

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

The role of the cuticle in the stress responses of aquatic beetles

El papel de la cutícula de escarabajos acuáticos en respuesta al estrés

Dª. María Botella Cruz

2022

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María Botella Cruz

PhD Thesis 2022



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*Dissertation submitted by **María Botella Cruz** to obtain the PhD degree with the International Mention by the University of Murcia*

Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM)

Doctoral programme "Biodiversity and Environmental Management"

2022

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CITA RECOMENDADA: Botella-Cruz, M. 2022. Papel de la cutícula de escarabajos acuáticos en respuesta al estrés. Tesis Doctoral. Universidad de Murcia, España.

RECOMMENDED CITATION: Botella-Cruz, M. 2022. The role of the cuticle in the stress responses of aquatic beetles PhD. Dissertation. University of Murcia, Spain.

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Biosketch and Funding

BIOSKETCH

I was born in 1992 in Murcia. During my undergraduate student period, I joined the Aquatic Ecology research group as a collaborator student. I helped in several tasks that the group was carrying at that moment, being involved in laboratory experiments for physiological research with water beetles and field sampling, assisting PhD students. Then, I discovered that I really enjoyed field and lab work and decided to take the leap into the scientific world. I developed my degree project "Thermal tolerance of *Enochrus politus* (Küster, 1849)" supervised by Josefa Velasco and Andrés Millán, which was presented in the National Congress CONAMA in Madrid in 2014 and was published as my first research article in 2016 in *Limnetica*.

In 2015, I achieved a M.Sc. in Biodiversity management in Mediterranean ecosystems at the University of Murcia. I joined the Doctorate Program "Biodiversity and Environmental Management" that led to the present thesis, and I presented the master thesis entitled "Salinity acclimation enhances desiccation tolerance in the aquatic beetle *Nebrioporus baeticus* (Schaum, 1864) (Dytiscidae) but not the other way", awarded as the best poster presentation in the 9th International Congress of Comparative Physiology and Biochemistry (Cracow, 2015). As part of the practical training of the master degree, I collaborated in the Department of Zoology and Physical Anthropology of the University of Murcia, in the research group of Animal Phylogeny and Evolution, under the supervision of Professor Pilar de la Rua. In 2016, I organized and coordinated 30 weekly research talks framed in the Doctorate program. That year, I also got a M.Sc. in "Formación del profesorado de Educación Secundaria Obligatoria y Bachillerato, Formación profesional y enseñanza de idiomas" at the Miguel Hernández University (Alicante).

I started my PhD thesis in 2016, but it was not until 2018 when I got a predoctoral contract funded by the University of Murcia (FPU-2018). The thesis objectives were framed within a research project of the Aquatic Ecology group from the Spanish Ministry of Science and Education entitled "The evolution of habitat transitions in aquatic Coleoptera" (CGL2013-48950-C2-2-P). As a PhD student, I enjoyed two short visitor research fellowships (three months each), funded by the University of Murcia, which

greatly contributed to my scientific formation and the development of the present thesis. First, in 2017, I visited the Department of Animal Physiology/systems Neurobiology and Neural computation in Humboldt University of Berlin (Germany), under the supervision of the head department Dr. Stefan Hetz. I learned respirometry techniques and performed the experiments that contributed to the 3rd chapter of this thesis. Second, in 2019, I visited the University of Plymouth (UK) under the supervision of Dr. David T. Bilton (Marine Biology and Ecology Research Centre, School of Marine Science and Engineering), where I developed the immune experiments that contributed to the 4th chapter of this thesis.

I have also participated in teaching tasks (210 hours) of the Department of Ecology and Hydrology, specifically in the subjects “Ecología” and “Gestión del Medio Natural”. I have also participated in the organization committee of national (Jornadas de Doctorado II, III, IV and V) and international congresses (Limnología 2020 Congress). In 2018, as member of the Asociación de Investigadores de la Universidad de Murcia (AJIUM), I participated in the organization of bimonthly informal scientific divulgation talks in pubs, called “Investigación + divulgación + cañas (I + D + c)”. Since 2021, I collaborate in the knowledge transfer group of the University of Murcia Avances en Biodiversidad”.

The chapters of this thesis have been presented in national (XXXIII Jornadas de la Asociación Española de Entomología, 2017; and III Jornadas Doctorales de la Universidad de Murcia, 2018) and international congresses (VIII International Symposium on Molecular Insect Science, 2019; and XX Congress of the Iberian Association of Limnology, 2020), and most of them are published in SCI-indexed journals at the time of presentation of this thesis (listed in the “General Introduction” section).

I am currently collaborating in the research projects “Dynamics of macroinvertebrate metacommunities in rockpools” (CGL2017-84157-P) and “CAVEheAT. Climate change, thermal niche and conservation of subterranean biodiversity” with the PhD students Juana María Mirón Gatón, Antonio José García Meseguer and Raquel Colado Manero. As a result of this and other previous collaborations, I have led or co-authored the following papers:

- **Botella-Cruz M**, Carbonell JA, Pallarés S, Millán A, Velasco J. 2016. Plasticity of thermal limits in the aquatic saline beetle *Enochrus politus* (Küster, 1849) under changing environmental conditions. *Limnetica*, 35: 131-142. DOI: 10.23818/limn.35.11.
- Pallarés S, **Botella-Cruz M,** Arribas P, Millán A, Velasco J. 2017. Aquatic insects in a multistress environment: cross-tolerance to salinity and desiccation. *Journal of Experimental Biology*, 220(7): 1277-1286. DOI: 10.1242/jeb.152108.
- Velasco J, Gutiérrez-Cánovas C, **Botella-Cruz M**, Sánchez-Fernández D, Arribas P, Carbonell JA, Millán A, Pallarés S. 2019. Effects of salinity changes on aquatic organisms in multiple stressor context. *Philosophical Transactions of the Royal Society B*, 374: 20180011. DOI: 10.1098/rstb.2018.0011.
- Arribas P, Gutiérrez- Cánovas C, **Botella-Cruz M**, Cañedo-Argüelles M, Carbonell JA, Millán A, Pallarés S., Velasco J, Sánchez-Fernández D. 2019 Insect communities in saline waters consist of realized but not fundamental niche specialists. *Philosophical Transactions of the Royal Society B,* 374: 20180008. DOI: 10.1098/rstb.2018.0008.
- Pallarés S, Millán A, Miron-Gatón JM, Velasco J, Sánchez-Fernández D, **Botella-Cruz, M**, Abellán P. 2020. Assessing the capacity of endemic alpine water beetles to face climate change. *Insect Conservation and Diversity,* 13(3): 271-282. DOI: 10.1111/icad.12394.
- Pallarés S, Colado R, **Botella-Cruz M**, Montes A, Balart-Garcia P, Bilton DT, Millán A, Ribera I, Sánchez-Fernández D. 2020. Loss of heat acclimation capacity could leave subterranean specialists highly sensitive to climate change. *Animal Conservation,* 24(3): 482-490. DOI: 10.1111/acv.12654.
- Colado R, García-Meseguer AJ, Mirón-Gatón JM., **Botella-Cruz M,** Pallarés S, Sánchez-Fernández D. 2021. Thermal tolerance and vulnerability to climate change in subterranean species: a case study using an Iberian endemic pseudoscorpion. *Insect Conservation and Diversity*. DOI: 10.1111/icad.12541.
- Millán A, **Botella-Cruz M**, García-Meseguer AJ, Mirón-Gatón JM Velasco J. 2021. El hemíptero acuático invasor *Trichocorixa verticalis verticalis* (Fieber, 1851)

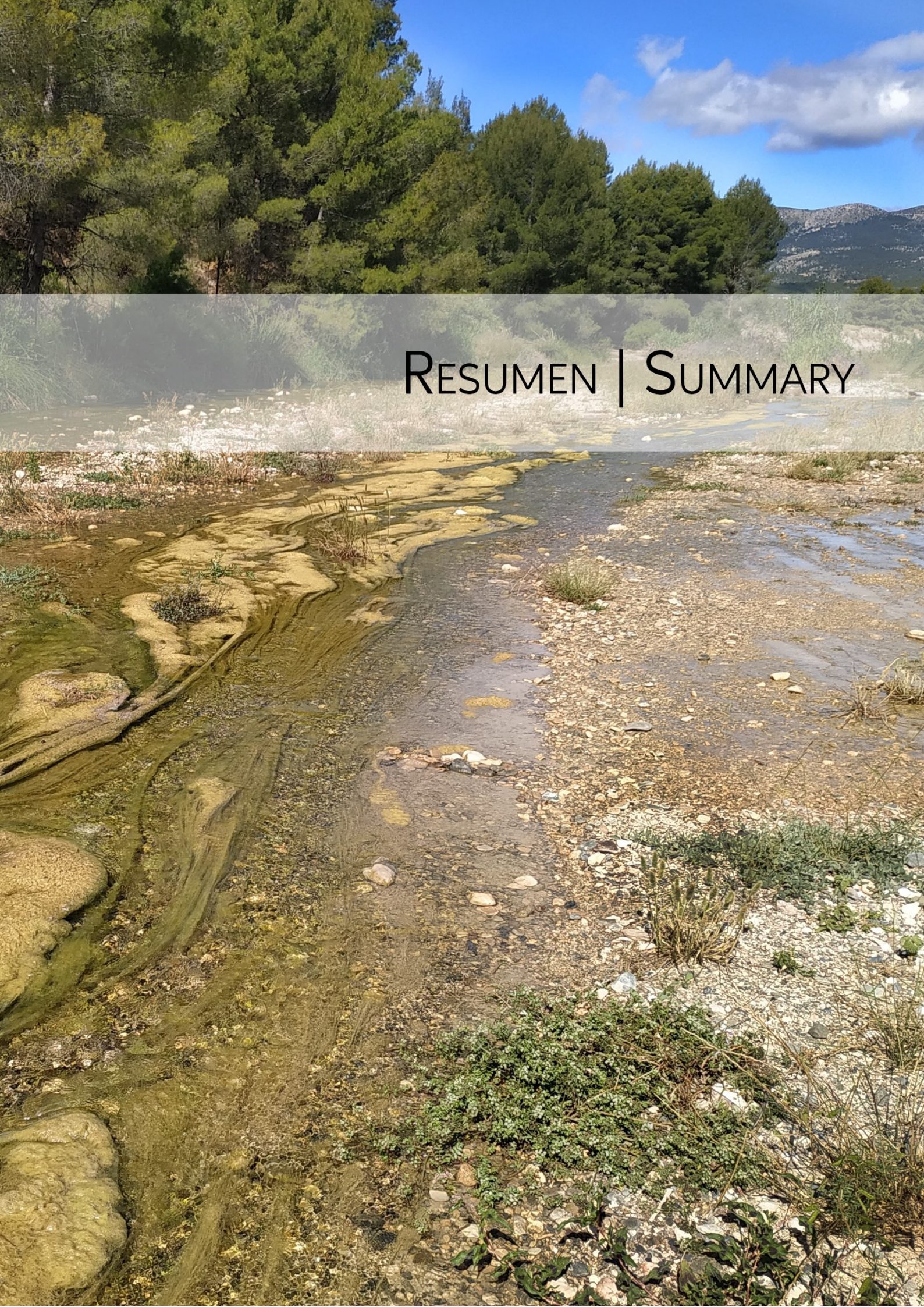
(Hemiptera: Corixidae) llega a la costa de Murcia (España). *Boletín de la Sociedad Entomológica Aragonesa,* 69: 241–242.

- Mirón-Gatón JM, **Botella-Cruz M**, García-Meseguer AJ, Millán A, Velasco J. 2022. Thermal tolerance differs among co-occurring and congener beetle species in marine coastal rockpools. *Marine Ecology Progress Series.* 681: 185-196. DOI: 10.3354/meps13916.
- Velasco J, Mirón-Gatón JM, García-Meseguer AJ, **Botella-Cruz M.** In press. Life cycle differences between two coexisting species of supratidal rockpools: *Ochthebius quadricollis* Mulsant, 1844 and *Ochthebius lejolisii* Mulsant and Rey, 1861 (Coleoptera, Hydraenidae). *Boletin de la Sociedad Española de Entomología*.

FUNDING

The work developed in this thesis, including several PhD stays in other foreign research centres, has been funded by the following institutions:

- Spanish Ministry of Economy and Competitiveness: research project no. CGL2013-48950-C2-2-P (co-funded by FEDER funds).
- FPU predoctoral contract from the University of Murcia (2018-2021).
- Aquatic Ecology research group (University of Murcia).
- Ayudas para estancias en el extranjero de jóvenes investigadores y estudiantes de doctorado en las líneas de actuación de campus Mare Nostrum 2016-2017.
- Ayudas para estancias en el extranjero para beneficiarios del programa de contratos predoctorales (FPU) de la Universidad de Murcia para 2019.



Resumen | Summary

RESUMEN

Los insectos han tenido un éxito extraordinario en la colonización de numerosos hábitats, tanto terrestres como acuáticos. Este éxito se debe en gran medida a una cutícula protectora, un fuerte material extracelular que sirve como piel y esqueleto y funciona como una primera línea física de defensa, proporcionando la principal estructura y barrera bioquímica contra estresores ambientales, daños mecánicos y penetración de organismos potencialmente infecciosos. En el contexto actual de cambio climático y aridificación global, los mecanismos de protección frente a la desecación y a potenciales agentes infecciosos son fundamentales para la supervivencia de los insectos, especialmente para especies de regiones áridas o semiáridas.

El mantenimiento del equilibrio hídrico no es solo importante para los insectos terrestres, sino también para muchos insectos acuáticos en zonas áridas, ya que también quedan periódicamente expuestos a la desecación cuando vuelan en búsqueda de cuerpos de agua permanentes durante las sequías estacionales. Los insectos de ambientes acuáticos salinos, además, tienen que hacer frente al estrés hiperosmótico en un medio con una alta salinidad (muchas veces mayor a las del mar) que se ve magnificado por la frecuente exposición a periodos de estiaje que causan una pérdida del agua, y el aumento de la concentración de sales interna.

Entre las diversas formas de minimizar esta pérdida de agua en insectos, el control de la permeabilidad de la cutícula es uno de los mecanismos más importantes. De las partes internas a las externas, la cutícula consta de dos capas principales diferentes, una procutícula más gruesa cubierta por una epicutícula delgada, ambas secretadas por células epidérmicas ubicadas justo debajo. La epicutícula está compuesta mayoritariamente por compuestos hidrofóbicos llamados hidrocarburos, cuya función principal es minimizar dicha pérdida. Los hidrocarburos cuticulares son excepcionalmente diversos e incluyen mezclas complejas de compuestos de cadena lineal (n-alcanos), alcanos ramificados y compuestos insaturados. Para la función impermeabilizante, las moléculas más viscosas o sólidas y de mayor peso molecular, con una mayor longitud de cadena como los n-alcanos, confieren a la cutícula una mayor resistencia a la pérdida de agua.

La salinidad y la aridez, dos estresores que ocurren de forma simultánea en los medios acuáticos salinos de zonas áridas y provocan efectos fisiológicos similares en los organismos, pueden haber supuesto importantes presiones evolutivas para la cutícula de los insectos acuáticos. Sin embargo, las características y la composición de la cutícula en insectos acuáticos han sido poco estudiadas en comparación con la de los homólogos terrestres. Se desconoce, por tanto, su posible papel en la evolución de la tolerancia a la salinidad y la desecación. A priori, las especies más tolerantes a la salinidad y a la desecación requerirían una cutícula más impermeable que especies menos tolerantes, lo que supondría un problema para los estadios inmaduros de dichas especies, por lo general con una cutícula más frágil y permeable que los adultos. Los estadios inmaduros pueden, por lo tanto, tener mecanismos de tolerancia menos eficientes que los adultos de la misma especie. Además, se espera que la salinidad y la desecación influyan en la capacidad de impermeabilización de la cutícula. Ambos factores podrían ejercer una presión selectiva sobre la composición de los hidrocarburos cuticulares, por lo que se podría esperar que las especies con mayor tolerancia a la salinidad y la desecación tuvieran una mayor abundancia relativa de compuestos que les confieren una mayor impermeabilidad. Se espera, por tanto, que estas especies tengan una cutícula altamente resistente que mejore su capacidad para hacer frente al estrés osmótico e hídrico. Como ya ha sido estudiado anteriormente, los mecanismos fisiológicos para hacer frente a la salinidad y la desecación son energéticamente costosos, lo cual puede ir en detrimento de la inversión energética en otros procesos, como las respuestas inmunitarias. Por lo tanto, esperamos que las respuestas inmunes estén menos desarrolladas en los escarabajos salinos en comparación con los de agua dulce debido a que la posible compensación (trade off) con el coste de la osmorregulación y también debido a la barrera física y bioquímica que proporciona la cutícula de los escarabajos salinos es más impermeable y más efectiva contra la entrada de parásitos y patógenos en comparación con la de los parientes de agua dulce.

En esta tesis se estudia la composición de hidrocarburos de la cutícula y su plasticidad como respuesta adaptativa a la salinidad y a la sequía ambiental, tanto en larvas como en adultos de coleópteros acuáticos, así como la relación de la salinidad con la respuesta inmune. Para ello, se usaron como modelo especies de dos géneros

(*Enochrus*: fam. Hydrophilidae y *Nebrioporus*: fam. Dytiscidae) que contienen especies típicas de distintos hábitats acuáticos en el área mediterránea dentro de un amplio gradiente de salinidad.

El estudio de estas respuestas nos permite identificar patrones de adaptación a la salinidad y a la aridez, contribuyendo a una mejor comprensión de los procesos evolutivos que han posibilitado la colonización y diversificación en ambientes tan extremos como los medios salinos de zonas áridas. Esta información es esencial para la conservación de estos frágiles ambientes salinos del área mediterránea, amenazados por el aumento de temperatura y creciente aridificación debido al cambio climático.

En el **capítulo 1**, titulado "*Hidrocarburos cuticulares en escarabajos acuáticos salinos*", se caracterizó el perfil de hidrocarburos cuticulares (CHC) de larvas y adultos de dos especies salinas de coleópteros acuáticos, *Nebrioporus baeticus* (Schaum, 1864) (Dytiscidae) y *Enochrus jesusarribasi* Arribas and Millán, 2013 (Hydrophilidae). Para ello se utilizó un cromatógrafo de gases acoplado a un espectrómetro de masas (GC-MS). El perfil de hidrocarburos cuticulares de adultos de ambas especies estuvo caracterizado por una alta abundancia de alcanos ramificados y baja abundancia de insaturados. Dicha composición parece ser más similar a la de algunos escarabajos terrestres del desierto (por ejemplo, Tenebrionidos) que a la de otros coleópteros acuáticos (distíscidos de agua dulce) cuyos perfiles de hidrocarburos cuticulares se han descrito en estudios previos. Los adultos de *E. jesusarribasi* presentaron compuestos de cadena más larga que los de *N. baeticus*, de acuerdo con su mayor resistencia a la salinidad y la desecación. La cutícula de las larvas se caracterizó por una menor diversidad de compuestos, carbonos con longitud de cadena más corta y una mayor proporción de hidrocarburos insaturados en comparación con la de los adultos, lo que está asociado a una mayor permeabilidad cuticular. Estos resultados sugieren que el estrés osmótico en insectos acuáticos podría ejercer una presión selectiva en el perfil de hidrocarburos cuticulares que supone la aridez para especies terrestres.

En el **capítulo 2**, titulado "*Papel de la composición de los hidrocarburos de la cutícula en la tolerancia a la salinidad de los escarabajos acuáticos*", se estudiaron los perfiles cuticulares de hidrocarburos de ocho especies pertenecientes a dos linajes de

escarabajos acuáticos (género *Nebrioporus*, familia Dytiscidae y género *Enochrus*, familia Hydrophilidae) con clara especificidad de hábitat a lo largo del gradiente salino (ambientes de agua dulce, hiposalinos, mesosalinos e hipersalinos) para: i) determinar la variación interespecífica de la composición de hidrocarburos cuticulares en relación con la tolerancia a la salinidad de las especies, y ii) explorar los ajustes plásticos en los perfiles de hidrocarburos de la cutícula en respuesta a los cambios de salinidad, a nivel intraespecífico, en las especies más tolerantes a la salinidad. Se emplearon las mismas técnicas analíticas que en el capítulo anterior. Los perfiles de hidrocarburos cuticulares fueron altamente específicos para cada especie, más complejos y diversos en composición, y caracterizados por compuestos de cadena más larga en aquellas especies con mayor tolerancia a la salinidad dentro de cada género. Estas características de los hidrocarburos cuticulares están relacionadas con una mayor impermeabilidad de la cutícula y sugieren que dicha capacidad impermeabilizante de la cutícula fue uno de los mecanismos clave en la adaptación a los medios acuáticos salinos. A nivel intraespecífico, las especies salinas también mostraron una capacidad extraordinaria para ajustar los perfiles de hidrocarburos cuticulares frente a condiciones cambiantes de salinidad en el laboratorio en un tiempo relativamente corto (7 días), lo que refleja una gran plasticidad y un alto potencial para hacer frente a las fluctuaciones ambientales diarias y estacionales en los hábitats salinos, altamente dinámicos.

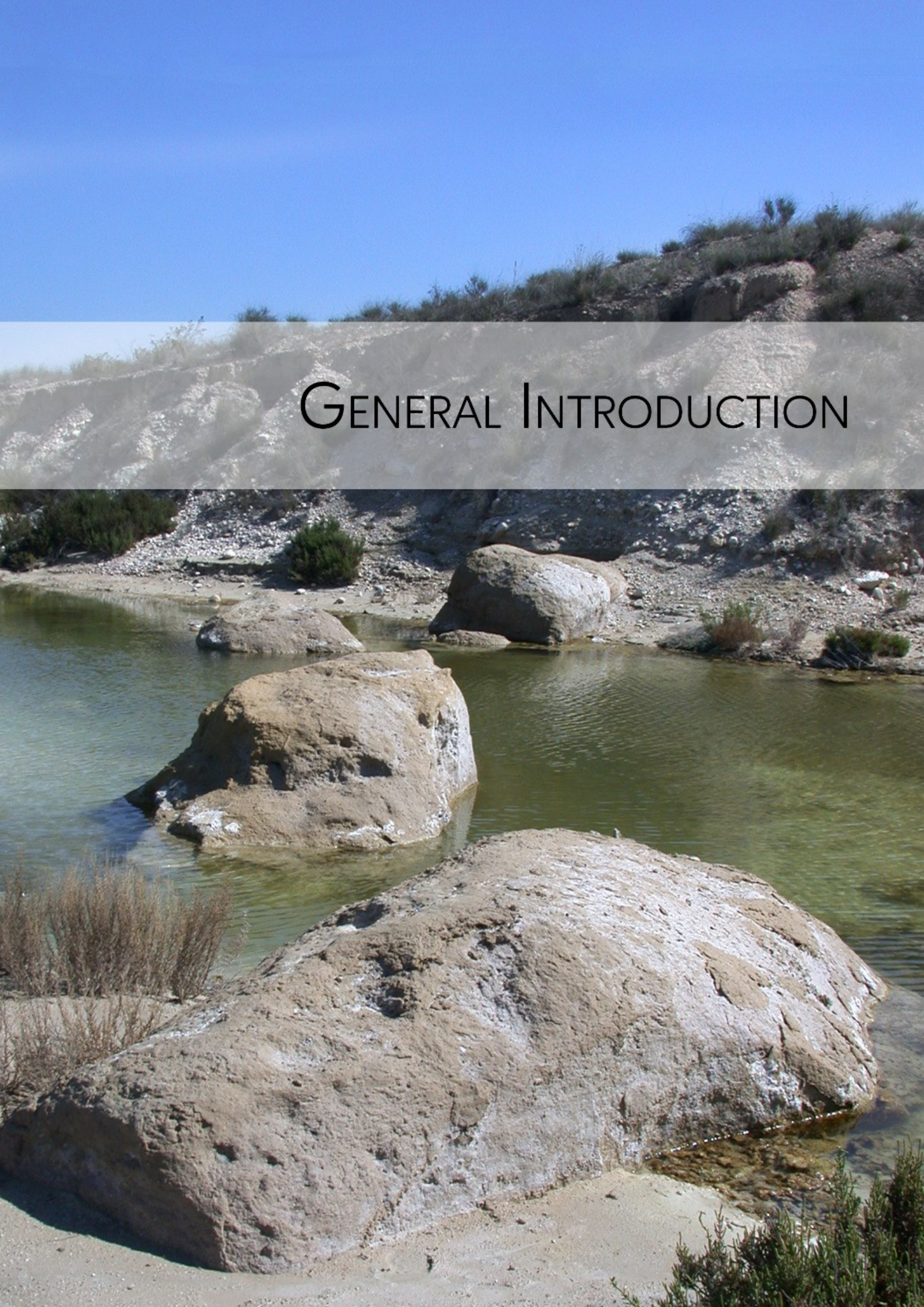
En el **capítulo 3**, titulado "*Los hidrocarburos cuticulares muestran variación plástica frente a la desecación en escarabajos acuáticos salinos*" se estudiaron las respuestas de aclimatación al estrés por desecación en adultos de dos especies endémicas de escarabajos de linajes evolutivos distantes, que viven en arroyos salinos intermitentes del área mediterránea: *Enochrus jesusarribasi* (Hydrophilidae) y *Nebrioporus baeticus* (Dytiscidae). Ejemplares adultos de ambas especies se expusieron a desecación (en condiciones no letales), y tras un periodo de recuperación en agua se expusieron a un segundo tratamiento de desecación. Otro grupo de individuos solo fue expuesto al segundo tratamiento de desecación. Se comparó la pérdida de agua cuticular calculada por gravimetría y la composición de hidrocarburos cuticulares, usando un cromatógrafo de gases acoplado a un espectrómetro de masas, entre los individuos expuestos y no expuestos a la desecación previa, con los individuos de un

grupo de control de referencia no expuestos a ningún tratamiento de desecación. *E. jesusarribasi* mostró una respuesta de aclimatación beneficiosa, ya que la tasa de pérdida de agua cuticular durante el segundo tratamiento de desecación fue menor en los individuos previamente desecados. Esa capacidad de reducir la pérdida de agua fue consistente con las diferencias observadas en la cantidad y composición de hidrocarburos cuticulares entre individuos previamente desecados y no desecados. También se observaron diferencias en el perfil de hidrocarburos cuticulares en *N. baeticus* entre los diferentes tratamientos, pero dichos cambios no se correspondieron con un mejor control de la pérdida de agua durante la segunda exposición a desecación. Por lo tanto, dicha especie tiene una menor capacidad de aclimatación para hacer frente al aumento de la aridez.

En el **capítulo 4**, titulado "*La colonización de aguas salinas se asocia con una reducción de las respuestas inmunitarias en escarabajos acuáticos*", se estudiaron las respuestas inmunitarias de pares de especies de agua dulce y salina. Se testó la hipótesis de que las especies hipersalinas tienen una capacidad inmunitaria menor que sus congéneres de agua dulce. Se midieron tres componentes clave de la función inmunitaria (actividad fenoloxidasa, capacidad de encapsulación y actividad peptídica antimicrobiana) en pares de especies de agua dulce/hipersalina de los géneros *Nebrioporus* (Dytiscidae) y *Enochrus* (Hydrophilidae), que difieren en la permeabilidad cuticular tal y como describe en el capítulo 2 de esta misma tesis. Las dos especies hipersalinas estudiadas (*N. ceresyi* (Aubé, 1838) y *E. jesusarribasi* Arribas and Millán, 2013) presentaron cantidades de péptidos antimicrobianos y fenoloxidasa significativamente más bajas que sus parientes de agua dulce (*N. clarkii* (Wollaston, 1862) y *E. salomonis* (Sahlberg)). La capacidad de encapsulación difirió entre los dos géneros. Estos resultados sugieren que la adaptación a ambientes salinos puede haber implicado un compromiso entre los mecanismos fisiológicos para hacer frente al estrés osmótico y la inversión en defensa inmune, ya que ambas funciones implican procesos energéticamente costosos. En este caso, si la evolución de la tolerancia a la salinidad se ha producido a costa de una capacidad inmunitaria reducida, los especialistas en salinidad podrían ser especialmente vulnerables a la dilución de las aguas salinas y los consiguientes cambios en la comunidad y la carga de patógenos tras la colonización por

microorganismos más generalistas. Las diferencias observadas entre especies con distinta tolerancia a la salinidad también son consistentes con una posible menor carga de infección microbiana y/o la cutícula más resistente de las especies que viven en aguas salinas.

Los resultados de esta tesis representan una importante contribución al campo de la ecofisiología en los hábitats acuáticos continentales, donde los aspectos que se estudian han permanecido en gran parte inexplorados hasta el momento. En esta tesis se demuestra que las propiedades de la cutícula (en particular, la composición de hidrocarburos) y las respuestas inmunitarias son rasgos cruciales en el contexto del estrés biótico y abiótico en los hábitats acuáticos, que han influido en gran medida en la fisiología, el comportamiento y la evolución de los insectos. Estos hallazgos ayudan a comprender cuestiones clave relacionadas con el proceso evolutivo de colonización de las aguas salinas continentales, pero también abren otras nuevas, animando a llevar a cabo más investigaciones en este campo. Creemos que los resultados aquí presentados servirán de base para construir nuevos conocimientos sobre tolerancia a la salinidad, resistencia a la desecación e inmunidad en especies acuáticas, lo cual es fundamental para el desafío que tenemos por delante de predecir los impactos del cambio climático y la aridificación sobre la biodiversidad acuática.



General Introduction

INTRODUCTION

Insects' cuticle: a key structure for their evolutionary success

Insects have been extraordinary successful in colonizing numerous habitats on earth, both terrestrial and aquatic (Bilton *et al.,* 2019). This success is in great extent due to a protective cuticle, a strong extracellular material that serves as a skin and skeleton and functions as a first physical line of defence, providing the primary structural and biochemical barrier against environmental challenges, mechanical damage, and penetration by potentially infectious organisms (Marmaras *et al*., 1996; Noh *et al*., 2016).

For small arthropods with a high surface area to volume ratio, the cuticle was essential for the colonization of terrestrial environments, where preserving water balance under the highly desiccating terrestrial conditions became a challenge (Gibbs and Rajpurohit, 2010). Their light but strong cuticles could have been one of the critical factors providing them the ability to fly and a great advantage in survival competition (Grimaldi and Engel, 2005; Bradley *et al*., 2009). Furthermore, the cuticle was essential for colonizing freshwaters from terrestrial media. In the aquatic media, the cuticle plays an essential role in maintaining the hydric and osmotic balance, which is especially challenging in saline waters (Bradley, 2009). An improved osmoregulation capacity, probably involving complex cuticular and rectal adaptations, enabled the colonization of extreme saline inland waters and promoted species diversification in some lineages of aquatic insects such us Coleoptera, Diptera and Hemiptera (Bradley, 2009; Arribas *et al*., 2014; Pallarés *et al*., 2017a; Villastrigo *et al*., 2018). Salinity tolerance acquisition was inferred to be associated with geological periods of increased aridification in some insect lineages, and may thus represent an exaptation from the adaptation to aridity favoured by a similar physiological mechanism (Pallarés *et al*., 2017b). However, the specific mechanisms behind such hypothesised exaptation are unknown and in particular, the role of the cuticle in coping with desiccation and salinity in aquatic insects has been poorly explored so far.

A better understanding of the cuticle of aquatic insects should therefore provide insights into the evolutionary processes of adaptation to saline waters. In addition,

improving our knowledge on the physiological mechanisms by which aquatic insects deal with different environmental stressors is pivotal in a context of increasing aridity and salinization of inland waters, especially in arid and semiarid regions (Cañedo-Argüelles *et al*., 2013), and an urgent issue given the worrying rates of biodiversity loss in aquatic ecosystems, which are higher than those from other ecosystems (Dudgeon, 2019; Albert *et al*., 2021).

Structure of insects' cuticle

The insect cuticle is a remarkable layer of biomaterial primarily formed from structural cuticle proteins and the linear polysaccharide chitin. Its structure is complex and varies among different taxonomic groups as well as through the ontogenetic development of an individual (e.g., larva versus adult). The cuticle ranges from thin and flexible in larvae, to rigid and armour-like in most adults (Crowson, 1981).

From internal to external parts, the cuticle consists in two different main layers, a thicker procuticle overlaid with a thin epicuticle, both secreted by epidermic cells located just below (Figure 1). The epidermis and cuticle together form the integument, the outer covering of the living tissues of the insect. The procuticle ranges between 10 µm to 5 mm of thickness and is formed mainly by chitin complexed with proteins. It differentiates into a thick and flexible endocuticle covered by a thinner exocuticle resulting from the sclerotization of proteins after moulting. The exocuticle is very variable in thickness, thin in soft bodied larvae and in the intersegmental membranes and very thick and rigid in hard sclerotised parts. The procuticle is the bulk of the cuticle, providing resistance to mechanical loads and a barrier to infection, mainly because of its thickness and architecture (Crowson, 1981; Moret and Morau, 2012).

In the outermost part, the epicuticle ranges from 1 to 4 µm in thickness and it is formed by four layers (Figure 1): an inner epicuticle and an outer epicuticle, a lipid or wax layer and a variable discrete cement layer. The inner epicuticle is formed by sclerotized structural lipids (cuticulin), while the rest of the lipids covering the outer epicuticle are mostly cuticular hydrocarbons (CHC). CHC are the principal hydrophobic compounds of this layer, representing in some cases more than 90% (Hadley, 1977; Malinski *et al*., 1986). They are exceptionally diverse and include complex mixtures of

straight-chain compounds (n-alkanes), branched alkanes and unsaturated compounds (Lockey, 1988).

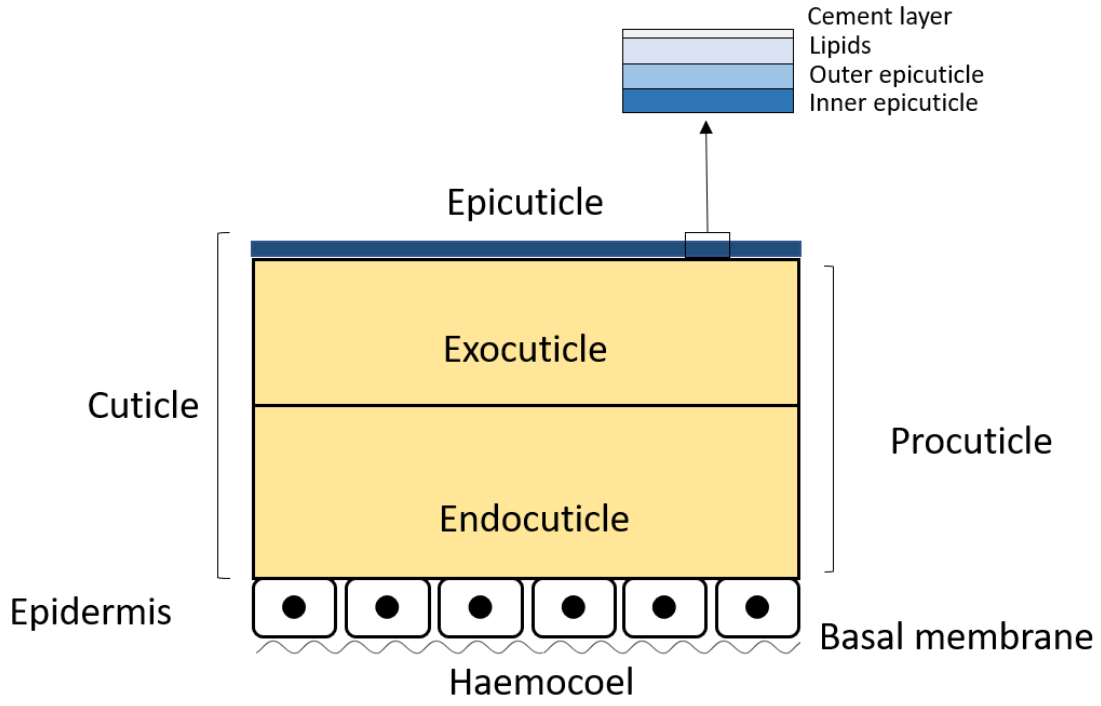


Figure 1. Schematic figure of the different structural layers of insect cuticle.

Functions of insects' cuticle

The cuticle provides the supportive structure, and defines the size and shape of the insect body, being also the space for muscle attachment. This covering protects organs against harmful damage and creates a physical and chemical barrier between the insect and the environment. Its main functions are to maintain the hydric and ionic balance in insect body and to prevent the entry of harmful microbes and chemicals. However, it is also involved other number of processes, such as chemical communication (e.g., Howard and Blomquist, 2005; Billeter *et al*., 2009; Savković *et al.,* 2012; Pattanayak *et al*., 2014; Zhang *et al*., 2014), sexual recognition (Carlson *et al*., 1971; Jacob and Hansen, 1986) and nestmates and castes identification in social insects (Menzel *et al*., 2019).

Waterproofing capacity

The cuticle acts as an impermeable barrier preventing water and ions from freely moving outward. Since the cuticle is the main via for water loss (e.g., usually accounting around 80-90% of the total water loss for most insects), a relatively water-impermeable cuticle is a major component of insect desiccation resistance (Hadley, 1994; Chown and Nicolson, 2004; Benoit, 2010; Gibbs and Rajpurohit, 2010). Its waterproofing capacity is related with the cuticle thickness and composition (Crowson, 1981; Reidenbach *et al*., 2014). Hydrocarbon composition is vital in preventing water loss and dehydration from the insects' body to the surrounding environment by controlling the transcuticular water flux, a function derived from their hydrophobicity (Benoit, 2010; Gibbs and Rajpurohit, 2010). Although cuticular hydrocarbons account for only about 0.1% of total mass of an insect, their presence can reduce the insects' permeability to water by up to 1300% (Edney, 1977). Each CHCs class serves to different functions depending on biophysical properties such as melting temperature, viscosity and molecular weight. For the waterproofing function, the molecules most viscous or solid and with higher molecular weight, such as n-alkanes, confer the cuticle a higher resistance to water loss (Chung and Carroll, 2015), while more fluid and volatile ones are better for communication purposes (Gibbs and Pomonis, 1995; Sprenger *et al*., 2018; Menzel *et al*., 2019). The chain length (size) and the saturation (absence of double bonds) have also a large impact on the melting point of the compounds and then in determining their major function. Melting points increase with size and decrease with double bonds, i.e., a high percentage of long chain hydrocarbons has been shown to confer impermeability to the cuticle in arthropods (e.g., Hadley, 1977; Toolson and Hadley, 1977; Lockey, 1980; Gibbs and Pomonis, 1995; Gibbs *et al.*, 2003; Gibbs and Rajpurohit, 2010). However, introducing a double bond into the molecule can drastically decrease the melting point and therefore, the waterproofing function (Gibbs and Pomonis, 1995).

Protection from infections

Insects are continuously exposed to a wide variety of viruses, bacteria and eukaryotic parasites and pathogens (Schmid-Hempel, 2005). The cuticular structure serves as a physical shield that protects insects against pathogens, and its chemical composition has

also shown to harbour antibacterial and cytolytic activity, which protects them from potential infections (Moret and Morau, 2012). However, if the external agent breaks the cuticle and a wound occurs, the activation of the immune system is necessary.

Despite the lack of an adaptive immune system in invertebrates, innate immunity plays a pivotal role in front line defence against pathogens (Schmid-Hempel, 2005). When the cuticle barrier is breached, the innate immune system, including humoral (Phenoloxidase cascade -PO-, and antimicrobial peptides -AMPs-) and cellular responses (e.g., encapsulation of the invader), are activated to prevent pathogen multiplication and spread inside the infected insect (Marmaras *et al*., 1996; Sugumaran *et al*., 2002; Schmid-Hempel, 2005; Moret and Moreau, 2012).

Evolution and plasticity of CHC profiles

Cuticular profiles are shaped by phylogenetic constraints; for example, CHC in Coleoptera display common features at superfamily or family levels, reflecting evolutionary tendencies (Jacob and Hansen, 1986). However, CHC composition is also an evolutionary labile, adaptive character that shows an important variation between species and populations reflecting local adaptation (Gibbs *et al*., 1997; Kwan and Rundle, 2010). For example, adaptation to aridity in desert Tenebrionids involves an exceptionally thick and impermeable epicuticular wax layer characterized by high proportions of long chain lengths of branched alkanes (Lockey, 1980; Crowson, 1981). On the contrary, ant species living in habitats with higher precipitation consistently show higher proportions of unsaturated CHC (van Wilgenburg *et al*., 2011; Menzel *et al*., 2017). In *Drosophila melanogaster,* CHC profiles varied and chain length increased in populations from warmer environments (Rajpurohit *et al*., 2017).

Furthermore, part of the intraspecific variability of insects cuticular composition is attributed to its physiological plasticity in response to daily or seasonal changes (Howard and Blomquist, 1982; Gibbs and Rajpurohit, 2010). Such changes can happen within short time periods, from hours to days, and may enhance survival under stressful conditions such as desiccation exposure (Stinziano *et al*., 2015; Menzel *et al.,* 2018; Sprenger *et al*., 2018). Many studies have reported differences in CHC profiles within species depending on sex (e.g., Beran *et al*., 2014; Pattanayak *et al*., 2014),

developmental stage (e.g., Bagnères *et al*., 1996), the feeding state of individuals (e.g., Jacob and Hansen, 1986; Alabi *et al*., 2011), environmental conditions (Toolson, 1982) or rearing temperature (e.g., Rouault *et al*., 2004; Rajpurohit *et al*., 2017). Despite not having much knowledge to date, some studies have also reported plastic changes in cuticle permeability in relation with water salinity (Stobbart, 1971; Nicholson and Leader, 1974) and other studies indirectly support such an association between cuticle properties of aquatic insects and salinity, as those showing different water loss rates (Cannings, 1981; Pallarés *et al*., 2016, 2017a) or changes in the expression of cuticle genes (Uyhelji *et al*., 2016) related with salinity conditions.

In semiarid regions, salinity and desiccation of aquatic media can be important drivers of the evolution of physiological mechanisms, favouring selection of CHC profiles that confer high waterproofing capacity. In several water beetle lineages (e.g., within the families Hydrophilidae and Dytiscidae), congeneric species show markedly different desiccation resistance and osmoregulatory abilities in relation to habitat occupation along the salinity gradient (Pallarés *et al*., 2015, 2017a), which might be in part related to adaptive changes (i.e., those arising by genetic changes over multiple generations) in CHC profiles. It has been suggested that tolerance to salinity and desiccation are physiologically linked, as mechanisms to avoid water loss are involved in the response to both factors. Indeed, cross-tolerance to both stressors has been shown in some insects (Elnitsky *et al*., 2009; Pallarés *et al*., 2017b). Evolutionary studies have proposed that the acquisition of the hyporegulation ability (i.e., the capacity to osmoregulate in hyperosmotic media) and the colonization of saline waters in beetle lineages was facilitated by ancestral mechanisms to cope with desiccation (Arribas *et al*., 2014; Pallarés *et al*., 2017a), likely conserved from terrestrial ancestors. Thus, the knowledge of aquatic insects CHC composition and its plasticity in response to desiccation and salinity may provide important information on many aspects of aquatic insect's physiology, ecology and evolution.

Coping with salinity and desiccation requires an important energetic investment (Rivera-Ingraham and Lignot, 2017), which may result in energetic trade-offs between different processes such as physiological adaptations and immune responses (Rantala, 2005; Adamo *et al*., 2008; Lazzaro and Little, 2009; Ardia *et al*., 2012). There is evidence

of evolutionary trade-offs between the immune function and other organismal traits, where the evolution of high performance in non-immune features (e.g., growth, survival or reproductive effort) is associated with a reduction in the immune function (Zuk and Stoehr, 2002; Schmid-Hempel, 2003; Lazzaro and Little, 2009). 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 *et al*., 2019). Also, a recent study showed that the saline water species *Trichocorixa verticalis* (Fieber, 1851) (Hemiptera, Corixidae), exhibited lower immune response than less salt-tolerant relatives, due to a possible trade-off between osmoregulation and investment in immune defences (Cespedes *et al*., 2019).

In aquatic habitats, both abiotic stress (e.g., salinity and desiccation) and biotic stress (pathogen load) may constitute important selective pressures on cuticle properties related with its waterproofing capacity and physical resistance against infectious agents. However, no studies to date have explored the role of abiotic stress as a selective pressure on the cuticle of aquatic beetles, and most studies on either the waterproofing function (e.g., Blomquist and Jackson, 1979; Lockey, 1980; Alabi *et al*., 2011; Pattanayak *et al*., 2014; Stinziano *et al*., 2015; Rajpurohit *et al*., 2017) or immune responses (e.g., Lawniczak *et al*., 2007; Tye *et al*., 2020) have been carried out on terrestrial insects. Among aquatic ones, CHC profiles have only been described for some freshwater dytiscids (Jacob and Hansen, 1986; Alarie *et al.*, 1998), and studies on immune function are extremely limited to date. Thus, the contribution of CHC in driving differences in stress tolerance needs to be further investigated, as well as the study on immune responses in aquatic taxa. This information is especially relevant in the context of global change and ongoing aridification in Mediterranean-climate regions.

Extreme environments

The semiarid and arid areas in the Mediterranean region hold a wide diversity of inland aquatic ecosystems, including saline streams, ponds and wetlands, encompassing a wide salinity gradient (Figure 2). These ecosystems are very rare in a European or global context and perhaps for this reason, they have been traditionally less studied than

freshwater ones. This is a miss opportunity, as inland saline waters provide an ideal system for studies related with insect's life adaptations, for different reasons: i) their particular physicochemical and biological characteristics (Herbst, 2001; Millán *et al*., 2011), ii) they represent a refuge of rare and/or endemic species compared to the generally more diverse freshwater environments (Millán *et al.,* 2011) and iii) life in extreme environments is associated with the evolution of novel traits that improve species' ability to cope with stress.

These habitats are characterized by a high degree of natural stress, with variable and extreme environmental conditions, where high temperature, high levels of salinity (sometimes even six times up to that of the sea) and desiccation are frequently co-occurring stressors. In summer, the salinity levels usually increase due to evaporation or flow cessation, flow connectivity is disrupted, and some water bodies may remain completely dry for long periods (Velasco *et al*., 2006). In addition, these naturally stressful conditions are accentuated by human pressures and climate change. Currently, many freshwater bodies are becoming salinized by anthropogenic impacts (e.g., salt mines, de-icing salt or water abstraction), whereas some naturally saline waters are being diluted by human activities (e.g., agricultural drainage), which in combination with other stressors, has significant effects on aquatic communities (Velasco *et al*., 2019). Besides, climatic models predict an increase in temperature and a decrease in rainfall in Mediterranean regions, and consequently dry events will become more intense, prolonged and unpredictable (IPCC, 2021), with the consequent reduction in available aquatic habitats and an increase in their salinity levels.

Such a high level of abiotic stress in inland saline waters acts not only as an environmental filter for colonization by invertebrate lineages, but also influences microbial survival, allowing only the presence of extremely and moderately halophilic organisms (Oueriaghli *et al*., 2018; Oren, 2011). As a result, saline waters are presumed to have considerably lower pathogen loads than freshwater ones, likely resulting in a reduced selection pressure on the immune responses of their insect inhabitants (e.g., Céspedes *et al*., 2019). Therefore, any change in natural salinity levels accentuated by human pressures could directly affect the aquatic fauna: an increment in salt levels (salinization) would increase the osmotic stress and a reduction of salinity (dilution)

could increase biotic stress, with the consequent proliferation of pathogenic microorganisms more generalists in terms of salinity tolerance.



Figure 2. Diversity of inland aquatic habitats in the Mediterranean region. From the upper left to the bottom right corner: *Río Corneros* (Murcia, Spain), *Rambla Salada*, (Murcia, Spain), *Río Chícamo* (Murcia, Spain) and *Rambla de Minglanilla* (Cuenca, Spain). Author: Aquatic Ecology Group.

Water beetles as study group

Water beetles are one of the most diverse insect orders in inland waters, having colonized water at least 20 times from separate terrestrial ancestors (Jäch and Balke, 2008). They are distributed in all continents except Antarctica (Bilton *et al*., 2019), and they represent a great contribution to aquatic biodiversity in the Mediterranean region, which hosts a large proportion of endemic beetle species (Sánchez-Fernández *et al*., 2008; Millán *et al*., 2014).

Specific cuticular modifications such as the appearance of elytra is considered to be one of the key innovations that has facilitated their unparalleled adaptive radiation,

becoming the species richest insect order (Ribera and Beutel, 2014). Adult beetles are characterized by a strongly sclerotized body with the forewings hardened into elytra, a greatly thickened and tanned dorsal cuticle secreted by the forewing epidermis, which serves to protect the more delicate hind wings (Noh *et al*., 2016). Indeed, their sturdy exoskeleton, in connection with a rather well sealed subelytral space that may contain an air reservoir, was likely a major preadaptation for the invasion of aquatic media (Jäch and Balke, 2008). This capability to create a gas store under their elytra thanks to the hydrophobicity of the cuticle enables them to breath beneath the water (Edney *et al*., 1977).

Aquatic beetles have also colonized saline waters, where the families Hydrophilidae, Hydraenidae and Dytiscidae are the most representative. Therefore, they provide an interesting study group in the fields of ecophysiology and evolutionary biology, because they have developed a variety of physiological, morphological and behavioural adaptations to successfully withstand the thermal, ionic and osmotic stress associated with the extreme environmental conditions of these habitats (e.g., Sánchez-Fernández *et al*., 2010; Pallarés *et al*., 2012; Céspedes *et al*., 2013; Pallarés *et al*., 2016). Indeed, in multiple water beetle lineages, congeneric species show markedly different osmoregulatory capacity and desiccation resistance (Pallarés *et al*., 2015, 2017a). However, the necessary cuticular adaptations to live in saline waters, as well as many aspects of the cuticle in such taxa have received little attention to date (Alarie *et al*., 1998).

Many water beetles are relatively locally abundant (especially saline ones) and adults are easy to collect and maintain under laboratory conditions being suitable target organisms for experimental work. Beetles are a major component of the aquatic macroinvertebrate community, and given their value as bioindicators (Bilton *et al.,* 2006; Sánchez-Fernandez *et al*., 2006), studying their responses to stress is essential to understand the effects of climate change in arid and saline aquatic environments.

The present thesis focuses on two of the richest water beetle families that have colonized the saline media (Hydrophilidae and Dytiscidae, Figure 3), as models to explore the role of the cuticle in salinity and desiccation tolerance, and immune

responses of aquatic beetles, from an ecological and evolutionary perspective. A more mechanistic and comprehensive understanding of the physiological adaptations by which aquatic beetles cope with multiple stressors is necessary given the significant knowledge gap on ecophysiology for these non-model organisms. Such knowledge would provide not only significant insights into the process of adaptation to saline waters, but also basic information to forecast the effects of climate change and the ongoing aridification in arid and semiarid areas, on aquatic biodiversity.



Figure 3. Upper left: *Enochrus jesusarribasi* (Hydrophilidae), upper right: *Enochrus politus* (Hydrophilidae), lower left: *Nebrioporus ceresyi* (Dytiscidae), lower right *Nebrioporus baeticus* (Dytiscidae). Photos: Antonio José García Meseguer.

Objectives and thesis outline

The present thesis focuses on i) the waterproofing role of the cuticle in aquatic beetles, in terms of composition and plasticity, as an adaptive response to cope with multiple natural stressors such as salinity and desiccation, and ii) the relationship between salinity and immune capacity. To explore such responses, our target organisms were species with contrasting habitat preferences along a salinity gradient, from two genera

of water beetles (*Enochrus* and *Nebrioporus*) belonging to different families (Hydrophilidae and Dytiscidae, respectively) and suborders (Polyphaga and Adephaga, respectively). The species studied represent independent evolutionary lineages that have separately colonized saline media. The thesis is arranged in four chapters (Figure 4) that correspond to the following specific objectives:

Chapter 1. *Cuticle hydrocarbons in saline aquatic beetles*

To determine if CHC profiles of saline water species from distant evolutionary lineages show a similar pattern of intraspecific variation (throughout ontogeny and between sexes) as an adaptation to saline media.

Chapter 2. *Role of cuticle hydrocarbons composition in the salinity tolerance of aquatic beetles*

To determine the interspecific variation of CHC composition of representative species of the fresh-hypersaline gradient in relation to their salinity tolerance, and to explore the intraspecific variation and salinity-induced plastic responses of CHC composition in salinity-tolerant species.

Chapter 3. *Cuticle hydrocarbons show plastic variation under desiccation in saline aquatic beetles*

To determine the plastic variation in the waterproofing capacity (cuticular water loss rate) and CHC profiles in response to desiccation stress in salinity-tolerant species.

Chapter 4*. The colonization of saline waters is associated with lowered immune responses in aquatic beetles*

To explore the immune capacity in freshwater/hypersaline species pairs with differing osmoregulatory capacity and cuticular waterproofing properties.

In relation with these objectives, we addressed the following hypotheses:

1. Adults have a higher waterproofing cuticle compared with larvae. Adults' cuticles are usually thicker and more chitinized than the cuticle of the larvae; therefore, adults are expected to have a higher proportion of those compounds that are more effective at preventing water loss than larvae.

2. Salinity and desiccation influence the cuticle' waterproofing capacity. Both factors could exert a selective pressure on cuticular hydrocarbon composition so that species with higher tolerance to salinity and desiccation could be expected to have a higher relative abundance of compounds that confer higher impermeability. These species are expected to have highly resistant cuticle that enhances their ability to cope with osmotic and hydric stress. Acclimation of individuals from low salinity localities at high salinities in the laboratory would induce changes in CHC composition resulting in similar CHC profiles than those of populations from high salinity sites.

3. Cuticle composition also show plasticity in response to salinity and desiccation in the studied genera. Exposure to non-lethal desiccation conditions would enhance desiccation resistance under a subsequent desiccation stress.

4. Immune responses are less developed in saline beetles compared to freshwater ones because the possible trade-off with the osmoregulation cost and 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.

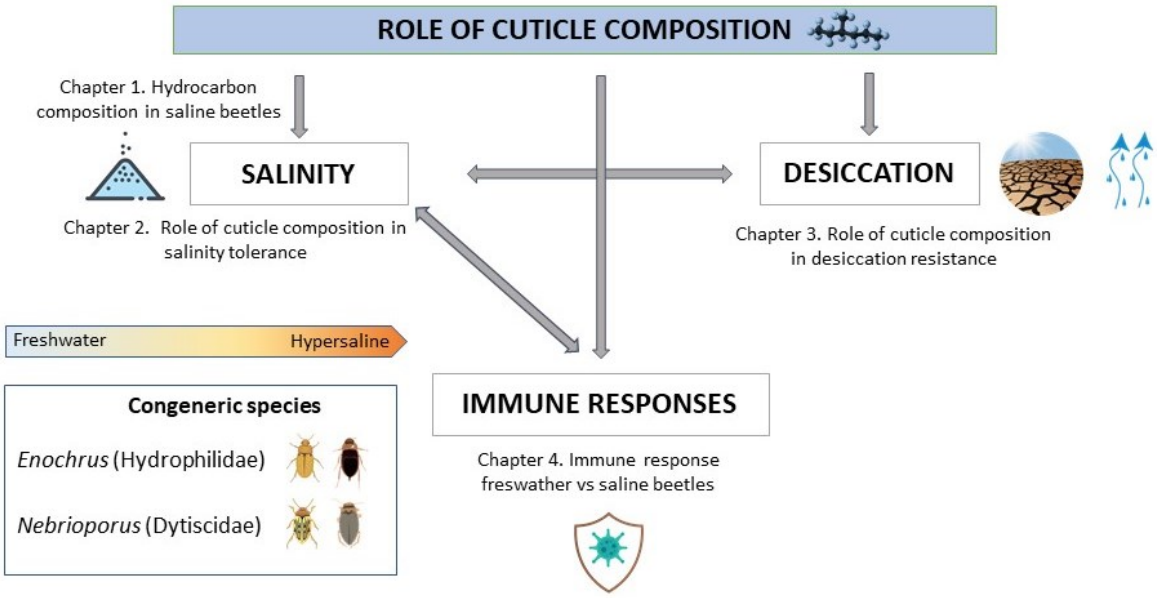


Figure 4. Scheme of the methodological approach and thesis structure.

Chapters 1, 2 and 3 are published in international peer-reviewed journals indexed in SCI, while Chapter 4 is under review for publication in *Freshwater Biology* at the moment of completion of this thesis. The four articles on which this thesis is based are:

- **Chapter 1**. Botella-Cruz M, Villastrigo A, Pallarés S, López-Gallego E, Millán A, Velasco J. 2017. Cuticle hydrocarbons in saline aquatic beetles. *PeerJ*, 5: e3562. IF: 2.118, Q1 (Agricultural and Biological Sciences).
- **Chapter 2**. Botella-Cruz M, Pallarés S, Millán A, Velasco J. 2019. Role of cuticle hydrocarbons composition in the salinity tolerance of aquatic beetles. *Journal of Insect Physiology*, 117: 103899. IF: 2.246, Q1 (Entomology).
- **Chapter 3**. Botella-Cruz M, Velasco J, Millán A, Hetz S, Pallarés S. 2021. Cuticle hydrocarbons show plastic variation under desiccation in saline aquatic beetles. *Insects*, 12: 1–14. IF: 3.046 Q1 (Entomology).
- **Chapter 4**. Botella-Cruz M, Pallarés S, Velasco J, Moody J, Billington R, Millán A, Bilton DT. 2022. The colonization of saline waters is associated with lowered immune responses in aquatic beetles. *Freshwater Biology* (Under second review).

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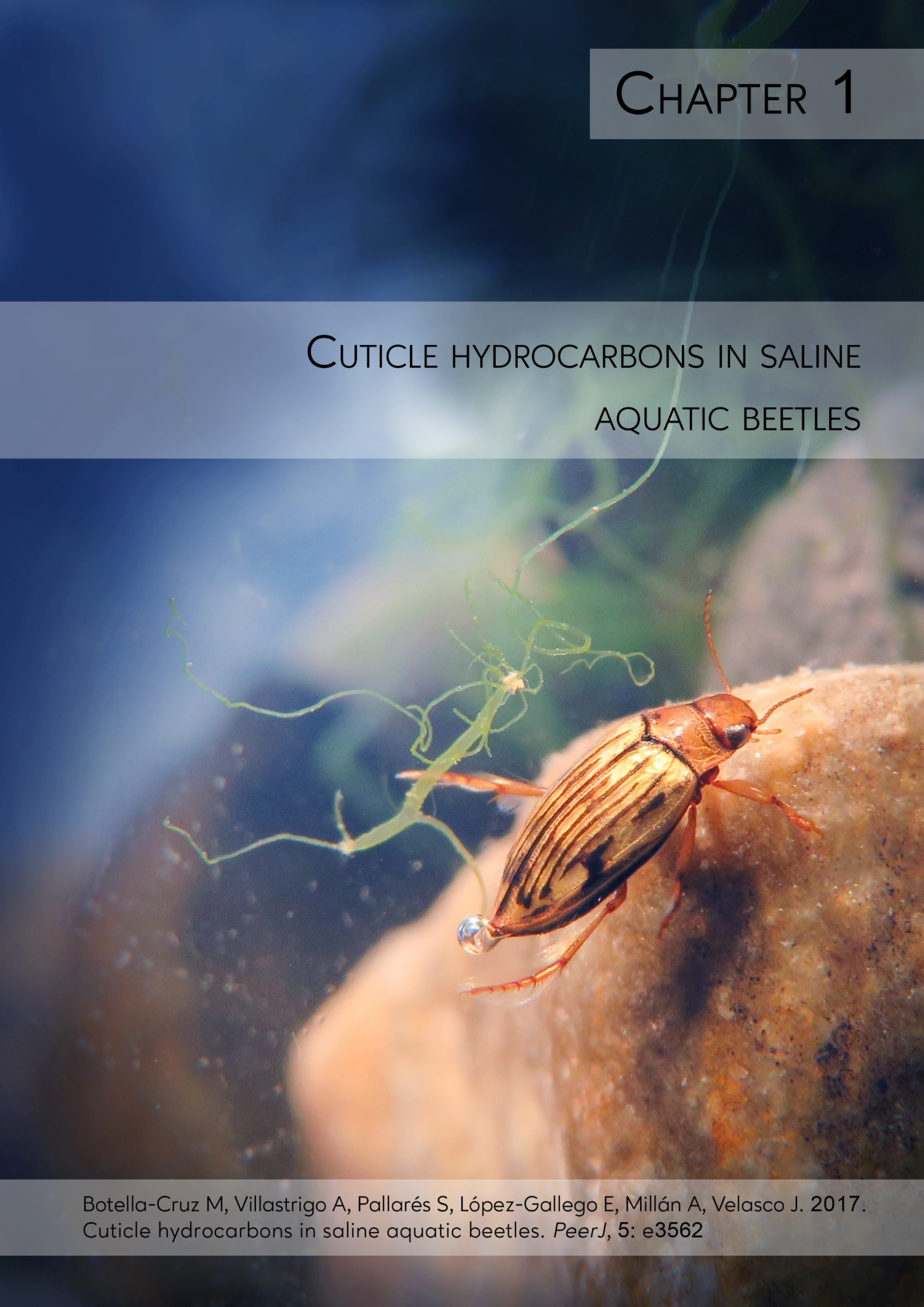
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Published Information

Chapter 1

Cuticle hydrocarbons in saline aquatic beetles



Botella-Cruz M, Villastrigo A, Pallarés S, López-Gallego E, Millán A, Velasco J. 2017. Cuticle hydrocarbons in saline aquatic beetles. *PeerJ*, 5: e3562

CHAPTER 1

Cuticle hydrocarbons in saline aquatic beetles

Journal: PeerJ

Abstract: Hydrocarbons are the principal component of insect cuticle and play an important role in maintaining water balance. Cuticular impermeability could be an adaptative response to salinity and desiccation in aquatic insects; however, cuticular hydrocarbons have been poorly explored in this group and there are no previous data on saline species. We characterized cuticular hydrocarbons of adults and larvae of two saline aquatic beetles, namely *Nebrioporus baeticus* (Dytiscidae) and *Enochrus jesusarribasi* (Hydrophilidae), using a gas chromatograph coupled to a mass spectrometer. The CHC profile of adults of both species, characterized by a high abundance of branched alkanes and low of unsaturated alkenes, seems to be more similar to that of some terrestrial beetles (e.g., desert Tenebrionidae) compared with other aquatic Coleoptera (freshwater Dytiscidae). Adults of E. jesusarribasi had longer chain compounds than *N. baeticus,* in agreement with their higher resistance to salinity and desiccation. The more permeable cuticle of larvae was characterized by a lower diversity in compounds, shorter carbon chain length and a higher proportion of unsaturated hydrocarbons compared with that of the adults. These results suggest that osmotic stress on aquatic insects could exert a selection pressure on CHC profile similar to aridity in terrestrial species.

URL: https://peerj.com/articles/3562/

Chapter 2

Role of cuticle hydrocarbons composition in the salinity tolerance of aquatic beetles



Botella-Cruz M, Pallarés S, Millán A, Velasco J. 2019. Role of cuticle hydrocarbons composition in the salinity tolerance of aquatic beetles. *Journal of Insect Physiology*, 117: 103899

CHAPTER 2

Role of cuticle hydrocarbons composition in the salinity tolerance of aquatic beetles

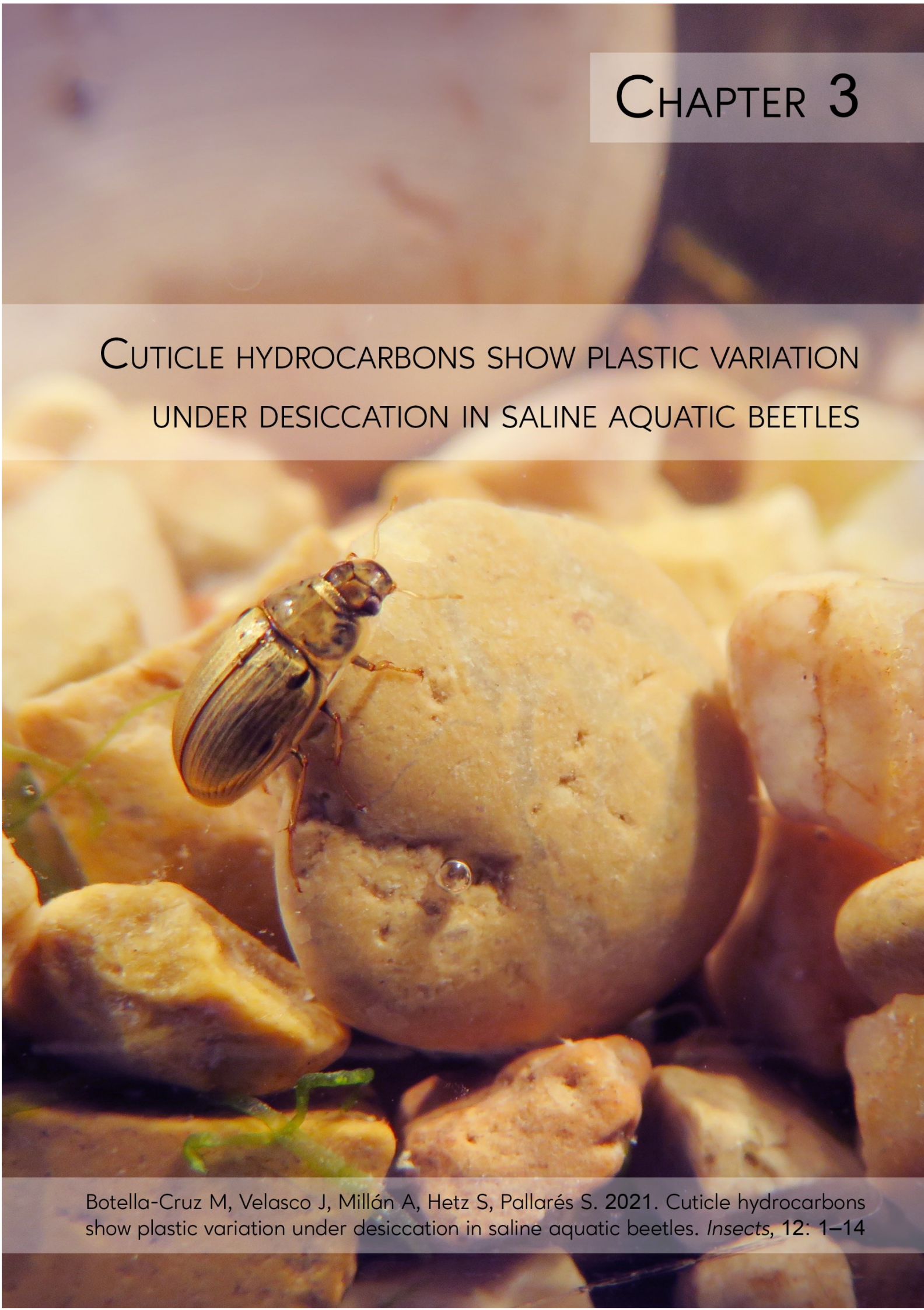
Journal: Journal of Insect Physiology

Abstract: Salinity tolerance has enabled the colonization of inland saline waters and promoted species diversification in some lineages of aquatic insects. However, the mechanisms behind this tolerance, particularly the role of cuticle hydrocarbons (CHCs), are not well-known. We characterized the CHC profile of eight species of two water beetle genera (Nebrioporus, Adephaga: Dytiscidae and Enochrus, Polyphaga: Hydrophilidae), which span the fresh-hypersaline gradient, to: i) determine the interspecific variation of CHC composition in relation to species' salinity tolerance; ii) explore plastic adjustments in CHC profiles in response to salinity changes at the intraspecific level in saline-tolerant species. CHC profiles were highly species-specific, more complex and diverse in composition, and characterized by longer-chain-length compounds in the species with higher salinity tolerance within each genus. Higher salinity tolerance in the Enochrus species was also associated with an increase in the relative abundance of branched alkanes, and with a lower proportion of n-alkanes and unsaturated compounds. These CHC characteristics are related with improved waterproofing capacity and suggest that reducing cuticle permeability was one of the key mechanisms to adapt to saline waters. Similar CHC composition patterns were found at the intraspecific level between populations from lower and higher salinity sites within saline-tolerant species of each genus. These saline species also displayed an extraordinary ability to adjust CHC profiles to changing salinity conditions in the laboratory in a relatively short time, which reflects great plasticity and a high potential to deal with daily and seasonal environmental fluctuations in the highly dynamic saline habitats.

URL: https://doi.org/10.1016/j.jinsphys.2019.103899

Chapter 3

Cuticle hydrocarbons show plastic variation under desiccation in saline aquatic beetles



Botella-Cruz M, Velasco J, Millán A, Hetz S, Pallarés S. 2021. Cuticle hydrocarbons show plastic variation under desiccation in saline aquatic beetles. *Insects*, 12: 1–14

CHAPTER 3

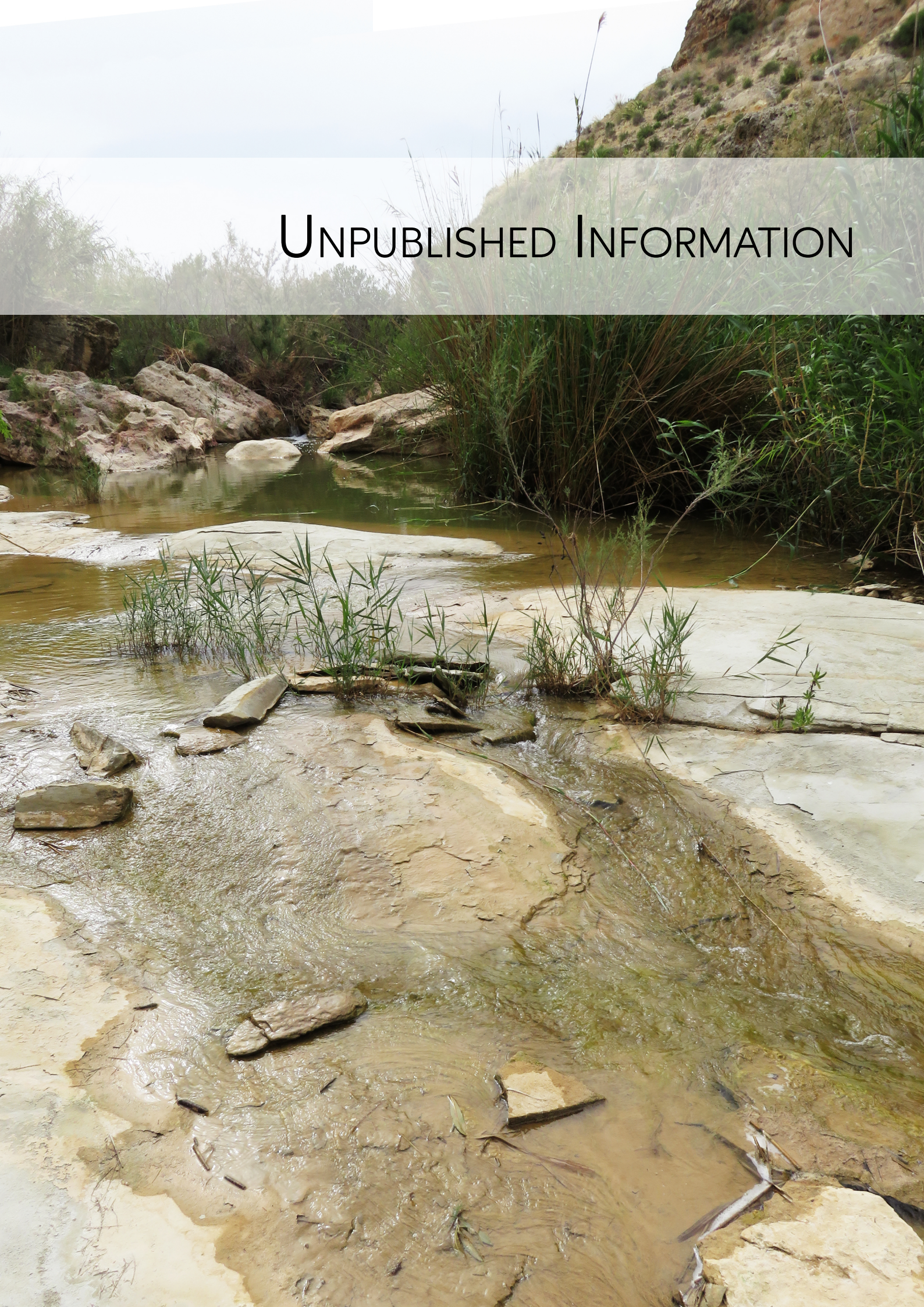
Cuticle hydrocarbons show plastic variation under desiccation in saline aquatic beetles

Journal: Insects

Abstract: In the context of aridification in Mediterranean regions, desiccation resistance and physiological plasticity will be key traits for the persistence of aquatic insects exposed to increasing desiccation stress. Control of cuticular transpiration through changes in the quantity and composition of epicuticular hydrocarbons (CHCs) is one of the main mechanisms of desiccation resistance in insects, but it remains largely unexplored in aquatic ones. We studied acclimation responses to desiccation in adults of two endemic water beetles from distant lineages living in Mediterranean intermittent saline streams: *Enochrus jesusarribasi* (Hydrophilidae) and Nebrioporus baeticus (Dytiscidae). Cuticular water loss and CHC composition were measured in specimens exposed to a prior non-lethal desiccation stress, allowed to recover and exposed to a subsequent desiccation treatment. E. jesusarribasi showed a beneficial acclimation response to desiccation: pre-desiccated individuals reduced cuticular water loss rate in a subsequent exposure by increasing the relative abundance of cuticular methyl-branched compounds, longer chain alkanes and branched alkanes. In contrast, N. baeticus lacked acclimation capacity for controlling water loss and therefore may have a lower physiological capacity to cope with increasing aridity. These results are relevant to understanding biochemical adaptations to drought stress in inland waters in an evolutionary and ecological context.

URL: https://doi.org/10.3390/insects12040285

Unpublished Information



Chapter 4

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

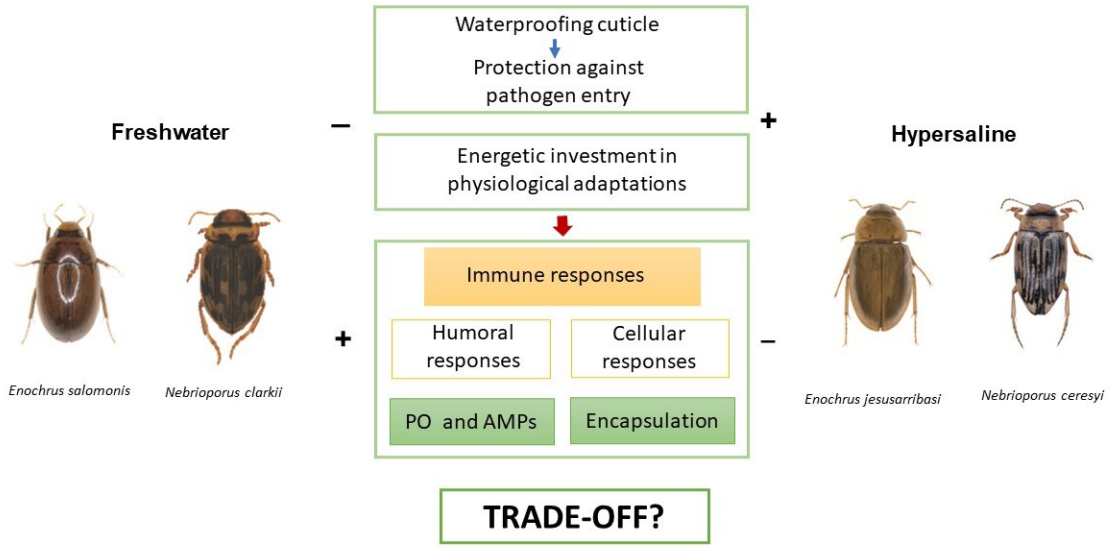


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The colonization of saline waters is associated with lowered immune responses in aquatic beetles

ABSTRACT

The immune response represents an evolved trait suite 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. 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. 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. Our results suggest that adaptation to saline environments may have involved a trade-off between osmoregulation and investment in immune defences and are also consistent with relaxed selection pressures on basal immune responses in saline waters due to lower microbial infection load and/or the more resistant cuticle of species occupying such habitats.



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 and Lu, 2018; Sheehan *et al.*, 2018; Cotter *et al.,* 2019; Mangahas *et al*., 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 *et al*., 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 and 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 and 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 and Moreau, 2012). As a result, haemolymph crystallizes, encapsulates and immobilises the invading organism

(Sugumaran *et al*., 2002). Since immune function is highly complex, with different elements acting synergistically, 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 and 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 and Bradley, 2011; Arribas *et al*., 2014; Pallarés *et al*., 2017). 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, shifts to saline waters in freshwater lineages require an important additional energetic investment in osmoregulatory processes (Edwards, 1982; Bradley, 2009). Both hyporegulation in hyperosmotic media and hyperegulation in hypo-osmotic conditions entail an increase in respiration rates of aquatic organisms (Rivera-Ingraham and Lignot, 2017). Such energetic costs increase as the difference between the osmotic pressure of the aquatic media and the organism's internal fluids increases, meaning that the energetic challenge of osmoregulation is especially important in hypersaline media (Bradley, 2009). Inland saline waters are concentrated in arid and semi-arid regions, meaning that aquatic organisms have developed physiological and morphological adaptations to deal not only with elevated salt concentrations, but also with the thermal, hydric and osmotic stress generated by high temperatures and exposure to desiccation when these habitats dry out (Velasco *et al.*, 2006; Millán *et al*., 2011; Chester *et al.*, 2015; Strachan *et al.*, 2015). All aspects of the insect immune response are also metabolically costly, which may result in energetic trade-offs between different processes (Rantala, 2005; Adamo *et al*., 2008; Lazzaro and Little, 2009; Ardia *et al*., 2012) as well as opportunity costs (fitness loss if other tasks cannot be met) (Schmid-Hempel, 2005). There is 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 (Schmid-Hempel, 2003; Lazzaro and Little, 2009; 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.

Also, of potential relevance here are recently discovered differences in cuticular composition between related fresh and salt water insects. 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 mean that other immune defence mechanisms are less developed in saline compared to freshwater taxa.

Finally, the high level of abiotic stress in inland saline waters acts not only as a filter for colonization by freshwater invertebrate lineages, but also influences microbial survival, allowing only the presence of extremely and moderately halophilic organisms (Oren, 2011; Oueriaghli *et al*., 2018). In hypersaline habitats, bacterial and archaeal diversity is considerably lower than in freshwaters (Ortega *et al.*, 2009; Auguet *et al*., 2010), comprising approximately 50% of the archaeal and less than 25% of the total inland aquatic microbial diversity (Ma and Gong, 2013). 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).

Here we explore possible links between immune function and habitat in two lineages of water beetle which 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 species studied within each genus have been shown to differ in their osmoregulatory capacity (Pallarés *et al*., 2015) and cuticular waterproofing (Botella-Cruz *et al*., 2019). 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.

MATERIAL AND METHODS

Study Species, Field 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 (Millán *et al*., 2011). 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 hyporegulate 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).

Table 1. Data on habitat preference of study species (ranges of electrical conductivity (EC, $mScm^{-1}$) and salinity (gL^{-1}) of occupied waters), species osmotic capacity (mOsmol Kg^{-1}), and EC, salinity and osmolality (mOsmol Kg^{-1}) of collection sites.

Species	Habitat	Field range EC Salinity[a]	Maximum osmotic capacity[b]	Collection sites	
				Locality	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	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	100 70 2470
Enochrus salomonis (Sahlberg, 1900)	Subsaline waters	0.70-2.16 0.49-1.51	-10.98 ± 10.29	Pétrola wetland, Spain	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	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.

Adults of each species were collected from their natural habitats in September 2019 (Table 1). Electrical conductivity values were measured *in situ* with a conductivity meter (HACHC/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, Cittadella, Padua, Italy) 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. 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 o 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

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}.

AMP activity

AMP activity was measured using the zone of inhibition assay adapted from Moret and Schmid-Hempel, 2000 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

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; Whitehorn *et al*., 2011). Images were analysed with Image J Sofware (Image J software v. 1.48, National Institute of Health, USA).

Statistical analyses

PO activity and encapsulation response (scab size and brightness) were compared between species using a nested ANOVA (species nested within genus), followed by Bonferroni post-hoc tests. 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

In both genera, dopachrome production rates were significantly higher ($F=14.86$, $df=3$ $p<0.001$) in the freshwater species, *E. salomonis* and *N. clarkii* than their saline relatives, *E. jesusarribasi* and *N. ceresyi* (Figure 1A).

AMP activity

Inhibition zones were only produced against Gram positive bacteria; there was no effect on *E. coli* in 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

Each encapsulation measurement (intensity and scab size) showed a different pattern across species and genera. Encapsulation intensity differed between species pairs in the two genera (Genus x Species: $F=9.31$, $df=2$ $p<0.001$; Figure 1B). In *Nebrioporus,* encapsulation intensity was higher in *N. clarkii* (freshwater) than *N. ceresyi* (hypersaline). In *Enochrus*, encapsulation intensity was significantly higher in *E. jesusarribasi* (hypersaline) than in *E. salomonis* (freshwater). The size of the scab also showed different response patterns in each genus (Genus x Species $F=31.67$, $df=2$, $p<0.001$; Figure 1C). No significant differences were observed between the *Nebrioporus* species, whilst within *Enochrus* the freshwater *E. salomonis* produced larger scabs than the hypersaline *E. jesusarribasi* (Figure 1C).

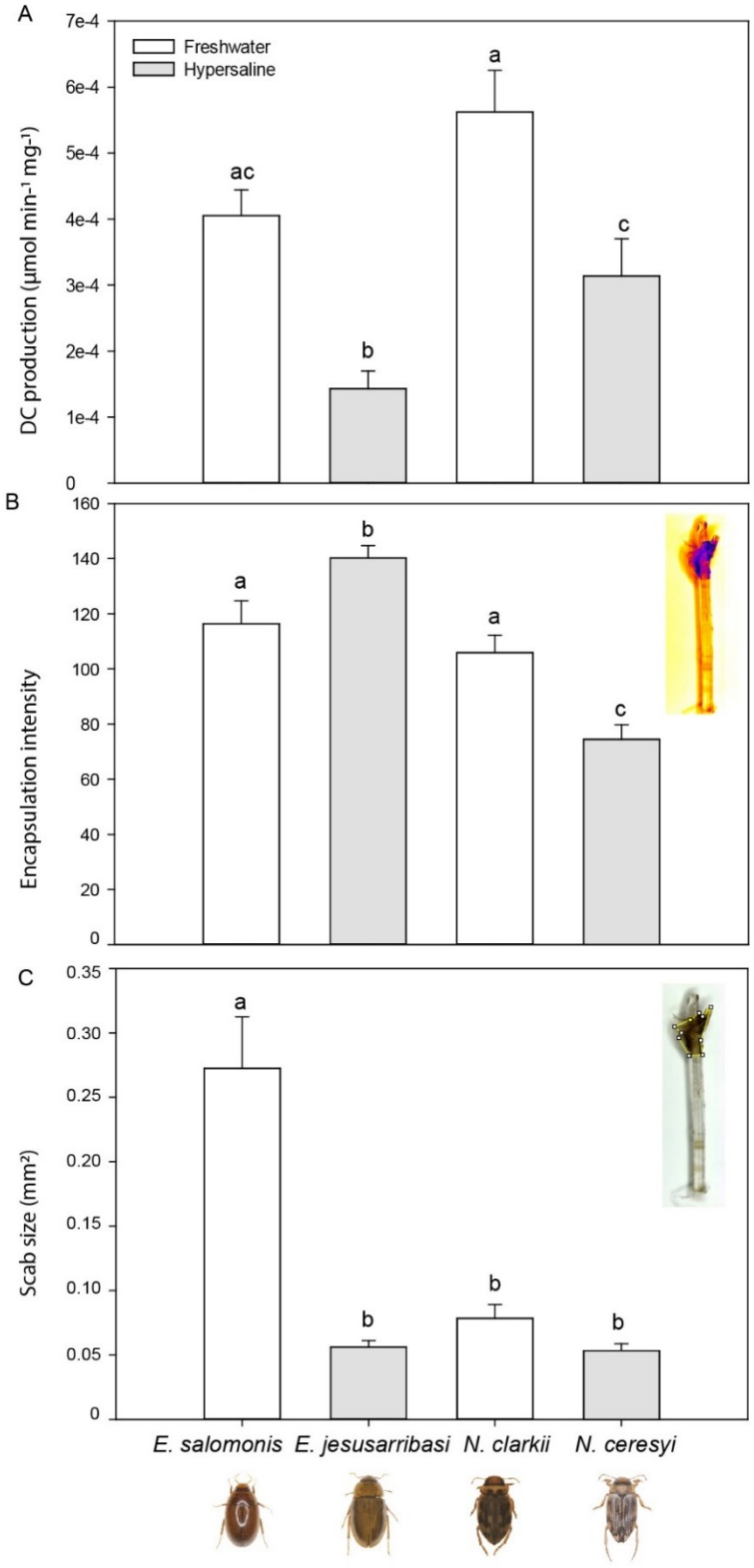


Figure 1. A) Phenoloxidase activity measured as the rate of dopachrome (DC) production; B) encapsulation intensity and C) encapsulation scab size. Bar-plots show mean ± S.E. Lowercase letters indicate significant differences (P<0.05) between species in Bonferroni post-hoc tests. Images on B and C show 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. This result is in line with growing evidence that immune responses entail trade-offs with other energetically costly physiological mechanisms, such as osmoregulation (Lazzaro and Little, 2009; Ardia *et al*., 2012; Adamo *et al*., 2017). Our findings are also consistent with relaxed selective pressure on basal immune responses in saline waters due to the lower microbial infection load in such habitats and/or the more waterproof cuticle of saline species acting as a more effective physical barrier to infection.

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 and Córdoba-Aguilar, 2012), lower basal PO activity is indicative of a reduced immune response (Marmaras *et al.*, 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 *et al*., 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 *et al*., 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 *et al*., 2019) but only a few studies have addressed such response in aquatic insects (Cioffi *et al.*, 2016; Mangahas *et al.*, 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, which would merit further research.

We suggest that the lower metabolic cost of osmoregulation in hypo-osmotic vs. highly hyperosmotic media, as well as 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 *et al*., 2019). These effects appear temporary, and disappear in the absence of osmotic stress (e.g., Mangahas *et al*., 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 salt-tolerant relatives, which may also be due to evolutionary trade-offs with other physiological functions (Demas *et al*., 2012; Cespedes *et al*., 2019). In the case of the water beetles, the cuticle composition of the saline species studied (*E. jesusarribasi* and *N. ceresyi*), characterized by a higher proportion of long chain hydrocarbons and complex methyl alkanes than their freshwater relatives, may result not only in higher waterproofing and desiccation resistance (Botella-Cruz *et al*., 2019; Botella-Cruz *et al*., 2021), but also provide a more effective physical and biochemical barrier against the entry of parasites and infectious agents (Marmaras *et al*., 1996; Noh *et al*., 2016). In effect, such cuticular changes may represent an exaptation against infection (Gould and Vrba, 1982).

Most saline insect species appear to be generalists, in terms of their fundamental salinity niche, as they show high performance and survival at both low and high salinity in the laboratory, but are rarely found in fresh or-low conductivity habitats in nature, and never breeding in such localities (Arribas *et al*., 2019; Lambret *et al.*, 2021). Our results suggest that the lower immune capacity of saline species could be one of the factors accounting for their absence from freshwaters in nature. 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 and Zamparas, 2010; Filipe *et al*., 2013; 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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Synthesis and Future Perspectives

SYNTHESIS AND FUTURE PERSPECTIVES

This thesis explored, for the first time, the role of cuticular permeability in aquatic beetles as an adaptive response to cope with the multiple natural stressors, such as salinity and desiccation, in arid inland aquatic ecosystems. Stress adaptation entails a significant energetic cost, in detriment of the immune response in saline species compared to those of freshwater ones. This research complements and adds novel insights into the knowledge about salinity tolerance in water beetles from previous studies (Arribas *et al*., 2014; Pallarés, 2017; Villastrigo *et al.,* 2019).

Results in Chapter 1 showed an important ontogenetic modification of the cuticular lipids composition in saline beetle species, and therefore in their cuticular permeability. As expected, larvae had a softer and more permeable cuticle than adults, which is therefore less effective against water loss (Chapman, 1975). Moreover, the cuticle hydrocarbons (CHC) profile described for two saline water beetles differed from that of other freshwater beetles (Jacob and Hansen, 1986; Alarie *et al.,* 1998), and showed common patterns to those generally attributed to adaptation to aridity in terrestrial Coleoptera (Lockey, 1979; Jacob and Hansen, 1986; Lockey, 1988; Nelson and Charlet, 2003), with an exceptionally thick and impermeable epicuticular wax layer (Crowson, 1981). Thus, these results point that salinity could impose a selective pressure on CHC profiles of aquatic insects similar to that exerted by aridity in terrestrial species, suggesting that the cuticle of aquatic coleopterans could have an important role in adaptation to salinity and desiccation in this group. Such aspects were explored in more details in Chapters 2 and 3.

These chapters confirmed that insect cuticle composition play a fundamental and rather complex protective function against salinity and desiccation stress and pointed that increasing the waterproofing capacity of the cuticle was one of the potential mechanisms that facilitated desiccation resistance and salinity tolerance in *Enochrus* and *Nebrioporus* genera. Freshwater species presented similar CHC profiles, but different from saline species ones. In *Enochrus*, these differences where clearly attributed to an increased cuticle impermeability in species with higher hyporegulation capacity and desiccation resistance (*E. jesusarribasi* and *E. fuscipennis*) compared with

the freshwater ones. However, no such clear patterns were found in the *Nebrioporus* genus. Further studies would be needed to improve the understanding on the relationship between CHC composition and salinity tolerance. For example, studies including more species with different habitat preferences in order to compare CHC profiles between freshwater and saline species across beetle lineages would be interesting.

This thesis also demonstrated that plasticity of insect cuticle composition plays a crucial protective role against salinity and desiccation stress in saline aquatic insect species. In the saline species studied (*E. jesusarribasi* and *N. baeticus*) salinity (Chapter 2) and desiccation (Chapter 3) induce changes in the cuticle, and the two species adjusted CHC profiles to changing conditions. Salinity involved the synthesis of new saturated n-alkanes and longer chain branched alkanes, resulting in a decrease of permeability in of both species (*E. jesusarribasi* and *N.baeticus*). The intraspecific differences found in the CHC profiles between populations from habitats with contrasting salinities could be attributed to local physiological acclimatization (i.e., reversible non-genetic phenotypic changes; Lande, 2009), given the high plasticity of CHCs shown by these species under laboratory acclimation conditions. However, interpopulation variation may also reflect local adaption (i.e., fixed genetic effects). Future reciprocal transplant experiments studies rearing individuals at different salinities to those of their habitat, across multiple generations, would be an interesting approach to test to what extent intraspecific differences in CHC profiles are environmentally or genetically driven (e.g., Kimberly and Salice, 2015). Although such transgenerational studies are currently limited for the species studied here given the difficulties to complete their whole life-cycle in the laboratory, other organisms could be appropriate models for such purposes (e.g., water bugs).

Desiccation triggered a similar response in *E. jesusarribasi*. This species showed a positive acclimation response to desiccation, showing an effective decrease in water loss also associated with CHC changes. Such changes are determinant for the waterproofing properties of the cuticle, as it has been observed in some terrestrial insects, like *Drosophila melanogaster* (Stinziano *et al*., 2015) and Tenebrionidae (Menzel *et al*., 2017) and *Rhodnius prolixus* (Dulbecco *et al*., 2020). However, desiccation did not

increase the waterproofing capacity of the cuticle of *N. baeticus*, in agreement with the inability of this species to reduce water loss rate when exposed to desiccation after a prior desiccation, so the ecological function of the CHC observed in this species under desiccation remains to be further explored. These results are also consistent with the higher desiccation resistance (Pallarés *et al*., 2017a), osmoregulatory ability and salinity tolerance (Pallarés *et al*., 2015) of *E. jesusarribasi* compared to *N. baeticus*. These interspecific differences reflect the importance of the evolutionary history and ecology in driving responses of species exposed to similar stressors (salinity and desiccation).

Taken together, these results suggest that *E. jesusarribasi*, the species with higher tolerance to salinity and desiccation and acclimation capacity, could have a better physiological capacity to deal with increasing aridification and be less vulnerable to climate change than *N. baeticus*. Accordingly, we found that *Enochrus* species have a more complex cuticles and more with diverse in composition than *Nebrioporus* ones. Indeed, the longest chain length among CHCs was conservative within species of each genus and higher in the *Enochrus* species (36C) than *Nebrioporus* ones (31C). Future transcriptomic studies of the gene expression patterns underlying the CHC changes related with the increased impermeability would be needed. Besides, estimations of cuticular water loss rates under different salinity and desiccation conditions with associated changes in CHC composition, would provide crucial information for a better mechanistic understanding of salinity tolerance in aquatic insects as well as it would help to elucidate the evolutionary constraints on these traits in these water beetle lineages.

Finally, Chapter 4 showed that the adaptation to salinity shapes the immune responses in aquatic beetles, an aspect not explicitly considered in physiological studies of water beetles to date. Our findings showed that saline species of two water beetle genera (*E. jesusarribasi* and *N. ceresyi*), have generally lower basal immune responses than their freshwater relatives (*E. salomonis* and *N. clarkii*), measured at the typical salinity of their natural habitats. This result is in line with growing evidence that immune responses entail trade-offs with other energetically costly physiological mechanisms, such as osmoregulation (Lazzaro and Little, 2009; Ardia *et al*., 2012; Adamo *et al*., 2017). These findings are also consistent with a relaxed selective pressure on basal immune responses in saline waters due to the lower microbial infection load in such habitats

and/or the more waterproof cuticle of saline species acting as a more effective physical barrier to infection. Lower immune capacity of saline species could be one of the factors accounting for their absence from freshwaters in nature (Arribas *et al*., 2019). In this case, 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). This opens emerging interesting research questions to further study 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.

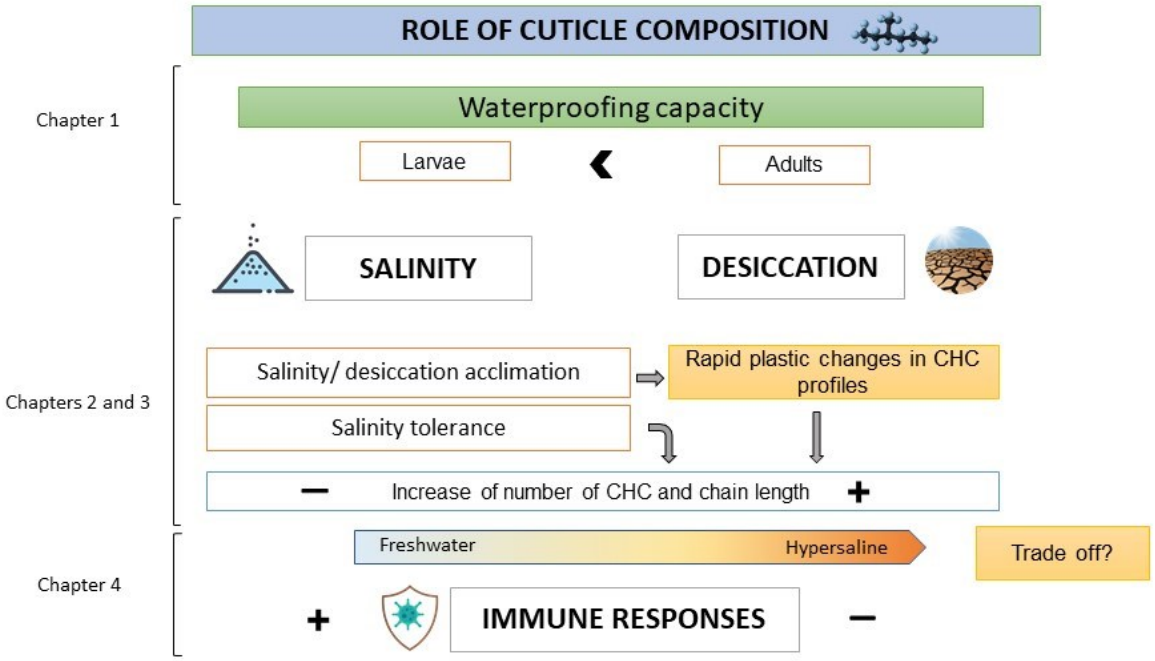


Figure 1. Scheme of thesis results.

The results from this thesis represent an important contribution to the field of ecophysiology in inland aquatic habitats, where these aspects have remained largely unexplored so far. This thesis demonstrated that cuticle properties (in particular, hydrocarbons composition) and immune responses are crucial traits in the context of biotic and abiotic stress in aquatic habitats, which have greatly influenced insect physiology, behaviour, and evolution. Our findings help to understand key questions

related with the evolutionary process of colonization of inland saline waters, but also open new ones, encouraging to carry out further research in this field. We believe that the results presented here will serve a base on which to build up new knowledge on salinity tolerance, desiccation resistance and immunity in aquatic species, which is fundamental for the challenge ahead of predicting the impacts climatic change and aridification on aquatic biodiversity.

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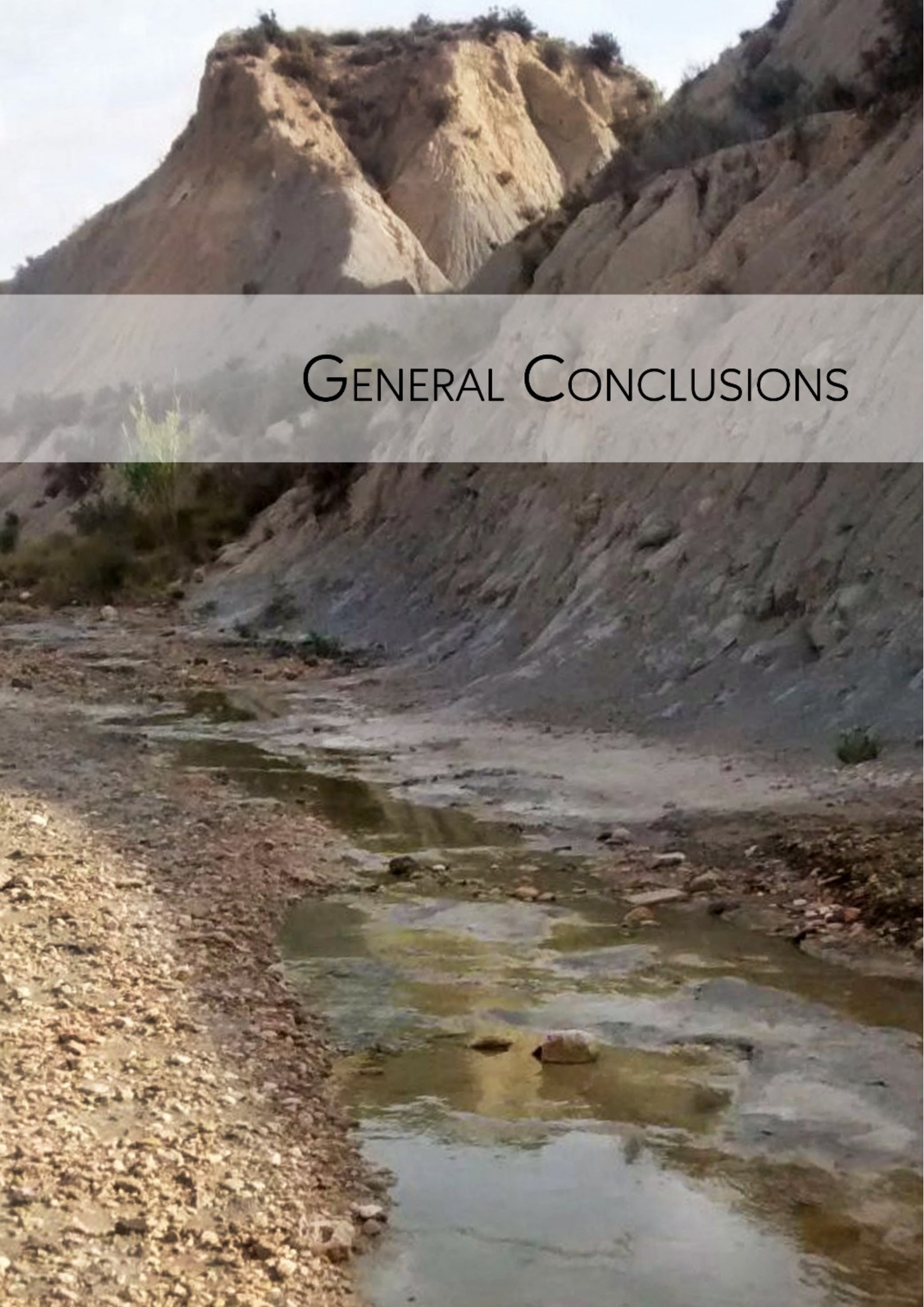
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General Conclusions

CONCLUSIONS

Chapter 1: Cuticle hydrocarbons in saline aquatic beetles

1. The CHC profile of adults of the two studied saline species (*Enochrus jesusarribasi* and *Nebrioporus baeticus*) are characterized by a high abundance of branched alkanes and low of unsaturated compounds. Such profile seems to be more similar to that of some terrestrial beetles (e.g., desert Tenebrionidae) than that of other aquatic Coleoptera (freshwater Dytiscidae)
2. Adults of *E. jesusarribasi* have longer chain compounds than *N. baeticus*, in agreement with their higher tolerance to salinity and desiccation.
3. Larvae have a more permeable cuticle than adults, characterized by a lower diversity in compounds, shorter carbon chain length and a higher proportion of unsaturated hydrocarbons than adults.
4. Interspecific differences, as well as between life-stages variation in CHC profiles within the study species, suggest that osmotic stress has an important role as a selection pressure on cuticular traits of aquatic insects.

Chapter 2: Role of cuticle hydrocarbons composition in the salinity tolerance of aquatic beetles

5. CHC profiles were highly species-specific across genera (*Enochrus* and *Nebrioporus*). Within each genus, the species with higher salinity tolerance showed a more complex and diverse in composition CHC profile, characterized by longer-chain-length compounds. These cuticular CHC characteristics are related with improved waterproofing capacity and suggest that reducing cuticle permeability was one of the key mechanisms to adapt to saline waters.
6. Higher salinity tolerance in the *Enochrus* species was also associated with an increase in the relative abundance of branched alkanes, and with a lower proportion of n-alkanes and unsaturated compounds.
7. Similar cuticular CHC composition patterns were found at the intraspecific level between populations from lower and higher salinity sites within saline-tolerant

species of each genus. These phenotypic changes in CHC composition involved the synthesis of new saturated n-alkanes and longer chain branched alkanes.

8. The saline species studied displayed an extraordinary ability to adjust CHC profiles to changing salinity conditions in a relatively short time, which reflects a great plasticity of this complex trait and a high potential to deal with daily and seasonal environmental fluctuations in the highly dynamic saline habitats.

Chapter 3: Cuticle hydrocarbons show plastic variation under desiccation in saline aquatic beetles

9. Desiccation exposure induces plastic changes in CHC in two saline aquatic species (*Enochrus jesusarribasi* and *Nebrioporus baeticus*).
10. In *E. jesusarribasi*, cuticular composition changes were effective in reducing cuticular permeability. This species showed a beneficial acclimation response, increasing its desiccation resistance after a prior exposure to desiccation.
11. In *N. baeticus,* cuticular composition changes were no related with improved waterproofing capacity.
12. Species living under similar environmental conditions might have different capacities to cope with increasing aridification in a climate change context.

Chapter 4: Lower immune response in saline vs freshwater beetles

13. Saline species of two water beetle genera (*Enochrus* and *Nebrioporus*) have generally lower basal immune responses than their freshwater relatives, measured at the typical salinity conditions of their natural habitats.
14. The lower metabolic cost of osmoregulation in hypo-osmotic vs. highly hyperosmotic media, the more waterproofing cuticle of saline species acting as a more effective physical barrier to infection as well as a relaxed selective pressure on basal immune responses in saline waters due to the lower microbial infection load in these habitats; may underlie their different basal immune capacities.
15. The lower immune capacity of saline species could be one of the factors accounting for their absence in freshwater in nature, despite being physiologically capable of surviving and reproducing in such conditions



Agradecimientos
Acknowledgements

AGRADECIMIENTOS

Desde el comienzo de mi etapa como investigadora ha formado parte mucha gente que no solamente me ha ayudado al desarrollo profesional, si no también personal. Un trocito de esta tesis también es suyo.

Quiero comenzar con un agradecimiento muy especial a mi familia, a mis padres, Teté y Paco, y también a mi hermano Paco por vuestro cariño, por creer y confiar siempre en mí, por apoyarme en todas mis decisiones. Gracias por todas las excursiones al campo desde bien pequeña, por los valores por la naturaleza que me habéis transmitido, sin vosotros esto no hubiera sido posible. También a mi abuela Tere, una segunda madre para mí, por todo su apoyo y confianza sin excepciones.

Por supuesto, a Alex, formamos un equipazo, por su ayuda en todos los aspectos, personal y profesional con salidas al campo incluidas, también por su apoyo incondicional y por saber sacar el lado positivo a cualquier situación.

En el ámbito investigador, quiero agradecer a mis directores, Pepa Velasco y Andrés Millán por haberme abierto las puertas del mundo de la investigación, por guiarme en este periodo de formación, con asesoramiento continuo, por haber estado siempre pendientes, por vuestro apoyo personal y por haberme acogido en vuestra maravillosa familia "Ecoaquática". Es de admirar el gran equipazo que habéis conseguido formar, con un ambiente sano de trabajo donde colaborar conjuntamente se vuelve sencillo, cosa que no es fácil lograr en un mundo tan competitivo.

En especial, quería agradecer a Susana Pallarés, su amistad, su apoyo científico y moral durante toda la tesis. A pesar de la solicitud de mis directores contando con el acuerdo de los miembros de las Comisiones Académicas y de la Comisión de la Garantía de Calidad del Programa de doctorado, no pudo ser incluida como codirectora debido a la nueva normativa que claramente perjudica a los nuevos doctores. Sin embargo, aunque no figure en los papeles, ella ha sido codirectora de mi tesis. Su ayuda directa en todos los experimentos (a veces hasta altas horas de la madrugada) e implicación desde el principio y en todos y en cada uno de los artículos ha sido fundamental para mí y para que esta tesis siguiera adelante. Estuviera en la ciudad donde estuviera trabajando, siempre ha tenido tiempo para escucharme y ayudarme. Incontables salidas

al campo (en ocasiones improvisadas), las carcajadas de después, los momentos de laboratorio e incontables anécdotas juntas, incluida la convivencia en Plymouth con sus buenos momentos que se quedarán para siempre.

A todos los coautores de los capítulos de esta tesis por sus contribuciones, en especial a David Bilton y a Stefan Hetz por haberme recibido en sus equipos de investigación durante mis estancias en el extranjero en la Universidad de Plymouth en Inglaterra y la Universidad de Humboldt en Berlín respectivamente.

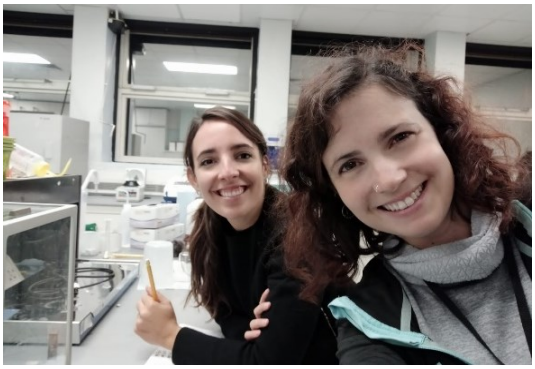
Por supuesto, muchas gracias a los Mengues, que se han convertido en grandes amigos, David Sánchez, Toni, Juani y Raquel por estos dos últimos años trabajando y compartiendo momentazos juntos, por todos los ánimos y por todos los desayunos en la cantina y en el campo con nuestras empanadillas. Toni, mi diseñador gráfico de cabecera, gracias por diseñar desinteresadamente todas las portadas de la tesis con ese cariño. Juani, mi compi de risas y cómplice desde el primer día que llegó al departamento y a David, todo un ejemplo a seguir. También a Nuria, compi de despacho, por tantas conversaciones mañaneras arreglando el mundo.

A Obdulia, por traer su alegría al despacho todos los días y por su disposición siempre a ayudar. También me gustaría agradecer al resto de la familia de ecoaquáticos, Jose, con el que me inicié en los experimentos de laboratorio, Simone y Adrián, con los que he compartido épocas de trabajo conjunto, Félix y Dani con los que compartí despacho durante sus últimos años de tesis, Paula, Pedro y Narci, Tano y Oscar.

A todos los compañeros del máster, con los que me inicié en el mundo investigador y que se convirtieron en amigos, José Manuel y Antonio Zamora, Pereñíguez, Ana Sánchez, Pablo, Diego y sobre todo a María Alcaraz, porque gracias a esa etapa nos dimos cuenta que formamos un gran equipo juntas (quien nos lo iba a decir en los muestreos por la costa). A mi compi de carrera Rosario, por su apoyo. También agradeceros los grandes momentos, viajes, fiestas y salidas a mis compañeros: Irene, Victor Zapata, Victor Orenes, Néstor, Merce, Paqui, Vicente, Lury, Maridol, María Sánchez-Tornel, Marta Alonso, Fátima y Antonio Guillén.

Por último, me gustaría dar las gracias a todos mis compañeros de la Asociación de Jóvenes Investigadores de la Universidad de Murcia (AJIUM), por su esfuerzo continuo para que las condiciones en el ámbito de la investigación mejoren.

¡MUCHAS GRACIAS A TODOS POR HABER FORMADO PARTE DE ESTA MARAVILLOSA ETAPA!







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