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Effective population size and heterozygosity-fitness correlations in a population of the Mediterranean lagoon ecotype of long-snouted seahorse *Hippocampus guttulatus*

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1 **Effective population size and heterozygosity-fitness correlations in a population**
2 **of the Mediterranean lagoon ecotype of long-snouted seahorse *Hippocampus***
3 ***guttulatus***

4
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24
25 **Abstract**

26 The management of endangered species is complicated in the marine environment
27 owing to difficulties to directly access, track and monitor in situ. Population genetics
28 provide a genuine alternative to estimate population size and inbreeding using non-
29 lethal procedures. The long-snouted seahorse, *Hippocampus guttulatus*, is facing
30 multiple threats such as human disturbance or by-catch, and has been listed in the red
31 list of IUCN. One large population is found in the Thau lagoon, in the south of
32 France. A recent study has shown this population belongs to a genetic lineage only
33 found in Mediterranean lagoons that can be considered as an Evolutionarily
34 Significant Unit (ESU) and should be managed with dedicated conservation
35 strategies. In the present study, we used genetic analysis of temporal samples to
36 estimate the effective population size of the Thau population and correlations between
37 individual multilocus heterozygosity and fitness traits to investigate the possible
38 expression of inbreeding depression in the wild. Non-invasive sampling of 172
39 seahorses for which profiles were pictured and biometric data recorded were
40 genotyped using 291 informative SNPs. Genetic diversity remained stable over a 7-
41 year time interval. In addition, very low levels of close relatedness and inbreeding
42 were observed, with only a single pair of related individuals in 2008 and two inbreds
43 in 2013. We did not detect departure from identity equilibrium. The effective
44 population size was estimated to be $N_e = 2742$ (~40 reproductive seahorses per km²),
45 larger than previously thought. No correlation was observed between heterozygosity
46 and fluctuating asymmetry or other morphometric traits, suggesting a population with
47 low variance in inbreeding. Together these results suggest this population does not
48 meet conventional genetic criteria of an endangered population, as the population
49 seems sufficiently large to avoid inbreeding and its detrimental effects. This study

50 paves the way for the genetic monitoring of this recently discovered ESU of a species
51 with patrimonial and conservation concerns.

52

53 **Key words:** marine endangered species, *Hippocampus guttulatus*, population
54 effective size, inbreeding, heterozygosity-fitness correlations

55

56

57 **Introduction**

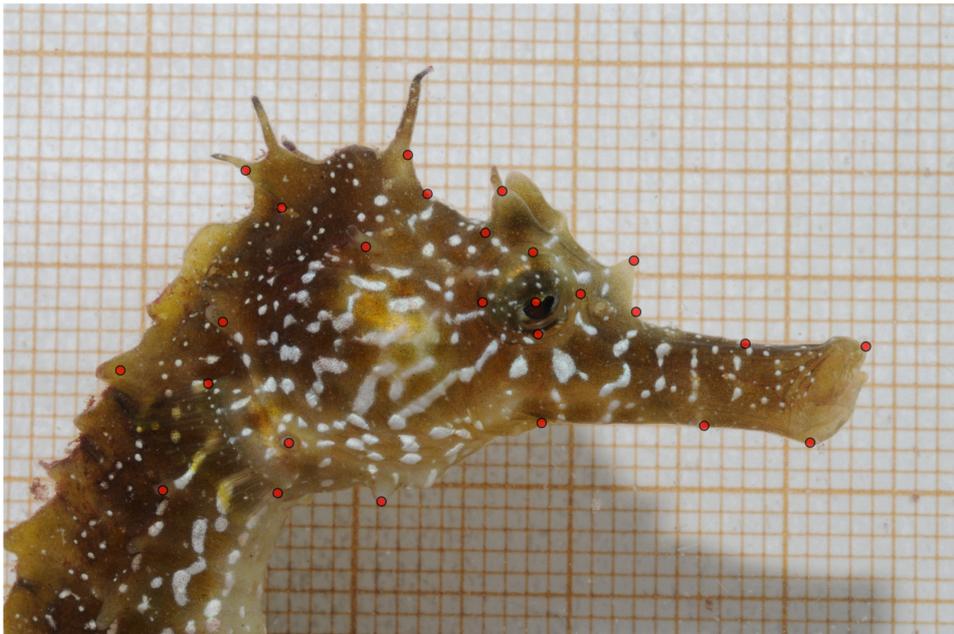
58 Marine conservation is lagging behind efforts on terrestrial ecosystems
59 (Hendriks et al. 2006). One reason is that the marine domain is more difficult to
60 access, making direct monitoring of populations by counting or capture-recapture
61 more complicated to realize. DNA analysis has grown substantially over the last two
62 decades as an alternative to determine genetic diversity and relatedness within
63 populations, connectivity among populations, infer demographic history of
64 populations, or even provide acute estimates of the effective population size
65 (Caughley 1994; Frankham 1995; Luikart et al. 2010). Seahorses (*Hippocampus* spp.)
66 are protected by the Convention on International Trade in Endangered Species of
67 Wild Fauna and Flora (Appendix II; 2014) against over-exploitation through
68 international trade, as well as by the EU Wildlife Trade Regulations (Annex B, 2014).
69 More specifically, *Hippocampus guttulatus* Cuvier, 1829 is classified as Data-
70 Deficient in the International Union for Conservation of Nature Red list of threatened
71 species, indicating the lack of knowledge of the species overall. In addition, *H.*
72 *guttulatus* is listed as near threatened, vulnerable or endangered in several European
73 countries and most European countries ratified the Berne Convention, Barcelona
74 Convention and OSPAR Convention, all aiming to protect *H. guttulatus*. Despite
75 legislations regarding its protected status, only a handful of studies on *H. guttulatus*
76 have been done so far, of which a few focused on patterns of population genetics
77 (López et al. 2015; Riquet et al. 2019; Woodall et al. 2015). Riquet et al. (2019)
78 identified four cryptic lineages maintained by partial reproductive isolation. These
79 lineages lie somewhere in the grey zone of speciation (Roux et al. 2016), but should
80 be considered Evolutionarily Significant Units (ESUs) with independent conservation
81 management plans. Within lineages, genetic homogeneity over large distances was
82 observed (Woodall et al. 2015; Riquet et al. 2019), suggesting populations are
83 sufficiently large and connected to maintain genetic panmixia despite sparse
84 distribution, low mobility and the absence of dispersive stage (Foster and Vincent
85 2004). One of the identified lineages exclusively inhabits Mediterranean lagoons
86 (Riquet et al. 2019). The largest population of this ESU is found in the Thau lagoon
87 (France). Isolated in the north of the Mediterranean Sea, this population has no other
88 evolutionary route than to adapt to global warming. It appears necessary to gather
89 data on the vulnerability of this lagoon ecotype, before introducing any conservation
90 management plan.

91 In the present study, we used three temporal samples (2006, 2008 and 2013) of
92 Thau *H. guttulatus* to assess genetic diversity and its variability over time, as well as
93 any demographic events. In addition, we assess inbreeding depression, by estimating
94 inbreeding coefficients and identity disequilibrium (*i.e.* correlations in homozygosity),
95 to characterize population inbreeding (David 1998; Slate et al. 2004; Balloux et al.
96 2004; Szulkin et al. 2010). We also tested correlations between heterozygosity and
97 fitness traits (David 1998; Chapman 2009; Szulkin et al. 2010) using weight, lengths
98 and fluctuating asymmetry as fitness traits.

99

100 **Materials and Methods**

101 *Sampling procedure, biometry and tissue acquisitions* - Tissue samples were collected
102 from *H. guttulatus* in the Thau Lagoon (France) in November 2005 and March 2006
103 (N=5+8, pooled as the 2006 sample), in December 2008 (N=110) and in July-August
104 2013 (N=49). Each individual was sexed, weighed, and snout and total lengths were
105 measured. In 2008 and 2013, right and left profiles were pictured in a standardized
106 way using the Micro Nikkor 60 mm lens of a Nikon D300. Before releasing the
107 sampled individuals, the dorsal fin was partially clipped, a non-lethal procedure
108 (Woodall et al. 2012). Each individualized fin-clip was preserved in 96% ethanol for
109 subsequent genetic analyses.
110



111 **Fig. 1** Localization of the 26 landmarks used to analyze bilateral symmetry in
112 *H. guttulatus*
113

114
115 *Morphometric analyses* – Shape analyses were based on geometric landmark
116 coordinates (Claude 2008). Landmarks were chosen for an optimal coverage of the
117 seahorse profile morphology, to compare right and left profiles, as well as their
118 homology and repeatability among individuals. Individuals were digitized twice, on
119 both sides, to test the significance of fluctuating asymmetry. A total of 26 landmarks
120 were digitized on each image (Fig. 1) using tpsDig v2.17 (Rohlf 2010), then imported
121 in R (R Development Core Team, 2011) and analyzed using Claude's R functions
122 (Claude 2008). To screen out all non-shape difference (e.g. scale, position and
123 orientation), we first conducted a partial Generalized Procrustes Analysis (GPA)
124 superimposition (Rohlf and Slice 1990; Dryden and Mardia 1998). We then
125 investigated whether fluctuating asymmetry (random deviations from perfect
126 bilateral), directional asymmetry (significantly biased deviations from perfect bilateral
127 towards one side) and symmetric inter-individual variation were present in our data.
128 To estimate component of symmetric and asymmetric variation in size (Palmer 1994),
129 we performed a mixed effect analysis of variance on centroid size with individual,
130 side and their interaction. To test shape asymmetry, we performed a PCA on the

131 coordinate matrix, then run a MANOVA (Wilks 1932) using the same factors as for
132 the size analysis.

133 Because these indices were present for just one subset, we later computed index of the
134 mean asymmetry (computed as shape difference between both sides, *i.e.* square root
135 of the sum of squared procrustes differences between sides) per individual. Departure
136 from equilibrium was tested as well as putative correlations with genetic stress such
137 as inbreeding.

138

139 *DNA extraction and Genetic Analyses* - DNA was extracted using a standard
140 cetyltrimethylammonium bromide chloroform:isoamyl alcohol (24:1) protocol (Doyle
141 and Doyle 1987). We checked quality and quantity of DNA extraction on an agarose
142 gel, and equalized to 35 ng.μL⁻¹ using Qubit Fluometric Quantitation (Invitrogen).
143 SNPs were genotyped using GoldenGate Genotyping Assay with VeraCode
144 technology at ADNid company (Montpellier, France). Details regarding SNP calling
145 are provided in Riquet et al. (2019). Allelic richness (A_r , *i.e.* the expected number of
146 alleles corrected for sampling size based on a rarefaction method) was estimated
147 using the R package *diveRsity* (Keenan 2013). We used *Genepop on the Web*
148 (Rousset 2008) to estimate allelic frequencies, expected heterozygosity (H_e), fixation
149 index (F_{IS}) and to test both departure from Hardy-Weinberg equilibrium (10000
150 dememorization steps, 500 batches and 5000 iterations per batch) and temporal
151 genetic structure.

152 Relatedness coefficients between all pairs of individuals were computed using the
153 approach described in Wang (2007), implemented in COANCESTRY 1.0.0.1 (Wang
154 2011). This approach uses maximum likelihood (ML) to infer the relatedness
155 coefficient between two individuals given population allele frequencies. We set error
156 rates of 10⁻⁶ per locus. Mean relatedness coefficients and their 95% confidence
157 intervals were estimated with a bootstrap procedure (500 bootstraps). Simulations
158 based on all genotypes were also run to compare calculated and expected relatedness
159 estimates.

160 Individual Multi-Locus Heterozygosity (MLH) was calculated for each individual. In
161 a population with variance in inbreeding, inbred individuals are less heterozygous (*i.e.*
162 lower MLH). Inbreeding variance generates Identity Disequilibria (ID) *-i.e.*
163 correlations in homozygosity across loci, a measure of departure from random
164 associations between loci (Szulkin et al. 2010). ID were measured in 2008 and 2013
165 specimens by calculating the parameter g_2 and its standard deviation using the
166 software RMES (David et al. 2007), but the sample size of specimens from 2006 was
167 too low to conduct such an analysis. The g_2 parameter measures the excess of double
168 heterozygotes at two loci relative to the expectation under a random association,
169 standardized by average heterozygosity (Szulkin et al. 2010), providing a measure of
170 genetic associations and inbreeding variance in the population. To test for the
171 significance of g_2 , random re-assortments of single-locus heterozygosities among
172 individuals were tested 1000 times (David et al. 2007).

173 Finally, we tested for heterozygosity-fitness correlations (Chapman 2009; Szulkin
174 2010) by looking for correlations between MLH and phenotypic data (measurements,
175 weight and Asymmetrical Index). Spearman correlations (Spearman 1904) were
176 computed using the software package R (R Development Core Team, 2011).

177

178 *Ne Estimations* - Estimating N_e is challenging, and large variance in performance
179 among methods is commonplace (Gilbert and Whitlock 2015; Riquet et al. 2016).
180 Therefore the use of several independent methods is recommended to improve

181 accuracy (Waples 2005; Fraser et al. 2007; Waples and Do 2010; Hare et al. 2011).
182 We used the two types of methods to estimate the effective population size that were
183 found to produce the best results when the study population experienced no migration
184 (Gilbert and Whitlock 2015). First, N_e was estimated by measuring temporal changes
185 in allele frequencies between two diachronic samples separated by 7 and 5 years
186 (Waples 1989). The pseudo-likelihood based approach (Wang 2001), implemented in
187 MLNe 2.3 (Wang and Whitlock 2003) was used. We set maximum N_e at 10 000,
188 higher than the census size (N_c) estimated by Louisy and Bérenger (2015). The mean
189 age of reproducing adults in a population that contributed to a cohort was estimated to
190 1.8 years, the reproductive output assumed constant through age with a life span
191 estimated to ca. 5 years (Curtis and Vincent 2006, Louisy and Bérenger 2015). With a
192 generation time unknown, we set it to 2 years and we also tested longer generation
193 times (3- and 5- year generation time). The effective number of breeders that
194 produced the sample (N_b) was also estimated (Waples 2005) with 2008 and 2013
195 samples. We measured the nonrandom association of alleles at different loci within a
196 sample (Hill, 1981) using a bias correction (Waples 2006; Waples and Do 2008)
197 implemented in NeEstimator v2 (Do et al. 2014). Following the recommendations of
198 Waples and Do (2010), we excluded alleles with frequencies below 0.02 and
199 according to *H. guttulatus* reproductive characteristics, monogamy was applied.

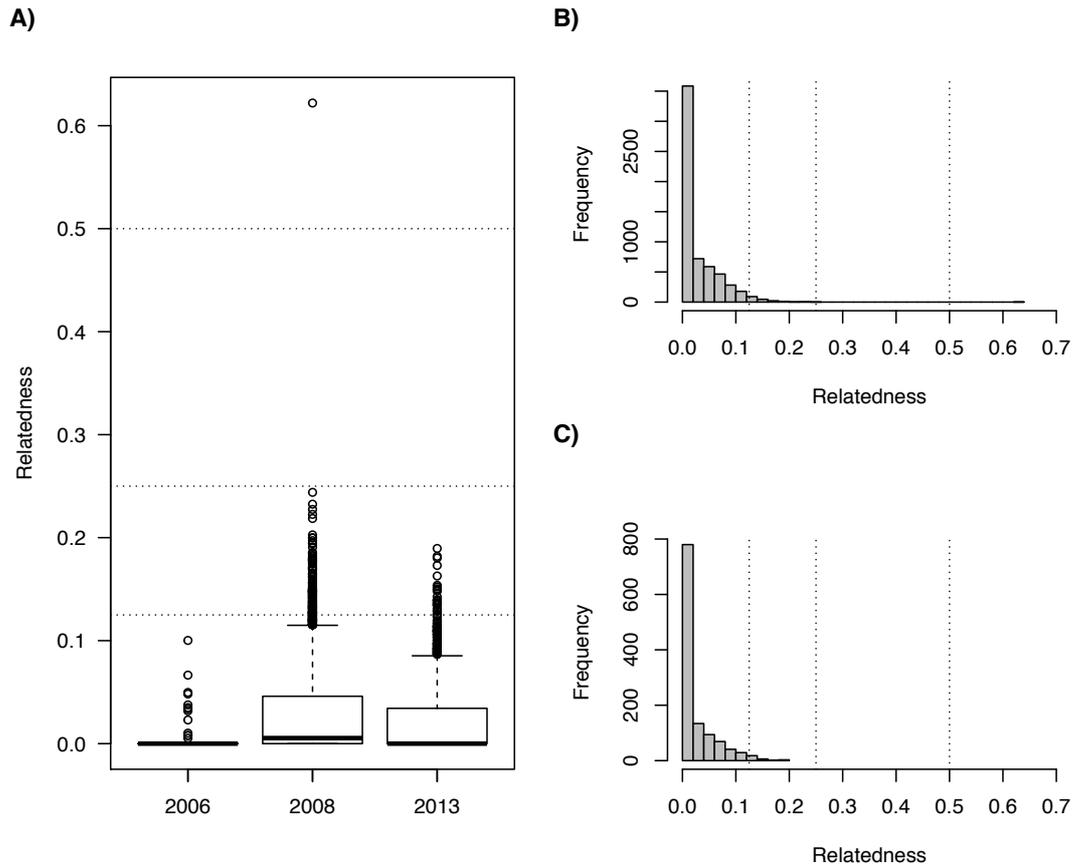
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201 **Results**

202 *Fluctuating asymmetry in the Thau lagoon population*

203 Individuals with only one usable image per profile were removed from the analyses,
204 resulting in 77 individuals from 2008 being analyzed using 26 landmarks. Significant
205 fluctuating and directional asymmetries on the size were observed (ANOVAs $F =$
206 4.62 and $F = 4.41$, p -value = 0.03 and p -value = 0.04, respectively). Shape fluctuating
207 asymmetry was still significant (MANOVA $F = 3.16$ p -value = $2.2 \cdot 10^{-16}$), while
208 directional asymmetry was not (MANOVA $F = 1.29$, p -value = 0.23).

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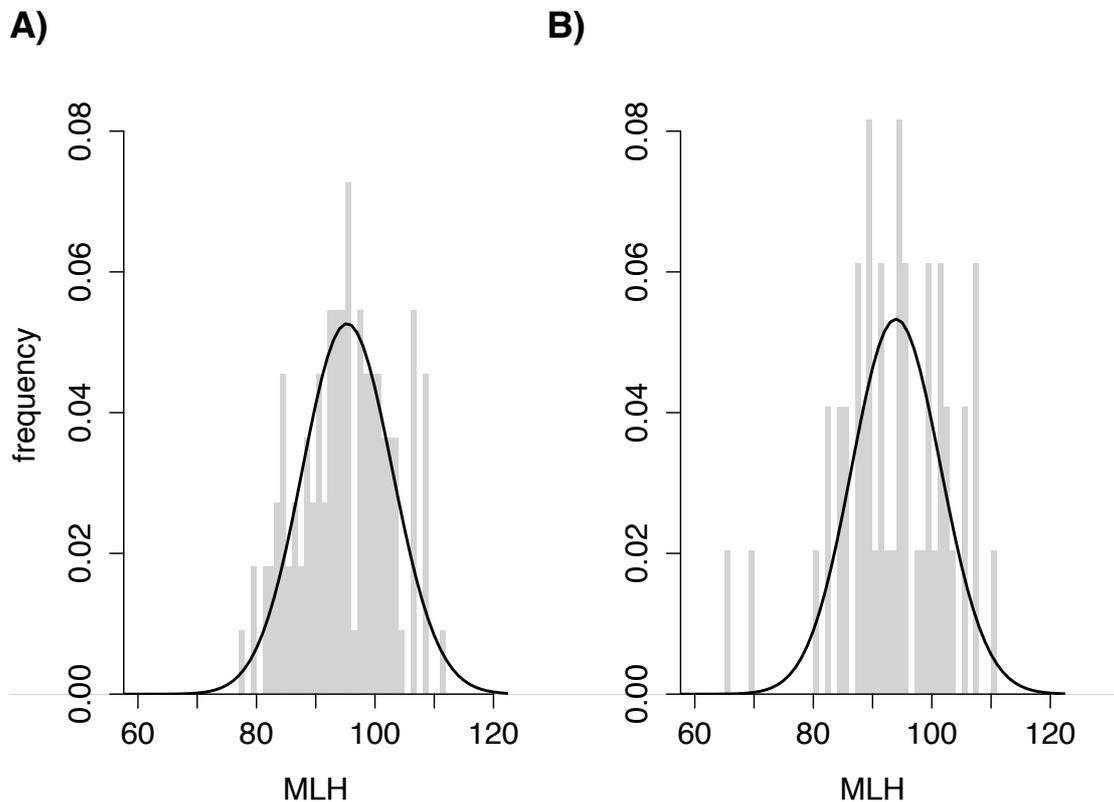
Fig. 2 Pairwise relatedness coefficient estimates among *H. guttulatus* sampled in 2006, 2008 and 2013. Dotted lines highlight a relatedness coefficient of 0.5 (e.g. full sibs or parent-offspring), 0.25 (half-sibs or grandparent-grandchild) and 0.125 (first cousins, aunt/uncle - offspring). A) Pairwise relatedness in each sample. B) Frequency of pairs of individuals distribution in 2008. C) Frequency of pairs of individuals distribution in 2013. Frequency of pairs of individuals distribution in 2006 is not shown (N = 13).

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Diversity and relatedness in the Thau lagoon population

Monomorphic loci among all individuals or loci with minor allelic frequencies lower than 5% were removed, so that we used 291 SNPs for all 172 individuals, with no missing data. All loci showed heterozygotes in every sample (2006, 2008 and 2013) and genetic diversity remained stable over time ($A_r-2006= 1.89$, $A_r-2008= 1.93$ and $A_r-2013= 1.92$; $H_e-2006= 0.336$, $H_e-2008= 0.328$ and $H_e-2013= 0.326$; p -values > 0.99). Note that 13 samples were used to estimate A_r , *i.e.* a measure of genetic diversity that takes into account variation of sample size. No departure from Hardy-Weinberg equilibrium was observed ($F_{IS-2006} = 0.0001$, $F_{IS-2008} = 0.006$, $F_{IS-2013}= 0.008$; p -values > 0.99) along with no temporal genetic differentiation ($F_{ST} = 0.0001$, p -value = 0.91). We estimated relatedness using the assumptions of inbreeding and no inbreeding (Wang 2007) and similar results were obtained. As there was little evidence for inbreeding (see below), we present results with no inbreeding. Relatedness coefficients ranged from 0 to 0.10 in 2006 (mean \pm sd = 0.006 ± 0.017), from 0 to 0.62 in 2008 (mean \pm sd = 0.027 ± 0.038) and from 0 to 0.19 in 2013 (mean \pm sd = 0.022 ± 0.034 ; Fig. 2). Relatedness was not significantly different between the three

236 study years (p -value = 0.93). Based on simulations, relatedness estimator comparisons
 237 between the calculated relatedness coefficient values and the expected relatedness
 238 values were not significantly different (expected levels of relatedness r = 0.005, 0.26
 239 and 0.21 in 2006, 2008 and 2013, respectively; Wilcoxon signed ranks test, Z = -
 240 1.807, p -value = 0.071). Over all dyad comparisons, only one (0.01%) was identified
 241 with a relatedness coefficient higher than 0.5, suggesting the presence of parent-
 242 offspring or full sibling pairs in 2008 (Fig. 2B). In 2008, most relatedness estimates
 243 (97.54 %) of relatedness coefficients values were lower than 0.125 (*i.e.* first cousins
 244 relatedness) while 2.45% ranged from 0.125 to 0.25 (*i.e.* halfsibs, avuncular,
 245 grandparent-grandchild relatedness), suggesting that most dyads remained mostly
 246 unrelated. In 2013, a similar pattern was seen with a maximum relatedness estimate of
 247 0.189; 97.8% of relatedness coefficients estimates were lower than 0.125 while 2.28%
 248 ranged from 0.125 to 0.25. While in 2006, all relatedness estimates were lower than
 249 0.125.
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 255 **Fig. 3** Distributions of observed multi-locus heterozygosity (in gray) and expected
 256 multi-locus heterozygosity under random mating (black lines) in 2008 (A) and 2013
 257 (B).
 258

259 *Heterozygosity-Fitness correlations*

260 Multi – Locus Heterozygosity (MLH) ranged from 85 to 111 in 2006 (mean \pm sd = 98
 261 \pm 8.0), from 77 to 111 in 2008 (mean \pm sd = 94 \pm 7.7) and from 65 to 110 in 2013
 262 (mean \pm sd = 93 \pm 9.3; Fig. 3, data of 2006 not shown). MLHs were normally
 263 distributed (Shapiro test p -values = 0.24, 0.61 and 0.16 in 2006, 2008 and 2013,

264 respectively; Fig. 3, data of 2006 not shown) and no difference in MLH distribution
265 was observed over time (t-test p -value = 0.38).
266 Identity disequilibrium, *i.e.* a measure of departure from random associations of
267 homozygosity between loci, was only identified in 2013 ($g_2= 0.003$, p -value = 0.015),
268 mainly due to two individuals with low MLH (Fig. 3). This suggests that these
269 individuals may be inbred. No identity disequilibrium remained when removing these
270 two individuals ($g_2=0.00002$, p -value = 0.46). No identity disequilibrium was
271 identified in 2008 ($g_2=0.0002$, p -value = 0.42).
272 Heterozygosity did not correlate with fitness traits, regardless of trait tested
273 (Asymmetry Index estimated in 2008 per individual with its replicate, snout length,
274 total length and weight; p -values on Spearman tests ranged from 0.11 to 0.75).
275

276 *Effective population size*

277 Temporal changes in allele frequencies estimated N_e per generation (2-year
278 generation time) of 2742 at Thau (Confidence Interval (CI) = (665, $+\infty$)). Larger
279 generation time (3- and 5-year generation time) basically lead to smaller point
280 estimates, but more uncertainties with higher CIs ($N_{e-3y \text{ generation time}} = 1329$, CI=(286,
281 $+\infty$) and $N_{e-5y \text{ generation time}} = 1008$, CI = (165, $+\infty$)). Smaller values were estimated with
282 single-sample method, with nonetheless overlapping CIs: N_b estimations varied from
283 959 (CI = (796, 1201)) in 2008 to 845 (CI = (601, 1401)) in 2013.
284
285

286 **Discussion**

287 Indirect study using predictions of population genetics produces particularly
288 valuable information for marine species (Waples 1998). In the present study, genetic
289 diversity of the isolated Thau population of the Mediterranean lagoon ecotype of *H.*
290 *guttulatus* proved to remain stable over a seven-year period. We did not identify
291 significant temporal genetic structure, departure from Hardy-Weinberg nor identity
292 equilibrium. Most individuals were likely unrelated and the population mainly
293 outbred, with a unique pair of related individuals identified in 2008, and two inbred
294 individuals in 2013. Heterozygosity did not significantly correlate with fitness traits,
295 reflecting a population with no strong variance in inbreeding, and therefore no
296 evidence of inbreeding depression (Szulkin et al. 2010). All results suggest that the
297 Thau lagoon *H. guttulatus* population is large and panmictic and therefore will not be
298 affected by the detrimental effects of genetic drift and inbreeding.
299

300 We observed only two inbred individuals in our sample of 172 seahorses. *H.*
301 *guttulatus* is usually described sedentary, with low mobility, although it may disperse
302 up to 150m in a single day (Caldwell and Vincent 2012) with home ranges of up to
303 400m² (Garrick-Maidment et al. 2010). However they are thought to undergo seasonal
304 migrations and have an ontogenic habitat shift. Regarding the inbred crosses found in
305 this study, relatives may have mated because dispersal is not effective or because they
306 dispersed collectively (Broquet et al. 2013; Yearsley et al. 2013). Inbreeding variance
307 was not observed in our samples using identity disequilibrium (g_2) and
308 Heterozygosity-Fitness Correlations (HFCs) were not identified. HFCs have been
309 widely examined in natural populations (review in Chapman et al. 2009), although
310 such correlations should only be detected in population with a high variance of
311 inbreeding (Bierne et al. 2000; Szulkin et al. 2010). Our results suggest the study
312 population of seahorse does not seem suffering from inbreeding depression, even if
313 inbreeding could occur in the Thau lagoon.

314 The census size of the Thau population of *H. guttulatus* is thought to have varied
315 by factors from 1 to 10 over time according to an 8-year SCUBA-diving survey
316 (Louisy and Bérenger 2015). Although protocols were modified throughout the
317 survey, making dives difficult to compare, this monitoring gave an overview of Thau
318 *H. guttulatus* fluctuations, with a minimum size observed in 2006 following lagoon
319 pollution events, and a maximum in 2009-2010 (Louisy and Bérenger 2015). As
320 expected the effective population size (N_e) remained stable from 2006 to 2013 and
321 reflects the harmonic mean of the census size over that period. Indeed, not all adults
322 effectively reproduce; hence, N_e estimates correspond to the smallest census size of
323 the study time series. Our estimate therefore corresponds to oscillation minima of the
324 population size. Therefore the population of *H. guttulatus* in this 75 km² lagoon (~50
325 individuals km⁻²) is likely large enough that demographic events would not impact
326 genetic diversity much, a result in agreement with large scale genetic studies (López
327 et al. 2015; Woodall et al. 2015; Riquet et al. 2019). Such fluctuations in census size
328 population were also observed in the Ria Formosa coastal lagoon (southern Portugal).
329 *H. guttulatus* declined by 94% over 5 years (Curtis and Vincent 2006; Caldwell and
330 Vincent 2012) due to a combination of habitat loss (Curtis 2007; Gamito 2008;
331 Correia et al. 2015) and coastal ocean warming (Teles-Machado et al. 2007), and is
332 now recovering (Correia et al. 2015). In Galicia, similar effective population size
333 estimates have been obtained (López et al. 2015). Although N_e estimates should be
334 considered with caution (Hare et al. 2011), *H. guttulatus* N_e estimates are larger than
335 most endangered species (e.g. Chapman et al. 2002; Hoarau et al. 2005), which
336 should ensure the maintenance of genetic variation across the longer term providing
337 appropriate management practices are maintained.

338

339 Seahorses are characterized by a strong parental investment, ensuring
340 reproductive success even at low densities. This was hypothesized to make them more
341 able to endure severe population demographic events such as bottleneck events
342 caused by environmental perturbations for instance, when compared to species with
343 low parental investment (Romiguier et al. 2014). Traill et al. (2010) showed that
344 exceeding the number of individuals required for management and conservation plans
345 will ensure the viability and longevity of the population. In this context, monitoring
346 the Thau population of *H. guttulatus* over time is important in order to foster the
347 results observed here, especially facing the global warming and other threats they will
348 have to face.

349 Genetic monitoring of this recently discovered conservation unit in *H.*
350 *guttulatus* should continue to quantify any temporal changes in population genetic
351 metrics in response to ever-increasing anthropogenic changes to natural ecosystems,
352 which may affect the long-term status of this lineage. Keeping *H. guttulatus* an
353 ambassador in marine conservation makes sense. Facing alarming statistics regarding
354 marine biodiversity decline and efforts (Hendriks et al. 2006; Lotze et al. 2006),
355 fascinating and iconic marine species, such as *H. guttulatus*, act efficiently as flagship
356 for other endangered species and ecosystems such as Mediterranean lagoons (Pérez-
357 Rufaza et al. 2011).

358

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