Gonad plasticity and gametogenesis in the endangered Spanish toothcarp Aphanius iberus (Teleostei: Cyprinodontidae)

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A B S T R A C T

The Spanish toothcarp Aphanius iberus is an endangered species which inhabits small rivers, creeks, salt marshes and marine salt pans in the Mediterranean coast of Spain. No differences in weights were observed among females or males taken from different environments. Analyses of the morphology of the gonads and the gametogenesis were performed in fish taken from different environments by comparing gamete development in females and in males and gonadal cell proliferation in the testis. A high degree of plasticity was observed in the gonad morphology of A. iberus. Females possess two ovaries which show non-restricted oogenesis with all germ cell stages within the same ovigerous lamellae, while males possess gonads without any clear division with the typical restricted pattern observed in cyprinodontid fish. Some females and males showed asymmetrically developed gonads. Proliferation of germ cells in testis is plastic in the gonad morphology of A. iberus was induced to spawn in different environmental conditions. Aphanius iberus, an eurythermic and euryhaline cyprinodontid fish, is an endemic fish which inhabits a wide range of environments along the SE Mediterranean coast of Spain, such as the brackish waters of salt marshes, coastal lagoons and river-mouths (García-Berthou and Moreno-Amich, 1992; Oliva-Paterna et al., 2006). The species is catalogued as endangered (EN A2ce; IUCN, 2007) and is of the few Iberian fish species protected by national and international laws (Elvira, 1995; Doadrio, 2002). However, due to the introduction of exotic fish (Elvira and Almodóvar, 2001; Caialoa and De Sostoa, 2005) and destructive human impacts on its natural habitats (Planelles, 1999), the geographical range of A. iberus is continuing to shrink. Nowadays it is found mainly in small fragmented saline localities (Doadrio, 2002; Araguas et al., 2007). Many previous studies have focused on the ecology of A. iberus and some aspects of its life history (Vargas and De Sostoa, 1997; Planelles, 1999). A. iberus show an extraordinary phenotypic plasticity and is well adapted to fluctuating environments (Oliva-Paterna et al., 2006; Alcázar et al., 2008).

1. Introduction

There is an urgent need to increase our knowledge of the life history and eco-physiological traits of fish species under threat, as a necessary tool for management and conservation programmes to be undertaken (for review see Wootton et al., 2000). Knowledge of reproduction is one of the bases of fish biology, and, therefore, for management and conservation (Meffe and Carroll, 1997). Cyprinodontiforms comprise species adapted to extreme conditions, such as high ranges of temperature and salinity (e.g. Aphanius spp.; García-Berthou and Moreno-Amich, 1999), tidal salt marshes (e.g. Fundulus heteroclitus; Kneib, 1986) or temporal ponds (e.g. Austrolebias charrua; Arezo et al., 2007). Survival in such diverse and extreme conditions implies a high degree of plasticity in the life cycle and general physiology of these small teleosts. It has previously been shown that the general metabolism of fish, especially in cyprinodontids, is affected by several factors including salinity (Jordan et al., 1993; Plaut, 2000; Wuenschel et al., 2004). Moreover, analyses of ammonia excretion rates in this species demonstrate a correlation between salinity and ammonia excretion, although it also depends on body size and reproduction stage (Oliva-Paterna et al., 2007). It might therefore be expected that salinity would interfere in reproduction. However, Oltra and Todolí (2000) observed no differences between the numbers of viable embryos when A. iberus was induced to spawn in different environmental conditions.

Aphanius iberus, an eurythermic and euryhaline cyprinodontid fish, is an endemic fish which inhabits a wide range of environments along the SE Mediterranean coast of Spain, such as the brackish waters of salt marshes, coastal lagoons and river-mouths (García-Berthou and Moreno-Amich, 1992; Oliva-Paterna et al., 2006). The species is catalogued as endangered (EN A2ce; IUCN, 2007) and is of the few Iberian fish species protected by national and international laws (Elvira, 1995; Doadrio, 2002). However, due to the introduction of exotic fish (Elvira and Almodóvar, 2001; Caialoa and De Sostoa, 2005) and destructive human impacts on its natural habitats (Planelles, 1999), the geographical range of A. iberus continues to shrink. Nowadays it is found mainly in small fragmented saline localities (Doadrio, 2002; Araguas et al., 2007). Many previous studies have focused on the ecology of A. iberus and some aspects of its life history (Vargas and De Sostoa, 1997; Planelles, 1999). A. iberus show an extraordinary phenotypic plasticity and is well adapted to fluctuating environments (Oliva-Paterna et al., 2006; Alcázar et al., 2008).
However, relatively little is known about the reproduction of A. iberus and, to the best of our knowledge, no information about its gametogenesis exists. Macroscopic studies (gonadosomatic index, GSI) were performed in A. iberus from the Ebro River (Vargas and De Sostoa, 1997) whose principal reproductive season is from May to August. The population in the Guadalquivir basin (Fernández-Delgado et al., 1988), which has recently been described as a separate species (A. baeticus, Doadrio et al., 2002), presents two peaks in the GSI, the major one occurring in spring (April–May) and a polymodal size-frequency distribution of oocytes during the rest of the spawning period. This oogenesis process reflects seasonally synchronous group ovary maturation (Fernández-Delgado et al., 1988). In the province of Murcia, the reproduction period occurs in summer (July–August) when animals are at the end of their gametogenesis processes and beginning to spawn (Oliva-Paterna, 2006).

The aim of the present work was to provide the first description of gonadal morphology, oogenesis and spermatogenesis in populations from habitats which principally differed in the degree of salinity. More specifically, we carried out a microscopic study of the gonads and an immunocytochemical method to detect proliferating cells. Histological method which has proved successful in determining the reproductive pattern of teleost fish (Parenti and Grier, 2004). In this way, we hoped to establish the influence of salinity (freshwater and high saline water) on gametogenesis.

2. Materials and methods

2.1. Fish

Twenty-eight wild mature specimens of A. iberus (Valenciennes, 1846) were captured in June and July (2006), the main reproductive season (Oliva-Paterna, 2006), using minnow-traps from three sampling sites in the south of Spain with the following salinity ranges: Chicamino (freshwater creek, 0–2‰) n = 10; La Hita (salt marsh, 35–40‰) n = 7 and Marchamalo (marine salt pans, 65–70‰) n = 11. All specimens belong to the same geographical and genetic group (sensu, Doadrio et al., 1996). Fish from all sites were kept in cooled aerated tanks and transported to the laboratory (University of Murcia), where they were anaesthetised with clove oil essence (Eugenia caryophyllata, Mon Deconatur S.L., Barcelona) at 20 ppm in fresh or saltwater according to García-Gómez et al. (2002), depending on the origin of the specimens. The animals were then weighed and decapitated. The trunks of the specimens (including the whole peritoneal cavity) were removed and processed for light microscopy, as described below, in order to analyse the position of the gonads and to describe the gametogenesis process. For cell proliferation analysis, some specimens were anaesthetised for 1 min and injected intraperitoneally with 50 μg·g⁻¹ body weight of 5-bromo-2′-deoxyuridine (BrdU, Sigma, Saint Louis, USA) 2 h before sampling, maintaining the specimens in natural photoperiod and temperature conditions. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Light microscopy

The samples were fixed for 24 h in Bouin’s solution or 4% paraformaldehyde solution, embedded in paraffin (Paraplast Plus; Sherwood Medical, Athy, Ireland), and serially sectioned (horizontal plane) at 5 μm. Some sections were stained with haematoxylin and eosin to determine the reproductive stage of each specimen and the degree of development of the gonads based on previous gametogenesis classifications in teleost fish (Jalabert, 2005; Meijide et al., 2005). Slides were examined with an Axioslab (Zeiss) light microscope. In order to determine oocyte growth, oocyte cell diameters were drawn manually and measured by image analysis using an Axioslab (Zeiss) light microscope, a CoolSNAP digital camera (RS Photometrics) and SPOT Advance 3.3 software (Diagnostic Instruments, Inc.).

2.3. Immunohistochemical staining

An indirect immunological method was performed to determine cell proliferation, according to Chaves-Pozo et al. (2005). Briefly, 4% paraformaldehyde-fixed sample sections from BrdU-treated specimens were incubated for 40 min in peroxidase-quenching solution [H₂O₂ (commercial solution at 30%, Panreac, Barcelona, Spain) in methanol, 1:9], for 30 min in 1% acidic acid at 60 °C and finally for 30 min in 5% bovine serum albumin (BSA, Sigma, Saint Louis, USA) in phosphate buffered saline (PBS, pH 7.4). Subsequently, they were incubated with a monoclonal antibody anti-BrdU (Becton Dickinson, San Jose, USA) at the optimal dilution of 1:100 in 1% BSA in PBS for 2 h at room temperature. Subsequently, sections were washed in PBS, and incubated with a peroxidase-conjugated rabbit anti-mouse IgG (whole molecule) at the optimal dilution of 1:100 in 1% BSA in PBS for 1 h at room temperature. The sections were then washed twice in PBS and in 0.05 M Tris-HCl buffer (pH 7.6) for 5 min each. The peroxidase activity was revealed by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Steinheim, Switzerland) in Tris–HCl buffer (pH 7.6) containing 0.05% H₂O₂ for 15 min at room temperature. The sections were slightly counterstained with Meyer’s haematoxylin and the specificity of the reactions was determined by omitting the first antiserum and by using tissue sections from fish that had not been injected with BrdU.

2.4. Measurements and statistical analyses

Total lengths (±1 mm) and weights (±0.1 mg) from fish between the three sampling sites (Table 1) and oocyte diameters until and including stage V (see Table 2) of oogenesis were recorded. Only oocytes in which the cut was at the equator of the cell and the nucleus was clearly visible at the centre of the oocytes were taken for this analysis. Comparison of fish sizes and oocyte diameters was performed using analysis of variance (ANOVA). The homogeneity of variance was previously checked and all the analyses were performed using the SPSS v. 16.0 (SPSS Inc., Chicago) statistical package. Significant differences were recorded at p < 0.05.

3. Results

No differences in weight were observed among females or males taken from the three different environments. However, standard lengths in females from Chicamino River (freshwater) were significantly higher compared than of those from the Marchamalo and La Hita (saltwater) populations (ANOVA; F(2,9) = 4.472; p < 0.05, Table 1).

3.1. Plasticity of the gonads

Horizontal sections of the whole and inviolate peritoneal cavity were performed from the trunk of the animals (Fig. 1). In general, the kidneys were found in the anterior and dorsal parts of the cavities (extra-peritoneal), the liver and gut covering the anterior parts of the peritoneal cavities, and the central parts were occupied by the gonads and gut (Fig. 1e, female and Fig. 1f, male). In the posterior parts, the dorsal and distal parts were covered by the gonads,
Table 1
Mean lengths (mm) and weights (g) of *Aphanius iberus* collected from three different locations: a freshwater creek, Chícamo (*n* = 10), an evaporation salt pan, Marchamalo (*n* = 11) and a salt marsh, La Hita (*n* = 7). ANOVA tests were employed. *F*-statistics (*F*), degrees of freedom (d.f.) and *p*-values (*p*) are presented. CH (Chícamo River), MCH (Marchamalo) and LH (La Hita).

<table>
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<tr>
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<th>Standard length (mm)</th>
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Table 2
Cell number (*n*) and diameter (μm, mean ± standard deviation, SD) of each oogenesis stage from 12 *Aphanius iberus* females collected in the three different localities (Chícamo, Marchamalo and La Hita) and classification of oogenesis stages in *A. iberus*. I, oogonia; II, pre-vitellogenic oocytes; III, early vitellogenic oocytes; IV, late vitellogenic oocytes; V, post-vitellogenic oocytes; VI, oocytes in maturation and VII, mature or hydrated oocytes. GVBD (germinal vesicle breakdown).

<table>
<thead>
<tr>
<th>Stages</th>
<th>Chícamo</th>
<th>Cell diameter μm ± SD (<em>n</em>)</th>
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<th>La Hita</th>
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<tr>
<td>I</td>
<td>34 ± 17 (67)</td>
<td>25 ± 7 (16)</td>
<td>31 ± 12 (13)</td>
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<td>II</td>
<td>70 ± 24 (796)</td>
<td>63 ± 22 (199)</td>
<td>80 ± 29 (89)</td>
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<tr>
<td>III</td>
<td>143 ± 35 (22)</td>
<td>135 ± 21 (17)</td>
<td>158 ± 23 (12)</td>
<td></td>
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<tr>
<td>IV</td>
<td>164 ± 32 (32)</td>
<td>185 ± 33 (22)</td>
<td>240 ± 57 (11)</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>334 ± 87 (30)</td>
<td>330 ± 77 (26)</td>
<td>398 ± 78 (19)</td>
<td></td>
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<tr>
<td>VI</td>
<td>574 ± 130 (5)</td>
<td>421 ± 92 (17)</td>
<td>445 ± 110 (9)</td>
<td></td>
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<tr>
<td>VII</td>
<td>669 (1)</td>
<td>609 ± 109 (4)</td>
<td>614 ± 80 (3)</td>
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</table>

Slightly basophilic cytoplasm and large spherical nucleus with marked nucleolus.

Slightly basophilic cytoplasm and nucleolus appeared in the nucleus.

Their nucleus contains multiple nucleoli closed to the nuclear envelope. Yolk vesicles appear in the cytoplasm, with presence of first lipid droplets.

Secondary yolk vesicles appear in the cytoplasm and more lipid droplets are visible.

Central nucleus or germinal vesicle and almost all the cytoplasm covered by lipid droplets and yolk vesicles.

Migration of the nucleus or germinal vesicle to the periphery of the cytoplasm. Proteolysis of yolk protein, coalescence of lipid droplets and hydration process.

Homogenous cytoplasm with some lipid droplets and peripheral cortical alveoli. GVBD occurs.

while the most ventral parts, until the anus, were occupied by the gut.

The gonads were located dorsally to the gut and sometimes covering a considerable space in the peritoneal cavity (Figs. 1 and 2). In females both ovaries showed a similar development and do not appear at lateral positions. The ovaries were in the central part of the cavity and separated by a distinguishable layer of connective tissue (Fig. 2a). Both ovaries are caudally connected in a common oviduct. In males, testes were located centrally with no clear division (Fig. 2b). However, some females (Fig. 2c) and males (Fig. 2d) showed asymmetrically developed gonads, where the right ovary and testis, respectively, were considerably larger than their counterparts on the left side, which could even be very small and difficult to distinguish and separate from the bigger one.

No differences in weight were observed among females or males taken from the three different environments. However, standard lengths in females from Chícamo River (freshwater) were significantly higher compared than of those from the Marchamalo and La Hita (saltwater) populations (ANOVA; *F*(2,9) = 4.472; *p* < 0.05, Table 1).
3.2. Oogenesis

The ovary of *A. iberus* was formed by folds of the germinal epithelium, named ovigerous lamellae, which surround an ovarian lumen or cavity (Fig. 2a), in which all germ cell stages of oogenesis were present. These ovigerous lamellae contained nests of oogonia, oocytes and follicles in various stages of development and growth, embedded in a smooth connective tissue and delimited by epithelial cells (Fig. 3). In order to define the germinal cell populations, the morphology and diameters of the cells were taken into account. Table 2 shows the oogenesis classification and the average of oocyte diameters at each stage. The earliest step in oogenesis is represented by oogonia (stage I), which were located throughout the ovary and appeared among more developed oocytes (Fig. 3a and b). Oogonia develop into perinucleolar or pre-vitellogenic oocytes (stage II). At the end of this stage, pre-vitellogenic oocytes were surrounded by a continuous follicular cell layer (the granulose layer) formed by flattened cells (Fig. 3a). Early vitellogenic oocytes (stage III) were characterized by a weaker basophilic cytoplasm, with yolk granules randomly distributed and first lipid droplets forming a ring (Fig. 3a). Surrounding the late vitellogenic oocytes (stage IV), the second follicular cell layer (the theca layer) became apparent. The cytoplasm of late vitellogenic oocytes was filled up with lipid vacuoles and yolk granules (Fig. 3b). Post-vitellogenic oocytes (stage V) (Fig. 3b) still presented the germinal vesicle or nucleus in a central position, and all the cytoplasm was homogenously covered by yolk granules and lipid droplets. Once the meiosis process, which had stopped in prophase I, reassembled, the maturation stage (stage VI) involved a rearrangement of the cytoplasm and migration of the nucleus (Fig. 3c). Finally, mature (hydrated) oocytes (stage VII) are ready to be ovulated and be released for external fertilization. In this stage germinal vesicle or nucleus break-down (GVBD). Degenerated oocytes (atresic) occasionally occur and had an irregular shape and showed a highly condensed and basophilic nucleus surrounded by cytoplasm filled with acidophilic and some basophilic granules (data not shown). No indicators of masculinisation (presence of testis in the ovaries) were observed. Furthermore, oocytes in stage II were the most abundant cells in the gonad of all the fish analysed (Table 2). Because it was expected that salinity could influence oocyte diameter, the diameters of oocytes from the three different environments were compared. No differences were observed
3.3. Oogenesis

In oogenesis for the different salinity conditions, \( p > 0.05 \) (data not shown).

3.3. Spermatogenesis

*A. iberus* males developed an asynchronous spermatogenesis characterized by the presence of spermatogonia stem cells and cysts of all germ cell types in the tubules of the testis (Fig. 2b). Thus, the tubules were formed by spermatogonia stem cells, and cysts of primary and secondary spermatogonia and spermatocytes (Fig. 4a and b). As spermatogenesis proceeded, the amount of free spermatozoa in the tubular lumen increased, at the same time as the cyst variability decreased. Both fresh- and saltwater specimens showed a similar degree of maturation and were in middle and late spermatogenesis, when most of the tubules are formed by spermatocytes and spermatid cysts and high amount of free spermatozoa are present in the lumen of the tubules and in the efferent duct (Figs. 2b and 4d). Surrounded by germinal epithelia, cysts of spermatogonia are observed in the testicular periphery, and successive stage of the spermatogenesis occurs in the tubules in direction to the centre of the testis (Fig. 2b).

3.4. Cell proliferation

In males, the cysts located at the periphery of the gonad, which correspond to primary and secondary spermatogonia cysts, proliferated during the spawning period (Fig. 5). The BrdU positive cells were localized at the periphery of the testis, corroborating the restricted spermatogonia-type testis. No immunostaining was observed in the control sections (Fig. 5d). In the case of females, they did not show clear immuno-positive reactions, indicating a low or absence of proliferative rate in the ovaries during the spawning period (data not shown).

4. Discussion

Histological examination showed a high degree of plasticity in the morphology and positioning of the gonads in the peritoneal cavity of *A. iberus*. The present data demonstrate that *A. iberus* possess two ovaries, both bound in the central part of their connective tissue, although each ovary has an independent lumen. This morphology differs from the regular elongated ovaries of other cyprinodontiforms, such as *A. charrua* (*Arezo* et al., 2007).
The oogenesis process appears to be non-restricted. All oocyte stages appeared homogeneously distributed along the ovary. As expected, pre-vitellogenic oocytes were the most abundant stage found, followed by vitellogenic oocytes, which increase in size due to the accumulation and storage of lipids and proteins (vitellogenins), carbohydrates and phosphate groups. Very few oocytes were found undergoing maturation or in a mature stage, indicating that ovulation and spawning occurs frequently but in few oocytes each time. However, post-ovulatry follicles were rarely or not observed, suggesting a rapid re-absorption of follicle tissues.

Final oocyte maturation involves proteolysis of the yolk and clarification, GVBD and a rapid increase in oocyte volume due to water entering into the oocytes, after osmotic disequilibrium generated by the proteolysis (Jalabert, 2005). This process differs according to whether fish produce marine pelagic, marine demersal or freshwater eggs. It has been suspected that the different habitats inhabited by A. iberus might influence the maturation of the oocytes, resulting in a variation in oocyte diameter. Pelagic marine eggs show a greater increase in oocyte size, while freshwater eggs such as those from rainbow trout show a small increase in size (Jalabert, 2005). However, the results did not show any significant difference in oogenesis, suggesting that salinity does not modify the growth of oocytes during the maturation process in A. iberus. García-Marín et al. (1990) described a genetic variation among the Mediterranean populations of A. iberus and nuclear (allozyme) and mitochondrial (cytochrome b) DNA revealed high genetic divergence in A. iberus from the Iberian Peninsula (Perdices et al., 2001). However, a unique geographical and genetic group of A. iberus was previously described from the three sampling sites (Doadrio et al., 1996). More studies at molecular level are being carried out in the same population analysed here, as a complement to this morphological approach.

In several males, a large and centrally positioned testis was observed with lateral extension to both sides and no morphological evidence for the presence of two clearly separated testes was obtained. However, in other specimens, the results suggested asymmetric testes and the testis shape varied among individuals indicating extreme plasticity. Polarization of spermatogenesis was not unexpected. The confinement of spermatogonia to the distal end of the lobules in restricted lobular patterned testis is an arrangement typically found in cyprinodontid fishes (Grier, 1981; Parenti and Grier, 2004). The localization of germ cells in the periphery of the testis has also been observed in other cyprinodontiforms such as Fundulus heteroclitus (Selman and Wallace, 1986). Cyprinodontiforms belong to Atherinomorpha, the only teleost group with spermatogonia cells confined to the most distal borders of the testis.
Fig. 5. Proliferative cells in the testis of *Aphanius iberus*. Germinal cells showing immuno-reaction to anti-BrdU (counterstained with haematoxiline). The reaction are specifically located in the spermatogonia area in the edge of the gonads (pictures a to c). (d) Control section without first antibody. SPG, spermatogonia; SPC, spermatocytes. Bars indicate: 100 μm (a and d); and 50 μm (b and c).

(Parenti and Grier, 2004). Moreover, the same authors pointed to the phylogenetic relevance of the restricted spermatogonial pattern as a unique derived character for Atherinomorpha. No differences were observed in the spermatogenesis process of males from the three different environments.

The presence of cell proliferation in fish gonads is not surprising. In contrast to mammals’ gonads, several works described the proliferation of cells taking place in gonads during fish gametogenesis (Grier, 2000; Chaves-Pozo et al., 2005). *A. iberus* showed a clear cell proliferation in the testis, a process that basically occurs in the periphery and was restricted to spermatogonia germ cell type.

Finally, *A. iberus* specimens were for the first time successfully anaesthetised with clove oil essence (*Eugenia caryophyllata*) an extract used for marine teleosts (García-Gómez et al., 2002) with effective anaesthetic properties and very low cost. The animals showed excellent recovery, indicating the potential use of this essential oil for anaesthetising cyprinodontid fishes.

In conclusion, *A. iberus* showed large morphological diversity of the gonads, with females and males presenting central or unequal lateral gonads. The gonads showed wide phenotypic plasticity, as expected for animals adapted to extreme conditions including high and low salinity levels. Interestingly, the final process of gametogenesis was quite similar in specimens inhabiting very different salinity conditions and no differences were observed during the oogenesis process. Decreases in population size of this endangered species in Chícamo River and La Hita saltmarsh do not seem to be a consequence of a dysfunction at gonadal level. No feminization or masculinisation of the gonads was observed. More studies should be carried out in order to understand the reproductive mechanisms of this species, the advantage of such gonad plasticity and its adaptation to extreme environments.

Acknowledgments

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References


