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The colonization of saline waters is associated with lowered immune responses in aquatic beetles

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Aquatic coleoptera, immunity, trade-off, hypersaline habitats, osmorregulation

AUTHOR CONTRIBUTIONS

DB and JV conceived the ideas; RB, JM, MBC and SP designed methodology; AM, SP, JV and MBC collected the data; MBC and SP analysed the data; MBC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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ABSTRACT

1. The immune response represents a suite of evolved trait that can involve energetic and evolutionary trade-offs with other energy-demanding and fitness-related processes. Here we tested the hypothesis that aquatic beetles living in inland hypersaline habitats have lower immune capacity than freshwater congeners.
2. Phenoloxidase activity, encapsulation response and antimicrobial peptide activity were compared in freshwater/hypersaline species pairs with differing osmoregulatory capacity and cuticular waterproofing properties in the genera *Nebrioporus* (Dytiscidae) and *Enochrus* (Hydrophilidae); independent evolutionary lineages that have colonized saline media separately.
3. Hypersaline species (*N. ceresyi and E. jesusarribasi*) showed significantly lower phenoloxidase activity and antimicrobial peptide responses than their freshwater relatives (*N. clarkii and E. salomonis*). Encapsulation response in freshwater species also appeared to be higher than in hypersaline relatives.
4. Our results reinforce the complex nature of immune responses and suggest that adaptation to saline environments may have involved a trade-off between osmoregulation and investment in immune defences, but they are also consistent with relaxed selection pressures on basal immune responses due to lower microbial infection load in saline habitats. In addition, the species occupying saline habitats have a more resistant cuticle that may conferring physical and biochemical protection against the entry of parasites. This may also relieve selection pressure on immune responses. Thus, if the evolution of salinity tolerance has come at the cost of reduced immune capacity, saline specialists could be particularly vulnerable to the dilution of saline waters and consequent

changes in pathogen community and load following colonization by more generalist microorganisms.

INTRODUCTION

The immune capacity to defend against parasites and pathogens represents a complex suite of coevolved traits, key to organismal fitness (Schulenburg *et al.,* 2009), which can be considered as a component of their physiological niche (Cioffi *et al.,* 2016). Despite the lack of an adaptive immune system in invertebrates, innate immunity plays a pivotal role in front line defence against a wide range of viruses, bacteria and eukaryotic parasites and pathogens (Schmid-Hempel, 2005). Notwithstanding the fact that an increasingly comprehensive understanding of insect immunity has been achieved in recent years (Adamo, 2017; Chen & Lu, 2018; Sheehan *et al.*, 2018; Cotter *et al.,* 2019; Mangahas, Murray & McCauley, 2019), many questions remain unanswered, particularly in terms of the interaction between immunity and other aspects of organismal physiology. In addition, knowledge of the comparative immunity of related insect taxa remains limited, particularly in an ecologically relevant context.

In insects, and indeed other arthropods, the cuticle is the first physical line of defence, providing the primary structural and biochemical barrier against environmental challenges, mechanical damage and penetration by potentially infectious organisms (Marmaras, Charalambidis & Zervas,1996; Noh *et al*., 2016). As a next line of defence, insects have developed responses that recognise common antigens on the cell surfaces of potential pathogens in ways that are analogous to those seen in the mammalian immune system (Iwanaga & Lee, 2005). Such basal immune functions can be separated into humoral and cellular responses. The humoral response (Sheehan *et al.,* 2018) is characterized by the phenoloxidase (PO) cascade and the action of antimicrobial peptides (AMPs). PO activity is an indicator of melanin production for cuticle pigmentation,

sclerotization, wound healing and encapsulation (Eleftherianos & Revenis, 2011). AMPs are soluble peptides and proteins targeting mainly Gram-negative and Gram-positive bacteria but also fungi and viruses (Schmid-Hempel, 2005). The cellular response is a nonspecific, multicellular defence mechanism which involves the formation of layers of haemocytes around foreign bodies (encapsulation; Strand, 2008) to limit the damage caused by the invader, and also to arrest the growth and spread of pathogens in the haemocele (Sugumaran *et al*., 2002; Moret & Moreau, 2012). As a result, haemolymph crystallizes, encapsulates and immobilises the invading organism (Sugumaran *et al*., 2002). Since immune function is highly complex, several aspects of the response must be measured if we are to understand the comparative immune strategies of different insect taxa (Moreno-García *et al*., 2013). These responses have been studied in a number of terrestrial insects (e.g. Lawniczak *et al*., 2006; Tye *et al*., 2020), but investigations of immune responses in aquatic taxa, particularly in ecologically realistic scenarios, are extremely limited.

Salinity is a major selection pressure shaping physiological mechanisms in aquatic organisms (Albers & Bradley, 2011), and a key factor determining assemblage composition in inland waters (e.g. Gutierrez-Canovas *et al*., 2013), where primarily freshwater lineages have evolved the capacity to live in saline habitats (e.g. Bradley *et al*., 2009; Albers & Bradley, 2011; Arribas *et al*., 2014; Pallarés *et al*., 2017). However, to date, whether adaptation to salinity shapes the immune responses of aquatic invertebrates has not been explicitly considered, but this may be expected due to three different, but non-mutually exclusive mechanisms: First, although osmoregulation cost has been long debated (Potts 1954; Croghan 1961; Sutcliffe 1984; Verberk, Buchwalter & Kefford 2020), both hypo-regulation and hyper-regulation may entail an increase in metabolic rate (Carbonell *et al.*, 2017; Rivera-Ingraham & Lignot, 2017; Buchwalter *et*

al., 2019; Orr & Buchwalter 2020), influencing overall energy budget as well as the allocation of energy to competing functions (Verberk Buchwalter & Kefford, 2020) in aquatic organisms. Such energy investment for coping with osmotic stress in saline waters might be to some extent compensated by the lower predation and interspecific competition occurring in these media (Southwood, 1988; Herbst, 2001; Arribas et al., 2019).

All aspects of the insect immune response are also metabolically costly, which may result in energetic trade-offs between different processes (Rantala and Roff, 2005; Adamo *et al*., 2008; Lazzaro & Little, 2009; Ardia *et al*. 2012), such as osmoregulation (Mangahas, Murray & McCauley, 2019), as well as opportunity costs (fitness loss if other tasks cannot be met) (Schmid-Hempel, 2005). There is also evidence in some cases of evolutionary trade-offs between immune function and other organismal traits, where the evolution of high performance in non-immune features is associated with a reduction in immune function (Lazzaro & Little 2009; Schmid-Hempel, 2003; Schwenke *et al.*, 2016; Rádai *et al.*, 2020), although no studies to date have explored this possibility in association with the colonization of saline waters.

Additionally, the high level of abiotic stress in inland saline waters acts not only as a filter for colonization by freshwater invertebrate lineages, but also could influence microbial survival, allowing only the presence of extremely and moderately halophilic organisms (Oren, 2011; Oueriaghli *et al*., 2018). Indeed, in hypersaline habitats, bacterial and archaeal diversity is considerably lower than in freshwaters (Ortega *et al.*, 2009; Auguet, Barberan & Casamayor 2010), comprising approximately 50% of the archaeal and less than 25% of the total inland aquatic microbial diversity (Ma and Gong, 2013), which may also be a reflect the lower diversity of hosts in such habitats. As a result, inland saline habitats seem likely to have considerably lower pathogen loads, resulting in reduced

selection pressure on the immune responses of their insect inhabitants (e.g., Céspedes *et al*., 2019).

Finally, even under high pathogen pressure and in the absence of trade-offs between immunity and osmoregulation, saline insects might show less developed basal immune responses than freshwater relatives because of their different cuticle properties. Saline water beetles have highly waterproof cuticles, with a lipid component which is more complex and diverse in composition than that of their freshwater congeners, characterized by a high abundance of branched alkanes and few unsaturated alkenes (Botella-Cruz *et al*., 2017; 2019). Such properties, along with the plasticity of epicuticular hydrocarbon composition, result in a highly resistant cuticle that has been shown to enhance the ability to cope with osmotic and hydric stress (Botella-Cruz *et al*., 2019) and to reduce water loss under desiccation (Botella-Cruz *et al*., 2021). The resistant cuticle of saline beetles may also constitute a more effective physical and biochemical barrier against the entry of parasites and pathogens compared to that of freshwater relatives, which could result in a relaxation of other immune defence mechanisms.

Here we explore possible links between immune function and habitat in two lineages of water beetle that have independently colonized inland saline waters from freshwater ancestors (Hunt *et al.*, 2007; Arribas *et al.*, 2014; Pallarés *et al.*, 2017). We hypothesised that basal immune responses may be less developed in saline beetles than freshwater ones. We compared the immune responses of freshwater/hypersaline species pairs from two genera of aquatic beetles (*Nebrioporus*, Family Dytiscidae and *Enochrus*, Family Hydrophilidae). The hypersaline species studied within each genus have very high osmoregulation capacities, maintaining osmotic gradients over 3500 mOsmol kg^{-1} (Pallarés *et al*., 2015). The strong osmotic gradients they face in hypersaline habitats almost certainly require high energetic investment in osmoregulation, something which

may be traded off against immune function (see above). These species also have much higher cuticular waterproofing than their freshwater relatives (Botella-Cruz *et al*., 2019). In light of these considerations, we hypothesised that basal immune responses may be less developed in saline beetles than their freshwater. To characterize the immune response of these species in nature, we measured three key components of immunity in laboratory mesocosms: 1) basal phenoloxidase activity, 2) encapsulation response, and 3) basal antimicrobial peptide activity.

Salinity levels in inland waters are changing rapidly worldwide, the secondary salinisation of freshwaters being a growing environmental concern (Cañedo-Argüelles et al. 2013; 2016). In contrast, many naturally saline inland waters are threatened by dilution due to agricultural intensification, which has important negative impacts on organismal performance (e.g., Velasco et al., 2019). In such a context, where salinity shifts will alter microbial community composition and therefore pathogenic pressures on aquatic insects, it is clearly important to have a better understanding of immune responses of aquatic taxa across the saline-freshwater divide.

MATERIAL AND METHODS

Study species and localities, collection and maintenance

The studied genera, *Enochrus* and *Nebrioporus*, have strictly aquatic larval and adult stages, and occur across the entire salinity gradient in the Mediterranean Region. Within each genus, two species were selected, which occupy the extremes of the salinity gradient (fresh and hypersaline waters, respectively), and have very divergent osmoregulation capacities and cuticular waterproofing (see Table 1 and Botella-Cruz *et al*., 2019). Within each genus, the hypersaline species (*N. ceresyi* and *E. jesusarribasi*) are effective hyper- and hypo-regulators, whilst the freshwater *N. clarkii* and *E. salomonis* are unable to hypo-

regulate in media that exceed their haemolymph osmotic concentration (Pallarés *et al*., 2015). In both genera, the species with higher salinity tolerance have more waterproof cuticles than the congeneric freshwater species (Botella-Cruz *et al*., 2019).

Species were collected from localities in the south and southeast of Spain (Table 1), a region with an arid Mediterranean climate where inland saline waters are very common (Williams, 1996; Millán *et al.*, 2011).

Table 1. Data on habitat preference of the study species (ranges of electrical conductivity (EC, $mScm^{-1}$) and salinity (gL^{-1}) of occupied water bodies), species osmotic capacity (mOsmol Kg^{-1}), and collection site information (latitude, longitude, elevation, mean annual temperature, flow regime, electrical conductivity (EC), salinity and osmolality (mOsmol Kg^{-1}) measured in the field.

Species	Habitat	Field range EC Salinity[a]	Maximum osmotic capacity[b]	Collection sites					
				Locality	Latitude - longitude	Elevation (m)	Flow regime	T[a] mean[c] (C°)	EC Salinity Osmolality
Nebrioporus clarkii (Wollaston, 1862)	Sub-hyposaline waters	0.11-9.00 0.10-6.3	89.94 ± 10.60	Corneros stream, Spain	37.7173 -1.9053	529	Intermittent	15.9	3 2.1 46
Nebrioporus ceresyi (Aube 1838)	Meso-Hypersaline waters	4.50-129.00 3.15-90.3	-2875.58 ± 34.86	Rambla Salada stream, Spain	38.1263 -1.1182	131	Perennial	17.6	100 70 2470
Enochrus salomonis (Sahlberg, 1900)	Subsaline waters	0.70-2.16 0.49-1.51	-10.98 ± 10.29	Pétrola wetland, Spain	38.8471 -1.5589	623	Intermittent	13.7	5.14 3.6 90
Enochrus jesusarribasi Arribas and Millán, 2013	Hypersaline waters	14.90-160.00 10.43-112	-3649.36 ± 35.27	Rambla Salada stream, Spain	38.1263 -1.1182	131	Perennial	17.6	100 70 2470

[a] Field data from Aquatic Ecology Research Group database, University of Murcia

[b] Data from Pallarés *et al*., (2015); osmotic capacity: difference between the osmotic concentration of the animal's body fluids and that of the external medium

[c] Mean annual temperature from Worldclim 2.0 database (Fick & Hijmans, 2017)

Adults of each species were collected from their natural habitats in September 2019 (Table 1) and transported to the laboratory with moist vegetation in aerated and refrigerated containers. Electrical conductivity values were measured *in situ* with a conductivity meter (HACH/Hq40d). In the laboratory, specimens of each species were placed in aerated 4 L aquaria with water at the corresponding salt concentration of their collection sites (Table 1). Saline solutions were prepared by dissolving an appropriate quantity of marine salt (Ocean Fish, Prodac) in distilled water. Specimens were kept at 20 (± 1) °C and under a 12:12 L:D cycle in temperature-controlled rooms for 5 days prior to the experiments, to allow acclimation to laboratory conditions and reduce the effects of recent thermal history, previous field conditions and transport. Food was provided *ad libitum* daily (frozen chironomid larvae for *Nebrioporus* and frozen *Ruppia* for *Enochrus* species). No deaths were recorded in culture.

Sample extraction and homogenization

Twenty specimens of each species were individually freeze-killed at -80˚C for determination of PO and AMP activity. Whole body extracts from individual beetles were obtained using 100 mM Hepes buffer (pH 6.9) at a ratio of 19 ml of buffer to 1 g of beetle mass, in a Potter homogenizer kept on ice. Extracts were centrifuged for 15 min at 21,380 g at 4°C, to obtain the supernatant, which was used for PO and AMPs measurements.

Measurement of phenoloxidase activity

In insects, phenoloxidase (PO) activity produces indole groups, which are subsequently polymerized to melanin (González-Santoyo & Córdoba-Aguilar, 2012), something which in itself is important in would healing and encapsulation, and is central to insect immune responses (Nakhleh, El Moussawi & Osta, 2017). These reactions also involve a complex suite of intermediates, including quinones, diphenols, superoxide, hydrogen peroxide, and

reactive nitrogen intermediates, which play an important role in defence against bacteria, fungi, and viruses. Phenoloxidase activity has a complex regulation, and is costly; many studies show, for example, the importance of protein in the diet for maintaining an adequate PO response (e.g. Srygley et al., 2009; González-Tokman et al., 2011).

Basal PO activity was measured as the rate of dopachrome formation from the substrate L-dopa (L-3,4-dihydroxyphenylalanine). The rate of absorbance change was measured using the difference in absorbance at two wavelengths, 475 and 600 nm, in a spectrophotometer, in order to distinguish levels of dopachrome from the further production of intermediates in melanin synthesis.

Ten microliter aliquots of extracts from each individual (n = 15) were pipetted into a flat-bottomed 96-well plate, and the reaction was initiated by adding 240 µl of substrate solution (5.2 mM L-dopa in 100 mM sodium Hepes buffer, pH 6.9) to each well, giving a final L-dopa concentration of 5 mM. Substrate was prepared fresh 5-10 min before each assay. At this concentration, L-dopa is close to its solubility limit, so the solution was centrifuged at 21,000 g for 30 s to remove any undissolved material before use. The plate was immediately transferred to a SpectraMax 190 plate reader (Molecular Devices) and the absorbance of each sample monitored at 475 and 600 nm, every 30 s for 30 min, at 25°C, using Softmax Pro 6.51 software. The instrument was set to shake the plate for 3 s between each read, to ensure wells were oxygenated, and to minimise noise associated with the formation of particles of insoluble melanin. The initial rate of production of dopachrome was estimated by fitting a quadratic equation to the first few minutes of the time courses of A_{475}-A_{600}, and using the slope of the linear component of the equation as the initial rate of change of A_{475}-A_{600}.

Quantifying antimicrobial peptide activity

AMPs are an important form of immune defence in eukaryotes, against bacterial, viral or fungal pathogens. AMPs range in size from > 20 to 100–200 amino acids and either disrupt the structure and function of microbial membranes, function as lytic enzymes, nutrient-binding proteins or target the function of specific microbial macromolecules (Ganz, 2003; Hoffmann, 2003; Bulet, Stöcklin & Menin 2004; Manniello et al., 2021). Whilst clearly diverse, most identified AMPs share common characteristics including a size of 12–50 amino acids, a net positive charge and an amphipathic structure (Sheehan et al., 2018).

AMP activity was measured using the zone of inhibition assay adapted from Moret & Schmid-Hempel (2001) and Datta *et al*. (2013). Potential AMP responses to three biologically-relevant bacteria were measured: the Gram-positive bacteria *Arthrobacter globiformis* (Conn, 1928) (NCIMB 8717) and *Bacillus thuringiensis* (Berliner, 1915) (DSM 2046), and the Gram-negative *Escherichia coli* (Migula, 1895) (K-12 strain EMG2, NCTC). Bacteria were grown for 48 h in 9 cm Petri dishes on Mueller-Hinton agar (Oxoid) at the optimal temperature for each taxon (37, 30 and 27°C, respectively). Multiple 50 mL conical flasks containing 5 mL Mueller-Hinton broth were inoculated with one colony per flask. Bacteria were again grown over 24 h in a shaking water bath at the optimal temperature for each bacterium and adjusted to 108 cells mL^{-1} using sterile Mueller-Hinton broth and measuring the optical density of the suspension by spectrophotometry. To measure AMP activity, 100 μL of these bacterial suspensions were added to 10 mL sterile Mueller-Hinton agar at 48˚C, and poured into a sterile 9 cm Petri dish. The dish was gently swirled to create a thin layer of agar and ensure even dispersal of bacteria. Eight evenly spaced 2 mm-wide wells were created in the agar, and 3 μL of insect extract sample added to each well (previously centrifuged for 2 min at 21,500 g to ensure that any residues from the extraction process were removed). Negative (3 μL

sterile Muller-Hinton broth) and positive (3 μL tetracycline) controls were added to each plate, the latter using minimum inhibition concentrations from Cioffi *et al*., 2016 (0.0075 mg mL^{-1} for *A. globiformis*; 0.0081 mg mL^{-1} for *B. thuringiensis* and 0.125 mg mL^{-1} for *E. coli*). Plates were then sealed with Parafilm to prevent desiccation and incubated over 96 h at each bacteria's optimal temperature until a bacterial lawn was visible. The number of zones of inhibition produced were recorded and the diameter of those zones measured using callipers at their widest and narrowest points.

Encapsulation ability

Encapsulation occurs when multiple haemocytes bind to relatively large invaders, including parasitoids and nematodes, that cannot be engulfed by a single cell, although this can also involve bacterial aggregations (Strand, 2008). The response is usually mediated through plasmatocytes and granulocytes, which may operate synchronously (e.g., Wiegand et al., 2000) or in sequence (e.g. (Pech and Strand, 2000)). Capsule formation concludes with apoptosis of an outer layer of granulocytes to form a basement membrane-like structure which is often then melanised via a PO cascade (e.g., Wertheim et al., 2005). This immune reaction is thought to act independently of the humoral response mounted against invading pathogens (Eleftherianos & Revenis, 2011).

The encapsulation response was measured in living specimens (n = 10) of each species by inducing a wound with a synthetic nylon monofilament of 0.0165 mm diameter (Koskimäki *et al*., 2004; Rantala and Roff, 2007; Whitehorn *et al*., 2011). Specimens were placed under a dissecting microscope (Leica MZ12, Milton Keynes, UK) at x 10-15 magnification and secured with Blu-Tack R (Bostik Ltd, Leicester, UK). The filament was inserted in the intersegmental membrane between the second and third ventrites using 7 mm titanium forceps (John Weiss, Milton Keynes, UK), and thus exposed to the circulating haemolymph for 24 h (Konig and Schmid-Hempel, 1995; Cioffi *et al*., 2016).

During this period, animals were kept individually, head-down, in perforated 0.2 mL pipette tips, to prevent removal of the filament with their hind legs. Later, the implant was carefully removed under the same dissecting microscope using fine forceps and mounted on a microscope slide, together with a clean monofilament used as a control for variation in lighting between measurements. Each monofilament was rotated and photographed twice under a Leica M205c microscope coupled to a digital camera with fixed light and contrast conditions. The area of the scab and the degree of melanisation were both assessed from digital images. The latter was measured as the mean grey scale darkness on a scale of 0–255 (encapsulation intensity; higher intensity values indicate higher encapsulation response) following Cioffi *et al.,* (2016), as this approach provided the best melanin band distinction against the background. The melanization score for each individual was calculated as the average difference of the two implant images subtracted from those of the controls (e.g. Konig and Schmid-Hempel, 1995; Gershman *et al*., 2010 and Whitehorn *et al*., 2011). Images were analysed with Image J Sofware (Image J software v. 1.48, National Institute of Health, USA).

Data analyses

PO activity and encapsulation response (scab size and brightness) were compared between species using a nested ANOVA (species nested within genus) in order to compare saline vs freshwater species within each genus. When the species term was significant, we used post-hoc tests with Bonferroni correction to check for differences in the response variables within each species pair. Normality and homoscedasticity assumptions were validated on model residuals by graphical inspection (Zuur *et al*., 2009).

AMP responses were measured as the percentage of individuals sampled that produced inhibition zones. All statistical analyses were conducted in R v.3.6.1 (R Core Team, 2020).

RESULTS

PO activity

The nested ANOVA indicated significant differences in dopachrome production rates between species pairs (Table 2) and these were significantly higher in the freshwater species, *E. salomonis* and *N. clarkii* than their corresponding saline relatives, *E. jesusarribasi* and *N. ceresyi* according to post hoc tests (Fig. 1A).

AMP activity

Inhibition zones were only produced against Gram positive bacteria; there was no effect on *E. coli* in any of the studied species. In *Enochrus*, only the freshwater species, *E. salomonis*, showed AMP production against both *A. globiformis* (91.66% of samples) and *B. thuringiensis* (58.33% of samples). In *Nebrioporus*, the AMP activity of the freshwater species (*N. clarkii*) was higher (83.33% of samples against *B. thuringiensis* and 75% of samples against *A. globiformis*) than that of the hypersaline *N. ceresyi*, which only produced AMPs against *B. thurigiensis* (16.66% of samples).

Encapsulation response

Both encapsulation measurements (intensity and scab size) differed significantly between congeneric species pairs (Table 2), but the response showed a different pattern in each genus. In *Nebrioporus*, encapsulation intensity was higher in *N. clarkii* (freshwater) than *N. ceresyi* (hypersaline), whilst in *Enochrus*, it was significantly higher in *E. jesusarribasi*

329 (hypersaline) than in *E. salomonis* (freshwater) (Fig. 1B). Regarding the size of the scab,
330 no significant differences were observed between the *Nebrioporus* species, whilst within
331 *Enochrus* the freshwater *E. salomonis* produced larger scabs than the hypersaline *E.*
332 *jesusarribasi* (Fig. 1C).

333 Table 2. Nested ANOVA results on the differences in phenoloxidase and encapsulation
334 measurements.

Immune response		Df	F value	P value
PO	Genus	1	11.52	0.0013
	Species (Genus)	2	14.86	< 0.001
	Residuals	53		
Brightness	Genus	1	50.767	< 0.001
	Species (Genus)	2	9.315	< 0.001
	Residuals	42		
Size	Genus	1	15.85	< 0.001
	Species (Genus)	2	31.67	< 0.001
	Residuals	42		

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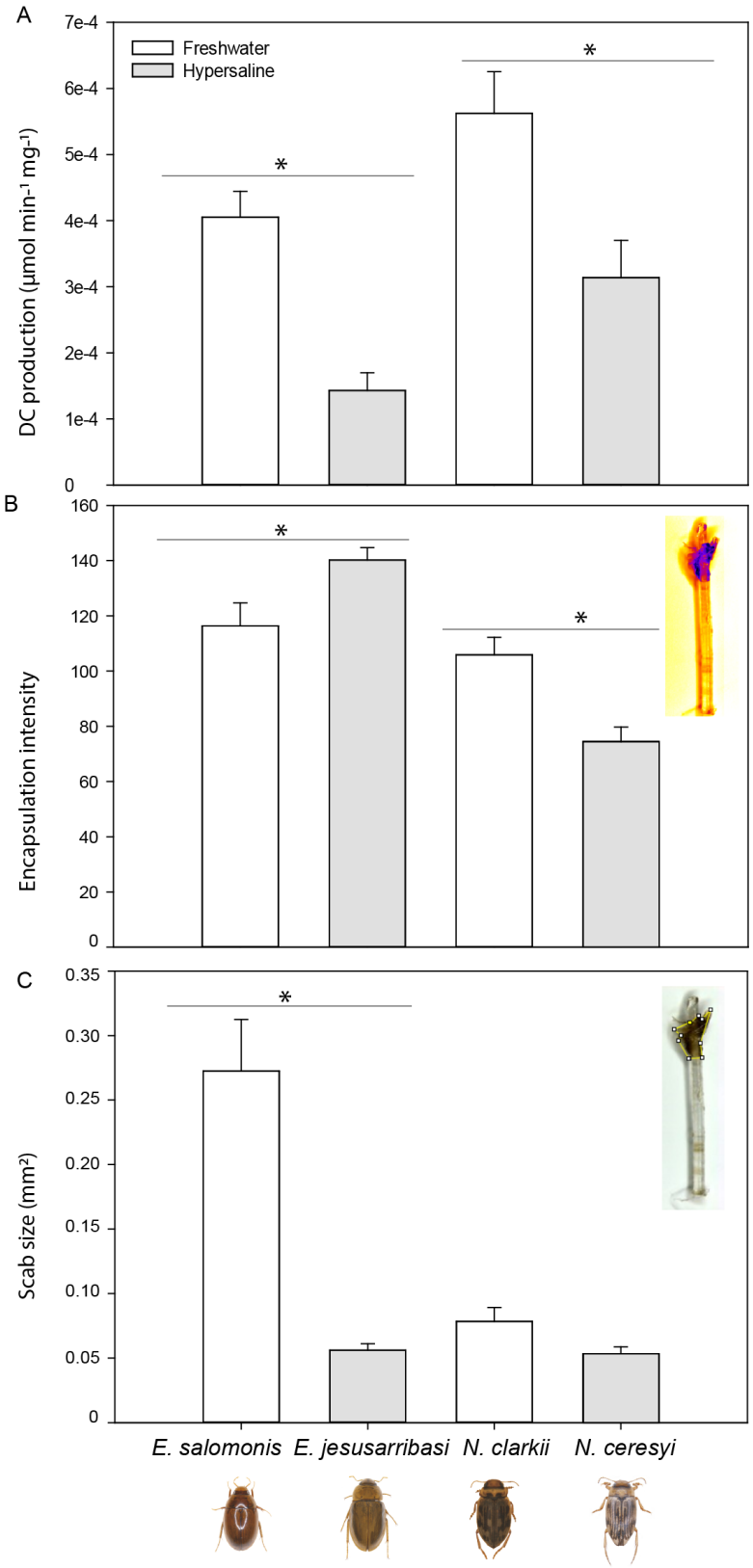

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338 Figure 1. A) Phenoloxidase activity measured as the rate of dopachrome (DC) production;

339 B) encapsulation intensity and C) encapsulation scab size. Bar-plots show mean ± S.E.

Asterisks indicate significant differences between species within each genus according to post hoc tests (Bonferroni corrected P-values, $P<0.05$). Images on B and C show encapsulated implants.

DISCUSSION

This study shows that saline species of two water beetle genera have generally lower basal immune responses than their freshwater relatives, measured at the typical salinity of their natural habitats. Our results are compatible with three different, but not mutually exclusive hypotheses. Firstly, such a pattern is in concordance with the growing evidence that immune responses entail trade-offs with other energetically costly physiological mechanisms, such as osmoregulation (Ardia *et al*., 2012; Lazzaro & Little, 2009; Adamo *et al*., 2017). Secondly, our findings are also consistent with the existence of relaxed selection pressures on basal immune responses in saline waters due to the lower microbial infection load in such habitats. Finally, the more waterproof cuticle of saline species may act as a more effective physical barrier to infection, leading to a reduction in investment in immunity in such species.

As predicted, saline species in both genera (*N. ceresyi* and *E. jesusarribasi*) showed lower basal PO activity and lower AMP responses than their freshwater relatives (*N. clarkii* and *E. salomonis*). As PO is the major component of the insect immune system (González-Santoyo & Córdoba-Aguilar, 2012), lower basal PO activity is indicative of a reduced immune response (Marmaras, Charalambidis & Zervas, 1996; Fedorka *et al.*, 2013). Céspedes *et al.*, (2019) found similar results when comparing PO activity amongst corixid species with different salinity tolerance. The higher humoral immune response shown by freshwaters species may be related to the greater bacterial richness and therefore infection risk in freshwaters (Ortega *et al.*, 2009; Auguet, Barberan & Casamayor 2010; Ma and

Gong, 2013), which would select for stronger AMP responses against pathogenic bacteria. In the diving beetle genus *Deronectes*, more southerly, range-restricted species showed stronger antibacterial activity than their more wide-ranging counterparts (Cioffi *et al.*, 2016), perhaps related to greater bacterial diversity at lower latitudes (Lear *et al.*, 2013). Haemolymph of the freshwater species studied here have AMPs against *A. globiformis* and *B. thurrigensis*, but no antibacterial effect against *E. coli*. This gram-negative bacterium is frequently used to assess antibacterial responses in insects (Arce *et al*., 2012; Murdock *et al*., 2013; Cioffi *et al*., 2016), but a similar lack of response has been observed in other water beetles (Cioffi *et al*., 2017), suggesting that this situation could be relatively widespread, at least in aquatic taxa.

The different metrics of encapsulation response examined (intensity and scab size), showed different response patterns between freshwater and saline species in each genus. In *Nebrioporus*, encapsulation intensity was lower in the hypersaline species than its freshwater relative and there were no differences in scab size. However, in *Enochrus*, the freshwater *E. salomonis* showed lower melanisation intensity but produced bigger scabs than the hypersaline *E. jesusarribasi*. In line with these results, salinity exposure significantly reduced the melanization response of dragonfly larvae (Mangahas, Murray & McCauley, 2019). Encapsulation responses have been relatively well studied in terrestrial insects (e.g., Rantala *et al.*, 2000, 2002, 2003; Koskimäki *et al.*, 2004; Rantala and Roff, 2007; Whitehorn *et al*., 2011; Mangahas, Murray & McCauley, 2019) but only a few studies have addressed such response in aquatic insects (Cioffi *et al.*, 2016; Mangahas, Murray & McCauley, 2019). Detailed understanding of the comparative biology of these responses in insects is generally lacking and the patterns observed here may reflect taxon-level differences in cuticular sclerotization processes, which would merit further research.

We suggest that either the lower metabolic cost of osmoregulation in hypo-osmotic vs. the highly hyperosmotic media which the saline species studied here inhabit, and/ or differences in cuticular waterproofing between freshwater and saline species, may underlie their different basal immune capacities. Previous studies have shown that the hypersaline species studied (*N. ceresyi* and *E. jesusarribasi*) are the most effective osmoregulators known within their genera, whilst *N. clarkii* and *E. salomonis* have no hypo-regulation capacity whatsoever (Pallarés *et al*., 2015). Since maintaining standing defences incurs significant energetic costs (Poulsen *et al*., 2002, Ardia *et al*., 2012), adaptation to saline environments in these taxa may have entailed a trade-off between physiological mechanisms to cope with osmotic stress and investment in immune defences.

Exposure to salinity has also been shown to result in reversible immunosuppression in a range of freshwater taxa, including fish (Cuesta *et al*., 2005), decapod Crustacea (Joseph and Philip, 2007) and insects such as dragonfly larvae (Mangahas, Murray & McCauley, 2019). These effects appear to be temporary, and disappear in the absence of osmotic stress (e.g. Mangahas, Murray & McCauley, 2019). However, the response could be irreversible in saline water specialists, where salinity tolerance is likely to have evolved at the expense of other traits, including immune responses (Schmid-Hempel, 2003). Whilst we have not explicitly examined the immune responses of saline water taxa across a range of salinities, we suspect that these specialists may not be capable of significantly upregulating their immunity at lower salinities. Additionally, it is important to remember that the responses we have observed are ecologically realistic, from a salinity perspective, and so reflect what would happen with these taxa in the field. Comparable data from other saline water insects are limited, but in water boatmen (Hemiptera, Corixidae), the saline water *Trichocorixa verticalis* (Fieber, 1851) exhibited a lower immune response than less

414 salt-tolerant relatives, which may also be due to evolutionary trade-offs with other
415 physiological functions (Demas *et al*., 2012; Céspedes *et al*., 2019).

416 Even if basal immune responses in saline species do not trade-off with other physiological
417 mechanisms, and if saline and freshwater species are exposed to similar infection
418 pressures in nature, the cuticle of saline water beetles might provide a relative advantage
419 in the face of infection challenges compared to their freshwater relatives. The cuticle
420 composition of the saline species studied (*E. jesusarribasi* and *N. ceresyi*), characterized
421 by a higher proportion of long chain hydrocarbons and complex methyl alkanes than their
422 freshwater relatives, may result not only in higher waterproofing and desiccation
423 resistance (Botella-Cruz *et al*., 2019, 2021), but also provide a more effective physical
424 and biochemical barrier against the entry of parasites and infectious agents (Marmaras,
425 Charalambidis & Zervas, 1996; Noh *et al*., 2016). In effect, such cuticular changes may
426 represent an exaptation against infection (Gould & Vrba, 1982). Nevertheless, the extent
427 to which such a resistant cuticle could compensate weaker basal immune responses when
428 fighting infection remains to be addressed.

429 Most saline insects appear to be generalists, in terms of their fundamental salinity niche,
430 as they show high performance and survival at both low and high salinity in the
431 laboratory, but are rarely found in fresh or-low conductivity habitats in nature, and never
432 breed in such localities (Arribas *et al*., 2019; Lambret *et al.*, 2021). Our results suggest
433 that the lower immune capacity of saline species could be one of the factors accounting
434 for their absence from freshwaters in nature (Céspedes *et al*., 2019). Changes to the
435 salinity of inland waters, currently accentuated by direct anthropogenic pressures and
436 climate change, affects aquatic organisms in several ways from increasing physiological
437 stress to causing outright mortality, all of which affect the viability of populations
438 (Cañedo-Argüelles *et al*., 2013, 2016). Furthermore, if the evolution of salinity tolerance

has come at the cost of reduced immune capacity, saline specialists could be particularly vulnerable to the dilution of saline waters and consequent changes in pathogen community and load following colonization by more generalist microorganisms (Gutierrez-Cánovas *et al*., 2009). Such issues are ongoing in many semi-arid regions, as a consequence of a combination of land-use and climatic changes (Zacharias & Zamparas, 2010; Filipe, Lawrence & Bonada, 2012; IPCC, 2021). Further studies on insect immune responses across salinity gradients, including exploration of whether the reduced immune responses observed in saline specialists are maintained in the absence of osmotic stress, or up and down-regulated as a function of stress level, would prove very illuminating.

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DATA AVAILABILITY

Data supporting this article are available online at Figshare. https://figshare.com/s/534814650391af445877

CONFLICT STATEMENT

No potential conflict of interest was reported by the authors

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