UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Comparative ecophysiological responses of supratidal rockpools *Ochthebius* species (Coleoptera: Hydraenidae) to multiple stressors

Respuestas ecofisiológicas de especies de *Ochthebius* (Coleoptera: Hydraenidae) en pozas rocosas supralitorales frente a múltiples factores de estrés

D. ª Juana María Mirón Gatón

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Autora: D. ª Juana María Mirón Gatón

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Biodiversidad y Gestión Ambiental

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Comparative ecophysiological responses of supratidal rockpools Ochthebius species (Coleoptera: Hydraenidae) to multiple stressors

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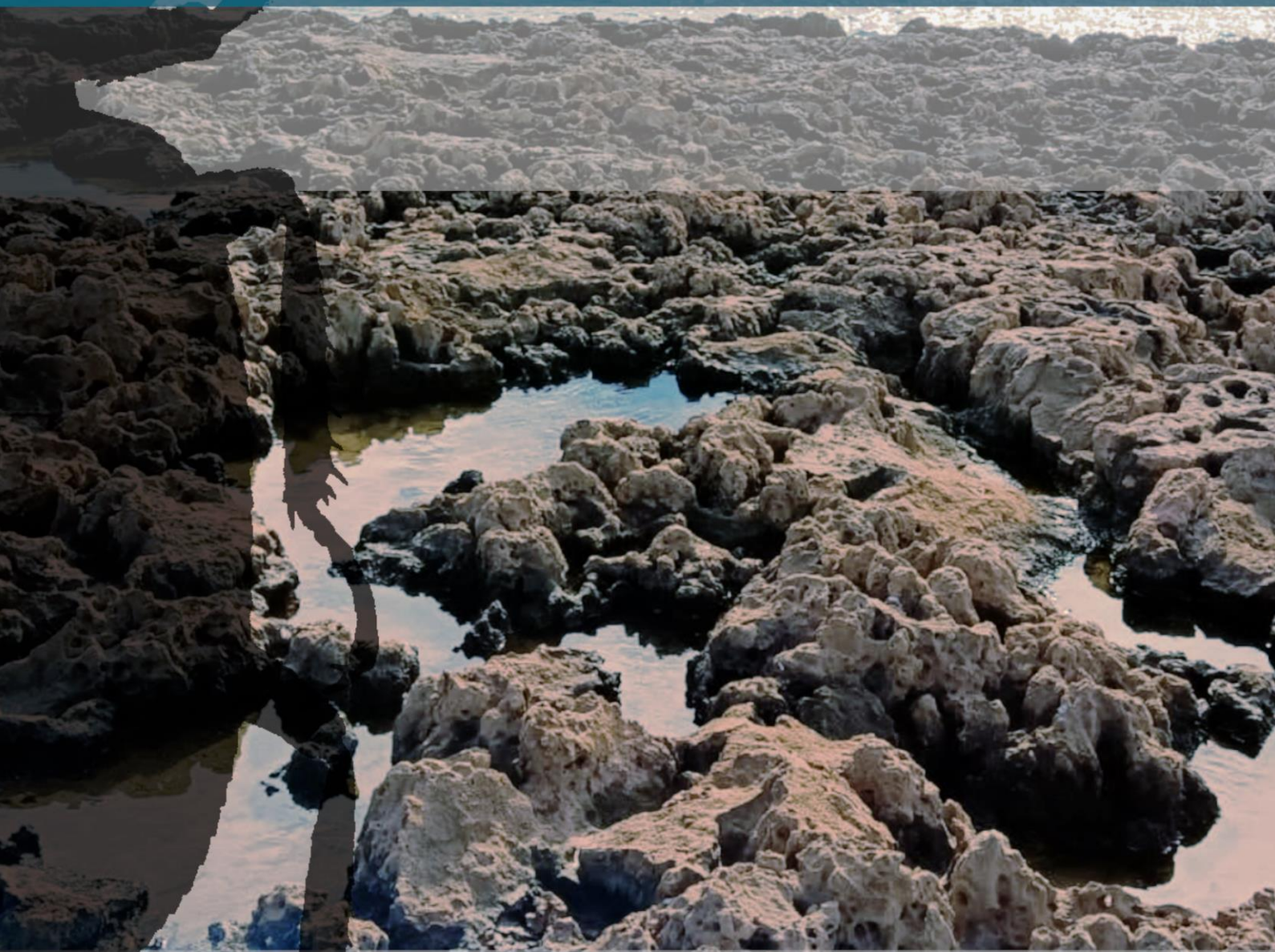
COMPARATIVE ECOPHYSIOLOGICAL RESPONSES OF SUPRATIDAL ROCKPOOLS *OCHTHEBIUS* SPECIES (COLEOPTERA: HYDRAENIDAE) TO MULTIPLE STRESSORS

RESPUESTAS ECOFISIOLÓGICAS DE ESPECIES DE *OCHTHEBIUS* (COLEOPTERA: HYDRAENIDAE) EN POZAS ROCOSAS SUPRALITORALES FRENTE A MÚLTIPLES FACTORES DE ESTRÉS



JUANA MARÍA MIRÓN GATÓN

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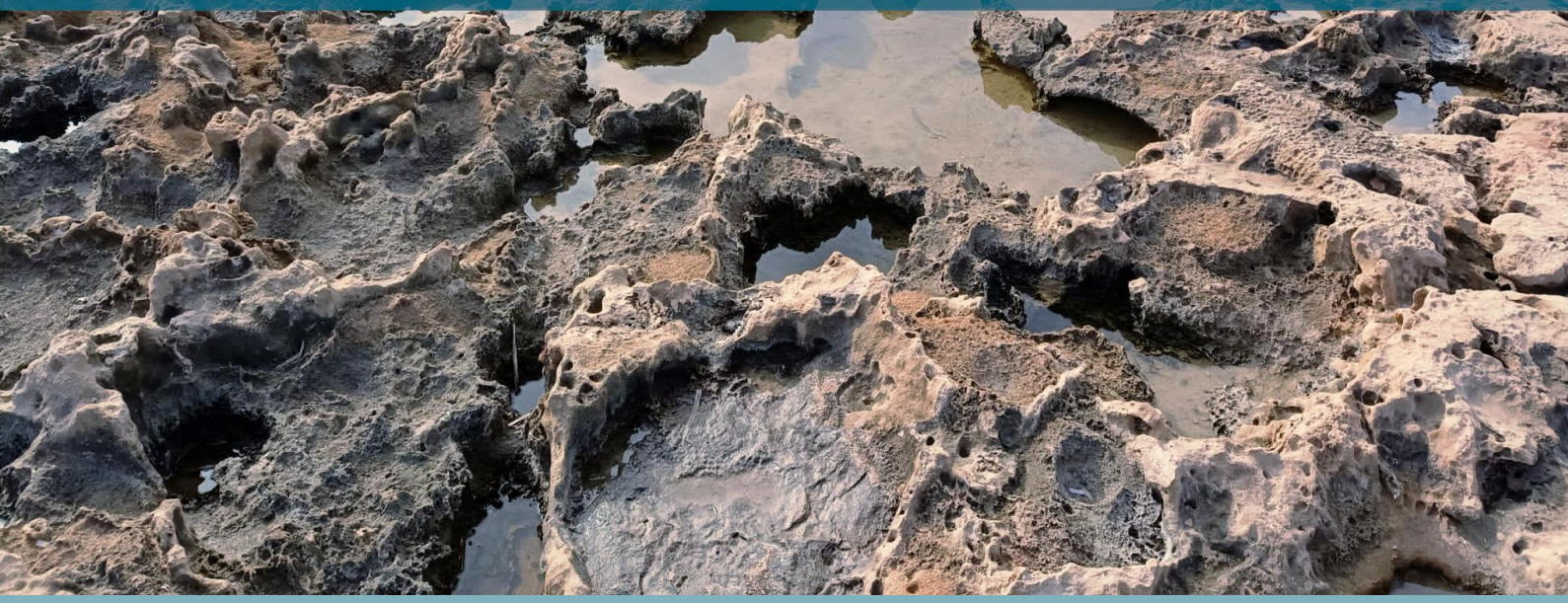

BIOSKETCH

I was born in 1989 in “Puerto de Mazarrón”, a coastal town in Murcia, where I spent all my childhood. At the age of 16, I decided not to continue my high school studies, and after 6 years, I resumed them. At that point, I was clear about wanting to study Environmental Sciences, so I began a degree at the University of Murcia (2013-2017). Subsequently, I pursued a master’s degree in Protected Areas, Natural Resources, and Biodiversity (2017-2018), which led me to start my research career in the Department of Ecology and Hydrology, where I completed my master's thesis under the supervision of Josefa (Pepa) Velasco and Andrés Millán. The title of my master's thesis was "Thermal tolerance of the aquatic beetles *Ochthebius quadricollis* and *Ochthebius subinteger* in highly changing environmental conditions."

After completing my master's thesis, I embarked on my doctoral thesis in the Biodiversity and Environmental Management doctoral program at the University of Murcia, under the supervision and direction of Pepa and Andrés, in the Aquatic Ecology research group. The thesis was framed in ecophysiology aspects within the “Rockpools Project”: Macroinvertebrate metacommunity dynamics in supralittoral rockpools (CGL2017-84157P). During the first year, I was employed as researcher in this project. In late 2019, I started a predoctoral contract within the University Teaching Staff Training Subprogram (FPU, by its acronym in Spanish) by the Ministry of Universities.

As a predoctoral student, I had the opportunity to spend three months at the University of Plymouth, conducting a study on metabolism and learning new respirometry techniques. This research was carried out under the supervision of Dr. David T. Bilton at the Marine Biology and Ecology Research Centre, School of Marine Science and Engineering.

Throughout my doctoral period, I have had the opportunity to participate in several international conferences, such as the “Eighth International Symposium on Molecular Insect Science” in Sitges; the “2nd Meeting of the Iberian Ecological Society (SIBECOL) and the XXI conference of the Iberian Association of Limnology (AIL)” in Aveiro; and the “13th Symposium for European Freshwater Sciences” in Newcastle. Additionally, I have taught 180 hours in various subjects, including Ecology and Environmental Impact Assessment, in the Department of Ecology and Hydrology of the University of Murcia. Additionally, I have participated in the first and second congresses on Teaching Innovation at the University of Murcia, and I

completed a training program titled "EXPERT IN UNIVERSITY TEACHING (EDU)" consisting of 260 hours.

Currently, I´m collaborating with the predoctoral student Antonio José García Meseguer within the “Rockpools project CGL2017-84157P”, conducting population studies using microsatellites. Furthermore, I have contributed to other research projects principally related to ecophysiology and biology of insects in extreme environments, resulting in co-authorship in the following publications:

- Pallarés, S., Millán, A., **Mirón, J.M**, 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*, 271-282. https://doi.org/10.1111/icad.12394

- Millán, A., Botella-Cruz, M., García-Meseguer, A.J., **Mirón-Gatón, J.M.** & 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 (S.E.A.), 69*: 241–242.

- Colado, R., García-Meseguer, A.J., **Mirón-Gatón, J.M.**, Botella-Cruz, M., Pallarés, S. and Sánchez-Fernández, D. (2022), Thermal tolerance and vulnerability to climate change in subterranean species: a case study using an Iberian endemic pseudoscorpion. *Insect Conservation and Diversity, 15*: 181–190. https://doi.org/10.1111/icad.12541

- Velasco, J., **Mirón-Gatón, J.M.**, García-Meseguer, A.J. & Botella-Cruz, M. (2022) Life cycle differences between two coexisting species of supratidal rockpools: *Ochthebius quadricollis* Mulsant, 1844 and *Ochthebius lejolisii* Mulsant and Rey, 1861 (coleoptera, Hydraenidae). *Suplementos del Boletín de la Asociación Española de Entomología, 4*, 131–136.

- García-Meseguer, A.J., Abellán, P., **Mirón-Gatón, J.M.**, Botella-Cruz, M., Guareschi, S., Millán, A. & Velasco, J. (2023). Fine-scale niche differences allow the co-existence of congeneric aquatic beetles in supratidal rockpools. *Hydrobiologia 851*, 471–485. https://doi.org/10.1007/s10750-023-05333-0

- García-Meseguer, A.J., Villastrigo, A., **Mirón-Gatón, J.M.**, Millán, A., Velasco, J., Muñoz, I. (2023). Novel Microsatellite Loci, Cross-Species Validation of Multiplex Assays, and By-Catch Mitochondrial Genomes on *Ochthebius* Beetles from Supratidal Rockpools. *Insects, 14*, 881. https://doi.org/10.3390/ insects14110881

- Villastrigo, A., Orenes-Salazar, V., García-Meseguer, A.J., **Mirón-Gatón, J.M.**, Mourre, B., Millán, A. & Velasco, J. (2023). Oceanic currents maintain the genetic structure of non-marine coastal taxa in the western Mediterranean Sea. *npj biodiversity, 2*, 25. https://doi.org/10.1038/s44185-023-00028-0

- Plaza-Buendía, J., **Mirón-Gatón, J.M.**, García-Meseguer, A.J., Villastrigo, A., Millán, A. & Velasco, J. (2024). Flight dispersal in supratidal rockpool beetles. *Insects, 15*(3), 140. https://doi.org/10.3390/insects15030140

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- Aquatic Ecology research group (University of Murcia).

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SUMMARY / RESUMEN

SUMMARY

The ecophysiologists face one of today's greatest challenges: understanding species' adaptation to environmental changes in an ever-transforming world. They use ecosystems with extreme environmental conditions as models, which present significant physiological challenges to the organisms inhabiting them. One interesting study system are the Mediterranean supratidal rockpools that suffer long periods of desiccation, resulting in significant environmental fluctuations with extreme conditions of high temperature and salinity.

This thesis focuses on the ecophysiological comparative responses to multiple stressors of three aquatic beetle species from the genus *Ochthebius*: *O. quadricollis*, *O. lejolisii*, and *O. subinteger*, exclusive of rockpools. The main goal was to seek their common or different response patterns.

Chapter 1. The temperature at which heat coma occurs was determined and compared among populations of the three *Ochthebius* species. The effect of acclimation salinity (at non-stressful and sublethal levels) on thermal tolerance in adults and larvae of *O. quadricollis* and *O. lejolisii* was studied. Furthermore, thresholds of temperature for escape responses (water emergence and flight) were identified. Significant differences were found in thermal tolerance between species and populations. *Ochthebius quadricollis* exhibited greater thermal tolerance without the effect of saline acclimation. In contrast, *O. lejolisii* showed effects on thermal tolerance at a sublethal salinity, increasing tolerance in larvae and decreasing in adults. Thresholds of temperature for escape responses varied between species in accordance with their physiological tolerance. Salinity affected the thresholds of temperature for water emergence in both species. An additive effect of temperature and salinity on the frequency of emergence and flight was observed in both species. These findings provide valuable information for developing survival models against climate change.

Chapter 2. The realized and fundamental saline niches of adults, larvae, and eggs of *O. quadricollis* and *O. lejolisii* were identified and compared. The realized niche was determined using field data on the abundance of adults and larvae, while the fundamental niche was analysed in laboratory experiments exposing adults, larvae, and eggs to different salinity levels. A discrepancy was found between the realised and fundamental niches. Both

species proved to be euryhaline, tolerating extreme salinities in their natural habitat, especially *O. quadricollis*. In the laboratory, *O. lejolisii* showed greater physiological tolerance than *O. quadricollis* in all life stages. Comparing life stages, both larvae and eggs were more tolerant than adults. These species exhibited a high physiological capacity to withstand extreme salinities, a factor that could be exacerbated by climate change.

Chapter 3. The cross-tolerance of *O. quadricollis* and O*. lejolisii* to salinity and desiccation was evaluated. Adults, larvae, and eggs of both species were acclimated to non-stressful and sublethal salinity levels, followed by exposure to extreme desiccation. Both species showed similar responses to desiccation throughout ontogeny, with larvae and eggs being more resistant than adults. *Ochthebius lejolisii* larvae were more desiccation-tolerant than *O. quadricollis* larvae, while *O. quadricollis* eggs had greater success following acclimation to non-stressful salinity. *Ochthebius lejolisii* eggs maintained similar hatching rates at both salinity levels.

Chapter 4. Two hypotheses were tested in populations of *O. lejolisii* from areas with different thermal variability: the Metabolic Cold Adaptation (MCA) and Climatic Variability Hypotheses (CVH). Reciprocal acclimation experiments under different fluctuating temperature regimes were conducted to evaluate phenotypic plasticity and local adaptation in metabolic rates and thermal limits. The population at higher latitude, featuring a colder climate, showed higher metabolic rates at low temperatures, confirming the MCA. The lower latitude population, with greater climatic variability, demonstrated higher thermal tolerance. Only the higher latitude population exhibited plasticity in its upper thermal limit. These results suggest trade-offs between tolerance and plasticity in the thermal adaptation, increasing the vulnerability of Mediterranean coastal populations against higher latitude Atlantic populations to extreme temperature increases by climate change.

All these findings contribute to understanding the effect of stress factor interactions on the studied species and their responses throughout their life cycle. The differences between species and life stages may be related to the different microenvironmental niches each species occupies.

RESUMEN

La ecofisiología es un área de investigación donde se utilizan parámetros fisiológicos para cuantificar la interacción entre el ambiente externo e interno de un organismo. Dentro de la ecofisiología, la fisiología comparativa estudia las diferencias y similitudes en cómo los cuerpos de los organismos de distintas especies cambian dependiendo de su interacción con su entorno, proporcionando información valiosa sobre su evolución y ecología. En los últimos años, la fisiología comparativa ha ido ganando reconocimiento científico debido a su capacidad de evaluar los impactos potenciales del cambio global sobre la biodiversidad, y consecuentemente, en la dinámica y relaciones de las especies con sus ecosistemas.

La mayoría de los ectotermos, como los insectos y crustáceos, son especialmente adecuados para el estudio de las respuestas fisiológicas a la variación ambiental debido, principalmente, a que: a corto plazo los ectotermos pueden responder a condiciones cambiantes mediante la "plasticidad fenotípica", lo que significa que pueden cambiar ciertos aspectos de su morfología, fisiología o comportamiento para adaptarse a las circunstancias; y a largo plazo, los ectotermos pueden adaptarse con el ajuste de sus respuestas fundamentales al medio ambiente o alterando el grado de plasticidad que exhiben. Esto significa que, a lo largo de generaciones, los ectotermos pueden evolucionar para adaptarse mejor a las condiciones cambiantes, ya sea a través de variaciones en su comportamiento o en sus características físicas. Además, en ectotermos, hay una evidente influencia del clima con la distribución de las especies, con una fuerte relación entre las variables climáticas y la abundancia y distribución de insectos.

Por ello, la ecofisiología ha puesto el foco en el estudio de las respuestas de las especies a la variabilidad e imprevisibilidad ambiental, la intensidad de las condiciones extremas y la frecuencia y duración de condiciones específicas. En este contexto, los organismos que viven en ecosistemas con condiciones ambientales extremas y cambiantes como algunos hábitats costeros (estuarios, zonas intermareales, etc.) representan un excelente modelo para abordar estas cuestiones.

Es el caso de las pozas rocosas de la zona supralitoral, zona situada por encima de la línea de marea alta y cubierta por agua sólo durante las tormentas. Estos hábitats son considerados como uno de los más extremos, especialmente en la costa mediterránea, donde

las bajas mareas y al oleaje moderado hacen que dichos hábitats estén más aislados del mar durante períodos prolongados. Como resultado, los organismos que habitan estos hábitats fragmentados y muy heterogéneos sufren temperaturas extremas más altas y fluctuaciones térmicas más rápidas en comparación con otras zonas litorales más expuestas al mar.

Junto con la temperatura, los principales factores abióticos que determinan las comunidades presentes en las pozas rocosas supralitorales del mediterráneo son la salinidad y la desecación. La facultad de los ectotermos para ajustar de manera flexible los mecanismos fisiológicos en respuesta a los cambios en la temperatura ambiental (p. ej., plasticidad fenotípica o aclimatación) determina su capacidad para amortiguar el efecto de la variación ambiental. Además, la temperatura ambiental juega un papel clave en la determinación de la distribución geográfica de los ectotermos ya que a menudo se correlaciona con sus límites superior e inferior de tolerancia térmica y sensibilidad fisiológica. En las pozas rocosas supralitorales, las sequías suelen estar precedidas por un aumento de la salinidad a medida que desciende el nivel del agua, por lo que muchos organismos acuáticos que viven en estos ambientes están sometidos de forma secuencial y repetidamente a estrés por salinidad y desecación. Si bien la mayoría de las respuestas fisiológicas a cada uno de los factores ambientales se han estudiado de forma aislada en organismos de zonas costeras o poco profundas, estos factores estresantes actúan simultáneamente. En un entorno natural, pueden concurrir múltiples factores estresantes, o cada factor estresante puede exhibir exposiciones continuas, cíclicas o recurrentes, lo que resulta en una multitud de escenarios de exposición ligeramente diferentes, cada uno de los cuales puede provocar respuestas fisiológicas únicas. Los estudios experimentales que involucran múltiples factores estresantes realizados hasta ahora indican que sus efectos dependen de la naturaleza específica de los mismos, así como de su intensidad y duración relativas.

Así, esta tesis compara las respuestas ecofisiológicas a múltiples estresores de tres especies de escarabajos acuáticos del género *Ochthebius*: *O. quadricollis*, *O. lejolisii*, y *O. subinteger* (Familia Hydraenidae), exclusivas de pozas rocosas supralitorales. El objetivo principal ha sido determinar la capacidad y estrategias de los diferentes estados del ciclo vida de estas especies para afrontar condiciones extremadamente estresantes y predecir su viabilidad en el marco de los cambios ambientales esperados en el contexto del cambio climático. Para ello se emplearon enfoques experimentales comparativos al objeto de analizar

las similitudes y diferencias en las respuestas de las especies en sus diferentes etapas del ciclo de vida a temperaturas extremas, salinidad, desecación y sus interacciones. De forma complementaria, en una de las especies objetivo y a nivel poblacional, se exploró su adaptación a las condiciones térmicas locales. Esta memoria de tesis se ha dividido en 4 capítulos:

Capítulo 1. El principal objetivo de este capítulo fue determinar la tolerancia térmica de las tres especies de *Ochthebius* utilizando respuestas fisiológicas y de comportamiento de regulación térmica para predecir su sensibilidad al cambio climático. Para ello, se determinó y comparó, entre poblaciones de las tres especies, el límite térmico superior; y se estudió el efecto de la salinidad de aclimatación (a niveles de no estrés y subletal) sobre la tolerancia térmica en adultos y larvas de *O. quadricollis* y O*. lejolisii*. Además, en adultos de estas dos especies, se identificaron los umbrales de temperatura que desencadenan respuestas de escape (emersión del agua y vuelo) bajo estrés salino. Se encontraron diferencias significativas entre las especies y poblaciones en la tolerancia térmica. La especie que mostró mayor tolerancia térmica sin efecto de la aclimatación salina fue *O. quadricollis*. Sin embargo, *O. lejolisii* mostró efectos en su tolerancia térmica a una salinidad subletal, aumentando su tolerancia en larvas y disminuyendo en adultos. Los umbrales de temperatura para las respuestas de escape variaron entre especies en concordancia con su tolerancia fisiológica. La salinidad afectó los umbrales de temperatura para la emersión en ambas especies. Además, se observó un efecto aditivo de la temperatura y salinidad en la frecuencia de emergencia y vuelo en ambas especies. Estos hallazgos proporcionan información relevante para estimar los márgenes de seguridad térmica y desarrollar modelos predictivos de la supervivencia de estas especies ante escenarios de cambio climático actuales y futuros.

Capítulo 2. En este capítulo, el principal objetivo fue determinar el nicho salino de *O. quadricollis* y *O. lejolisii*, para comprender su distribución espacial y su coexistencia, y así predecir su capacidad para enfrentarse a la creciente inestabilidad ambiental provocada por el cambio climático. El nicho realizado se determinó mediante datos de campo sobre la abundancia de adultos y larvas, mientras que el nicho fundamental se analizó con experimentos de laboratorio exponiendo adultos, larvas y huevos a diferentes niveles de salinidad (35, 60, 90, 110, 140 and 170 gL^{-1}). Ambas especies mostraron ser eurihalinas, soportando fluctuaciones extremas de salinidad desde 1.80 hasta casi 140 gL^{-1}, y las larvas de

O. quadricollis en campo soportando hasta 180 gL^{-1}. Sin embargo, la tolerancia fisiológica en el laboratorio fue mayor en *O. lejolisii* que en *O. quadricollis*, pudiendo los adultos de *O. lejolissi* sobrevivir a 170 gL^{-1} durante más de 3 días con un 30% de éxito en la eclosión de huevos, mientras que *O. quadricollis* mostró un máximo tiempo de supervivencia de 2 días y ningún éxito en la eclosión de huevos a esa salinidad. Las larvas y huevos de ambas especies fueron más tolerantes que los adultos. Estas especies mostraron una elevada capacidad fisiológica para soportar salinidades extremas, un factor que podría verse agravado por el cambio climático.

Capítulo 3. Los principales objetivos de este capítulo fueron determinar si la exposición a una concentración salina subletal tenía un efecto protector o perjudicial en una exposición posterior a la desecación en *O. quadricollis* y *O. lejolisii*, y determinar cómo esos efectos varían entre sus diferentes etapas de la vida. Para ello, se evaluó la tolerancia cruzada de *O. quadricollis* y *O. lejolisii* a la salinidad y desecación. Adultos, larvas y huevos de ambas especies se aclimataron a niveles de salinidad de no estrés y subletales y, posteriormente se sometieron a condiciones de desecación a 15 ± 2 % de humedad relativa. Ambas especies mostraron respuestas similares a la desecación en su ontogenia, siendo las larvas y los huevos más resistentes que los adultos. Las larvas de *O. lejolisii* fueron más tolerantes a la desecación que las de *O. quadricollis*, mientras que los huevos de *O. quadricollis* tuvieron mayor éxito, previa aclimatación, a una salinidad no estresante. En cambio, los huevos de *O. lejolisii* mantuvieron tasas de eclosión similares a ambas salinidades. La tolerancia cruzada se encontró sólo en adultos de *O. quadricollis*, que mostraron una mayor supervivencia bajo desecación después de la aclimatación a la salinidad más alta. Las dispares tolerancias al estrés observadas podrían estar relacionadas con la temporalidad de los microhábitats que ocupa cada especie. Estos resultados contribuyen a una mejor comprensión de la interacción entre los factores de estrés ambiental concurrentes y los mecanismos de adaptación en ambientes acuáticos dinámicos y altamente estresantes.

Capítulo 4. El principal objetivo de este capítulo fue determinar la adaptación local y la plasticidad fenotípica en las tasas metabólicas y los límites térmicos en poblaciones de *O. lejolisii* de áreas con diferente variabilidad térmica y se testaron dos de los principales paradigmas de la ecofisiología espacial: la adaptación metabólica al frío (MCA) y la hipótesis de la variabilidad climática (CVH). Para ello, se realizaron experimentos con dos poblaciones

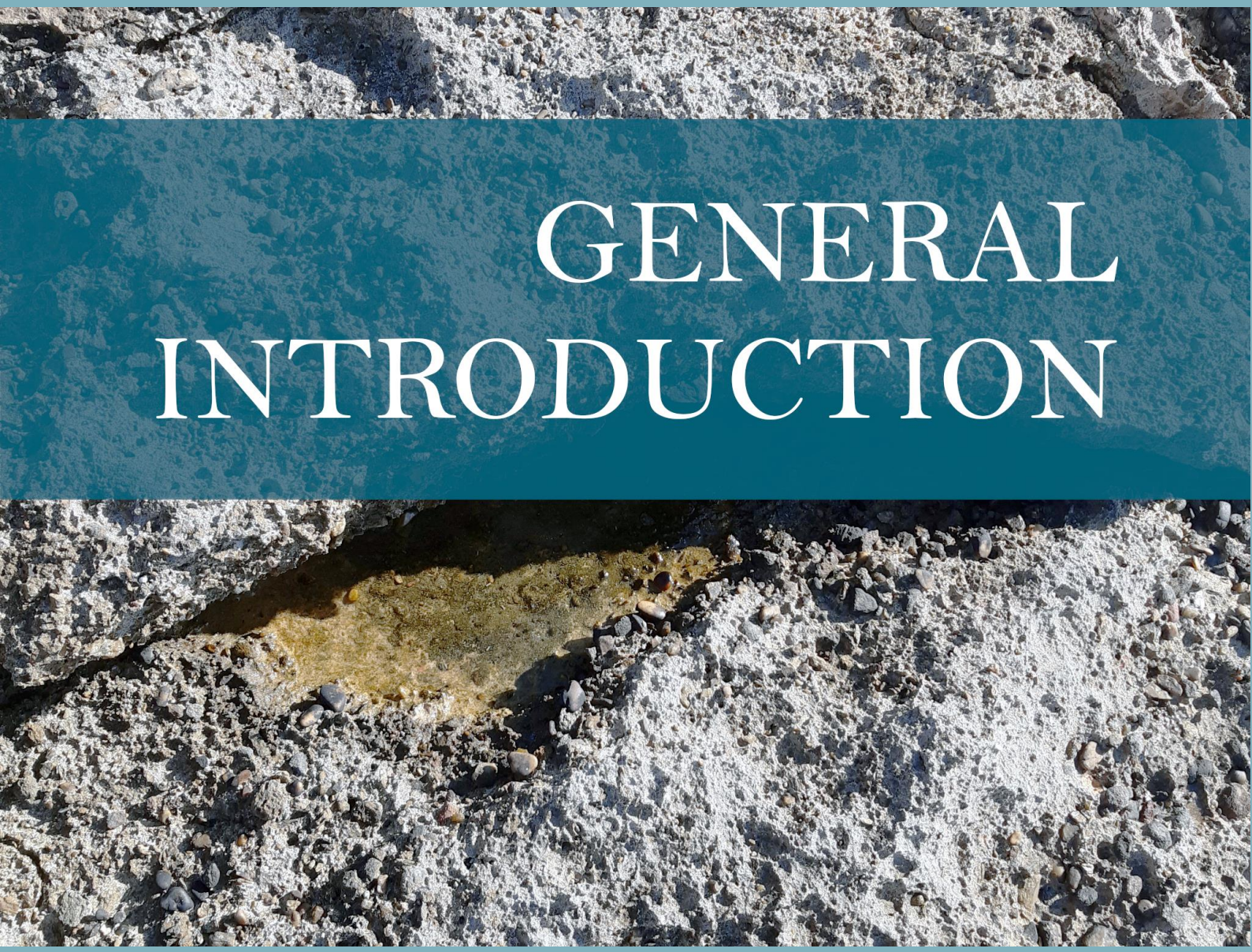
de diferente latitud (sur de Inglaterra y sureste ibérico) que se sometieron a aclimatación recíproca bajo regímenes de temperatura representativos de primavera de cada localidad, incorporando la variación diurna local. Las tasas metabólicas se midieron mediante respirometría cerrada y los límites de tolerancia térmica se estimaron mediante termografía. De acuerdo con la MCA, la población de latitudes más altas (clima más frío) mostró tasas metabólicas y Q10 más altas a temperaturas más bajas que la población de latitudes más bajas. De acuerdo con la CVH, la población de latitudes más bajas (clima más variable) mostró una mayor tolerancia térmica superior, pero solo la población de latitudes más altas pudo aclimatarse a los límites térmicos superiores. Estos resultados sugieren compensaciones entre tolerancia y plasticidad en la adaptación térmica de la especie, por lo que podría aumentar la vulnerabilidad de las poblaciones costeras mediterráneas frente al aumento de temperaturas por el cambio climático.

En general, los hallazgos encontrados durante la elaboración de esta tesis contribuyen a la comprensión del efecto de los factores ambientales de estrés y la interacción de estos, en las especies estudiadas de *Ochthebius*, y sus respuestas ontogenéticas. Se observa un patrón común en las respuestas ontogénicas entre *O. quadricollis* y *O. lejolisii*, donde los estados de vida temprana, huevos y larvas, presentan una mayor tolerancia a los factores de estrés estudiados que el estado adulto. La importancia de estos resultados radica en que la mayoría de los estudios fisiológicos sobre invertebrados marinos muestran una respuesta contraria, presentando una mayor sensibilidad a los factores ambientales de estrés en etapas de vida temprana. Por tanto, estos resultados abren una puerta hacia la investigación de respuestas a lo largo de la ontogenia en organismos que habitan las pozas rocosas supralitorales y la relación de sus respuestas con las adaptaciones evolutivas que hayan podido tener.

Por otro lado, las diferencias entre especies y estados de vida encontrados podrían relacionarse con los distintos nichos microambientales que ocupa cada especie, por lo que es de especial relevancia en estudios ecofisiológicos, considerar la variación ambiental y la estabilidad del hábitat a escalas muy locales (microhábitat), ya que pueden dar determinar diferencias en las respuestas fisiológicas en especies estrechamente relacionadas.

Por último, los hallazgos de esta tesis enfatizan el hecho de que una comprensión más integral de la relación entre la variabilidad climática local, el metabolismo, la tolerancia

térmica y la plasticidad puede mejorar las predicciones de las respuestas de los organismos al cambio climático e identificar qué especies y poblaciones deberían ser el foco de los esfuerzos de conservación específicos.



GENERAL INTRODUCTION

GENERAL INTRODUCTION

Due to the inherently variable external environment, all living organisms must maintain a separate and distinct internal environment. Ecophysiology, or ecological physiology, is an area of research where physiological parameters are used to quantify the interaction between the external and internal environment of the organism (Ferry-Graham & Gibb, 2008). One of the fields of ecophysiology is comparative physiology (Basile et al., 2021), which study the differences and similarities in how organisms' bodies of different species change depending on their interaction with their environment to infer evolution and ecology (Basile et al., 2021). Two of the main approaches used in the study of comparative physiology (Mykles et al., 2010), upon which the theoretical framework of this thesis is based, are: i) vertical integration of physiological processes: referring to understanding how physiological processes are integrated and related across different hierarchical levels within an organism, including the comparison between different developmental stages, such as larvae and other life phases, and; ii) horizontal integration of physiological processes: referring to understanding how physiological processes interact and relate among different organisms within an ecosystem. This involves comparing functions across species to find common physiological principles.

In a context of global change, comparative physiology is gaining recognition for its ability to provide valuable insights into the potential impact of global changes on biodiversity and, consequently, on the dynamics and relationships of species within their ecosystems (Somero, 2011; Basile et al., 2021). By understanding the fundamental mechanisms underlying sublethal and lethal stress and recognizing disparities among species in their ability to manage environmental stressors, it is possible to establish the groundwork for predicting how well or poorly organisms might adapt to alterations in critical abiotic factors, such as temperature, salinity, oxygen levels, and pH, all of which contribute to global changes (Somero, 2011).

Many ectotherms, such as insects or crustaceans, are particularly well-suited for the study of physiological responses to environmental variation, for a number of reasons. They can often be observed in large numbers in the field, can be bred in the laboratory, and the ethical considerations of using these organisms for physiological research are less complicated than for vertebrates (Harrison et al., 2012). Responses and adaptations to changing environmental conditions in ectotherms can principally occur through two non-mutually

exclusive mechanism (Newell, 1976; S. Chown & Nicolson, 2004; Ghalambor et al., 2007): i) in the short term, ectotherms can respond to changing conditions through "phenotypic plasticity," which means they can change certain aspects of their morphology, physiology or behaviour to fit the circumstances; ii) in the long term, ectotherms can adapt through "adaptation," which can manifest adjusting their fundamental responses to the environment or altering the extent of plasticity they exhibit. This means that over generations, ectotherms can evolve to be better suited to changing conditions, either through variations in their behaviour or their physical characteristics.

In ectotherms, the influence of the climate on species distribution is evident, as well as the strong relationships between climatic variables and the abundance and distribution of insects (Chown & Gaston, 1999; Chown & Terblanche, 2006). It has been widely demonstrated that not only the mean conditions of local environments, but also the magnitude of environmental variation at different spatio-temporal scales, is an important selective force for organisms in the wild (Bozinovic et al., 2016). Therefore, ecophysiological research has put a great focus on the study of species' responses to environmental variability and unpredictability (Angilletta, 2006; Kingsolver & Buckley, 2017), the intensity of extreme conditions and the frequency, and the duration of specific conditions (Chown & Terblanche, 2006). In this context, organisms living in ecosystems with extreme and changing environmental conditions (Ferry-Graham & Gibb, 2008), such as some coastal habitats (estuarine or intertidal zones), represent an excellent model to address these questions.

Coastal habitats

The term "coastal habitats" encompasses all types of habitats found within the transition zone between land and sea. Coastal habitats are very dynamic environments due to their simultaneous exposure to terrestrial, oceanic, and atmospheric processes (Parvizi et al., 2022). Although organisms living in these habitats have adapted to withstand frequent changes in sea level, temperatures, salinity, oxygen concentration and desiccation stress (Przeslawski, 2005; Brandes et al., 2015; Hawkins et al., 2023), coastal taxa are often ecologically specialized, frequently restricted to localized habitats (e.g., narrow intertidal bands) and, therefore, are potentially vulnerable to the effects of environmental disturbances. A strong environmental stress gradient occurs perpendicular to shore (Figure 1), related to desiccation,

temperature and salinity, exhibiting their most extreme values towards the upper limit of the littoral zone (Chappuis et al., 2014).

This ecotone has a long history of ecological studies dating back nearly 200 years when Audouin & Edwards (1833) described zoning patterns on the rocky coasts of northern France. Subsequently, studies on these habitats followed a long period of qualitative description and classification of broad distribution patterns of organisms on rocky shores. The way zoning patterns were modified by the influence of tides and wave exposure locally and how they differed geographically were documented in the work of Stephenson & Stephenson (1949, 1972), who proposed a universal zoning scheme (Figure 1). A typical rocky shore can be divided into the supratidal zone (also called the splash, spray, or supralittoral zone), which is above the high-tide line and is covered by water only during storms, and an intertidal zone, which lies between the high and low tidal extremes. Along most shores, the intertidal zone can be separated into the following subzones: high tide zone, middle tide zone, and low tide zone. The low intertidal zone, which borders on the shallow subtidal zone, is only exposed to air at the lowest of low tides and is primarily marine in character. The mid-intertidal zone is regularly exposed and submerged by average tides. The high intertidal zone is only covered by the highest of the high tides and spends much of its time as a terrestrial habitat (Figure 1).

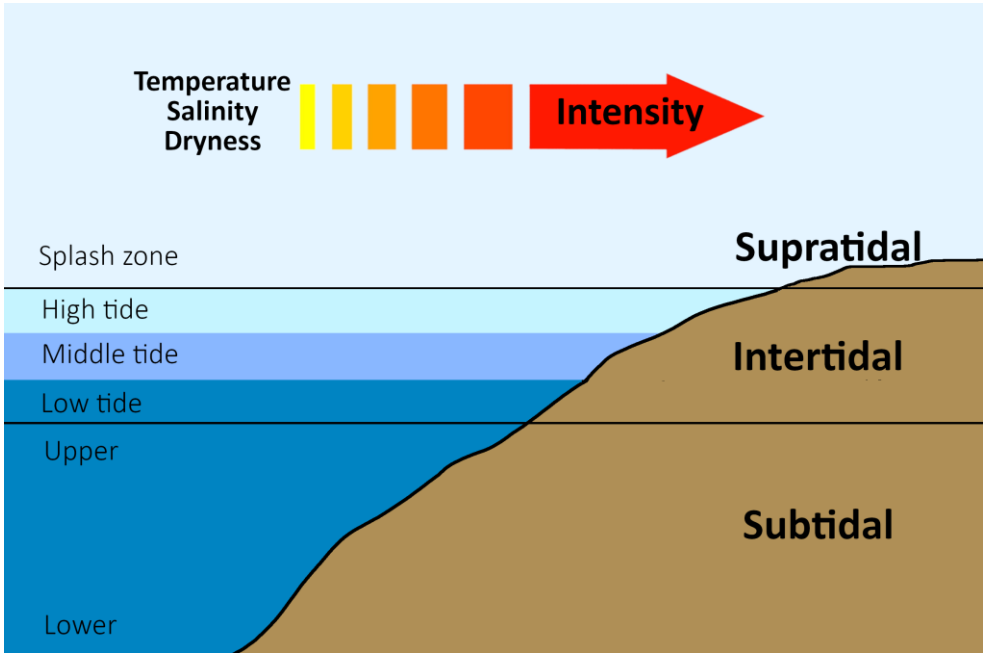


Figure 1. Environmental gradient and zonation scheme in the high coast

In the second half of the twenty century, qualitative studies gave way to more quantitative descriptions of intertidal organism's distribution patterns (Southward & Orton, 1954; Moyse & Nelson-Smith, 1963). In parallel with these descriptions, laboratory experiments were employed to explore the tolerances of algae species (Baker, 2011) and invertebrates (Broekhuysen, 1940; Foster, 1971) from different intertidal zones at different elevations. It was demonstrated that organisms from higher zones were more tolerant to desiccation and temperature than those from lower zones along the coast (see Hawkins et al., 2020). In recent years, these habitats have gained increased attention as they are considered sentinel systems for monitoring broader climate-driven changes in biodiversity and marine ecosystems (Helmuth et al., 2006; Hawkins et al., 2023). Furthermore, these habitats offer several advantages for scientific research such as accessibility and their inhabitants are well-suited models for experimental manipulation given their generally short lifespans and rapid response times. Most algae and sessile and sedentary animals compete for space to live, a two-dimensional resource that is easily quantifiable. These attributes allow for non-destructive sampling and experimentation (Hawkins et al., 2020). However, recognizing the environmental mechanisms that support biodiversity in coastal habitats is a challenge, primarily due to the complexity of the physical processes at the land-sea interface (Parvizi et al., 2022).

Mediterranean supratidal rockpools

Among coastal habitats, supratidal rockpools are recognized as one of the most challenging vital ecosystems worldwide, characterized by rapid changes in harsh environmental conditions (Brandes et al., 2015; Parvizi et al., 2022), especially along the Mediterranean coast, with low to moderate wave energy, small tidal ranges (from 0.9 m at Gibraltar to typically less than 0.2 m in the Balearic Sea), weak tidal currents, and rivers with low or intermittent flow (Furlani et al., 2014). On the Iberian Mediterranean coast, supratidal rockpools form fragmented and highly heterogeneous habitats (Figure 2) with conditions varying along the distance gradient from the sea.

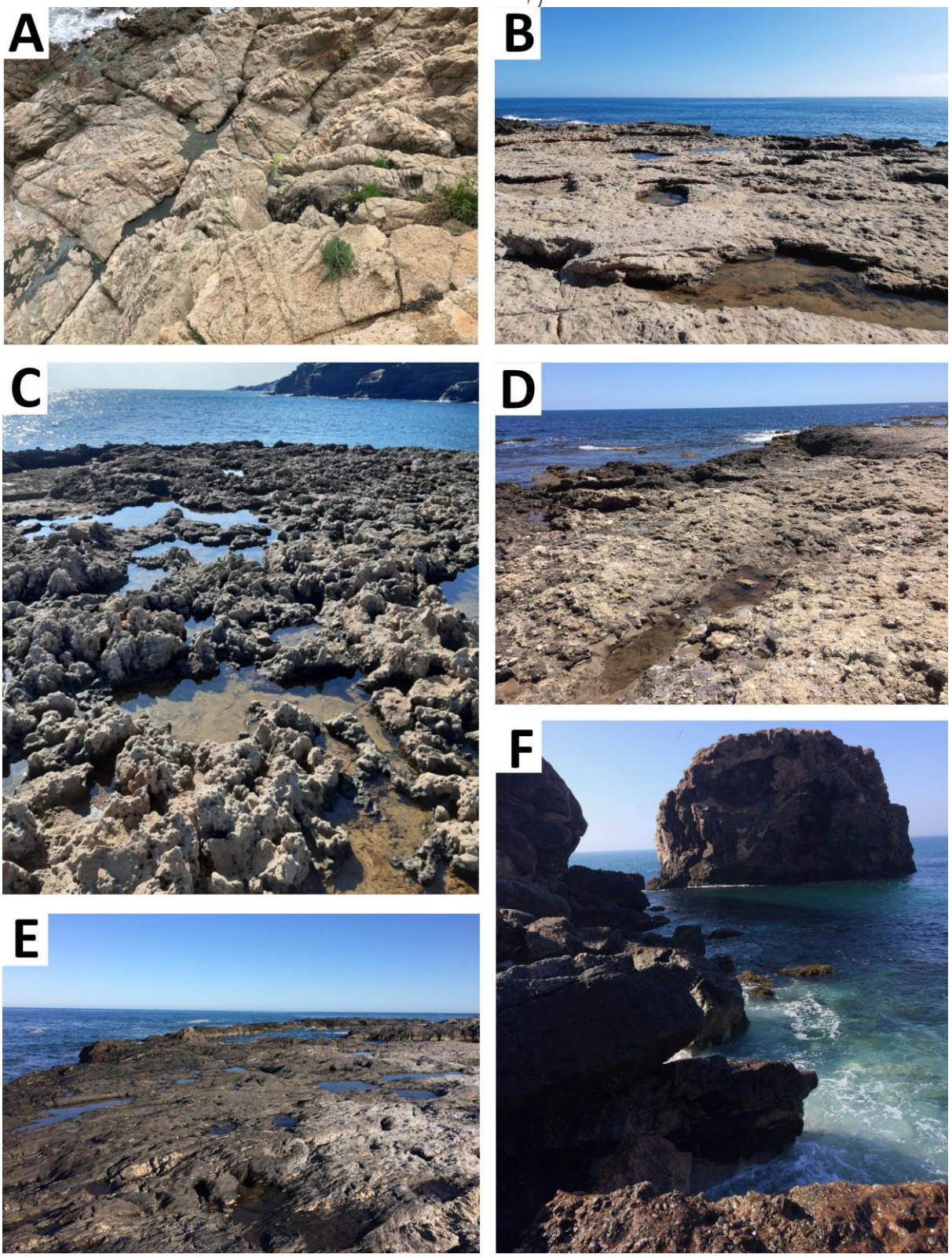


Figure 2. Diversity of habitats of supratidal rockpools along the Iberian Mediterranean coast. A) Blanes, Girona; B) La Illeta, Castellón; C) Cala Reona, Murcia; D) Cabo Cope, Murcia; E) Cala Panizo, Almería; F) Cala de la Rijana, Granada

Conditions range from relatively constant in pools near the sea-line but highly exposed to waves and sea-level changes, to those farther from the coast and, usually, at higher elevations with significant environmental variation (García-Meseguer et al., 2023). Following Margalef´s characterization of Mediterranean coastal rockpools (1949), there are four types of pools based on their distance from the sea (Figure 3):

- Type A. Located close to sea level with relatively easy access to the sea, characterised by small daily thermal variation, high and relatively stable oxygen levels, limited phytoplankton, various alga types and remains of *Posidionia* leaves, as well as no significant detritus accumulation on the bottom.

- Type B. Pools with greater independence from the sea, but protected by rocks, preventing excessive heating and organic matter input, remains of Posidonia leaves, limited phytoplankton, and slight detritus accumulation.

- Type C. Pools somewhat separated from the sea, exposed to insolation, with moderate organic matter input, significant daily thermal variation, oxygen-saturated water during peak insolation hours and accumulation of detritus on the bottom. Generally, no macroscopic algae or remains of Posidonia leaves.

- Type D. Very similar to the previous type but with higher organic matter input and more detritus accumulation on the bottom. They are more ephemeral and remain saturated with salts for a significant part of the year.

As a result of these fluctuating extreme conditions, macroinvertebrate communities of Mediterranean supratidal rockpools are characterised by an exclusive fauna composed of low species richness (García-Meseguer et al., 2023), where Crustacea and Mollusca of marine origin, and Insecta (principally Diptera and Coleoptera) of terrestrial origin, are the dominant inhabitants (Margalef, 1949).

Physiological stressors in Mediterranean supratidal rockpools

In the Mediterranean supratidal rockpools, organisms are more isolated from the sea for extended periods due to the low tides experienced (Furlani et al., 2014). As a result, they suffer higher extreme temperatures and faster thermal fluctuations compared to those in the

intertidal zone (Raffaelli & Hawkins, 1996; Chappuis et al., 2014). Furthermore, since seawater has five times greater heat capacity than air, these thermal challenges are amplified in species inhabiting these zones (Leeuwis & Gamperl, 2022). Considering that body temperature is one of the most important variables in ectotherms and is primarily determined by environmental temperature (Muñoz & Bodensteiner, 2019), we can conclude that heat temperature is, probably, the primary stress factor in supratidal rockpools.

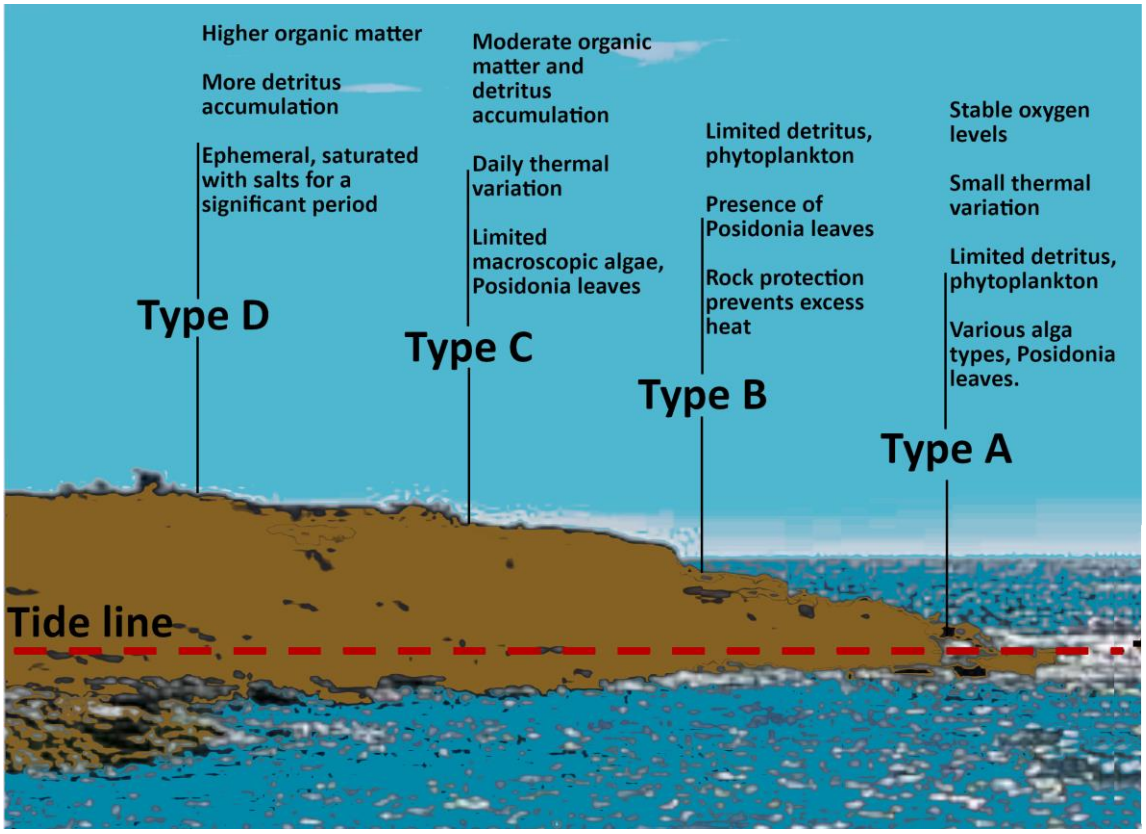


Figure 3. Vertical distribution of the types of pools presents in the Mediterranean supratidal rocky shore regarding their distance to the sea

Practically all aspects of the behaviour and physiology of ectotherms are sensitive to body temperature (Angilletta, 2009). The ability of ectotherms to flexibly adjust physiological mechanisms in response to changes in environment temperature (e.g., phenotypic plasticity or acclimation) determines their capacity to buffer performance and fitness from environmental variation (Seebacher et al., 2015). Also, environment temperature plays a key role in determining the geographic distribution of ectotherms (Pörtner, 2001; Sunday et al., 2012), as it is often correlated with their upper and lower thermal tolerance limits and physiological sensitivity (Sunday et al., 2010).

Alongside temperature, salinity, and desiccation are crucial abiotic factors that constrain aquatic communities in coastal habitats (Ganning, 1971; Ranta, 1982). In supratidal rockpools, droughts are often preceded by an increase in salinity as the water level drops (Hershkovitz & Gasith, 2012), hence, many aquatic organisms living in these environments sequentially and repeatedly face salinity and desiccation stress (Tomanek, 2002). Daily and sometimes hourly changes in water depth and mixing due to waves and wind also influence salinity levels in coastal habitats. Evaporation or droughts can raise the saline concentration well above that of seawater (hypersaline conditions) (Telesh et al., 2013). The initial mechanisms to confront salinity and desiccation challenges are behavioural. These are particularly important for osmoconformers, although osmoregulators also utilize them, likely because they are cost-effective and do not require significant physiological adjustments. Behavioural responses revolve around seeking refuge in a more favourable microhabitat or reducing the exposed surface (Leeuwis & Gamperl, 2022). At a physiological level, both salinity and desiccation disrupt the water and ion balance of internal fluids, both critical for cellular function (Evans, 2019) as both stresses trigger shared physiological mechanisms (Pallarés et al., 2017).

While most physiological responses to each of the environmental factors have been studied isolated (Blewett et al., 2022), in natural environments, especially those organisms in shallow and coastal settings discussed earlier, they are simultaneously exposed to multiple stressors (Petitjean et al., 2019). Results from experiments involving multiple stressors conducted thus far indicate that the effects of multiple stressors depend on the specific nature of these stressors, as well as their relative strength and duration. For instance, in the natural environment, multiple stressors can occur simultaneously, or each stressor may exhibit continuous, cyclical, or recurrent exposures, resulting in a multitude of slightly different exposure scenarios, each of which can elicit unique physiological responses (Gunderson et al., 2016; Velasco et al., 2018; Blewett et al., 2022).

Aquatic beetles of supratidal rockpools as model experimental organisms

Beetles constitute the group of insects with the highest number of known species (Stork, 2018; Bilton et al., 2019), capable of inhabiting most habitats (Parker & Lawrence, 2001; Colado et al., 2022), with representatives widely distributed in all geographic regions. The transition to

aquatic environments from terrestrial ancestors has occurred multiple times independently throughout the evolution of Coleoptera, leading them to develop morphological and behavioural adaptations for various aquatic environments (Jäch & Balke, 2008). This adaptability has made aquatic beetles one of the most specialized groups of insects (Short, 2018), along with dipterans and trichopterans, although the latter two orders do not include aquatic adults. There are more than 13,000 described species of aquatic beetles, which are abundant and ecologically significant in almost all non-marine aquatic habitats, from small ponds to larger lakes and rivers, on all continents except Antarctica. Their extensive geographical and ecological distribution, along with relatively stable and accessible taxonomy, makes these insects excellent models for addressing a variety of ecological and evolutionary questions (Short, 2018; Bilton et al., 2019).

However, while no species with a strictly open marine lifestyle is known, it is understood that some species of beetles are capable of spending at least part of their life associated with seawater, particularly in the ecotone between marine and terrestrial environments (Cheng, 1976). Aquatic beetles, along with some dipterans and heteropterans, are among the few insects capable of tolerating hypersaline waters (Bilton et al., 2019). The genus *Ochthebius* Leach, 1815 (Family Hydraenidae), is one of the few lineages of aquatic beetles capable of living and even diversifying in supratidal rockpools (Villastrigo et al., 2020). This genus included some species restricted exclusively to supratidal rockpools. Villastrigo et al. (2022a) highlighted the presence, of at least, 21 species that are exclusively in this habitat. The extreme dynamics of supratidal rockpools, with continuous and rapid changes, appear to favour their heterogeneity and, consequently, may facilitate many species in the Western Mediterranean region (Villastrigo et al., 2020; Villastrigo et al., 2022a).

In this study, we have focused on three co-occurring species of supratidal rockpool beetles (Figure 4) belonging to the genus *Ochthebius* (Coleoptera: Hydraenidae). These include two cryptic species from the subgenus *Cobalius*: *O. lejolisii* Mulsant & Rey 1861 and *O. subinteger* Mulsant & Rey 1861. The first one is mainly distributed in the Nord Oriental Atlantic coast, but also the southern and eastern Iberian Mediterranean coast; and the second one, appears mainly in the Occidental coast of the Mediterranean basin (Villastrigo et al., 2022). The third species is *O. quadricollis* (Mulsant 1844), which is included in the subgenus *Ochthebius* (Villastrigo et al., 2019), widely distributed in the Mediterranean and Atlantic coast

(Villastrigo et al., 2022a). Despite living exclusively in habitats with challenging environmental conditions, there are few studies on the physiology, ecology, and biology of these species. There are some old observational and experimental data showing the wide thermal and salinity tolerance of *O. quadricollis* in the Mediterranean basin (see Hase, 1926; Margalef, 1949; Jacquin 1956; Balfour-Browne, 1958). Currently, different studies have been carried out in parallel to this thesis, defining the environmental niches of *O. quadricollis* and *O. lejolisii* (García-Meseguer et al., 2023), describing their life cycles (Velasco et al., 2022), phylogeny (Villastrigo et al., 2019) and phylogeographic structure in the western Mediterranean (Villastrigo et al., 2022b). Although these species of aquatic beetles coexist in the same area, they may occupy different environmental niches. For example, *O. quadricollis* is usually associated with pools closer to the sea, experiencing lower environmental fluctuations, whereas *O. lejolisii* is found in more ephemeral pools with significant environmental variations (García-Meseguer et al., 2023). Additionally, *O. quadricollis* has a higher number of generations per year, an overall shorter life cycle, and more successful egg hatching than *O. lejolisii*, which confers greater demographic success. Theses earlier ecological studies showed that even in a very simple but extreme habitat, the species of this genus can show different niches and seasonal patterns. This also means that more species-level investigations are needed to understand the diversity of responses to environmental change of these water beetles. The identification of similar or different responses to stressful environmental factors at different stages of their life cycle is essential to understand the main strategies to live in these habitats and to assess their viability in the face of global change.

Objectives and thesis outline

The present thesis explores the physiological and behavioural responses of aquatic beetles of the genus *Ochthebius* that coexist in Mediterranean supratidal rockpools to cope with the multiple natural stressors and explore their interactions. The main objective was to determine the capacity and strategies of the different life stages of these species to face extremely stressful conditions and to predict the species' viability in the framework of the expected environmental changes, following the objective 2 of the “Rockpools Research Project CGL2017-84157P”. Comparative experimental approaches are employed to investigate response differences and similarities among species and life cycle stages to extreme temperature, salinity, desiccation, and their interactions (Figure 5, chapters 1, 2 and 3,

respectively). Finally, we explored, at the population level, the adaptation to local thermal conditions (chapter 4).

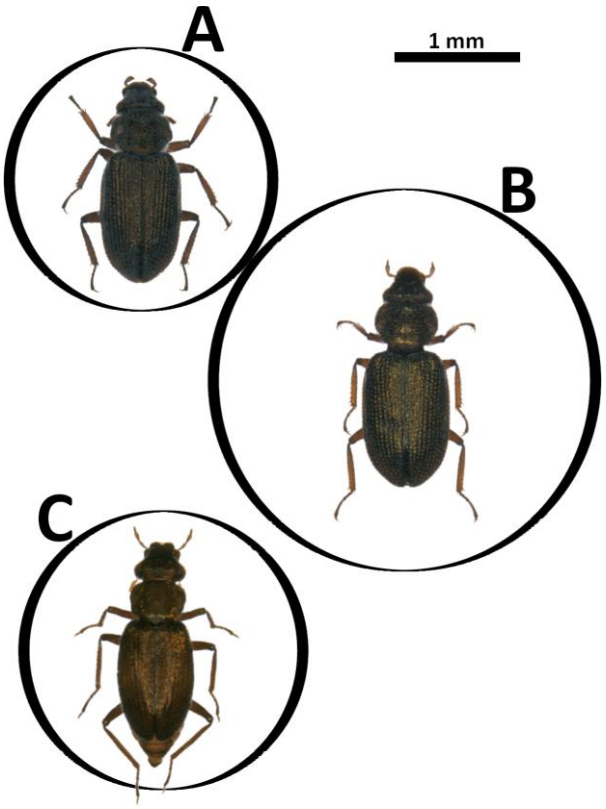


Figure 4. Aquatic beetle species of the genus *Ochthebius* (Family Hydraenidae) studied: A) *O. lejolisii*; B) *O. subinteger*; C) *O. quadricollis*

The specific objectives of each chapter were:

Chapter 1. To determine the thermal tolerance by investigating both physiological and behavioural responses to predict the vulnerability to global warming of three species of *Ochthebius*. Firstly, the inter-specific and inter-populational variation in the heat coma of the adult stage of the focal species were explored. Secondly, for two of the species, the effect of acclimation to salinity on the upper thermal limit was analysed for both adult and larval stages. Finally, for these two species, the temperature thresholds that trigger avoidance responses (water emergence, escape) in adults and how they vary with salinity were quantified.

Chapter 2. To determine the salinity tolerance of two coexisting species of aquatic beetles in supratidal rockpools to predict their viability in the face of expected changes in salinity in their habitats. The study involves comparing the realized salinity niches of adults

and larvae of each species (derived from field distribution data) and the fundamental niches of adults, larvae, and eggs (determined from laboratory experiments on salinity tolerance).

Chapter 3. To determine if cross-tolerance to salinity and desiccation stress arise across life stages (adults, larvae, and eggs) of two *Ochthebius* species: *O. quadricollis* and *O. lejolisii*. We compared various physiological responses after sequential exposure to both stressors.

Chapter 4. To assess the role of thermal adaptation to local environmental conditions, testing both the metabolic cold adaptation and the climatic variability hypothesis in an integrated manner. It explores the effects of acclimation to different thermal regimes on metabolic rates and thermal tolerances of two populations of *O. lejolisii* spanning a wide latitudinal distribution.

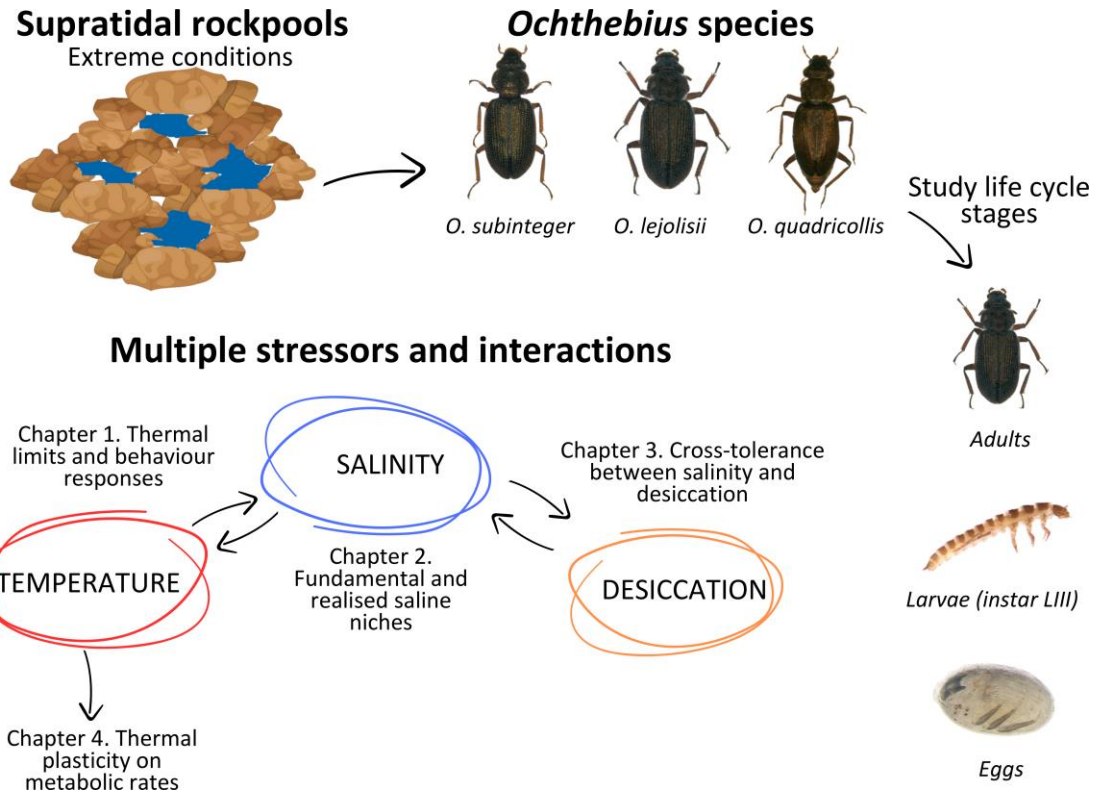


Figure 5. Scheme of thesis structure and the methodological approaches used

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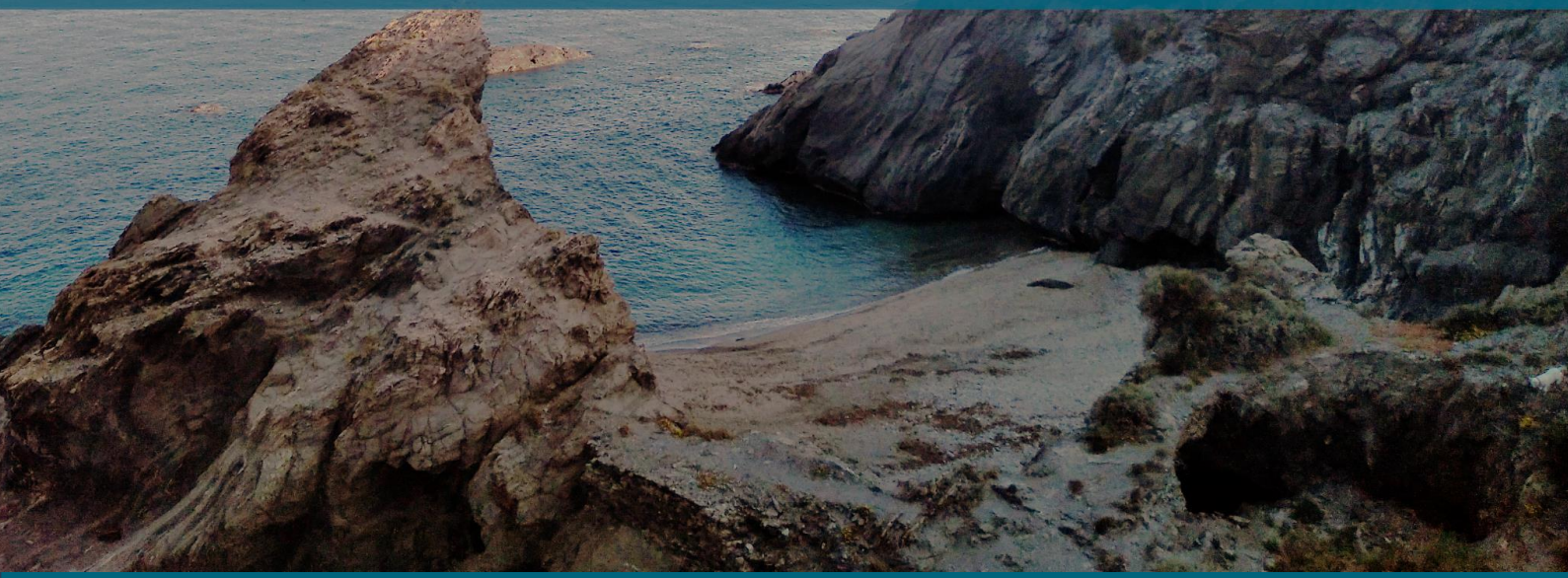
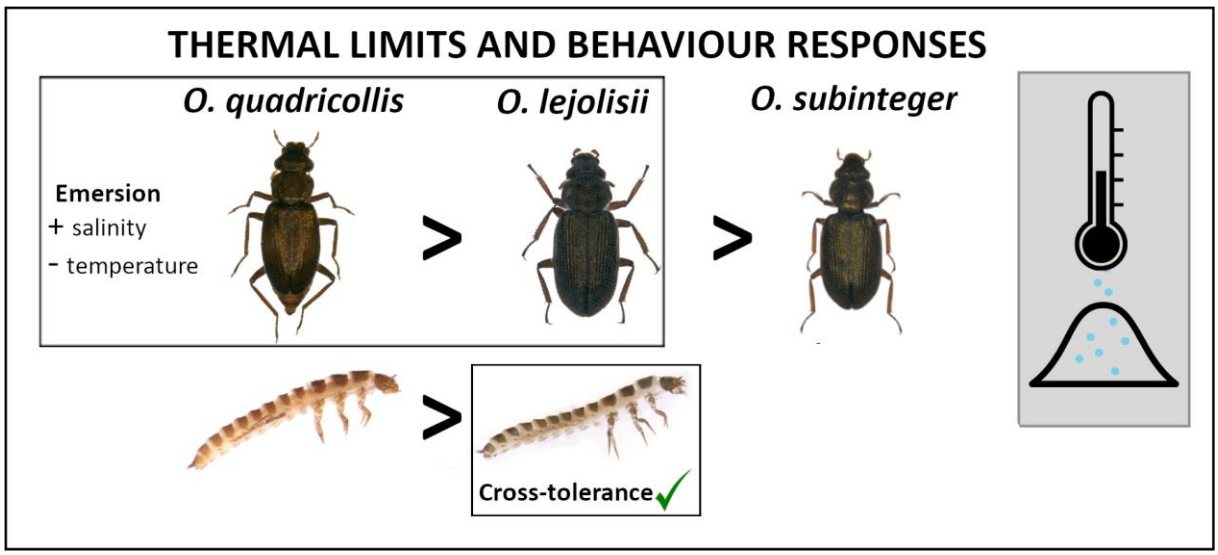


CHAPTER 1: Thermal tolerance differs between co-occurring congeneric beetle species in marine supratidal rockpools

Mirón-Gatón, J.M., Botella-Cruz, M., García-Meseguer, A.J., Millán, A. & Velasco. J. (2022). Thermal tolerance differs between co-occurring congeneric beetle species in marine supratidal rockpools. *Marine Ecology Progress Series 681*: 185-196. https://doi.org/10.3354/meps13916

ABSTRACT

In the context of global change, it is important to know the physiological limits and behavioural responses of species to environmental changes, especially for those living in extreme environments such as supratidal rockpools. Our main study objective was to determine the thermal tolerance of 3 beetle species (*Ochthebius quadricollis*, *O. subinteger* and *O. lejolisii*) co-occurring on the western Mediterranean coast using physiological and behavioural thermoregulatory responses to predict their sensitivity to climate change. To this end, we: i) compared the heat coma (HC) among species and populations; ii) explored the effect of salinity on HC in adults and larvae of *O. quadricollis* and *O. lejolisii*; and iii) determined the temperature thresholds for avoidance responses (water emersion and flight) of adult stages against heat and salinity stressors. We found significant interspecific and interpopulation differences in HC. *Ochthebius quadricollis* larvae and adults were the most heat tolerant, showing similar HC values under different salinity conditions. In *O. lejolisii*, high salinity (90 gL^{-1}) conferred greater thermal tolerance in larvae but lower tolerance in adults. The temperature thresholds for avoidance responses were generally lower than 40 °C, but interspecific variation followed their obtained HC patterns. In both of these species, salinity affected the sublethal temperature thresholds for water emersion. An additive effect of temperature and salinity was observed for the frequency of emergence and flight in both species. Our results provide relevant information for estimating thermal safety margins and developing mechanistic predictive models for the survival of these species when faced with current and future climate change scenarios.



INTRODUCTION

At the land−sea interface, supratidal rockpools are one of the most dynamic extreme environments and are particularly vulnerable to habitat loss by the projected sea level rises caused by global change (Kaplanis et al., 2020). The organisms living in these habitats face severe thermal challenges given their exposure to both aquatic and terrestrial environmental conditions (Vinagre et al., 2015). Hence, coastal rockpools have been considered good model systems for investigating global climate change impacts, and their inhabitants are considered to be sentinels of warming (Helmuth et al., 2006; Kaplanis et al., 2020).

The Mediterranean Sea has tides with low amplitude (averaging a few centimetres) because of the narrow channel connecting with the Atlantic Ocean (Izquierdo & Mikolajewicz, 2019). In these conditions, supratidal rockpools are vastly variable habitats because of the effect of sea spray, waves, wind, precipitation, solar radiation and evaporation. This variability may also increase as a result of global change (Tomanek & Helmuth, 2002; Anadón et al., 2005; Martinez et al., 2012; Legrand et al., 2018), by affecting habitat availability, spatial distribution, water permanence and salinity (Denny & Gaines, 2007; Antonini et al., 2010; Sabatelli et al., 2016). Temperature, salinity, and desiccation are the main abiotic factors that constrain aquatic communities in marine rockpools (Ganning, 1971; Ranta, 1982). Environmental temperature determines species development, fecundity and survivorship, and can impose thermal constraints on the survival of life-cycle stages (Angilletta, 2009). Thermal variation may strongly affect organisms given its direct effects on the rates of physiological and biochemical reactions (Vereshchagina et al., 2016). Rising temperature occurs concomitantly with changes in other stressors, such as salinity and desiccation, which lead to dehydration and osmotic stress and are critical problems in cellular terms (Pallarés et al., 2017). The mechanisms underlying tolerance to thermal and osmotic stresses have a high energetic cost, and complex interactions might arise when organisms are exposed to sublethal levels of both stressors (Sinclair et al., 2013).

Salinity can affect the thermal tolerance of species. Some studies have found that high salinity increased heat tolerance in some aquatic beetle species (e.g. Sánchez-Fernández et al., 2010; Arribas et al., 2012), but it decreased in less salinity-tolerant species (Botella-Cruz et al., 2016). The combined effect of temperature and salinity stressors is usually additive, i.e.

effects equalling the sum of individual effects (Velasco et al., 2018), although a stronger (synergism) or milder (antagonism) effect than the sum of individual effects is also frequent in marine environments (Spicer & Gaston, 1999; Gaston, 2003; Gunderson et al., 2016). As a result of these environmental stressors, macroinvertebrate communities of Mediterranean supratidal rockpools are characterised by an exclusive fauna composed of low species richness (Villastrigo et al., 2020), where Crustacea and Mollusca of marine origin, and Insecta (Diptera and Coleoptera) of terrestrial origin, are the dominant inhabitants (Margalef, 1949).

Although the insects living in this habitat have adapted to tolerate extreme temperature and salinity conditions (Cheng, 1976), most are unable to survive in open habitats by physiological thermal tolerance alone and, therefore, must possess behavioural thermoregulation mechanisms to cope with current and future conditions (Sunday et al., 2014). Beetles from rockpools can move locally among pools by either flying or walking when environmental conditions are unfavourable or by looking for thermal refuges in microhabitats (crevices or below sediments) (Hase, 1926; Jacquin, 1956). Therefore, physiological and behavioural responses influence their survival and species co-existence (Marais & Chown, 2008; Angilletta, 2009; Pallarés et al., 2012a), and both aspects are critical for determining the vulnerability of species to climate warming and extreme events (Williams et al., 2008; Arribas et al., 2012; Sunday et al., 2014).

The adult stages of species from higher intertidal zones, which are more frequently exposed to high air temperatures, are often more tolerant to extreme conditions of temperatures and desiccation than those occurring in lower intertidal zones (Kensler, 1967; Stillman & Somero, 1996; Miller et al., 2009; Mislan et al., 2014). Moreover, thermal stress can differently affect species depending on their life stage. The adult phase can display a considerably higher tolerance threshold than the early benthic phase of the same species (Gosselin, 1997). The interspecific variation in the tolerance thresholds of the early benthic phase is generally related to the upper limit of species intertidal distribution and the microhabitat occupied in this phase (Hamilton & Gosselin, 2020). Thus, it is crucial to know the most temperature-sensitive life stage to determine species vulnerability to climate change (Carbonell et al., 2012; Hamilton & Gosselin, 2020; van der Lee et al., 2020).

Thermal plasticity, defined as any phenotypic response (physiological, behavioural or morphological) to environmental temperatures that alters performance (survival) and probably enhances fitness (Angilletta, 2009), can also play a key role in enabling the persistence of populations (Chevin et al., 2010), and it is a determining factor of species distributions in novel climate landscapes (Barria et al., 2018). However, all species exhibit some genetic variation in thermal physiology terms, which should cause populations to differently respond over space and time, even if their environments are similarly warm (Angilletta, 2009).

Behavioural changes in response to temperature variation often occur earlier than physiological changes (Snell-Rood, 2013) because an organism's activity is constrained to a subset of temperature ranges that they can tolerate physiologically. This results in a decoupling of behavioural and physiological thermal performance curves in species that regularly experience significant fluctuation in their thermal environment (Monaco et al., 2017). Accordingly, studies into lethal (upper thermal limit) and avoidance (e.g. water emergence, flight) responses are relevant for defining the physiological limits of species and the temperature thresholds for avoidance activity (Pörtner & Farrell, 2008; Williams et al., 2008). Empirical estimates of critical thermal limits and acclimation capacity are now available for a range of intertidal species, especially Crustacea and Mollusca (Stillman, 2003; Madeira et al., 2012; Barria et al., 2018; Vinagre et al., 2013, 2015b, 2016, 2018, 2019; Hamilton & Gosselin, 2020); research has also been conducted into the thermoregulatory behaviour (e.g. Monaco et al., 2016) and the relationship between physiological and behavioural traits of these species (e.g. Monaco et al., 2017). However, the thermal physiology of supratidal rockpool insect species has been poorly studied to date (Hase, 1926; Margalef, 1949; Jacquin, 1956), and to our knowledge, no study has examined behavioural traits and their relationships to physiological limits.

This study focused on 3 water-exclusive beetle species (*Ochthebius quadricollis*, *O. subinteger*, and *O. lejolisii*) that are inhabitants of supratidal rockpools (Villastrigo et al., 2019). We had 3 main objectives. Firstly, the main objective was to determine their thermal tolerance by studying both physiological and behavioural responses to predict their vulnerability to global warming. The interspecific and interpopulation variation in the heat coma (HC) of the adult focal species was first explored. Secondly, for *O. quadricollis* and

O. lejolisii, the effect of salinity acclimation on the upper thermal limit in both adult and larval stages was analysed. Finally, for these 2 species, the temperature thresholds that trigger avoidance responses (water emergence, flight) in adults and how they vary with salinity were quantified. The obtained results provide relevant information for developing mechanistic predictive models for the survival of these species when faced with current and future climate change scenarios (Angilletta, 2009) by considering the most sensitive life-cycle stage (Chown & Gaston, 2008; Williams et al., 2008; Wiens et al., 2009; Terblanche et al., 2011). Based on theoretical and empirical data, our predictions were: i) *O. quadricollis*, the most abundant and widely distributed species on the west Mediterranean coast, would have higher thermal tolerance than *O. lejolisii* and *O. subinteger*; ii) interpopulation variation in the HC in *O. quadricollis* would exist between higher-latitude and southern populations; iii) at the species level, the adult stage would be more thermally tolerant than the larval stage; iv) thermal tolerance would increase in high salinity conditions; and v) sublethal temperature thresholds for avoidance responses would differ between species according to their physiological tolerance and would be more sensitive to the salinity effect.

MATERIALS AND METHODS

Study species

The 3 co-occurring beetle species used in this study inhabit west Mediterranean supratidal rockpools and belong to the genus *Ochthebius* (family Hydraenidae). They comprise 2 cryptic species of the subgenus *Cobalius* - *O. lejolisii* Mulsant & Rey 1861 and *O. subinteger* Mulsant & Rey 1861 - and *O. quadricollis* (Mulsant 1844), which is included in the subgenus *Ochthebius* (Villastrigo et al., 2019). *Ochthebius quadricollis* and *O. lejolisii* have an Atlantic−Mediterranean distribution, but the first species is more common on the Mediterranean coast and the latter is found more commonly on the Atlantic coast. *Ochthebius subinteger* is mainly west Mediterranean (Millán et al., 2014) but there are some imprecise records from the coasts of the Black Sea and the Indian Ocean. They have been found to co-exist in the same localities, and even in the same pool on the southeast Mediterranean Iberian coastline (authors' unpublished data).

Very little is known about the physiology, biology, and ecology of the studied species. There are some old observational and experimental data showing the wide thermal and

salinity tolerances of *O. quadricollis* in the Mediterranean Basin (Hase, 1926; Margalef, 1949; Jacquin, 1956). Recent laboratory experiments performed by the authors reveal that *O. quadricollis* adults tolerated higher salinities (lethal concentration at which 50% of the individuals die [LC50] at 7 days and 20 °C; 124.10 gL^{-1}) than *O. lejolisii* (LC50: 89.69 gL^{-1}) (authors' unpublished data). In the study localities, species of the subgenus *Cobalius* were generally found in shallow and ephemeral pools on conglomerate rocks with a compact biofilm of cyanobacteria on the sand, located more distantly from the coastline than those pools occupied by *O. quadricollis*, which lie closer to the sea (Balfour-Browne, 1958; authors' field observations). All the species are macropterous with flight capacity and can move locally among pools by either flying or walking when environmental conditions are unfavourable (Hase, 1926; Jacquin, 1956). *Ochthebius quadricollis* appears to fly more frequently in the field, while *O. lejolisii* is often found burying below pool sediment, where water loss and temperature are lower than on exposed surfaces. Adults and larvae have also been seen walking along the shoreline (authors' field observations).

Study area

Seven Iberian Mediterranean rockpool localities were sampled in spring or autumn (2018 and 2019) to analyse interspecific and interpopulation variations in thermal tolerance (Table 1, Figure 1). The males and females of the representative populations of each species were field collected with soft entomological forceps and brushes. Two nearby southeast localities, with similar climate conditions, were selected to study the effect of salinity on both lethal and behavioural thermal responses: Cala Reona (Cartagena, Murcia Region, Spain) for *O. quadricollis*; and Cabo Cope (Águilas, Murcia Region) for *O. lejolisii*. Adult individuals were collected in spring in both localities. To capture the daily and monthly variations in water temperature in the pools of both localities during an annual cycle, the temperature was monitored every 5 min for 24 h, once a month with data logger sensors (HOBO Pendant® MX Water Temperature Data Logger, MX2201). The obtained data showed a daily temperature range of 23.8 to 36.6 °C in summer and 7.3 to 29.2 °C in winter, and 37.88 °C and 7.25 °C were the absolute maximum and minimum temperatures, respectively (Table 1). Water conductivity was also measured in 10 pools (portable WTW conductivity meter) on each sampling date and in each locality and ranged from 51.6 to 222 mS cm^{-1} (equivalent salinity: 42.3 to 179.8 gL^{-1}) all year long.

Table 1. Coordinates of the study sites (UTM: Universal Transverse Mercator coordinate), maximum air temperature (MxAT) of the warmest month and minimum air temperature (MnAT) of the coldest month (BIO5 and BIO6 respectively; www.worldclim.org), and maximum and minimum water temperatures (MxWT, MnWT) recorded in the warmest and coldest months, respectively. Also shown are conductivity (C) and salinity ranges (S; salinity equivalence is in gL^{-1} using a conversion ratio of 0.82 from conductivity values for seawater; IEEE 1980, Lewis 1981)

Locality	Species	UTM	MxAT (°C)	MnAT (°C)	MxWT (°C)	MnWT (°C)	C ($mS.cm^{-1}$)	S (gL^{-1})
El Playazo (Almería)	*O quadricollis*	30S 588651 4079848	30.4	7.3				
Cabo Cope (Murcia)	*O. lejolisii*	30S 634182 4144025	31.4	5.5	35.82	7.25	31.6 – 234	25.9 – 191.9
Cala Reona (Murcia)	*O quadricollis*	30S 701818 4165830	31.4	6.2	37.88	8.49	19.7 – 222	16.15 – 182
Denia (Alicante)	*O. quadricollis* *O. subinteger*	31S 249045 4303025	30.6	5.1				
El Perelló (Valencia)	*O. quadricollis*	30S 734898 4351084	28.5	4.5				
Sa Posta del Sol (Menorca)	*O. subinteger*	31S 288612 4816775	27.3	4.2				
La Escala (Girona)	*O. quadricollis* *O. subinteger*	31T 511158 4662395	27.1	4.5				

Specimen maintenance

The live adults collected in the field were transported to the laboratory in small aerated aquaria with 2-3 cm of seawater and filter paper as a substrate. In the laboratory, the *O. quadricollis* individuals were morphologically identified and differentiated from the subgenus *Cobalius* species under a binocular microscope. The *Cobalius* species were distinguished by genetic analysis of the mitochondrial COI gene from 3 individuals obtained from each sampled population (authors' unpublished data), which was carried out before the experimental trials. Then, groups of 20 to 25 individuals (males and females) from the different collected species/populations (Figure 1) were acclimated for 7 days to 20 °C and 35 gL^{-1} salinity under a 12 h light:12 h dark cycle in a climate chamber (Sanyo MLR-351). These temperature and salinity conditions are considered non-stressful within the tolerance range of the study species and are used frequently in thermal tolerance experiments with hypersaline aquatic beetles (Sánchez-Fernández et al., 2010; Pallarés et al., 2015; Botella-Cruz et al., 2016; Pallarés et al., 2017). Individuals were fed *ad libitum* with green microalgae *Tetraselmis chuii*. In spring 2019, reproductive pairs of *O. quadricollis* and *O. lejolisii* were collected in Cala Reona and Cabo Cope, respectively, and were reared in the laboratory under the same previously described conditions for egg production and larval development. The last instar larvae (LIII) were used to determine the HC, but behavioural responses could not be studied due to the high mortality of the first instar larvae during moulting to the second instar, especially in *O. lejolisii*.

Heat coma

HC, the temperature at which the individual becomes completely paralysed before dying (Gallego et al., 2016), was considered as the critical thermal maximum (Chown & Nicolson, 2004). A dynamic method was followed at an incremental ramping rate of 1 °C min^{-1}. Fifteen individuals were randomly selected from the previously acclimated adults of each species/ population, washed with distilled water and dried with filter paper. Then, they were glued dorsally to a slide with non-toxic glue (Pritt stick), leaving their legs free to move. Later, they were placed inside a temperature-controlled chamber (BINDER MK53), coupled with a video (Sony DCR-DVD110E) and a thermographic camera (FLIR SC305), to simultaneously record individuals' movement and temperature during the trial. The ramping programme started at

the temperature to which individuals had been acclimated (20 °C) until 65 °C, the end point that exceeded the thermal lethal limits of other studied *Ochthebius* species (Arribas et al., 2012). Thermal images were analysed using ThermaCAM Researcher Professional 2.10 software.

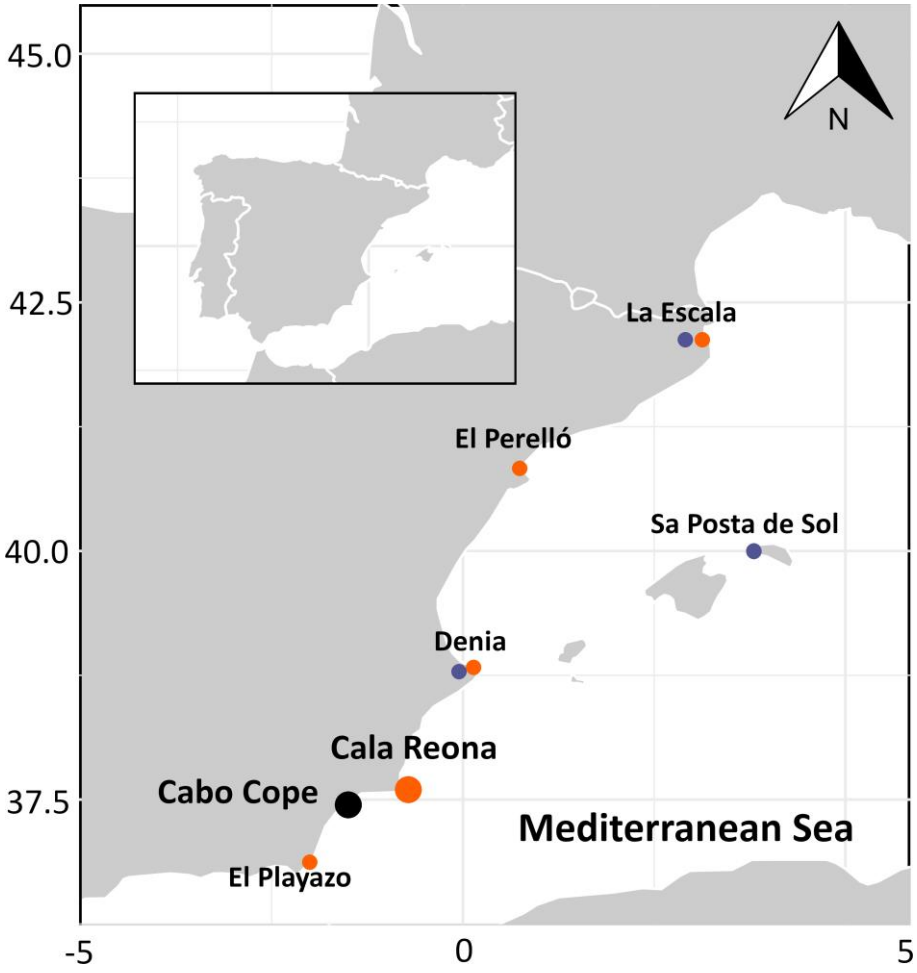


Figure 1. Iberian Mediterranean coast localities where adults of *Ochthebius quadricollis* (orange dots), *O. subinteger* (purple dots) and *O. lejolisii* (black dot) were collected to study their thermal tolerance. Larger orange and black dots correspond to the localities where reproductive pairs of each species were also obtained to rear larvae. Coordinates in decimal degrees

A piece of aluminium foil was placed near individuals to correct reflected temperatures. A cuticle emissivity of 0.95 was used to analyse thermal images, as in other coleopteran species (Gallego et al., 2016). To determine the effect of salinity on HC in the 2 selected species (*O. quadricollis* and *O. lejolisii*), groups of 30 adults and larvae were acclimated at 20 °C for 7 days under a 12 h light:12 h dark cycle at 2 salinities within their tolerance range by representing non-stressful (35 gL^{-1}) and stressful conditions (90 gL^{-1}).

These salt concentrations were representative of the salinity range within which species were present, and the upper value was close to the LC50 estimated in previous salinity tolerance experiments. To estimate the HC, the same previously described ramping methodology was followed. All the salinity solutions were prepared by dissolving an appropriate quantity of sea salt (Ocean Fish, Prodac) in distilled water.

Behavioural responses

In adults, avoidance responses (water emergence and flight) to increased temperature were measured in 2 species (*O. quadricollis* and *O. lejolisii*) after a 7 days acclimation period to the same above-described salinitiy, temperature and light conditions. Ten individuals from each salinity treatment were placed inside a glass container with 100 ml of the corresponding saline solution. An artificial stone stuck to the bottom partially emerged from the water to allow individuals to leave the water or fly. Containers were immersed in a water bath by increasing the temperature at a 1 °C min^{-1} heat rate, starting from 20 °C. Every 5 min, the water temperature, and the number of individuals of each species that emerged, flew or died were recorded. The experiment was replicated 3 times for each species and salinity treatment.

Data analysis

An ANOVA was used to analyse: i) the interspecific (species as a fixed factor) and interpopulation variation in HC (populations of each species as fixed factors); and ii) the effect of salinity acclimation on the selected species' HC according to the life-cycle stage by taking species, stage (larval and adult) and salinity (35 and 90 gL^{-1}), and their interactions, as fixed factors. Bonferroni post hoc tests were used for the pair-wise comparisons of HC between all the combinations of species, populations, and stages. Normality and homoscedasticity assumptions were validated on model residuals by graphical inspection (Zuur et al., 2009). Non-parametric tests (the Kruskal-Wallis test as the equivalent to an ANOVA and the Wilcoxon test as the post hoc analysis) were used to determine the interspecific differences in the threshold sublethal temperatures that triggered the water emergence and flight responses between salinity treatments (35 and 90 gL^{-1}) in *O. quadricollis* and *O. lejolisii* (salinity and species as factors). The isolated effects of temperature and salinity and their interaction on the frequency of the avoidance responses were calculated using effect sizes according to Crain et al., (2008). Non-stressful salinity and temperature (35 gL^{-1} and 20–25 °C) were used as

controls in this way: the effect of the salinity stressor alone (stressful salinity and non-stressful temperature: 90 gL^{-1}, 20−25 °C), the effect of the temperature stressor alone (non-stressful salinity and stressful temperature: 35 gL^{-1}, 40−45 °C) and both stressors combined (stressful salinity and temperature: 90 gL^{-1} and 40−45 °C). The percentages of individuals that emerged or flew with the number of live individuals present in each stressor combination treatment were taken as the variable responses. Individual factors were determined to be statistically significant if the bias-corrected 95% confidence interval did not overlap zero (Hedges & Olkin, 1985). Additive interactions were those whose 95% confidence interval overlapped zero. Synergistic interactions were those in which both individual effect sizes were negative, or one was negative and the other was positive, and the interaction effect size was significantly below zero. Antagonism occurred when the interaction effect size exceeded zero, and at least one individual effect size was negative (Crain et al., 2008). All analyses were carried out using R version 3.6.1 (2019-07-05, R Development Core Team 2019).

RESULTS

Interspecific and interpopulation variation in HC

The ANOVA and post hoc results showed interspecific differences in HC (Table 2A, Figure 2), with *O. quadricollis* more tolerant to higher temperatures (mean ± SE HC: 48.78 ± 0.06 °C) than *O. subinteger* and *O. lejolisii* together (mean ± SE HC: 47.68 ± 0.08 °C). The HC of the *O. quadricollis* adults differed among populations (Table 2B, Figure 2), and the southernmost population was more tolerant (El Playazo, Almería, Spain 49.08 ± 0.14 °C) than the northernmost one (L'Escala, Gerona, 48.3 ± 0.07 °C), with the remaining populations presenting intermediate values. *Ochthebius subinteger* also showed differences in HC among populations, of which the least tolerant was Sa Posta de Sol (Menorca Island, the Balearics, Spain) (Table 2C, Figure 2).

Table 2. ANOVA results of the (A) effect of species on heat coma and (B,C) population effect on heat coma in *Ochthebius quadricollis* and *O. subinteger* adults, respectively. Bold indicates a significant effect

	Origin	d.f.	SS	MS	F	p value
A	Species	2	30.490	15.240	55.760	**<0.001**
	Residuals	96	26.250	0.270		
B	*O. quadricollis* populations	4	3.440	0.860	5.480	**0.001**
	Residuals	47	7.390	0.160		
C	*O. subinteger* populations	2	1.640	0.820	5.780	**0.007**
	Residuals	29	4.110	0.140		

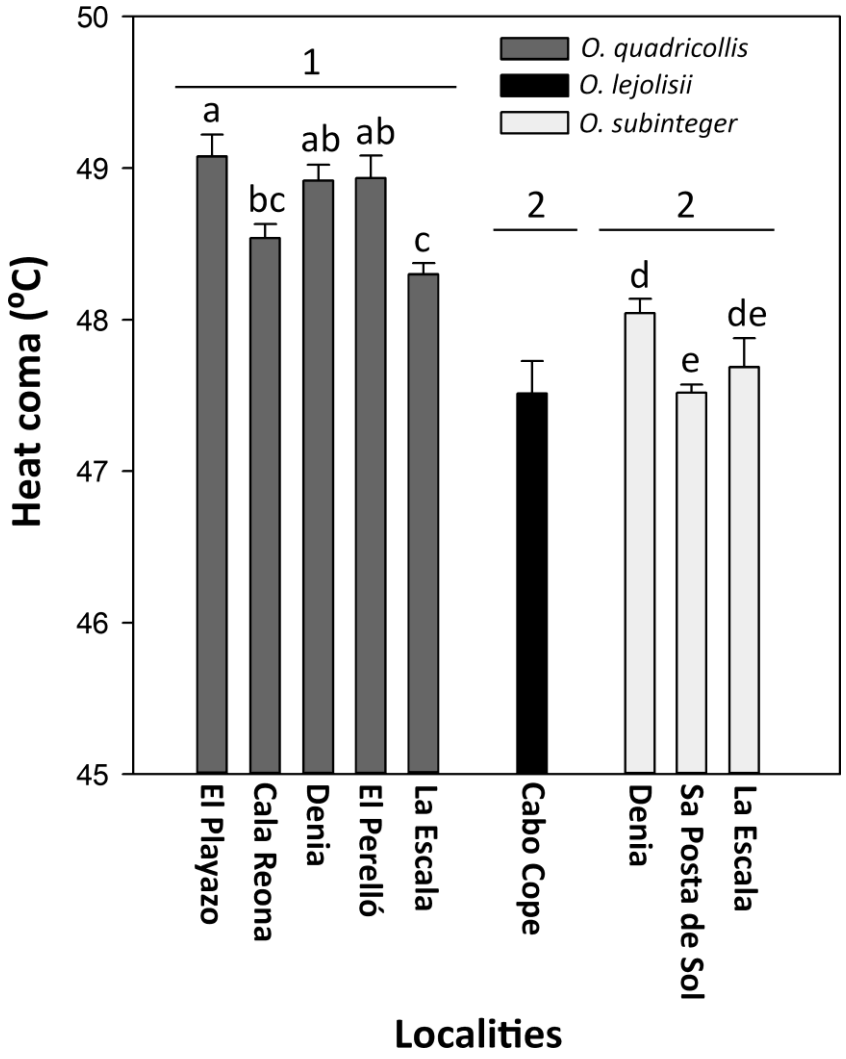


Figure 2. Mean ± SE temperature at which heat coma occurred in *Ochthebius quadricollis*, *O. lejolisii* and *O. subinteger* adults from different locations along the Iberian Mediterranean coast. Bonferroni post hoc significant differences ($p \leq 0.05$) are indicated with different lowercase letters on the bars; different numbers denote differences among species

Effect of salinity acclimation on adult and larval HC

Salinity also had contrasting effects depending on species and life stage (species × stage × salinity interaction, $p < 0.001$; Table 3A). In *O. quadricollis*, salinity had a positive effect on HC, but no differences were found between larvae and adults in the 2 salinity treatments (Table 3B). The HC obtained in the adults and larvae of *O. quadricollis* was higher than 48 °C (Figure 3). However, differences in HC between the adult and larvae stages depending on salinity treatments were observed for *O. lejolisii* (Table 3C). Adults were more tolerant at 35 gL^{-1} (47.84 ± 0.15 °C), and larvae more tolerant at 90 gL^{-1} (47.86 ± 0.18 °C), but the mean HC was lower than 48 °C in both stages and in different salinity treatments.

Table 3. ANOVA results of the (A) effect of species, life-cycle stage, salinity treatment and their interaction on heat coma and (B,C) effect of life-cycle stage, salinity treatment and their interaction on heat coma in *Ochthebius quadricollis* and *O. lejolisii*, respectively. Bold indicates a significant effect

	Origin	d.f.	SS	MS	F	P value
A	Species	1	53.18	53.18	161.15	**<0.001**
	Stage	1	0.32	0.32	0.98	0.324
	Salinity	1	1.12	1.12	3.41	0.068
	Species x stage	1	0.35	0.35	1.05	0.308
	Species x salinity	1	1.15	1.15	3.47	0.065
	Stage x salinity	1	5.95	5.95	18.03	**<0.001**
	Species x stage x salinity	1	8.27	8.27	25.06	**<0.001**
	Residuals	98	32.34	0.33		
B	***O. quadricollis***					
	Stage	1	0.73	0.73	2.06	0.156
	Salinity	1	2.17	2.17	6.11	**0.016**
	Stage x salinity	1	0	0	0	0.994
	Residuals	58	20.59	0.35		
C	***O. lejolisii***					
	Stage	1	0.02	0.02	0.06	0.809
	Salinity	1	0.02	0.02	0.08	0.782
	Stage x salinity	1	14.22	14.22	48.42	**<0.001**
	Residuals	40	11.75	0.29		

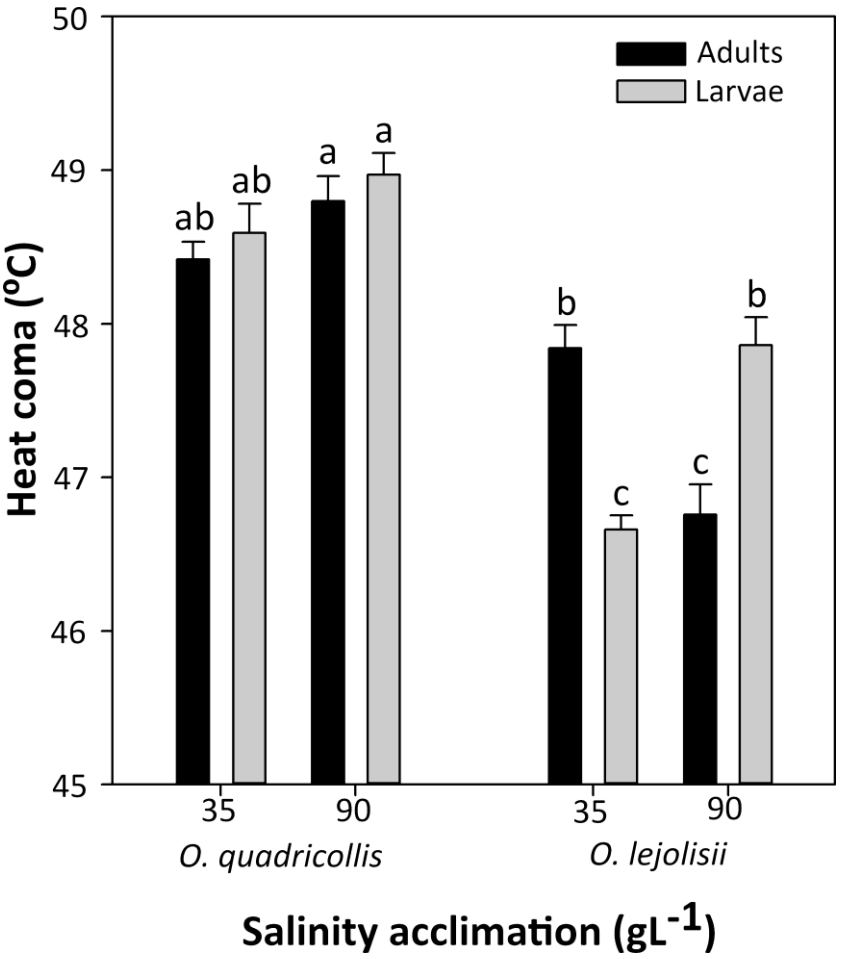


Figure 3. Mean ± SE heat coma of *Ochthebius quadricollis* and *O. lejolisii* adults and larvae acclimatised to different salinities. Bonferroni post hoc significant differences ($p \leq 0.05$) are indicated by different lowercase letters

Behavioural responses to combined thermal and saline stressors

In both *O. quadricollis* and *O. lejolisii*, salinity had a significant effect on the sublethal temperature thresholds for water emersion (*O. quadricollis*: $\chi2 = 6.9$, $p < 0.01$; *O. lejolisii*: $\chi2 = 23.03$, $p < 0.001$). At 90 gL^{-1}, *O. lejolisii* emerged at lower temperatures (35.62 ± 0.81 °C) than *O. quadricollis* (38.42 ± 0.67 °C) (Figure 4), following its lower HC.

However, no effect of salinity was found at the temperature thresholds that triggered the flight response in both species (Figure 4). Our effect size analyses revealed variable temperature and salinity effects on avoidance responses. Warming (40−45 °C temperatures) increased the frequency of avoidance responses (flight and emergence) in both species. Higher salinity (90 gL^{-1}) decreased flight frequency in *O. quadricollis* and increased flight

frequency in *O. lejolisii* and had no effect on the emergence of either species (see Table S1 in the Supporting information). In addition, the joint effect types, based on the effect size estimates of the 2 stressors on avoidance responses, were positive (increase) and additive in all cases.

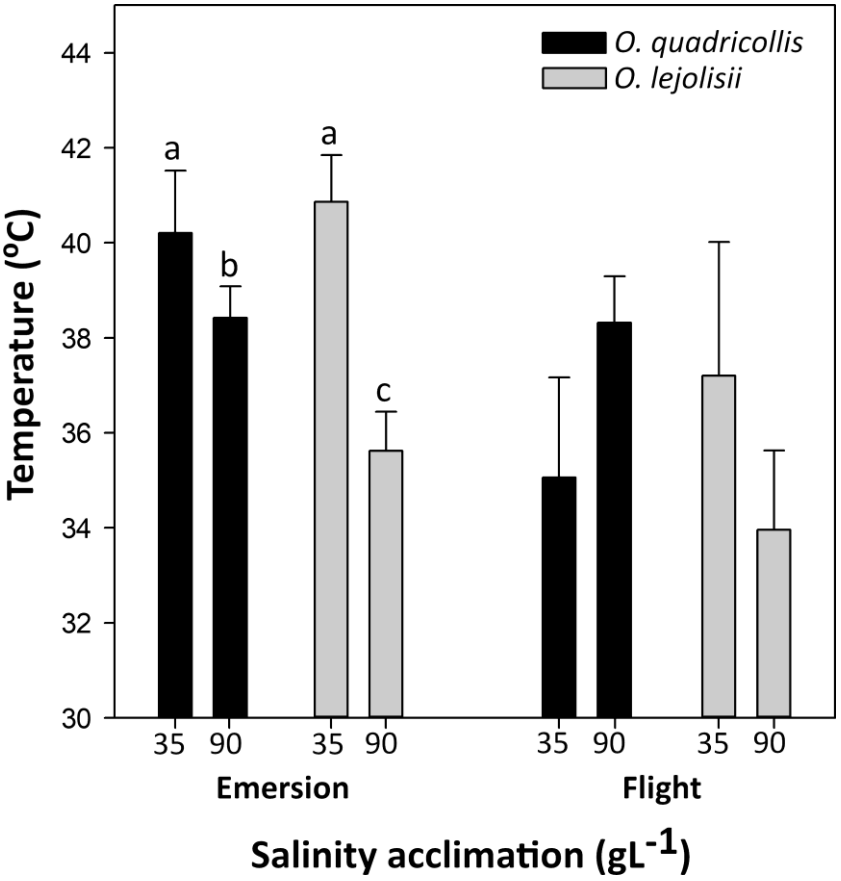


Figure 4. Mean ± SE sublethal temperature thresholds at which *Ochthebius quadricollis* and *O. lejolisii* adults emerged and initiated flight during different salinity treatments. Wilcoxon significant differences ($p \leq 0.05$) are indicated by different lowercase letters

DISCUSSION

In accordance with the high-water temperatures and the wide thermal variation of Mediterranean supratidal rockpools, the studied *Ochthebius* species showed very high HC values (47−49 °C). The thermal tolerance differed among species, populations and life-cycle stages, as did the salinity effect, despite them being able to coexist in the same habitat. The temperature thresholds for the avoidance responses in our thermal tolerance experiments were generally lower than 40 °C, but the interspecific variation followed the obtained HC patterns, i.e. the threshold temperatures of the behavioural responses were higher in

O. quadricollis than in *O. lejolisii*. These thermal tolerance dissimilarities could promote the co-existence of the species of this genus and may also reflect their geographic range and their different vulnerability to climate change.

Interspecific and interpopulation variations in thermal tolerance

Ochthebius quadricollis, the most widely distributed species on our study's spatial scale (the Spanish Mediterranean coast), was the most heat tolerant. This species display clear interpopulation variation in heat tolerance, and an increasing HC along the north–south coastal gradient following environmental thermal variation. *Ochthebius subinteger*, despite a more restricted distribution in the northern half of the Spanish Mediterranean coast, showed interpopulation differences in heat tolerance, but did not display a geographic pattern, which could be due to the short climatic gradient considered in this work. The lower thermal tolerance found in *O. lejolisii* could be related to ancestral adaptation to low temperatures in those rockpools subjected to high tides, in coincidence with the main Atlantic distribution of this species (Millán et al., 2014). In summary, although the study *Ochthebius* species can coexist in many locations on the Mediterranean coast, their dissimilar geographical distribution and isolation of their populations might have conferred them different physiological tolerances related to ancestral and recent thermal variation in their habitats. Thus, the local responses of each species must be considered when predicting global warming impacts (Angilletta, 2009). The HC values reported herein were similar to those found when applying the same experimental approach to other species of the genus, such as *O. glaber* (47.98 °C) and *O. notabilis* (48.61 °C), inhabitants of Mediterranean hypersaline streams and wetlands, respectively (Arribas et al., 2018). These values exceeded not only the average lethal limit known for insects (41.6 °C, Araújo et al., 2013), but also those obtained for different groups of coastal marine organisms from temperate and tropical areas at a lower heating rate of 1 °C 15 min^{-1} (Vinagre et al., 2018, 2019). So, although our values are likely overestimated because of faster ramping rates (Terblanche et al., 2007; Jørgensen et al., 2019), the temperature tolerance of *Ochthebius* species, of terrestrial origin, seems higher than that of species of marine origin, probably due to the higher thermal extremes on land than on sea (Pinsky et al., 2019).

Thermal sensitive of larval and adult stages and their response to salinity

Previous studies on the thermal limits of aquatic saline beetle species have shown that acclimation conditions (temperature, salinity and their interaction) can significantly influence their thermal limits (Sánchez-Fernández et al., 2010; Arribas et al., 2012; Botella-Cruz et al., 2016). Our results indicate that the thermal sensitivity of the life-cycle stages of the studied species could be related to their different salinity tolerance. A better osmoregulation capacity can enhance thermal tolerance by osmolyte accumulation in the haemolymph, and a consequent reduction in protein denaturation during heat stress (Harada et al., 2011), which in turn, would provide salinity and heat cross-tolerance (Pallarés et al., 2012). Adults and larvae of *O. quadricollis*, the most tolerant species to temperature and salinity, showed similar HC values under different salinity conditions. In *O. lejolisii*, high salinity (90 gL^{-1}) conferred greater thermal tolerance in larvae but lower tolerance in adults. The beneficial effect of salinity on heat tolerance in the LIII of *O. lejolisii* can be explained by its higher salinity tolerance compared to the adult stage (at 90 gL^{-1}, larvae survived a mean of 330 h vs. 86 h for adults; authors' unpublished data). Furthermore, the different thermal response to salinity of *O. lejolisii* larvae compared with those of *O. quadricollis* could be a result of different evolutionary lineages. In fact, *O. lejolisii* belongs to the *Cobalius* subgenus, with terrestrial representation in the case of *O. serratus* Rosenhauer, 1856, typically found in coastal wetlands. In contrast, all species included in the *quadricollis* gr. are exclusively from supratidal rockpools (see Villastrigo et al., 2019). The plastic thermal response to salinity confers *O. lejolisii* larvae higher resistance against stressful conditions when pools dry out or when they move to terrestrial microhabitats (e.g. crevices) to enter the pupae stage. This increase in heat tolerance was observed in other intertidal species of Mollusca and Crustacea that resort to different microhabitats during ontogeny (Hamilton & Gosselin, 2020). However, the negative effect of salinity on thermal tolerance in the *O. lejolisii* adults was probably because its tolerance limit came close to the tested salinity stress (LC50, 7 days: 89.69 gL^{-1}). Although the plastic response to temperature acclimation has not yet been examined in the studied species, we expected a loss of ability to acclimatise in species with high absolute temperature tolerances, such as that observed in congeneric species of intertidal porcelain crabs (Stillman, 2003). This can pose a serious problem in the climate change context; for example, species whose habitat and climate preferences come close to their upper thermal limit are unlikely to

develop higher physiological temperature tolerance, which makes them more sensitive to climate change (Tomanek, 2008; Araújo et al., 2013).

Behavioural responses

Limited physiological plasticity can be compensated for by behavioural plasticity to buffer organisms from experiencing environmental variation, and by potentially reducing selection pressure on other phenotypic physiological traits (Bogert, 1949; Huey et al., 2003; Gunderson & Stillman, 2015; Gunderson et al., 2016). In the laboratory, *O. quadricollis* and *O. lejolisii* displayed increasing avoidance responses (water emergence, flight) with rising temperature, just like other saline species of the genus such as *O. glaber* and *O. notabilis* (Pallarés et al., 2012). The water emersion response was more sensitive to the salinity increase than the flight and lethal responses. At 90 gL^{-1}, the emergence response in *O. lejolisii* initiated at lower stress thresholds (35.62 ± 0.81 °C) than *O. quadricollis* (38.42 ± 0.67 °C), according to the lower HC. These water temperature thresholds are reached frequently in the studied rockpools in summer (Table 1). Hence, the emergence response provides information about the sublethal stress limits that organisms can tolerate under combined temperature and salinity stressors. Although the synergistic effect of multiple stressors is widespread in marine systems (Crain et al., 2008; Harvey et al., 2013; McBryan et al., 2013; Przeslawski et al., 2015), in our study, an additive effect (the combined effect equalling the sum of the effects of each stressor in isolation) of temperature and salinity was observed on the water emergence and flight of both species. Temperature, salinity, and desiccation are co-occurring stressors in supratidal rockpools, and *Ochthebius* species are frequently exposed to stressful conditions that come close to their physiological limits, in which thermoregulatory behavioural responses can increase their survival and fitness in more favourable thermal refuges. Field observations of adults of *O. quadricollis* flying and colonising new favourable pools, and larvae and adults of *O. lejolisii* burying below pool sediment or in crevices where water loss and temperature are lower than on exposed surfaces, are examples of these species' thermoregulatory responses.

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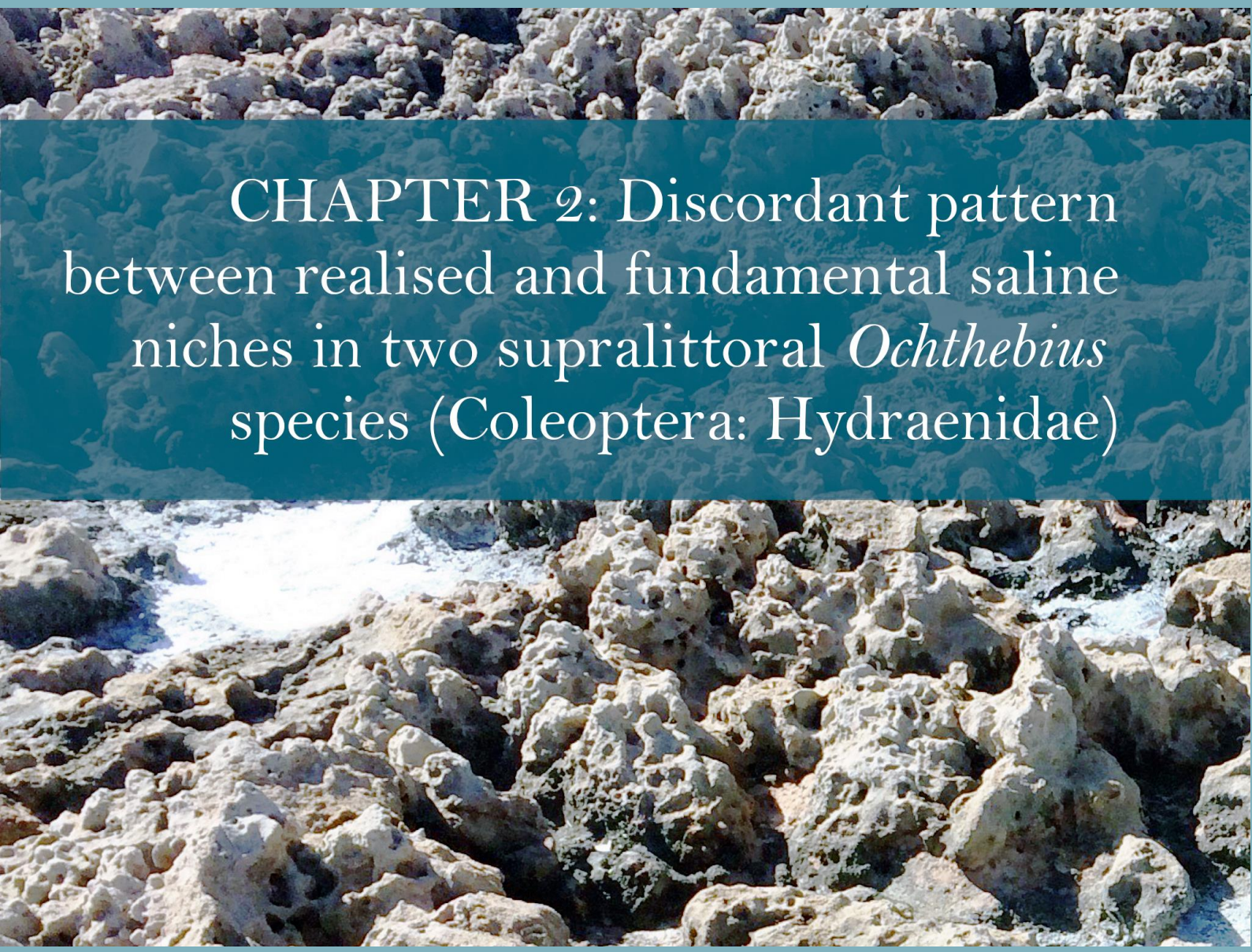
SUPPORTING INFORMATION

Table S1. Individual and interaction effects of salinity and temperature on the frequency of behavioural responses. Salinity × temperature treatments are as follows: control: 20−25°C, 35 gL^{-1}; salinity stressor (A): 20−25°C, 90 gL^{-1}; temperature stressor (B): 40−45°C, 35 gL^{-1}; salinity × temperature stressors (A×B): 40−45°C, 90 gL^{-1}. da: individual effect of salinity; db: individual effect of temperature; dA: main effect of salinity; sA2: variance of the salinity main effect; dB: main effect of temperature; sB2: variance of the main effect of temperature; dAB: interactive effect of salinity × temperature; sAB2: variance of the main effect of interaction; CI: confidence interval; CIlow: lower confidence interval limit; CIup: upper confidence interval limit; Add (additive): sum of the individual salinity and temperature effects (da + db); Int-class: sign of individual effects

Species	RV	da	db	dA	sA2	dB	sB2	dAB	sAB2	CI	CIlow	CIup	Add	Int-class	Interaction type
O. quadricollis	Emergence	0.00	1.55	0.30	0.33	2.04	0.38	0.59	1.35	2.28	–1.69	2.87	1.55	0+	Additive
O. quadricollis	Flight	–0.36	0.46	0.13	0.33	1.06	0.35	1.09	1.38	2.30	–1.22	3.39	0.09	–+	Additive
O. lejolisii	Emergence	–0.01	0.81	0.02	0.33	0.95	0.34	0.06	1.33	2.26	–2.20	2.32	0.81	–+	Additive
O. lejolisii	Flight	0.24	0.80	-0.05	0.33	0.59	0.34	-0.63	1.35	2.28	–2.91	1.64	1.04	++	Additive

Individual effects reflect the response to one stressor alone in relation to the control. Main effects represent the individual stressor effect plus its contribution to the interaction effect, calculated in the presence and absence of the other stressor. Interaction effects are classified using effect sizes according to Crain et al. (2008): additive interactions are those whose 95% confidence interval (95% CI) include a zero value. Synergistic interactions are those in which both individual effect sizes were negative, or one is negative and the other positive, and the interaction effect size is significantly smaller than zero. Antagonism occurred when the interaction effect size is greater than zero and at least one individual effect size is negative.

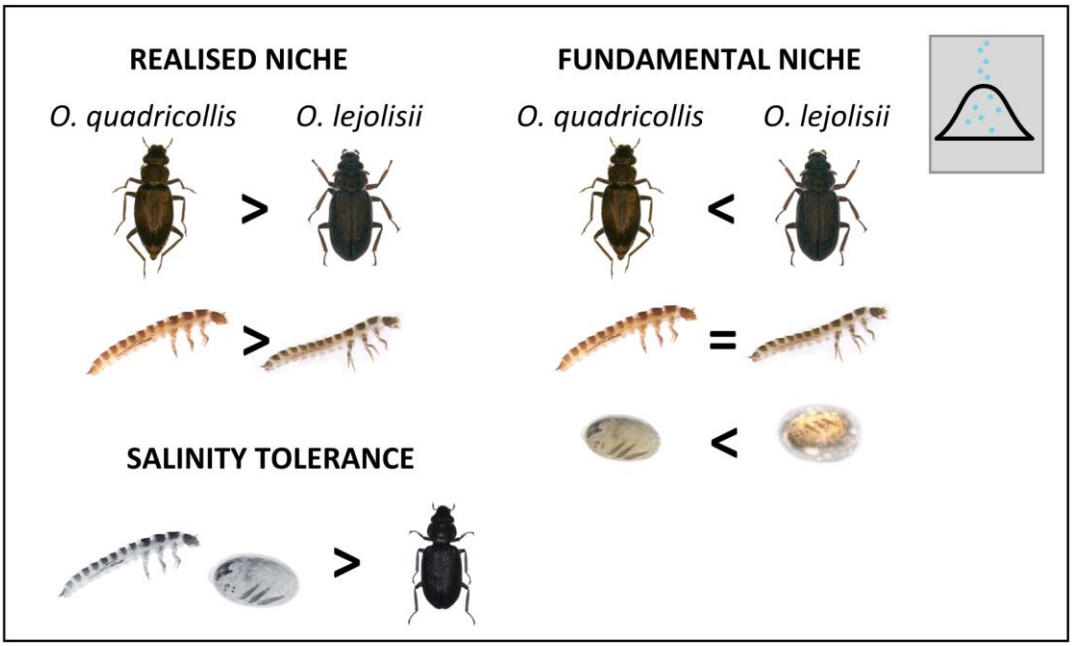


CHAPTER 2: Discordant pattern between realised and fundamental saline niches in two supralittoral *Ochthebius* species (Coleoptera: Hydraenidae)

Mirón-Gatón, J.M., Botella-Cruz, M., García-Meseguer, A.J., Millán, A. & Velasco. J. (2023). Discordant pattern between realised and fundamental saline niches in two supralittoral *Ochthebius* species (Coleoptera: Hydraenidae). *Ecological Entomology* *48*(3): 284-294. https://doi.org/10.1111/een.13220

ABSTRACT

Determining the saline niche of the congeneric and co-existing species that inhabit supralittoral rockpools subjected to extremely fluctuating saline conditions is a crucial concern to understand their spatial distributions and occurrence, and to predict their ability to face increasing environmental instability due to climate change. Therefore, we compared the realised and fundamental saline niches of *Ochthebius quadricollis* and *O. lejolisii* (Coleoptera: Hydraenidae) on the Mediterranean coast of the Iberian Peninsula. A realised niche was determined using field data on the abundance of the adults and larvae of both species. A fundamental niche was addressed with laboratory experiments by measuring the survival of the adults and larvae and the egg-hatching success exposed to different salinity treatments (35, 60, 90, 110, 140 and 170 gL^{-1}). Our study found a discordant pattern between realised and fundamental niches. Both species are euryhalines and support extreme salinity fluctuations from 1.80 to almost 140 gL^{-1}, and *O. quadricollis* larvae in the field withstand up to 180 gL^{-1}. However, physiological tolerance was greater in *O. lejolisii* than in *O. quadricollis*, and *O. lejolissi* adults survived in the laboratory at 170 gL^{-1} for more than 3 days with 30% egg-hatching success, whereas *O. quadricollis* showed a maximum survival time of 2 days and no egg-hatching success at that salinity. The larvae and eggs of both species were more tolerant than adults. The two studied species exhibited high physiological capacity to tolerate extremely fluctuating salinity, which will be exacerbated by climate change.



INTRODUCTION

Fluctuating salinity is one of the major natural stress factors for aquatic biota because it determines their habitat suitability and distribution (Lambret et al., 2021). In land-sea transition zones, such as supralittoral rockpools, salinity fluctuation is intense because of a wide variation range from freshwater to concentrated seawater as a result of the combined effect of marine and terrestrial water inputs (Telesh et al., 2013; Vinagre et al., 2015). This splash zone is also influenced by wave action and wind, with surf and salt spray wetting rocks. These fluctuations correlate with other environmental changes (Ganning, 1971). Thus, increasing salinity, temperature, dissolved oxygen and UV occur as aerial exposure time prolongs (McAllen et al., 1998; Telesh et al., 2013; Mirón-Gatón et al., 2022), and is more marked on the Mediterranean coast due to low tide amplitude (Izquierdo & Mikolajewick, 2019). During drier seasons, Mediterranean rockpools can be salt-saturated for several days to months, and concentrations can exceed 200 gL^{-1} (author´s field data). These fluctuations may be exacerbated by the climate change effect (Smale et al., 2019; Kaplanis et al., 2020; Balogh & Byrne, 2021), and by other non-climatic drivers, such as infrastructure development, agriculture, industrialisation and anthropogenic habitat degradation (Newton et al., 2012; IPCC, 2019, 2021; Cavraro et al., 2022). For all these reasons, rockpools have been considered good model systems for investigating climate change impacts and their inhabitants as sentinels of global warming (Kaplanis et al., 2020). In these habitats, organisms are physiologically adapted to cope with drought and osmotic stress, mainly to the increase of salinity in drying pools. Therefore, studies of tolerance responses of the species living in these extreme environments have shown considerable interest in them for ecological and conservation purposes.

The macroinvertebrate communities that inhabit rockpools are characterised by poor species richness of exclusive halotolerant and halophilic fauna (Villastrigo et al., 2020), where Crustacea and Mollusca of marine origin, and Insecta (Diptera, Coleoptera and Hemiptera) of continental origin, are the dominant inhabitants (Margalef, 1949). Remarkably, some species show extreme euryhalinity and cope with rapid transitions between freshwater and saltwater (McAllen et al., 1998; Harrison et al., 2012). They must possess effective hypo-osmoregulation mechanisms to live in hyperosmotic media, such as reduced extracellular water loss, maintaining body water content by drinking, ion excretion (Pallarés et al., 2015; Velasco et al.,

2018), and hyper-osmoregulatory capability in dilute media (Bradley, 2009).

Despite the importance of physiological salinity tolerance studies and their relations with vertical zonation patterns, most current knowledge has been acquired by focusing on intertidal organisms (e.g., Hoyaux et al., 1976; McMahon, 2003; Iwabuchi & Gosselin, 2020), and very little attention has been paid to supralittoral species. Of them, crustacea have been the most widely studied, such as some harpacticoid copepod species of *Tigriopus* Norman (e.g., Ranade, 1957; McAllen et al., 1998; McAllen & Taylor, 2001; Bonello et al., 2018), the crab *Armases miersii* (Rathbun) (e.g., Charmantier et al., 1998; Anger et al., 1996, 2000; Torres et al., 2007), and several species belonging to the genus *Ligia Linnaeus* (e.g., Todd, 1963; Wilson, 1970, Zhang et al., 2016). Other salinity tolerance studies have been conducted on mollusc littorinid species (e.g., Sundell, 1985; Muraeva et al., 2017) and, to a lesser extent, on insects, like those carried out on the larvae of some mosquito species (Margalef, 1949), the collembola *Anurida maritima* (Guerín) (Witteveen & Joosse, 1987), and the beetle *Ochthebius quadricollis* (Hase, 1926; Jacquin, 1956).

Ochthebius (Coleoptera: Hydraenidae) is one of the most specious water beetle genera (more than 500 species) to live across the complete salinity gradient (Millán et al., 2014; Villastrigo et al., 2019, 2020), and only a few are known to spend their entire life cycle in supralittoral rockpools worldwide (Sabatelli et al., 2016; Villastrigo et al., 2022), and different congeneric species are able to co-exist in the same pools (Mirón-Gatón et al., 2022; Villastrigo et al., 2022). As comparative studies into the thermal tolerance (Mirón-Gatón et al., 2022) and life cycle (Velasco et al., 2022) of two co-existing species (*O. quadricollis* and *O. lejolisii*) in the western Mediterranean coast have recently highlighted thermal niche and life-cycle differences, we also expect them to display differences in salinity tolerance. These and other potential differences in niche specialisation can help to understand their spatio-temporal co-existence patterns and to assess the viability of the populations of both species when faced with present and future global changes (Bozinovic, Calosi & Spicer, 2011; Carbonell et al., 2012). We applied the Grinellian concept of niche, where the fundamental niche is defined as the range of abiotic conditions that influences a species' positive intrinsic growth rates (physiological tolerance) and, within it, the species can only effectively occupy a subset of the entire range of favourable conditions, which is referred to as the species' realised niche (Soberón, 2007; Arribas et al., 2019).

The aim of the present study was to compare the realised and fundamental saline niches of water beetles *O. quadricollis* and *O. lejolisii* to predict their viability against expected changes in salinity in their habitats. Our specific objectives were to: i) define from field distribution data the saline realised niches of adults and larvae in each species; ii) define the fundamental niches of the adults, larvae and eggs of both species with the data obtained from salinity tolerance laboratory experiments; iii) determine the concordance between the realised and fundamental niches of both life-cycle stages and species. We predicted that *O. quadricollis* would show greater salinity tolerance than *O. lejolisii* in concordance with its greater thermotolerance (Mirón-Gatón et al., 2022), and the eggs and larvae of both species would be more halotolerant than adults due to their null or lower mobility, respectively. We also expected to find some differences between the realised and fundamental saline niches in the studied species and stages due to the diverse response to the combined effect of salinity and other environmental stressors in natural media.

MATERIALS AND METHODS

Studied species

The two co-occurring Hydraenidae species, *Ochthebius lejolisii* Mulsant & Rey 1861, and *O. quadricollis* Mulsant 1844, represent different evolutionary lineages among the *Ochthebius* that have colonised supralittoral rockpools (Villastrigo et al., 2019, 2022). *Ochthebius lejolisii* is a cryptic species of the subgenus *Cobalius*, while *O. quadricollis* is included in the subgenus *Ochthebius* (Villastrigo et al., 2019). Both species have an Atlantic-Mediterranean distribution, and *O. quadricollis* is commoner on the Mediterranean coast and *O. lejolisii* is on the Atlantic coast (Villastrigo et al., 2022). In the study localities, both the adults and larvae of *O. lejolisii* were found generally in shallow and ephemeral pools with a compact cyanobacteria biofilm on sand or rock, generally located more distantly from the coastline than those pools occupied by *O. quadricollis* (Balfour-Browne, 1958; authors' field observations), which lie closer to the sea. Eggs were not usually detected in the field due to their small size (mean size of 0.6 mm), but were easily observed to be adhered to hard surfaces (stones, filter paper) in the laboratory.

Field sampling and realised niche

The adults and larvae of both species were sampled in autumn and spring (October 2018 and

May 2019), which is when species abundance was higher (Velasco et al., 2022) to record their abundance in the 11 localities from the southern and south-eastern Spanish Mediterranean coast (Figure 1, Table S1). Both larvae and adults were collected with soft forceps from 10 random pools in each locality following a gradient of distance to the sea level by recording the salinity gradient that they occupied. Water electrical conductivity, as a surrogate of salinity, was measured in each pool with standard portable equipment (WTW conductivity meter).

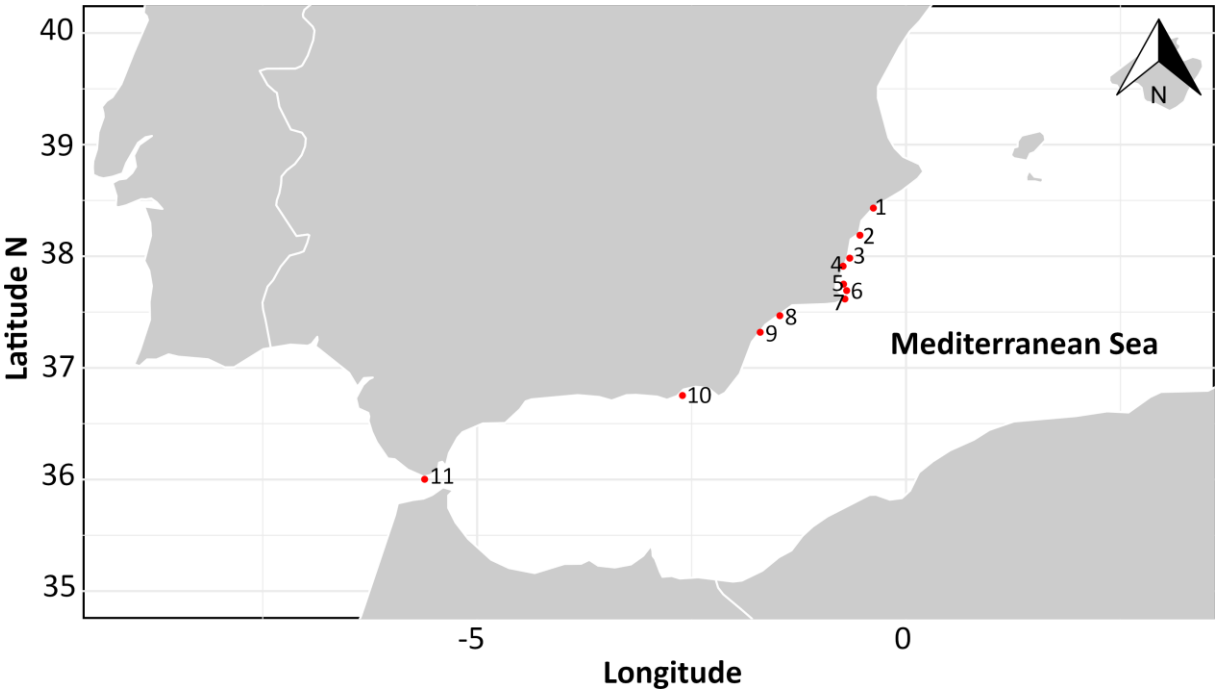


Figure 1. *Ochthebius quadricollis* and *O. lejolisii* rockpool sampling locations along the Spanish Mediterranean coast

The saline realised niche of each stage and species was assessed by applying the Outlying Mean Index (OMI) analysis (Dolédec et al., 2000) following the niche procedure in ADE-4 (Thioulouse et al., 1997). The OMI, or Species Marginality Index, measures the distance between the mean habitat conditions used by species (species centroid) and the mean habitat conditions of the sampling area (origin of the niche hyperspace). An OMI analysis places species under habitat conditions. Species' position depends on their niche deviation from a reference, which represents neither the mean nor the most abundant species, but a theoretical ubiquitous species that tolerates the most general habitat conditions (i.e., a hypothetical species uniformly distributed under habitat conditions). This analysis also calculates niche breadth (tolerance) as a measure of the amplitude in the distribution of each species along the sampled gradient (Carbonell et al., 2012; Céspedes et al., 2013).

Laboratory tolerance experiments

The saline fundamental niche of each species was obtained from the laboratory experiments as survivorship curves for adults and larvae, and as hatching success for eggs, along a salinity gradient. The living *O. quadricollis* adults were collected in Cala Reona, and those of *O. lejolisii* in Cabo Cope (Region of Murcia, Spain) in summer and autumn 2020. Individuals were transported to the laboratory in small aerated aquaria with 2−3 cm of seawater and filter paper as substrate. In the laboratory, both species were morphologically identified under a binocular microscope (Leica M165C with a Leica MEB10 fibre optic illuminator) and placed in a climatic chamber inside different aquaria with water at 35 gL^{-1} salinity, 20 °C temperature and a photoperiod of 12 h light:12 h dark. These salinity, temperature and photoperiod conditions were considered to be non-stressful and were more prone to the reproduction of both species (Mirón-Gatón et al., 2022). Individuals were weekly fed green microalgae *Tetraselmis chuii* Butcher *ad libitum*, and water was renovated every 2 weeks for egg production and larval development.

Salinity tolerance of larvae and adults

Six water salinity treatments (35, 60, 90, 110, 140 and 170 gL^{-1}) were selected according to the range of salinities frequently measured in pools. All the salinity solutions were prepared by dissolving an appropriate quantity of sea salt (Ocean Fish, Prodac, Cittadella, Padua, Italy) in distilled water. The third instar larvae (LIII) and adults of each species were collected from the breeding aquaria. For each treatment, one specimen was placed inside a plastic recipient with 40 ml of saline solution (n=10 for each salinity treatment) and was maintained in a climatic chamber under the above-described temperature/light conditions. Each recipient contained a small piece of filter paper used as substrate for larvae. Adults were placed inside empty tea bags, with a corner emerged from the water to permit them to breathe atmospheric oxygen, but to prevent them from escaping. During previous experiments, a behavioural escape response in adults was observed, which spent long periods outside water to avoid stress salinity. So bags keep individuals in contact with saline water, and provide more accurate estimates of the lethal salt concentration. Mortality of individuals was measured every 24 h for 1 week in adults and for 2 weeks in larvae due to larvae's greater salinity tolerance compared to adults.

Kaplan-Meier survival curves (Altman, 1992) were used to estimate the conditional survival probabilities for each salinity treatment of the larval and adult stages of each species. Survival curves were compared between salinity treatments by the log-rank (Mantel-Cox) test (e.g., Folguera et al., 2011; Kefford et al., 2012). Kruskal-Wallis tests and post hoc analyses by the BH (Benjamini-Hochberg) p-adjustment method were used to determine interspecific and intraspecific variation in survival time at different salinities.

Salinity tolerance of eggs

We collected *O. quadricollis* and *O. lejolisii* eggs from their respective breeding aquaria. We selected 2 or 3 day-old eggs, distinguishable by being a greyish colour and with no differenced embryos. For each salinity treatment, one egg was added to an Eppendorf tube with 1 ml of saline solution (n=10 per salinity treatment). Eggs were left inside the climatic chamber under the same temperature and light conditions as adults and larvae. Egg hatching was checked on experiment days 7 and 14. A logistic regression model (logit model) and the post hoc analyses with the BH p-adjustment method were used to determine intraspecific and interspecific variation in hatching success at different salinities. All the analyses were carried out with the R software, version 3.6.1 (2019-07-05, Development Core Team, 2019).

RESULTS

Realised niche of larvae and adults

The electrical conductivities of the sampled rockpools ranged from 2.19 to 234 $mS.cm^{-1}$ (1.80 to 192 gL^{-1} equivalent salinity). Both species were found over most of this range, but the *O. lejolisii* adults and larvae were located in the pools with the lowest conductivity (2.19 $mS.cm^{-1}$), while the *O. quadricollis* adults and larvae remained in pools with higher conductivities, and larvae were found up to 221 $mS.cm^{-1}$ (Table 1). The *O. lejolisii* larvae obtained the highest OMI and niche breadth values (Table 1 and Figure 2), while the *O. quadricollis* adults had the lowest niche breath values and less deviation from the most general conductivity conditions of the sampling sites. In this latter species, the niche breadth values of the larvae and adults were similar, but there were differences in the niche breadth between the O. lejolisii larvae and adults.

Table 1. Conductivity (C) and salinity ranges (S; salinity equivalence expressed as gL^{-1} using a conversion ratio of 0.82 from conductivity values for seawater; IEEE, 1980, Lewis, 1981) of natural habitats. Outlying mean index (OMI) and niche breadth (Tol) of the studied stages of the *Ochthebius* species along the conductivity gradient

Species	Stage	C ($mS.cm^{-1}$)	S (gL^{-1})	OMI	Tol	P value
O. quadricollis	adults	2.19 – 182	1.8 – 149	< 0.01	0.64	0.93
	larvae	8.27 – 221	6.78 – 181	0.06	0.77	0.28
O. lejolisii	adults	2.19 – 168.40	1.8 – 138	0.08	0.94	0.08
	larvae	2.19 – 168.40	1.8 – 138	1.12	1.73	0.05

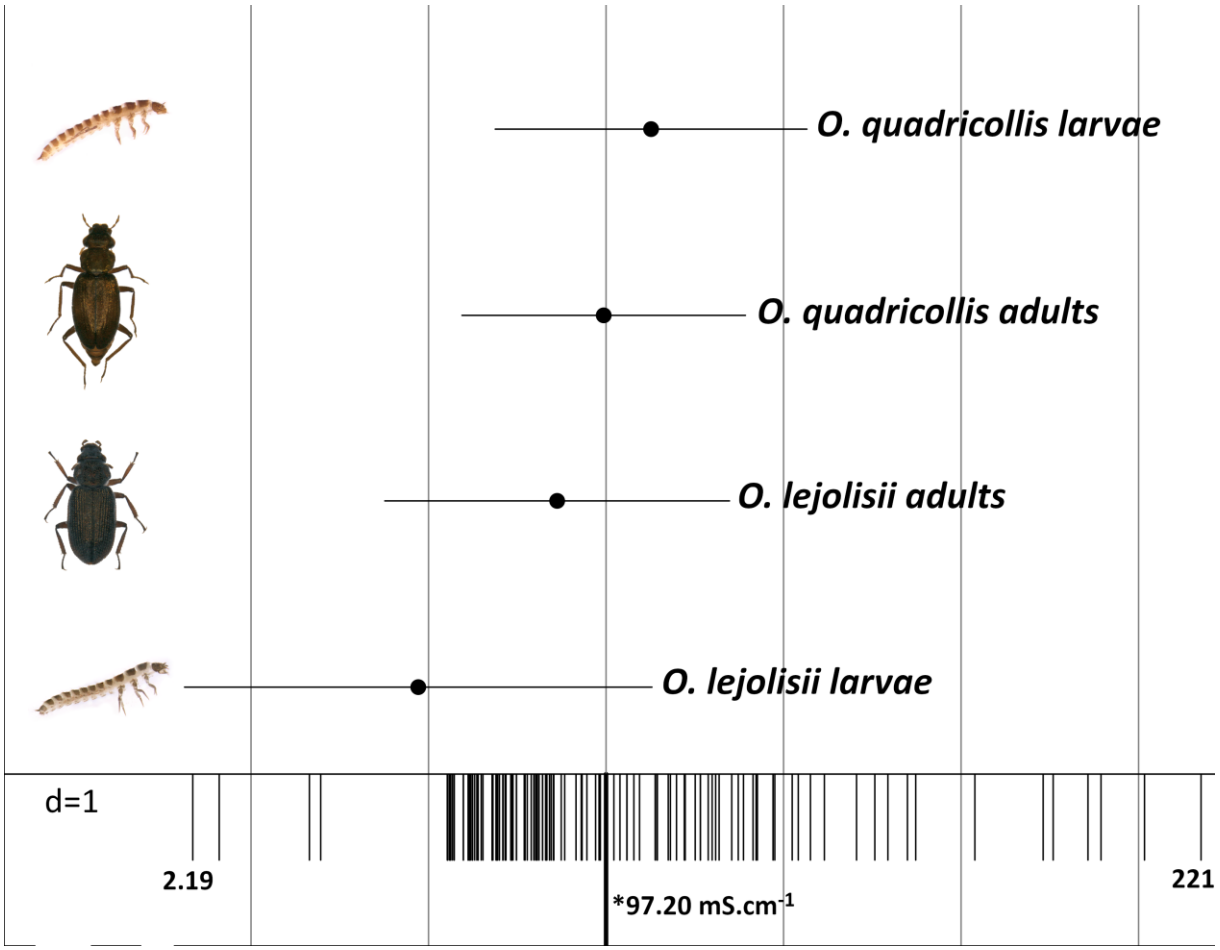


Figure 2. Outlying mean index (dot) and realised niche breadth (horizontal bars) for the adults and larvae of *Ochthebius quadricollis* and *O. lejolisii* across the water conductivity gradient in the sampling area (x-axis represents the conductivity range in the study area; *Mean conductivity value of sampling sites)

Fundamental niche

Physiological tolerance of larvae and adults

When comparing treatments (Figure 3), the cumulative survival of the *O. quadricollis* adults and larvae and the *O. lejolisii* larvae significantly decreased under the tested higher salinity conditions (140 and 170 gL^{-1}). However, the survival of the *O. lejolisii* adults significantly decreased only at 170 gL^{-1} (Figure 3).

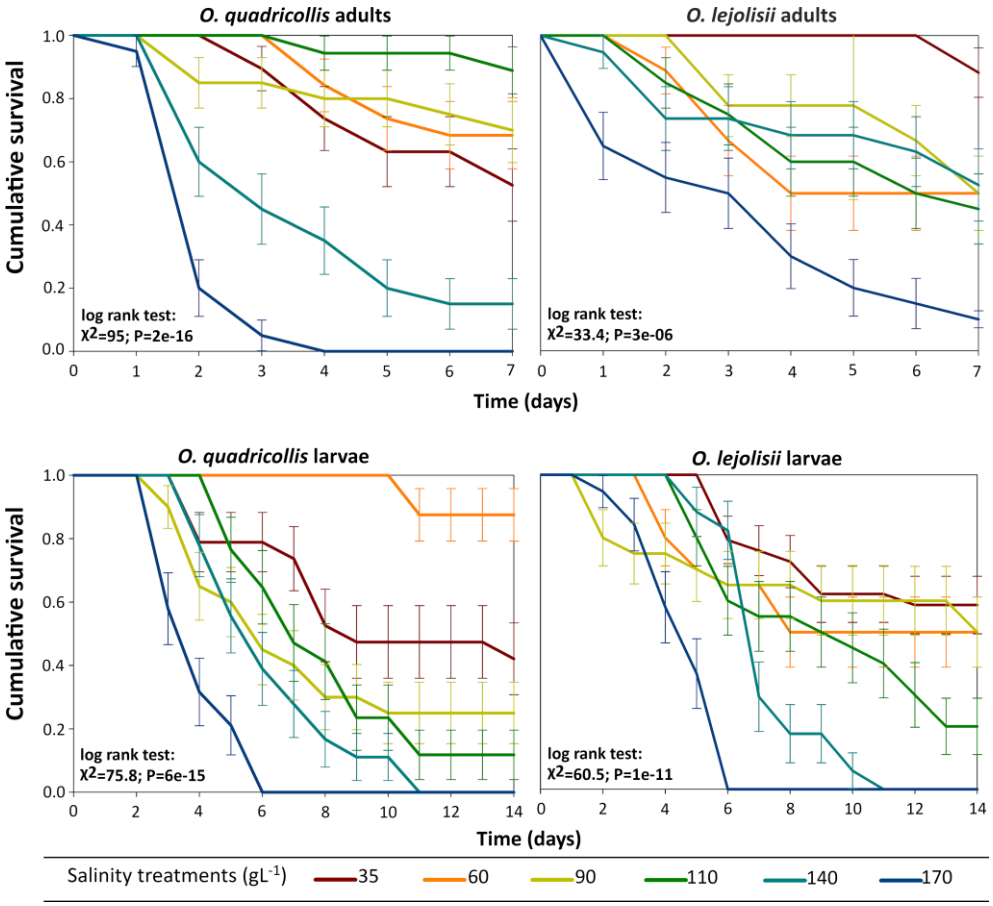


Figure 3. Kaplan–Meir survival curves of the larvae and adults of *Ochthebius lejolisii* and *O. quadricollis* for each salinity treatment. Each data area represents probability of survival (mean ± SE), and the log rank test results are indicated

The mean survival time at 170 gL^{-1} was significantly shorter in the *O. quadricollis* adults (2.20 ± 0.14 days) than in the *O. lejolisii* adults (3.35 ± 0.50 days) (Figure 4, Table 2). The larvae of both species showed longer survival than adults and similar cumulative survival curves (Figure 3), with a mean survival time at 170 gL^{-1} of 4.11 ± 0.27 days in *O. quadricollis* and 4.67 ± 0.28 days in *O. lejolisii*, (Figure 4).

Table 2. Kruskal–Wallis results of the effect of salinity, species, stage, and their interaction, on survival time

	χ^2	df	P value
Salinity	133.49	5	**< 0.001**
Species	2.35	1	0.13
Stage	8.45	1	**< 0.01**
Salinity x Species x Stage	187.65	23	**< 0.001**

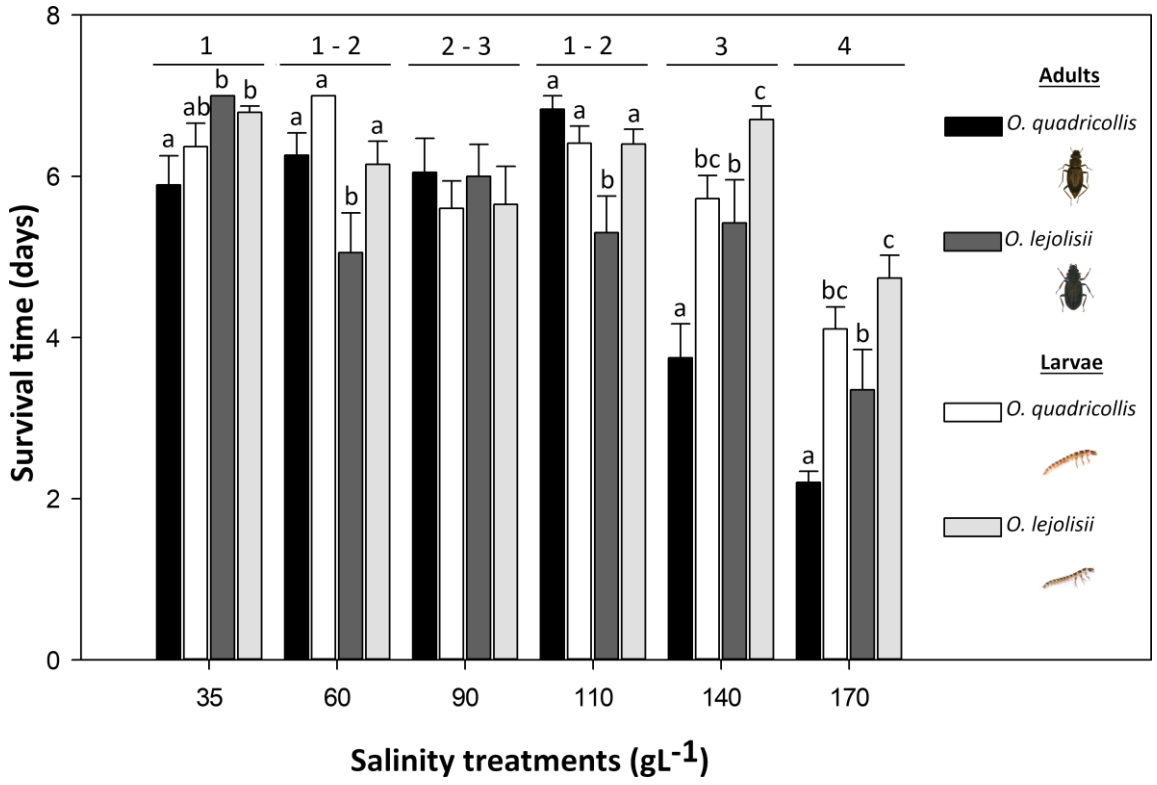


Figure 4. Mean ± SE survival time of the larvae and adults of *Ochthebius quadricollis* and *O. lejolisii* after 7 days of being exposed to different salinity treatments. The BH post hoc significant differences ($p \leq 0.05$) among salinity treatments are indicated with different numbers; a lowercase letter denotes significant differences in stages/species for each salinity treatment

Physiological tolerance of eggs

In both species, egg hatching success tended to decrease with increasing salinity (Figure 5). The logit model and the post hoc results analyses showed interspecific differences in hatching success in the 60, 140 and 170 gL^{-1} salinity treatments (Figure 5, Table 3). At 60 gL^{-1}, the hatching success of the *O. quadricollis* eggs was better than for *O. lejolisii* (Figure 5). However,

at 140 gL^{-1}, the hatching success of the *O. lejolisii* eggs was better than for *O. quadricollis*. Furthermore at 170 gL^{-1}, the *O. quadricollis* eggs did not hatch, while about 30% of hatching occurred in *O. lejolisii*.

Table 3. Logistic regression model results of the effect of salinity and species, and their interaction, on egg-hatching success

	Estimate	Std. Error	P value
Intercept	-9.48	1.98	< **0.001**
Salinity	0.09	0.02	< **0.001**
Species	4.08	1.07	< **0.001**
Salinity x Species	- 0.04	0.01	< **0.001**

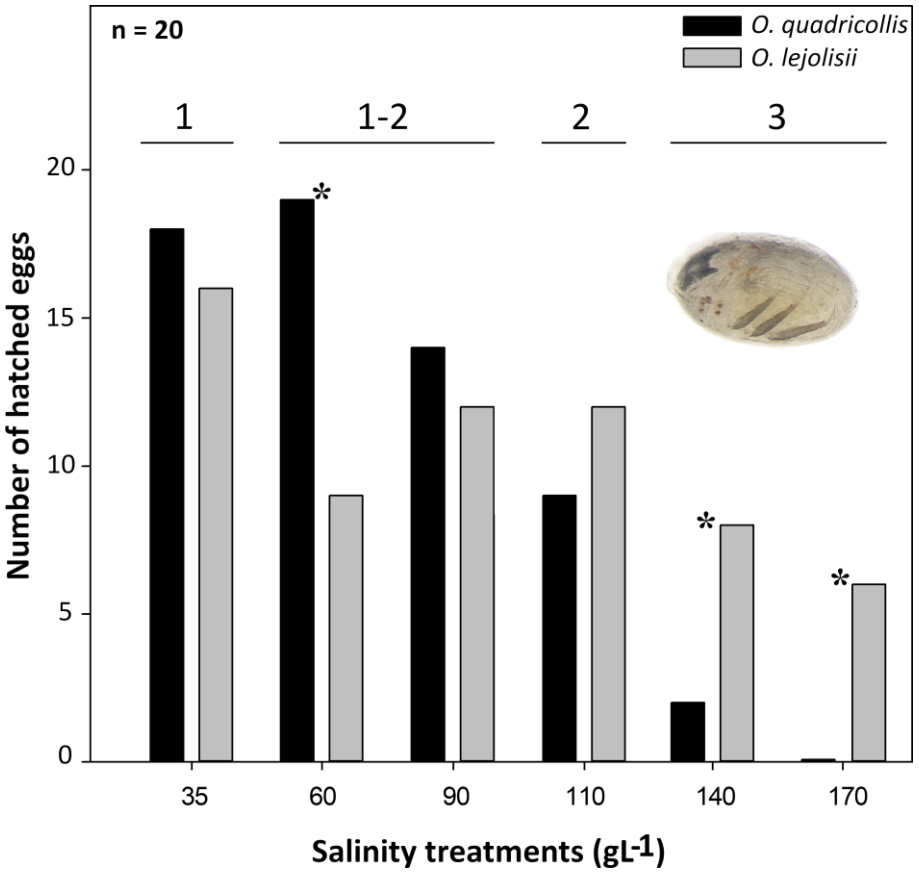


Figure 5. Percentage of the hatched eggs of *Ochthebius quadricollis* and *O. lejolisii* in each salinity treatment. The BH post hoc significant differences ($p \leq 0.05$) among salinity treatments are indicated with different numbers; asterisks denote significant differences among species for each salinity treatment. A photograph of *O. quadricollis* egg is shown on the right side of the figure

DISCUSSION

Both *Ochthebius* species demonstrated good tolerance to extreme salinity conditions, and were able to inhabit and reproduce in the field from subhaline to hyperhaline waters up to almost 140 gL^{-1} in response to the fluctuating and extreme conditions of the Mediterranean supralittoral rockpools where they lived. Previous studies performed with other species of the genus *Ochthebius* from hypersaline inland waters, such as *O. glaber* and *O. notabilis*, also report a highly halotolerant response when living within salinity ranges of 16−205 gL^{-1} (Pallarés et al., 2012). Although no specific works that have focused on osmoregulation mechanisms in the *Ochthebius* species are available, both study rockpool species can be considered effective osmoregulator; i.e., they are able to maintain body fluid volume, osmotic pressure and ionic composition when exposed to changes in extracellular osmolality (Rivera-Ingraham & Lignot, 2017). Waterproofing cuticles, water ingestion and salt excretion through Malpigian tubules and rectum might allow these species to survive in hyperosmotic environments, as reported for other aquatic insect species living in hypersaline inland water bodies (Smith et al., 2008; Pallarés et al., 2015; Botella et al., 2017, 2019). Similarly to other saline beetle species, they can show hyperosmotic regulation in hypoosmotic media (Céspedes et al., 2013; Pallarés et al., 2015). Other syntopic species, such as the copepod *Trigriopus brevicornis*, have been found in pools with saline concentrations up to 200 gL^{-1} (Damgaard & Davenport, 1994), and are considered hypo-osmoregulators (McAllen & Taylor, 2001), unlike marine crustacea that are osmoconformers and are restricted to habitats with minor salinity variability (Péqueux, 1995; Bradley, 2009). Overall, those species that inhabit saline waters (realised niche specialist) are generalists in their fundamental niches (physiological salinity tolerance) and display a predominant high survival pattern in freshwater or under low-salinity conditions, where their performance tends to be similar, or even better, than in saline waters (Arribas et al., 2019).

This study noted a discordant pattern between the realised and fundamental niches in the studied species. In the field, the *O. lejolisii* adults and larvae had higher realised niche breadth values, but within a narrower salinity range than the *O. quadricollis* adults and larvae. The last species occupied the pools with the highest salinities (181 gL^{-1} for larvae), while the *O. lejolisii* larvae and adults were found at lower maxima values (138 gL^{-1}). Conversely in the laboratory, *O. lejolisii* was more physiologically tolerant than *O. quadricollis*. Both studied species generally showed maintained survival response curves in adults and larvae at salinities

ranging from 35 gL^{-1} to 110 gL^{-1} (or 140 gL^{-1} in the *O. lejolisii* adults), which quickly decreased in the higher salinity treatments. Similarly, egg hatching success also diminished with increasing salinity. This response has also been found in several insects living in freshwater (Kefford et al., 2004) and saltwater (Carbonell et al., 2012).

As we predicted, the larval and eggs stages of both species were more resistant to salinity than adults. Larvae's greater resistance to extreme conditions may be the result of their amphibian behaviour (Millán et al., 2014), and their clearly lower dispersal ability compared to adults, which can fly when pool conditions are unfavourable (Williams, 2006; Mirón-Gatón et al., 2022). This result agrees with the greater thermotolerance of the larval stage versus the adult one found in the studied species (Mirón-Gatón et al., 2022). However, this finding contrasts the general tendency indicated in the literature, which refers to the tolerance of young life stages (Covernton & Harley, 2020; Lambret et al., 2021; Balogh & Byrne, 2021), considered to be generally more sensitive to increasing salinity than adult stages. We did not acquire any information about osmoregulation mechanisms in the eggs of the insects living in rockpools. However, in some Crustacea, egg envelops act as barriers and provide osmoprotection to embryos or their membranes function as osmoregulatory organs (or form part of one) (Morritt & Spicer, 1996; Charmantier & Charmantier-Daures, 2001; Bass et al., 2022).

The greater tolerance of eggs to salinity is common in species that live in pools subjected to drought, which is the case of the corixid *Sigara selecta* (Carbonell et al., 2012), and suggests the presence of resistance mechanism in this life phase to cope with drying conditions. In the genus *Enochrus* (Coleoptera: Hydrophilidae), a strong correlation between adults' salinity and desiccation tolerances has been recorded (Arribas et al., 2014), and desiccation resistance mechanisms firstly evolved and provided the physiological basis for hyporegulation ability to develop by allowing these insects to colonise and diversify across meso- and hypersaline habitats (Pallarés et al., 2017). A recent preliminary laboratory experiment has shown greater resistance to the desiccation of *O. lejolisii* larvae and eggs than for *O. quadricollis* (authors' unpublished data), which supports their greater physiological tolerance to salinity.

The tolerance mechanisms to salinity and/or desiccation of the eggs and larvae in these

species are unknown, but a highly waterproofing cuticle in larvae (Botella et al., 2021) and a thick adhesive coating of mucilage enveloping eggs, as found in *O. lejolisii* (Velasco et al., 2022), may act as primary barriers to water loss. Other mechanisms, such as anhydrobiosis, involve the organism's ability to survive water loss (Stubbington et al., 2017). Certain protozoans, rotifers, nematodes and tardigrades are able to tolerate anhydrobiosis in all their life-cycle stages (Watanabe, 2006), while other organisms are only able to resist anhydrobiosis as the eggs, like as some crustacea (e.g., Artemia) (Crowe, 1971), or as the larvae in some diptera (e.g., *Polypedilum vanderplanki* Hinton) (Hinton, 1960; Lees, 1961). Anhydrobiosis appears to be induced by reduced water availability and then resumes the reactivation of metabolic and development processes when rehydrated (Crowe, 1971; Williams, 2006). Further research into this topic is crucial to identify the taxa and stages with a specific physiological mechanism to tolerate the salinity and desiccation stressors in these insects.

The discordant pattern found between the realised and fundamental saline niches could be due to the combined effect of salinity and other environmental stressors, such as temperature and water availability, which can be additive, or can interact synergistically or antagonistically, to determine a species' field distributions (Gunderson et al., 2016; Velasco et al., 2019). A previous comparative study of thermal tolerance has shown that *O. lejolisii* is less heat-tolerant than *O. quadricollis*, but high salinity (90 gL^{-1}) confers the former species greater thermal tolerance in larvae, but lower tolerance in adults, while do not affect the heat tolerance of *O. quadricollis* (Mirón-Gatón et al., 2022). Thus, stressful temperature and salinity conditions in pools can be avoided by *O. lejolisii* adults flying to more favourable pools or taking refuge by burrowing under sediment or in crevices as both adults and larvae (Mirón-Gatón et al., 2022; Velasco et al., 2022). These behavioural responses can be exacerbated in drying pools, where temperature and dehydration are lower in microrefuges than in exposed surfaces, and allow *O. lejolisii* adults and larvae to resist until pools have refilled with rains. These results collectively suggest that the greater heat tolerance of *O. quadricollis* and its lower resistance to desiccation allow it to occupy pools that are more exposed to seawater with a long hydroperiod within a wide salt concentration range. *Ochthebius lejolisii* can occupy more temporary pools and resist extreme salinity conditions by hiding as larvae or adults under sediment or in rock crevices, and produce resistant desiccation eggs. Further studies to understand how multiple stressors interact, as well as species patterns responses to these

interactions, are crucial to explain these species' field distributions and adaptation processes to climate change.

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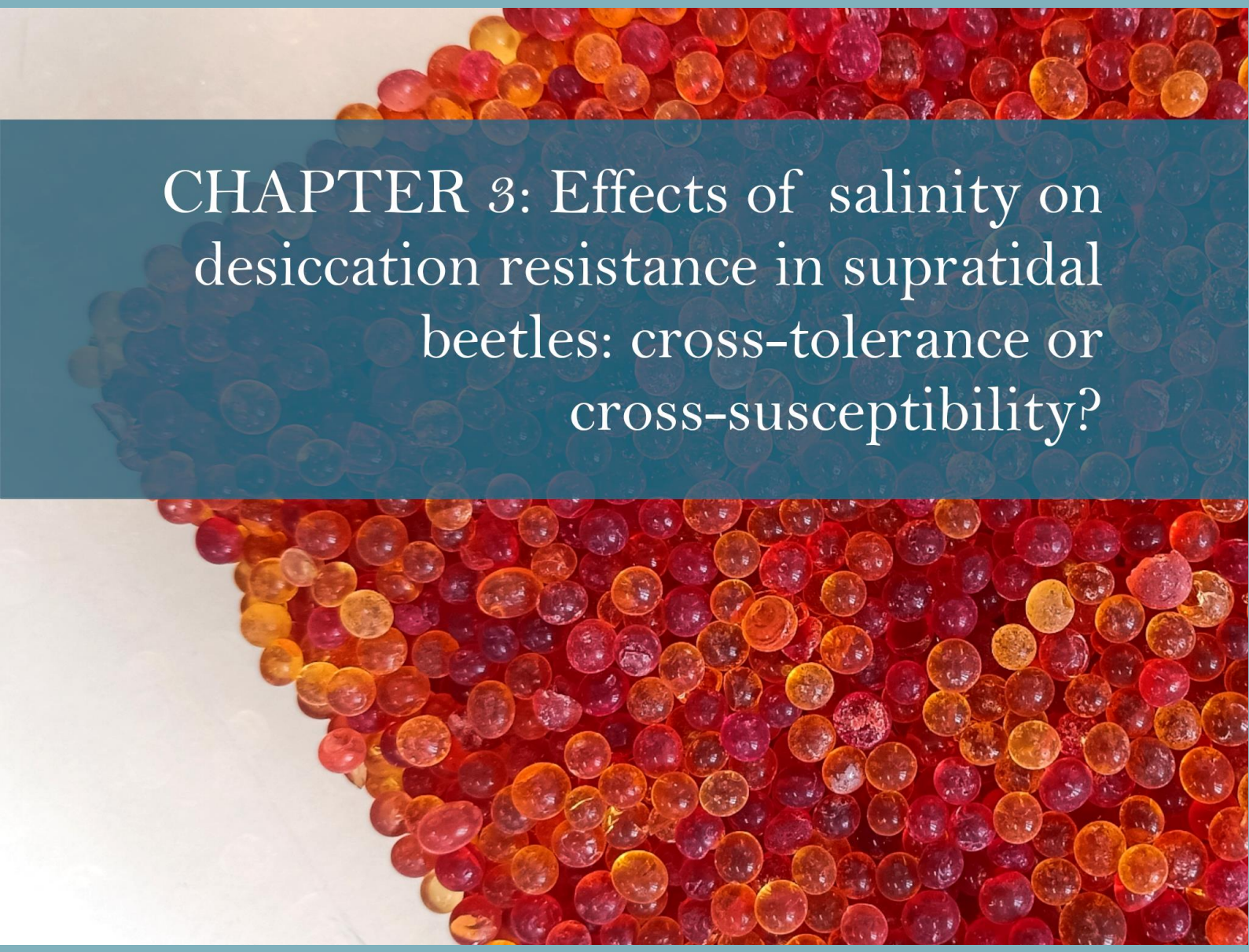
SUPPORTING INFORMATION

Table S1. Geographical coordinates of the rockpools sampling locations of *Ochthebius quadricollis* and *O. lejolisii* along the Spanish Mediterranean coast

Locality	**Longitude (°)**	**Latitude (°)**	**Locality**	**Longitude (°)**	**Latitude (°)**
1- La Illeta	-0.380682	38.431633	7- Campoamor	-0.733682	37.911118
2- Santa Pola	-0.537224	38.188259	8- Cabo Cope	-1.471246	37.467716
3- Torrevieja	-0.656448	37.983053	9- Cala Panizo	-1.700164	37.318990
4- Punta del Cocedor	-0.728286	37.749961	10-Roquetas de Mar	-2.606268	36.752597
5- Cala Túnez	-0.692300	37.692300	11- Isla de Tarifa	-5.612443	36.001918
6- Cala Reona	-0.712855	37.617327			

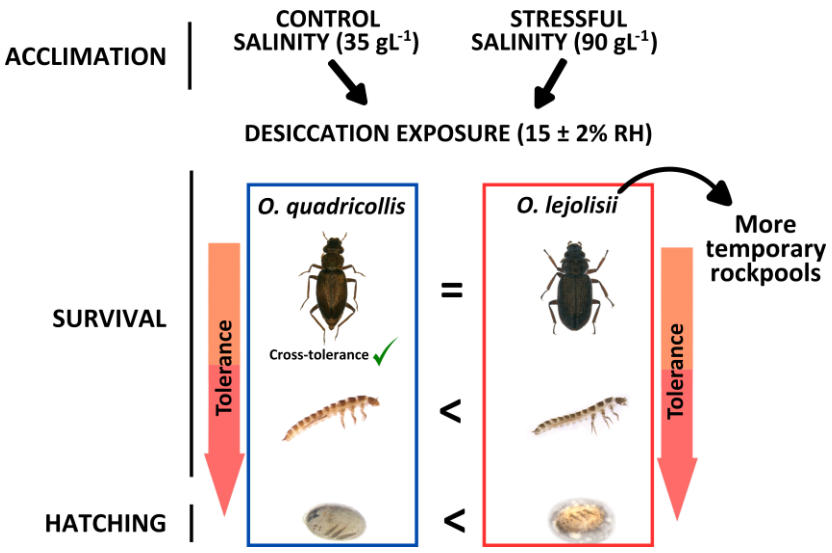
UNPUBLISHED CHAPTERS



CHAPTER 3: Effects of salinity on desiccation resistance in supratidal beetles: cross-tolerance or cross-susceptibility?

ABSTRACT

Organisms in dynamic environments like supratidal rockpools have evolved diverse strategies to manage desiccation and osmotic stress, involving various physiological and behavioural adaptations. Under a multiple-stress context, exposure to a particular stressor might enhance (cross-protection) or decrease (cross-susceptibility) tolerance to a subsequent stressor. Our aims were to determine: i) whether exposure to a sublethal saline concentration had a protective or detrimental effect in a subsequent exposure to desiccation in the coexisting aquatic beetles *Ochthebius quadricollis* and *O. lejolisii* from rockpools and ii) how such effects vary across their different life-stages. Adults, larvae, and eggs of both species were acclimated at 35 gL^{-1} (non-stressful salinity) and 90 gL^{-1} (sublethal salinity) and subsequently exposed to desiccation conditions at 15 ± 2 % relative humidity. Both species exhibited similar ontogenetic responses to desiccation, being earlier life stages more tolerant than adults. In species comparisons, the larvae of *O. lejolii* were significantly more tolerant to desiccation than those of *O. quadricollis*. Eggs of *O. quadricollis* exposed to desiccation showed higher hatching success after hydration at 35 gL^{-1} than those of *O. lejolisii*, whereas at 90 gL^{-1}, *O. quadricollis* eggs did not hatch and those of *O. lejolisii* maintained similar hatching rates than at lower salinity. Cross-tolerance was found only in *O. quadricollis* adults, which showed a higher survival under desiccation after acclimation at the highest salinity. These contrasting stress tolerances between species could be related to the temporality of the microhabitats each species occupies. Our results contribute to our understanding of the interplay between co-occurring environmental stress factors and adaptation mechanisms in highly stressful and dynamic aquatic environments.



INTRODUCTION

Desiccation resulting from temperature and humidity fluctuations is one of the predominant stress factors in nature, with pronounced effects on aquatic insects (Chown et al., 2011; Thorat & Nath, 2015). These organisms employ diverse strategies, involving behavioural and physiological responses, to cope with desiccation in their different life stages (Edney, 1977; Chown & Nicolson, 2004). Some aquatic insects face droughts in situ in microrefugia (e.g., Stubbington et al., 2016), whereas others with flying adults disperse to more favourable wet habitats (Strachan et al., 2015; Bilton, 2023). Some species rely on desiccation-resistant eggs or on the production of protective compounds to prevent dehydration and cellular damage (see Strachan et al., 2015 for a detailed review of desiccation responses in freshwater invertebrates). Improving our understanding of such responses is fundamental to predicting the impact of the increasing aridification ongoing in many regions of the world on aquatic biota (Kernan et al., 2010; Ibáñez & Caiola, 2013).

In nature, aquatic species do not experience desiccation in isolation, as they are also exposed to other stressors during seasonal droughts, such as elevated temperatures, high levels of ultraviolet light as well as fluctuations in salinity, pH or oxygen levels (Strachan et al., 2015). In recent years, experimental biologists have shifted from single to multiple-stressor approaches, providing significant advances in our understanding on how organisms respond to multiple stressors. Exposure to a particular stressor may trigger protective mechanisms that help in coping with a subsequent or simultaneous stressor of a different nature (cross-protection) or increase the negative effect of the second stressor (cross-susceptibility) (Sinclair et al., 2013; Todgham & Stillman, 2013; Rodgers & Gomez Isaza, 2021, 2023). More specifically, two types of cross-protection can be identified: i) cross-tolerance, where stress factors share protective mechanisms (Steinberg, 2012) and ii) cross-talk, where stress factors share signalling/regulatory pathways that activate independent protective mechanisms (Velasco et al., 2018; Rodgers & Gomez Isaza, 2023).

A better understanding of the mechanistic bases driving the responses to multiple stressors is of crucial concern to predict when multiple stressors may result in cross-tolerance or cross-susceptibility, and understanding the adaptive capacity of organisms to respond to future multifactorial environmental change (Sinclair et al., 2013; Verberk et al., 2013). In the

case of desiccation, its combined effect with temperature has been relatively well studied (e.g., Adhikari et al., 2010; Guareschi & Wood, 2020), while other stressor combinations such as salinity and desiccation have received limited attention, at least in aquatic species (see Pallarés et al., 2017; Velasco et al., 2018). Both salinity and desiccation lead to dehydration and osmotic stress, posing critical challenges at the cellular level (Bradley & Bradley, 2008; Cohen, 2013). Consequently, the stress induced by salinity and desiccation in insects triggers similar and even shared physiological mechanisms. As observed in certain insects, the pre-activation of osmoregulatory mechanisms during exposure to salinity appears to minimize water loss during subsequent desiccation exposure (e.g., Elnitsky et al., 2009; Pallarés et al., 2017). For example, certain dipteran larvae can accumulate organic osmolytes to delay and/or limit cellular dehydration while preserving metabolic function (Elnitsky et al., 2009).

Supratidal rockpools are ideal systems for studying the combined effects of salinity and desiccation on aquatic organisms. These habitats, which can be considered among the most fluctuating ecosystems worldwide (Brandes et al., 2015; García-Meseguer et al., 2023), are characterized by extraordinary daily and seasonal fluctuations in temperature, salinity and water levels (Denny & Dowd, 2022), particularly those located along the Mediterranean coast (Izquierdo & Mikolajewicz, 2019). These pools are subjected to extremely variable hydroperiods due to the combined effect of marine (splash, waves) and terrestrial water inputs (Madeira et al., 2012; Vinagre et al., 2015), which cause intense salinity variations. During the hottest months, these pools can dry out completely, reaching salinity concentrations that can exceed 200 gL^{-1} (Mirón-Gatón et al., 2023). Therefore, organisms inhabiting these environments frequently experience increasing salinity and desiccation sequentially or simultaneously, and therefore, represent an excellent model for studying cross-related responses between both stressors.

Ochthebius (Coleoptera: Hydraenidae) is one of the most specialised genera of aquatic beetles, with exclusive species of supratidal rockpools (Velasco et al., 2022; Villastrigo et al., 2022), and different congeneric species are capable of coexisting within the same pools (Mirón-Gatón et al., 2022; Villastrigo et al., 2022). This is the case of the species *O. quadricollis* Mulsant 1844 and *O. lejolisii* Mulsant & Rey 1861 (Villastrigo et al., 2019, 2020). Both species inhabit pools with extreme conditions, where water temperatures can reach up to 38 °C (Mirón-Gatón et al., 2022) and, frequently, with salinity concentrations of 140 gL^{-1} (larvae of

O. quadricollis have been found at a salinity of up to 180 gL^{-1}) (Mirón-Gatón et al., 2023). In laboratory experiments, these species have shown distinct tolerances to temperature (Mirón-Gatón et al., 2022) and salinity across adult and juvenile life stages (Mirón-Gatón et al., 2023). Larvae of both species have greater thermotolerance than the adults (Mirón-Gatón et al., 2022), and tolerate higher salinity concentrations and for longer period (Mirón-Gatón et al., 2023). Eggs are also highly tolerant to salinity, being able to hatch at saline concentrations of up to 170 gL^{-1} in *O. lejolisii* and 140 gL^{-1} in *O. quadricollis* (Mirón-Gatón et al., 2023). Moreover, *O. lejolisii* larvae showed greater heat tolerance after acclimation at high saline concentrations, showing a cross-protection response to salinity and temperature (Mirón-Gatón et al., 2022).

The main objective of this study was to determine whether *O. quadricollis* and *O. lejolisii* exhibit cross-tolerance to salinity and desiccation and to assess how the responses to such stressors vary among life-stages. We compared several physiological responses (survival in larvae and adults and hatching success in eggs) after sequential exposure to both stressors between life-stages within each species and between species in each life-stage.

METHODS

Specimen collection and maintenance

Adults of both species were collected from supratidal rockpool coastal areas in the Region of Murcia (southeast of Spain). Specimens of *O. quadricollis* were collected from Cala Reona (30S 701844E 4165819N), and *O. lejolisii* from Isla Plana (30S 657571E 4160139N), in February 2021. Beetles were collected with soft forceps and brushes and transported to the laboratory in small, aerated aquaria with 2–3 cm of seawater and filter paper as substrate. In the laboratory, both species were morphologically identified under a binocular microscope (Leica M165C with a Leica MEB10 fibre optic illuminator) and placed in a climatic chamber in different aquaria with water at 35 gL^{-1} salinity, 20 °C temperature and a photoperiod of 12 h light:12 h dark. These conditions have previously been shown to be non-stressful and favourable for the reproduction of both species (see Mirón-Gatón et al., 2022, 2023). Individuals were fed *ad libitum* with green microalgae (*Tetraselmis chui* Butcher, 1959). Additionally, a breeding aquarium was set up for each species under the same conditions, with breeding pairs collected from the field at the time of copulation, to obtain larvae.

Cross-tolerance experiments

Salinity acclimation

Thirty adults and thirty larvae (instar LIII) of each species were randomly selected and acclimated for 7 days at two salinity treatments (35 gL^{-1} as not stressful and 90 gL^{-1} as a sublethal stress) and 20 °C and 12h light:12h dark photoperiod, following the approach of Mirón-Gatón et al. (2023). For the salinity acclimation of eggs, breeding pairs of each species were placed in aquariums at each salinity concentration, and after spawning, eggs with a smooth dark colour (without a defined embryo) were collected (Figure 1). All the salinity solutions were prepared by dissolving an appropriate quantity of sea salt (Ocean Fish, Prodac) in distilled water.

Survival of adults and larvae to desiccation

Salinity acclimated individuals (adults and larvae) of both species were gently dried with paper and individually placed into glass test tubes (9 ml). The tubes were sealed with a mesh to prevent escape while allowing aeration. These test tubes were placed inside a container of 7 litres with 1 kilograms of silica gel. The container was sealed with plastic wrap and adhesive tape, creating an environment with a constant relative humidity (15 ± 2 %). The relative humidity was monitored using a data logger placed inside the container. Adult survival was assessed at 3–6–9–12 and 24 hours, as previous tests indicated survival times did not exceed 24 hours. In larvae, survival was checked every 12 hours until death, as we had previously observed they can survive for several days under these conditions. Another group of 30 individuals of each species and life stage were left in the aquariums at both salinities as a control, not exposed to desiccation, to monitor mortality during the same experimental period (Figure 1).

Eggs hatching after desiccation

Eggs were exposed to four desiccation treatments at 15 ± 2 % relative humidity during 24–48–72 and 96 hours, in addition to a control treatment (i.e., submerged in water at the same salinity concentrations to which the corresponding breeding pairs laying the eggs were exposed). Twenty eggs reared at each salinity were subject to each desiccation treatment and the control. Eggs were gently dried with paper, and using a dry brush, they were individually placed into 2 ml Eppendorf tubes. The tubes were placed inside a container (7 L) with desiccant

salts (1 kg) to maintain the desired relative humidity. The control eggs were kept in 2 ml Eppendorf tubes with saline water at concentrations of 35 and 90 gL^{-1}. After the desiccation treatment, the eggs were hydrated by immersing them under the corresponding water salinities at which they were acclimated. Hatching success was checked at 7 and 14 days (Figure 1).

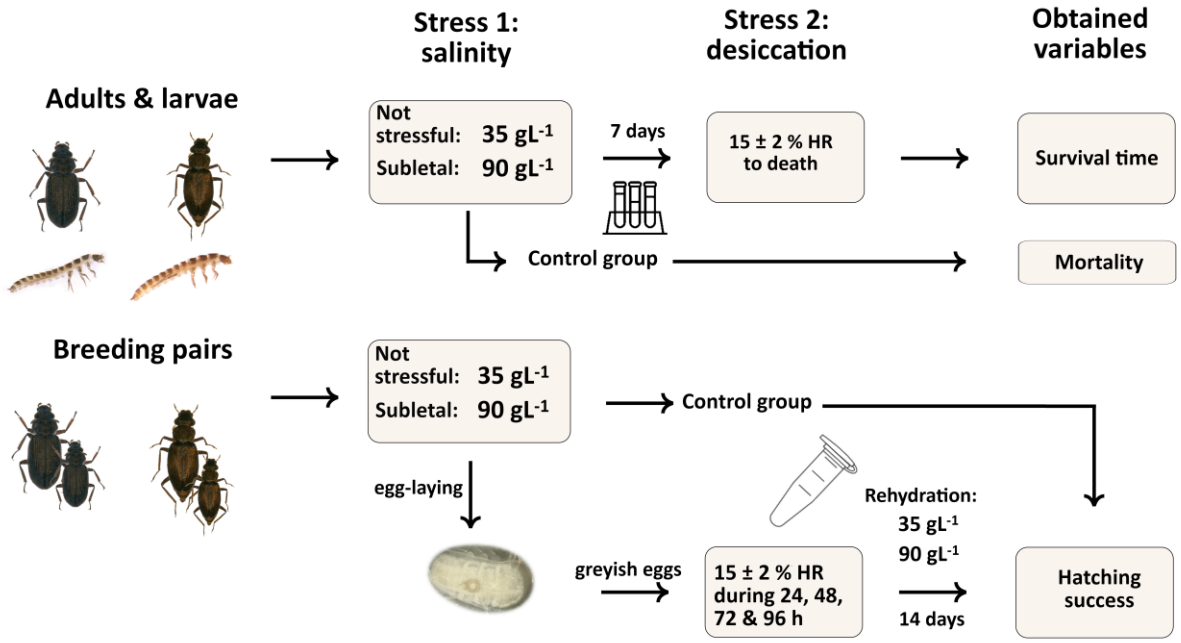


Figure 1. Schema of experimental design of cross-tolerance between salinity and desiccation and the variables measured

Data analyses

Kaplan–Meier survival curves were used to estimate the survival probabilities to desiccation of adults and larvae for each species and salinity acclimation treatment. Survival curves of adults and larvae were compared between life-stages, species, salinity acclimations, and their interactions by the log-rank (Mantel–Cox) test.

For eggs, a GLM (logit model) and post hoc analyses with the "Bonferroni" p-adjustment method were used to determine differences in hatching success between species, salinity acclimation treatment, desiccation treatments (i.e., different exposure times), and their interactions. All the analyses were carried out with the R software, version 4.2.3 (2023-03-16, Development Core Team, 2023).

RESULTS

Survival of adults and larvae under desiccation

Log rank test revealed different survival patterns between species, life-stages, and salinity acclimations (see Table 1). There were significant differences in survival probability under desiccation between species only in the larval stage, at both salinities (Figure 2) ($\chi^2 = 89.5$; $p < 0.001$). The larvae of *O. lejolisii* exhibited a higher mean survival time (143.17 ± 10.37h at 35 gL^{-1}; 144.00 ± 12.25h at 90 gL^{-1}) than those of *O. quadricollis* (60.46 ± 5.13 h at 35 gL^{-1}; 55.38 ± 3.9 h at 90 gL^{-1}). Acclimation salinity did not show a significant effect on the probability of survival, except from the adults of *O. quadricollis* (Figure 2), in which individuals previously acclimated at the higher salinity showed longer survival times (9.89 ± 0.42 h) than those at lower salinities (8.79 ± 0.31 h). In both species, the mortality of adults and larvae in the control group was negligible during the experiment.

Table 1. Log-rank test results on differences in survival probability among species, life-stages, salinity acclimation treatments and their interactions

	χ^2	P value
Species	30.500	**< 0.001**
Salinity	0.200	0.700
Life-stage	236	**< 0.001**
Species x salinity	30.600	**< 0.001**
Species x life-stage	277	**< 0.001**
Salinity x life-stage	244	**< 0.001**
Species x salinity x life-stage	284	**< 0.001**

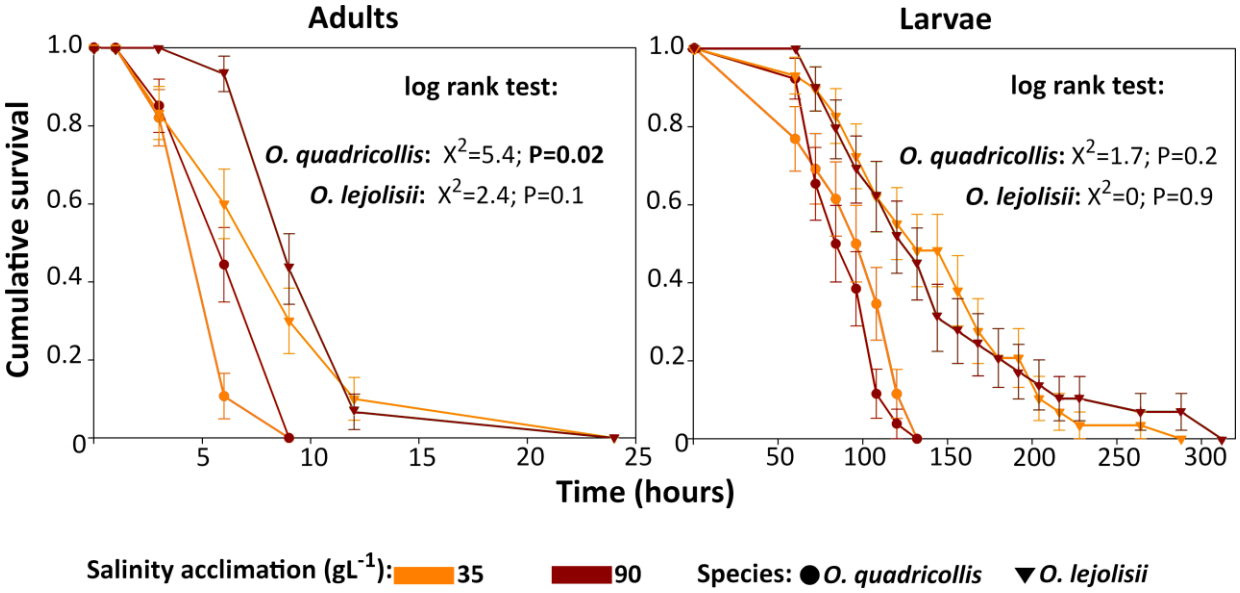


Figure 2. Kaplan–Meir survival curves of the larvae and adults of *Ochthebius lejolisii* and *O. quadricollis* for each salinity treatment. Each data point represents the probability of survival (mean ± SE). The log rank test results comparing salinity acclimations for adults and larvae of each species are indicated (significant differences are shown in bold)

Hatching success of eggs under desiccation

GLMs revealed differences in hatching success among species, salinities, and their interaction (Table 2). There was also a significant effect of exposure time on hatching success in both species, decreasing as the desiccation time increased (Figure 3). Eggs of *O. quadricollis* exhibited higher hatching success under desiccation than those of *O. lejolisii* at 35 gL^{-1}. On the contrary, in the saline acclimation treatment at 90 gL^{-1}, eggs of *O. quadricollis* did not hatch neither in the control (not subject to desiccation) or any desiccation time treatment, except from eggs exposed to desiccation for 24 h (15 ± 2 %). At 90 gL^{-1}, 15 % of the eggs of *O. lejolisii*, subjected to desiccation for 96 h did hatch (Figure 3) and there was not significant differences in the hatching success between salinity acclimation treatments (post-hoc tests: Bonferroni adjusted p-value = 0.22).

Table 2. GLM results on differences in eggs hatching success between species, salinity acclimation treatments, desiccation time and their interactions

	Df	Deviance	Resid. Df	Resid. Dev	P value
Null			389	524.130	
Species	1	11.430	388	512.690	< **0.001**
Salinity	1	26.220	387	486.470	< **0.001**
Desiccation time	4	62.810	383	423.670	< **0.001**
Species x salinity	1	69.980	382	353.670	< **0.001**
Species x desiccation time	4	6.260	378	347.410	0.180
Salinity x desiccation time	4	9.240	374	338.180	0.060
Species x salinity x desiccation time	4	1.620	370	336.550	0.800

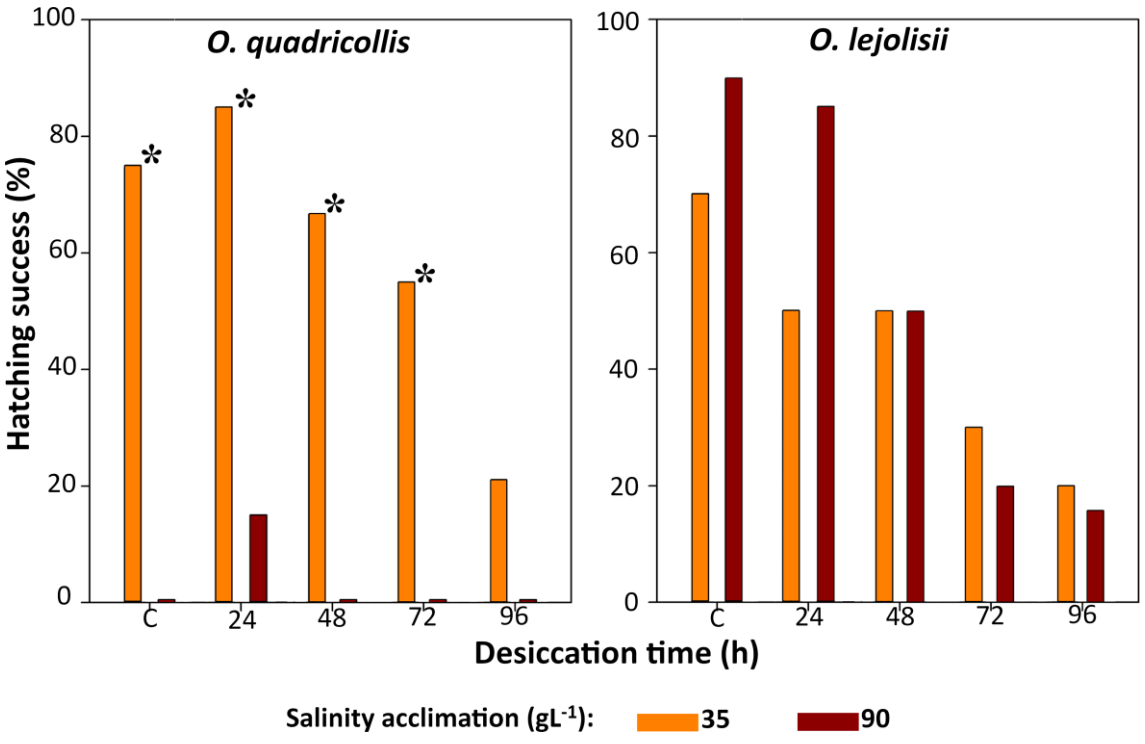


Figure 3. Percentage of the hatched eggs of *Ochthebius quadricollis* and *O. lejolisii* in each salinity acclimation and desiccation time (C: control, eggs not subject to desiccation treatment). Significant differences between salinity treatments within each species, according to Bonferroni post hoc tests ($p \leq 0.05$), are indicated with asterisks

DISCUSSION

Ontogenetic and interspecific variation in desiccation resistance

We found that both species exhibited a similar ontogenetic pattern in the response to desiccation, with eggs and larvae showing a notably higher resistance than adults. Larvae (instar LIII) were able to survive desiccation for a significant longer time than the adults (over 100 hours more) and eggs were able to hatch after 96 hours of desiccation at a relative humidity of 15 ± 2 %. The highly desiccation-resistance of the eggs in these species' contrasts with most true aquatic beetles (sensu Jäch & Balke, 2008), which generally do not have desiccation-resistant egg or larval stages (Millán et al., 2014). This finding also differs from the relatively general pattern found in marine invertebrates, in which larvae are more sensitive to environmental stress than adults (Verween et al., 2007; Pineda et al., 2012; Miller et al., 2013). However, this result is consistent with the extraordinary tolerance to other stressful factors, such as salinity and temperature, that early stages of the study species have previously shown (Mirón-Gatón et al., 2022, 2023). In the highly dynamic habitats that the study species inhabit, adults might exhibit stress-avoidance behaviour by flying or moving to another pools when conditions turn adverse, at the expense of a lower physiological capacity to resist in situ. The persistence of populations in situ would therefore rely on less or non-mobile stages with higher physiological resistance/tolerance. Similar responses were found in *Tigriopus californicus*, a copepod inhabiting supratidal pools, in which early life stages exhibit greater resistance to temperature than adults (Tangwancharoen & Burton, 2014).

When comparing both species, it was noteworthy that the larvae of *O. lejolisii* endured a considerably longer desiccation period than those of *O. quadricollis* (more than double). This is consistent with the higher salinity tolerance that larvae of *O. lejolisii* have shown, compared to those of *O. quadricollis* (Mirón-Gatón et al., 2023). These results suggest that *O. lejolisii* has evolved more efficient osmoregulation mechanisms, which could confer higher tolerance or resistance to both salinity and desiccation. Furthermore, in *O. lejolisii*, the transformation period from instar LIII to pupa, which takes place out of water, is longer than in *O. quadricollis* (approximately 10 days longer) (Velasco et al., 2022). This may allow this species to develop more effective stress protection mechanisms. The same positive relationship between salinity tolerance and desiccation resistance seems to be present in eggs, as those of *O. lejolisii*, with higher tolerance to high saline concentrations (Mirón-Gatón et al., 2023), exhibited greater

hatching success under desiccation conditions after acclimation to 90 gL^{-1} salinity compared to *O. quadricollis* eggs. It is likely that the mucilaginous layer covering the eggs of *O. lejolisii*, which appears to be rare in *O. quadricollis* (Velasco et al., 2022), is responsible for providing greater protection against these stressors.

The differences in stress responses between the coexisting species studied here could be linked to subtle differences in their ecological niches at the microhabitat level, related to the seasonality of their habitat (García-Meseguer et al., 2023). For example, *O. quadricollis* has been observed in permanent pools closer to the sea (Balfour-Browne, 1958; Mirón-Gatón et al., 2023), with more stable salinity levels. In contrast, *O. lejolisii* is frequently found in pools further from the sea, subject to greater desiccation and salinity variation (Mirón-Gatón et al., 2022, 2023, García-Meseguer et al. 2023). Therefore, we could conclude that the species inhabiting temporary environments more exposed to environmental fluctuations and stressful conditions, *O. lejolisii*, has developed a greater desiccation (and salinity) tolerance during its early life-stages. This finding is consistent with observations in other intertidal organisms, in which desiccation tolerance is associated with the type of microhabitat (Hamilton & Gosselin, 2020).

Differing cross-tolerance responses across species and life-stages

Survival curves revealed a cross-tolerance response to salinity and desiccation in adults of *O. quadricollis*. In adults of *O. lejolisii*, there was also a trend towards higher survival rates in individuals previously acclimated to higher saline concentrations, but such difference was not statistically significant (Figure 2). Pallarés et al., (2017) observed a similar pattern when exploring cross-tolerance between salinity and desiccation in adults of two species of aquatic beetles from inland saline environments. They suggested that the greater ability to withstand desiccation following exposure to high salinity could be due to the activation of different osmoregulatory responses and behaviours. For example, insects increase the water drinking rates during exposure to hyperosmotic conditions, excrete accumulated salts and reabsorb water through the activity of Malpighian tubules and specialized parts of the hindgut, such as rectal pads, to counteract osmotic stress (Bradley & Phillips, 1975; Patrick & Bradley, 2000). The pre-activation of these osmoregulatory organs and tissues to maintain water and osmotic balance during salinity exposure is likely the primary mechanism enhancing the control of

water loss during subsequent desiccation exposure. Furthermore, cuticular hydrocarbons (CHCs), which show a plastic composition in saline water beetles under salinity (Botella-Cruz et al., 2019) and desiccation stress (Botella-Cruz et al., 2021), likely play also a fundamental and protective role against these stressors.

The existence of cross-tolerance responses in these organisms is likely to have an adaptive advantage in supratidal rockpools, in which they are often simultaneously exposed to a wide range of co-occurring environmental stress factors (Leeuwis & Gamperl, 2022). Indeed, cross-tolerance has been observed in other supratidal species, as the rockpool copepod *Tigriopus californicus*, in which increased salinity enhanced thermal tolerance (Denny & Dowd, 2022). However, in the larvae and eggs of the species studied here, this cross-tolerance was not observed. It should be noted that cross-tolerance responses strongly depend on stress intensity and duration and often enhance survival under a stressor after exposure to sublethal stressful conditions of other stressor (Adhikari et al., 2010; Gotcha et al., 2018; Ben-Yosef et al., 2021). Lower stress level might not activate cross-protection responses, while higher stress could be detrimental. Hence, it is possible that the salinity concentration of 90 gL^{-1} was closer to lethal levels in adults, but less stressful for larvae, as the latter have shown a higher salinity tolerance (Mirón-Gatón et al., 2023). In line with these results, previous studies analysing the effect of salinity acclimation on heat tolerance in *O. lejolisii*, showed that high salinity (90 gL^{-1}) conferred greater thermal tolerance in larvae (cross-tolerance) but lower tolerance in adults (cross-susceptibility) (Mirón-Gatón et al. 2022). The negative effect of salinity on thermal tolerance observed in adults was probably due to a previous exposure to salinity levels close to their tolerance limit. Other authors have found different cross-responses in function of the intensity of the first stressor. For example, Todgham et al. (2005) observed, in the tidepool sculpins *Oligocottus maculosus* Girard, 1856, that a pre-treatment at a 12°C thermal shock increased stress tolerance to osmotic and hypoxic stressors, while a 10°C thermal shock had no effect, and a 15°C thermal shock was detrimental.

In contrast to the cross-tolerance between salinity and desiccation found in adult of *O. quadricollis*, a decrease in hatching success (with almost no hatching) was observed in eggs of the same species under desiccation after pre-exposure to a salinity of 90 gL^{-1}. Although such result could be interpreted as a cross-susceptibility response, it is also possible that the

previous salinity exposure was detrimental for the eggs of this species. In fact, eggs in the control treatment (not exposed to desiccation conditions) also showed no hatching responses at 90 gL^{-1}. Such as response was unexpected because in a previous study, this species did exhibit a high hatching percentage (approximately 70 %) at the same salinity (Mirón-Gatón et al., 2023). The fact that breeding pairs were collected at different times of the year in such previous study could be a determining factor in explaining the different salinity sensitivities observed. Seasonality may lead to differentiation in life strategies (Vanschoenwinkel et al., 2010; Teets & Denlinger, 2013), highlighting the importance of incorporating it as a crucial factor in these types of studies.

The specific mechanisms underlying the salinity and/or desiccation tolerance in the eggs of these species are unknown. As indicated above, a thick adhesive mucilage layer surrounding the eggs, as found in *O. lejolisii* (Velasco et al., 2022), may play a crucial role. In general, in many aquatic invertebrates, embryonic development takes place inside gelatinous masses or egg capsules, and embryonic development largely depends on the protection that this capsule can provide against environmental factors (Pechenik, 1986). The different microhabitats in which our study species are found could determine that *O. lejolisii* eggs need to develop protective mechanisms to withstand higher stress and more fluctuating conditions than those of *O. quadricollis*, which occupies more permanent pools closer to the sea.

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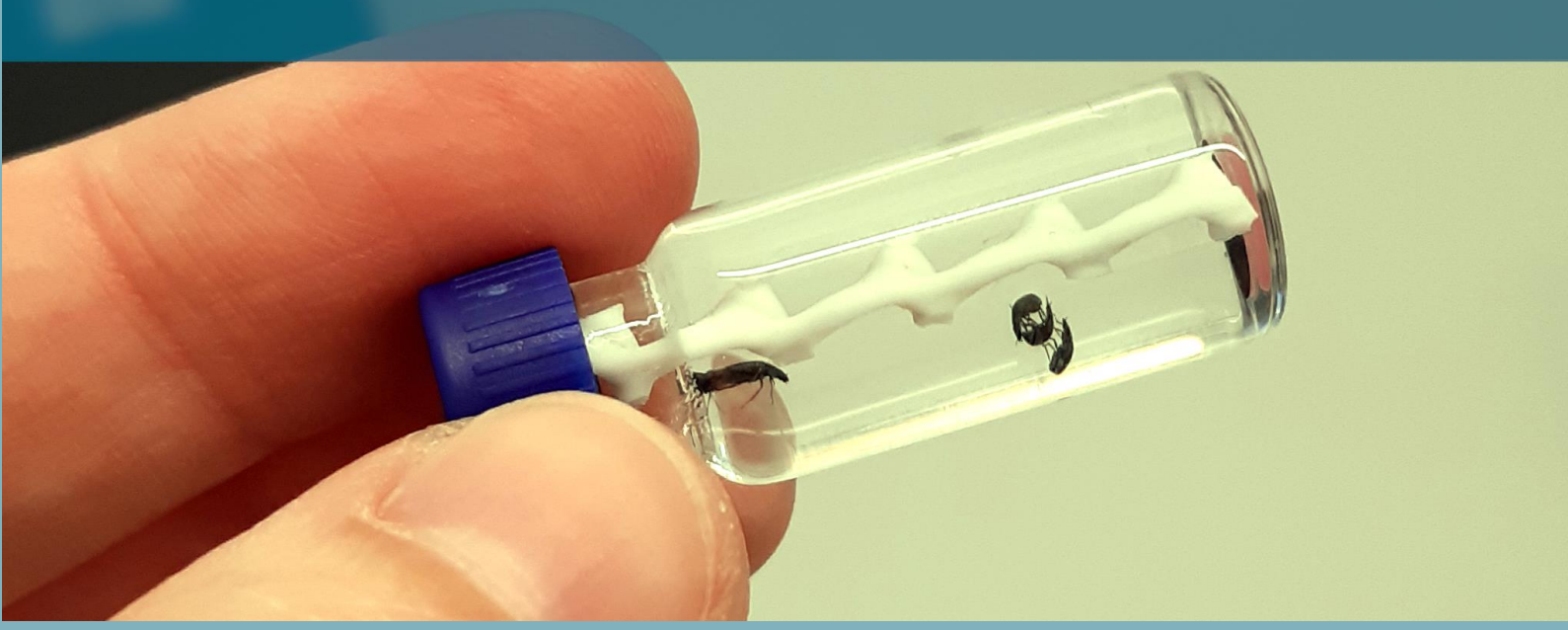
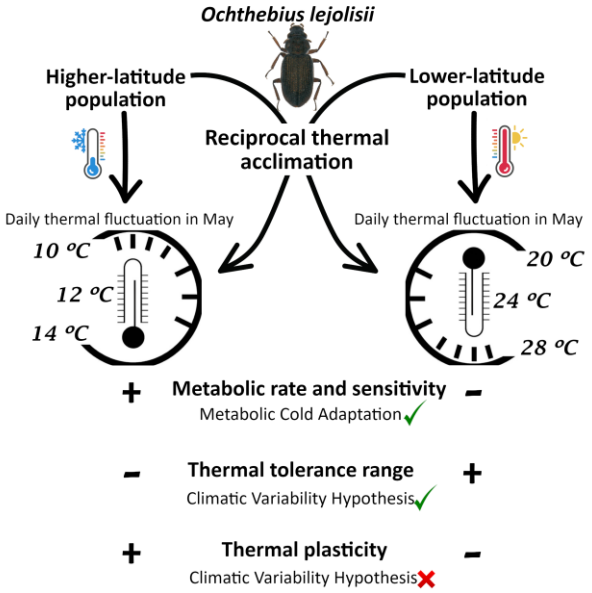
CHAPTER 4: Testing metabolic cold adaptation and the climatic variability hypotheses across the latitudinal range of a widespread, supratidal water beetle



ABSTRACT

Temperature significantly impacts ectotherm physiology, with thermal tolerance and metabolic traits typically varying with latitude across species ranges. The drivers of this variation remain unclear, however, despite obvious consequences for population persistence and conservation in the face of ongoing global change. This study explores local adaptation and phenotypic plasticity of metabolic rates and thermal limits in the supratidal rockpool beetle *Ochthebius lejolisii* from localities experiencing contrasting thermal variability. Using populations from localities at different ends of the species range, experiencing contrasting thermal variability, we simultaneously test two of the major paradigms of spatial physiological ecology; metabolic cold adaptation (MCA) and the climatic variability hypothesis (CVH). Reciprocal acclimation was conducted under spring temperature regimes of both localities, incorporating local diurnal variation. Metabolic rates were measured by closed respirometry, and thermal tolerance limits estimated through thermography. In line with MCA, the higher-latitude population (colder climate) showed higher metabolic rates and Q_{10}s at lower temperatures than the lower-latitude population. As predicted by the CVH, the lower-latitude population (more variable climate) showed higher upper thermal tolerance but only the higher-latitude population was able to acclimate upper thermal limits. This pattern suggests the existence of trade-offs in thermal adaptation in this species, which likely increase the vulnerability of populations on Mediterranean coasts to the projected increases in extreme temperatures under ongoing climate change.



INTRODUCTION

A major challenge in global change biology is to understand how thermal tolerance and acclimation capacity vary within and between species, since these factors shape the way organisms respond to ongoing climate change (Seebacher & Franklin, 2012; Gunderson & Stillman, 2015; Verberk et al., 2023). Local adaptation to different thermal regimes can lead to differences in thermal tolerance and physiological plasticity between species and between populations of widespread species (Lardies et al., 2010; Gaitán-Espitia et al., 2013, 2014; Barria et al., 2018). Metabolic rate integrates the energy required for vital functions (Messamah et al., 2017; Barria et al., 2018; DeLong et al., 2018; Shah et al., 2021) and can reflect the energetic costs of adaptation to a specific thermal environment (Barria et al., 2018). In ectotherms, metabolic rates are temperature sensitive and the relationship between metabolism and temperature has been shown to vary across the range of widespread species, following environmental gradients (Addo-Bediako et al., 2002; Barria et al., 2018).

A number of hypotheses have been developed which attempt to relate the metabolic adaptations of species to temperature (Barria et al., 2018; Shokri et al., 2022), and although often at least partly complimentary, these are usually considered in isolation. One of the most tested, and yet controversial, is metabolic cold adaptation (MCA) (Addo-Bediako et al., 2002; Lardies et al., 2004; Messamah et al., 2017; Shokri et al., 2022). This suggest that ectotherms living at higher latitudes, where climates tend to be colder, show higher metabolic rates or higher metabolic sensitivity to temperature than organisms at lower latitudes with warmer climates (Hodkinson, 2003; Terblanche et al., 2009). Such higher metabolic rates may be advantageous in ectotherms, especially insects living at higher latitudes, because it allows them to metabolise food faster, develop more rapidly and therefore completing their life cycle over a relatively short growing season (Chown & Gaston, 1999; Lardies et al., 2004). Similarly, species inhabiting cold climates whose metabolic rate is relatively sensitive to temperature, can take better advantage of short-term temperature increases (Nielsen et al., 1999; Nespolo et al., 2003) than warm climate species (Hodkinson, 2003). Addo-Bediako et al., (2002) suggested that this sensitivity may be higher in Northern Hemisphere temperate taxa that those in the Southern Hemisphere, where relatively stable, cool temperatures may have selected for reduced sensitivity (i.e. a lower slope) of the metabolic rate-temperature curve (Rao & Bullock, 1954; Young, 1979; Messamah et al., 2017).

Empirical tests have provided mixed support for MCA at the interspecific level (Addo-Bediako et al., 2002; Sokolova & Pörtner, 2003), something which likely results from other biological differences amongst species (Messamah et al., 2017). Comparing metabolic rates across populations of a single species with a wide latitudinal range is a potentially powerful way around such difficulties (Sømme et al., 1989; Nylund, 1991; Lardies et al., 2004). To date, however, intraspecific variation in metabolism has received limited attention, despite its importance in understanding geographical variation in performance (Lardies et al., 2004) and the evolution of life-history strategies in different environments (Addo-Bediako et al., 2002).

Local thermal variability, in addition to mean temperature, represents a strong selective pressure on traits shaping the thermal niches of organisms (Gaston et al., 1998; Barria et al., 2018). Indeed, according to the Climatic variability hypothesis (CVH) (Stevens, 1989), species from more stable environments are predicted to have lower thermal tolerance (i.e., narrower tolerance breadths and lower acclimation capacity) than those from more variable habitats (Magozzi & Calosi, 2015; Shah et al., 2017; Pallarés et al., 2021). Thus, according to the CVH, thermal specialists are expected show higher metabolic sensitivity to temperature, with metabolic rates that change rapidly in response to temperatures outside their normal range (Shah et al., 2021). In contrast, thermal generalists, capable of functioning effectively over a wide range of temperatures (Angilletta et al., 2002), are predicted to have relatively low metabolic rates across a wider range of temperatures and be less sensitive to increasing temperatures to keep their energy demands low (Shah et al., 2021). As with the MCA hypothesis, however, such a pattern has not always been supported by empirical studies (Seebacher et al., 2015). In some species, evolutionary trade-offs instead seem to have constrained the evolution of different components of thermal tolerance, such that species that have evolved the greatest thermal limits (particularly upper ones) may have done so at the expense of the plasticity of such limits and therefore show limited acclimation capacity (Stillman, 2003).

Although ecologists and evolutionary biologists clearly recognise the importance of local environmental variability (e.g. Bozinovic & Pörtner, 2015; Vázquez et al., 2017), research on thermal tolerance and global change biology has mostly focused on the ecological impact of changes in the average values of climatic variables. Clearly, under global change, variability itself is changing in both frequency and intensity, not just average conditions (Vázquez et al.,

2017). In this context, more studies which explore the impact of thermal variability on the performance of individuals and their plastic responses to it are clearly needed (e.g. Bozinovic et al., 2016). Situated at the land−sea interface, supratidal rockpools represent one of the most dynamic, extreme environments on earth (Kaplanis et al., 2020). These habitats experience significant daily and seasonal fluctuations in temperature and other correlated stressors (e.g., salinity and dissolved oxygen) and they frequently dry out completely during summer. As a consequence, these systems present an ideal model for testing the effects of environmental variability on organismal performance (da Silva et al., 2019). Only a small number of organisms are able to endure these extreme conditions on a permanent basis (McAllen, 1999), such as Crustacea and Mollusca of marine origin, and Insects (Diptera, Coleoptera and Hemiptera) of continental origin (Margalef, 1949).

In the present study, we used the widespread supratidal specialist beetle *Ochthebius lejolisii* Mulsant & Rey, 1861 as a model organism to test both MCA and the CVH hypotheses in an integrated manner, exploring the effects of acclimation at different thermal regimes on metabolic rates and thermal tolerances. We compared two populations spanning the wide latitudinal distribution of this beetle (one from a higher latitude, with colder and more thermally stable conditions and another from a lower latitude with warmer, more widely fluctuating daily and seasonal temperatures) to assess the role of thermal adaptation to local environmental conditions. We predicted that i) in line with MCA, the higher-latitude population (colder climate) would exhibit higher metabolic rates and higher sensitivity to temperature change than its lower-latitude relatives; ii) according to the CVH, the more thermally stable (higher-latitude) population would display not only a higher thermal sensitivity of metabolic rates, but also a narrower thermal tolerance breadth and lower plasticity of thermal limits and metabolic rates than the (lower-latitude) population from the more thermal variable environment.

METHODS

Study species and specimen collection

Ochthebius lejolisii (Coleoptera: Hydraenidae) is a small aquatic beetle with an Atlantic-Mediterranean distribution, which exclusively inhabits supratidal rockpools (Villastrigo et al., 2022a; Villastrigo et al., 2022b). Comparatively speaking, the species has relatively high

thermal and salinity tolerance, as would be predicted given its habitat. Adults have an upper thermal limit of 47.84 ± 0.15°C (Mirón-Gatón et al., 2022) estimated by a dynamic method with a heating rate of 1 °C min^{-1} and a salinity tolerance range between 1.8 and 138 gL^{-1} (Mirón-Gatón et al., 2023). Adults use bimodal breathing, storing air under their elytra and having functional spiracles. They extract oxygen from the water using an air film on the ventral surface, supported by hydrofuge hairs, which functions as a physical gill and is connected to the subelytral air store (Perkins, 1997). Gas exchange at the water surface is facilitated by breaking surface tension with the hydrophobic antennae, creating an air channel to the ventral air bubble (see Pallarés et al., 2021).

We selected two genetically and geographically distant populations of *O. lejolisii* (Villastrigo et al., 2022a) subjected to contrasting temperature regimes: an Atlantic population from Plymouth (50.32 °N; -4.12 °E, higher-latitude population) in the southwest of England, which occupies colder (mean sea surface temperature: 13 °C, http://climate-data.org_last accessed 12 July 2023) and more thermally stable habitats, and a Mediterranean population from Murcia (37.57 °N; -1.21 °E, South Population) in the southeast of Spain, with a warmer mean temperature (mean sea surface temperature: 18.9 °C, http://climate-data.org_last accessed 12 July 2023), and widely fluctuating daily and seasonal temperatures (Figure 1). These latitudinal extremes were previously used to test metabolic and reproductive plasticity of core and marginal populations of a eurythermic saline water bug (Carbonell et al. 2017). We collected adult beetles directly with soft tweezers and brushes from both populations during May and October 2022 (with similar climatic conditions) and transported them to the laboratory in plastic jars filled with moist paper to avoid desiccation. In the laboratory, individuals of each population were kept at 20 ± 1 °C with a 12 h light: 12 h dark photoperiod, in an aquarium with seawater and rocks as substrate for 2 days before starting the experiments. They were fed *ad libitum* with the microalgae *Tetraselmis chuii*.

Experimental design

We carried out a common garden laboratory experiment, with reciprocal acclimations between populations, and measured the metabolic rates and thermal limits of specimens following acclimation. Acclimation treatments were chosen to reproduce the range of daily temperature fluctuations that each population typically experiences in May (when maximal

adult activity and reproduction take place) in the supratidal zone of the two localities (Figure 2).

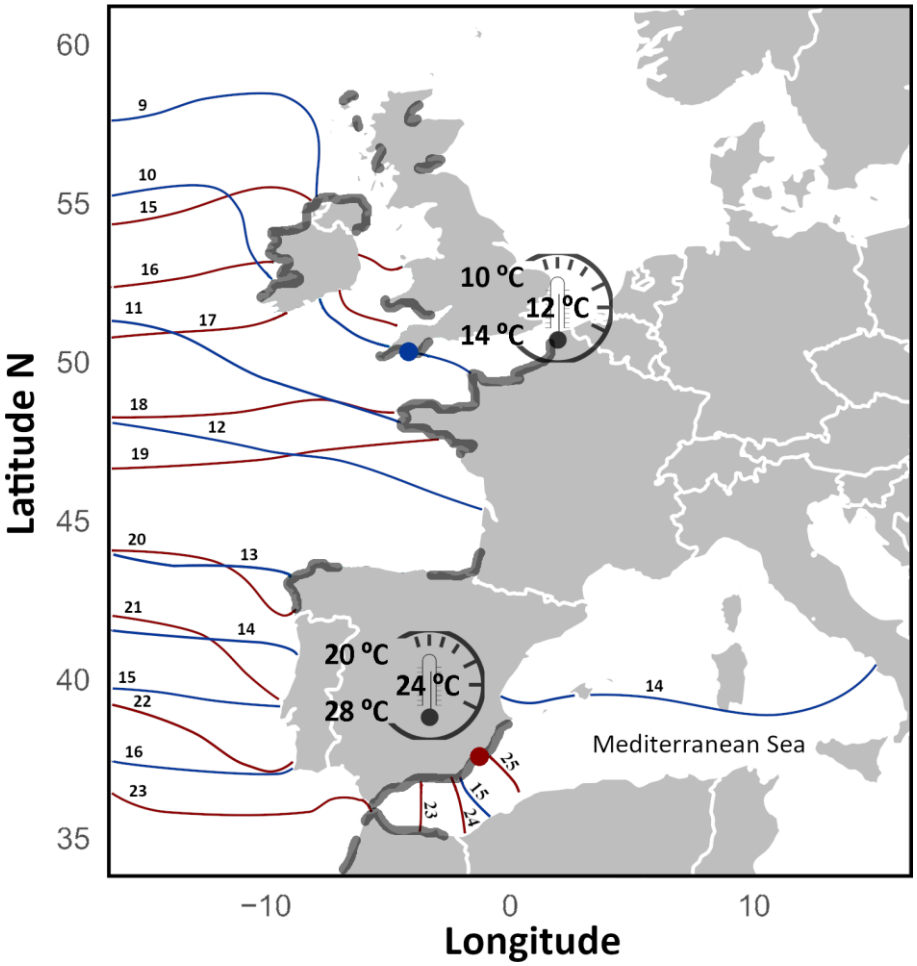


Figure 1. Distribution area of *Ochthebius lejolisii* in the western Palaearctic (shading) (modified from Villastrigo et al., 2022b). Sampling locations: higher-latitude population (blue point): Mount Batten Beach/Heybrook Bay (Plymouth); and lower-latitude population (red point) Isla Plana (Murcia). Mean minimum winter and the mean maximum summer sea surface temperatures for the period 2000- 2015 (Hawkins et al., 2019) are represented by the blue and red isotherms, respectively. The temperature icons illustrate the daily mean, min, and max temperatures in May in the two localities, used as a reference for the acclimation treatments

Acclimation

Beetles were held in 150 ml glass jars (ten per population), each containing 30 individuals. Jars were filled with 40 ml of seawater (salinity = 35 gL^{-1}) and contained a piece of filter paper and small stones as substrate. Five jars for each population were acclimated to daily cyclic temperature variation of 10 to 14 °C (mean 12 °C – higher-latitude acclimation, Table S1)

following rockpools water temperature data provided in Southward, (1958) and Amstutz et al., (2021). The other five jars were acclimated to daily cyclic temperature variation of 20 to 28 °C (mean 24 °C – lower-latitude acclimation, Table S1) following our own measurements in 2019 (HOBO Pendant MX Water Temperature Data Logger). Acclimation was carried out for 8 days, with a 12 h light: 12 h dark photoperiod at 20 ± 1 or 10 ± 1 °C (Figure 2)., for the lower-latitude and higher-latitude acclimations, respectively, using a programmable recirculating water bath (Grant R5 TXF200, Grant Instruments Ltd, UK).

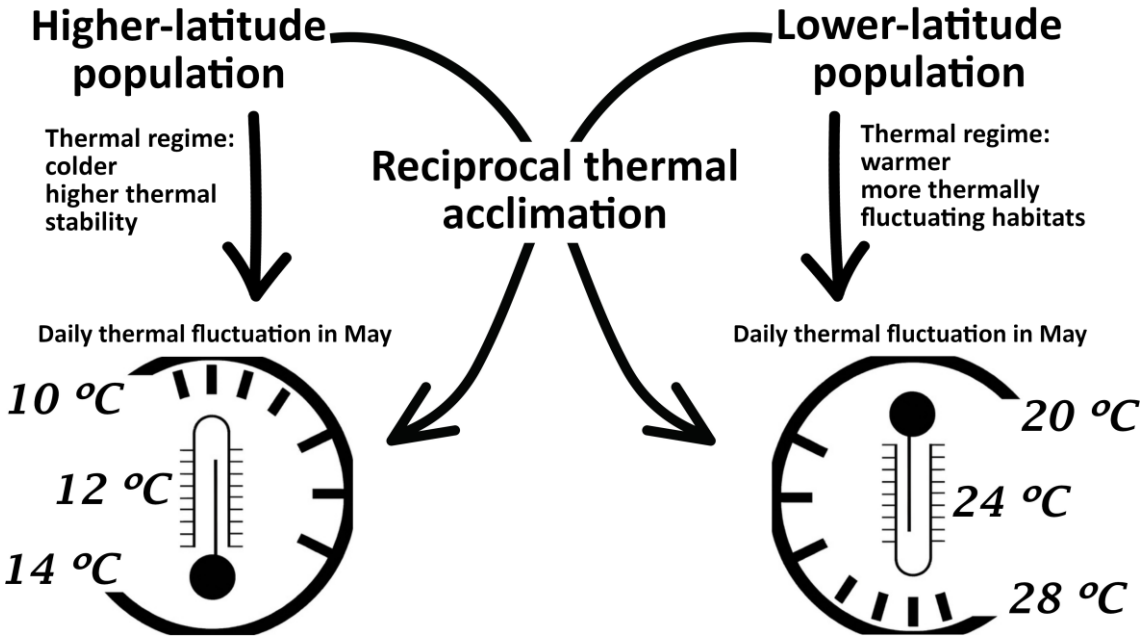


Figure 2. Schematic representation of the experimental design for acclimation. The icons with the thermometer represent daily thermal cycles

Metabolic rates

We estimated the metabolic rates of individuals from each population and acclimation treatment across a physiologically optimal range of temperature (10-30 °C). Tests were also conducted at a temperature of 35 °C, but we discarded them because individuals from both populations died during the experiment. Individuals were deprived of food for 24 h before measurement. Routine metabolic rate was measured using closed respirometry. We used 2 ml respiratory chambers, filled with 1.85 ml of sterile saline solution (35 gL^{-1}) previously oxygenated with an air pump. This left an air bubble at the top of the chamber which provided sufficient space for animals to perform aerial gas exchange. A piece of mesh was also inserted along the length of the respiratory chamber for beetles to rest on. Five individuals were

introduced into each chamber as it was found in previous studies that oxygen consumption was not detectable with fewer individuals, due to the species' small size (body length approx. 2 mm). Dissolved oxygen measurements were made at five temperatures (10, 15, 20, 25 and 30 °C) by immersing the chambers in a water bath (Grant R5 TXF200, Grant Instruments Ltd, UK). Five replicates per test temperature and five blanks (chambers without individuals to control for background respiration) were measured. Beetles were allowed to rest for 20 min before starting measurements to habituate and reach thermal equilibrium. Oxygen consumption in the aquatic compartment was measured at 10-minute intervals using micro-optics connected to a Fibox 4 fibre-optic oxygen meter (PreSens instruments, Germany). After two and a half hours, chambers were removed from the thermal baths and individuals dried with absorbent paper and then weighed on a microbalance (precision level of 0.0001g) to obtain fresh mass.

Thermal limits

Fifteen individuals from each acclimation/population were used to estimate thermal tolerance, using supercooling point and heat coma as lower and maximum thermal limits, respectively. One day before determining thermal limits, individuals were deprived of food. Ramping experiments were made in a climatic chamber (BINDER MK53) starting at the mean temperature of each acclimation treatment and increasing or decreasing the temperature at a rate of ± 1 °C/min. The supercooling point was estimated as the body temperature of the organisms just before the exothermic reaction that precedes the freezing of internal fluids. The heat coma temperature is the temperature at which the individual becomes completely paralyzed before dying (Gallego et al., 2016). For this, we used a video recorder (Sony DCR-DVD110E) and a thermographic infrared camera (FLIR SC305), to simultaneously record individuals' movement and body temperature during trials. Thermal images were analysed using ThermaCAM Researcher Professional 2.10 software, following the methodology of Mirón-Gatón et al., (2022).

Data analysis

Linear regressions were fitted to calculate oxygen consumption rates in relation to temperature. Rates were corrected for mean background respiration at each test temperature. We used the Arrhenius transformation of metabolic rates, which presents the

logarithmically transformed rates as a function of inverse temperature, $(kT)^{1}$, where k is the Boltzmann constant (eV K_1), and T is the absolute temperature (K). Metabolic sensitivity to temperature changes was estimated by the slope of this relationship, which is the activation energy (Ea), and the Q10 between correlative pair temperature treatments and between the minimum and maximum temperatures tested.

Q10 values were obtained using the following equation:

$$Q10 = K_2/K_1^{\ 10/(t2-t1)}$$

where: K_1 is the mean metabolic rate at temperature t1; K_2 is the mean metabolic rate at temperature t2.

A generalized linear regression model was used to compare metabolic rates between populations and acclimation treatments and their interaction. The body mass of the five individuals' group was included as covariate. Upper and lower thermal limits were compared between populations and acclimation treatments (including their interaction) using an ANOVA and post hoc analyses with Bonferroni p-adjustment. Normality and homoscedasticity of the data were first checked using Shapiro-Wilk and Levene tests, respectively.

All the analyses were carried out using R software, version 4.2.3 (2023-03-01, Development Core Team, 2023).

RESULTS

Metabolic rates

Metabolic rates of both populations increased significantly with temperature and differed between populations but not between acclimation treatments in any population (Table 1, Figure S1). Overall, metabolic rates were higher in the higher-latitude population (Figure S1 and Figure 3), with a lower activation energy (Mean Ea = 0.36 eV) compared to the lower-latitude population (Mean Ea = 0.66 eV). Post-hoc tests revealed significant differences between populations at 15 and 20 °C but not at the remaining temperatures (Figure 3). Body mass did not significantly affect metabolic rates in either population.

Table 1. Logistic regression model results of the effect of temperature (Temp), population (Pop), acclimation (Acc), and their interactions, and body mass (BM) on metabolic rates of both populations

Predictor	Df	Deviance	Resid. Df	Resid. Dev	F value	P value
Temp	1	3.092	89	17.048	17.338	**< 0.001**
Pop	1	1.908	88	15.140	10.700	**< 0.005**
Acc	1	0.096	87	15.044	0.540	0.464
BM	1	0.004	86	15.040	0.021	0.884
Temp * Pop	1	0.222	85	14.818	1.245	0.268
Temp * Acc	1	0.149	84	14.669	0.835	0.363
Pop * Acc	1	0..009	83	14.660	0.049	0.826
Temp * Pop * Acc	1	0.035	82	14.625	0.196	0.659

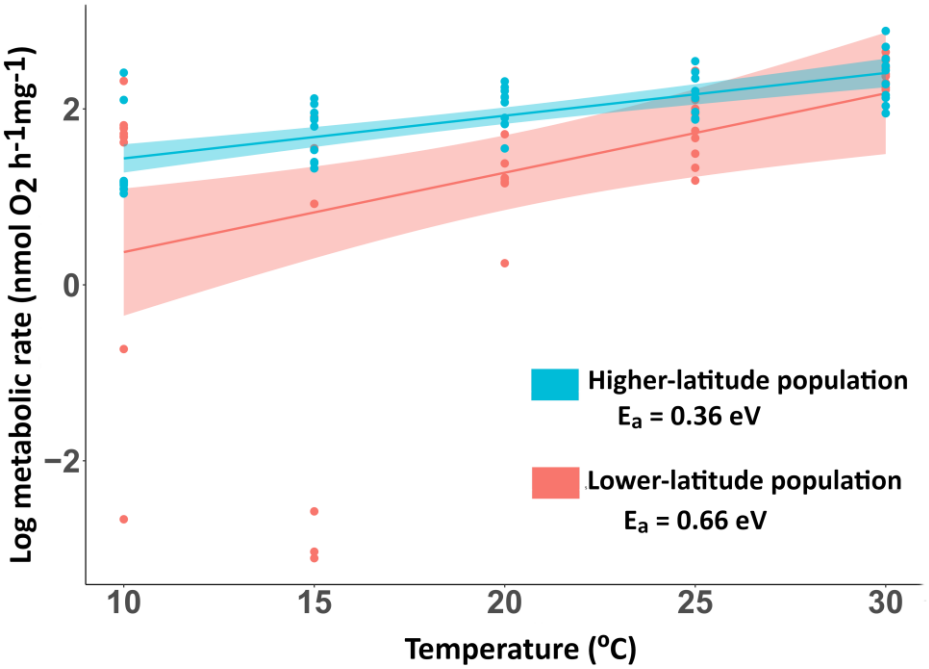


Figure 3. Mean metabolic rates of the two *Ochthebius lejolisii* populations across the range of temperature tested. Shaded areas represent the 95 % confidence intervals of fitted models. Points indicate raw values for each group tested. Ea is the activation energy for each population. Asterisks indicate significant differences (post hoc analysis) in metabolic rates between populations at those temperatures

Both populations showed high variation of Q10 values across the studied temperature range (Figure 4). Q10 mean values across the entire temperature range tested (10-30 °C) were slightly LARGE in the higher-latitude population (1.65) compared to the lower-latitude one (1.51). Q10 results revealed that the higher-latitude population exhibited higher thermal sensitivity in metabolic rates at colder temperatures (Q10 = 2.38 at 10-15 °C). This slowed (Q10 < 2) as temperature increased (15-25 °C), and then rose again (25-30 °C) (Figure 4). In contrast, the lower-latitude population showed higher sensitivity in metabolic rates at 20-25 °C (Q10 = 5.32) and 25-30 °C (Q10 = 3.63).

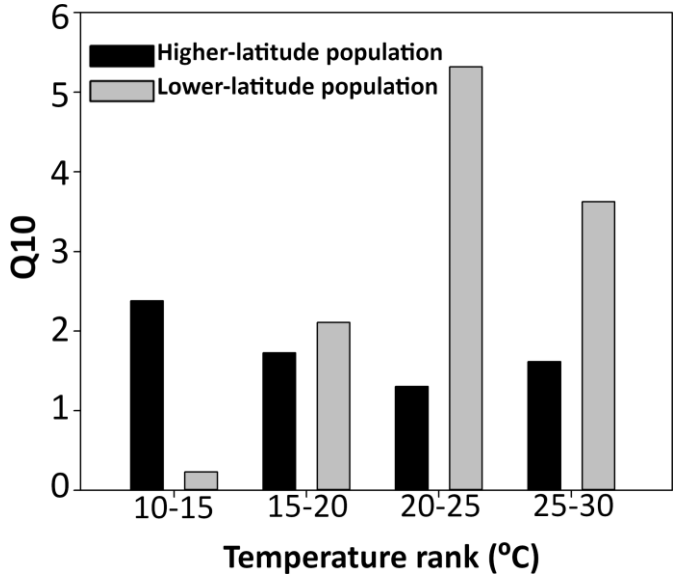


Figure 4. Temperature coefficients (Q10s) obtained for metabolic rates at interval temperatures for each population

Thermal limits

The lower-latitude population exhibited significantly higher heat coma in both acclimations compared to the higher-latitude population (Figure 5). The effect of acclimation treatment was not consistent across populations, as it was significant for the higher-latitude population but not for the lower-latitude one (Table 2, S2; Figure 5). The former showed a higher heat coma after reciprocal acclimation (lower-latitude regime) than under its natural thermal regime. Supercooling points showed greater inter-individual variability than heat coma temperature and did not differ either between populations, thermal acclimations, or their interactions (Table 2, S2; Figure 5). The lower-latitude population exhibited a wider thermal range compared to the higher-latitude population in both acclimation regimes. However, the

higher-latitude population increased its thermal range following lower-latitude acclimation (Table S2, Figure 5).

Table 2. ANOVA results for the effects of population (Pop), acclimation treatment (Acc) and their interaction on heat coma and supercooling temperature

Variable	Predictor	Df	Sum Sq	Mean Sq	F value	P value
Heat coma temperature	Pop	1	76.300	76.300	589.240	**< 0.001**
	Acc	1	8.380	8.380	64.750	**< 0.001**
	Pop x Acc	1	6.220	6.220	48.030	**< 0.001**
	Residuals	47	6.090	0.130		
Supercooling point	Pop	1	6.970	6.970	2.450	0.120
	Acc	1	1.660	1.660	0.580	0.450
	Pop x Acc	1	0.220	0.220	0.080	0.780
	Residuals	44	125.040	2.840		

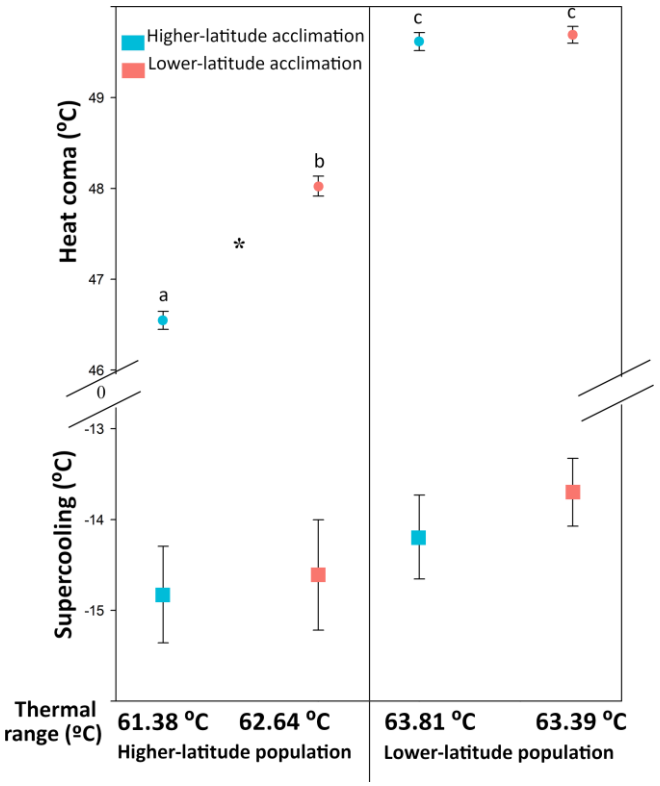


Figure 5. Heat coma temperature and supercooling point for both populations and acclimations (mean ± SE). Asterisks indicate significant differences between acclimations within each population ($p<0.05$). The letters denote differences between populations. The thermal range (difference between the mean heat coma temperature and supercooling point) is given for each population and acclimation regime on the x axis

DISCUSSION

Environmental variability represents a strong selection pressure that results in either thermal adaptation or the evolution of phenotypic plasticity, or a combination of the two, outcomes that depend on an interplay between this variability and organismal traits/evolutionary history. Both theoretical and empirical studies suggest that adaptation to local thermal regimes may shape metabolic responses, thermal tolerance, and physiological plasticity (Stevens, 1989; Addo-Bediako et al., 2002; Burton et al., 2011; Shokri et al., 2022; Dwane et al., 2023), traits that set distributional limits and determine how populations respond to global change. Although usually considered in isolation, metabolic cold adaptation and the climatic variability hypothesis represent complementary frameworks for interpreting emergent patterns and should be considered together in studies of responses to global change. Our results show that local adaptation to climates which differ in both average temperature and thermal variability has driven intraspecific variation in physiological traits in a widely distributed, supratidal water beetle species, providing support for both MCA and the CVH.

In terms of metabolic rates, our results were consistent with MCA (Addo-Bediako et al., 2002; Hodkinson, 2003), in contrast to most previous studies of coastal organisms (Sokolova & Pörtner, 2003; M. Lardies et al., 2004; Monaco et al., 2010; Lardies et al., 2011; Gaitán-Espitia et al., 2014; Barria et al., 2018) although some other studies do support the hypothesis (see Pulgar et al., 2006; Dwane et al., 2023). Here, the higher-latitude population of *O. lejolisii*, adapted to a colder climate, showed higher metabolic rates than the lower-latitude population at temperatures within the range experienced in nature during late spring/summer (15-20 °C). Such cold adaptation could clearly be advantageous for higher-latitude populations, which experience a shorter favourable temperature period for reproduction/development than their lower-latitude counterparts. The elevated metabolic rates we observed would allow higher-latitude beetles to meet the high ATP costs of growth and development required for completion of life cycles in the comparatively short growing season in this higher latitude location (Somme & Block, 1991; Gaston & Chown, 1999; Addo-Bediako et al., 2002). In contrast, at lower latitudes with warmer climate, *O. lejolisii* reproduces and develops during most of the year, being able to have at least three generations per annum (Velasco et al., 2022). In this case, high metabolic rates are less important, and could indeed incur fitness costs associated with rapid growth (Addo-Bediako et al., 2002;

Lardies et al., 2004).

The higher-latitude population exhibited an overall lower thermal sensitivity of metabolic rate, in terms of Ea, than the lower-latitude population. A number of authors have suggested that adaptation to cold conditions is associated with a lowering of activation energy, meaning that Q10 values well above 2 are likely at lower temperatures (Young, 1979; Aunaas et al., 1983; Somme & Block, 1991) as we found in our study. Such high Q10 values at relatively low temperatures would clearly allow cold adapted populations to respond rapidly to small rises in temperature (Hodkinson, 2003). We found no general effect of acclimation treatment on metabolic rates in any population, in stark contrast to the general pattern found in aquatic organisms, where acclimation typically shifts the thermal sensitivity of metabolism (Seebacher et al., 2015; Pallarés, Verberk, et al., 2021; Dwane et al., 2023). Carbonell et al., (2017) studied populations of the coastal saline water bug, *Sigara selecta*, across the same latitudinal and climatic gradient to our study. Whilst they found no clear interpopulation differences in metabolic rates, the higher-latitude population showed higher levels of plasticity of both metabolic and reproductive traits following acclimation to different temperatures/salinities.

Our results also provide partial support for the CVH, suggesting that climatic variability is an important factor shaping the physiological traits of *O. lejolisii* across its range, particularly heat tolerance. As predicted by the CVH, the lower-latitude population, experiencing higher annual temperature variation, had higher heat tolerance and a wider thermal range than the higher-latitude population, from a colder and more thermally stable environment. In contrast to CVH predictions, however, thermal limits in the lower-latitude population did not shift significantly following acclimation, whilst the higher-latitude population did increase its heat tolerance following acclimation to warmer temperatures. Such findings are consistent with trade-offs in thermal adaptation (Stillman, 2003), including recent findings on other water beetles (Hydrophilidae). In these hydrophilids, populations from more thermally variable environments appear to have evolved higher upper thermal limits than those from more climatically stable areas, but have done so at the expense of the plasticity of such limits (Pallarés et al., 2023). A lack of such thermal plasticity has also been reported in a range of species from intertidal zone, including porcelain crabs, shrimp and nudibranch molluscs (Stillman, 2003; Magozzi & Calosi, 2015; Armstrong et al., 2019). In the intertidal, organisms

are clearly subjected to rapid and frequent temperature changes that are both predictable (e.g., associated with circadian, tidal, and seasonal cycles) and unpredictable (i.e., short-term changes that are particularly pronounced during e.g., low tide or wind-driven upwelling intensity). Although predictable environmental variation is believed to enhance thermal plasticity, unpredictable environmental variability does not favour plasticity, as environmental cues can be deceptive (Barria et al., 2018; da Silva et al., 2019). Such unpredictable changes are also present in the supratidal, and are more pronounced on the Mediterranean coast, which is subject to greater temperature variability and more extreme events than Atlantic localities at higher latitudes (Izquierdo & Mikolajewicz, 2019; Parvizi et al., 2022). The apparent lack of plasticity in Mediterranean *O. lejolisii*, despite their broad thermal limits, may mean that these populations will be more vulnerable to future warming than their Atlantic counterparts (Seebacher et al., 2015). In the lower-latitude population, more extreme heatwaves will undoubtedly exert a strong selection pressure in the future, by exposing the species to temperatures closer to its physiological limit more frequently. Given the apparent lack of plasticity, this could result in selection of individuals with higher basal heat tolerance (Parvizi et al., 2022), although (as with almost all other organisms) the species' evolutionary limits in this regard remain unclear.

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SUPPORTING INFORMATION

Table S1. Programming of daily acclimation cycles.

Higher-latitude acclimation		**Lower-latitude acclimation**	
Time	**Temperature (ºC)**	**Time**	**Temperature (ºC)**
9:00	12	9:00	22
12:00	13	10:00	23
13:00	14	11:00	24
18:00	13	11:30	25
19:00	12	12:00	26
20:00	11	12:30	27
21:00	10	13:00	28
8:00	11	17:00	27
		18:00	26
		19:00	25
		20:00	24
		21:00	23
		1:00	22
		4:00	21
		7:00	20
		8:00	21

Table S2. Upper Thermal Limit (UTL), Lower Thermal Limit (LTL), and thermal range of both populations and acclimations.

	Higher-latitude population		Lower-latitude population	
Acclimation	Cold	Warm	Cold	Warm
UTL (ºC)	46.55 ± 0.09	48.03 ± 0.11	49.62 ± 0.09	49.69 ± 0.09
LTL (ºC)	-14.83 ± 0.53	-14.61 ± 0.61	-14.19 ± 0.46	-13.7 ± 0.37
Thermal range (ºC)	61.38	62.64	63.81	63.39

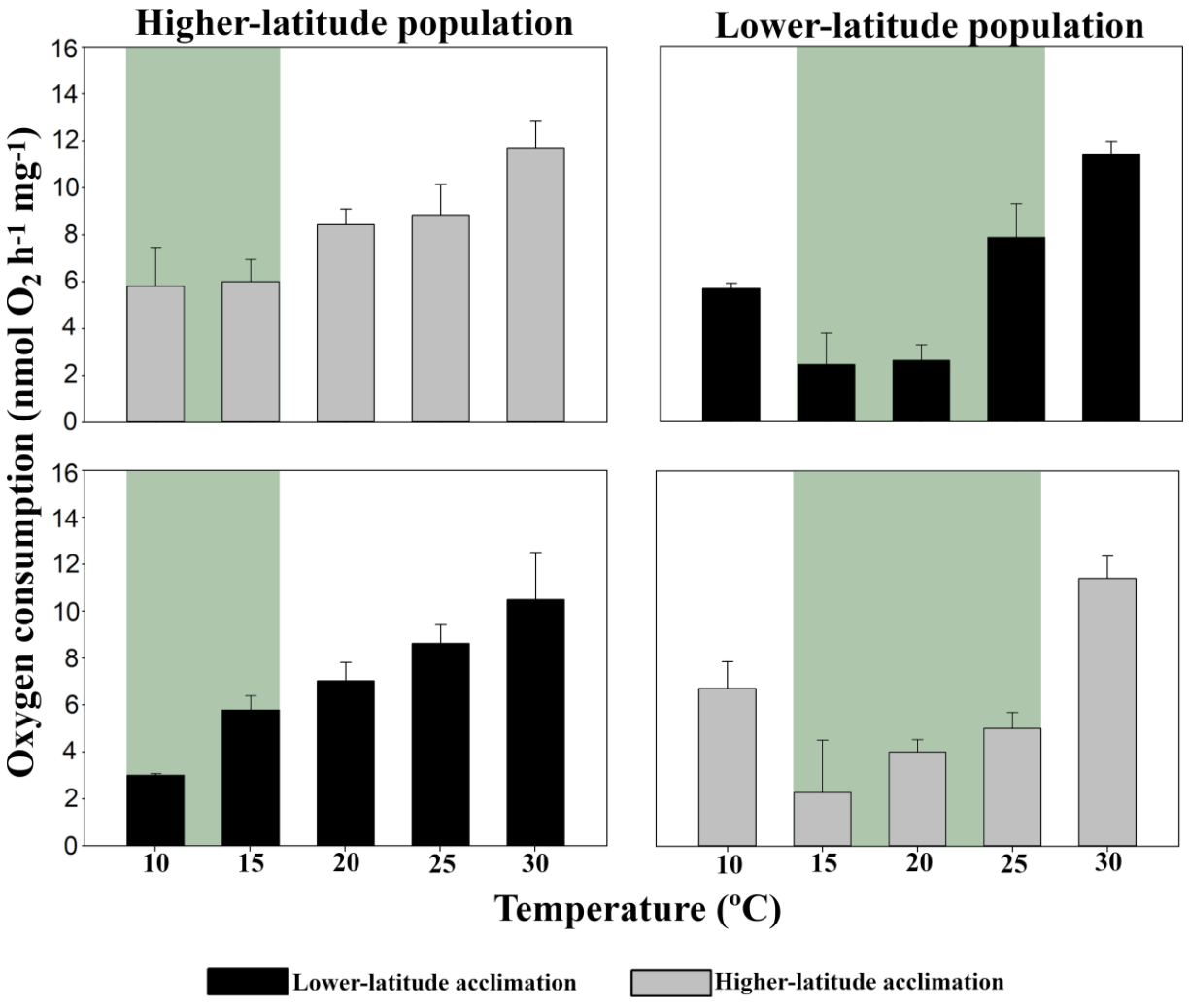

Figure S1. Mean metabolic rates ± SE of both populations and acclimations of *Ochthebius lejolisii*. The shaded green rectangles represent the range between minimum mean winter and maximum mean summer sea surface temperatures for the period 2000-2015 (Hawkins et al., 2019) for each population.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Chapter 1: Thermal tolerance differs between co-occurring congeneric beetle species in marine supratidal rockpools

1. *Ochthebius quadricollis* could be more resistant to environmental changes caused by climate change than *O. lejolisii*, because of its higher thermal tolerance.

2. Sensitivity to climate change in *O. lejolisii* appears higher, but its ability to adjust its thermal physiological limit to salinity changes could confer a higher adaptive capacity, especially in the larval stage.

3. The temperature thresholds for the adult avoidance responses were more sensitive to the salinity effect and were lower than the Heat Coma, but interspecific variation followed their physiological tolerances.

4. Physiological and behavioural responses obtained in this study for supratidal *Ochthebius* species can be useful for estimating thermal safety margins and for the implementation of mechanistic models to understand ecological climate change impacts.

Chapter 2: Discordant pattern between realised and fundamental saline niches in two supralittoral *Ochthebius* species (Coleoptera: Hydraenidae)

5. Physiological tolerance to salinity (fundamental niche) is greater in *O. lejolisii* than *O. quadricollis*, although the inverse pattern appears in their realised niches, probably due to the interaction effect of salinity with other correlated environmental stressors (e.g., temperature, drought) in nature.

6. In both species, larvae and eggs tolerate salinity more than adults probably due to their lower or null mobility and greater resistance to desiccation.

Chapter 3: Effects of salinity on desiccation resistance in supratidal beetles: cross-tolerance or cross-susceptibility?

7. This study reveals a similar ontogenetic variation in the response to desiccation in two congeneric, coexisting beetle species *O. quadricollis* and *O. lejolisii*, in which early

stages (eggs and larvae) are more resistant to desiccation than adults.

8. Results show interspecific differences in desiccation resistance and contrasting effects of salinity on the response to desiccation, likely related to differential microhabitat occupation. Such findings highlight the relevance of environmental variation and habitat stability at very local scales (microhabitat), which may shape the physiological responses in closely related species.

Chapter 4: Testing metabolic cold adaptation and the climatic variability hypotheses across the latitudinal range of a widespread, supratidal water beetle

9. The higher-latitude population of *O. lejolisii*, which inhabits colder and more stable thermal conditions, exhibited higher metabolic rates and higher metabolic sensitivity at cold temperature ranges than the lower-latitude population, with a warmer and more variable temperature regime, according to the Metabolic Cold Adaptation.

10. The differences found in heat tolerance limits between populations point to local adaptation to more variable temperatures in the lower-latitude population. Although such results suggest that the study species has a relatively high adaptive potential, the lack of plasticity found in metabolic rates in response to reciprocal acclimation and the apparent trade-offs between basal heat tolerance and thermal plasticity, could pose a risk for lower-latitude populations, probably living already near their physiological limits, in the face of more extreme events under climate change.

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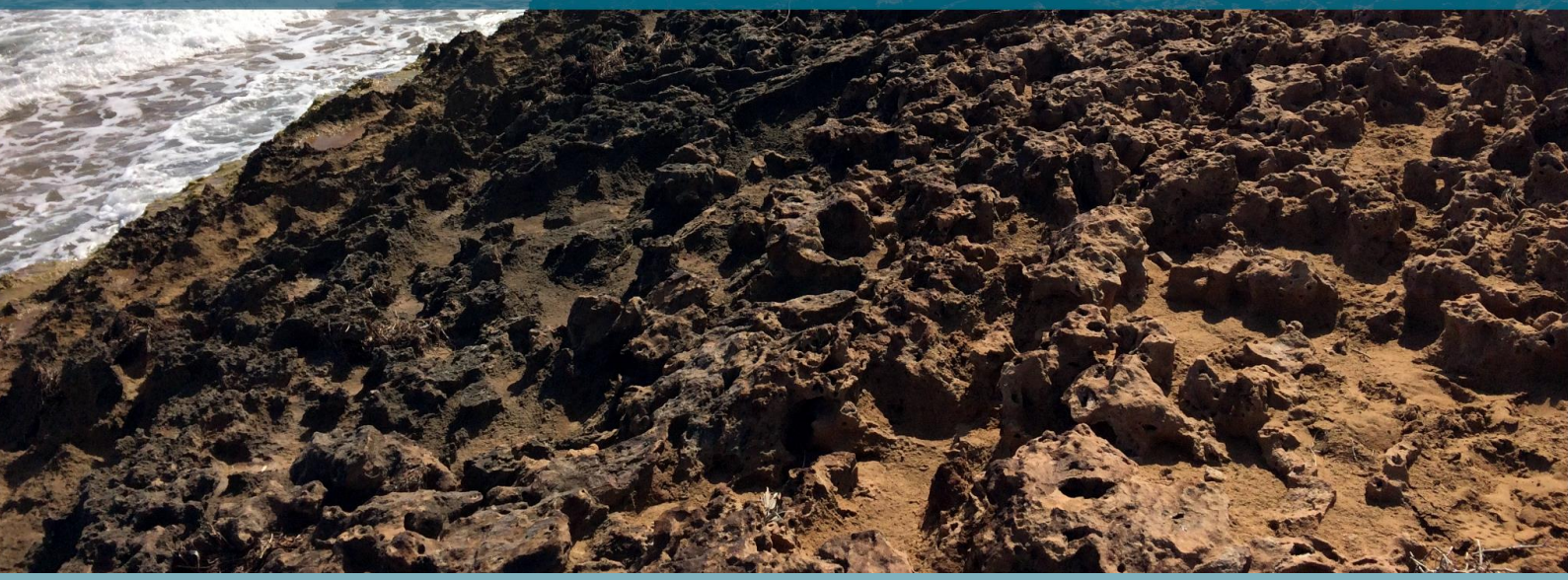

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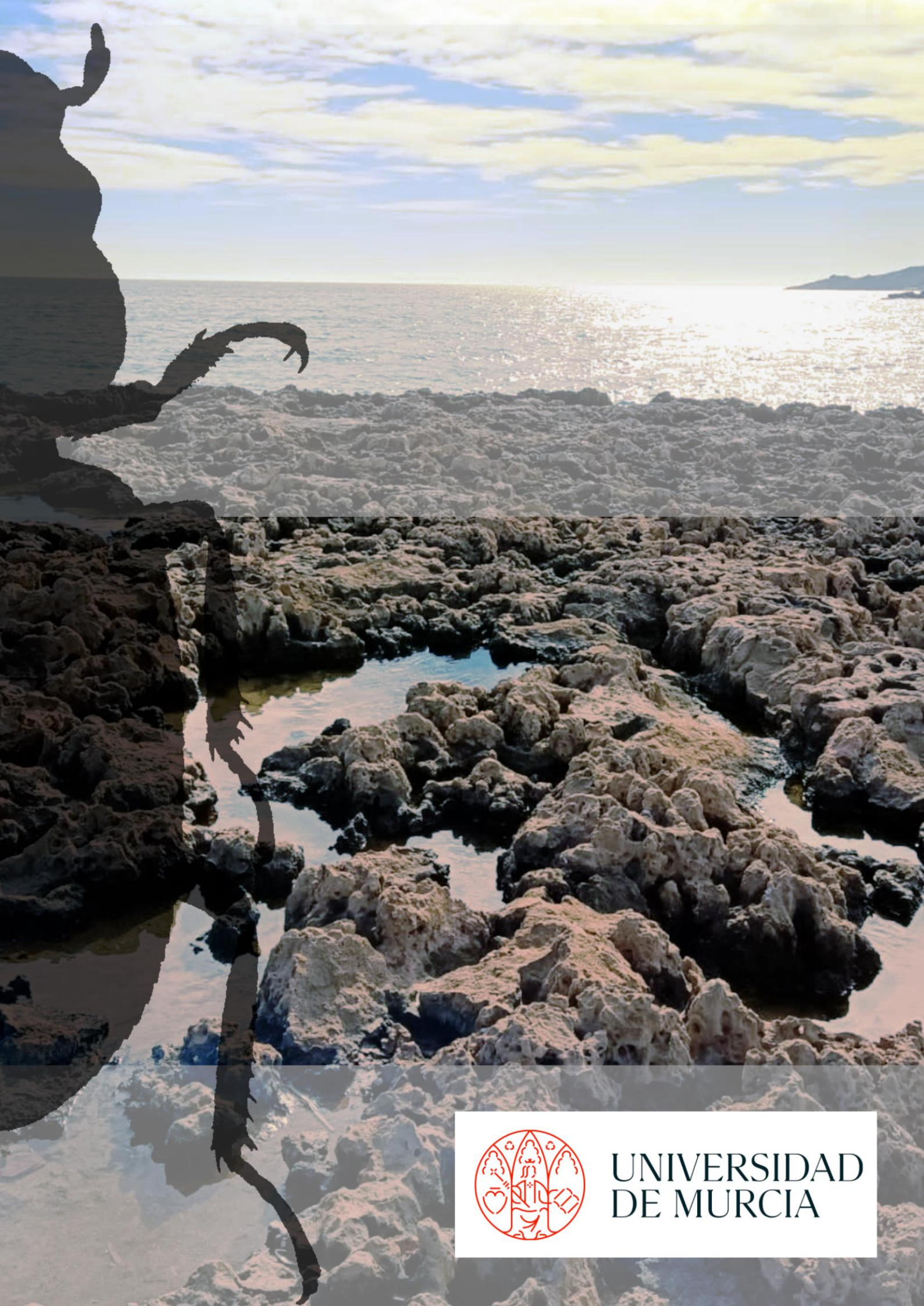

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