they require to survive. Yet some species have a wider range of tolerance toward certain factors than do others [2]. One example of this trait would be the marine copepod - *Tigriopus californicus*.

T. californicus is a microscopic marine organism that resides in tide pools (Fig. 1). Individuals are able to live in waters from 0.35ºC [3] up to approximately 34 °C [4], demonstrating a high adaptability to temperature changes. Tigriopus californicus may be used as a model for the future situations of other species that are not currently exposed to such a large range of temperatures. The large range of their habitat makes them the ideal model for studies environmental genomics [5].

In this work, we focused on two specific genes: a toll-like receptor gene and hsp70. The toll-like receptor gene functions within the body's immune system [6]. The second gene, hsp70, is part of a family of proteins called the "Heat Shock Proteins" and is known to be transcribed when a specimen is exposed to thermal stress [7].

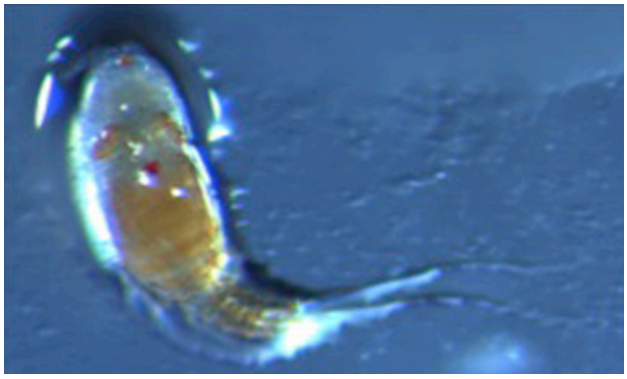

Figure 1

To study the difference in these genes during thermal stress, we grouped the *Tigriopus californicus* population into groups of five mating pairs and then subjected them to hour-long treatment in 34ºC seawater. As a result, the gene(s) that allow *T. californicus* to survive when immersed in 34ºC water were isolated and identified. We can gain insight into thermal resistance for species beyond the *Tigriopus californicus* and inform predictions regarding the effect of atmospheric and temperature change to marine ecosystems.

Therefore, identifying the gene expression changes in *T. californicus* when exposed to thermal stress is a significant part of addressing the rapid change in climate. Not only will it allow for a better understanding of this species, it will also give us a look into how other species could potentially widen their range of tolerable temperatures.

RESULTS AND DISCUSSION

Our findings suggest that although the amount of hsp70 being expressed had a more impressive increase than the amount of TLR following thermal stress, both hsp70 and TLR play important roles in permitting *Tigriopus californicus* to survive in a variety of temperatures. The presence of hsp70 increased almost 10 fold and TLR increased approximately 4 fold. It is apparent that more than one gene is responsible for the versatility of *Tigriopus californicus*.

Following analysis of our real time qPCR results, we were able to calculate the average number of additional cycles either TLR or hsp70 were required to cross the cycle threshold in comparison to the reference gene of ßactin. To do this for each gene, we calculated the ΔC_t s of all the trials – as explained in the Methods section of this paper. The average ΔC_t s for both genes were almost consistently lower in thermally stressed individuals than ambient organisms (Fig. 4). This means that it took fewer cycles for the thermally stressed DNA to cross the cycle threshold; in other words, there were higher levels of both genes in the thermally stressed individuals. For example, the mean ΔC_t for TLR in the ambient copepods was 8.5. On the other hand, the mean ΔC_t for the thermally stressed organisms was 6.5. So we know that it took about 2 more cycles for TLR to cross the cycle threshold in ambient individuals.

To determine if this difference in ΔC_t values represented a significant change in the level of gene expression between the ambient and thermally stressed *Tigriopus californicus,* we used a T-test and the Mann Whitney U-test. For TLR, the fold change was found to be 3.97, which means the gene is nearly 4 times more active in thermally stressed copepods than in the ambient temperature ones (Fig. 3). In support of this conclusion, the T-test returned a p-value of 0.00399 and the Mann Whitney returned a p-value of 0.0016. In other words, the p-value indicates that there is a significant correlation between the type of treatment the

	Mean ΔCt	Q-Value ($2^{-\Delta Ct}$)	ΔΔCt	Fold Change (QThermal/QAmbient)
Ambient TLR	8.497	0.003	0.000	1.000
Thermal TLR	6.507	0.011	1.990	3.972
Ambient hsp70	4.670	0.039	0.000	1.000
Thermal hsp70	1.379	0.385	3.291	9.791

Figure 4

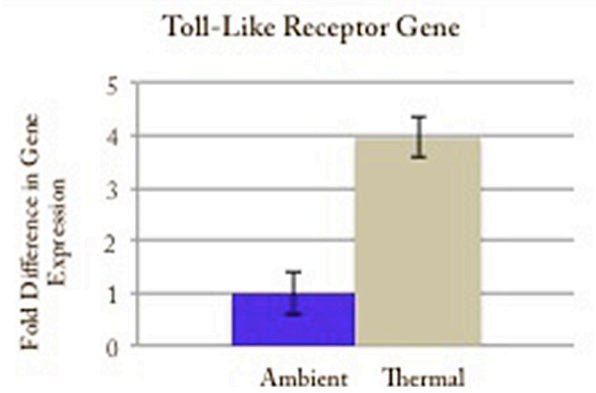

Figure 3a

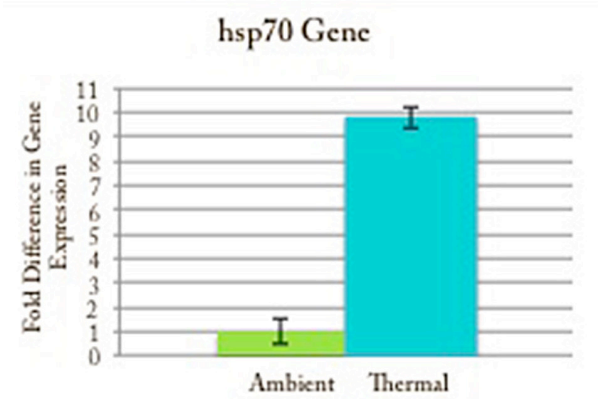

Figure 3b

individual received (ambient vs. thermal) and the magnitude of gene expression since it is <0.05. Similarly for hsp70, the p-values were 0.00005 and 0.0035 for the T-test and Mann Whitney respectively and the gene was almost 10 times more highly expressed in the thermally stressed *T. californicus* (Fig. 3).

The more conservative nonparametric Mann Whitney U-test also yielded significant p-values, lending support that a significant change in the two genes occurred when the organisms were exposed to 34ºC for one hour. These data support the hypothesis that TLR and hsp70 play an important role in allowing the *Tigriopus californicus* to live in a wide range of temperatures because both were more active when individuals were placed in 34ºC water.

This study has brought to light the importance of TLR and hsp70 in *T. californicus* with regards to surviving sudden temperature changes. Not only does the data support work previously published by emphasizes the role of hsp70 when an organism experiences a change in temperature [7], it also shows that more than one family of genes mediates this trait. This is significant because of its applicability to other species.

Since *T. californicus* has been exposed to a wide range of temperatures, they have been able to develop the genes they require to thrive in such a situation. As climate change continues to occur, *T. californicus* is a potentialmodel for the futures of these organisms and for the evolutionary paths that they will take.

FUTURE DIRECTIONS

We found that both the toll-like receptor and hsp70 gene contribute to the resilience of the *Tigriopus californicus* toward extreme changes in the temperature of its habitat. As expected, the hsp70 gene was much more highly expressed in the thermally stressed individual because it is part of the "heat shock protein" family. By increasing sampling effort, this study confirms initial findings from a *T. californicus* pilot study on gene expression changes. In this case, 34 replicates were used – 17 ambient replicates and 17 thermally stressed replicates.

However, questions and problems still remain before the information found here can be applied to other species. This study revealed that both genes were more active when the copepods were thermally stressed, but are these the only two genes that contribute to the variability of this species? Other genes may also be involved in allowing these microscopic marine creatures to survive an astounding range of 34ºC. Another question is how the *Tigriopus californicus* developed these genes. We cannot simply take genes from one species and "inject" them into individuals from another species, so how do we transfer the thermal resistance of these copepods?

Despite these issues, the study has still given us more insight into how *Tigriopus californicus* can thrive in extremely cold and hot waters. This insight can be applied to our constantly changing world as global warming becomes more severe. We are yet another step closer to

being able to sustain and protect the thousands of species that call this planet their home.

MATERIALS AND METHODS

Specimen Collection

Tigriopus californicus specimens were collected from tide pools on the coast of Long Beach, Los Angeles. The specimens were drawn out of the water using disposable pipettes and placed into deli containers with unfiltered seawater. This species is quite hardy and fairly easy to maintain so no specific volume of seawater is required. In this study, there was a population density of approximately 1 individual per 5 mL of seawater. The seawater cultures had an average salinity of 38 ppt and an average temperature of 20ºC. The population was given a single food pellet of 50mg Aquadine ground spirulina fish food each week [4].

RNA Extraction

Copepods were equally distributed into two groups of ten individuals. One group was placed into a 1.5 mL Microcentrifuge tube and the other into a 0.2 mL PCR tube. The PCR tube was put into the thermocycler (Eppendorf) for one hour at 34º C. After one hour, the copepods were removed from the PCR tube and transferred into a Microcentrifuge tube. Superfluous water was removed via pipette and 250 μL of TRIzol Reagent (Invitrogen) were added to each Microcentrifuge tube. RNA extraction was performed in an RNase-free environment to maximize the yield of the reaction and preserve the RNA that was present within the tubes [8]. The solution was homogenized using a pestle and 50μL of chloroform were added to the solution. The rest of the steps for RNA Extraction were followed from the TRIzol® Reagent Protocol by Ambion [9]. After RNA was re-suspended in RNase-free water, it was quantified using a Qubit fluorometer. RNA was then stored in a freezer at -30ºC.

Single Stranded Complementary DNA Synthesis

cDNA synthesis was performed as described by BioRad for their iScript Select Synthesis Kit [10]. Briefly, for each reaction used to yield cDNA, 4μL of 5x Buffer Master Mix (BioRad), 12μL of Nuclease-free water (BioRad), 10pg-5μg of RNA, and 1μL of 200units per μL Reverse Transcriptase were placed into a PCR tube. The PCR tube was placed into the thermocycler for 5 minutes at 25 ºC, then 30 minutes at 42 ºC, 5 minutes at 85 ºC, and held at 4 ºC.

qPCR Amplification

To identify relative changes in gene expression, gene specific primers were added to the solution and attached to the single stranded DNA. The genes were chosen from an initial pilot study that indicated a change in the amount of TLR and hsp70 that were present when an organism was submerged in 34ºC seawater. For each reaction, 12.5μL of 2X SYBR GoTaq ® Master Mix (Promega), 11μL of Nuclease-free H2O, 0.5 μL of 10μM primers, and 1μL of cDNA – the concentration of template varied depending on the yield of the previous reaction of complementary DNA synthesis – were added into each well of a plate. The protocol for this real time PCR reaction was found in the SYBR GoTaq® qPCR Master Mix kit [11]. The reactions were carried out in an iCycler (BioRad) where the amount of product was quantified by the number of cycles it must go through before it reaches the cycle threshold (Ct) (Fig. 2) – the PCR cycle at which a minimum fluorescence is detected in the reaction. ßactin was used as the reference gene because of its constitutive expression in Tigriopus californicus. Since large

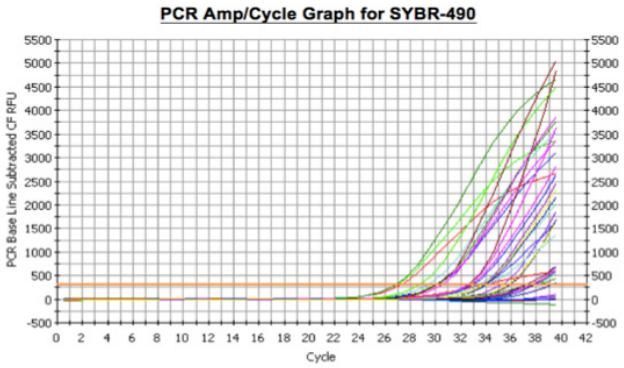

Figure 2

amounts of ßactin were constantly present, it crossed the cycle threshold earlier than both TLR and hsp70, making it easy to compare against.

Data Analysis

The data collected from the qPCR was analyzed in Excel using the delta C_t method [12]. Since each reaction was done in triplicate, the average C_t of the 3 was calculated. To permit relative quantification of hsp70 and TLR abundances, gene Cts were standardized using the C_t from the reference gene ßactin. The delta C_t was calculated by subtracting the C_t of the reference gene, beta Actin, from the C_t of the gene of interest. A T-test was run on the delta C_t values to calculate the corresponding p-value. A nonparametric alternative test, the Mann Whitney U-test, was also conducted for a more conservative p-value. Fold change for each gene was found by using $2^{-\Delta\Delta Ct}$ – where $\Delta\Delta C_t$ is the ΔC_t of the ambient individual subtracted from the ΔC_t of the thermally stressed specimen.

ABBREVIATIONS

Abbreviation	Full Form
T. californicus	*Tigriopus californicus*
T. cal	*Tigriopus californicus*
qPCR	Quantitative Polymerase Chain Reaction
Hsp70	Heat Shock Protein 70
TLR	Toll-like Receptor

KEY WORDS

Tigriopus californicus; genetics; thermal stress; climate change

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REVIEW OF: "IDENTIFYING THE GENE(S) THAT ALLOW TIGRIOPUS CALIFORNICUS TO SURVIVE UNDER THERMAL STRESS"

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Associate Professor , Tier 2 Canada Research Chair
Department of Chemistry and Biochemistry
Laurentian University

The authors present a study examining the expression of a pair of genes in a marine invertebrate under thermal stress. They extrapolate their results out to possible general changes in gene expression as organisms experience changes in environment with the warming trend associated with global climate change. The authors demonstrate and interesting correlation of gene expression with environmental change and claim to have identified two genes that allow the organism to survive thermal stress, but this claim is not substantiated.

The authors quantify the changes in expression of a pair of genes, hsp70 and TLR, in the marine copepod, Tigriopus californicus, in response to thermal stress. The general question of possible changes in gene expression in response to stress response, and specifically thermal stress, is both interesting and timely. The organism and genes selected are appropriate and make this an interesting system of study. The experiment is simple and well designed, and the data appropriately analyzed. The study shows a strong correlation in expression of both genes, in both cases up regulation, with thermal stress. The study does not, however, show causation. Correlation is not causation. Many genes that are not directly involved in, or responsible for differences in, thermal tolerance will have expression patterns that are correlated with changes in temperature. Determining causation will require further experiments testing the function, and biological effects, of changes in the expression of these genes.

Overall, the work is well done and the paper is generally well written. I have included an edited copy of the manuscript with a series of minor wording and editorial comments in Track Changes. I like the inclusion of Future directions, although these need to be expanded to reflect the extension of this work from correlations in gene expression to possible causation of biological differences. I think this section would also benefit from a discussion of thermal stress versus stress in general. The Methods are clear and well described. I do think that the Results and Discussion should be reorganized to more clearly flow from results to conclusions. For this work to be publishable, I think the authors have two options. They can perform experimental manipulations to show that the genes that they are studying actually confer or modify thermal tolerance. Alternatively, they can re-write their manuscript to more accurately indicate that their results demonstrate correlation – and the potential for causation. While this rewrite will not be trivial, even the title will need to be reconsidered, I strongly recommend this second option. The experimental work is sufficient as done to justify publication, if the manuscript is re-written to more accurately reflect the scope of the results of the work conducted.