Zygoparity and sex steroid hormone profiles in bluemouth *Helicolenus dactylopterus*

V. Sequeira [1*], E. Couto [3], A. Neves [1,2], A. R. Vieira [1,2], R. B. Paiva [1,2], A. Canario [3] and L. S. Gordo [1,2]

[1] MARE – Marine and Environmental Sciences Centre, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[2] Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[3] CCMAR – Centre for Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Running headline: *H. DACTYLOPTERUS* ZYGOPARITY AND SEX STEROIDS

* Author to whom correspondence should be addressed. Tel.: + 351 217 500 824; email: vlsequeira@fc.ul.pt

Abstract

Bluemouth *Helicolenus dactylopterus* (Scorpaeniformes, Sebastidae) is a commercially important deep water species with an unusual reproductive strategy. 207 individuals (103 females and 104 males) from the western Atlantic ranging from 13.9 cm to 37.5 cm total length (L_T) were analysed from September 2011 to October 2012 for gonad maturity stages and blood plasma levels of estradiol-17β (E_2), 11-ketotestosterone (11-KT), 17,20β-dihydroxypregn-4-en-3-one (17,20β-P). Results confirmed the existence of an annual reproductive cycle with asynchrony between females and males and a spawning season from January to May. A pronounced peak in 17,20β-P in October for both sexes was associated with possible mating behavior and recent copula. Levels of E_2 increased preceding the elevation of gonadosomatic index during ovarian growth, and were lower during regression and regeneration. The frequency distribution of oocyte/embryonic stages and variation of hormone levels suggest the existence of daily rythms. Fertilization was detected between 2000–0000 and 0800–1200 hours period and spawning took place throughout the day peaking between 2000–0000 hours. The cyclic pattern of sex steroids and ovarian recruitment provides a new insight into the reproductive strategy of this species.

Key words: Reproductive strategy; estradiol-17β; 11-ketotestosterone; 17,20β-dihydroxypregn-4-en-3-one.

Introduction

Teleost fishes are characterized by a diversity of reproductive strategies with the majority being oviparous ovuliparous; nevertheless ~2.5% are viviparous (Wourms, 1991). Knowledge of reproductive strategies and spawning patterns is essential for a comprehensive understanding of the population dynamics of any fish species (Rinchard & Kestemont, 2003) allowing future management and assessment of fishery resources. Scorpaeniformes are particularly interesting as their reproductive strategies vary between oviparity and viviparity (Wourms, 1991).

Bluemouth *Helicolenus dactylopterus* (Delaroche, 1809) is a benthopelagic (100-1000m) scorpaeniform with a wide distribution in the eastern Atlantic (from Norway to South Africa, and around the Azores, Madeira and the Canary islands) and in the Mediterranean (Hureau & Litvinenko, 1986). It is an important commercial species in ICES waters (~ 10,500 tons average annual landings since 2006) as bycatch of demersal trawl and as target for longline fisheries (ICES, 2012). Female and male reproductive cycles are out of phase, with internal fertilization and storage of spermatozoa in cyst-like structures inside the ovaries for several months (Muñoz *et al.*, 1999; Sequeira *et al.*, 2012a; Sequeira *et al.*, 2015). Fecundity is relatively low highlighting the vulnerability of the species to the fishery. Furthermore, fecundity appears to be of the indeterminate type in the western Iberian Peninsula (Sequeira *et al.*, 2012b; Sequeira *et al.*, 2015) and of determinate type in the northwestern Mediterranean Sea (Muñoz *et al.*, 2010). Oocyte development is centripetal (Muñoz *et al.*, 1999) and asynchronous (Muñoz *et al.*, 2010; Sequeira *et al.*,

2012b). Multiple spawning of individual females occurs in the winter-early spring (Muñoz *et al.*, 2010; Sequeira *et al.*, 2012b; Sequeira *et al.*, 2015) and consists of early celled embryos (zygoparity) wrapped in a gelatinous matrix produced by the ovarian wall and peduncular epithelia (the structure that supports each oocyte) (Sequeira *et al.*, 2011).

Considering the complexity of the reproductive cycle and spawning pattern of *H. dactylopterus*, a combined analysis of gonadal stages and sex hormones can provide insights into the physiology and timing of reproductive processes and thereby better support management decisions about the fishery. Sex steroids, produced in the gonads, are under control of pituitary gonadotrophins and regulate key processes during germ cell development. Estradiol-17β (E_2) produced by the ovarian follicle regulates vitellogenin synthesis in the liver, which is then transported in the blood stream and incorporated into the oocyte where it is responsible for most of secondary growth and for the provision of key nutrients for the developing embryo; 11-ketotestosterone (11-KT) produced by the Leydig cells is necessary for spermatogenesis to proceed; and 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) is the maturation inducing steroid in most fish species and is important for spermiation and milt hydration (Nagahama, 1994; Scott *et al.*, 2010; Schulz *et al.*, 2010).

The objectives of the present study were: 1) to examine morphological changes in the annual reproductive cycle of gametogenesis and spawning of male and female *H. dactylopterus*, and 2) to relate the individual levels of sex steroids (E2, 11-KT, 17,20β-P) in order to clarify aspects of the species reproduction and the potential role of hormones.

Materials and methods

Sampling

Helicolenus dactylopterus individuals were collected monthly on board bottom-trawlers operating along the western Atlantic (center region of Portugal 39° 21'N; 9°23'W; average depth 100 m) between September 2011 and October 2012, except April due to adverse weather conditions. Trawls lasted 1.30 to 3.00 h and time of day was recorded; five intervals of four hours between 0400 h and 0000 h were created to analyse data (there were no trawls between 0000h and 0400 h). 207 individuals (103 females and 104 males) (Table I) ranging in size from 13.9 cm to 37.5 cm in L_T were sampled.

Blood samples (~1 ml) were collected from the caudal vein with heparinised syringes. Plasma was separated by centrifugation (13 000 rpm for 5 min) and stored at -20° C until hormone analysis. Fish were tagged, stored in ice and taken to the laboratory where total length (L_T; 0.1 mm), total and eviscerated masses (W_T and W_E; 0.01 g), gonad mass (W_G; 0.01 g) and sex were recorded. The gonadosomatic index (I_G) ($I_G = W_G / W_E \times 100$) was determined. In imminent spawning females, the gelatinous matrix was extracted according to Sequeira *et al.* (2011), and 1 ml of each fresh sample was observed on a light microscope for embryo staging. All gonads were preserved in 10% buffered formaldehyde immediately after sampling and maturity stages were assigned by histological examination.

Histology

Fixed fragments of the mid ovarian and testicular region were dehydrated with ethanol, embedded in metacrylate, sectioned at 3-5 *μ*m, stained with toluidine blue and examined on a Leica DM 2000 light microscope with a Leica DFC 290 digital camera (http://www.leica-microsystems.com/home/).

Ovarian follicles and testicular germ cells were classified based on histological criteria (Grier, 1981; Wallace & Selman, 1981; West, 1990; Grier, 2012). Advanced oocytes and embryos in spawning females were staged according to Grier (2012) and Sequeira *et al.* (2015) as follows: secondary growth oocyte, full-grown step (SGfg), mature oocyte, eccentric germinal vesicle step (OMegv), mature oocyte, germinal vesicle migration step (OMgvm), mature oocyte, meiosis resumes step (OMmr), fertilized (f), early celled (ec), blastula (b).

The universal terminology proposed by Brown-Peterson *et al.* (2011) was used to describe gonadal maturity phases and subphases: developing (D), spawning capable (SC) (and subphase actively spawning (AS), for females), gestation (G) (and subphases fertilized (F), early celled (EC) and blastula (B), for females), regressing (RE) and regenerating (R).

Histological sections were searched for the presence of spermatozoa cysts (CSz) (prevalence of CSz = number of females with CSz/total number of females x 100) and interlamelar free spermatozoa.

Sex steroid analysis

Individual plasma samples (50 μl) were extracted with diethyl ether (2x4 ml), the solvent evaporated under nitrogen and the residue re-suspended in 1 ml 0.05 M phosphate buffer, pH 7.6. Steroid hormones were measured by radioimmunoassay as previously described: E_2 (Guerreiro *et al.*, 2002), 17,20β-P (Canario *et al.*, 1989) and 11-KT (Kime & Manning, 1982). The limits of detection were between 10 (E_2) and 100 (17,20β-P and 11-KT) pg/ml.

Statistical analysis

Data were log transformed whenever necessary to meet assumptions of analysis of variance (ANOVA). One-way ANOVA was used to compare sex steroids monthly, daily and between maturity stages, followed by Duncan's honestly significant difference post-hoc test. If assumptions of ANOVA were not met even after transformation nonparametric tests (Kruskal-Wallis (*H)* and Mann-Whitney *U*-test (*U*)) were used. The latter was performed to determine statistical significance in average monthly I_G for both sexes. Pearson correlations between steroid levels and I_G were estimated.

All values are expressed as the mean ± standard error of the mean (± S.E.) and statistical significance was inferred at $P < 0.05$. Statistica Software version 12 (http://www.statsoft.com/Products/STATISTICA-Features/Version-12) was used for all statistical analyses.

Results

166

167 Reproductive cycle

168

169 The females' I_G ranged from 0.33 ± 0.02% in June to 5.34 ± 0.48% in
170 February, while the males' I_G varied between 0.03 ± 0.02% in May and 0.52 ±
171 0.22% in September. In both, the I_G changed significantly throughout the year
172 (females: H = 69.866, d.f. = 10, P < 0.001; males: H = 47.375, d.f. = 10, P <
173 0.001) with higher I_G values in females between December and March ($P \leq$
174 0.034) and in males between July and December ($P \leq 0.043$) (Fig. 1(a)).

175 Females at the D stage were observed between September and January,
176 followed until May by predominance of females at late vitellogenesis, maturation
177 and gestation (SC and G) coinciding with the spawning period. Regarding G
178 females, 44% were at the F subphase, 33% at B subphase and 22% at the EC
179 subphase. Post-spawning females (Re and R) were sampled between May and
180 October (Fig. 1(b)). In males, the D stage lasted from May until August and
181 individuals in active spermatogenesis (SC) were observed between July and
182 February. Males with residual spermatozoa in regression and regenerating
183 condition (RE and R) were sampled between January and June (Fig. 1(c)).

184 Cysts of spermatozoa were observed at the centre of ovary around the
185 connective tissue at the base of the ovigerous lamellae and in the interlamellar
186 space in 84% of the females (Fig. 2). Their prevalence was over 75% between
187 October and May. Free sperm was also observed at the base of the ovigerous
188 lamellae and in the interlamellar space between August and January. All
189 females sampled in October and November were in this condition. Empty cysts

were observed between February and August coinciding with spawning and post-spawning periods.

Sex steroid hormone profile

Sex steroids were analysed in 163 individuals (88 females and 75 males) (Table I).

E_2 levels ranged between 0.12 ± 0.02 ng ml^{-1} in August and 0.97 ± 0.61 ng ml^{-1} in February. Significant monthly changes were observed (ANOVA, $F_{10,81}$ = 4.565, P < 0.001) with higher values between December and February compared to May through November (P ≤ 0.048) corresponding to active vitellogenesis during the pre-spawning/spawning period. In males, the lowest level of 11-KT was measured in March (0.15 ± 0.07 ng ml^{-1}) and the highest in October (0.94 ± 0.49 ng ml^{-1}). The monthly changes were significant (ANOVA, $F_{10,75}$ = 2.839, P = 0.006) with the period from May to December showing significantly higher values than February and March (P ≤ 0.035) (Fig. 3(a)). Both E_2 and 11-KT started to increase one month before I_G. In males I_G and 11-KT were significantly positively correlated (Pearson's correlation, r = 0.52, n = 73, P < 0.001) but there was no significant correlation between I_G and E_2 in females (Pearson's correlation, r = 0.21, n = 76, P = 0.075).

For most of the year 17,20β-P varied between 0.25 ± 1.15 ng ml^{-1} (November) and 1.17 ± 0.33 ng ml^{-1} (July) in females, and between 0.37 ± 0.09 ng ml^{-1} (August) and 0.88 ± 0.05 ng ml^{-1} (March) in males. However, in October 3 in 8 females had values ≥ 9.49 ng ml^{-1} and 2 in 5 males ≥ 3.82 ng ml^{-1} (Fig. 3(b)). Given the high variability, the average levels in October (4.48 ± 5.22 ng

215 ml^{-1} in females, and 2.45 ± 3.02 ng ml^{-1} in males) were not statistically
216 significant from other months. Nevertheless, plasma 17,20β-P was significantly
217 elevated in February compared to January, May and November (*U*, $P \leq 0.048$)
218 for females and in March compared to August for males (*U*, $P = 0.024$).

219 E_2 levels varied significantly between maturation phases (ANOVA, $F_{4,80,}$
220 $= 4.253$, $P = 0.004$) increasing from post spawning females (RE and R) to
221 vitellogenesis and spawning (SC and G) ($P < 0.045$). 11-KT did not vary
222 significantly although regressing males presented lower values (11-KT: ANOVA,
223 $F_{3,75,} = 2.227$, $P = 0.093$) ((Fig. 4(a)). Mean plasma 17,20β-P levels did not vary
224 significantly between maturity stages in both sexes (females: *U*, $P = 0.540$;
225 males: *U*, $P = 0.340$) (Fig. 4(b)).

226 The relative frequency of the f stage increased from 2000–0000 to 0800–
227 1200 hours period, of the ec stage from 0400–0800 to 1200–1600 hours period
228 and of the b stage from 0400–0800 to 2000–0000 hours period (Fig. 5(a)). In
229 the same individuals an apparent increase in E_2 and 17,20β-P levels was
230 observed in females sampled between 0020–0000 and 0800–1200 hours
231 period, and a decrease in the subsequent period between 1200–1600 hours
232 (Fig. 5(a)). Nevertheless, these variations were not statistically significant (E_2:
233 ANOVA, $F_{4,70} = 1.052$, $P = 0.387$; 17,20β-P: *U, P* = 0.942). In mature males, no
234 pattern was observed for 11-KT (ANOVA, $F_{4.69} = 0.538$, $P = 0.709$). 17,20β-P
235 was only analysed for part of the day, with elevated levels registered in the
236 1200–1600 hours period but without statistical significance (*U*, $P = 0.522$) (Fig.
237 5(b)) due to the high variability.

238 To help clarify the ovarian development pattern during the spawning
239 season, E_2 and 17,20β-P levels were related to the stage of the two most

advanced cohorts of oocytes of spawning females (Fig. 6). A similar apparent cyclic pattern for both sex steroids could be observed. E_2 and 17,20β-P levels were lower in ovaries containing ec embryos and OMegv oocytes and higher when oocytes were at maturation stage and included OMmr oocytes. Higher levels of E_2 were observed in ovaries with OMgvm and SGfg as the two most advanced cohorts (Fig. 6). Nevertheless these variations were not statistically significant (E_2: ANOVA, $F_{9,42,}$ = 0.290, P = 0.071; 17,20β-P: H = 3.875, d.f. = 9, P = 0.920).

Discussion

This study analysed sex steroid hormones and gonadal changes during the reproductive cycle of *H. dactylopterus*. While observations of the reproductive cycle to a large extent confirmed previous observations, the hormonal profile allowed a more detailed characterization of the timing of the underlying processes. Overall, changes in 11-KT and E_2 preceded $I_{G,}$ and 17,20β-P variations are suggested to be associated with oocyte maturation, ovulation and mating events.

The histological analysis and the I_G confirmed the spawning season of *H. dactylopterus* from January to May with a small delay compared to previous reports for the same geographic area (Sequeira *et al.*, 2012a; Sequeira *et al.*, 2015). Males had an out of phase and more extended active reproductive period than previously found (Muñoz *et al.*, 1999) lasting eight months. This result suggests the availability of females to be fertilized over a long period.

Indeed, 84% of the females collected had spermatozoa cysts in their ovaries with over 75% prevalence covering the period of active spermatogenesis in males and spawning in females. Moreover free spermatozoa were observed inside the ovaries for several months, suggesting the possibility of multiple copulations throughout the year. The presence of empty cysts inside the ovaries during the spawning and post-spawning periods is indicative of recent fertilization, as suggested by Vila *et al.* (2007).

From the analysis of relative frequency of the last oocyte/embryonic stage in spawning females, the increase of the relative frequency of the f stage from 2000–0000 to 0800–1200 hours period followed by the increase of the relative frequency of successive embryonic stages (ec and b) from 0400–0800 hours period onwards suggests that fertilization occurs from around dusk throughout the night. Based on the fact that blastula (b) is the most advance embryonic stage that can be observed in *H. dactylopterus* ovaries (Sequeira *et al.*, 2011; Sequeira *et al.*, 2015), females should spawn (release embryos) throughout the day reaching their maximum around dusk between 2000–0000 hours. A nocturnal preference for reproductive activity is not unusual within the same family: yellowtail rockfish *Sebastes flavidus* (Ayres, 1862) and rockfish *Sebastes inermis Cuvier, 1829*, releases their embryos during the night (Eldridge *et al.*, 1991) and copulate at dusk (Shinomiya & Ezaki, 1991), respectively.

The annual profile of E_2 and 11-KT confirmed the overall pattern of the reproductive cycle emphasizing that female and male are out of phase. A significant positive correlation between 11-KT and I_G supports the role of this steroid in active spermatogenesis (SC) (Nagahama, 1994; Barcellos *et al.*,

2002; Schulz *et al.*, 2010; Shimizu, 2014). Also, highest values of E_2 were present in vitellogenic females (SC and G). The delay of one month observed between the increase of E_2 and I_G was expected as a time interval mediates estrogen stimulation of vitellogenin production by the liver and the beginning of vitellogenesis (Takano *et al.*, 1991).

As for 17,20β-P, there was very little variation throughout the year, except in October when females were in RE/D maturation phase and males in SC. Some individuals of both sexes had high levels resulting in a peak of high variability. This variability is not unexpected since 17,20β-P is normally produced at higher levels during oocyte maturation, or in relation to sperm duct hydration and possibly mating behavior and copula (Liley *et al.*, 1986, Scott *et al.*, 2010) which are relatively short lived events (from minutes to hours). In support of this hypothesis is the fact that all females sampled in October and November had free spermatozoa in the ovaries suggesting recent fertilization. Therefore, the probability that all individuals do not go through the processes simultaneously resulted in high variation in hormone levels. A consequence of the increased variability is that larger samples would be required to achieve statistical significance.

For the same reason it was not possible to demonstrate significant daily hormone peaks. However, a daily rhythm is suggested for E_2 and 17,20β-P as both hormones had an apparent elevation between 2000–0000 and 0800–1200 hours period decreasing thereafter. These hormonal changes could be related to the secretion from two or more cohorts of oocytes under the control of gonadotrophin(s), one secreting E_2 and undergoing vitellogenesis and the other secreting 17,20β-P and undergoing oocyte maturation and ovulation. Short term

315 fluctuations in E_2 have been previous observed in multiple batch spawners
316 (Takano *et al.*, 1991; Dahle *et al.*, 2003 and references therein). *H.*
317 *dactylopterus* is an asynchronous multiple spawner species (87 batches per
318 season) with a short spawning interval (1.73 days) (Sequeira *et al.*, 2015)
319 requiring continuous vitellogenesis during the spawning period to recruit new
320 batches into maturity, which is consistent with the present results. Parallel
321 changes for the two hormones have been shown in goldfish *Carassius auratus*
322 (L. 1758) (Kobayashi *et al.*, 1987). Also, levels of E_2 and 17,20β-P in relation to
323 the two most advanced oocyte cohorts of spawning females is consistent with
324 their roles in oocyte development and maturation in *H. dactylopterus*. Lower
325 levels of both steroids were present in females with ec embryos-OMegv oocytes
326 with a subsequent apparent increase of hormonal levels as the two most
327 advanced cohorts continued to develop. The cohorts consisting of ec and b
328 embryos can be considered to originate a “between batches” period; the levels
329 of E_2 were lower because there are fewer vitellogenic oocytes producing it. As
330 development proceeds more and larger mature oocytes become available for
331 the next batch and E_2 levels increase. Lower peak concentrations of E_2 have
332 also been detected in group-synchronous Atlantic cod *Gadus morhua* L. 1758
333 (Dahle *et al.*, 2003) and turbot *Scophthalamus maximus* (L. 1758) (Howell &
334 Scott, 1989) and suggested to be characteristic of fishes with asynchronous
335 oocyte development (Matsuyama *et al.*, 1990) as is the case of *H.*
336 *dactylopterus*. The decrease of 17,20β-P after ovulation could indicate that it is
337 the maturation inducing steroid of *H. dactylopterus*, but further studies are
338 needed to confirm this hypothesis. In any case, 17,20β-P seems to play an

important role in both sexes, particularly in relation to sexual behavior, as levels seem to be higher during matting.

In males, few studies have analyzed daily cycles in sex steroids. However, in *H. dactylopterus*, 11-KT did not show any particular daily pattern, probably reflecting differences in the way meiosis proceeds in the two sexes.

In conclusion, the hormonal profile of sex steroids was associated with important events in the reproductive cycle of *H. dactylopterus*. This is particular relevant for a wild commercial species which lacks physiological data to interpret its peculiar reproductive strategy. Nevertheless, given the diversity of reproductive processes involved, further studies are required to understand the importance and function of gonadal steroids, namely those associated with mating behavior, gestation and continuous oocyte recruitment in this species.

Acknowledgments

The authors would like to thank P. Gomes and the crew of the trawler Sagittarius for providing the specimens used in this study. This study had the support of Fundação para a Ciência e Tecnologia (FCT), through the strategic project UID/MAR/04292/2013 granted to MARE, and the grants attributed to V. Sequeira (SFRH/BPD/70200/2010 and SFRH/BPD/108917/2015), A. Neves (SFRH/BD/92769/2013), and R. B. Paiva (SFRH/BD/80268/2011). The authors would also like to thank two anonymous reviewers for their comments and suggestions.

References

Barcellos, L., Wassermann, G. F., Scott, A. P., Woehl, V. M., Quevedo, R. M., Ittzés, I., Krieger, M. H. & Lulhier, F. (2002). Plasma steroid concentrations in relation to the reproductive cycle of cultured male *Rhamdia quelen*. *Journal of Fish Biology* **61**, 751–763.

Brown-Peterson, N. J., Wyanski, D. M., Saborido-Rey, F., Macewicz, B. J. & Lowerre-Barbieri, S. K. (2011). A standardized terminology for describing reproductive development in fishes. *Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science* **3**, 52–70.

Canario, A. V. M., Scott, A. P. & Flint, A. P. F. (1989). Radioimmunoassay investigations of 20β-hydroxylated steroids in maturing/ovulating female rainbow trout (*Salmo gairdneri*). *General and Comparative Endocrinology* **74**, 77–84.

Dahle, R., Taranger, G. L., Karlsen, Ø., Kjesbu, O. S. & Norberg, B. (2003). Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (*Gadus morhua* L.). *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **136**, 641–653.

Eldridge, M. B., Whipple, J. A., Bowers, M. J., Jarvis, B. M. & Gold, J. (1991). Reproductive performance of yellowtail rockfish, *Sebastes flavidus*. *Environmental Biology of Fishes* **30**, 91–102.

Grier, H. J. (1981). Cellular-organization of the testis and spermatogenesis in fishes. *American Zoologist* **21**, 345–-357.

389 Grier, H. J. (2012). Development of the follicle complex and oocyte staging in
390 red drum, *Sciaenops ocellatus* Linnaeus, 1776 (Perciformes, Sciaenidae).
391 *Journal of Morphology* **273**, 801–829.
392 Guerreiro, P. M., Fuentes, J., Canario, A. V. M. & Power, D. M. (2002). Calcium
393 balance in sea bream (*Sparus aurata*): the effect of oestradiol-17β. *Journal*
394 *of Endocrinology* **173**, 377–385.
395 Howell, B. R. & Scott, A. P. (1989). Ovulation cycles and postovulatory
396 deterioration of eggs of the turbot (*Scophthalmus maximus* L.). *Rapports*
397 *et procès-verbaux des réunions / Conseil permanent international pour*
398 *l'exploration de la mer* **191**, 21–26.
399 Hureau, J. C. & Litvinenko, N. I. (1986). Scorpaenidae. In *Fishes of the North-*
400 *eastern Atlantic and the Mediterranean* (Whitehead, P. J. P., Bauchot, M.
401 L., Hureau, J. C., Nielsen, J. & Tortonese, E., eds.), pp. 1211–1229. Paris:
402 FAO.
403 ICES (2012). Report of the Working Group on the Biology and Assessment of
404 Deep-sea Fisheries Resources (WGDEEP). *ICES CM 2012/ ACOM: 17*.
405 Kime, D. E. & Manning, N. J. (1982). Seasonal patterns of free and conjugated
406 androgens in the brown trout *Salmo trutta*. *General and Comparative*
407 *Endocrinology* **48**, 222–231.
408 Kobayashi, M., Aida, K. & Hanyu, I. (1987). Hormone changes during ovulation
409 and effects of steroid hormones on plasma gonadotropin levels and
410 ovulation in goldfish. *General and Comparative Endocrinology* **67**, 24–32.
411 Liley, N. R., Breton, B., Fostier, A. & Tan, E. S. P. (1986). Endocrine changes
412 associated with spawning behavior and social stimuli in a wild population

of rainbow trout (*Salmo gairdneri*): I. Males. *General and Comparative Endocrinology* **62**, 145–156.

Matsuyama, M., Adachi, S., Nagahama, Y., Maruyama, K. & Matsura, S. (1990). Diurnal rhythm of serum steroid hormone levels in the Japanese whiting, *Sillago japonica*, a daily-spawning teleost. *Fish Physiology and Biochemistry* **8**, 329–338.

Muñoz, M., Casadevall, M. & Bonet, S. (1999). Annual reproductive cycle of *Helicolenus dactylopterus dactylopterus* (Teleostei: Scorpaeniformes) with special reference to the ovaries sperm storage. *Journal of the Marine Biological Association of the United Kingdom* **79**, 521–529.

Muñoz, M., Dimitriadis, C., Casadevall, M., Vila, S., Delgado, E., Lloret, J. & Saborido-Rey, F. (2010). Female reproductive biology of the bluemouth *Helicolenus dactylopterus dactylopterus*: spawning and fecundity. *Journal of Fish Biology* **77**, 2423–2442.

Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. *International Journal of Developing Biology* **38**, 217–229.

Rinchard, J. & Kestemont, P. (2003). Liver changes related to oocyte growth in roach, a single spawner fish, and in bleak and white bream, two multiple spawner fish. *International Review of Hydrobiology* **88**, 68–76.

Schulz, R. W., de Franca, L. R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R. H. & Miura, T. (2010). Spermatogenesis in fish. *General and Comparative Endocrinology* **165**, 390–411.

Scott, A. P., Sumpter, J. P. & Stacey, N. (2010). The role of the maturation-inducing steroid, 17,20β-dihydroxypregn-4-en-3-one, in male fishes: a review. *Journal of Fish Biology* **76**, 183–224.

438 Sequeira, V., Vila, S., Neves, A., Rifes, P., Nunes, M. L., Vieira, A. R., Paiva, R.
439 B., Muñoz, M. & Gordo, L. S. (2011). The gelatinous matrix of the teleost
440 *Helicolenus dactylopterus dactylopterus* (Delaroche, 1809) in the context
441 of its reproductive strategy. *Marine Biology Research* **7**, 478–487.
442 Sequeira, V., Neves, A., Paiva, R. B., Lima, J., Vieira, A. R. & Gordo, L. S.
443 (2012a). Life history parameters as possible discrimators of bluemouth,
444 *Helicolenus dactylopterus* (Delaroche, 1809) populations in Portuguese
445 waters *Fisheries Research* **125-126**, 69–76.
446 Sequeira, V., Neves, A., Paiva, R. B., Vieira, A. R. & Gordo, L. S. (2012b). Is
447 the fecundity type of the zygoparous fish species *Helicolenus*
448 *dactylopterus* determinate or indeterminate? *Vie et Milieu - Life and*
449 *Environment* **62**, 37–42.
450 Sequeira, V., Neves, A., Vieira, A., Paiva, R., Canário, A. & Gordo, L. (2015).
451 Estimating fecundity in the zygoparous species *Helicolenus dactylopterus*
452 (Actinopterygii, Scorpaeniformes): adaptation of the gravimetric method.
453 *Hydrobiologia* **758**, 211–222.
454 Shimizu, A. (2014). Reproductive Physiology of the Mummichog *Fundulus*
455 *heteroclitus* - An Excellent Experimental Fish. *Aqua-BioScience*
456 *Monographs* **7**, 79–116.
457 Shinomiya, A. & Ezaki, O. (1991). Mating habits of the rockfish *Sebastes*
458 *inermis*. *Environmental Biology of Fishes* **30**, 15–22.
459 Takano, K., Takemura, A., Furihata, M., Nakanishi, T. & Hara, A. (1991). Annual
460 reproductive and spawning cycles of female *Sebastiscus marmoratus*.
461 *Environmental Biology of Fishes* **30**, 39–48.

462 Vila, S., Muñoz, M., Sabat, M. & Casadevall, M. (2007). Annual cycle of stored
463 spermatozoa within the ovaries of *Helicolenus dactylopterus dactylopterus*
464 (Teleostei, Scorpaenidae). *Journal of Fish Biology* **71**, 596–609.
465 Wallace, R. A. & Selman, K. (1981). Cellular and dynamic aspects of oocyte
466 growth in teleosts. *American Zoologist* **21**, 325–343.
467 West, G. (1990). Methods of Assessing Ovarian development in Fishes: a
468 Review. *Marine and Freshwater Research* **41**, 199–222.
469 Wourms, J. (1991). Reproduction and development of *Sebastes* in the context
470 of the evolution of piscine viviparity. *Environmental Biology of Fishes* **30**,
471 111–126.

Table I. Total number and total length range of *Helicolenus dactylopterus* females and males sampled by month and subject of sexual steroid analysis.

	Females				Males			
	n	L_T range (cm)	E_2	17,20β-P	*n*	L_T range (cm)	11-KT	17,20β-P
Jan	17	18.7 - 23.7	12	12	25	17.9 - 24.8	19	18
Feb	19	17.7 - 27.0	19	19	2	20.5 - 26.4	2	2
Mar	14	17.0 - 22.9	13	13	3	18.8 - 23.5	3	3
Apr	-	-	-	-	-	-	-	-
May	7	19.3 - 23.9	6	7	6	20.5 - 23.0	6	6
Jun	10	18.6 - 20.9	4	6	5	19.6 - 22.5	3	3
Jul	5	17.1 - 20.4	3	4	13	13.9 - 25.2	7	7
Aug	3	19.0 - 19.5	3	2	10	19.6 - 24.1	6	6
Sep	4	21.4 - 31.5	4		8	21.4 - 27.6	8	
Oct	8	20.4 - 31.9	6	8	5	20.0 - 23.7	5	5
Nov	3	21.6 - 29.3	2	2	12	21.2 - 37.5	5	5
Dec	13	18.6 - 28.6	8	8	15	19.2 - 25.2	11	11
Total	103	17.0 - 31.9	80	81	104	13.9 - 37.5	75	66

11-KT, 11-ketotestosterone; 17,20β-P, 17,20β-dihydroxypregn-4-en-3-one; E_2, estradiol-17β; L_T, total length; *n*, sample size

Figure captions

Fig. 1

Monthly variation of (a) gonadosomatic index (I_G) (mean ± S.E.) (○, females; ■, males) and relative frequency of reproductive phases (⊡, development; ■, gestation; □, regenerating; ⊠, regressing; ▣, spawning capable) in *Helicolenus dactylopterus* (b) females and (c) males.

Fig. 2

Histological sections of *Helicolenus dactylopterus* female and male gonads: (a) mature female in the gestation phase, early celled subphase showing oocytes in different stages and embryos in the early celled stage (sampled in January 2012); (b) mature male in the spawning capable phase showing sexual cells in different stages (sampled in September 2011); (c) mature female presenting spermatozoa inside a cyst and freely next to the ovarian lamellae (sampled in October 2011). CSz, spermatozoa cyst; ec, early celled embryo; FSz, free spermatozoa; gv, germinal vesicle; od, oil drop; OMegv, mature oocyte, eccentric germinal vesicle step; PGca, primary growth oocyte, cortical alveolar step; Sc, spermatocyte; Sg, spermatogonia; SGe, Secondary growth oocyte, early growth step; SGfg, secondary growth oocyte, full-grown step; SGl, Secondary growth oocyte, late growth step oocyte; St, spermatid; Sz, spermatozoa.

Fig. 3

Monthly changes of plasma sex steroids (mean ± S.E.) in *Helicolenus dactylopterus*: (a) estradiol-17β (○) and 11-ketotestosterone (■); (b) 17,20β-dihydroxypregn-4-en-3-one (○, females; ■, males).

Fig. 4

Steroid hormones concentrations according to *Helicolenus dactylopterus* reproductive maturity phase: (a) estradiol-17β (□) and 11-ketotestosterone (■), and (b) 17,20β-dihydroxypregn-4-en-3-one in females (□) and males (■). Bars represent mean ± S.E. * denotes a significant difference from all other maturity phases ($P < 0.05$). D, development; G, gestation; R, regenerating; RE, regressing; SC, spawning capable.

Fig. 5

Daily rhythm of (a) estradiol-17β (——) and 17,20β-dihydroxypregn-4-en-3-one (----) in association with female relative frequency of the last oocyte/embryonic stage (⊡, b, blastula; ▨, ec, early celled; ■, f, fertilized; □, OMegv, mature oocyte eccentric germinal vesicle step; ⊟, OMgvm, mature oocyte germinal vesicle migration step; ▣, OMmr, mature oocyte meiosis resumes step), and (b) 11-ketotestosterone (□) and 17,20β-dihydroxypregn-4-en-3-one (■) in males. Bars represent mean ± S.E.

Fig. 6

Rhythm of estradiol-17β (-□-) and 17,20β-dihydroxy-4-pregnen-3-one (-■-) according to the oocyte stage of the two most advanced cohorts present in ovaries of *Helicolenus dactylopterus* spawning females. Bars represent mean ±

S.E. 2nd LC, second last cohort; b, blastula; ec, early celled; f, fertilized; LC, last cohort; OMegv, mature oocyte, eccentric germinal vesicle step; OMgvm, mature oocyte germinal vesicle migration step; OMmr, mature oocyte meiosis resumes step; SGfg, secondary growth oocyte full-grown step.

Fig 1_R3

Click here to download Figure MS 16-097R3_Fig 1.tif

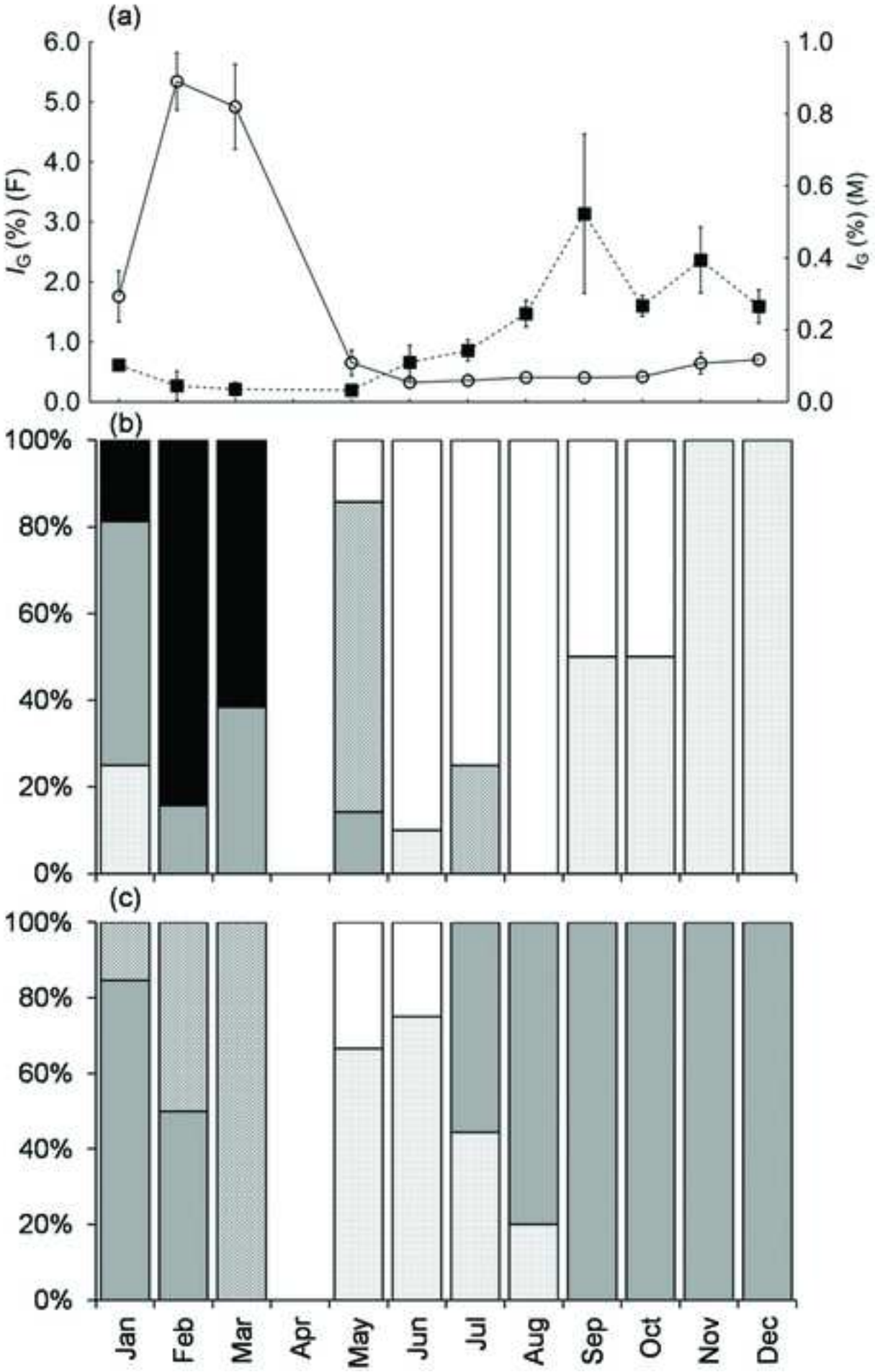


Fig 2_R3

Click here to download Figure MS 16-097R3_Fig 2.tif

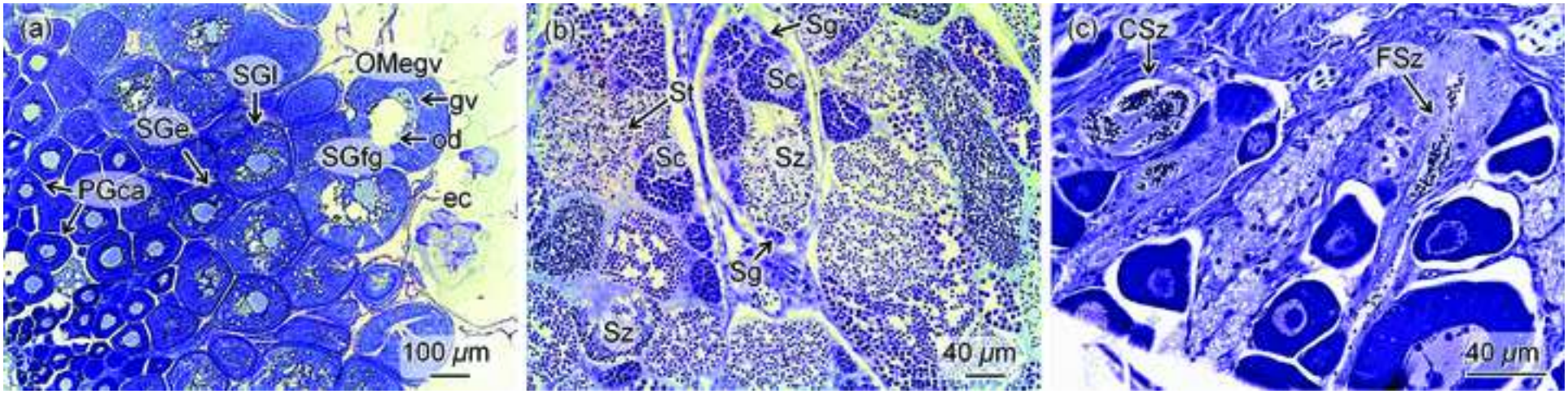
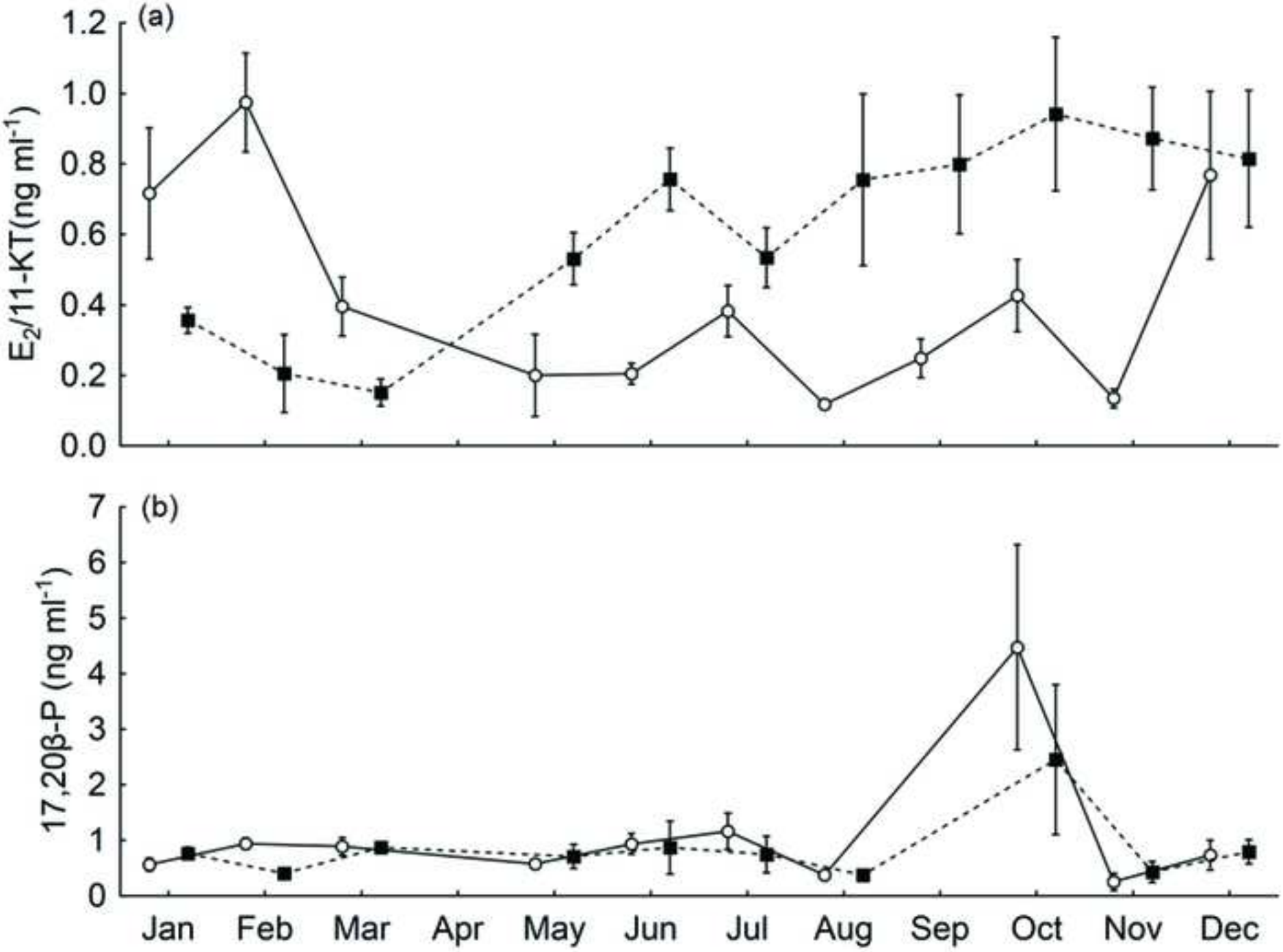


Fig 3_R3

Click here to download Figure MS 16-097R3_Fig 3.tif



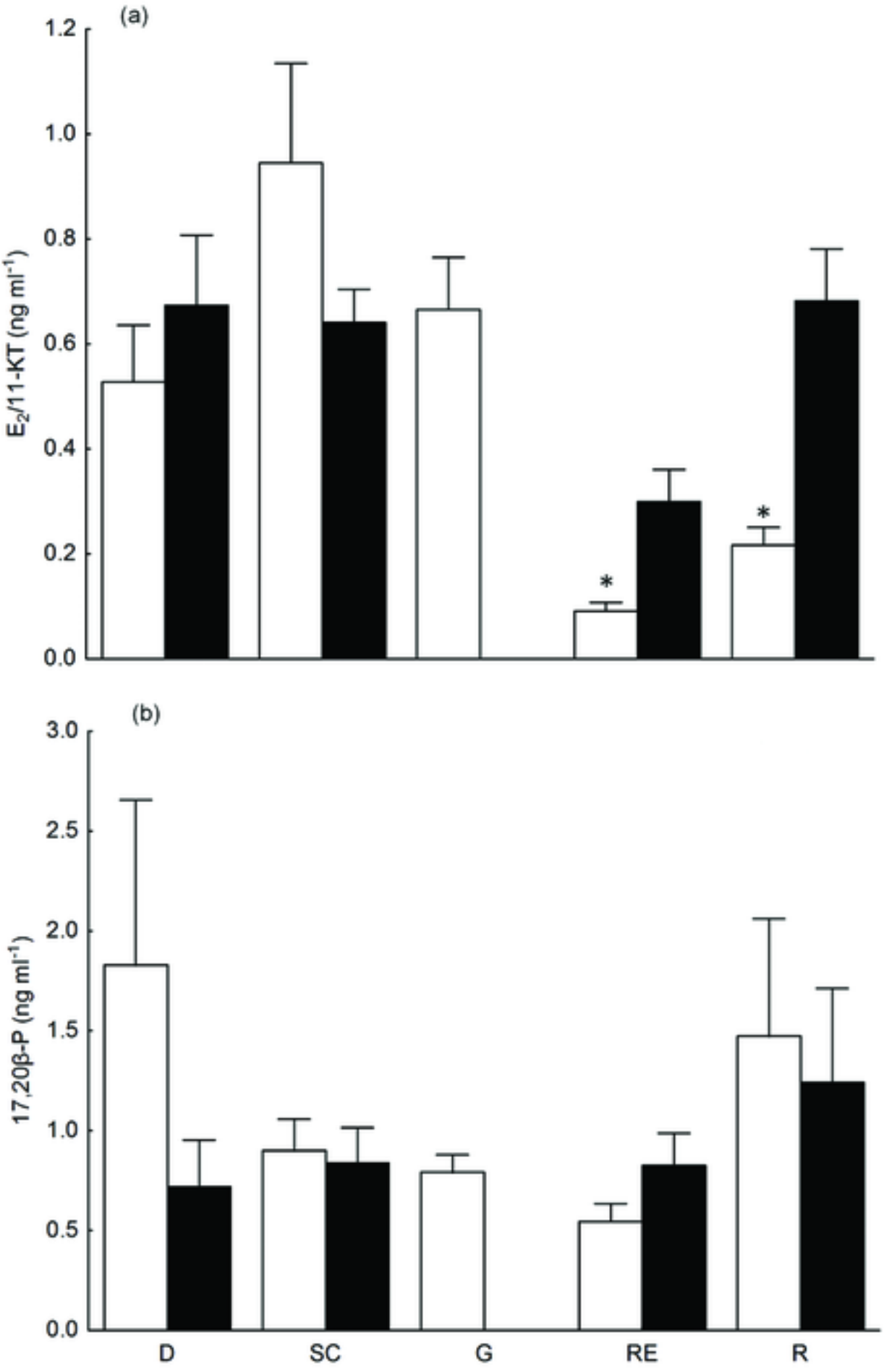


Fig 5_R3

Click here to download Figure MS 16-097R3_Fig 5.tif

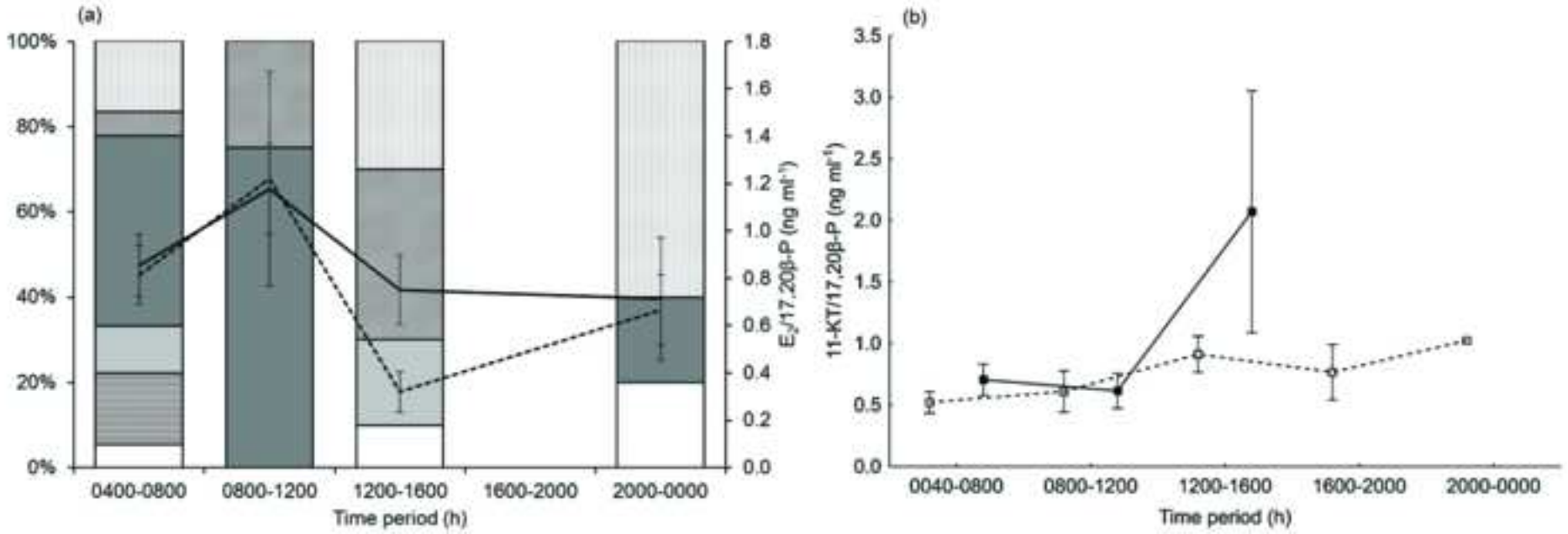


Fig 6_R3

Click here to download Figure MS 16-097R3_Fig 6.tif