**Assessment on the effects of confinement in the spider crab
Maja brachydactyla broodstock**

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2 **Assessment on the effects of confinement in the spider crab *Maja brachydactyla***
3 **broodstock**

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16 metabolic enzymes, stress biomarkers.

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18 **Running title:** spider crab broodstock under confinement.

19 Abstract

20 Adults from spider crab, *Maja brachydactyla*, Balss, 1922 were kept in captivity for up to two years.
21 Haemolymph of ten females was extracted monthly for monitoring several metabolites and the
22 presence of Vibrionacea bacteria. A neurotoxic marker and hepatic metabolic and digestive enzyme
23 capacities were contrasted between wild specimens and those reared in captivity. As results, no
24 differences were observed in hepatosomatic index (HSI) between males and females, but captive
25 animals presented lower HSI than the wild ones. After two years of confinement, no changes in
26 quantities of haemolymph microflora and metabolites were observed and this was considered
27 indicative of adaptation of the broodstock to confinement. Spider crab also showed a large plasticity
28 in their hepatopancreatic digestive capabilities suggested by the adaptation to the food supplied in
29 captivity. Moreover, captivity conditions did not seem to negatively alter their antioxidant defenses,
30 xenobiotic capacity or neuromuscular activity as no enhanced oxidative stress damage was shown in
31 either males or females. These results indicate that although the confinement conditions used in this
32 study do not mimic those from the wild, they could be considered as adequate for rearing *M.*
33 *brachydactyla* in captivity, at least with regards to the set of parameters assessed.

34 Introduction

35 The spider crab *Maja brachydactyla*, Balss, 1922 is considered as an alternative species within
36 aquaculture diversification programs in Western Europe and particularly in Spain. The species
37 possesses some interesting biological features, such as high growth rate and plasticity, and great
38 adaptability to reproduction in captivity, showing high fecundity and successive egg clutches during
39 the breeding season, allowing seeds production during the whole year (González-Gurriarán,
40 Fernández, Freire & Muiño 1998). Traditionally, research on this species has been focused on larval
41 and juvenile development (Andrés, Estévez & Rotllant 2007, Andrés, Estévez, Anger & Rotllant
42 2008; Guerao & Rotllant 2009) and reproduction (Rotllant, González-Gurriarán, Fernández,
43 Benhalima & Ribes 2007; Simeó, Ribes & Rotllant 2009; Simeó, Kurtz, Chiva, Ribes & Rotllant
44 2010a; Simeó, Kurtz, Rotllant, Chiva & Ribes 2010b). Up to now, the culture of this species is based
45 on the production of seeds from wild mature animals that are just kept for one season for spawning
46 purposes (González-Gurriarán *et al.* 1998; Andrés, Estévez, Hontoria & Rotllant 2010a), which
47 might compromise the conservation of this overexploited species (Freire, Bernárdez, Corgos,
48 Fernández, González-Gurriarán, Sampedro & Verísimo 2002). Hence, its sustainable culture requires
49 long-term maintenance of broodstock in order to be independent from wild caught animals. For this
50 reason it is relevant to assess the condition of animals kept in captivity at least for two years, which
51 is considered the minimal time necessary to reach maturity from hatching (Iglesias, Sánchez,
52 Moxica, Fuentes, Otero & Pérez 2002).

53 Several physiological and metabolic parameters might be used for monitoring the condition
54 of animals kept in captivity. Studies in crabs and shrimps have shown that haemolymph metabolites
55 can be used as valuable biomarkers for assessing the physiological condition of wild and cultured
56 animals (Durand, Devillers, Lallier & Regnault 2000; Frederich & Pörtner 2001; Palacios 2000;
57 Sánchez, Pascual, Sánchez, Vargas-Albores, LeMoullac & Rosas 2001; Pascual, Gaxiola & Rosas
58 2005). Although the presence of bacteria in the haemolymph of Crustacea was initially interpreted as

59 a disease indicator (Lightner 1975), this is currently viewed as a natural process (Gómez-Gil, Tron
60 Mayen, Roque, Turnbull, Inglis & Guerra-Flores 1998). In this sense, recent findings have suggested
61 that bacterial populations found in the haemolymph of the apparent healthy spider crabs could be use
62 as an indicator of the health status of captive animals (Gómez-Gil, Roque, Lacuesta & Rotllant
63 2010).

64 Digestive enzymes can be also used as reliable physiological biomarkers in captive animals,
65 since the rate of digestion may limit the uptake of nutrients to the haemolymph and potentially
66 compromise the growth of the whole organism and in the particular case of the broodstock, directly
67 affect the spawning performance and the nutritional quality of eggs (Harrison 1997). Several authors
68 have shown that digestive enzymes are a remarkable tool for studying the adaptations of wild and
69 captive organisms to changes in food quality and quantity (Sánchez-Paz, García-Carreño,
70 Hernández-López, Muhlia-Almazán & Yepiz-Plascencia 2007; Rotllant, Moyano, Andrés, Estévez,
71 Díaz & Gisbert 2010). Among the different biochemical and nutritional condition indices frequently
72 used, digestive enzymes are considered reliable indicators of the nutritional state of individuals due
73 to their species and age specificity, sensitivity and short latency (Harms, Anger, Klaus & Seeger
74 1991; Harms, Meyer-Harms, Dawirs & Anger 1994; Johnston, Ritar & Thomas 2004a; Rotllant,
75 Moyano, Andrés, Díaz, Estévez & Gisbert 2008).

76 Besides the use of digestive enzymes as reliable markers of the animal condition, there is a
77 set of metabolic enzymes that can also be used for the same purpose. In the context of our study, a
78 measure of the antioxidant defenses has been applied in studies aiming to contrast the stress
79 condition between wild and reared fish (Ferrante, Ricci, Aleo, Passi & Cataudella, 2008), since
80 cultured stressed animals were seen to be more vulnerable to disease and produced lower quality
81 flesh (Martínez-Álvarez, Morales & Sanz 2005). In addition to antioxidant defenses, a broader suite
82 of biomarkers had been also applied to wild and cultured fish but, in this latter case, in relation to
83 pollutants exposure (Fernandes, Porte & Bebianno 2007; Ferreira, Antunes, Costa, Amado, Gil,

84 Pousão-Ferreira, Vale & Reis-Henriques 2008a; Fernandes, Caetano, Costa, Pousão-Ferreira, Vale &
85 Reis-Henriques 2008b; Ferreira, Caetano, Antunes, Costa, Gil, Bandarra, Pousão-Ferreira, Vale &
86 Reis-Henriques 2010). The use of biomarkers of stress has also been applied to crustaceans. In
87 particular shrimps have been tested for their health status performance under actual aquaculture
88 practices (Tu, Silvestre, Scippo, Thome, Phuong & Kestemont 2009), in relation to viral
89 (Mohankumar & Ramasamy 2006) and temperature-linked infections (Holmblad & Söderhäll 1999;
90 Cheng, Wang & Chen, 2005).

91 As a general feature, all aerobic processes generate reactive oxygen species (ROS), as
92 molecules needed for physiological functions, but ROS are also a result of enhanced metabolic
93 reactions. The key is to reach a compromise so that the ROS levels attained do not become toxic.
94 Organisms have the ability to maintain ROS balance thanks to a suite of antioxidant molecules and
95 antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase, glutathione peroxidase (t-GPX),
96 glutathione reductase (GR) as those selected for this study). Superoxide dismutase (EC 1.15.1.1) is an
97 antioxidant enzyme that changes anion superoxide (O_2^-) to H_2O_2 which, in turn, is further
98 transformed by catalase. Catalase (EC 1.11.1.6) transforms H_2O_2 into H_2O and O_2 , and t-GPX (EC
99 1.11.1.9) reduces H_2O_2 as well as other organic hydroperoxides to water and alcohols respectively.
100 GR (EC 1.6.4.2) maintains the balance $GSH \leftrightarrow GSSG$ towards the reduced form. Other hepatic
101 enzymes such as glutathione S-transferases (GSTs; EC 2.5.1.18) were also considered for its
102 antioxidant GPX role as they can conjugate hydroperoxides with endogenous GSH in order to make
103 them more readily excretable (Livingstone 2001). Hepatic carboxylesterase (CbEs; EC 3.1.1.1) were
104 included as they are involved in the hydrolysis of a broad range of endogenous esters and they play
105 an important role during molting in crustacean (Homola & Chang 1997). Lipid peroxidation (LP)
106 was selected as an indicator of lipid membrane damage as a consequence of exposure to excess of
107 ROS and insufficient antioxidant defenses. Protein yield (PY) was adopted as an unspecific marker
108 of protein synthesis/degradation. Acetylcholinesterase (AChE; EC 3.1.1.7) and the

pseudocholinesterase propionilcholinesterase (PrChE; EC 3.1.1.8) activities in muscle were characterized and measured as potential biomarkers of neurotoxicity.

Under the former context, there is no information on the impacts of short and long-term confinement on *M. brachydactyla*. Therefore, the aim of the present study was to evaluate the effects of captivity on spider crab by means of the assessment of nutritional (digestive enzymes) and stress biomarkers (haemolymph parameters and antioxidant enzymes).

Materials and methods

Broodstock maintenance and sampling schedule

Adults of *M. brachydactyla* (30 females and 20 males; 144 ± 14 mm of carapace length) were captured from the NE Atlantic Ocean (Galicia, Spain) and transported by road in polystyrene boxes to the IRTA facilities (Sant Carles de la Ràpita). Animals were kept in 2000 L tanks connected to a recirculation unit for a maximum of two years as described in Andrés *et al.* (2007). Briefly, spider crabs were maintained at a constant salinity of 36 ‰ and temperature of $18 \pm 1^\circ\text{C}$, under natural photoperiod, and fed *ad libitum* with a combination of fresh mussel (Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819, 5 times per week) and frozen crab (*Liocarcinus sp.* Stimpson, 1871, twice a week). During the confinement, animals moved, ate and copulated, and several hatching events were observed (Andrés *et al.* 2010a).

The effect of confinement in spider crab was evaluated at short-term (<1 year) by bacteria and metabolite levels measured every three weeks in the haemolymph of the captive females, whereas long-term (2 years) effects were determined by monitoring the activity of muscular and hepatopancreatic, as well as digestive enzymes in both sexes.

134 **Haemolymph extraction, bacteria counting and metabolites analyses**

135 Ten females were sampled every three weeks during one year. They were caught with a dipnet and
136 anaesthetized on ice for 15 minutes. Before sampling, the base of the periopod (coxa) was
137 disinfected with 70 % ethanol, and *ca.* 200 μ L of haemolymph were drawn with a 1 mL syringe
138 attached to a needle of 25G x5/8". One hundred μ L were inoculated onto a Petri dish with TCBS
139 agar (thiosulphate citrate bile sucrose salt, Scharlau, Spain) and incubated at 28°C for 24 h to count
140 the colonies formed (yellow-saccharolytic and green-non saccharolytic).

141 For the quantification of selected haemolymph metabolites, another sample of 2 mL of
142 haemolymph was taken with a 5 mL syringe (previously washed with 10 mM EDTA) attached to a
143 needle 21Gx9/16 and processed immediately. Glucose and cholesterol were assessed according to
144 the methods of Allain et al. (1974) and Trinder (1969), respectively. They were quantified by means
145 of Termotrace ® kits (Melburne, Australia). These kits are based on the incubation of 50 μ L of
146 haemolymph with the respective substrates at 37°C for 5 minutes and the resulting absorbance was
147 read at 500 nm.

148 Lactate was measured with Biotechnica® kit (Madrid, Spain) according to Marbach and Weil
149 (1967). Haemolymph samples (25 μ L) were incubated with 200 μ L of NAD mixture at 37°C for 5
150 minutes. After incubation, absorbance was read at 340 nm. Lactate recovery ranged from 97 – 117%.
151 Vitamin C was measured following the method of Beutler and Beinstingl (1980) with a Biotechnical
152 ® kit (Martinsried, Germany). For this test, the haemolymph was previously cleaned from lipids and
153 proteins by transferring 200 μ L of sample into a clean eppendorf containing 200 μ L of absolute
154 ethanol. The mixture was allowed to stand for 1 h to precipitate lipids and proteins and centrifuged
155 for 10 min at 13,500 rpm. The supernatant was transferred to a new tube and left open for 2 h in
156 order to assure ethanol evaporation, and then incubated (10 μ L) at 37°C for 6 minutes with reagent
157 solutions provided by the kit. After incubation absorbance was read at 578 nm. All methods were
158 adapted to microplate reading.

The above-mentioned haemolymph parameters were quantified by quadruplicate for each sample point. In each assay zero was made with 10 mM EDTA and the standards used were those provided by the kits. Absorbance readings were conducted by means of a microplate reader (Sinergy HT, BIO-TEK®).

Sample preparation for enzyme analyses

Wild and one and two years captive spider crab adults were anesthetized on ice for at least 10 min, sacrificed and dissected. Samples of muscle, hepatopancreas and intestine were removed and immediately frozen (-80°C) until posterior enzymatic analyses. Hepato-somatic index (HSI) was calculated by dividing the weight the hepatopancreas by the body weight and multiplying by 100.

Digestive enzyme activities

The enzymes assayed were total proteases, trypsin, chymotrypsin, pepsin-like (acid proteases like enzymes belonging to the aspartic peptidases family, such as cathepsin D and E), amylase, esterase, aminopeptidase N and alkaline phosphatase. Preparation of samples for hepatopancreatic enzymes were conducted as previously described by Rotllant *et al.* (2008) and Andrés, Gisbert, Díaz, Moyano, Estévez & Rotllant (2010b). In brief, frozen samples were homogenized 5 min in 30 volumes (v/w) of ice-cold Tris-Mannitol (50 mM), HCl (2 mM) buffer at pH=7 using an Ultra Turrax T-25 (IKA® WERKE, Germany). Aliquots were centrifuged for 5 min at 13,000 g (4°C) and the supernatant stored after homogenization at -20°C until enzymatic analyses were performed.

The aliquots were thawed and briefly centrifuged (10,000 rpm for 15 s) prior to enzymatic analysis and every sample was assayed in triplicate. All samples for a single enzymatic assay were run the same day. Blank controls in which reaction did not take place were introduced when needed. All enzymatic activities were read in a Sinergy HT, BIO-TEK® spectrophotometer using either 48

183 (Nunc) 48, Nunc, Denmark) or 96 (F96 MicroWell™ Plates, Nunc) well flat bottom microplates
184 and data were expressed as specific enzyme activity (U/g protein or mU/g protein).

185 Total protease activity was estimated in crude homogenates using azocasein 0.5% as substrate
186 in 50 mM Tris-HCl buffer, pH=8, following the method by García-Carreño & Haard (1993). One
187 unit of protease activity corresponded to a 1 μ mol of substrate hydrolyzed in 1 min per mL of
188 enzymatic extract ($\epsilon_{366}=900 \text{ M}^{-1} \text{ cm}^{-1}$). Trypsin-like enzyme activity was assayed using 0.1 M N α -
189 benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate in 50 mM Tris-HCl-20 mM CaCl_2 buffer,
190 pH 8.2, for 50 μ L extract (Holm et al., 1988). One unit of trypsin activity corresponded to 1 μ mol of
191 4-nitroaniline liberated in 1 min per mL of extracellular enzymatic extract, based on the extinction
192 coefficient of the substrate ($\epsilon_{407}=8,200 \text{ M}^{-1} \text{ cm}^{-1}$). Chymotrypsin activity was assayed using Suc-
193 Phe-4-nitroanilide as substrate in 200 mM triethanolamin (TEA), 20 mM CaCl_2 buffer pH 7.8
194 (Geiger, 1988). One unit of chymotrypsin activity corresponded to 1 μ mol of substrate hydrolyzed in
195 1 min per mL of enzymatic extract, based on the extinction coefficient of the substrate ($\epsilon_{405}=10,200$
196 $\text{M}^{-1} \text{ cm}^{-1}$). Pepsin-like activity quantification followed the Anson (1938) method as modified by
197 Worthington (1982). One unit of pepsin activity was defined as the μ g of tyrosine released at 37°C
198 $\text{min}^{-1} \text{ mL}^{-1}$, considering the extinction coefficient ($\epsilon_{280}=1250 \text{ M}^{-1} \text{ cm}^{-1}$).

199 α -Amylase activity was assayed by means of a starch-iodine detection following Metais &
200 Bieth (1968). One unit (UW= Unit Wohlgemut) of α -amylase activity was defined as the mg of
201 starch hydrolyzed per min at 37°C per mL of enzymatic extract. Lipase activity was measured using
202 p-nitrophenyl myristate as a substrate dissolved in Tris-HCl 0.25 M, metoxyetanol 0.25 mm, sodium
203 cholate 5 mM buffer (Iijima, Tanaka & Ota 1998).

204 Aminopeptidase N activity was analyzed using 0.1 M L-leucine-p-nitroanilide dissolved in
205 DMSO as a substrate in 80 mM $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$ buffer, pH 7.0 (Maroux, Louvard & Baratti 1973).
206 Aminopeptidase N activity was defined as the amount (μ mol) of substrate hydrolyzed in 1 min per
207 mL of the enzymatic extract ($\epsilon_{410}=8,200 \text{ M}^{-1} \text{ cm}^{-1}$). Alkaline phosphatase activity was assayed using

208 5mM p-nitrophenyl phosphate as a substrate in 30 mM Na₂CO₃-H₂O, 1 mM MgCl₂-6H₂O buffer, pH
209 9.8 (Bessey, Lowry & Brock 1946; Hausamen, Helger, Rick & Gross 1967). One unit of phosphatase
210 alkaline activity corresponded to a 1 µmol of the substrate hydrolyzed in 1 min per mL of the
211 enzymatic extract ($\epsilon_{407}=18,300 \text{ M}^{-1} \text{ cm}^{-1}$).

212

213 **Muscle and hepatopancreas biomarkers**

214 Two different groups of biomarkers were assayed: a) muscular metabolic biomarkers
215 (cholinesterases – ChEs, lactate dehydrogenase – LDH and lipid peroxidation levels – LP), and b)
216 hepatic enzymes (superoxide dismutase – SOD, catalase – CAT, total glutathione peroxidase – t-
217 GPX, glutathione reductase – GR, glutathione S-transferases – GST, carboxylesterases – CbEs). For
218 muscular parameters analyses, a portion of this tissue (*ca.* 0.2 g) was homogenized in a 50 mM
219 buffer phosphate pH 7.4 in a 1:5 (w:v) ratio using a Polytron® blender. The homogenate was
220 centrifuged at 10,000 g x 30 minutes at 4°C and the supernatant used for measuring activities of
221 cholinesterases and LDH. A portion of hepatopancreas (1-2 g) was homogenized using a Teflon
222 homogenizer in a 1:4 (w:v) of 100 mM buffer phosphate pH 7.4 containing 0.1 M KCl and 1 mM
223 EDTA as well as freshly added 1 mM dithiothreitol (DTT), 0.1 mM phenantroline and 0.1 mg/L trypsin
224 inhibitor. The homogenate was centrifuged at 10,000 g x 30 minutes at 4°C and the supernatant (S10)
225 used for biochemical determinations. All assays were carried out in triplicate at 25°C using either
226 undiluted or diluted S10 supernatant depending on the assay.

227 Determination of ChEs activities was done according to the microplate modified method of
228 Ellman, Courtney, Andres & Featherstone (1961), using acetylthiocholine iodide, S-
229 butyrylthiocholine iodide and propionylthiocholine iodide as substrates. Reading was performed in
230 triplicate at 405 nm in a microplate reader (TECAN Infinite M200) during 5 minutes. Activity was
231 expressed in nmol/min/mg prot. Selective inhibitors were used for identifying different ChEs: (1)
232 eserine (physostigmine) sulphate, as inhibitor of specific versus non-specific ChEs, (2) iso-OMPA

(tetraisopropyl pyrophosphoramidate), inhibitor of butyrylcholinesterase (BChE), and (3) BW253c51 (1,5-bis(4-allyldimethyl-ammonimphenyl) penta-3-one dibromide), as inhibitor of true acetylcholinesterase (AChE). In all cases, incubation of muscle S10 with the three selective inhibitors (at 10^{-3} M) was made at room temperature (22°C) for 30 minutes. Hundred twenty μ L of S10 were incubated with 5 μ L of each specific inhibitor. After the 30 minutes incubation, ChEs determination was carried out as described before using 1 mM ATC as substrate. A blank without inhibitor and with either bidistilled water (in the case of BW284c51) or with ethanol (in the case of eserine sulphate and iso-OMPA) was also performed.

Lactate dehydrogenase (LDH) was measured using 25 μ L of undiluted S10 and measured the absorbance decrease over 3 min at 340 nm using in well the concentrations of pyruvate (1 mM) and NADH (200 μ M) following the method of Vassault (1983).

Lipid peroxidation levels (LP) were determined in muscle and hepatopancreas using 200 μ L of the same supernatant homogenate (S10) and mixed with 650 μ L of 1-methyl-2-phenylindole in acetonitrile:methanol (3:1) and 150 μ L of HCl. This mixture was incubated at 65°C for 60 minutes, the reaction was stopped in ice and further centrifuged at 13,000 rpm x 10 minutes to precipitate proteins. Absorbance was read at 586 nm versus a standard solution of 1,1,3,3-tetramethoxypropane treated similarly. LP content was expressed as nmol malondialdehyde/g wet weight as described in Antó, Arnau, Buti, Cortijo, Gutiérrez & Solé (2009).

Superoxide dismutase was measured following the McCord and Fridovich (1969) protocol adapted to microplate as described in (Kopecka-Pilarczyk & Coimbra 2010). Conditions in the well were 50 μ M hypoxanthine, 1.8 mUml⁻¹ xanthine oxidase and 10 μ M cytochrome c. A variable amount of sample was selected to achieve 50% inhibition of the cytochrome c reduction under the assay conditions. The reading was done for 3 minutes at 550 nm. A seven point standard of commercial SOD (1-40 U m⁻¹) was used for the quantification.

Catalase activity was measured in 3 mL cuvettes using 50 μL of S10 and 50 mM H_2O_2 as substrate in 100 mM phosphate buffer pH 6.5 by the decrease in absorbance during 1 minute ($\epsilon_{240} = 40 \text{ M}^{-1}\text{cm}^{-1}$) (Aebi 1974). Total Glutathione peroxidase activity was measured by the NADPH consumption ($\epsilon_{340} = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$) during the formation of reduced glutathione by commercial GR using 5 mM cumene hydroperoxide (CHP) as substrate. Conditions in the well were 2.5 mM GSH, 0.29 mM NADPH, one unit of GR and 5 μL of sample. The reaction was started by adding 30 μL of CHP (0.625 mM in well) and lasted for 3 minutes. Glutathione reductase activity conditions for the assay were 1 mM GSSG, 0.63 mM NADPH and 0.16 mM DTNB and 10 μL of sample. Reading was also performed on a microplate for 3 minutes at 405 nm according to Cribb, Leeder & Spielberg (1989). Glutathione S-transferases activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the final reaction mixture containing 1 mM CDNB and 1mM reduced glutathione (GSH) according to Habig, Pabst & Jakoby (1974). The activity rate was measured as change in OD/min during 3 minutes ($\epsilon_{340} = 9600 \text{ M}^{-1}\text{cm}^{-1}$) using 25 μL of (1/100 diluted sample) in the microplate reader Infinite M200 TECAN. Carboxylesterases were measured using 25 μL of 1/20 diluted S10. Concentrations in the well were 1 mM PTA (*S*-phenyl thioacetate) as substrates, and 180 μM DTNB. Reading was carried out in the same conditions as ChEs.

273

274 **Total protein content**

Protein content in haemolymph, hepatopancreatic, intestinal and muscular tissues was determined by the Bradford (1976) method using bovine serum albumin as standard (BSA 0.1-1 mg mL^{-1}). Protein Yield (PY) in muscle and hepatopancreas homogenates (S10) was expressed as protein content per gram of wet weight.

279

280

281

282 Statistical analysis

283 Data were checked for normality and homogeneity of variance by means of Kolmogorov-Smirnov
284 and Bartlett's tests, respectively. Temporal changes in the assessed biomarkers were conducted with
285 one-way ANOVA, followed by the Holm-Sidak multiple range test, when significant differences
286 were found ($p < 0.05$). Comparisons between males and females were conducted by a t-test. Data are
287 presented as mean $\pm$ SD. Pearson correlation coefficients were applied to establish relationships
288 between HSI and biomarkers. The statistical treatment of the data was performed using a SigmaStat
289 3 (Systat Software Inc., USA) software package.

290

291 Results

292 Short-term confinement effect in the physiological status of the broodstock

293 Bacteria and metabolite content was monitored in the haemolymph from ten female during one year
294 and expressed in a monthly and seasonal basis, respectively. The counts of total Vibrionaceae
295 showed a 4-fold increase in the first three months of confinement and remained high onwards, until a
296 significantly decrease in September. Saccharolytic Vibrionaceae presented the same pattern as total
297 Vibrionaceae, whereas the counts of non-saccharolytic Vibrionaceae started to decrease after March
298 and were significantly different from the saccharolytic population in summer (Fig. 1). Large inter-
299 individual variability was observed in all the metabolites analyzed in the haemolymph, among
300 females and/or sampling periods. Non significant differences were observed for protein, glucose,
301 vitamin C, cholesterol and lactate levels presented in the haemolymph of the female spider crabs
302 among seasons (Fig. 2).

303

Long-term confinement effect in the physiological status of the broodstock

Wild animals showed higher HSI values than captive ones ($\varnothing_W = 5.2 \pm 0.7$, $\varnothing_{C1y} = 3.8 \pm 0.8$, $\varnothing_{C2y} = 2.8 \pm 0.9$; $\sigma_W = 5.9 \pm 1.1$, $\sigma_{C2y} = 3.4 \pm 1.4$). In females, the HSI was negatively correlated with the length of the confinement period ($r = -0.995$, $p < 0.05$). No differences were observed in hepatosomatic indices (HSI) between wild and captive males and females (t-test, $p > 0.05$). Whenever no significant differences were observed between males and females, data from both digestive and metabolic enzymes and sexes was pooled together.

Results regarding hepatopancreatic enzymes are shown in Figure 3. Mean values of total protease specific activity were 2-fold higher in wild males than in females (t-test, $p < 0.05$). However, after two years of captivity not statistical significant differences were observed between both sexes and values were similar than those recorded before confinement (t-test, $p > 0.05$). Trypsin, chymotrypsin and lipase specific activities showed no changes neither between sexes or confinement (t-test, $p > 0.05$). After two years in captivity, amylase specific values were significantly higher than those measured in wild animals. No significant differences in amylase activity were observed between both sexes wild animals, although males showed significantly higher values than females after two years of confinement. No changes in alkaline phosphatase and aminopeptidase-N were observed between sexes and after two years of confinement (t-test, $p < 0.05$).

Data from muscular and hepatopancreatic biomarkers are summarized in Table 1. The neuromuscular activity markers AChE and PrChE did not vary either between sexes or due to captivity, and both presented similarly variations ($r = 0.82$; $p < 0.01$). BChE activity was not detected as the activity was very low and inhibition with iso-OMPA did not support its presence in the muscle. During the homogenization of the muscular tissue, 0.1% Triton X-100 was added to the process, but ChEs activities did not increase. LDH measurement was attempted in muscle tissue but it was undetectable in most samples except in a female under 2 years captivity and 1 wild and 1 captive male (data not presented).

As hepatic biomarkers concerns, the antioxidant enzyme catalase varied due to captivity and sex (Fig. 4a) although due to a high individual variation, statistical significance was not always found. SOD activity also presented high individual variability and therefore differences between males and females were not significant either. Then, SOD activities are presented together and no differences were observed due to captivity (Table 1). Other antioxidant enzymes t-GPX and GR showed no differences between captive and wild animals and between sexes (Table 1). A positive relationship was observed between both antioxidants (t-GPX and GR; $r=0.63$; $p<0.01$) but not with catalase. The phase I esterase CbE and the phase II conjugating GST also remained similar per sex and duration of captivity. Moreover, both enzymes evolved similarly ($r=0.64$; $p<0.01$). As a marker of effect LPO levels in hepatopancreas were also unaffected (Fig. 4b), and a negative and low but significant relationship was seen between catalase activity and LPO levels ($r=-0.30$; $p<0.05$). Measurements of LPO levels in muscle were also attempted but they were too low, so only LPO levels in hepatopancreas are reported. PY in muscle and hepatopancreas as an unspecific marker of protein synthesis was similar in muscle but significantly depressed in females' hepatopancreas after 2 years of captivity (Fig. 5).

Discussion

In order to attain independence from wild caught animals in seeds production and to achieve a profitable aquaculture of the spider crab *M. brachydactyla*, the short and long-term effects of broodstock confinement were evaluated. Presence of Vibrionaceae is frequent in crustaceans since their circulatory system is open (Welsh & Sizemore 1985). Results from this presence follow up indicated that females of adult spider crab can be maintained in captivity without any disadvantage to them from a bacteriological point of view. Although presence of bacteria in the haemolymph of Crustacea was previously interpreted as a disease indicator (Lightner 1975; Lightner & Lewis 1975),

now is viewed as a natural process (Gómez-Gil *et al.* 1998). In this sense, bacteria have been isolated from the haemolymph of apparently healthy spider crab (*M. brachydactyla*, Gómez-Gil *et al.* 2010), in other decapod crustaceans as the Pacific white shrimp (*Litopenaeus vannamei* (Boone, 1931); Gómez-Gil *et al.* 1998), the American lobster (*Homarus americanus* H. Milne Edwards, 1837; Cornick & Steward 1966; Bartlett, Wooster, Sokolowski, Dove & Bowser 2008), the blue crab (*Callinectes sapidus* M. J. Rathbun, 1896; Haskell, Sizemore & Colwell 1975; Tubiash 1975; Welsh & Sizemore, 1985; Davis & Sizemore 1982), the red swamp crawfish (*Procambarus clarkii* (Girard, 1852); Scott & Thune 1986), and the freshwater prawn (*Macrobrachium rosenbergii* (De Man, 1879); Brady & Lasso de la Vega 1992). Bacterial density does not often exceed 10^3 - 10^4 CFU mL⁻¹ of haemolymph (Tubiash 1975; Welsh & Sizemore 1985; Gomez-Gil *et al.* 1998) in 88.3 % of the analyzed organisms (Bartlett *et al.* 2008). All the above mentioned studies clearly support the need to know the bacterial populations in the haemolymph of healthy crustaceans in order to correctly interpret diagnostic results. Under the present stocking conditions of the current experiment, the counts of total Vibrionaceae increased from December to March and onwards. Although animals were kept under recirculation at a constant water temperature of 18°C and between 10 - 20% of the total volume of water kept in the system was daily renewed (Carbó, Estévez & Furones 2002). As water was not sterilized at the entrance of the system, microbiological composition might be more affected during summer months in relation to the rest of the year (Miguez & Combarro 2003), due to higher water temperature. Water temperature during the summer months in Alfacs Bay, where the water is pumped from, can exceed the 30°C (Ramón, Fernández & Galimany 2007). In addition, the residence time of bacteria in the haemolymph of crustaceans is from minutes to a few hours (Martin, Poole, Poole, Hose, Arias, Reynolds, McKrell & Whang, 1993; Alday-Sanz, Roque & Turnbull 2002). Therefore, it can be suggested that the bacterial population in the haemolymph could originate from the surrounding environment (Karthiayani & Iyer 1975;). For the summer months the presence of non-sacharolytic bacteria is proportionally higher, this may be explained again by water

379 conditions, when Vibrionacea belonging to sacharolytic species have been observed to predominate
380 in waters where the temperature is over 20°C (Maeda, Matsuo, Furushita & Shiba 2003; Urakawa &
381 Rivera 2006).

382 Metabolites (protein, glucose, vitamin C, cholesterol and lactate) monitored in the
383 haemolymph of *M. brachydactyla* presented a high individual variability and did not differ
384 significantly over one year confinement, whereas short-term stressed spider crabs presented changes
385 in these parameters (Durand *et al.* 2000; Frederich & Pörtner 2000). In the reported studies, lactate
386 and glucose levels and oxygen consumption decreased following one month starvation and after
387 emersion ammonia and urea excretion were reduced and glucose content increased the first three
388 hours (Durand *et al.* 2000). Moreover, Frederich & Pörtner (2000) found that high temperatures
389 (33.3°C) increased lactate and succinate levels in the haemolymph, musculature, hepatopancreas and
390 heart tissues and that extreme temperatures drop oxygen pressure in the haemolymph and increased
391 ventilation rates. Haemolymph metabolites have also been used to detect stress in shrimp produced
392 by serotonin injection (Racotta & Palacios 1998), temperature increase (Chen & Chen 1998),
393 reproductive effort in captivity (Palacios 2000) or osmotic pressure (Sánchez *et al.* 2001).
394 Consequently, no differences in these stress parameters may indicate that one year confinement did
395 not affect the broodstock population as corroborated by no deaths and the continuous spawning
396 during one year under the same environmental conditions (Andrés *et al.* 2010a). As in the present
397 study, Durand *et al.* (2000) did not observed differences in the haemolymph parameters
398 (hemocyanin, ammonia, urate, lactate and glucose) in summer between males and females, even
399 females were ovigerous; and neither Frederich & Pörtner (2000) in oxygen consumption and heart
400 and ventilation rates.

401 Diet of *M. brachydactyla* juveniles and adults is very diverse; in their natural environment
402 they eat mainly macro-algae, molluscs and echinoderms (Bernárdez, Freire & González-Gurriarán
403 2000) as it happens in another spider crab, *Chionocetes opilio* (Brêthes, Desrosiers & Coulombe

1984). Although carbohydrates are not essential in crustacean's diet, they can cost-effectively contribute to the accumulation of glycogen stores in the hepatopancreas, and these stores can be an important source of energy for biosynthetic processes during maturation (Harrison 1997). Digestive enzymes functionally link the process of ingestion and assimilation. They are responsible for the biochemical transformation of ingested organic matter and, in turn, they are regulated by food intake and metabolic needs. Amylase and protease activities are modulated by the composition of the diet in shrimps (Laubier-Bonichon, Van Wormhoudt & Sellos 1977; Van Wormhoudt, Ceccaldi & Martin 1980; Lucien-Brun, Van Wormhoudt, Lachaux & Ceccaldi 1985; Le Moullac, Van Wormhoudt & AQUACOP 1994). Similarly, spider crabs produce amylases to digest carbohydrates in adults (Brêthes *et al.* 1984) and in larvae and juveniles (Andrés *et al.* 2010b). In wild animals it is understandable that amylase activity is high because they ingest large quantities of macro-algae, and then wild animals have low quantities of amylase in the hepatopancreas because this enzyme is largely being used. In our study captive animals were fed with a fresh diet poor in carbohydrates (mussels and crabs) and therefore amylases were less used and accumulated in the hepatopancreas. After two years, the level of amylases accumulated in the hepatopancreas was higher in males because females have to use the glycogen stores for producing eggs and larvae as reported for the first year by Andrés *et al.* (2010a). Ovigerous females should also accumulate more lipids than proteins for gonad maturation when compared to males. This different strategy might explain why total proteases were higher in wild males than in wild females, however no significantly different lipase activities were found between sexes. Other analyzed enzymes did not presented variation neither between sexes or time of confinement. We suggest that the differences observed in digestive enzyme activities reflect mainly the type of diet offered to the crabs and that confinement did not negatively affect the digestive capacities of the crabs.

There is no available data in *M. brachydactyla* concerning the muscular and hepatopancreatic biomarkers studied in here either in wild or captivity conditions. In a comparative attempt with other decapod crustacean that used the same analytical procedures, it appears that *M. brachydactyla* expresses, in general, lower enzymatic activities than the Norway lobster *Nephrops norvegicus* (Linnaeus, 1758) and the rose shrimp *Aristeus antennatus* (Risso, 1816) as far as antioxidant defenses concerns, while its GST activity is intermediate to that in *A. antennatus* and *N. norvegicus* (Antó *et al.* 2009). The lack of BuChE in this species seems to be a common feature to other decapod crustaceans (Solé, García de la Parra, Alexandre-Grimaldo & Sardá 2006; Solé, Hambach, Cortijo, Huertas, Fernandez & Company 2009, Antó *et al.* 2009) as well as a good relationship between AChE and PrChE. Thus in further works the study of only one form is recommended. Hepatic CbE was about 4 times lower in *M. brachydactyla* than in the other two contrasted crustacean mentioned above whereas muscular esterases are similar (Antó *et al.* 2009). In another comparative study contrasting xenobiotic metabolism activities in hepatopancreas in marine invertebrates including this species (referred as *M. squinado*) from UK waters, Solé & Livingstone (2005) also revealed that total cytochrome P450 and some associated reductases were lower in *M. brachydactyla* than in other crustaceans. Present results and those from the literature evidence a low hepatic xenobiotic metabolism in this species. As far as muscular AChE concerns, this activity was much lower in *M. brachydactyla* than in another brachyuran species the swimming crab *Polybius* Leach, 1820 (Signa, Cartes, Solé, Serrano & Sánchez 2008). Despite an overall reduced xenobiotic metabolism activities and antioxidant defenses, specimens from *M. brachydactyla* under captivity for up to two years (males and females) did not seem to suffer any additional stress due to confinement conditions. Sex- and size-related differences in antioxidant defenses and LPO levels were reported in brain of wild-caught *A. antennatus* (Mourete & Díaz-Salvago 1999). In the present comparative study, even if antioxidant defenses (*e.g.* catalase) were lowered in confined specimens from both sexes, other antioxidants (*e.g.* SOD, t-GPX and GR) were not. As opposed to catalase, males (wild and confined)

tended to express higher SOD activities than females, however due to the high individual variability observed statistical differences in relation to sex could not be confirmed. Overall, confinement did not cause oxidative stress damage as LPO levels remained unaffected and no sign of neurotoxicity was evidenced either.

In conclusion, captive broodstock of the spider crab showed a large plasticity in their digestive capabilities as indicated by the adaptation to the food supplied in captivity (low diversity of feed items and monotonous diet). After two years of confinement, no signs of nutritional or metabolic distress were observed in animals kept in captivity neither changes in composition of haemolymph microflora and metabolites that could be indicative of unsuited adaptation to confinement. Although a lower xenobiotic metabolism and antioxidant defenses capacity was evidenced in this species when contrasted to other crustaceans, captivity conditions or sex did not seem to negatively affect their adaptation to captivity as indicated by selected biomarkers response and no enhanced oxidative stress damage (measured as LPO levels). These results indicated that although confinement conditions used in this study do not mimic those from the wild, they could be considered as suitable for rearing this species, at least with regards to the set of parameters assessed.

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714 **Figure captions**

715 Figure 1. *Maja brachydactyla*. Differential Vibrionaceae bacteria counts from the haemolymph of a
716 batch of adult female spider crabs maintained in captivity for one year. Total (▼), saccharolytic (●)
717 and non-saccharolytic (○) bacteria.

718
719 Figure 2. *Maja brachydactyla*. Metabolites measured from the haemolymph of a batch of adult
720 female spider crabs maintained in captivity for one year. a) Vitamin C, b) Cholesterol, c) Glucose, d)
721 Lactate and e) Protein. No significant differences were observed among season in the evaluated
722 metabolites ($p < 0.05$).

723
724 Figure 3. *Maja brachydactyla*. Digestive enzyme levels in the hepatopancreas of wild and captive (1
725 or 2 years) crabs. Letters indicate significant differences ($p < 0.05$) among captivity time and asterisks
726 between sexes.

727
728 Figure 4. *Maja brachydactyla*. Metabolic enzyme levels of wild and captive (1 or 2 years) crabs. a)
729 Catalase in the hepatopancreas and b) lipid peroxidation (LPO) levels in muscle. Letters indicate
730 significant differences ($p < 0.05$) among captivity time and asterisks between sexes.

731
732 Figure 5. *Maja brachydactyla*. Protein yield (PY) in muscle (a) and hepatopancreas (b) of wild and
733 captive (1 or 2 years) crabs. Letters indicate significant differences ($p < 0.05$) among captivity time
734 and asterisks between sexes.

Table 1. Muscular and hepatic biomarkers values from the muscle and hepatopancreas from wild and captive spider crabs *Maja brachydactyla*. Data expressed as mean (in nmol/min/mg prot) $\pm$ SEM. $P>0.05$. AChE: acetylcholinesterase; CbE: carboxylesterases; GR: glutathione reductase; GST: glutathione S-transferases; PrChE: propionylcholinesterase; t-GPX: total glutathione peroxidase; SOD: superoxide dismutase.

	Wild	1 year	2 years
Muscular			
AChE	6.4 ± 0.5	5.6 ± 0.3	5.6 ± 0.4
PrChE	4.9 ± 0.3	4.2 ± 0.3	4.6 ± 0.3
Hepatic			
t-GPX	44.7 ± 2.2	37.0 ± 2.4	53.5 ± 7.4^a
GR	2.5 ± 0.3	2.5 ± 0.3	3.5 ± 0.6
GST	1351 ± 92	1012 ± 93	1456 ± 165
CbE	131.0 ± 9.8	119.3 ± 5.6	142.9 ± 26.3
SOD	11.7 ± 8.6	8.3 ± 2.7	9.4 ± 4.5

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