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Behavioral thermoregulation and critical thermal limits of giant keyhole limpet *Megathura crenulata* (Sowerby 1825) (Mollusca; Vetigastropoda)[☆]

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ABSTRACT

The thermoregulatory behavior of the giant keyhole limpet *Megathura crenulata* was determined in a horizontal thermal gradient during the day at 18.9 °C and 18.3 °C for the night. The final preferendum determined for giant keyhole limpets was of 18.6 ± 1.2 °C.

Limpets' displacement velocity was 10.0 ± 3.9 cm h⁻¹ during the light phase and 8.4 ± 1.6 cm h⁻¹ during the dark phase. The thermotolerance (measured as CTMax at 50%) was determined in a keyhole limpet in three acclimation temperatures 17, 20, and 23 °C. Limpets were subjected to water increasing temperatures at a rate of 1 °C every 30 min, until they detached from the substrate. The critical thermal maximum at 50% was 27.2, 27.9 and 28.3 °C respectively.

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1. Introduction

Temperature is one of the principal abiotic factors influencing aquatic organisms. Marine ectotherms are especially affected by this variable, it exerts its effect at different levels of organization, for instance molecular, biochemical, physiological and behavioral (Stillman and Somero, 2000; Mora and Ospina, 2001). Thermal stress leads to changes in energy allocation for organisms' activities such as growth, reproduction and foraging, with consequences in their performance and fitness (Pörtner et al., 2008).

The intertidal ectotherms body temperature can vary drastically in low tides, making the intertidal zone of wave-washed rocky shores one of the most thermally stressful habitats; causing interspecific differences in thermal environment tolerance, which have been implicated in establishing the vertical zonation

characteristics of intertidal zones (Harley, 2008; Miller et al., 2009; Tepler et al., 2011). Organisms living in greater vertical shore heights, evolved specific adaptations that allow them to cope with environmental stress due to exposure to terrestrial conditions; this characteristic makes intertidal organisms great models for ecology, evolution stress and physiology studies (Stillman, 2002).

Many ectotherms are able to buffer the effects of space–time heterogeneity in thermal stress, these occurring in the intertidal zone in relation to diel and tidal cycles. More specifically, survival is warranted by adjustment of their body temperatures through a range of physiological, behavioral, and morphological adaptations (Somero, 2002; Lee and Lim, 2009). The tolerance window for each species is described as a favorable range of temperature or performance breadth. It includes an optimal and suboptimal zone, above or below that range, performance is negatively affected and the species cannot survive unless it is for a limited period of time (Madeira et al., 2012). Nonetheless, all species do not exhibit the same thermoregulatory ability. Specifically, tropical species inhabit stable and nonseasonal environments and have physiological acclimation abilities to thermal stress, as their thermal windows are narrower than those of temperate species (Compton et al., 2007; Chapperton and Seuront, 2011).

[☆]This article belongs to the Special Issue dedicated to Prof. Ken Bowler for on-going, long-term contributions to thermal biology; WHAT SETS THE LIMIT? HOW THERMAL LIMITS, PERFORMANCE AND PREFERENCE IN ECTOTHERMS ARE INFLUENCED BY WATER AND ENERGY BALANCE

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Aquatic ectotherms are not entirely at mercy of their thermal environment. Although most have only limited abilities to thermoregulate via physiological adjustment, many use behavioral adjustment to regulate body temperatures at remarkably narrow species-specific levels (Huey, 1991; Madeira et al., 2012). Behavioral thermoregulation can be adaptive in two complementary ways: (1) it can help an organism avoid extreme heat or cold that could be damaging or lethal (Kelsh and Neill, 1990), (2) it can increase the time an animal spends at optimal physiological temperatures (Huey, 2003). The thermal preference of a species corresponds closely with temperatures that maximize growth, and other physiological processes (Huey, 1991; Tepler et al., 2011).

Few studies have measured thermal preferences in marine gastropods as those conducted by: Casterlin and Reynolds (1980) in *Nassarius trivittatus*; Hecht (1994) in *Haliotis midae*; Díaz Herrera et al. (1996) in *Bulla gouldiana*; Soto and Bozinovic (1998) in *Nodillitorina peruviana*; Gilroy and Edwards (1998) in *Haliotis rubra* and *Haliotis laevis*; Díaz et al. (2000) in *Haliotis rufescens*; Muñoz et al. (2005) in *Echinollitorina peruviana*; Díaz et al. (2006) *Haliotis fulgens* and *Haliotis corrugata*; Díaz et al. (2011) in *Megastrea (Lithopoma) undosa*; Tepler et al. (2011) in *Chlorostoma funebris* and Chapperon and Seuront (2011) in *Littoraria scabra*.

The study of critical thermal limits provides important insight regarding ecology and physiology distribution in marine animals that are influenced by climate; these limits constitute the end-points of thermal performance curves in animals (Lutterschmidt and Hutchison, 1997; Crusella-Trullas et al., 2011). The critical thermal maximum (CTMax) is a common measure of ectotherm thermal resistance. The CTMax for aquatic species is typically determined by a process of water heating at a constant increasing rate up to "the thermal point at which the locomotory activities become disorganized and an animal loses its ability to escape from conditions that will promptly lead to its death" (Cowles and Bogert, 1944). The critical thermal maximum is widely used to define the range of animals' thermal tolerance within their environments (Huey and Stevenson, 1979).

Megathura crenulata is endemic to rocky intertidal and subtidal areas (to 25 m deep) along the coast of California, from Monterey Bay (36.6°N; 121.9°W) to Isla Asuncion, Baja California (27°N; 115°W). Interval temperatures that species experiment along distribution go from 16 to 20 °C (Morris et al., 1980). This species has commercial importance as its blood has hemocyanin which is being studied for its clinical application in cancer, allergies and immunosuppression treatment. (Nestle et al., 1998; Díaz-Sánchez et al., 1999; Harris and Markl, 1999). During the last decade, this species has been studied for a variety of reasons such as reproductive characteristics, effect of captivity and diet on KHL isoform ratios, non lethal method for extracting hemocyanin, structure and function of hemocytes, morphology of epithelial cells of the digestive tract, synthesis of hemocyanin in rhogocytes, production of digestive enzymes in the gut and characterization of metallothionein (Beninger et al., 2001; Oakes et al., 2004; Oakes, 2005; Martin et al., 2007, 2010, 2011, 2012).

Despite the great number of studies of this species, none today have focused on the thermal physiology aspect of this animal. This study is the first to evaluate the effect of different acclimation temperatures on giant keyhole limpet *M. crenulata* thermal preference and tolerance, in order of gathering knowledge of this species and its ability to resist environmental warming.

2. Material and methods

M. crenulata adults (weighing 111.0–524.0 g) were collected by deep sea hook diving at a depth of 5–10 m in the area of Tres

Hermanas in Punta Banda, Baja California, México (31°42' Lat. N; 116°45' Long. W.). Organisms ($n=110$) were kept for 30 days in laboratory conditions in four 2000 L reservoirs, at a 35‰ salinity and at 16 ± 1 °C in a flow-through water system (these being the same conditions measured at capture). The photoperiod was maintained in 12–12 h light–dark cycles with 30 min transition periods between them. Giant keyhole limpets were fed with a natural diet, such as when they were naturally in raw seawater (natural biota/biofilms). The reservoirs were washed weekly to reduce the accumulation of fecal matter and associated bacteria.

Forty-eight hours before beginning each experiment, limpets were tagged with a plastic mark. The preferred temperature experiments were performed in a trough, as described by Díaz et al. (2006). A Neslab thermoregulator model HX 150 (Newington, NH) was connected to the right end of the trough to cool the water to 10 °C. A 1000 W heater was placed at the left end of the trough, which was connected to a temperature-controlled heater in order to reach a water temperature of 30 °C, generating a stable temperature gradient. An aeration system that consisted of a high efficiency diffuser hose was placed along the gradient to maintain dissolved oxygen concentrations of 6.4–8.5 mg L⁻¹ and eliminate vertical thermal stratification from the water column. The water column depth in the trough was 8 cm and to maintain high water quality, it was refilled at a rate of 11.0–14.0 L h⁻¹.

The gravitation method described by Reynolds and Casterlin (1979), was used to determine the preferred temperature. Six individually labeled limpets with the same weight range, were introduced to the gradient at a virtual segment where water temperature was the same as the acclimatization temperature. They were exposed to a day light intensity of 0.30×10^{15} quanta - sec⁻¹ cm⁻² and a night light intensity of 0.02×10^{15} quanta - sec⁻¹ cm⁻². For each experimental condition, four repetitions were done (N total=24). The limpets were not fed 24 h prior to testing (Nelson et al., 1985; Beamish and Trippel, 1990). Observations taken of the number of limpets, in each segment, were made from reflections in a mirror oriented at a 45° angle and recorded hourly over 24 h. Simultaneously, temperature was measured with digital thermometers that were distributed equidistantly along the gradient.

Limpets' velocity displacement in the gradient, was determined by tracking the individual tags of each organism in each segment of the trough (every hour during the 24 h period, expressed in cm h⁻¹). A Mann–Whitney test was used to determine differences limpets' preferred temperature and their displacement in light and dark cycle (Zar, 1999). Temperature preference data was processed with Exploratory Data Analysis (Tukey, 1977) and plotted as parallel boxes.

To determine the critical thermal maxima (CTMax), 44 limpets were acclimated in three 2000 L reservoirs at temperatures of 17, 20 and 23 ± 1 °C, and a salinity of 35‰, for four weeks. Twenty four hours before trials started, limpets were not fed and a loop of monofilament was attached to their shell's surface, using epoxy clay close to the key hole. *M. crenulata* adults were then placed on acrylic plates (30 × 25 cm) in 40 L aquaria with aeration and were left undisturbed overnight. According to Morley et al. (2011) mono filament fishing line was hooked onto the loop attached to the limpets' shell, and then a 200 g mass was also placed at the other end of the line, in order to determine the temperature of loss attachment of plates. Each aquarium contained a thermostatically controlled 1000 W heater and an air stone. The temperature was raised from the acclimation temperatures of 17.0, 20 and 23 ± 1 °C at a rate of 1 °C every 30 min, to allow the core of temperature equilibrate to the surrounding water temperature (Cox, 1974; Díaz et al., 2000, 2006, 2011).

Determinations of CTMax were made between 0900 and 1400 h., visual monitoring was performed and recorded to note

limpet responses, and the time and temperature at which *M. crenulata* lost attachment. Immediately after losing attachment, limpets were transferred into 2000 L tanks during 96 h for recovery at acclimation temperature (organisms were used only once). The data obtained from limpets, that did not fully recover after returning to acclimation temperatures, was discarded. The Kruskal–Wallis test was used to determine differences between groups (Zar, 1999). The binomial data (percentage) at which 50% of the organisms remained attached, was transformed using arcsine, and subsequently it was calculated using linear regression Sigma-Stat, version 3.1 (Systat Software Inc., San Jose, CA) on all points below 100%.

3. Results

The preferred temperature by *M. crenulata* during the day was 18.9 °C and 18.3 °C for night cycles. The final preferendum determined for giant keyhole limpets was of 18.6 ± 1.2 (Fig. 1). The organisms were never found below 11 °C or above 26.5 °C in trough temperatures. There was not a significant difference in preferred temperatures of giant keyhole limpets between day and night cycle ($P > 0.05$) (Fig. 1).

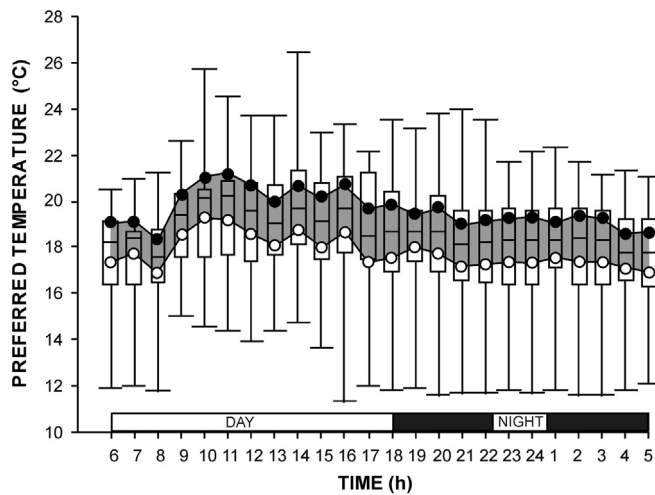


Fig. 1. Thermoregulatory behavior of adults of *Megathura crenulata* in a day–night cycle. The shadow zone bordered by triangles represents the 95% confidence interval of the median. The bars include 50% of the distribution and the vertical lines represent the quartiles.

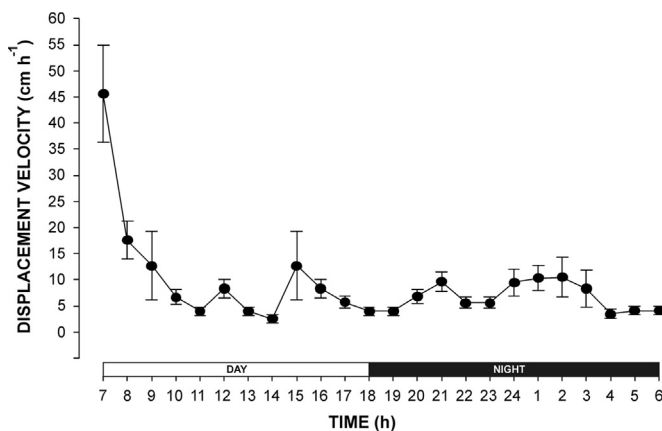


Fig. 2. Displacement velocity of giant keyhole limpet *Megathura crenulata* in a horizontal thermal gradient, in a day–night cycle. Median \pm 95% confidence interval.

During the first hour, after being placed in the trough, the displacement velocity of giant keyhole limpets diminished from 46.0 to 17.9 cm h⁻¹. In the third hour, once the organisms detected the preferred temperature, the displacement velocity decreased to an interval from 12.0 to 6.0 cm h⁻¹. During the light cycle, limpets displaced an average velocity of 10.0 ± 3.9 cm h⁻¹ (Fig. 2). In the dark cycle giant keyhole limpets displaced an average velocity of

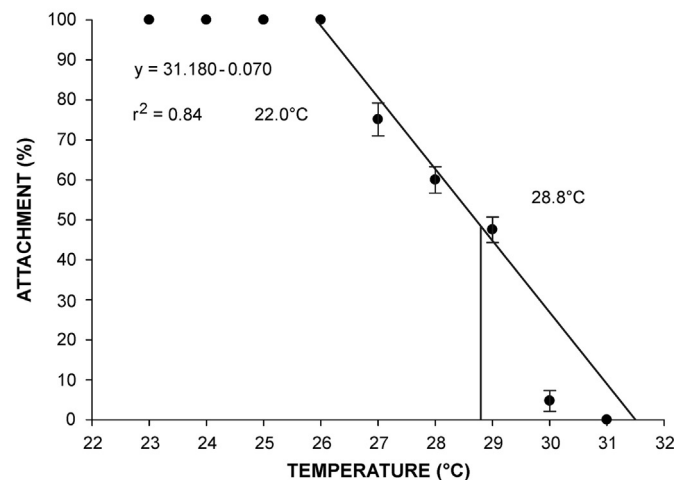
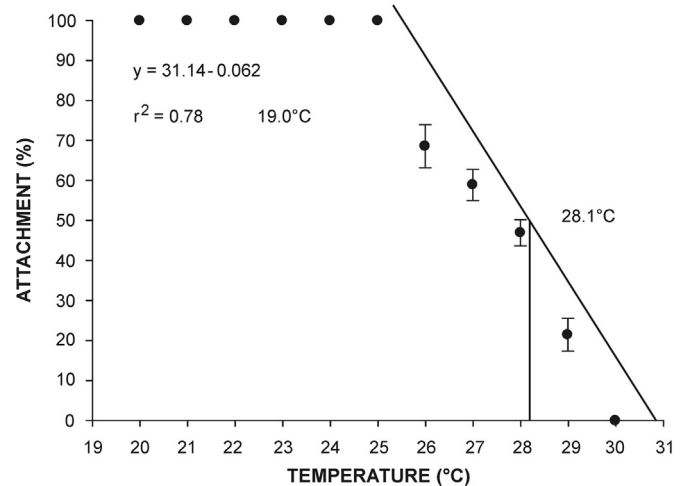
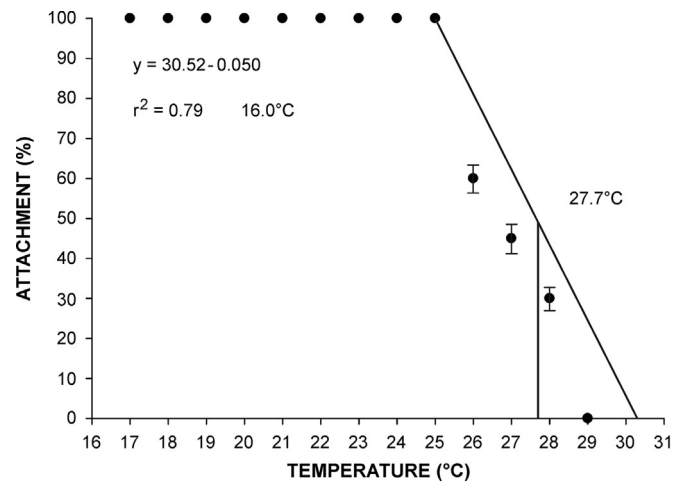


Fig. 3. Percentage of giant keyhole limpet *Megathura crenulata* remaining attached plotted against different acclimation temperatures. The continuous line denotes the critical thermal maxima at 50%.

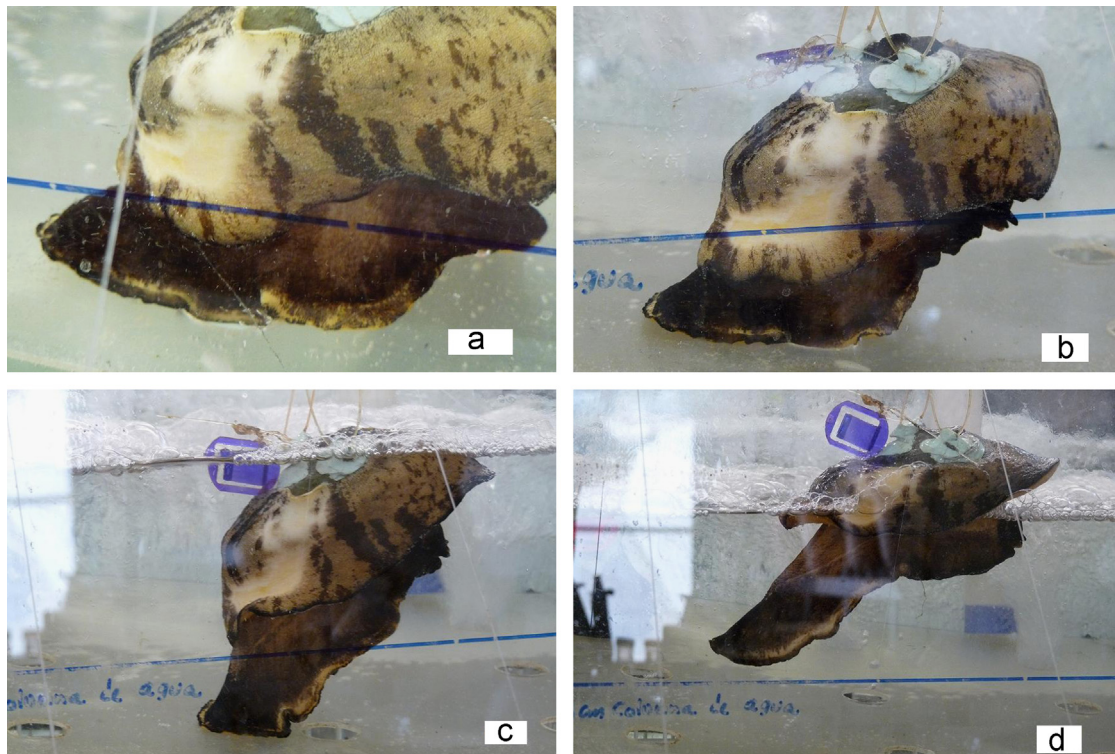


Fig. 4. Sequence the behavioral responses in *Megathura crenulata* due to thermal stress until lost attachment. (A) Foot totally attached to the acrylic, (B) initially detachment of foot, (C) foot partially attachment to substrate, and (D) foot completely lost attachment.

$8.4 \pm 1.6 \text{ cm h}^{-1}$. There were no relevant differences ($P > 0.05$) in terms of displacement velocity between day and night cycles.

For *M. crenulata* the relationship between percentage of attached organisms on the plate and acclimation temperature is represented as follows. For organisms acclimated to 17°C was given by:

$$y = 30.52 - 0.050x \text{ with an } r^2 = 0.79$$

In limpets acclimated to 20°C this relationship was:

$$y = 31.14 - 0.062x \text{ with an } r^2 = 0.78$$

In great keyhole limpets acclimated to 23°C this relationship was:

$$y = 31.18 - 0.070x \text{ with an } r^2 = 0.84$$

where y = temperature and x percentage of attached organisms. For organisms acclimated to 17°C the first limpet which lost holding was registered at 26°C , and for the ones that were acclimated at 23°C the last one at detaching was at 31°C (Fig. 3). The CTMax was defined as the temperature where 50% of the limpets remained attached, and was 27.7 , 28.1 and 28.8°C in organisms acclimated to 17 , 20 and 23°C respectively (Fig. 3). After 96 h at acclimation temperature, following the exposure to determine CTMax, 50% of giant keyhole limpets had a 98% survival rate.

Once the mass attached was applied to *M. crenulata*, it clamped down and started to resist the pull mass as temperature was increasing. Limpet's adductor and foot muscle worked to resist this force, maintaining a foot shape on the plate and therefore a suction force. Finally when the foot muscle became fatigued as a result of the increased temperature, the limpet lost its grip (Fig. 4).

4. Discussion

The preferred temperatures found in this study were 18.9°C in day cycle and 18.3°C , in night cycle. According to Fraenkel

and Gunn (1961), giant keyhole limpet used an orthothermokinetic orientation mechanism. The preferred temperatures for giant keyhole limpet are probably those that offer the greatest scope of activity (Fry, 1947). In addition, it has been mentioned by Beitinger and Fitzpatrick (1979) and McCauley and Casselman (1981) that the temperature that an organism prefers, commonly coincides with the optimum temperature that it requires to move, grow or reproduce, because it allows the organism to maximize its energy efficiency (Kelsch, 1996). The preferred temperature obtained in this study for *M. crenulata* according to Oakes et al. (2004), allows the optimization of the cultivation of this species, resulting not only in alleviating impacts on natural populations, but also potentially resulting in a less variable product as KHL.

Thus, limpet will remain within a relatively narrow temperature range, decreasing the displacement velocity to 12.0 – 6.0 cm h^{-1} , in temperatures that maximize their available energy (Kelsch and Neill, 1990; Bryan et al., 1990; Kelsch, 1996). Therefore, the greatest amount of available energy that will be channeled into movement, reproduction, survival and growth functions (Kelsch, 1996). The marine snail *B. gouldiana*, red abalone *H. rufescens*, green and pink abalone, wavy turban snail *M. (Lithopoma) undosa* and *L. scabra* exhibited a similar response when it was close to the preferred temperature: their displacement velocity decreased respectively. (Díaz Herrera et al., 1996; Díaz et al., 2000, 2006, 2011; Chapperon and Seuront, 2011).

This result demonstrates that *M. crenulata* was able to behaviorally thermoregulate in the gradient selecting advantageous temperatures available within the mosaics of environmental conditions that were offered. We conclude that like many other ectotherms the vetigastropoda are able to regulate their body temperature by using behavioral responses of thermoregulation, which may partially explain their pattern of spatial distribution in the rocky intertidal zone along the coast of California and Baja California.

The CTMax of *M. crenulata* was determined by using a methodology that involved dynamic changes in temperature. Díaz et al. (2006) presented a summary of the critical thermal maxima at 50% for various species of gastropods of genus *Haliotis*, which were acclimated to different temperatures and salinities. In the determination of CTMax to 50% giant keyhole limpet were subjected to a continuous and constant linear increase in temperature until an endpoint was reached. Smith (1991) and Morley et al. (2011) mention that attachment capacity in marine gastropods is the result from the sum of two main components: the proteinaceous or mucopolysaccharide secretions from the foot and also from muscular activity that maintains shape and suction force from the foot. It has been shown temperature affects the capacity of attachment in different species such as limpets, sea urchins sea cucumbers, abalones and wavy turban snail (Grenon and Walker, 1981; Davenport, 1997; Santos and Flammang, 2007; Flammang et al., 2002; Díaz et al., 2006, 2011).

According to Cowles and Bogert (1944) the CTMax endpoint at 50% reflects a temperature at which locomotory movements become disorganized and the limpet loses its ability to hold to the acrylic plate due to neuromuscular blockade and presynaptic failure (White, 1983). When these responses are present, organisms cannot escape from conditions that might ultimately lead to their death. The thermal dependence of attachment response in limpets is therefore likely to reflect an integrate measure of the effect of temperature on several organ systems.

The behavioral responses of limpets due to thermal stress, especially the ones which started to lift their shells and exposing the mantle, have also been observed in other abalone species (Hines et al., 1980; Hahn, 1989; Hecht, 1994; Gilroy and Edwards, 1998; Díaz et al., 2000, 2006) and wavy turban snail (Díaz et al., 2011). Hines et al. (1980) considered that these responses, from an adaptive point of view, were used as an evaporative cooling system that could increase desiccation and leave limpets more vulnerable to predation or disease.

The knowledge of critical thermal maxima provides a relevant ecological index; in this case giant keyhole limpet in nature can find spatial temperatures temporarily with acute fluctuations outside of their limits of tolerance. This information is vital in order to determine the suitability in different areas for the growth of marine gastropod species, the environmental requirements for keeping them in captivity, and understanding their distribution. The behavior of ectotherms challenges the predicted shifts in species distribution patterns, therefore knowledge concerning these unpredicted conducts in animals, teaches us that animals like limpets in this case, still have a lot to show us concerning global warming and climate changing models.

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