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Plasticity of thermal limits in the aquatic saline beetle *Enochrus politus* (Küster 1849) (Coleoptera: Hydrophilidae) under changing environmental conditions

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ABSTRACT

Plasticity of thermal limits in the aquatic saline beetle *Enochrus politus* (Küster 1849) (Coleoptera: Hydrophilidae) under changing environmental conditions

Information on the physiological tolerance of species is essential when forecasting their responses to climatic change, especially for those with restricted distributions, as well as those inhabiting extreme environments, such as inland saline waters. Temperature and salinity are expected to increase in these ecosystems under climate change, and both factors are known to affect the thermal tolerances of aquatic insects. The objective of this study was to determine, in the laboratory, the thermal limits and their plasticity to changes under the environmental conditions of the aquatic beetle *Enochrus politus*, a typical species of saline streams from the southeast of Spain. *Supercooling* temperature (point before the freezing temperature) and *Heat coma* (point of paralysis prior to death) were used as the lower and upper thermal limits, respectively. They were determined by a dynamic method combined with infrared thermography in groups of individuals previously acclimated at different conditions of temperature and salinity. *E. politus* showed a wide range of thermal tolerance ($-10.38 \pm 0.32\,°C$-$57.37 \pm 0.19\,°C$) and was higher in specimens acclimated at $20\,°C$ and 12 g/L, coinciding with the mean environmental conditions of the habitats where the species often lives. At lower temperatures and salinities, its thermal tolerance range decreased. The temperature and salinity of acclimation both had significant effects on the thermal limits. Elevated salinity decreased the heat tolerance and increased the freezing tolerance. The lower thermal limit of the species showed more plasticity than the upper limit. The species would potentially be able to tolerate temperature increase according to its wide thermal range, when compared to other insects; however, the salinization of its habitat might reduce its heat tolerance.

Key words: Saline streams, Coleoptera, thermal limits, phenotypic plasticity, *Supercooling, Heat coma*.

RESUMEN

Plasticidad de los límites térmicos en un escarabajo acuático, **Enochrus politus** *(Küster 1849) (Coleoptera: Hydrophilidae)en diferentes condiciones ambientales*

La información sobre la tolerancia fisiológica de las especies es esencial para preveer sus respuestas al cambio climático, especialmente en especies de distribución restringida o que habitan ambientes extremos como las aguas salinas continentales. En estos ecosistemas se prevé un aumento de temperatura y salinidad como consecuencia del cambio climático, y ambos factores influyen en la tolerancia térmica de insectos acuáticos. El objetivo de este trabajo fue determinar bajo una aproximación experimental los límites térmicos y su sensibilidad frente a distintas condiciones ambientales en una especie de coleóptero acuático, Enochrus politus, *común en ríos salinos del sureste de la Península Ibérica. Como límite térmico inferior se determinó el* Supercooling, *o temperatura previa a la congelación, y como límite superior se consideró el* Heat coma, *o punto de parálisis anterior a la muerte, combinando un método dinámico y termografía infrarroja, previa aclimatación de los ejemplares a diferentes condiciones de temperatura y salinidad. Los resultados mostraron que* E. politus *tiene un amplio rango de tolerancia térmica ($-10.38 \pm 0.32\,°C$ y $57.37 \pm 0.19\,°C$), siendo mayor en individuos aclimatados a $20\,°C$ y 12 g/L, condiciones similares a las de los hábitats donde frecuentemente vive la especie. A bajas temperaturas y salinidad, su rango de tolerancia disminuyó. Tanto la temperatura como la salinidad tuvieron un efecto significativo en ambos límites térmicos. Salinidades elevadas provocaron una disminución de la tolerancia al calor e incrementaron la tolerancia al frío. El límite térmico inferior de la especie mostró mayor plasticidad que el superior. Debido a su amplio rango térmico en comparación*

con otros insectos, esta especie sería potencialmente capaz de hacer frente a un incremento de temperatura, aunque la salinización de su hábitat podría reducir su tolerancia a altas temperaturas.

Palabras clave: *Ramblas salinas, Coleoptera, límites térmicos, plasticidad fenotípica, Supercooling, Heat coma.*

INTRODUCTION

The Mediterranean basin has a wide diversity of aquatic inland ecosystems. Among these ecosystems, saline streams ecosystems are considered especially singular, due to their variable and extreme environmental conditions, their halo-tolerant/halophilic biota and the high number of endemic species that they sustain (Millán *et al.,* 2011). In these ecosystems, high levels of salinity and temperature are frequent; therefore, only a limited number of organisms with specialized adaptations have been able to colonize them. Among these, the insect orders Diptera, Coleoptera and Hemiptera are the most representative among the macroinvertebrate community in saline streams, both in species richness and abundance (Millán *et al.,* 2011).

Although inland saline water ecosystems are globally distributed in arid lands, they are rare in a European context. Despite their high conservation interest, these systems are globally threatened by human pressures, which induce changes in the natural hydrology and salinity levels with a corresponding loss of biodiversity, affecting halophilic biota (Velasco *et al.,* 2006). In addition, climatic models predict an increase in temperature and a decrease in rainfall in Mediterranean regions (IPCC, 2013), with the consequent reduction in available aquatic habitats and an increase in their salinity levels (Sánchez-Fernández *et al.,* 2013). In this context of increasing stress, some traits, such as thermal and salinity tolerances, together with the dispersal ability, determine the species' capacity to persist in their current localities or escape to more suitable habitats (Arribas *et al.,* 2012a; b).

Thermal physiology received a great deal of empirical and theoretical investigations (Angilletta, 2009), given its informative potential to understand the diversity of the physiological adaptations of organisms (Lutterschmidt & Hutchison, 1997), the abundance and geographical distribution of species and its variation (Chown & Terblanche, 2006; Terblanche *et al.,* 2011). Furthermore, it provides insights into the conditions that are limiting for life over the course of its evolution (Pörtner, 2002; Boussau *et al.,* 2008). Growing concerns about the biodiversity impacts of global environmental change stimulated research on the various factors that influence the thermal limits of species and their plasticity (Terblanche *et al.,* 2011, Hofmann & Todgham, 2010).

Thermal tolerance has two components 1) an intrinsic or genetic component and 2) an acquired component or dependent of acclimation (in the laboratory) or acclimatization (in the field) conditions. Both of the components are good examples of phenotypic plasticity, which reflects the malleability of the organism in response to the environmental conditions experienced (Chown & Nicolson, 2004). In general, it has been assumed that acclimation is advantageous when organisms are exposed to similar conditions to those at which they were acclimated (Beneficial Acclimation Hypothesis-BAH, Leroi *et al.,* 1994). However, this hypothesis has been questioned because the acclimation or acclimatization also has a high physiological cost (Hoffmann, 1995) and therefore, does not necessarily provide an organism with a performance advantage (e.g., Gilchrist & Huey, 2001; Stillwell & Fox, 2005; Deere & Chown, 2006). Woods & Harrison (2002) argued that exposure to suboptimal temperatures may decrease survival in all environments (Detrimental Acclimation Hypothesis-DAH). As a consequence, explicit tests for beneficial or deleterious effects should always be undertaken when studying acclimation responses (Marais & Chown, 2008). Stillman (2003) proposed that those species that have evolved grea-

ter absolute temperature tolerances have done it at the expense of their ability to acclimatize. However, Calosi *et al.* (2010) showed that there is a positive relationship between higher temperature tolerance and acclimation capacity in freshwater beetles of the genus *Deronectes* (F. Dytiscidae). On the other hand, some studies suggest that acclimation capacity seems to be higher in animals living in moderately variable environments and limited in animals in very stable (e.g., Hoffmann & Harshman, 2000) or highly variable environments (e.g., Sanders *et al.,* 1991; Hofmann & Somero, 1995).

In aquatic ecosystems, other environmental factors, such as salinity, play an important role and may act additively, synergistically or antagonistically with temperature (Spicer & Gaston, 1999; Gaston, 2003). Therefore, the thermal tolerance of the organisms may be influenced by other characteristics of the environment, such as osmotic stress (Chown & Nicolson, 2004). Previous studies show that despite the wide thermal tolerance of some aquatic saline beetle species, acclimation conditions (temperature, salinity and their interaction) may have a significant influence on their thermal limits (Sánchez-Fernández *et al.*, 2010; Arribas *et al.*, 2012b). In this context, research on the thermal tolerance of saline species and acclimation capacity is an issue of great interest to understanding the processes of adaptation to climate change (Terblanche *et al.*, 2011).

The main goal of this study was to experimentally determine the thermal tolerance of the saline beetle species *Enochrus politus* (Küster 1849) and its plasticity under different acclimation conditions of temperature and salinity. *E. politus* is a euryhaline species, living in running saline waters and with a circum-Mediterranean range distribution (Millán *et al.*, 2014). Based on the theoretical and empirical background, we predicted that 1) *E. politus* would present a wide thermal tolerance like other species of the genus; 2) *E. politus* acclimation at high temperatures would increase heat tolerance; 3) *E. politus* acclimation at low temperatures would increase tolerance to cold; and 4) *E. politus* acclimation at elevated salinities would increase tolerance to heat and cold. We also predicted that the species would possess a low acclimation capacity, due to the extreme and variable environmental conditions in which they live.

METHODS

Adults of *E. politus* were collected from Chícamo stream, a saline and intermittent tributary of the Segura River, located in southeast Spain (30S 670 522 E- 4 231 172 N), in the most arid area of the province of Murcia (Spain). The climate is characterised by a mean annual precipitation below 300 mm and a mean annual temperature of 18 °C. Annual and interannual variations in rainfall can lead to high discharge variability, with extreme conditions of flooding and drying. The highest flows normally occur in early autumn, followed by a second peak in spring. Chícamo stream is characterized by wide annual, seasonal and diel variabilities in environmental conditions (Vidal-Abarca *et al.,* 2001, 2002). The salinity ranges from 4 g/L to 18.3 g/L, and the temperature ranges seasonally from 7 °C to 34 °C, with daily variations of up to 18 °C. Basin lithology is dominated by highly impermeable materials (sedimentary marls).The natural cover of the watershed is open Mediterranean scrub, although much is dedicated to agriculture.

Laboratory works

The specimens were maintained in the laboratory for one day at 20 °C in 5 L aquaria containing water and substrate from the collection site to simulate natural conditions. Groups of 50-60 specimens were later acclimated at different combinations of temperature (15 °C, 20 °C and 25 °C) and salinity (1 g/L, 12 g/L, 35 g/L and 60 g/L) in 2 L aquaria for 7 days under a 12 h light:12 h dark cycle in a climatic chamber (Sanyo MLR-351). The chosen temperatures and salinities were representative of the habitat range where the species is present. Saline solutions were prepared by dissolving an appropriate quantity of marine salt (Ocean Fish, Prodac) in distilled water. Organisms were fed with aquatic macrophytes (*Cladophora fracta* and *Enteromor-*

pha intestinalis) *ad libitum*, but no food was provided 48 h prior to the determination of thermal limits.

Among the number of potential end-points that exist for both tolerance to heat and cold (Chown & Nicolson, 2004), here the *Heat coma* point (*HC*) or point of paralysis prior to death was considered the upper thermal limit, and the *Supercooling* point (*SCP*) or point before the freezing temperature was the lower thermal limit.

Following the acclimation period, individuals from each of the 12 combined treatments were randomly assigned to two equal subgroups (*N* = 20); one was used to measure *HC* and the other to determine *SCP*. Specimens were washed with distilled water and dried on absorbent paper, and then placed downside up on a clean, dry rectangular piece of pottery using nontoxic glue (ErichKrause®). This procedure ensured that individuals were immobile during the trial.

Thermal limits trials were carried out in air, employing a dynamic method using a ramping rate of $\pm 1\,°C\,min^{-1}$ (Chown & Nicolson, 2004)

in a controlled-temperature chamber (BINDER MK53, BINDER GmbH, Tuttlingen, Germany) and infrared thermography. The ramping program started at the temperature at which individuals of a given subgroup had been acclimated, until the end points (70 °C for *HC* and −30 °C for *SCP*) that exceeded the thermal limits in other similar species of water beetles (Sánchez-Fernández *et al.*, 2012). A thermographic camera (FLIR SC305) was coupled to the climatic chamber, and thermal images of each individual during the trial were analysed with the software Therma-CAM Researcher Professional 2.10. A video camera (Sony DCR-DVD110E. Sony Co., Tokyo, Japan) was also used to record the movement of individuals during the experiments. *HC* was estimated using both video and thermal images. From the video images, the moment at which the movement of legs, antennae and palps ceased was determined, and the body surface temperature at that moment was obtained from infrared thermal images. Such cessation of movement was preceded by spasmodic movements of legs, antennae and palps. The *SCP* of each individual
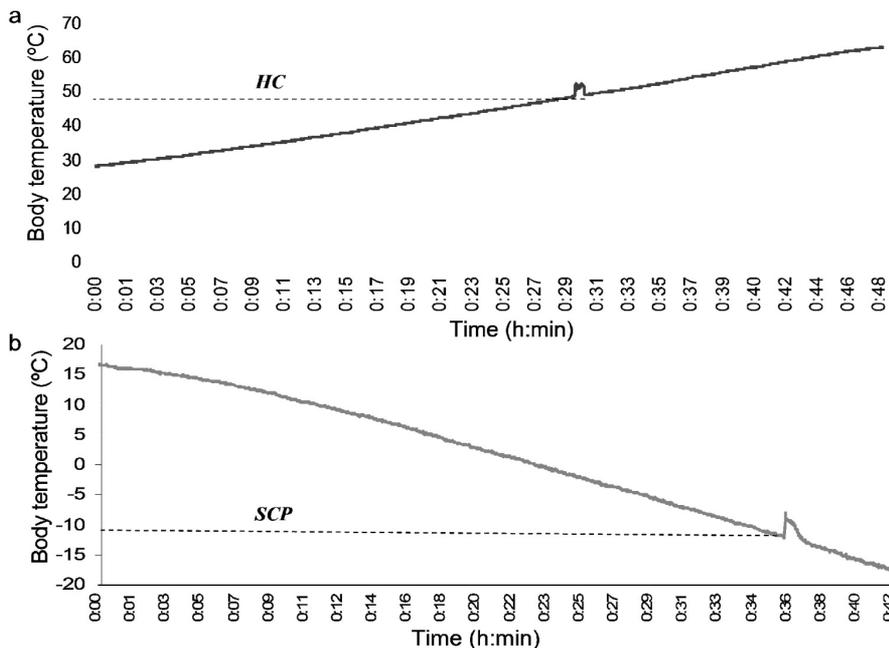
Figure 1.   Variation in body temperature of an individual during a) the *Heat coma* trial and b) the *Supercooling* trial. *Variación de la temperatura corporal de un individuo durante los experimentos a)* Heat coma *y b)* Supercooling.

was easily identified because individuals experienced a sudden rise in body temperature due to an exothermic reaction caused by the freezing of the animal's body fluids (Wilson *et al.*, 2003). All of the specimens were sexed by examining their genitalia.

Data analysis

An analysis of variance (ANOVA) was used with untransformed data to investigate the effects of salinity and temperature of acclimation and their interactions on both thermal limits. Previous analyses showed no differences between sexes; therefore, sex was excluded from the subsequent analyses. The data met the assumptions of normality and homogeneity of variances. A post-hoc Bonferroni analysis was implemented to identify significant differences in the response variables among acclimation treatments (Underwood, 1997; Rutherford, 2001). The acclimation capacity in thermal limits was estimated as the magnitude of the variation (increment or decline) in *HC* and *SCP* in response to the increase of the acclimation temperature (Calosi *et al.*, 2008). The relationship between both thermal limits was analysed by Pearson correlation. All of the

statistical analyses were conducted using SPSS, version 19.0.

RESULTS

Heat coma (HC)

During the heat trials, the movement of legs, antennae and palps of organisms increased with increasing temperature. When body temperature reached approximately 48 °C, spasmodic movements alternated with short periods of immobility, until the total cessation of movements. *HC* was preceded by accelerated movements of legs and an abrupt increase of body temperature, as a result of the intense heat generated (Fig. 1a).

The highest mean value of *HC* (54.24 ± 0.29 °C) was reached in those individuals acclimated at 25 °C and 1 g/L, and the lowest mean value of *HC* (52 ± 0.26 °C) was reached at 20 °C and 60 g/L (Fig. 2a). Significant differences in *HC* were found among acclimation temperatures, although the *p* value was close to the significance level (Table 1). Heat tolerance was lower in the individuals acclimated at 20 °C than in those acclimated at 10 °C and 25 °C (Fig. 2a). Regarding

Table 1. ANOVA results for *Heat coma* and *Supercooling*. *Resultado del ANOVA para el* Heat coma *y* Supercooling.

	Origin	$\sum$ square type III	df	Mean square	F	*p*
Heat coma	Corrected model	97.92	11	8.90	6.45	< 0.001
	Intercept	697 338.28	1	697 338.28	504 830.29	< 0.001
	Temperature	8.99	2	4.50	3.26	0.040
	Salinity	70.74	3	23.58	17.07	< 0.001
	Temperature * Salinity	14.55	6	2.42	1.76	0.109
	Error	341.19	247	1.38		
	Total	737 224.78	259			
	Total corrected	439.11	258		$R^2 = 0.34$ (R^2 corrected $= 0.31$)	
Supercooling	Corrected model	271.93	11	24.72	10.93	< 0.001
	Intercept	25 919.51	1	25 919.51	11 455.39	< 0.001
	Temperature	148.25	2	74.12	32.76	< 0.001
	Salinity	47.78	3	15.92	7.04	< 0.001
	Temperature * Salinity	52.09	6	8.68	3.84	0.001
	Error	538.51	238	2.26		
	Total	28 082.61	250			
	Total corrected	810.44	249		$R^2 = 0.22$ (R^2 corrected $= 0.19$)	

salinity, there were no significant differences between acclimation treatments of 1 g/L and 12 g/L and between 35 g/L and 60 g/L; however, there were differences between both groups, as the individuals acclimated at lower salinities had a greater heat tolerance (Fig. 2a). The interaction of salinity and temperature of acclimation was not significant (Table 1).

Supercooling (SCP)

During cold ramping experiments, individuals vigorously beat their legs in temperatures ranging between 7 °C and 4 °C. From there, the movements decreased, ceasing at temperatures between −4 °C to −7 °C. Body temperature gradually decreased until the *SCP*; at this point, a sudden increase of 0.7-2.5 °C in body temperature was registered (Fig. 1b).

The lowest mean *SCP* (−11.61 ± 0.35 °C), i.e., the highest cold tolerance, was observed in individuals acclimated at 20 °C and 12 g/L, and the highest mean *SCP* (lowest cold tolerance, −7.86 ± 0.34 °C) was observed at 15 °C and 1 g/L (Fig. 2b). *SCP* was significantly influenced by temperature, salinity and their interaction (Table 1).

Regarding temperature, the maximum mean

SCP was measured in individuals acclimated at 15 °C (−9.22 ± 0.19 °C), and no significant differences were found between the 20 °C and 25 °C treatments. Individuals acclimated at 1 g/L were less cold tolerant (−9.53 ± 0.27 °C) than those acclimated at higher salinities, although no significant differences were detected between the 12 g/L, 35 g/L and 60 g/L treatments (Fig. 2b).

The effect of the interaction between both factors was observed only at the lowest temperature tested (15 °C), where cold tolerance was higher as the acclimation salinity increased (Fig. 2b).

Thermal range and plasticity of thermal limits

The upper and lower thermal limits were not significantly correlated (Pearson $r = 0.208$, $p = 0.496$). The highest tolerance range for this species was found in individuals acclimated at 20 °C and 12 g/L, whereas the lowest tolerance range was found at 15 °C and 1 g/L acclimation conditions (Table 2).

The mean overall acclimation capacity in response to temperature (i.e., the variation of thermal limits among the acclimation treatments) was −1.9 °C for *SCP* and 0.16 °C for *HC*. The

(a)   (b)
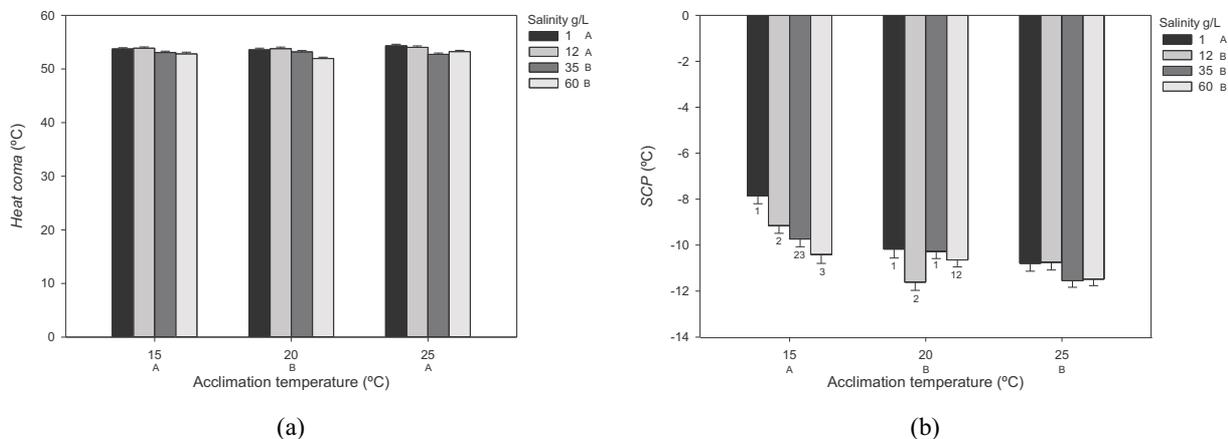
Figure 2. a) The mean ± SE *Heat coma* and b) *Supercooling* points of *E. politus* acclimated to different temperatures and salinities. Significantly different means ($p \leq 0.05$) measured at different acclimation temperatures and salinities are indicated by different letters in the temperature axis and salinity legend, respectively. Significantly different means measured at different salinities within the same temperature treatment are indicated by different numbers below the bars. *a) Media ± ES para el* Heat coma *y b)* Supercooling *de* Enochrus politus *aclimatado a diferentes tratamientos de temperatura y salinidad. Las diferencias significativas para las temperaturas de aclimatación están indicadas con letras en el eje y para las salinidades están en la leyenda. Diferencias entre salinidades para cada tratamiento de temperatura están indicadas con números bajo las barras.*

plasticity of *SCP* to increasing temperatures differed at the different acclimation salinities. At freshwater and low salinities (1 g/L and 12 g/L), the plasticity of *SCP* was higher from 15 °C to 20 °C than from 20 °C to 25 °C (Table 2). However, at higher salinities (35 g/L and 60 g/L), the acclimation capacity of *SCP* was higher from 20 °C to 25 °C. The greatest changes in *HC* occurred when the species was acclimated at 60 g/L, resulting in a decline of 0.79 °C when changing from 15 °C to 20 °C and an increase of 1.25 °C from 20 °C to 25 °C (Table 2).

DISCUSSION

E. politus can be considered a thermal generalist species, given its wide temperature tolerance range (−10.38±0.32 °C and 57.37±0.19 °C), similar to other species of the genus, such as *E. bicolor* (Fabricius) (−11.57 °C and 52.45 °C) and *E. jesusarribasi* Arribas & Millán (named as *E. falcarius* IP in Arribas *et al.*, 2012b) (−11.57 °C and 53.07 °C), although in these species, the lower thermal limits were measured as lethal points and not as *SCP* (Arribas *et al.*, 2012b). The upper thermal limits reported here were likely overestimated, since the ramping rates used in dynamic experiments (±1 °C min⁻¹) do not represent realistic thermal changes experienced by organisms in nature, although ramping assays are thought to be more relevant to natural conditions than other approaches (Terblanche *et al.*, 2011). However, the high upper thermal limit of *E. politus,* which was consistent with those of its congener species (> 50 °C), exceeded the temperature tolerated by most insects. The average upper thermal limit for insects is 41.6 °C, and temperatures above 47.8 °C are thought to be tolerated only temporarily by animals (Araújo *et al.*, 2013) or by those from extreme environments, such as desert species (e.g., Wehner *et al.,* 1992). Preliminary trials at a constant temperature showed that *E. politus* can tolerate up to 46 °C for 6 h (unpublished data).

This species also showed a higher thermal tolerance than other beetle saline species from different genera and families occupying similar habitats, such as *Nebrioporus* (Dytiscidae), with ranges from −5.20 °C to 45.24 °C in *N. baeticus* (Schaum) and −6.07 °C to 45.42 °C in *N. ceresyi* (Aubé) (Sánchez-Fernández *et al.,* 2010), and *Octhebius* (F. Hydraenidae), with ranges from −13.79 to 47.98 °C in *O. glaber* Montes & Soler and −12.57 to 48.61 °C in *O. notabilis* Rosenhauer (Arribas *et al.*, 2009). Tolerance to heat is

Table 2. Thermal range and plasticity of thermal limits. *Rango térmico y plasticidad de los límites térmicos.*

Acclimation conditions						
Temperature (°C)	Salinity (g/L)	SCP	ΔSCP	Heat coma	ΔHC	Thermal range
15	1	−7.86		53.68		61.54
20	1	-10.17	−2.31	53.72	0.04	63.89
25	1	−10.79	−0.62	54.29	0.57	65.08
15	12	−9.15		53.92		63.07
20	12	−11.69	−2.54	53.78	−0.14	65.47
25	12	−10.75	0.94	53.98	0.2	64.72
15	35	−9.66		53.26		62.92
20	35	−10.37	−0.71	52.99	−0.27	63.36
25	35	−11.52	−1.15	52.8	−0.19	64.33
15	60	−10.33		52.78		63.11
20	60	−10.7	−0.37	51.99	−0.79	62.69
25	60	−11.55	−0.85	53.24	1.25	64.79
Mean thermal limits and acclimation capacity		−10.61	−1.9	53.34	0.16	63.95

largely conserved across lineages (Araújo *et al.*, 2013). Thus, the wide thermal tolerance in the *Enochrus* species could be a conservative character of the genus, and the thermal differences with cohabiting species from different genera probably reflect the thermal heterogeneity that organisms with different adaptations to the aquatic environment experience at a microhabitat scale. In this sense, although *Enochrus* species are known as truly water beetles (Jäch & Balke, 2008), they usually occupy the shorelines of water bodies, where changing and more extreme temperatures are frequent when compared with those experienced by benthos and water column species.

The thermal tolerance of the species was influenced by both salinity and temperature, as previous studies on saline insect species found (Sánchez-Fernández *et al.*, 2010; Arribas *et al.*, 2009, 2012b; Coccia *et al.*, 2013). Concerning *E. politus*, its thermal range was maximized in middle acclimation conditions of temperature (20 °C) and salinity (12 g/L), similar to the mean conditions in the species' habitat.

Heat tolerance

The acclimation temperature and salinity had significant effects on the heat tolerance in *E. politus*. However, contrary to the expected result, there was no clear pattern of increasing *HC* with the acclimation temperature (2nd prediction rejected), and the heat tolerance decreased with the salinity of acclimation (4th prediction rejected). However, in the sister species *E. jesusarribasi*, the mean in the upper thermal limit (UTL) increased with both temperature and salinity of acclimation (Arribas *et al.*, 2012b), and similar results were found in *Nebrioporus* species (Sánchez-Férnandez *et al.*, 2010) and other aquatic insects, such as the corixid *Trichocorixa verticalis* (Fieber) (Coccia *et al.*, 2013). The mentioned beetle species showed greater salinity tolerance than *E. politus*, both from experimental and field data (e.g., Céspedes *et al.*, 2013), as the former were more efficient osmoregulators (Pallarés *et al.*, 2015). This could explain why the observed negative effect of high salinities (i.e., above 35 g/L) on thermoregulatory mechanisms in *E.*

politus was not found in other saline species under the same range of acclimation salinities.

The combination of high temperature and salinity caused a decrease in the upper thermal limit in *E. politus*, which suggested that a *trade-off* between tolerances to both stressors may exist (Angilletta *et al.*, 2003). The mechanisms underlying tolerance to osmotic and thermal stress are highly energetically costly, and complex interactions might arise when organisms are exposed to sublethal levels of both stressors.

Osmoregulation mechanisms may breakdown under high temperatures (e.g., Segal & Burbanck, 1963; Punzo, 1989). By contrast, the osmoregulatory effort in hyperosmotic media may result in decreased energy derived for thermoregulatory mechanisms, such as the production of heat shock proteins (Hsp). Under conditions of high heat and salinity stress, the temperature from which Hsp production is induced may decrease, reducing the upper thermal limit (Tomanek *et al.*, 2011). The role of Hsp in heat tolerance was widely documented (e.g., see Haranda *et al.*, 2011), especially in model organisms, such as *Drosophila* spp. (e.g., Welte *et al.*, 1993, Feder *et al.*, 1996, Bettencourt *et al.*, 2008, Krebs & Feder, 1998). Aquatic saline species also constitute a good model for the study of Hsp under multiple stressors, since these species are adapted to naturally stressful conditions, and some of them are able to tolerate extreme temperatures, as shown by our study and previous work (Arribas *et al.*, 2009, 2012b; Sánchez-Férnandez *et al.*, 2010).

Freezing tolerance

E. politus showed a higher freezing tolerance (lower *SCP* values) when acclimated at higher (20 °C and 25 °C) rather than lower temperature (15 °C), contrary to our 3rd prediction and to the pattern found in *E. jesusarribasi* (Arribas *et al.*, 2012b). However, regarding salinity, the studied species increased its freezing tolerance when subjected to higher salinity acclimation conditions, as was expected (4th prediction). Similar salinity acclimation effects over lower lethal thermal limits were also found by Sánchez-Fernández *et al.* (2010) in two dytiscid saline

beetle species (*Nebrioporus ceresyi* and *N. baeticus*) and by Coccia *et al.* (2013) in some corixid species. These positive effects of water salinity on freezing tolerance can be related to the organism's osmotic regulation ability to maintain ion homeostasis. A higher *SCP* (lower freezing tolerance) in freshwater (hypoosmotic conditions) is probably due to some dilution of the internal medium of the organism, i.e., a low osmolality of the haemolymph, which increases the temperature of the freezing point (Bradley, 2009). On the contrary, a higher haemolymph osmotic concentration in more saline media (Pallarés *et al.*, 2015) would increase the freezing point of internal fluids.

Acclimation capacity

As expected, *E. politus* presented a limited ability for thermal acclimation of the upper thermal limit, as other congeneric species (Arribas *et al.*, 2012b), but some degree of phenotypic plasticity of the lower thermal limit was found. These results agreed with some studies that investigated the effects of acclimation temperature on the thermal limits conducted at rates $\geq 0.25\,°C$, which found that the maximum thermal limit was less responsive to treatments than the minimum thermal limit (e.g., Klok & Chown, 2003; Terblanche *et al.*, 2006). However, as the acclimation capacity depends on the rates of change of the temperature used in the experimental determination of the thermal limits (Chown *et al.*, 2009), making comparisons is difficult.

Some studies suggest that acclimation capacity seems to be higher in animals living in moderately variable environments and limited in animals of very stable (e.g., Hoffmann & Harshman, 2000) or highly variable environments (e.g., Sanders *et al.*, 1991; Hofmann & Somero, 1995). Some examples of the absence of thermal acclimation in the upper thermal limit can be found in insects associated with extreme environments, such as *Stratiomys japonica* Wulp (Garbuz *et al.*, 2008) and larvae of the Antarctic fly *Belgica antarctica* Jacobs (Rinehart *et al.,* 2006), but also in the freshwater dytiscid species (Calosi *et al.*, 2008).

Species whose habitat and climatic preferences are close to their upper thermal limit probably cannot develop physiological temperature increased tolerances, so they will be more sensitive to climate change (Tomanek, 2008; Araújo *et al.*, 2013). In the case of *E. politus,* its high heat tolerance ($57.37 \pm 0.19\,°C$) is far from the maximum water temperature reached in their habitats ($35\,°C$) and, despite the species' low acclimation capacity, it might be able to survive the temperature rise of several degrees expected under climate change scenarios in the medium- and long-term (IPCC, 2013). However, according to our results, the combination of increased temperature and salinity and the persistence of more extreme conditions in inland water bodies could decrease the species' thermal range.

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