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Under pressure: Assessing the ecological boundaries of the epipelagic goose barnacle *Lepas anatifera* using ocean gliders and laboratory experiments

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ABSTRACT

Epipelagic barnacles have been considered good bioindicators since they are abundant and broadly distributed but with apparent tolerance restrictions to temperature and salinity, and also bioaccumulate pollutants. However, *Lepas anatifera* was found attached to the oceanic gliders, thriving through drastic and unreported environmental fluctuations. This study aimed to assess the resistance and oxidative stress responses of *L. anatifera* collected from gliders and attached to floating litter to temperature, salinity, and pressure. Barnacles withstood all tested pressure, temperature, and salinity ranges, except the extreme salt concentration. The activities of antioxidant enzymes – catalase and superoxide dismutase – were significantly increased under high temperature, high pressure, and low salinity. Malondialdehyde levels significantly increased only under high pressure. In conclusion, *L. anatifera* can be considered resistant organisms to extreme environmental changes. However, the instauration of oxidative stress under certain circumstances makes them vulnerable to predicted future trends in marine environments.

1. Introduction

The goose barnacles *Lepas* (Linnaeus, 1758) are marine cirriped crustaceans characterized by presenting a peduncle fixed to a surface and a capitulum consisting of five calcareous plates that protect them from predators (Hinojosa et al., 2006). They are epipelagic drifters that live and disperse through the ocean surface attached to different natural substrata such as wood and, in recent times, also in plastics and other synthetic objects (Inatsuchi et al., 2010). Although their dispersal link to the main surface ocean currents, the different species seem to show temperature preferences, generally distributed in broad latitudinal gradients around the globe which, together with their abundance, makes them good candidates as bioindicator organisms (Schiffer and Herbig, 2016). Furthermore, barnacles are good indicators of environmental pollution due to their high capacity to bioaccumulate pollutants (Lima et al., 2024, Preprint). There are currently nine species of *Lepas* considered valid (WoRMS, 2023), four of them cited from the western Mediterranean Sea (Ten et al., 2019): *L. anatifera* (Linnaeus, 1758), *L. anserifera* (Linnaeus, 1767), *L. pectinata* (Spengler, 1793) and *L. hillii* (Leach, 1818).

The present study focuses on the Balearic Sea, in the western Mediterranean, characterized by its oligotrophy and high evaporation rates (Bas i Peired, 2009). Sea Surface Temperature (SST) can vary seasonally and locally from 13 °C in winter to over 31 °C in summer. In recent years, the Mediterranean Sea has become one of the most affected regions by climate change, where the significant increase in the frequency, duration, and magnitude of marine heatwaves affect the coastal ecosystems and socio-economic activities. As the western Mediterranean is a vulnerable region to climate change, it responds rapidly to global warming with strong spatial variations between sub-basins (Schroeder et al., 2017). The high temperature values have progressively increased in the last recorded years (1982–2019) at 0.035 °C/year (Pastor et al., 2020). During the summer of 2022, some remarkable abnormal high values up to 3.3 °C above average were observed due to the marine heatwaves. These high-temperature values significantly increase surface stratification and affect the vertical mixing processes. Usually, in the

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upper 100 m of the water column, temperature tends to drop up to 0.05 °C/m. The permanent thermocline is located between 100 m to 1000 m of the water column, where minor variations occur (~0.02 °C/m). Below 1000 m, the temperature remains almost constant, with only a variation of 0.001 °C/m (Brown, 2016). The average surface values of salinity for the Balearic Sea during the year are ~37.8 *psu*. The vertical distribution of salinity varies significantly in the upper 400 m (Vargas-Yáñez et al., 2020).

Since 2018, large clusters of *L. anatifera* have been found attached to the surface of Autonomous Underwater Vehicles (AUV) (Personal observation), also known as *gliders*, operated in the Western Mediterranean by the Balearic Islands Coastal Observing and Forecasting System (SOCIB). Given that these organisms are epipelagic (only larvae have been documented in deep waters by Conway et al., 1990), it is noteworthy that they frequently become attached to gliders, where they spend the majority of their time in deep waters, withstanding significant variations of pressure, salinity, and temperature. Consequently, those conditions could be stressful for them, understanding *stress* as the external forces acting on an organism and disrupting its homeostasis (Stott, 1981). One way in which this internal balance is disturbed is through excessive production of reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, or superoxide anion (Lesser, 2006). To deal with these reactive species, organisms are provided with a complex antioxidant defence system, comprising endogenous and exogenous enzymatic and non-enzymatic elements. Among them, the most commonly used as biomarkers in marine organisms are the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and as non-enzymatic compounds vitamins C and E, glutathione (GSH), uric acid and malondialdehyde (MDA) (Zanette et al., 2015; Quetglas-Llabrés et al., 2020; Pinya et al., 2021).

The study aims to evaluate the tolerance of *L. anatifera* to different environmental conditions, measure the oxidative stress accumulated under those conditions, and compare the physiological state of unaltered individuals with those subjected to environmental pressures. The individuals of *L. anatifera* were collected in 2022 on floating debris and oceanic gliders monitoring the Mallorca and Ibiza Channels to examine these objectives and link them with the associated heatwave episodes in this region during the summer of 2022.

2. Materials and methods

2.1. Sample collection

Individuals of *L. anatifera* were recognised for being the only Mediterranean species with smooth calcareous plates (or with weak growth lines), of which the carina is usually bifurcated at its base, the right scutum has an internal umbonal tooth ant the tergum is slightly convex and quadrangular. In addition, there are two filamentary appendages on each side of the body, a short one near the base of the first cirri and a large one near the prosoma (Hinojosa et al., 2006). They were carefully detached from glider surfaces after different missions along the Ibiza and Mallorca Channels (called *GF126*, *GF130*, and *GF135*; Fig. 1). Gliders collect physical and biogeochemical data from the surface to 1000 m. They perform a vertical profile in a cycle of ~3.5 h, according to the operational target depth and bathymetry, and are able to stay in the sea for up to 3 months, depending on the sampling frequency, sensor configuration, and real-time communication. In addition, every few hours, they have been programmed to spend ~15 min on the surface for transmitting their location via GPS and a sub-set of the measured observations in real-time (Zarokanellos and Jones, 2021). The gliders can move vertically at an average speed of ~0.1 m/s and horizontally up to ~1 km/h. During their missions, they move daily through strong vertical gradients of hydrostatic pressure (0–100 bar), light, salinity (35.8–37.8 *psu*), and temperature (13.0–30.3 °C).

The duration of the missions *GF126*, *GF130*, and *GF135* was 59, 61, and 84 days, and they were performed during winter, spring, and summer. Seven, three, and five individuals appeared and were collected in these missions. After collection, individuals were transported immediately in labelled containers with sea water to the Laboratory of Ecological Biochemistry, Department of Biology, at the University of the Balearic Islands (Mallorca, Spain) and 10 of them were immediately frozen at −20 °C whereas the five remaining were used for resistance to high hydrostatic pressure (HHP).

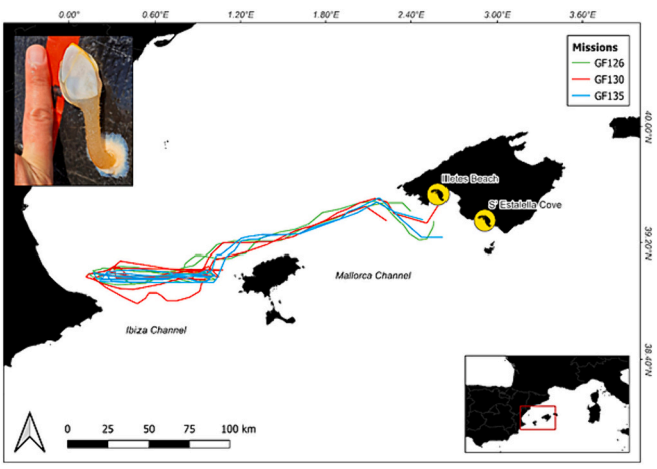

Fig. 1. Geographical location of the washed-up objects with *L. anatifera* adhered (yellow dots) along with the route of the three glider missions in which attached individuals appeared. NOTE: One of the collected specimens is shown in the upper left corner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In addition, fresh *L. anatifera* samples in good conditions were also obtained opportunistically from floating debris (plastics and ropes) washed up on the Illetes' Beach ($N = 37$) and S'Estalella Cove ($N = 28$), both located in southwest Mallorca (Fig. 1). Eight barnacles were frozen, as described above, considering that these individuals were unstressed and used as a reference control group. The other specimens still attached to their substratum were introduced in 20–50 l tanks with sea water and continuous oxygenation for further experiments.

2.2. Resistance test

A total of 57 individuals (3.07 cm ± 0.19 total length) were attached to the floating debris and subjected to resistance tests of salinity and temperature. In addition, five individuals collected from gliders were tested for resistance to high hydrostatic pressure (HHP). The organisms were exposed to extreme environmental conditions (compared to average values in the Balearic Sea) in 7.5 l tanks with continuous oxygen supply and natural light conditions for 72 h (except for the HPP test, as explained below). Given the number of individuals available, this duration was intended to balance the trade-off between observing reliable results and maintaining enough live organisms until the end of the experiments for further biochemical analysis. The 72 h of resistance tests were performed following the guidelines established by the United States Environmental Protection Agency (2002) for measuring effluent toxicity in marine organisms but also in line with previous similar experiments (Ebrahimpour et al., 2010; Sohn et al., 2015). After this procedure, survivors from each experimental group were immediately frozen. All samples were uniformly stored at −20 °C for seven days to prevent variations associated with storage conditions, since antioxidant enzymes and MDA concentration remain stable when maintained in such conditions (Jung et al., 1993; Miranda et al., 2004).

All control experiments were carried out in marine water collected together with specimens and maintained under a constant temperature of 27 °C, salinity of 37.6 *psu*, and with continuous fine bubble airflow. The environmental conditions were tested as follows:

- Salinity. Four increasing concentrations: Hyposalinity (31.6 *psu*), Control (37.6 *psu* = Average value in the Balearic Sea during the sampling period), Hypersalinity (50.1 *psu*) and Hypersalinity + (62.6 *psu*). Hyposalinity conditions were obtained adding by freshwater and hypersaline conditions by adding marine salt to natural seawater.
- Temperature. Three different temperature levels were tested: Cold (temperatures between 7 and 10 °C), Control (27 °C = Average value in the Balearic Sea during the sampling period) and Warm (32 °C). The low temperature was reached by cooling the tank with ice around it, replaced when needed. Temperature control was maintained by air conditioning the tank room temperature. As for the elevated temperature treatment, this was achieved by providing a continuous heat source by means of a heater inside the water tank. All aquaria were fitted with an internal thermometer to monitor the desired temperature.
- Pressure. After their collection from gliders, individuals were placed in a hyperbaric chamber at 100 bar (equivalent to 1000 m) for 1 h and 45 min. This process allowed us to expose *L. anatifera* to twice the pressure they were exposed to on the ocean gliders. Furthermore, a decompression from 100 to 0 bar place occurred in just 2 min and 30 s, 42 times faster than the decompression of gliders during their missions.

The values tested have reflected possible natural conditions but have also sought this species' maximum resistance and physiological limits.

In order to group the processes described above, Table 1 shows relevant information about the number of individuals per test, their sizes, their source/locality where they were collected, and their subsequent management treatments:

2.3. Preparation of tissue extracts for biochemical analyses

At least 0.1 g of tissue from the peduncle and cirri were homogenized in 10 vol (*w/v*) of 100 mM Tris–HCl buffer pH 7.5. Each homogenate was centrifuged at 9000 ×*g* at 4 °C for 15 min and supernatants were collected and immediately stored at −80 °C. The activities of the antioxidant enzymes – SOD, CAT – and the concentration of malondialdehyde (MDA) were then determined in the supernatants. Results were finally referred to the total protein content of the samples (Biorad® Protein Assay) using bovine serum albumin (BSA) as standard.

2.4. Enzymatic activities

CAT activity (K (s − 1)/mg protein) was measured following Aebi's method (Aebi, 1984). It is based on the decomposition of H_2O_2 leading to a decrease in absorbance at 240 nm. SOD activity (pmol/s/mg protein) was determined following the assay developed by Flohé and Otting (1984). It measures the rate of inhibition of cytochrome C reduction by superoxide anion generated through the xanthine oxidase/hypoxanthine system. Both activities were determined with a ShimadzuUV-2100 spectrophotometer at 25 °C.

Table 1
Experimental groups analysed in the oxidative stress tests within the number of samples used, size, source and treatment of the samples.

Experimental group	Test	N	Source/locality	Treatment
Pressure	Control	8	Illetes' Beach	−20 °C
	Gliders	10	Mallorca and Ibiza Channels	
	Gliders + HHP	5	Mallorca and Ibiza Channels	Resistance test - 20 °C
Salinity	Control	3	Illetes' Beach	
	Hyposalinity	10		
	Hypersalinity	7		
	Hypersalinity+	9		
Temperature	Control	6	S'Estalella Cove	
	Cold	8		
	Warm	6		

2.5. Malondialdehyde (MDA) determination

The levels of MDA, a lipid peroxidation (damage indicator) compound, were determined by a colorimetric assay. This is based on the reaction that takes place between MDA and a chromogenic reagent to yield a stable chromophore with an absorbance maximum at 586 nm. This test was carried out as soon as the samples were homogenized to avoid overestimation of the MDA. Promptly, samples were collected in glass tubes containing *n*-methyl-2-phenyl-indole (10.3 mM) in acetonitrile:methanol (3:1). Then, HCl (12N) was added, and the samples were incubated for 1 h at 45 °C. Absorbance was measured at 586 nm in a microplate reader (Bio-Tek®, PowerWaveXS).

2.6. Statistical analyses

Statistical analyses were conducted using the software RStudio v1.4.1717 for Windows (RStudio Team, 2020). Since the source, locality, and time of collection of the individuals differed for each environmental factor tested (pressure, salinity, and temperature), an experimental and analytical framework was considered for each. Shapiro-Wilk and Levene's tests were carried out to check for a normal distribution of the response variables and the homogeneity of variances between levels, as these are requirements for the hypothesis testing that was subsequently carried out.

Subsequently, one-factor analysis of variance type II (F-Test ANOVA) was applied to assess whether there were significant differences within each experimental group. Since experimental groups had more than two stressor levels tested, they were also subjected to the *t*-test Tukey HSD (Honestly Significant Difference). This is a multiple comparisons test that allows for the comparison of the means of the *t* levels of a factor after rejecting the null hypothesis of the ANOVA test. In this way, we were able to find out between which of the levels tested in each experimental group there were significant differences. It is based on the distribution that follows the difference between the maximum and minimum data in a normalized sample (studentized range distribution) (Abdi and Williams, 2010).

3. Results

3.1. Resistance

Every individual was found to survive the high hydrostatic pressure chamber assays under the described conditions. The tested *L. anatifera* have also proved to be resistant (over 50 % survival rate) to all salinity conditions, except for the highest concentration of 62.6 *psu* (hypersalinity +); there were no survivors in this group after 24 h. Finally, regarding the temperature conditions, goose barnacles were able to withstand both warm and cold temperatures. However, the physical appearance of the individuals was noteworthy. Individuals exposed to cold temperatures showed signs of latency (deficient activity or paralysed, with cirri mainly retracted inside plates). In contrast, those that endured warm temperatures, showed significant signs of stress (e.g., scarce or no movements and with cirri widely spread outside plates). These results are detailed in Table 2.

3.2. Biochemical analyses

The activity of antioxidant enzymes (SOD and CAT) and lipid peroxidation levels (MDA) of *L. anatifera* are displayed in colour-coded bar charts. Each experimental group was obtained from different sources and at different times, so that comparisons should only be made within each group.

Table 2
Survival rates observed for the different conditions tested.

Experimental group	Test	Survival rate
Pressure	HHP chamber	100 %
Salinity	Control	100 %
	Hyposalinity	80 %
	Hypersalinity	100 %
	Hypersalinity+	0 %
Temperature	Control	100 %
	Cold	80 %
	Warm	50 %

Catalase and superoxide dismutase activities (Fig. 2) were significantly higher in those *L. anatifera* that were exposed to high temperatures and low salinities ($p < 0.05$). At the same time, pressure has also proved to be a stressor. Organisms that endured attached to gliders showed significantly higher activities of these enzymes compared to their control group ($p < 0.001$). Even more significant and remarkable results were shown by individuals who were subjected to the additional hydrostatic pressure in the chamber ($p < 0.001$).

On lipid peroxidation, MDA concentrations (Fig. 3) were very significantly higher in specimens that were aboard gliders together with those under additional experimental hydrostatic pressure when compared to the epipelagic control group ($p < 0.001$). The results generated by the low salinity and high-temperature conditions, although they did not reach the minimum significance level for this study ($\alpha = 0.05$), showed a considerable increase in lipid damage compared to their control group.

4. Discussion

Based on the survival rates, the observations of their physical appearance after the assays, and the biochemical analyses, certain

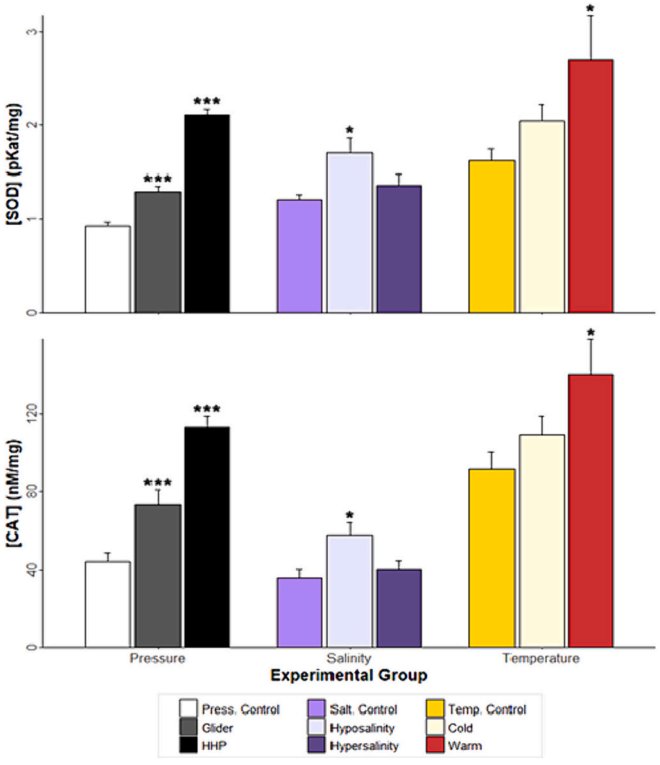

Fig. 2. Enzyme activities (SOD and CAT) in *Lepas anatifera* from floating debris (controls), gliders, and after 72 h exposure to different environmental conditions (pressure, salinity and temperature). Significance codes: *<0.005, ***<0.001.

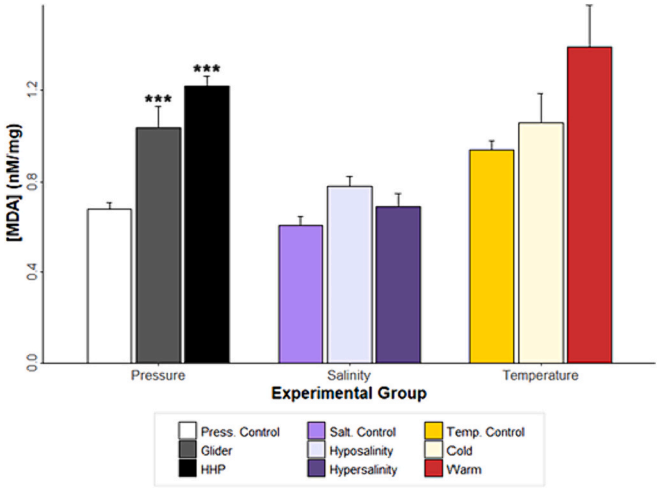

Fig. 3. MDA accumulation (lipid peroxidation) in *Lepas anatifera* obtained from floating debris (controls), gliders, and after 72 h exposure to different environmental conditions (pressure, salinity and temperature). Significance codes: ***<0.001.

physiological characteristics about *L. anatifera* have been elucidated. Their resistance to the wide variations in salinity (from 31.6 *psu* to 50.1 *psu*) is remarkable. Even greater hyposaline resistance has been reported in other pelagic barnacle species, such as *Chelonibia patula* (Ranzani, 1818), which have thrived in salinities as low as 16 *psu* (Sundell et al., 2019). However, to our knowledge, this is the first barnacle surviving in such high salinity conditions (50.1 *psu*). Nevertheless, different mortality ranges have been found in our experiments, and dissolved oxygen (DO) seems to have been a determining factor. There is a negative correlation between DO and salinity (Sherwood et al., 1991; Kielmas, 2018), meaning that individuals subjected to maximum salinity concentration (62.6 *psu*) could lack enough oxygen to carry out cellular respiration, resulting in death. In contrast, the other salinity levels tested would have had acceptable DO concentrations to survive. Despite this, a lower survival rate has been observed under low salinity conditions, which could be explained if oxidative stress is considered. As oxygen is essential for the formation of ROS (Birben et al., 2012), hyposalinity (and, consequently, high DO) specimens will be more prone to suffer from oxidative stress since, for the correct functioning of the organisms, there must be a balance between enough DO to carry out the vital processes and not favour the overproduction of ROS (Birben et al., 2012).

To interpret the results of the temperature gradient assays, the metabolic rate and the DO must be considered. Dissolution of oxygen in water decreases with increasing temperature (Truesdale and Downing, 1954). If we consider the relationship between DO and oxidative stress, there should be a greater tendency to produce it as temperature decreases (Lushchak, 2011). In contrast, mortality rates and oxidative stress levels were higher at the warmest temperature tested (32 °C). These results are consistent with those obtained in *Pollicipes pollicipes* (Gmelin, 1789), an Atlantic species of goose barnacle (Ramos et al., 2016). The metabolic rate of organisms could explain this apparent contradiction. Higher temperature stimulates most of the metabolic processes, such as oxygen consumption (despite the lower concentration in the environment) (Clarke and Fraser, 2004), leading to increased ROS production (Lushchak, 2011). Furthermore, enzyme activity rates double (e.g., SOD and CAT) for every 10 °C increase (Razavi et al., 2015). This same argument would explain why there was no remarkable oxidative stress and their survival at temperatures between 7 and 10 °C, even below the 15 °C threshold reported by Schiffer & Herbig in 2016 for this species. Another congener, *L. anserifera*, has also demonstrated the ability to survive under temperatures down to 11 °C (Inatsuchi et al.,

2010). Nevertheless, an alternative explanation is that the elevated rates of oxidative stress under low DO conditions (high temperature and salinity) could be due to "hypoxia-induced oxidative stress". The latter will increase the antioxidant responses under low oxygen concentrations as a preventive measure so that ROS are not produced when DO increases (Lushchak, 2011). Evidence that catalase activity increases under these conditions have been shown in Vidal et al. (2002), which indicates the ability of *L. anatifera* to withstand large environmental variations. When analysing oxidative stress markers in *L. anatifera*, it is remarkable that the antioxidant response was able to prevent a significant increase in MDA levels, as an indicator of lipid peroxidation both under tested salinity and temperature conditions.

Regarding hydrostatic pressure, both glider missions and subsequent hyperbaric chamber tests have led to a significant increase in CAT and SOD activities but, unlike temperature and salinity tests, they were not sufficient to protect barnacles against oxidative damage. Aboard gliders, *L. anatifera* are exposed to continuous variations in temperature (Brown, 2016), salinity (Vargas-Yáñez et al., 2020), and hydrostatic pressure ($P = \rho gh$) due to their displacement in the water column, which would explain the significant oxidative damage observed in these organisms. Nevertheless, barnacles that were subsequently exposed to additional pressure in the hyperbaric chamber showed the highest activities of SOD and CAT, and the highest levels of MDA. These results seem to indicate that hydrostatic pressure caused the greatest oxidative damage. Increases in pressure may involve rigidity of cell membranes, altered protein folding or difficulty in binding ligands inducing a high degree of physiological stress (Yancey, 2020). The observed high resistance of barnacles to this factor might be a sign that these organisms contain *piezolytes-type* molecules, which are present in other crustaceans, teleost fishes, sharks, or amphipods (Yancey, 2020). There are no previous studies on the effects of pressure variation in barnacles since they are typically epipelagic. However, it has been shown that exposure to high pressure induces genes involved in pathways of excitotoxic damage to neurons and heat shock in the shrimp *Palaemonetes varians* (Morris et al., 2015).

Temperature is the most concerning factor in the present study's survival rates and vulnerability to *L. anatifera*, an outcome that is relevant in a climate change context. The warming trend of the ocean in the upper layer, together with more frequent heatwave episodes (Smale et al., 2019; Pastor et al., 2020; Juza et al., 2022), could indeed be the main cause of an observed decline in numbers of *L. anatifera* over 2022, a year in which heatwaves and SST have been particularly intense in the Balearic Sea (Juza and Tintoré, 2020). Our results show that the higher temperature registered in 2022 may not only cause an increase in oxidative stress but also control the presence (attachment) of *L. anatifera* to floating objects on the surface. A previous study has already indicated that *Balanus trigonus* (Darwin, 1854), another cirriped crustacean, has reduced its attachment under high-temperature conditions (above 28 °C) (Thiyagarajan et al., 2003).

5. Conclusions

This study used laboratory experiments and specimens attached to gliders, and consequently, subjected to repetitive biophysical changes to determine the resistance of the goose barnacle *L. anatifera* to a range of variations in selected environmental conditions and the oxidative stress these changes can cause to individuals. After analysing the generated data and the subsequent interpretation of the results, we highlight the great resistance of *L. anatifera* to main oceanic factors (thermohaline and pressure characteristics in the study area). These results are consistent with the wide distribution and natural conditions in which this species is found (Schiffer and Herbig, 2016) but also indicate a greater resistance to even wider temperature and salinity ranges. However, they maintain other restrictions associated with the availability of substrate or the presence of oceanic currents. Exposure to high temperatures, low salinities, and high hydrostatic pressure incurs significant levels of oxidative stress. Therefore, the increase in sea surface temperature due to the global warming trend and the combination of the extreme heatwave episodes in duration and magnitude that we observed in 2022 in the Balearic Sea may negatively affect *L. anatifera* survival, attachment, and even abundance in this area. Future and detailed molecular studies could describe some molecular characteristics of *L. anatifera* to enlighten the origin of their resistance to different environmental factors. In addition, long-term monitoring of these individuals and surface temperatures could provide information on the effects of global change on marine organisms.

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CRediT authorship contribution statement

Yago Iván-Baragaño: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Nikolaos D. Zarokanellos:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Antoni Sureda:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **María Capa:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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