1   Survival on a semi-arid island: submersion and desiccation tolerances of fiddler crabs from

2   the Galapagos Archipelago

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5   Mariana V. Capparelli[a], Carl L. Thurman[b], Paloma Gusso Choueri[c,d], Denis Moledo Abessa[c],

6   Mayana Karoline Fontes[c], Caio Rodrigues Nobre[c], John Campbell McNamara[e,f*]

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9   [a]Facultad de Ciencias de la Tierra y Agua, Universidad Regional Amazónica Ikiam, km 7 vía

10   Muyuna, Tena, Napo, Ecuador

11   [b]Department of Biology, University of Northern Iowa, Cedar Falls IA 50614-0421, United

12   States of America

13   [c]Universidade Estadual Paulista, Campus de São Vicente, São Vicente 11380-972, SP, Brazil

14   [d]Departamento de Ecotoxicologia, Universidade Santa Cecília, Santos 11045-907, SP, Brazil

15   [e]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto,

16   Universidade de São Paulo, Ribeirão Preto 14040-901, SP, Brazil

17   [f]Centro de Biologia Marinha, Universidade de São Paulo, São Sebastião 11600-000, SP,

18   Brazil

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20   *Corresponding author: mcnamara@ffclrp.usp.br

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27   Running head: Ecophysiology of Galapagos fiddler crabs

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Abstract

During tidal cycles, semi-terrestrial fiddler crabs are subject to alternating periods of submersion and desiccation. Here, we compare physiological and biochemical adjustments to forced submersion and desiccation in two fiddler crabs from the Galapagos archipelago: the indigenous *Leptuca helleri*, and *Minuca galapagensis*. We examine ecological distributions and habitat characteristics using transect analysis; survival after 6 h forced submersion at different salinities (0, 21 and 42 ‰S), and after 6 or 12 h desiccation challenge, including alterations in hemolymph osmolality; and, oxidative stress responses in the gills and hepatopancreas, accompanying glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione reductase (GR) activities, and lipid peroxidase (LPO). We provide an integrated biomarker response (IBR) index for each species based on oxidative stress in each tissue and condition. Our transect study revealed that *L. helleri* occupies an intertidal niche while *M. galapagensis* is supralittoral, *L. helleri* being less resistant to submersion and desiccation. After 6 h submersion, *L. helleri* survived only at 21 ‰S while *M. galapagensis* survived at all salinities. Hemolymph osmolality decreased at 0 ‰S in *M. galapagensis*. After 6 h desiccation, osmolality decreased markedly in *L. helleri* but increased in *M. galapagensis*. Enzyme assays were not performed in *L. helleri* owing to high mortality on submersion/desiccation challenge. After submersion in *M. galapagensis*, hepatopancreas GPx activities decreased in 0 and 21 ‰S while GR activity was strongly inhibited at all salinities. Gill LPO decreased in 42 ‰S. On desiccation in *L. helleri*, GPx activity was inhibited in the hepatopancreas but increased in the gills. GST activity increased while LPO decreased in both tissues. After desiccation in *M. galapagensis*, hepatopancreas GPx activity increased. Both hepatopancreas and gill GST and GR activities and LPO were strongly inhibited. The IBR indexes for *L. helleri* were highest in fresh caught crabs, driven by gill and hepatopancreas LPO. For *M. galapagensis,* submersion at 21 ‰S contributed most to IBR, LPO in both tissues responding markedly. *Leptuca helleri* appears to be a habitat specialist adapted to a narrow set of niche dimensions while *M. galapagensis* survives over a much wider range, exhibiting little oxidative stress. The species' physiological flexibilities and limitations provide insights into how fiddler crabs might respond to global environmental change on semi-arid islands.

Introduction

Fiddler crabs are typical inhabitants of the estuarine environments of tropical and temperate zones (Crane, 1975). Many biological factors such as vegetation cover and resource competition, and abiotic attributes like edaphic characteristics, sediment grain size, organic content, and salinity and temperature influence their distribution (Nobbs, 2003; Thurman et al., 2013; Mokhtari et al., 2015; Checon and Costa, 2017). Such heterogeneous habitats range from salt marshes and exposed, dry sandy beaches to shaded, muddy mangrove forests (Thurman, 1984; Thurman et al., 2013), which can be physiologically challenging to fiddler crabs (Allen and Levinton, 2014; Munguia et al., 2017, Thurman et al., 2017). During tidal cycles, many fiddler crab species are exposed to temperature and salinity variation (Helmuth et al., 2006; Schneider, 2008; Somero, 2002) and face desiccation (Allen et al., 2012; Chapman and Underwood, 1996; Miller et al., 2009; Thurman, 1998).

Nevertheless, fiddler crabs are resilient components even of degraded ecosystems and some can tolerate severely polluted habitats (Capparelli et al., 2016). Certain species are more generalist in their ecological demands, inhabiting a diversity of environments while others are more specialized, exhibiting restricted habitat preferences. Given predicted alterations to mangrove communities, owing to habitat loss and climate change (Saintilanet al. 2014), some fiddler crab species may not be able to survive in novel habitats, despite their tolerances of variation in ambient parameters. Many minimize the effects of desiccation, for example, by inhabiting burrows as a refuge (Powers and Cole, 1976), albeit with fewer opportunities to forage and reproduce (Allen and Levinton, 2014). Desiccation and submersion tolerance varies among fiddler crabs and is an indicative of their resistance to aerial exposure during low tide (Thurman, 1998; Levinton et al., 2015; Principe et al., 2018). Large scale landscape changes, such as mangrove deforestation or altered ocean levels, may cause physiological changes resulting from submersion and desiccation challenge that affect ecological distribution patterns (Araújo et al., 2007, Wilson et al., 2005) and survival.

Fiddler crab ecology has been of much interest (Landstorfer et al., 2010, Cuellar-Gempeler and Munguia, 2013; Costa and Soares-Gomes, 2015, Thurman et al. 2003, 2005, 2017), but the effects of desiccation and submersion on their physiology is poorly known (Levington et al., 2015). Species distributed within the upper tidal zone like *Minuca rapax* are more resistant to dissection than intertidal species such as *Leptuca thayeri* (Principe et al., 2018). Osmoregulatory ability and aerial exposure show no general trend (Gilles and Péqueux, 1983; Borecka et al., 2016) although aerial exposure can increase hemolymph

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93 osmolality due to dehydration, physiological responses varying among species (Thurman,
94 1998).

95 Aerial exposure represents a major challenge for intertidal organisms, since it leads to
96 dehydration and metabolic stress (Allen et al., 2012; Chapman and Underwood, 1996; Miller
97 et al., 2009). Cellular oxidative stress occurs when the rate of production of reactive oxygen
98 species (ROS) exceeds their decomposition by antioxidant systems, increasing oxidative
99 damage such as lipid peroxidation, enzyme inactivation, DNA base oxidation and protein
100 degradation (Halliwell, 1993; Lemaire and Livingstone, 1993). Cellular protection against
101 ROS includes the glutathione system of specific antioxidant enzymes such as glutathione
102 peroxidase (Sies et al., 1979; Keeling and Smith, 1982; Sies, 1993) and glutathione S-
103 transferases (Tan et al., 1987), together with complementary enzymes like glutathione
104 reductase that produce glutathione and NADPH, maintaining cellular antioxidant status
105 (Reed, 1986). Antioxidant enzymes and oxidative damage levels as indicators of oxidative
106 stress have been evaluated in *M. rapax* from contaminated environments (Capparelli et al.,
107 2019). However, little is known with regard to modulation of oxidative stress in fiddler crabs
108 from pristine habitats during submersion and desiccation challenge.

109 The two fiddler crab species found on the Galapagos Archipelago, *M. galapagensis*
110 Rathbun 1902 and *L. helleri* Rathbun 1902, were originally considered endemic (Rathbun,
111 1902), and their ecological preferences are very sketchy. Boone (1927) quoting Beebe (1924)
112 stated that *M. galapagensis* typically occupied "salt marshes and tidal flats", with burrows at
113 the high-tide mark of about 2 cm diameter and 20-30 cm deep. Garth (1948) described *M.
114 galapagensis* as burrowing near brackish-water lagoons or on mud flats with iron-red or gray
115 colored substrate; *L. helleri* was usually found on sandy-mud among mangrove roots, the
116 species supposedly separated by their habitat preferences. Von Hagen (1968) considered *M.
117 galapagensis* as very flexible in occupying habitats with widely differing substrates. Peck
118 (1994) reported *M. galapagensis* to inhabit soft mud in mangroves, and *L. helleri* to occur on
119 sandy or muddy intertidal flats, with no ecological overlap.

120 The present study aims to compare the effect of submersion and desiccation challenge
121 in *L. helleri* and *M. galapagensis* from mangrove areas on Santa Cruz Island, in the
122 Galapagos Archipeligo. We hypothesize that *L. helleri*, an ecologically demanding species,
123 would be more sensitive to submersion and desiccation than *M. galapagensis*, a generalist
124 species, such tolerances subsidizing their ecological preferences. Understanding the
125 differential effects of extreme conditions of desiccation and submersion on fiddler crabs from
126 areas with different degrees of exposure to water helps to predict how these species behave in

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127 possible environmental change scenarios. Specifically we evaluate the species': (1) density

128 distributions as a function of selected ambient parameters; (2) tolerance of submersion and

129 desiccation, and hemolymph osmoregulatory ability; and (3) oxidative stress response to

130 submersion and desiccation.

131

132 **Materials and methods**

133 **Study area**

134 The study area was located in a stretch of arid lowland on the southern coast of Santa

135 Cruz Island, one of the 13 major islands that constitute the Galapagos Archipelago, Ecuador.

136 Santa Cruz Island is the second largest in the archipelago, is 986 km^2 in area and has a

137 maximum altitude of 864 m. Recent temperatures and precipitation have ranged from lows of

138 21-22 °C (mean 22 °C) and 0.5 mm in September 2018 to highs of 25-29 °C (mean, 27 °C)

139 and 64.9 mm in April 2019 (www.worldweatheronline.com/santa-cruz-weather-

140 averages/galapagos/ec.aspx). Around 90% of the terrestrial area is protected as part of the

141 Galapagos National Park (Servicio Parque Nacional Galapagos, 2006; Moity et al., 2019).

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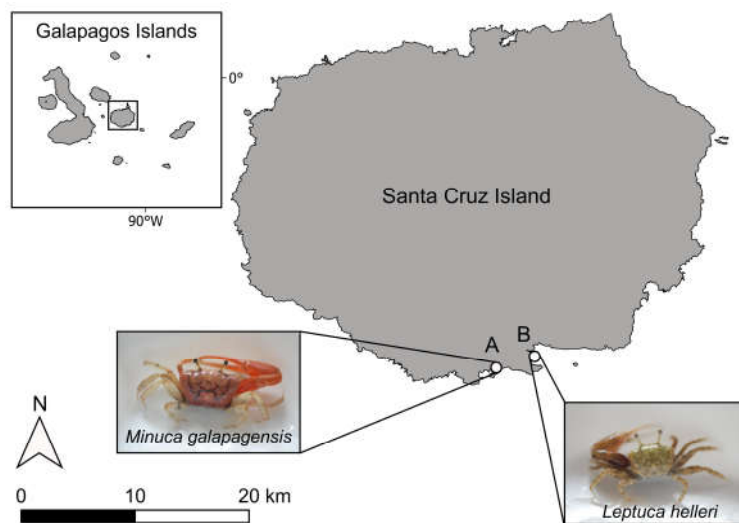151 **Figure 1**. The study area. Santa Cruz Island is one of the 13 main islands that form the

152 Galapagos Archipelago, Ecuador. It is the second largest island and has an area of 986 km^2

153 and a maximum altitude of 864 m. Approximately 90% of the island is protected as part of

154 the Galapagos National Park. The Galapagos fiddler crabs *Leptuca helleri* and *Minuca*

155 *galapagensis* abound in the coastal mangrove ecosystems. A, Bahia Tortuga collecting site

156   (00.76174° S, 90.34111° W). B, Playa de los Alemanes collecting site (00.75243°
157   S, 90.31087° W).

158

159   **Study organisms and ecological transects**

160   The two fiddler crab species found on the Galapagos Archipelago are *Minuca*
161   *galapagensis*, and the endemic *Leptuca helleri* (Rathbun, 1902). Originally placed in the
162   genus *Uca*, they now belong to separate genera: *Minuca* (Bott) and *Leptuca* (Bott) (Shih et
163   al., 2016). There is no information on the conservation status of either species in the IUCN
164   Red List of Threatened Species (https://www.iucnredlist.org, May, 2020).

165   Potential habitats on Santa Cruz Island were reconnoitered for fiddler crab colonies
166   and areas for transect analyses were selected for *Leptuca helleri and Minuca galapagensis*
167   based on salinity, abundance and ease of access. Most sites had only one species.

168   Among the eight possible locations, the best site for characterizing *L. helleri* was on
169   the eastern edge of the Playa de los Alemanes (00.75243° S, 90.31087° W), a beach
170   consisting of coarse coralline sand with sea shells and broken coral, near a mangrove stand
171   (*Rhizophora* sp.) (Figures 1 and 2). For *M. galapagensis*, the optimum area was a large sand
172   flat named "Tortuga Flats" by us, enclosed by mangroves on the northern shore of Bahia
173   Tortuga, a fine white sand beach (00.76174° S, 90.34111° W). The transects were established
174   orthogonally to the nearest water source. The "Tortuga Flats" transect ran 30 m inland from
175   the supralittoral sand berm towards a large shallow pond while the Playa de los Alemanes
176   transect ran 16 m down the beach from the supralittoral zone to the low water mark (Figures
177   1 and 2).

178   Vegetation coverage and the number of crab burrows in 1 m^2 quadrats were recorded
179   at 2 m intervals along the transects. On the *M. galapagensis* transect, percentage soil moisture
180   was taken at a depth of 15 cm with an electronic soil probe. Soil temperatures were measured
181   on the substrate surface and at 10 cm depth. Each burrow was excavated, the inhabitant
182   identified, a water sample collected, and the water table depth measured. The osmolality of
183   the water samples (in mOsm/kg H_2O) was measured using a vapor pressure micro-osmometer
184   (Wescor 5520, Logan, UT).

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Figure 2. Sites of transect analyses for the Galapagos fiddler crabs. For *Minuca galapagensis* (left panel), the optimum area was an extensive, sheltered sand flat ("Tortuga Flats") amongst the mangroves behind the sand berm on the shore of Bahia Tortuga (00.76174° S, 90.34111° W). For the endemic species *Leptuca helleri* (right panel), the best area was adjacent to an exposed mangrove stand on the eastern edge of Playa de los Alemanes (00.75243° S, 90.31087° W). Dotted lines indicate approximate transects taken orthogonally to the nearest water source.

Crab collections

Adult, intermolt specimens of *M. galapagensis* and *L. helleri* of either sex were collected in June and July 2019 (dry season) from the "Tortuga Flats" at Bahia Tortuga (00.76174° S, 90.34111° W) and the sandy beach at Playa de los Alemanes (00.75243° S, 90.31087° W), Santa Cruz Island, Galapagos (see Figures 1 and 2) (collecting permit #083-2019 from the Dirección del Parque Nacional Galápagos to MVC). Only *L. helleri* occurred at Playa de los Alemanes while both species were abundant at "Tortuga Flats".

The crabs were transported to the Fabricio Valverde laboratory at the Charles Darwin Research Station in plastic boxes containing sponge cubes moistened with seawater from the collecting sites. Only non-ovigerous, intermolt crabs of carapace width greater than 10 mm for *M. galapagensis* and 3 mm for *L. helleri* were used.

To acclimatize to laboratory conditions before use, the crabs were maintained unfed for 2 days after collection at 25 °C under a 12 h light: 12 h dark natural photoperiod, with

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208   free access to a dry surface, in plastic boxes containing seawater from the respective

209   collection sites (29 ‰S).

210

211   **Submersion and desiccation protocols**

212   To examine the effects of forced submersion, crabs were maintained fully submerged,

213   simulating the high tide covering their burrows. Groups of 10 crabs each were submerged for

214   6 h at salinities of 0 ‰S [distilled H_2O, hypo-osmotic medium], 21 ‰S [isosmotic reference

215   medium] or 42 ‰S [hyper-osmotic medium] in individual plastic jars containing 250 mL of

216   medium. Saline media were prepared by diluting seawater with bottled water or adding

217   Instant Ocean sea salts. Salinities were checked using a hand-held refractometer (American

218   Optical Company, MA). Six hours is roughly the period a crab would be submerged naturally

219   between the pre- and post-high tide (Batista, 2010; Capparelli et al., 20017).

220   To examine the effects of desiccation, previously blotted crabs were held in individual

221   dry containers for 6 or 12 h. Mortality was totaled at the end of both experiments. Crabs were

222   considered dead if they could not right themselves.

223   After the submersion and desiccation experiments, the crabs were cryo-anesthetized

224   in crushed ice for 10 min. A hemolymph sample was then drawn through the arthrodial

225   membrane at the base of the posterior-most pereiopod into a 1 mL syringe, and all gills and

226   the hepatopancreas were dissected, placed in individual, labeled micro-Eppendorf tubes and

227   frozen at -80 °C for posterior analysis. Two-day acclimatized crabs, dissected at the

228   beginning of the experiments (Time = 0 h), were used as reference control crabs (fresh caught

229   crabs).

230   Samples were transported in dry ice by air to laboratories in Brazil. The entrance of

231   samples into Brazil at Guarulhos Airport was authorized by the Ministério da Agricultura,

232   Pecuária e Abastecimento (licence #000014.0020214/2019 to JCM).

233

234   **Measurement of hemolymph osmolality**

235   Hemolymph osmolality in both crab species was measured in 10 μL aliquots or

236   occasionally employing 2-3 hemolymph pools, using a vapor pressure micro-osmometer

237   (Wescor 5500, Logan, UT).

238

239   **Oxidative stress assays**

240   Oxidative stress activities were measured in hepatopancreas and gill homogenates

241   from *M. galapagensis* and *L. helleri*. Immediately before the assays, samples were thawed on

242 ice and homogenized in a Tris-HCl buffer solution (4% w/v, in mmol L^{-1}, Tris 50,

243 Ethylenediamine tetra acetic acid 1, Dithiothreitol 1, Sucrose 50, KCl 150,

244 Phenylmethylsulfonyl fluoride 1, pH 7.6). Aliquots were separated to analyze lipid

245 peroxidation (LPO). The remaining homogenates were centrifuged (Eppendorf model 5804R,

246 Eppendorf North America, Hauppauge, NY) at 12,000 × g and 4 °C for 20 min, and the

247 supernatants used for the enzyme assays.

248 Glutathione S-transferase (GST) and glutathione peroxidase (GPx) and glutathione

249 reductase (GR) activities were assayed following the protocols described by Keen et al.

250 (1976), Sies et al. (1979) and Mcfarland et al. (1999), respectively. All enzyme activities

251 were measured spectrophotometrically at 340 nm. LPO analyses were performed by

252 fluorescence spectroscopy (excitation 516 nm, emission 600 nm) using the thiobarbituric acid

253 reactive substances (TBARS) method (Wills, 1987).

254 All assays were performed using a BioTek Synergy HT Multi-Detection Microplate

255 Reader (BioTek, Winooski, VT). Enzyme activities were normalized by total protein content,

256 measured using Bradford's (1976) method.

257

258 **Integrated biomarker response indexes**

259 The overall effects of 6-h forced submersion at the different salinities or desiccation

260 for 6 or 12 h on biomarker activities in each tissue were quantified using integrated

261 biomarker response indexes (Beliaeff and Burgeot, 2002; Liu et al. 2013; Perussolo et al.,

262 2019). To create each index, each individual biomarker response value was normalized by

263 subtraction from the grand mean value for all replicates and divided by its standard deviation.

264 Each normalized value was then added to the minimum absolute value obtained for each

265 biomarker (Z score). Each mean biomarker Z score was then multiplied by a weighting (GST,

266 GPx, GR = 1; LPO = 2), designated according to the systemic importance each activity

267 (biomarker response score). To obtain the integrated biomarker response index (IBR), the

268 sum of all biomarker response scores for each condition and tissue was then divided by the

269 sum of all weightings, providing the final degree of effect for each condition.

270 Biomarker response scores were then plotted as Radar charts using Microsoft Excel

271 software (Microsoft Corp., Redmond, WA, USA).

272

273 **Statistical Analyses**

274 After verifying the data for normality of distribution and homoscedasticity by

275 applying the Shapiro-Wilks and Bartlett tests, respectively, the effect of salinity or of

276   desiccation time on hemolymph osmolality and enzyme activities was evaluated using one-
277   way analyses of variance. Differences between means within each parameter were located
278   using the Student-Newman-Keuls *post-hoc* multiple comparisons procedure. A minimum
279   significance level of P = 0.05 was employed throughout.
280
281   **Results**
282   **Ecological transect sampling**
283   Figure 3 shows the variation in burrow densities and in abiotic parameters like
284   interstitial moisture content, salinity and burrow temperature measured along the respective
285   transects taken at the Playa de los Alemanes and Bahia Tortuga collecting sites where the
286   fiddler crabs *L. helleri* and *M. galapagensis* were abundant. The two species were sympatric
287   at the Bahia Tortuga site.
288   *Leptuca helleri* showed the highest burrow densities (120 burrows/m²) closest to the
289   low water mark. For *M. galapagensis*, burrow density was highest near the shoreline berm
290   (40 burrows/m²). Burrow densities decreased progressively along the transects to minima of
291   36 and 6, respectively, at 16 and 24 meters from the low water mark and berm. Burrow
292   salinity (34 to 37 ‰S), temperature (23.0 to 25.1 °C) and moisture (100%) were fairly
293   constant for *L. helleri*. However, for *M. galapagensis*, burrow moisture decreased from 66 to
294   25% while salinity increased from 56 to 115 ‰S along the transect; burrow temperature
295   ranged from 25.0 to 28.0 °C. Substrate surface temperatures were 26 to ≈29 °C for both
296   species while burrow temperatures (23 to 25 °C) were slightly less for *L. helleri*.
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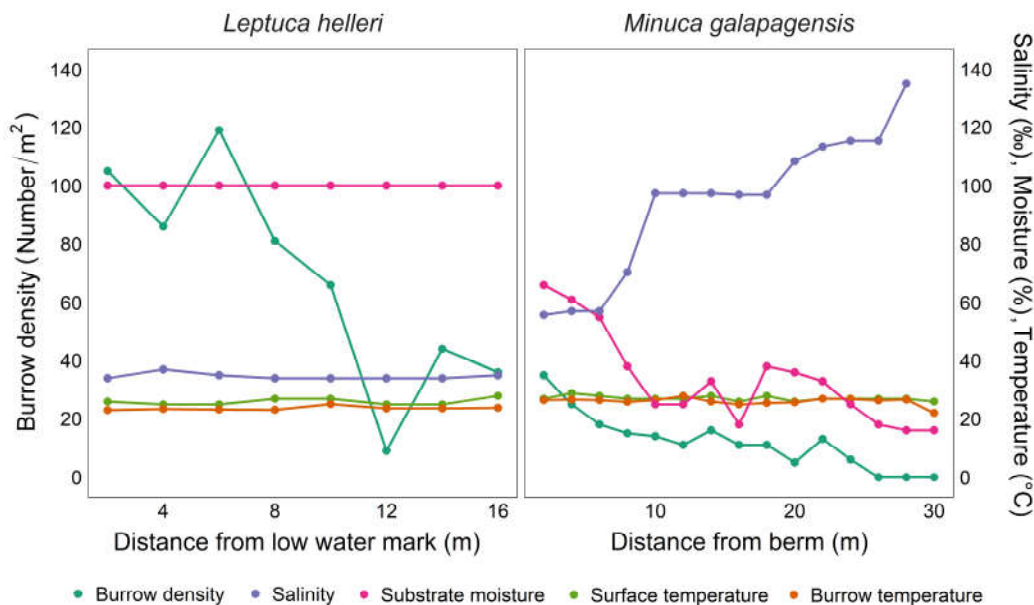
Figure 3. Variation in burrow densities and abiotic parameters (salinity ‰S, substrate moisture %, and surface and burrow temperatures °C) measured along the respective transects made at the Playa de los Alemanes (00.75243° S, 90.31087° W) (fine white sand) and Bahia Tortuga (00.76174° S, 90.34111° W) (coarse coralline sand with sea shells and broken coral) sites on Santa Cruz Island, Ecuador, where the fiddler crabs *Leptuca helleri* and *Minuca galapagensis* were abundant.

305

Effects of forced submersion and desiccation

Survival

Minuca galapagensis showed no mortality in either condition (Table 2). In contrast, *L. helleri* did not survive at all on submersion at 0 and 42 ‰S. At 21 ‰S, survival was 40%. During desiccation, *L. helleri* showed 30% and 0% survival after 6 and 12 h, respectively (Table 2).

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313

Table 2. Percentage survival of the Galapagos fiddler crabs *Leptuca helleri* and *Minuca galapagensis* from Santa Cruz Island, Ecuador, during forced submersion for 6 h at different salinities and up to 12 h desiccation.

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| Treatment	Percentage survival	
	Leptuca helleri	*Minuca galapagensis*
Submersion (‰S) 0	0	100
21	40	100
42	0	100
Desiccation (h) 6	30	100
12	0	100

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320

Hemolymph osmoregulatory ability

Hemolymph osmolality in *L. helleri* was unchanged after 6 h of forced submersion at 21 ‰S compared to fresh caught control crabs (Figure 4) and was strongly hyper-regulated (Δ = +405 mOsm/kg H_2O). In *M. galapagensis*, osmolality decreased after 6 h at 0 ‰S compared to fresh caught controls while at 21 and 40 ‰S osmolality was unaltered. In these salinity-challenged crabs, hemolymph osmolality increased progressively and was hyper-regulated (Δ = +230 mOsm/kg H_2O) in 21 ‰S but hypo-regulated (Δ = -310 mOsm/kg H_2O) in 42 ‰S (Figure 4).

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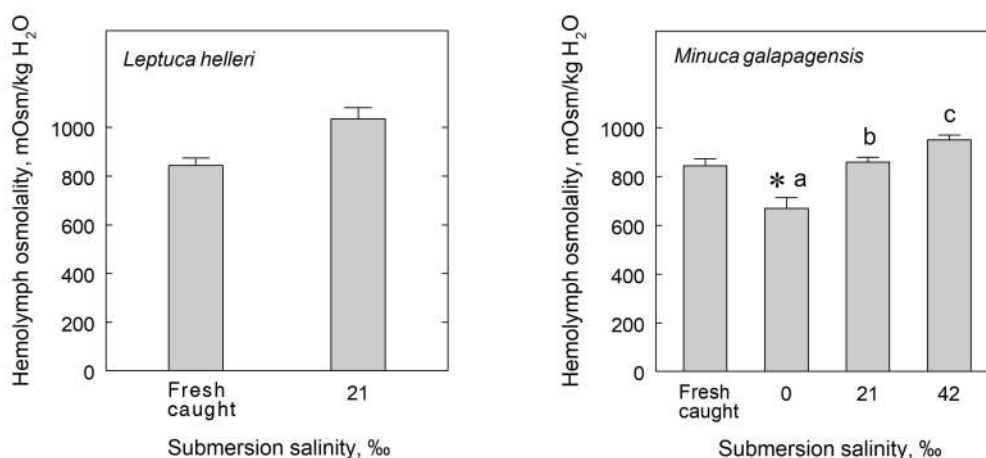
Figure 4. Osmoregulatory ability in the Galapagos fiddler crabs *Leptuca helleri* and *Minuca galapagensis* when maintained fully submerged for 6 h at different salinities (0 ‰S [distilled H_2O, hypo-osmotic challenge], 21 ‰S [630 mOsm/kg H_2O, isosmotic reference medium] or 42 ‰S [1,260 mOsm/kg H_2O, hyper-osmotic challenge) after 2 days held at 29 ‰S (670 mOsm/kg H_2O, fresh caught crabs). Data are the mean ± SEM (N=10). *P<0.05 compared to fresh caught crabs; different letters indicate significantly different groups

336   Hemolymph osmolality in *L. helleri* decreased 0.4-fold after 6 h desiccation compared

337   to fresh caught control crabs (Figure 5). In *M. galapagensis,* hemolymph osmolality

338   increased 1.7-fold after 6 h, decreasing after 12 h to fresh caught control values (Figure 5).

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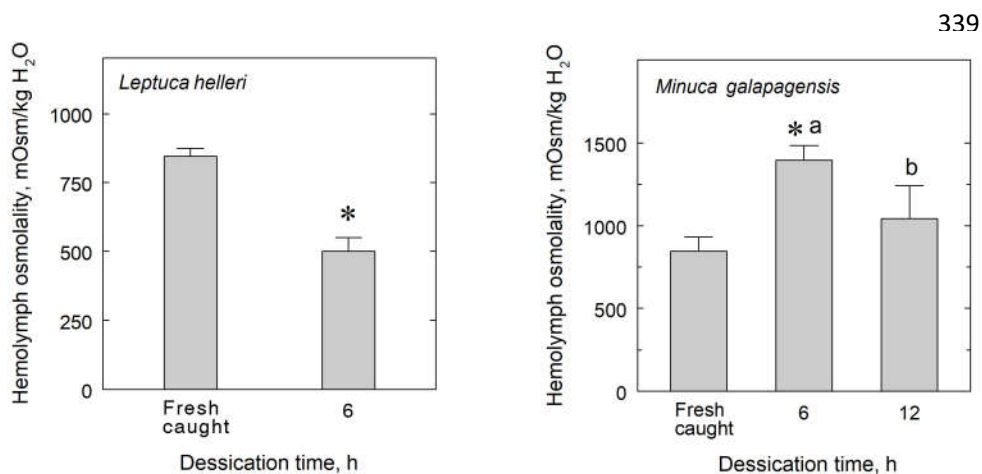
346   **Figure 5.** Changes in the hemolymph osmolality of the Galapagos fiddler crabs *Leptuca*

347   *helleri* and *Minuca galapagensis* when kept emerged without access to water for up to 12 h,

348   after 2 days held at 29 ‰S (670 mOsm/kg H_2O, fresh caught crabs). Data are the mean ±

349   SEM (N=10). *P<0.05 compared to fresh caught crabs; different letters indicate significantly

350   different groups.

351

352   *Oxidative stress enzymes*

353   After forced submersion of *M. galapagensis* for 6 h, hepatopancreas GPx activities

354   decreased in 0 and 21 ‰S compared to fresh caught control crabs (Figure 6). In 42 ‰S,

355   activity increased to control crab levels. GST activities were unaffected compared to fresh

356   caught crabs, although activity decreased in 42 ‰S compared to 0 and 21‰S.

357   Hepatopancreas GR activity was strongly inhibited at all salinities (Figure 6) while LPO

358   activities were unaffected by salinity compared to fresh caught crabs (Figure 6). However,

359   LPO activities increased in 21 and 42 ‰S compared to 0 ‰S.

360   There was no effect of submersion salinity on gill GPx, GST or GR activities

361   compared to fresh caught crabs (Figure 6). Gill LPO activities increased in 21 ‰S but

362   decreased in 42 ‰S to values below the fresh caught controls (Figure 6).

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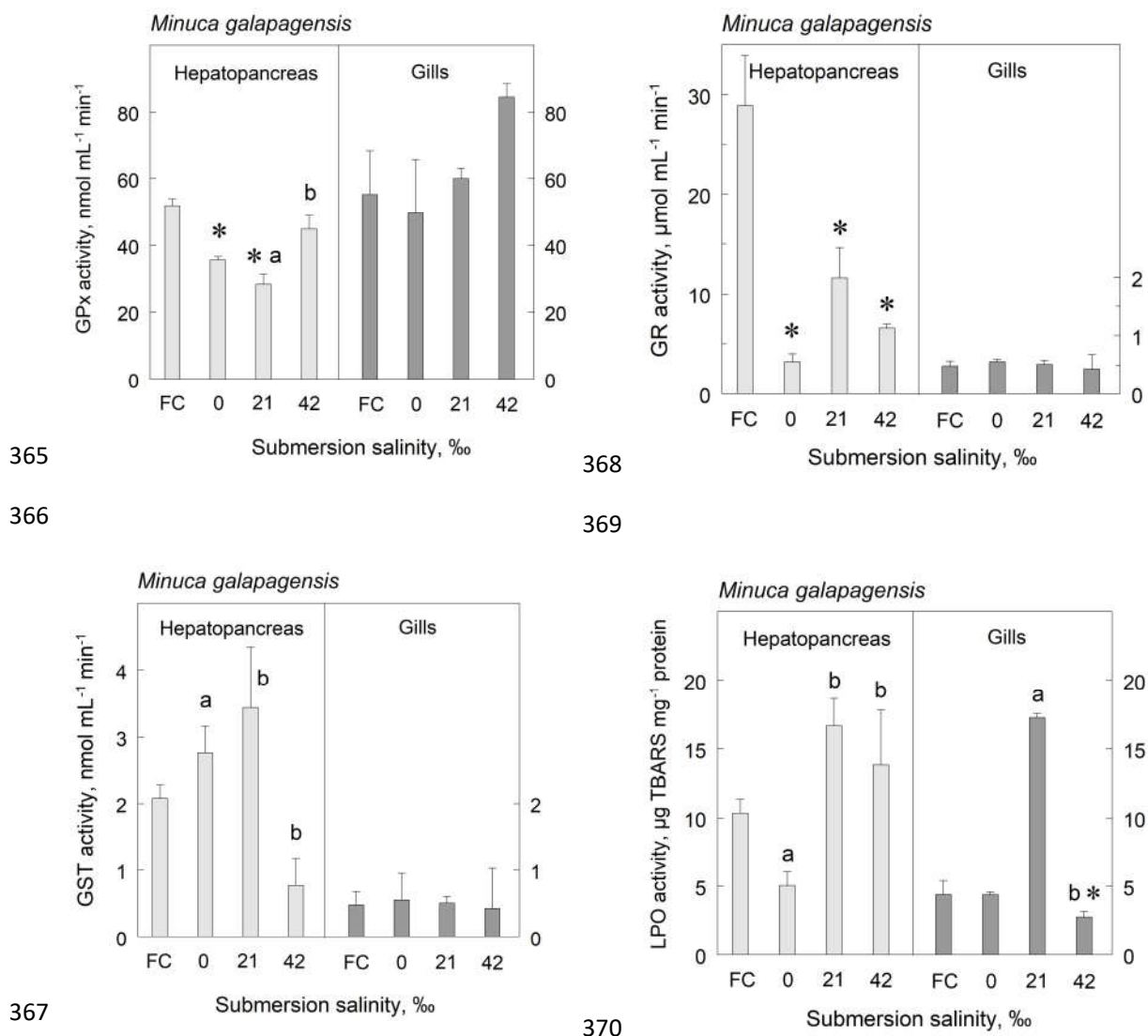
Figure 6. Enzyme activities (Glutathione peroxidase [GPx], Glutathione S-transferase [GST], Glutathione Reductase [GR]) and Lipid peroxidation [LPO] in hepatopancreas and gill homogenates from the Galapagos fiddler crab *Minuca galapagensis* maintained fully submerged for 6 h at different salinities (0 ‰S [distilled H_2O, hypo-osmotic challenge], 21 ‰S [isosmotic reference medium] or 42 ‰S [hyper-osmotic challenge) after 2 days held at 29 ‰S (fresh caught crabs, FC). Data are the mean ± SEM (N=10). *P<0.05 compared to fresh caught crabs; different letters indicate significantly different groups.

During desiccation in *M. galapagensis*, hepatopancreas GPx activity increased after 6 and 12 h compared to fresh caught control crabs (Figure 7). Gill GPx activities were unaltered (Figure 7). Both hepatopancreas and gill GST, GR and LPO activities were strongly inhibited after 6 and 12 h desiccation (Figure 7).
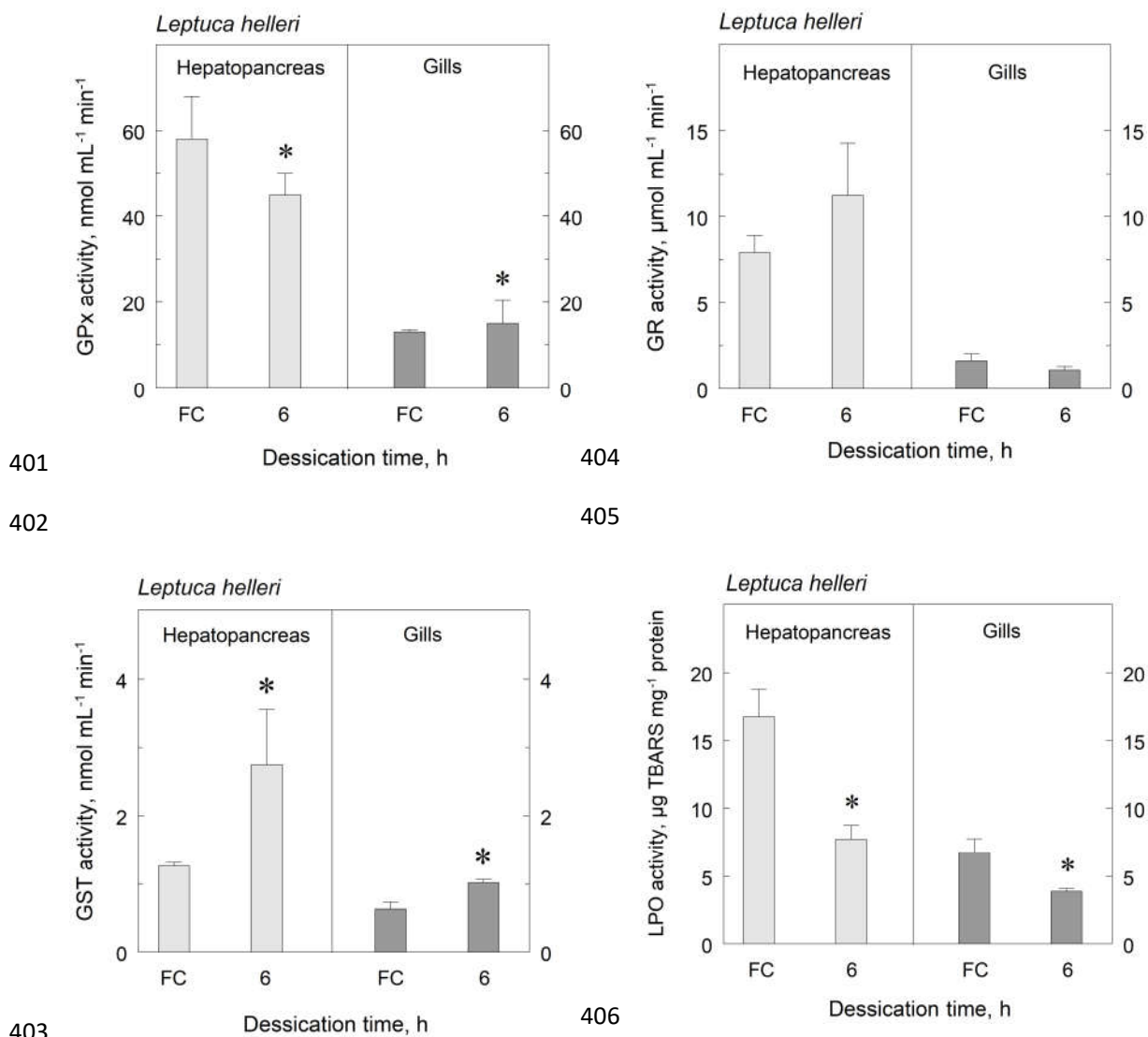
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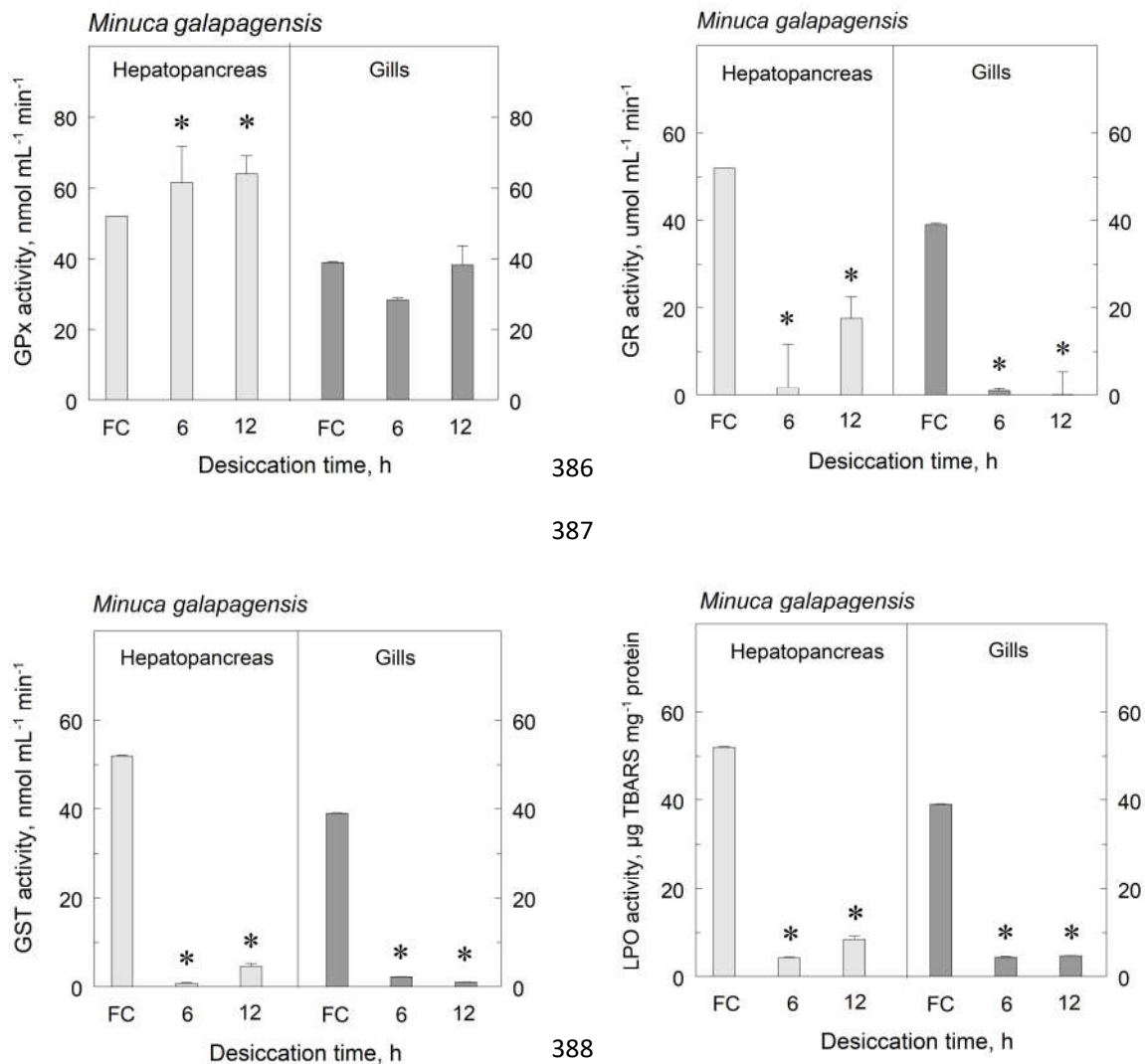
Figure 7. Enzyme activities (Glutathione peroxidase [GPx], Glutathione S-transferase [GST], Glutathione Reductase [GR] and Lipid peroxidation [LPO]) in hepatopancreas and gill homogenates from the Galapagos fiddler crab *Minuca galapagensis* kept emerged without access to water for up to 12 h, after 2 days held at 29 ‰S (fresh caught crabs, FC). Data are the mean ± SEM (N=10). *P<0.05 compared to fresh caught crabs.

In *L. helleri*, forced submersion resulted in high mortality (40%) and enzymatic assays could not be performed.

During desiccation, *L. helleri* survived only for 6 h. GPx activity was inhibited in the hepatopancreas but increased in the gills compared to fresh caught control crabs (Figure 8). GST activity increased in both tissues (Figure 8). GR activities were unaltered by desiccation (Figure 8) while LPO activities decreased in both tissues (Figure 8).

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Figure 8. Enzyme activities (Glutathione peroxidase [GPx], Glutathione S-transferase [GST], Glutathione Reductase [GR] and Lipid peroxidation [LPO]) in hepatopancreas and gill homogenates from the endemic Galapagos fiddler crab *Leptuca helleri* kept emerged without access to water for 6 h after 2 days held at 29 ‰S (fresh caught crabs, FC). Data are the mean ± SEM (N=10). *P<0.05 compared to fresh caught crabs.

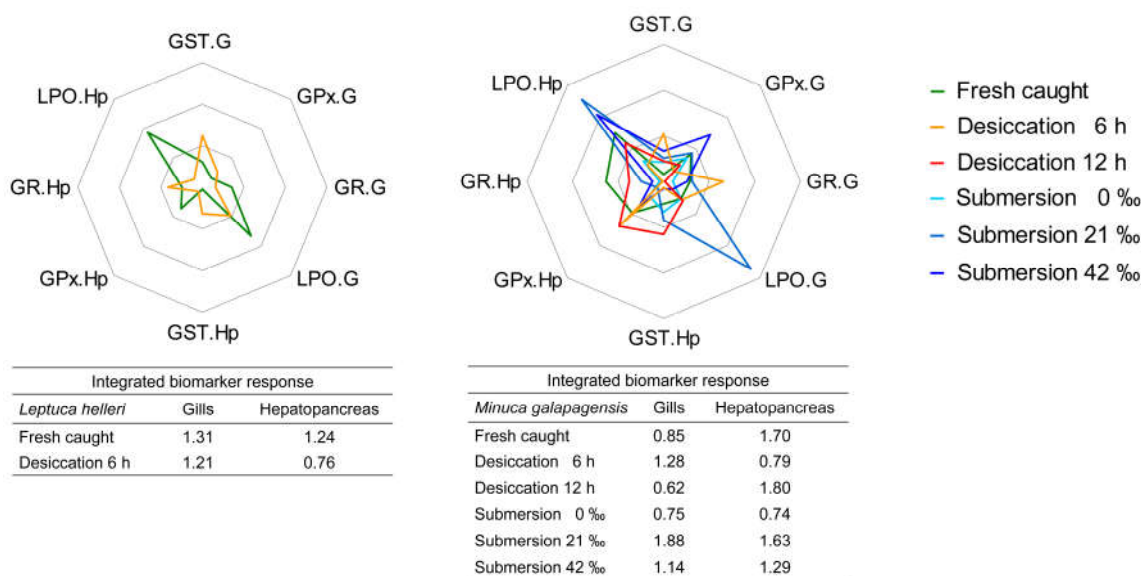**Integrated biomarker response indexes**

The response scores for each biomarker and the integrated biomarker response indexes are given in Figure 9 and associated tables.

For *L. helleri* (Figure 9, left panel), fresh caught crabs showed the highest LPO indexes in both the gills and hepatopancreas (table below left panel). Desiccation for 6 h had little effect.

16

419   For *M. galapagensis*, submersion at 21 ‰S and desiccation for 6 h were the most

420   relevant effectors of response (Figure 9, right panel). In the gills, LPO activity in 21‰ S, and

421   GST and GR activity after 6 h desiccation were the main determinants. In the hepatopancreas,

422   12 h desiccation and fresh caught crabs predominated as effectors (Figure 9 and table below

423   right panel).

424

Integrated biomarker response		
Leptuca helleri	Gills	Hepatopancreas
Fresh caught	1.31	1.24
Desiccation 6 h	1.21	0.76

Integrated biomarker response		
Minuca galapagensis	Gills	Hepatopancreas
Fresh caught	0.85	1.70
Desiccation 6 h	1.28	0.79
Desiccation 12 h	0.62	1.80
Submersion 0 ‰	0.75	0.74
Submersion 21 ‰	1.88	1.63
Submersion 42 ‰	1.14	1.29

425

426   **Figure 9.** Integrated biomarker response indexes for the Galapagos fiddler crabs *Leptuca*

427   *helleri* (left panel and table) and *Minuca galapagensis* (right panel and table) when

428   maintained fully submerged for 6 h at salinities of 0 ‰S [distilled H$_2$O, hypo-osmotic

429   challenge], 21 ‰S [630 mOsm/kg H$_2$O, isosmotic reference medium] or 42 ‰S [1,260

430   mOsm/kg H$_2$O, hyper-osmotic challenge) after 2 days held at 29 ‰S (670 mOsm/kg H$_2$O,

431   fresh caught crabs), or held emerged without access to water for up to 12 h.

432

Discussion

434   Our findings reveal clear differences in habitat characteristics, burrow densities,

435   survival ability on submersion and desiccation challenge, osmoregulatory ability and

436   oxidative stress enzyme behavior between the chosen populations of the two species of

437   Galapagos fiddler crabs investigated on Santa Cruz Island.

438   The population of *Leptuca helleri* studied inhabits the intertidal zone where it

439   encounters little variation in salinity and substrate moisture across the shore (Figure 3).

440   Burrow densities, highest on the low shore, decline rapidly and markedly by 80% within just

441   10 meters, and enigmatically, cannot be attributed to variation in measured transect

17

442 parameters. This population of *L. helleri* is abundant a fair distance from a mangrove stand

443 where daily tidal submersion likely confers protection against water loss. In contrast, *M.*

444 *galapagensis* inhabits the supralittoral zone, subject to only occasional seasonal tidal

445 inundation, and is distributed across marked salinity and substrate moisture gradients. Burrow

446 densities decline only slowly and progressively with increasing salinity and decreasing

447 moisture (Figure 3), suggesting better salinity and desiccation tolerance than *L. helleri*. The

448 burrows of *M. galapagensis* tend to be associated with mangrove vegetation, which together

449 with thermoregulatory behavior (Smith and Miller, 1973) may alleviate temperature stress

450 and evaporative water loss (McGuinness, 1994). This population of *M. galapagensis* is

451 encountered farther from the nearest water source than *L. helleri* and may be more subject to

452 water loss.

453   The two species exhibit striking differences in desiccation and submersion tolerances.

454 *Leptuca helleri* cannot survive more than 6 h without water under experimental conditions,

455 neither does it tolerate rigorous hypo- or hyper-osmotic challenge under forced submersion,

456 surviving only at a salinity (21 ‰S) moderately dilute compared to burrow salinity (34 to 37

457 ‰S) and at which it hyper-regulates strongly. Forced submersion together with severe

458 salinity challenge leads to death likely through a synergistic effect on osmoregulatory ability

459 such as hypoxia, and insufficient energy available owing to a putative shift to anaerobic

460 metabolism (Teal and Carey, 1967).

461   In contrast, *M. galapagensis* tolerated substantial experimental desiccation and

462 submersion, showing no mortality. The crab hyper/hypo-regulates well, showing little if any

463 effect of forced salinity submersion. *Uca rapax*, exposed to salinities between 40 and 63 ‰S

464 (1,200 to 1,890 mOsm/kg H_2O) hypo-regulates hemolymph osmolality between 1,069 and

465 1,085 mOsm/kg H_2O; however, the submerged crabs osmoconform (Zanders and Rojas,

466 1996b) as also seen in *Uca pugilator* (D'orazio and Holliday, 1985). Forcibly submerged *M.*

467 *rapax* shows a diminished ability to hypo-regulate hemolymph osmolality and [Na^+] and [Cl^-]

468 at 60 ‰S (Capparelli et al., 2017), which may be due partly to limited oxidative ATP

469 production (Teal and Carey, 1967).

470   Osmoregulatory ability in *M. galapagensis* was unaffected during submersion in

471 roughly isosmotic or hyper-osmotic media (21 and 42 ‰S), although *L. helleri* could not

472 survive severe hypo- or hyperosmotic challenge (0 and 42 ‰S) for 6 h. Anaerobic lactate

473 metabolism predominates on submersion in fiddler crabs (Teal and Carey, 1967) and gill

474 Na^+/K^+-ATPase activity increases in fiddler crabs submerged at low salinities (D'orazio and

475 Holliday, 1985; Capparelli et al., 2017). *Leptuca helleri* may be unable to generate energy

18

476 sufficient to osmoregulate and tolerate the synergic stress of forced submersion in severely
477 hypo- and hyper-osmotic media.

478 Reactive oxygen species (ROS) are produced as result of normal cellular metabolism
479 and can be induced by various environmental factors. They are highly reactive and damage
480 molecules such as DNA, carbohydrates, lipids and proteins, altering their functions (Birben et
481 al., 2012). Water flow through the gills is maintained during submersion, increasing O_2
482 availability: ROS concentrations can subsequently increase, augmenting oxidative stress. The
483 ROS defense system (GPx, GST and GR) in *M. galapagensis* is altered during submersion,
484 together with increased oxidative stress (increased LPO levels) at 21 and 42 ‰S, particularly
485 in the hepatopancreas, and at 21 ‰ S in the gills, possibly increasing tissue permeability
486 (Birben et al., 2012). Elevated salinities generate oxidative stress and affect antioxidant
487 mechanisms in fiddler crabs (Zanders and Rojas, 1996; Capparelli et al., 2017). Thus, salt
488 secretion may induce metabolic shifts that generate ROS. Coherently, the integrated
489 biomarker indexes for *M. galapagensis* reveal an increase in antioxidant defenses and
490 oxidative stress in the gills of crabs at 21 and 42 ‰S and a decrease at 0 ‰S.

491 When crustaceans are exposed to desiccation, gas exchange may decrease since the
492 gill lamellae collapse, reducing the diffusional surface area available (Withers, 1992; Morris
493 and Oliver, 1999a), oxidative challenge consequently diminishing. In the gills, desiccation
494 decreases the overall biomarker response in *L. helleri* and increases in *M. galapagensis.*
495 Coherently, the integrated biomarker indexes show that antioxidant defense activity and
496 oxidative stress diminish on desiccation in the hepatopancreas of both species.However, on
497 longer aerial exposure, as seen for *M. galapagensis* after 12 h, the decreased oxygen supply
498 can gill antioxidant defense activity manifest in the markedly reduced integrated biomarker
499 response index, and severely affect energy balance and reserves, and may shorten survival
500 time in aquatic animals (Martínez-Álvarez *et al.*, 2005; Abele *et al.*, 2007; Paital, 2013).
501 Hemolymph volume in the crab *Lithodes santolla* decreases with desiccation, reducing
502 oxygen titers and leading to systemic effects (Urbina *et al.*, 2013). *Leptuca helleri* does not
503 survive desiccation for much more than 6 h, when curiously, its hemolymph osmolality
504 decreases. In contrast, *M. galapagensis* survives 12 h or more, its hemolymph osmolality
505 increasing transiently, revealing water loss, returning incongruously to initial values.

506 Glutathione plays a major role in different cellular compartments, promoting
507 elimination of xenobiotics and acting within the antioxidant system. It reacts directly with
508 ROS, providing protective functions such as reduction, conjugation and interaction with other
509 non-enzymatic antioxidants like vitamins E and C (Forman *et al.*, 2019). Glutathione

510   peroxidase (GPx) is responsible for scavenging organic and inorganic peroxides, glutathione

511   S-transferase (GST) catalyzes the biotransformation of xenobiotics, and glutathione reductase

512   (GR) reduces glutathione disulfide to glutathione (GSH), the sulfhydryl form, an important

513   cellular antioxidant (Morris et al., 2014). The decreased hepatopancreas GPx and GR

514   activities seen in *M. galapagensis* during submersion may conserve energy then made

515   available during aerial exposure. Antioxidant enzymatic activities also decrease after 8 h

516   aerial exposure in *Neohelice granulata* (de Oliveira *et al*., 2005). The glutathione system

517   seems to be important during the emersion/submersion transition since all activities are

518   elevated in fresh caught crabs, and GST and GR activities become much reduced during

519   desiccation. This enzymatic defense system in the hepatopancreas and gills of *M.*

520   *galapagensis* may prevent oxidative damage during desiccation, as suggested by the low lipid

521   hydroperoxide activities.

522   The gills and hepatopancreas of the two fiddler crabs exhibited distinct oxidative

523   responses. In *M. galapagensis*, forced submersion induced pronounced alterations in all

524   biomarkers in the hepatopancreas. Oxidative stress during desiccation remained fairly

525   unchanged or diminished in both tissues, with a likely reduction in ROS titers ensuing. In *L.*

526   *helleri*, the oxidative stress system seems to be effective up to 6 h desiccation, with a

527   decrease in oxidative stress titers (LPO) in both tissues. Such tissue-specific differences may

528   be widespread since each responds differently to environmental parameters such as salinity

529   challenge in the mud crab *Scylla serrata* (Paital and Chainy, 2010). This is corroborated by

530   the integrated biomarker index, where each tissue exhibited a different score, based on

531   treatment.

532   In conclusion, our findings reveal marked differences in tolerance of forced

533   submersion and desiccation in the two fiddler crabs that inhabit the Galapagos Islands,

534   *Minuca galapagensis* being much more resistant than *Leptuca helleri*, owing to its

535   physiological and biochemical adjustments. *Minuca galapagensis* is a generalist species,

536   manifesting few physiological and oxidative stress effects while the more ecologically

537   demanding species, *Leptuca helleri*, cannot survive such conditions for long.

538

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553

Author contributions

555   *Mariana Vellosa Capparelli*: Conceptualization, Methodology, Validation, Formal analysis,

556   Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision,

557   Project administration. *Carl Leo Thurman*: Methodology, Resources, Writing - Review &

558   Editing, Funding acquisition. *Paloma Gusso Choueri*: Validation, Writing - Review &

559   Editing. *Denis Moledo Abessa*: Validation, Resources, Writing - Review & Editing. *Mayana*

560   *Karoline Fontes*: Validation, Writing - Review & Editing. *Caio Rodrigues Nobre*: Software,

561   Formal analysis, Writing - Review & Editing. *John Campbell McNamara*: Methodology,

562   Validation, Formal analysis, Investigation, Resources, Data curation, Writing - Original

563   Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

564

Declaration of competing interests

566   The authors declare that they have no known competing financial interests or personal

567   relationships that could influence the investigation reported in this article.

568

Compliance with Ethical Standards

570   This study complies with all Ecuadorian, Brazilian, institutional and international guidelines

571   on the use of invertebrate animals in scientific research.

572

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