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Parents' presence affects embryos' development in Salaria fluviatilis (Asso 1801), a fish with parental care

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Abstract:	In fishes, the parents' presence improves embryos' survival through parental care but it is also associated with some disadvantages such as clutch cannibalism and male physical condition loss. Captive breeding of the river blenny Salaria fluviatilis might improve if these disadvantages were avoided by artificially replacing parental care benefits in the lab. Before accepting this procedure, it should be studied whether embryo development is dependent or not on any other unknown effect related to the parents' presence. In this study, the ontogenetic sequence and some morphological structures (standard length, head height, jaw length and yolk-sac volume) from embryos reared both in the presence and in the absence of the parents were compared. In the parents' absence treatment, well-developed embryos were obtained, but a smaller size of the yolk-sac, a greater head height and a greater jaw length than in the parents' presence treatment were found at day 11 after oviposition.
Keywords:	Captive breeding; embryonic growth; freshwater blenny; ontogeny
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Joris M Koene, Ph.D.
Editor in Chief
Animal Biology

Dear Dr. Koene,

Attached to this cover letter you will find the manuscript of the article "**Parents' presence affects embryos' development in *Salaria fluviatilis* (Asso 1801), a fish with parental care" (AB-D-14-00042R1)"**, which we submit as a revision.

Thank you for the opportunity to make a few small changes in our manuscript before acceptance in your Journal. We are grateful to both Reviewers. Their indications have been very useful and we are satisfied with the final version of the MS. In the "Response to Reviews" document you will find our answer to each one of the comments. As suggested we provide the figures in high resolution and in TIFF format. We also supply a monochrome version for Figure 3 for the printed version. Moreover, some small format mistakes and improvements have been indicated and corrected both in the MS and in the tables. Finally, we have followed the recommendations and we have answered one last doubt about methodology from Reviewer 2.

We hope that the corrected MS solves all this small concerns. In case there is still any question, please do not hesitate to contact us for more information.

Looking forward to hearing from you soon,

Yours sincerely

Dr. Dolors Vinyoles

RESPONSE TO REVIEWERS COMMENTS

Article: "Parents' presence affects embryos' development in *Salaria fluviatilis* (Asso 1801), a fish with parental care" (AB-D-14-00042R1)

General comment: The numbers of text lines cited (in the replies) in all the comments below are in reference to the new MS version. In order to facilitate the revision we have also included a note for each correction in the MS which contains the correspondent Reviewer comment.

RESPONSE TO EDITOR (INDICATED IN YELLOW IN THE MS)

1	Editor *Please upload your figures in high resolution, and preferably in TIFF (or JPEG) format.* Answer Followed suggestion. We also supply a monochrome version for Figure 3 for the printed version.
2	Editor *To avoid delays in the further processing of your paper, please make sure that you have adhered to all the formatting instructions as found in the journal's Instructions for Authors. For example, pay attention to the fact that there are spaces around a = and > symbol.* Answer Followed suggestion. Improvements in the MS: The following small format changes have been directly accepted (and they are not highlighted) in the new version of the MS: - We added spaces associated to = and > symbols. - We changed "a.m" and "p.m" to "a.m." and "p.m." - We changed "(fig.2, fig.3)" to "(figs. 2, 3)". The following changes affecting the tables are indicated: - We corrected the two tables' headings and legends. The content is the same than in the previous version but we have reordered it to adjust to the Journal format (which requires that Abbreviations and symbols are listed below the table). -We have shortened the abstract to adjust it to the 150 words limit.

RESPONSE TO REVIEWER 2 (INDICATED IN GREEN IN THE MS)

1	Reviewer *Line222. "The measuring of" should be "the measurement" or "measuring"* Answer Followed suggestion, see line 222 page 10.
2	Reviewer *Were all larvae measured at Day 13 or just whenever they were hatched? The hatch time may vary for each embryo and may affect the results.* Answer Larvae from Experiment 2 were all measured on the same day (day 11 after oviposition). For this reason hatching was artificially induced that day. This protocol guaranteed that hatch time was the same for all the embryos. It is on Experiment 1 (observational) where larvae hatched naturally at day 13 of development. Improvements in the MS: We decided to clarify this in the MS by completing the sentence in lines 208-209 page 9: *"(thus guaranteeing that the hatch time was the same for all the embryos)".*
3	Reviewer *Figure 3. A scale bar should be included into the figures.* Answer Followed suggestion. A scale bar was included in this figure.

Parents' presence affects embryos' development in *Salaria fluviatilis* (Asso 1801), a fish with parental care

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Abstract

In fishes, the parents' presence improves embryos' survival through parental care but it is also associated with some disadvantages such as clutch cannibalism and male physical condition loss. Captive breeding of the river blenny *Salaria fluviatilis* might improve if these disadvantages were avoided by artificially replacing parental care benefits in the lab. Before accepting this procedure, it should be studied whether embryo development is dependent or not on any other unknown effect related to the parents' presence. In this study, the ontogenetic sequence and some morphological structures (standard length, head height, jaw length and yolk-sac volume) from embryos reared both in the presence and in the absence of the parents were compared. In the parents' absence treatment, well-developed embryos were obtained, but a smaller size of the yolk-sac, a greater head height and a greater jaw length than in the parents' presence treatment were found at day 11 after oviposition.

Keywords

Captive breeding; embryonic growth; freshwater blenny; ontogeny

Introduction

Although fish parental care is associated with an increase in the embryos' survival (Clutton-Brock, 1991) it entails some disadvantages. For example, some behaviours such as filial cannibalism often produce significant looses in the number of embryos both in nature (Manica, 2002) and under captivity conditions (Schwanck, 1986). Furthermore, the energetic expenditure associated with parental care produces a decrease in the physical condition of the progenitors involved that might compromise their future reproduction (Sabat, 1994; Smith & Wootton, 1995). These circumstances introduce an interesting question. Does the presence of the parents still benefits embryo development under controlled conditions (absence of both predators and pathogens and suitable oxygen supply)? If the answer is no, parents could be removed from the aquaria containing clutches, thus avoiding egg cannibalism, preventing male condition loss and increasing the possibility of further matings. There are no studies addressing these topics so far. Information related to the care of the eggs has a practical application for the captive breeding of endangered species.

Fish eggs show certain permeability to external substances (Potts & Rudy, 1969). Consequently, embryo development could potentially be affected by pheromones released by conspecifics. Testosterone and other hormones secreted by the parental male (Katsel et al., 1992; Stacey & Cardwell, 1997) might penetrate the eggs. It has been shown that, in some vertebrates, androgen levels before birth have an effect on development (Staub & De Beer, 1997). In fishes, testosterone has been described to have an effect on yolk utilization rates (McCormick, 1999). Several substances released through the skin or through specialized structures like the anal glands in blennies (Serrano et al., 2008) might contain compounds with an effect on embryo development.

Even the sperm, that some parental males release repeatedly on clutches (e.g., Marconato et al., 1996), might have some kind of effect (Kekäläinen et al., 2010). Similarly, female presence could affect embryo development directly through the multiple hormones they release (Sorensen & Stacey, 2004) or indirectly through the effects on the male behaviour which increases parental care in the presence of females (Pampoulie et al., 2004). All these aspects suggest that the mere presence of parents in the nest could play a role in the growth of the embryo.

The river blenny *Salaria fluviatilis* (Asso 1801) is a freshwater fish from the Blenniidae family that lives in rivers and lakes in the Mediterranean basin and in Portugal. This species is classified as vulnerable or endangered in many of the countries where it occurs (see Vinyoles & Sostoa, 2007). During the breeding season, males excavate a nest cavity under a stone, and females lay monolayer clutches of eggs under the stone. Females are multiple spawners and they lay from 600 to more than 3000 eggs (depending on their body size) in clutches of 300-600 eggs (Vinyoles & Sostoa, 2007). After fertilization, only males provide care to the eggs by fanning and defending them until they hatch. Several females might spawn with one male, which guards the eggs at different stages of development (Neat et al., 2003). Eggs are demersal and adhesive; embryos are sensitive to low oxygen concentration and they hatch in about 14 days at 20 ºC (Wickler, 1957).

For *S. fluviatilis*, embryo development and larvae description have been reported by Gil et al. (2010). These authors proposed an efficient method to breed this species. However, they found a high mortality of larvae in the first stages of development (85% of the individuals). Fry recruitment in captivity might be improved by increasing embryo production. One possibility to attain this goal would be to maintain the clutches separated from the parents in fish species presenting egg care, thus avoiding egg

cannibalism. Filial cannibalism and heterocannibalism occur in *S. fluviatilis* (Vinyoles et al., 1999). However, before accepting this possibility, parental presence effects on embryo development need to be better understood.

This study examines, for the first time in fishes, the potential effects of the absence of parents on the development of embryos under controlled conditions in the laboratory. The main objective was to analyze if there were morphological differences between embryos reared in the presence and in the absence of the parents. Two experiments were conducted in order to determine the effects of parental presence on the ontogenetic sequence of embryos and on the embryos' morphological development, respectively. Results will be discussed focusing on captive breeding improvement and future conservation programmes.

Material and methods

Methodological procedure was based on the experimental design proposed by Gil et al. (2010) who studied the embryo and larvae development of *S. fluviatilis* from eggs maintained in the parents' presence. Two experiments were conducted from April to July 2011 (Experiment 1: Parental presence effect on the embryonic ontogenetic sequence) and from April to July 2012 (Experiment 2: Parental presence effect on the embryonic morphological development). Wild fish were used in both experiments. Fish were caught in the River Segre (a tributary of the Ebro Basin), close to the locality of Camarasa (Spain) both in November 2010 (Experiment 1) and November 2011 (Experiment 2). Experiment 1 permitted the comparison of the ontogenetic sequence of embryos reared "with parents" (treatment W) with that of embryos reared "without

parents" (treatment W/O) and allowed to determine: (1) the physicochemical and antiseptic conditions needed to guarantee embryos survival, (2) a detailed experimental procedure (including, for example, the clutch division for the experimental treatments: W and W/O), (3) the time of the day when females lay eggs more frequently, and (4) the most suitable day for the induction of egg hatching. After the experience gained in Experiment 1, Experiment 2 permitted the comparison between treatments of some specific morphological characteristics at the end of embryonic development. After the experiments, fish were returned to the same place where they had been caught. The protocol of this study was approved by the Research Ethics Committee of the University of Barcelona (Registration nº 220111) and was in accordance with decree 214/97 from the Government of the Generalitat de Catalunya.

Experiment 1: Parental presence effect on the embryonic ontogenetic sequence

Five males (with total lengths between 100 and 135 mm) and 15 females (with total lengths from 75 to 100 mm) were maintained in a 260 l aquarium (hereafter referred to as general aquarium) supplied with a biological filter and PVC refuges. Eight 30 l aquaria were settled (hereafter referred to as experimental aquaria), four of them with the purpose of maintaining clutches under the treatment W and the other four intended for maintaining clutches under the treatment W/O. All these aquaria were located in a climatized room (20 ºC) and under a light regime of 12 h L: 12 h D (hours Light: hours Darkness). Each experimental aquarium had a biological filter, sand substratum (composed of a mixture of sand, gravel and coral), one artificial nest (already accepted by fish in previous essays) and an air-diffuser. Every nest consisted of a transparent plastic box (13.5 cm large x 7 cm height x 12 cm deep) opened at one side (this opening

acting as the entrance) and with the ceiling substituted by a squared glass (to prevent the structure from floating). The nest inner walls (including the ceiling) were covered with black acetate sheets that were fixed with plastic clips. This configuration permitted the removal of the sheets from the nests in order to manipulate the clutches easily.

Physicochemical water conditions were controlled daily in all the experimental aquaria. Oxygen concentration at the nest entrance over the study (mean ± SD = 8.3 ± 0.2 mg l^{-1}) was similar to the one found throughout the reproductive period of *S. fluviatilis* in natural conditions (Vinyoles et al., 1999). Other physicochemical parameters were also controlled: pH (mean ± SD = 7.5 ± 0.5), water temperature (mean ± SD = 23.1 ± 0.2 ºC), NO_2 (mean ± SD = 0.04 ± 0.05 mg l^{-1}), NO_3 (mean ± SD = 10.0 ± 0.0 mg l^{-1}) and NH_4^+ (mean ± SD = 0.06 ± 0.02 mg l^{-1}). The mean value for all these parameters was not significantly different between treatments W and W/O (all Mann-Whitney *U*-test, $P > 0.05$) in this experiment. Throughout the study (both in the general and experimental aquaria) fish were fed once a day with frozen quironomidae larvae and, once a week, with fresh mussels.

When, in the general aquarium, a fish couple presented sexual activity (i.e., the male showed courtship behaviour and the female was gravid and had stripped colouration) it was moved to one of the four 30 l experimental aquaria assigned to the treatment W. In these aquaria, egg laying was controlled twice a day (at 8:00 a.m. and at 8:00 p.m.). When a clutch was found it was split into two similar halves (one to be kept by the parents and the other to be kept alone) following the next steps: (1) careful removal of the acetate sheet containing the clutch from the nest and its placing inside one of the aquaria of the W/O treatment (this move was done inside a small container full of water to prevent air contact and consequential infections), (2) once inside this aquarium W/O, we split the acetate sheet into two similar halves using surgical scissors

(making sure not to break the eggs and following a random cut direction) and we took a picture (through the aquarium wall) which made it possible to define the clutch borders and to distinguish eggs added afterwards, (3) one of the two halves was randomly chosen and was left in the nest of the aquarium W/O in an equivalent position to the one found in the aquarium W , (4) the other half clutch was returned to the aquarium W in its original position (the hole generated in the sheet by the clutch division was substituted by a new piece of sheet to maintain continuity), and (5) the addition of a methylene blue solution in both aquaria (one dose of 4 ml from a dilution of methylene blue with concentration 6.8 g l^{-1}). Methylene blue is a common treatment to prevent fungal infection in fishes' eggs and fingerlings (Bolívar et al., 2001) and in low doses it is not harmful to embryos (Hayes, 1930). Clutch division was done in the aquaria W/O to reduce fish stress during manipulation. Although in natural conditions many females lay eggs in one male nest (Neat et al., 2003), only one female per aquarium was provided to control for the possible maternal effect on embryos' development. This female was left in the W treatment for the 11 days clutch development period in order to simulate a realistic environment. During reproduction, female presence in the proximity of nests is the prevalent condition (Neat et al., 2003) and we expected that this is required to both stimulate male parental care and prevent male total clutch cannibalism behaviour (Kvarnemo et al., 1998). In this experiment, all the females laid eggs between 8:00 a.m. and 8:00 p.m. (this timeframe coincided with the 12h L period of the light cycle). A sample of five eggs was collected daily from each half clutch (treatments W and W/O) since the day the eggs were detected for the first time until they hatched. Eggs were removed by suctioning with a pipette. Embryo manipulation and ontogenetic description were done following Gil et al. (2010). Fish couples who finished successfully a clutch were removed from the experiment (and maintained in a different

aquarium, similar to the general one, until the end of the study). Experimental aquaria were reused for new couples, after being cleaned. Fish couples that cannibalized their clutches were returned to the general aquarium to start again the selective process.

Eggs were removed until day 12 after oviposition and it was found that hatching occurred between day 12 and day 13 (at water temperature = 23 ºC) as described by Gil et al. (2010). As environmental stress accelerates hatching in fishes (e.g., Czerkies et al., 2001) we induced hatching artificially through immersion in water at 13 ºC during 5 minutes on day 11 (after oviposition). At that date embryos' development was almost complete and the morphological structures were well defined and easy to compare.

Experiment 2: Parental presence effect on the embryonic morphological development

11 males (ranging from 75 to 90 mm in total length) and 11 females (from 60 to 100 mm total length) were used in this experiment. The protocol was the same as in Experiment 1 but, in this case, a single sample of eggs per couple was collected. Hatching was induced day 11 after oviposition (thus guaranteeing that the hatch time was the same for all the embryos). Physicochemical parameters (see Experiment 1 for more details) were not significantly different between treatments W and W/O (all Mann-Whitney *U*-test, $P > 0.05$).

About 20-25 just-hatched embryos from each experimental treatment (W and W/O) were submitted to a lethal dose (150 mg l^{-1}) of tricaine methanesulfonate (MS-222) in a Petri plate. Pictures were taken in the lab (using a digital camera - optika microscopes Italy, 7M- fixed to a stereo microscope) and structures were measured afterwards using the software Sigma Scan Pro 5. Morphological variables measured in newborn embryos were: standard length (distance comprised between the tip of the snout and the posterior

edge of the hypural plate), head height (vertical line measured at the level of the operculum), jaw length (distance from the extreme caudal end to the tip of the Meckel's cartilage), and yolk-sac volume. The latter variable was measured following Heming & Buddington (1988) formula: $YSV = 0.1667\ \pi\ LH^2$, where *H* is the yolk-sac minimum diameter and *L* the yolk-sac maximum diameter. After measuring just-hatched embryos, males and females were anesthetized (with MS-222) and measured (total length) before being removed from the experiment (and maintained in an aquarium similar to the general one until the end of the study).

Statistical analysis

Before the statistical analyses a log (x + 1) transformation of the morphological variables was done in order to adjust to normality. All analyses were performed using the free software R version 2.15.2 (R Core Team, 2012).

In order to study the effect of the experimental condition on embryo development, and taking into account the fact that clutch number is a random factor, it is adequate to analyze these data with a linear mixed model. The following model was considered for each one of the embryos' morphological variables (see a similar example in Pinheiro & Bates, 2000): $y_{ijk} = \beta_j + b_i + b_{ij} + \epsilon_{ijk}$, where y_{ijk} is the morphological variable measured for the *k*th egg from the *i*th clutch under the *j*th treatment. Thus, β_j is the fixed effect for treatment (W or W/O). This model has random effects at two levels: the effects b_i for clutch and the effects b_{ij} for the type of treatment within each clutch (this allows to assess the presence of interactions between clutch and treatment).

Models were carried out with the lme function from the R package nlme (Pinheiro et al., 2012). Residual distribution fit to normality was verified by visual inspection of

normal probability plots. The conditional coefficient of determination (Cond. r^2, which describes the proportion of variance explained by both the fixed and random factors) and the marginal coefficient of determination (Mar. r^2, which describes the proportion of variance explained by the fixed factor alone) were calculated following Nakagawa & Schielzeth (2013). When multiple tests were performed, significance levels were corrected using the sequential Bonferroni method (Rice, 1989). Real probability values are reported throughout.

Within each experiment, clutches that provided the embryos came from independent parental pairings (once the protocol completed for one clutch, the progenitors involved were separated in a different aquarium and could not be selected again). However, data concerning cannibalism were obtained from pairings in which the male, the female, or both, could have intervened in a previous unfinished clutch. This repetition was necessary to obtain enough clutches.

Results

Fish in both experiments were sexually active from April onwards. All males exhibited paternal care: anal gland rubbing on the clutches, nest guarding and egg fanning. In both experiments all the eggs presented a normal development and succeeded to hatch. No deteriorated or infected eggs (by fungus or bacteria) were found.

Experiment 1: Parental presence effect on the embryonic ontogenetic sequence

Only five of the 19 clutches laid in the artificial nests were available to obtain a complete ontogenetic sequence under the two experimental treatments. This was because 74% of clutches were cannibalized in the aquaria under the experimental treatment W. Physicochemical and antiseptic conditions established in the experimental design proved to be adequate. The sequence of ontogenetic events observed by visual inspection in embryos under the treatment W was not different than that maintained under the treatment W/O (fig. 1). The ontogenetic sequence found was identical to that described by Gil et al. (2010).

Experiment 2: Parental presence effect on the embryonic morphological development

13 clutches were obtained but only five could be used for the analyses (the other eight were rejected because they were cannibalized). A similar number of embryos from each half clutch (one half for each experimental treatment) were photographed (table 1). All morphological variables were correlated to each other except jaw length and yolk-sac volume (Pearson correlations; standard length and head height: $r_p = 0.79$, $P < 0.05$; standard length and jaw length: $r_p = 0.57$, $P < 0.05$; standard length and yolk-sac volume: $r_p = -0.21$, $P < 0.05$; head height and jaw length: $r_p = 0.64$, $P < 0.05$; head height and yolk-sac volume: $r_p = -0.35$, $P < 0.05$; jaw length and yolk-sac volume: $r_p = -0.02$, $P > 0.05$).

Apart from standard length, the remaining morphological variables (head height, jaw length and yolk-sac volume) showed differences related to the presence or absence of parents (table 2). Both head height and jaw length presented higher values in the treatment W/O, while the yolk-sac volume showed higher values under the treatment W (figs. 2, 3). The yolk-sac volume was the morphological variable in this study which

presented the higher variability between clutches (fig. 2). Although the models indicated development differences between the two experimental treatments (W and W/O), the general low values of the marginal r^2 (table 2) express the small contribution of parents presence to the variability as compared to the one explained by clutch (conditional r^2). Among all the variables, jaw length showed the lowest variability between clutches.

Discussion

Parents' absence did not prevent the obtainment of well-developed embryos without malformations. However, embryos 11 days old showed differences in their morphological traits (yolk-sac volume, head height and jaw length) depending on whether they had been kept with or without the parents. This result represents a novelty because, so far, eggs were supposed to receive the hormones related to embryo development solely in the female's ovary (Sampath-Kumar et al., 1997). Although this study did not prove external hormone transmission from parents to eggs, results suggest that this might be possible. Embryos kept in the "with parents" treatment had a more developed yolk-sac at day 11 than embryos kept under the "without parents" treatment. Yolk-sac absorption rate is closely related to environmental factors and it accelerates under stress conditions, such as, for example, when temperature increases (Fukuhara, 1990) or oxygen concentration drops (Hamor & Garside, 1977). However, in this study, temperature and oxygen concentrations were similar in all aquaria (as were the other physicochemical conditions unrelated to progenitor fish), which means that the differences found must be attributed to the experimental treatments. In the peacock blenny (*S. pavo*) males release a species-specific odor that attracts reproductively

competent females (Serrano et al., 2008). This odor consists of hydrophilic odorants from the anal gland that the male releases in a slowly and sustained pattern (peptides and peptide-derivates) and less hydrophilic odorants that possibly originate from the testes or blind pouches (glycoproteins and steroids such as 11-ketotestosterone and glucuronides). An effect of such hormones released by the male (androgens) seems plausible. In a previous work, McCormick (1999) found that eggs injected with testosterone had a slower yolk absorption rate than eggs without manipulation. In accordance with this author's findings, in this study, embryos reared in the presence of the parents (treatment that might have been influenced by 11-testosterone and other steroids since the male is in close contact with the eggs) presented more developed yolk-sac than embryos without parents. In another study, Kekäläinen et al. (2010) found that many males releasing sperm simultaneously increased environmental steroid concentration (compared to the treatment with just a single male) with a similar effect on yolk-sac absorption. It is not known, however, how hormones released by the female could have influenced yolk-sac development. The father (sole carer of the eggs in this species) is probably the parent that contributes to help embryos to make a more efficient use of their yolk-sac and to have greater energetic resources at birth (thus increasing survival opportunities). However, other studies are needed to confirm this, as well as to differentiate between male and female presence effects. These approaches should ideally keep the male alone with the clutches but their design should solve first the problem of maintaining such situation without affecting male's behaviour (i.e., parental care, cannibalism and desertion).

There are many hypotheses that could explain the greater head height and jaw length development in embryos reared without the parents. A first explanation considers that male's androgens might affect the embryos´ growth rate. Supporting this,

Srivastava & Brown (1993) found that embryos treated with testosterone grow slower before hatching. Afterwards, during the fry phase, they grow faster than individuals in the control group. It seems that the yolk-sac accumulates extrinsic hormones and, as suggested by Piferrer & Donaldson (1994), the effect of these hormones is not immediate and appears later during the development. A second possibility could be that parents' presence affects embryos' sexual determination. *Salaria fluviatilis* is a species with sexual dimorphism and, among other morphological characteristics, head size and jaw length are bigger in males than in females (Vinyoles, 1986). In blennies, no sexual chromosomes have been found for the moment (e.g., Devlin & Nagahama, 2002). In fishes, sexual determination is often dependent on environmental factors, especially temperature (Baroiller et al., 2009) and pH (Römer & Beisenherz, 1996), and frequently appears early in the developing embryo (Seki et al., 2005). Additionally, the timing and duration of exposition to certain hormones is essential for sex determination (Piferrer, 2001). In some experiments performed with teleostean fishes' eggs it was found that egg immersion into hormonal solutions affected gonad development and sexual determination (e.g., Koger et al., 2000). Usually, contact with androgens is associated to masculinisation and contact with estrogens to feminisation (Yamamoto, 1969).

Results revealed a greater variability between clutches than between the treatments within a clutch in the second experiment. This could be attributed to maternal (e.g., Marteinsdottir & Steinarsson, 1998) and paternal (e.g., Butts & Litvak, 2007) effects related to the particular traits of the parents used in this experiment. Although the present study was not designed to relate the parents' characteristics to the embryos' development, the results encourage future investigations to delve deeper into this aspect.

There are many factors that have been described to affect parental care investment such as temperature (Shuter et al., 1980), oxygen (Lissåker et al., 2003) and the

presence of predators (Steinhart et al., 2005). An increase in cannibalism has been associated to both clutch reduction (Lindström & Sargent, 1997; Lissåker et al., 2003) and female scarcity (Kvarnemo et al., 1998). Our design required the division of each clutch into two experimental conditions (with and without the parents) and this reduced clutches' size. The presence of only one female was considered also necessary to homogenize embryo variability all across the clutch. Such conditions did not suppress parental care or promote male desertion throughout the experiments. Although male behaviour has probably been altered to a certain extent by this experimental design, the fact that parental care still persisted allows us to assume that the effect on embryo development is similar to that in natural conditions, although it may be less pronounced. Apart from the presence or absence of the parental fish, all the clutches were under similar experimental conditions and the differences found are not attributable to differences in manipulation.

It should be noted that in both experiments a high occurrence of total clutch cannibalism was observed (more than a half of the clutches obtained were discarded for this reason). This result must be interpreted with prudence since there was not an individual identification of the fish in the general aquarium and some of them could have intervened in more than one attempt to obtain useful clutches. This situation might have inflated the proportion of the cannibalism observed. Proportion of egg cannibalism attributable to the male or to the female was not possible to discern. However, its high occurrence probably was due to the limited size of the clutches after being divided into two parts. Cannibalized clutches did not participate in the analysis avoiding the possible effect of male removing specific eggs on the observed differences. Furthermore, it must be said that, in this species, cannibalism is probably not selective. In a previous study

based on the analysis of gastrointestinal contents (see Vinyoles et al., 1999) authors found that eggs consumed by the males were healthy and well-developed.

In summary, results from this study suggest that parents' presence has an effect on the embryonic development and possibly also on the sexual determination of progeny. This circumstance makes it advisable to maintain parents with their clutches. Future investigations are needed to describe paternal and maternal effects on the size and the phenotype of embryos, the survival rate of larvae reared with and without parents, and the male hormonal effect on clutches.

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FIGURE CAPTIONS

Figure 1. Morphological structures in the embryos of *Salaria fluviatilis* are listed in the boxes corresponding to the day of first apparition in Experiment 1. In both treatments (W and W/O the parents) the sequence was the same. This description followed Gil et al. (2010) and obtained equivalent results.

Figure 2. Standard length, yolk-sac volume, head height and jaw length (mean ± 95% CI) measured in 11 days old *Salaria fluviatilis* embryos reared with (Treatment W) and without (Treatment W/O) the parents for each clutch, are shown for Experiment 2.

Figure 3. Comparison of *Salaria fluviatilis* embryos on day 11 of development between the two experimental treatments (W and W/O the parents) in Experiment 2. It can be appreciated from the images that greater head heights and jaw lengths are found in the Treatment W/O, whereas a greater yolk-sac volume is found for Treatment W. In the first image, the lines indicate how some of the variables were obtained: HH (head height), JL (jaw length) and H, L (diameters required to calculate yolk-sac volume).

Table 1.

Fish total length and sample sizes (*n*) of measured embryos in Experiment 2. Embryos 11 days old are provided for each clutch and experimental treatment.

Clutch	Male length (mm)	Female length (mm)	Treatment W (*n*)	Treatment W/O (*n*)
1	90.0	91.2	25	27
2	75.5	68.3	20	21
3	80.6	62.2	20	26
4	78.8	84.9	25	25
5	89.1	63.6	27	26

Abbreviations and symbols: Treatment W, Treatment with parents; Treatment W/O, Treatment without parents.

Table 2.

Linear mixed effects models predicting embryos' development depending on the experimental treatment in Experiment 2. All variables were previously log (x+1) transformed.

	Estimates ± SE		ANOVA			r^2	
Variable	Treatment W	Treatment W/O	*F*	d.f.	*P*	Cond.	Mar.
Standard length (mm)	0.72 ± 0.02	0.72 ± 0.00	2.18	1	0.140	0.76	0.00
Head height (mm)	0.22 ± 0.01	0.23 ± 0.00	20.89	1	4.9e-06*	0.66	0.12
Jaw length (mm)	0.09 ± 0.00	0.10 ± 0.00	5.77	1	0.016*	0.47	0.02
Yolk-sac volume (mm^3)	0.03 ± 0.01	0.02 ± 0.00	15.02	1	1.0e-04*	0.69	0.04

Abbreviations and symbols: Treatment W, Treatment with parents; Treatment W/O, Treatment without parents; Cond. and Mar., Conditional and Marginal coefficients of determination values (r^2); *, $P < 0.0125$ (after Bonferroni correction).

Figure 1
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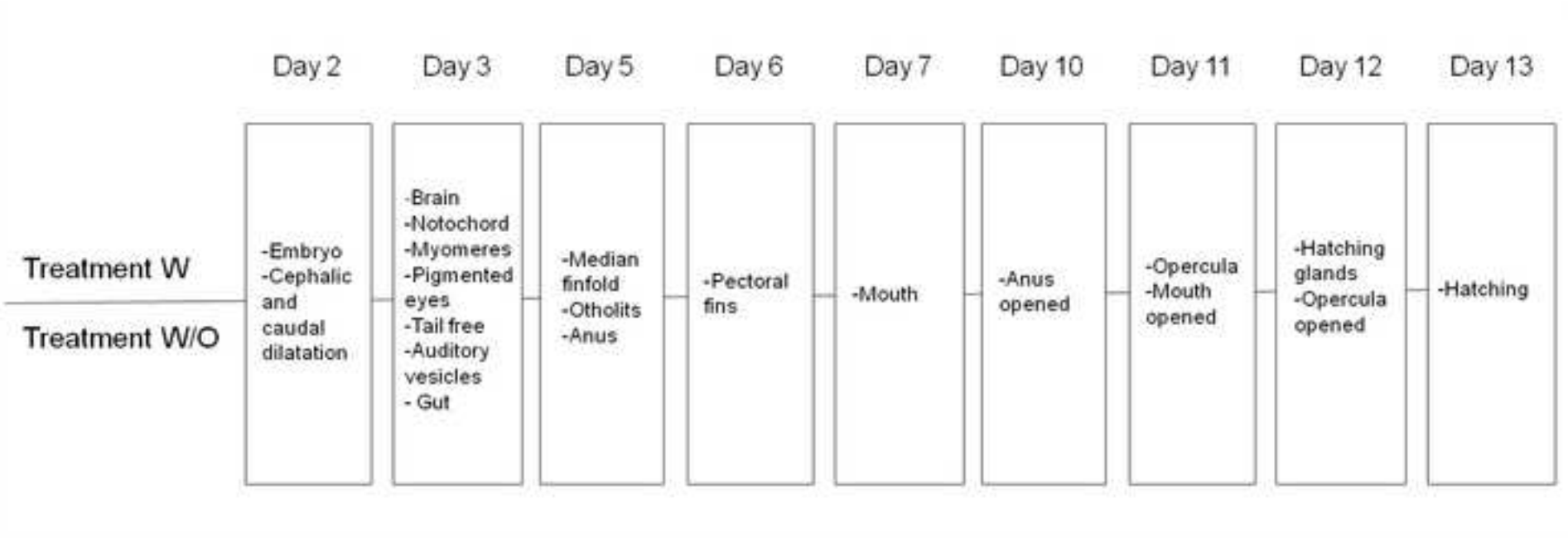

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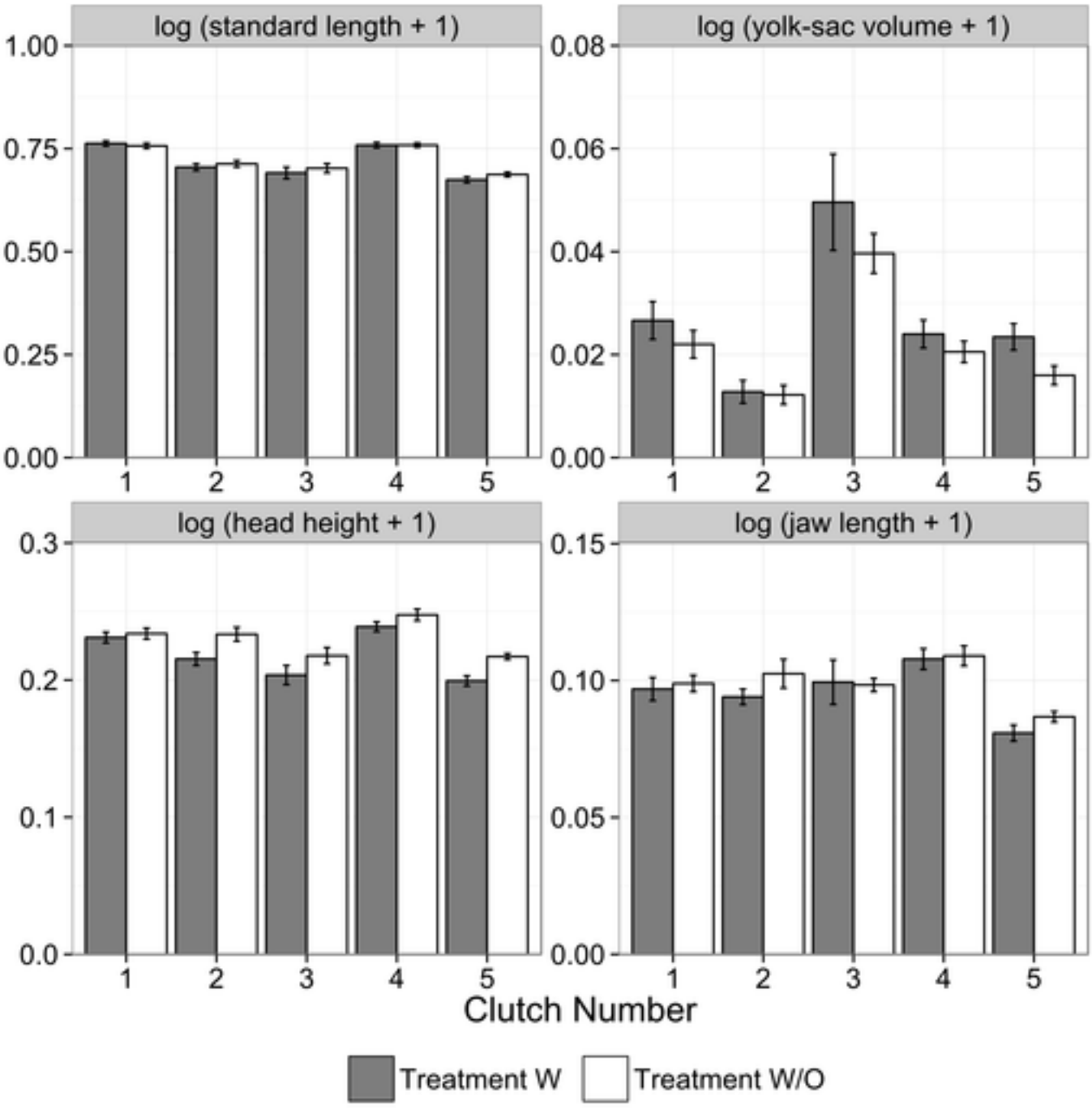

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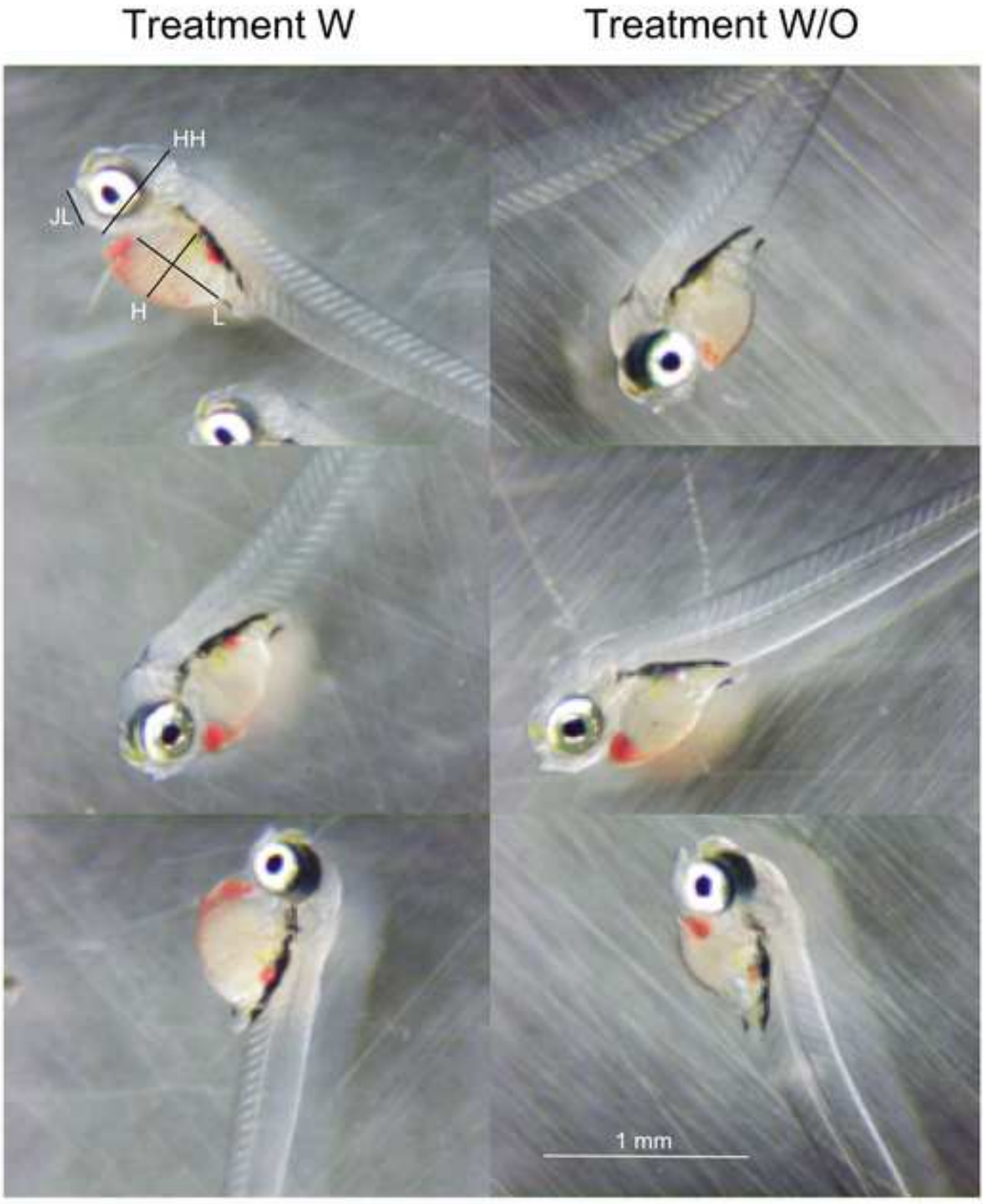

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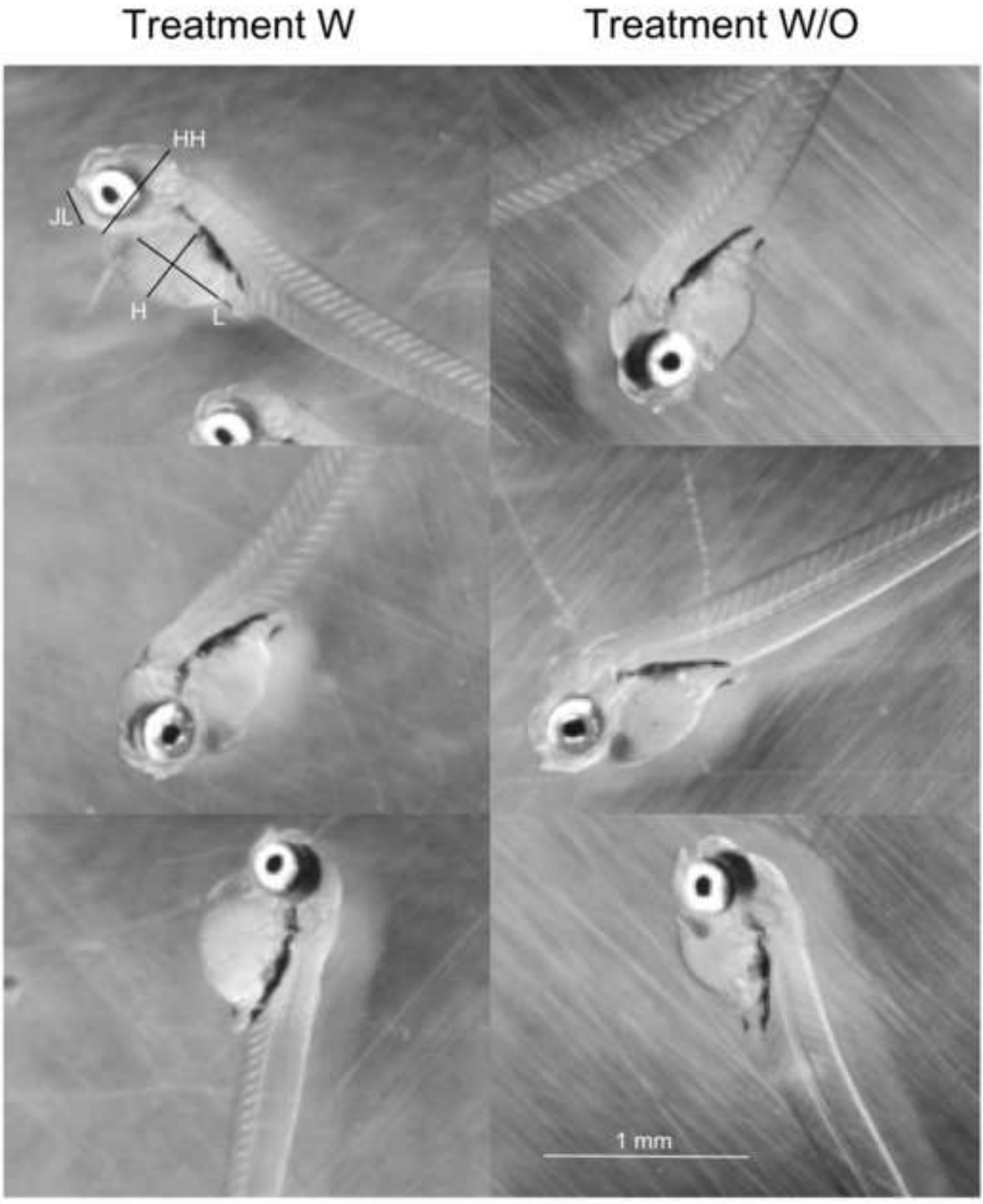

Parents' presence affects embryos' development in *Salaria fluviatilis* (Asso 1801), a fish with parental care

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Abstract

In fishes, the parents' presence improves embryos' survival through parental care but it is also associated with some disadvantages such as clutch cannibalism and male physical condition loss. Captive breeding of the river blenny *Salaria fluviatilis* might improve if these disadvantages were avoided by artificially replacing parental care benefits in the lab. ~~However, b~~Before accepting this procedure, it should be studied whether embryo development is dependent or not on any other unknown effect related to the parents' presence. ~~In order to do so,~~ In this study, the ontogenetic sequence and some morphological structures (standard length, head height, jaw length and yolk-sac volume) from embryos reared both in the presence and in the absence of the parents were compared. In the parents' absence treatment, well-developed embryos were obtained, but a smaller size of the yolk-sac, a greater head height and a greater jaw length than in the parents' presence treatment were found at day 11 after oviposition. ~~These results suggest that the parents' presence might affect embryo development and perhaps offspring sexual determination. Therefore, the practise of depriving clutches from their parents in captivity breeding programmes should be questioned.~~

Comment [MD1]: Editor Comment 2

Keywords

Captive breeding; embryonic growth; freshwater blenny; ontogeny

Introduction

Although fish parental care is associated with an increase in the embryos' survival (Clutton-Brock, 1991) it entails some disadvantages. For example, some behaviours such as filial cannibalism often produce significant looses in the number of embryos both in nature (Manica, 2002) and under captivity conditions (Schwanck, 1986). Furthermore, the energetic expenditure associated with parental care produces a decrease in the physical condition of the progenitors involved that might compromise their future reproduction (Sabat, 1994; Smith & Wootton, 1995). These circumstances introduce an interesting question. Does the presence of the parents still benefits embryo development under controlled conditions (absence of both predators and pathogens and suitable oxygen supply)? If the answer is no, parents could be removed from the aquaria containing clutches, thus avoiding egg cannibalism, preventing male condition loss and increasing the possibility of further matings. There are no studies addressing these topics so far. Information related to the care of the eggs has a practical application for the captive breeding of endangered species.

Fish eggs show certain permeability to external substances (Potts & Rudy, 1969). Consequently, embryo development could potentially be affected by pheromones released by conspecifics. Testosterone and other hormones secreted by the parental male (Katsel et al., 1992; Stacey & Cardwell, 1997) might penetrate the eggs. It has been shown that, in some vertebrates, androgen levels before birth have an effect on development (Staub & De Beer, 1997). In fishes, testosterone has been described to have an effect on yolk utilization rates (McCormick, 1999). Several substances released through the skin or through specialized structures like the anal glands in blennies (Serrano et al., 2008) might contain compounds with an effect on embryo development.

Even the sperm, that some parental males release repeatedly on clutches (e.g., Marconato et al., 1996), might have some kind of effect (Kekäläinen et al., 2010). Similarly, female presence could affect embryo development directly through the multiple hormones they release (Sorensen & Stacey, 2004) or indirectly through the effects on the male behaviour which increases parental care in the presence of females (Pampoulie et al., 2004). All these aspects suggest that the mere presence of parents in the nest could play a role in the growth of the embryo.

The river blenny *Salaria fluviatilis* (Asso 1801) is a freshwater fish from the Blenniidae family that lives in rivers and lakes in the Mediterranean basin and in Portugal. This species is classified as vulnerable or endangered in many of the countries where it occurs (see Vinyoles & Sostoa, 2007). During the breeding season, males excavate a nest cavity under a stone, and females lay monolayer clutches of eggs under the stone. Females are multiple spawners and they lay from 600 to more than 3000 eggs (depending on their body size) in clutches of 300-600 eggs (Vinyoles & Sostoa, 2007). After fertilization, only males provide care to the eggs by fanning and defending them until they hatch. Several females might spawn with one male, which guards the eggs at different stages of development (Neat et al., 2003). Eggs are demersal and adhesive; embryos are sensitive to low oxygen concentration and they hatch in about 14 days at 20 ºC (Wickler, 1957).

For *S. fluviatilis*, embryo development and larvae description have been reported by Gil et al. (2010). These authors proposed an efficient method to breed this species. However, they found a high mortality of larvae in the first stages of development (85% of the individuals). Fry recruitment in captivity might be improved by increasing embryo production. One possibility to attain this goal would be to maintain the clutches separated from the parents in fish species presenting egg care, thus avoiding egg

cannibalism. Filial cannibalism and heterocannibalism occur in *S. fluviatilis* (Vinyoles et al., 1999). However, before accepting this possibility, parental presence effects on embryo development need to be better understood.

This study examines, for the first time in fishes, the potential effects of the absence of parents on the development of embryos under controlled conditions in the laboratory. The main objective was to analyze if there were morphological differences between embryos reared in the presence and in the absence of the parents. Two experiments were conducted in order to determine the effects of parental presence on the ontogenetic sequence of embryos and on the embryos' morphological development, respectively. Results will be discussed focusing on captive breeding improvement and future conservation programmes.

Material and methods

Methodological procedure was based on the experimental design proposed by Gil et al. (2010) who studied the embryo and larvae development of *S. fluviatilis* from eggs maintained in the parents' presence. Two experiments were conducted from April to July 2011 (Experiment 1: Parental presence effect on the embryonic ontogenetic sequence) and from April to July 2012 (Experiment 2: Parental presence effect on the embryonic morphological development). Wild fish were used in both experiments. Fish were caught in the River Segre (a tributary of the Ebro Basin), close to the locality of Camarasa (Spain) both in November 2010 (Experiment 1) and November 2011 (Experiment 2). Experiment 1 permitted the comparison of the ontogenetic sequence of embryos reared "with parents" (treatment W) with that of embryos reared "without

parents" (treatment W/O) and allowed to determine: (1) the physicochemical and antiseptic conditions needed to guarantee embryos survival, (2) a detailed experimental procedure (including, for example, the clutch division for the experimental treatments: W and W/O), (3) the time of the day when females lay eggs more frequently, and (4) the most suitable day for the induction of egg hatching. After the experience gained in Experiment 1, Experiment 2 permitted the comparison between treatments of some specific morphological characteristics at the end of embryonic development. After the experiments, fish were returned to the same place where they had been caught. The protocol of this study was approved by the Research Ethics Committee of the University of Barcelona (Registration nº 220111) and was in accordance with decree 214/97 from the Government of the Generalitat de Catalunya.

Experiment 1: Parental presence effect on the embryonic ontogenetic sequence

Five males (with total lengths between 100 and 135 mm) and 15 females (with total lengths from 75 to 100 mm) were maintained in a 260 l aquarium (hereafter referred to as general aquarium) supplied with a biological filter and PVC refuges. Eight 30 l aquaria were settled (hereafter referred to as experimental aquaria), four of them with the purpose of maintaining clutches under the treatment W and the other four intended for maintaining clutches under the treatment W/O. All these aquaria were located in a climatized room (20 ºC) and under a light regime of 12 h L: 12 h D (hours Light: hours Darkness). Each experimental aquarium had a biological filter, sand substratum (composed of a mixture of sand, gravel and coral), one artificial nest (already accepted by fish in previous essays) and an air-diffuser. Every nest consisted of a transparent plastic box (13.5 cm large x 7 cm height x 12 cm deep) opened at one side (this opening

143 acting as the entrance) and with the ceiling substituted by a squared glass (to prevent the
144 structure from floating). The nest inner walls (including the ceiling) were covered with
145 black acetate sheets that were fixed with plastic clips. This configuration permitted the
146 removal of the sheets from the nests in order to manipulate the clutches easily.

147 Physicochemical water conditions were controlled daily in all the experimental
148 aquaria. Oxygen concentration at the nest entrance over the study (mean ± SD = 8.3 ±
149 0.2 mg l^{-1}) was similar to the one found throughout the reproductive period of *S.*
150 *fluviatilis* in natural conditions (Vinyoles et al., 1999). Other physicochemical
151 parameters were also controlled: pH (mean ± SD = 7.5 ± 0.5), water temperature (mean
152 ± SD = 23.1 ± 0.2 ºC), NO_2 (mean ± SD = 0.04 ± 0.05 mg l^{-1}), NO_3 (mean ± SD = 10.0
153 ± 0.0 mg l^{-1}) and NH_4^+ (mean ± SD = 0.06 ± 0.02 mg l^{-1}). The mean value for all these
154 parameters was not significantly different between treatments W and W/O (all Mann-
155 Whitney *U*-test, $P > 0.05$) in this experiment. Throughout the study (both in the general
156 and experimental aquaria) fish were fed once a day with frozen quironomidae larvae
157 and, once a week, with fresh mussels.

158 When, in the general aquarium, a fish couple presented sexual activity (i.e., the
159 male showed courtship behaviour and the female was gravid and had stripped
160 colouration) it was moved to one of the four 30 l experimental aquaria assigned to the
161 treatment W. In these aquaria, egg laying was controlled twice a day (at 8:00 a.m. and at
162 8:00 p.m.). When a clutch was found it was split into two similar halves (one to be kept
163 by the parents and the other to be kept alone) following the next steps: (1) careful
164 removal of the acetate sheet containing the clutch from the nest and its placing inside
165 one of the aquaria of the W/O treatment (this move was done inside a small container
166 full of water to prevent air contact and consequential infections), (2) once inside this
167 aquarium W/O, we split the acetate sheet into two similar halves using surgical scissors

168 (making sure not to break the eggs and following a random cut direction) and we took a
169 picture (through the aquarium wall) which made it possible to define the clutch borders
170 and to distinguish eggs added afterwards, (3) one of the two halves was randomly
171 chosen and was left in the nest of the aquarium W/O in an equivalent position to the one
172 found in the aquarium W , (4) the other half clutch was returned to the aquarium W in
173 its original position (the hole generated in the sheet by the clutch division was
174 substituted by a new piece of sheet to maintain continuity), and (5) the addition of a
175 methylene blue solution in both aquaria (one dose of 4 ml from a dilution of methylene
176 blue with concentration 6.8 g l^{-1}). Methylene blue is a common treatment to prevent
177 fungal infection in fishes' eggs and fingerlings (Bolívar et al., 2001) and in low doses it
178 is not harmful to embryos (Hayes, 1930). Clutch division was done in the aquaria W/O
179 to reduce fish stress during manipulation. Although in natural conditions many females
180 lay eggs in one male nest (Neat et al., 2003), only one female per aquarium was
181 provided to control for the possible maternal effect on embryos' development. This
182 female was left in the W treatment for the 11 days clutch development period in order to
183 simulate a realistic environment. During reproduction, female presence in the proximity
184 of nests is the prevalent condition (Neat et al., 2003) and we expected that this is
185 required to both stimulate male parental care and prevent male total clutch cannibalism
186 behaviour (Kvarnemo et al., 1998). In this experiment, all the females laid eggs between
187 8:00 a.m. and 8:00 p.m. (this timeframe coincided with the 12h L period of the light
188 cycle). A sample of five eggs was collected daily from each half clutch (treatments W
189 and W/O) since the day the eggs were detected for the first time until they hatched.
190 Eggs were removed by suctioning with a pipette. Embryo manipulation and ontogenetic
191 description were done following Gil et al. (2010). Fish couples who finished
192 successfully a clutch were removed from the experiment (and maintained in a different

aquarium, similar to the general one, until the end of the study). Experimental aquaria were reused for new couples, after being cleaned. Fish couples that cannibalized their clutches were returned to the general aquarium to start again the selective process.

Eggs were removed until day 12 after oviposition and it was found that hatching occurred between day 12 and day 13 (at water temperature = 23 ºC) as described by Gil et al. (2010). As environmental stress accelerates hatching in fishes (e.g., Czerkies et al., 2001) we induced hatching artificially through immersion in water at 13 ºC during 5 minutes on day 11 (after oviposition). At that date embryos' development was almost complete and the morphological structures were well defined and easy to compare.

Experiment 2: Parental presence effect on the embryonic morphological development

11 males (ranging from 75 to 90 mm in total length) and 11 females (from 60 to 100 mm total length) were used in this experiment. The protocol was the same as in Experiment 1 but, in this case, a single sample of eggs per couple was collected. Hatching was induced day 11 after oviposition (thus guaranteeing that the hatch time was the same for all the embryos). Physicochemical parameters (see Experiment 1 for more details) were not significantly different between treatments W and W/O (all Mann-Whitney *U*-test, $P > 0.05$).

Comment [MD2]: Reviewer 2 Comment 2

About 20-25 just-hatched embryos from each experimental treatment (W and W/O) were submitted to a lethal dose (150 mg l^{-1}) of tricaine methanesulfonate (MS-222) in a Petri plate. Pictures were taken in the lab (using a digital camera - optika microscopes Italy, 7M- fixed to a stereo microscope) and structures were measured afterwards using the software Sigma Scan Pro 5. Morphological variables measured in newborn embryos were: standard length (distance comprised between the tip of the snout and the posterior

edge of the hypural plate), head height (vertical line measured at the level of the operculum), jaw length (distance from the extreme caudal end to the tip of the Meckel's cartilage), and yolk-sac volume. The latter variable was measured following Heming & Buddington (1988) formula: $YSV = 0.1667\ \pi\ LH^2$, where H is the yolk-sac minimum diameter and L the yolk-sac maximum diameter. After measuring~~the measuring of~~ just-hatched embryos, males and females were anesthetized (with MS-222) and measured (total length) before being removed from the experiment (and maintained in an aquarium similar to the general one until the end of the study).

Comment [U3]: Reviewer 2 comment 1

Statistical analysis

Before the statistical analyses a log (x + 1) transformation of the morphological variables was done in order to adjust to normality. All analyses were performed using the free software R version 2.15.2 (R Core Team, 2012).

In order to study the effect of the experimental condition on embryo development, and taking into account the fact that clutch number is a random factor, it is adequate to analyze these data with a linear mixed model. The following model was considered for each one of the embryos' morphological variables (see a similar example in Pinheiro & Bates, 2000): $y_{ijk} = \beta_j + b_i + b_{ij} + \epsilon_{ijk}$, where y_{ijk} is the morphological variable measured for the *k*th egg from the *i*th clutch under the *j*th treatment. Thus, β_j is the fixed effect for treatment (W or W/O). This model has random effects at two levels: the effects b_i for clutch and the effects b_{ij} for the type of treatment within each clutch (this allows to assess the presence of interactions between clutch and treatment).

Models were carried out with the lme function from the R package nlme (Pinheiro et al., 2012). Residual distribution fit to normality was verified by visual inspection of

normal probability plots. The conditional coefficient of determination (Cond. r^2, which describes the proportion of variance explained by both the fixed and random factors) and the marginal coefficient of determination (Mar. r^2, which describes the proportion of variance explained by the fixed factor alone) were calculated following Nakagawa & Schielzeth (2013). When multiple tests were performed, significance levels were corrected using the sequential Bonferroni method (Rice, 1989). Real probability values are reported throughout.

Within each experiment, clutches that provided the embryos came from independent parental pairings (once the protocol completed for one clutch, the progenitors involved were separated in a different aquarium and could not be selected again). However, data concerning cannibalism were obtained from pairings in which the male, the female, or both, could have intervened in a previous unfinished clutch. This repetition was necessary to obtain enough clutches.

Results

Fish in both experiments were sexually active from April onwards. All males exhibited paternal care: anal gland rubbing on the clutches, nest guarding and egg fanning. In both experiments all the eggs presented a normal development and succeeded to hatch. No deteriorated or infected eggs (by fungus or bacteria) were found.

Experiment 1: Parental presence effect on the embryonic ontogenetic sequence

Only five of the 19 clutches laid in the artificial nests were available to obtain a complete ontogenetic sequence under the two experimental treatments. This was because 74% of clutches were cannibalized in the aquaria under the experimental treatment W. Physicochemical and antiseptic conditions established in the experimental design proved to be adequate. The sequence of ontogenetic events observed by visual inspection in embryos under the treatment W was not different than that maintained under the treatment W/O (fig. 1). The ontogenetic sequence found was identical to that described by Gil et al. (2010).

Experiment 2: Parental presence effect on the embryonic morphological development

13 clutches were obtained but only five could be used for the analyses (the other eight were rejected because they were cannibalized). A similar number of embryos from each half clutch (one half for each experimental treatment) were photographed (table 1). All morphological variables were correlated to each other except jaw length and yolk-sac volume (Pearson correlations; standard length and head height: $r_p = 0.79$, $P < 0.05$; standard length and jaw length: $r_p = 0.57$, $P < 0.05$; standard length and yolk-sac volume: $r_p = -0.21$, $P < 0.05$; head height and jaw length: $r_p = 0.64$, $P < 0.05$; head height and yolk-sac volume: $r_p = -0.35$, $P < 0.05$; jaw length and yolk-sac volume: $r_p = -0.02$, $P > 0.05$).

Apart from standard length, the remaining morphological variables (head height, jaw length and yolk-sac volume) showed differences related to the presence or absence of parents (table 2). Both head height and jaw length presented higher values in the treatment W/O, while the yolk-sac volume showed higher values under the treatment W (figs. 2, 3). The yolk-sac volume was the morphological variable in this study which

presented the higher variability between clutches (fig. 2). Although the models indicated development differences between the two experimental treatments (W and W/O), the general low values of the marginal r^2 (table 2) express the small contribution of parents presence to the variability as compared to the one explained by clutch (conditional r^2). Among all the variables, jaw length showed the lowest variability between clutches.

Discussion

Parents' absence did not prevent the obtainment of well-developed embryos without malformations. However, embryos 11 days old showed differences in their morphological traits (yolk-sac volume, head height and jaw length) depending on whether they had been kept with or without the parents. This result represents a novelty because, so far, eggs were supposed to receive the hormones related to embryo development solely in the female's ovary (Sampath-Kumar et al., 1997). Although this study did not prove external hormone transmission from parents to eggs, results suggest that this might be possible. Embryos kept in the "with parents" treatment had a more developed yolk-sac at day 11 than embryos kept under the "without parents" treatment. Yolk-sac absorption rate is closely related to environmental factors and it accelerates under stress conditions, such as, for example, when temperature increases (Fukuhara, 1990) or oxygen concentration drops (Hamor & Garside, 1977). However, in this study, temperature and oxygen concentrations were similar in all aquaria (as were the other physicochemical conditions unrelated to progenitor fish), which means that the differences found must be attributed to the experimental treatments. In the peacock blenny (*S. pavo*) males release a species-specific odor that attracts reproductively

competent females (Serrano et al., 2008). This odor consists of hydrophilic odorants from the anal gland that the male releases in a slowly and sustained pattern (peptides and peptide-derivates) and less hydrophilic odorants that possibly originate from the testes or blind pouches (glycoproteins and steroids such as 11-ketotestosterone and glucuronides). An effect of such hormones released by the male (androgens) seems plausible. In a previous work, McCormick (1999) found that eggs injected with testosterone had a slower yolk absorption rate than eggs without manipulation. In accordance with this author's findings, in this study, embryos reared in the presence of the parents (treatment that might have been influenced by 11-testosterone and other steroids since the male is in close contact with the eggs) presented more developed yolk-sac than embryos without parents. In another study, Kekäläinen et al. (2010) found that many males releasing sperm simultaneously increased environmental steroid concentration (compared to the treatment with just a single male) with a similar effect on yolk-sac absorption. It is not known, however, how hormones released by the female could have influenced yolk-sac development. The father (sole carer of the eggs in this species) is probably the parent that contributes to help embryos to make a more efficient use of their yolk-sac and to have greater energetic resources at birth (thus increasing survival opportunities). However, other studies are needed to confirm this, as well as to differentiate between male and female presence effects. These approaches should ideally keep the male alone with the clutches but their design should solve first the problem of maintaining such situation without affecting male's behaviour (i.e., parental care, cannibalism and desertion).

There are many hypotheses that could explain the greater head height and jaw length development in embryos reared without the parents. A first explanation considers that male's androgens might affect the embryos´ growth rate. Supporting this,

342 Srivastava & Brown (1993) found that embryos treated with testosterone grow slower
343 before hatching. Afterwards, during the fry phase, they grow faster than individuals in
344 the control group. It seems that the yolk-sac accumulates extrinsic hormones and, as
345 suggested by Piferrer & Donaldson (1994), the effect of these hormones is not
346 immediate and appears later during the development. A second possibility could be that
347 parents' presence affects embryos' sexual determination. *Salaria fluviatilis* is a species
348 with sexual dimorphism and, among other morphological characteristics, head size and
349 jaw length are bigger in males than in females (Vinyoles, 1986). In blennies, no sexual
350 chromosomes have been found for the moment (e.g., Devlin & Nagahama, 2002). In
351 fishes, sexual determination is often dependent on environmental factors, especially
352 temperature (Baroiller et al., 2009) and pH (Römer & Beisenherz, 1996), and frequently
353 appears early in the developing embryo (Seki et al., 2005). Additionally, the timing and
354 duration of exposition to certain hormones is essential for sex determination (Piferrer,
355 2001). In some experiments performed with teleostean fishes' eggs it was found that
356 egg immersion into hormonal solutions affected gonad development and sexual
357 determination (e.g., Koger et al., 2000). Usually, contact with androgens is associated to
358 masculinisation and contact with estrogens to feminisation (Yamamoto, 1969).

359 Results revealed a greater variability between clutches than between the treatments
360 within a clutch in the second experiment. This could be attributed to maternal (e.g.,
361 Marteinsdottir & Steinarsson, 1998) and paternal (e.g., Butts & Litvak, 2007) effects
362 related to the particular traits of the parents used in this experiment. Although the
363 present study was not designed to relate the parents' characteristics to the embryos'
364 development, the results encourage future investigations to delve deeper into this aspect.

365 There are many factors that have been described to affect parental care investment
366 such as temperature (Shuter et al., 1980), oxygen (Lissåker et al., 2003) and the

presence of predators (Steinhart et al., 2005). An increase in cannibalism has been associated to both clutch reduction (Lindström & Sargent, 1997; Lissåker et al., 2003) and female scarcity (Kvarnemo et al., 1998). Our design required the division of each clutch into two experimental conditions (with and without the parents) and this reduced clutches' size. The presence of only one female was considered also necessary to homogenize embryo variability all across the clutch. Such conditions did not suppress parental care or promote male desertion throughout the experiments. Although male behaviour has probably been altered to a certain extent by this experimental design, the fact that parental care still persisted allows us to assume that the effect on embryo development is similar to that in natural conditions, although it may be less pronounced. Apart from the presence or absence of the parental fish, all the clutches were under similar experimental conditions and the differences found are not attributable to differences in manipulation.

It should be noted that in both experiments a high occurrence of total clutch cannibalism was observed (more than a half of the clutches obtained were discarded for this reason). This result must be interpreted with prudence since there was not an individual identification of the fish in the general aquarium and some of them could have intervened in more than one attempt to obtain useful clutches. This situation might have inflated the proportion of the cannibalism observed. Proportion of egg cannibalism attributable to the male or to the female was not possible to discern. However, its high occurrence probably was due to the limited size of the clutches after being divided into two parts. Cannibalized clutches did not participate in the analysis avoiding the possible effect of male removing specific eggs on the observed differences. Furthermore, it must be said that, in this species, cannibalism is probably not selective. In a previous study

based on the analysis of gastrointestinal contents (see Vinyoles et al., 1999) authors found that eggs consumed by the males were healthy and well-developed.

In summary, results from this study suggest that parents' presence has an effect on the embryonic development and possibly also on the sexual determination of progeny. This circumstance makes it advisable to maintain parents with their clutches. Future investigations are needed to describe paternal and maternal effects on the size and the phenotype of embryos, the survival rate of larvae reared with and without parents, and the male hormonal effect on clutches.

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536

537 **FIGURE CAPTION**

538

539 **Figure 1.** Morphological structures in the embryos of *Salaria fluviatilis* are listed in the
540 boxes corresponding to the day of first apparition in Experiment 1. In both treatments
541 (W and W/O the parents) the sequence was the same. This description followed Gil et
542 al. (2010) and obtained equivalent results.

543

544 **Figure 2.** Standard length, yolk-sac volume, head height and jaw length (mean ± 95%
545 CI) measured in 11 days old *Salaria fluviatilis* embryos reared with (Treatment W) and
546 without (Treatment W/O) the parents for each clutch, are shown for Experiment 2.

547

548 **Figure 3.** Comparison of *Salaria fluviatilis* embryos on day 11 of development between
549 the two experimental treatments (W and W/O the parents) in Experiment 2. It can be
550 appreciated from the images that greater head heights and jaw lengths are found in the
551 Treatment W/O, whereas a greater yolk-sac volume is found for Treatment W. In the
552 first image, the lines indicate how some of the variables were obtained: HH (head
553 height), JL (jaw length) and H, L (diameters required to calculate yolk-sac volume).

Table 1.

Fish total length and sample sizes (*n*) of measured embryos in Experiment 2. Embryos 11 days old are provided for each clutch and experimental treatment.

Comment [U1]: Editor Comment 2

Clutch	Male length (mm)	Female length (mm)	Treatment W (*n*)	Treatment W/O (*n*)
1	90.0	91.2	25	27
2	75.5	68.3	20	21
3	80.6	62.2	20	26
4	78.8	84.9	25	25
5	89.1	63.6	27	26

Abbreviations and symbols: ~~Embryos 11 days old are provided for each clutch and experimental treatment (with parents =~~ Treatment W, Treatment with parents; ~~without parents =~~ Treatment W/O, Treatment without parents~~)~~.

Supplemental Table 2 with Track Changes

Click here to download Supplemental: Table 2_Fabre et al_S.fuviatilis_Sub3_with Track Changes.docx

Table 2.

Linear mixed effects models predicting embryos' development depending on the experimental treatment ~~(with parents = Treatment W, without parents = Treatment W/O)~~ in Experiment 2. All variables were previously log (x+1) transformed.

	Estimates ± SE		ANOVA			r^2	
Variable	Treatment W	Treatment W/O	*F*	d.f.	*P*	Cond.	Mar.
Standard length (mm)	0.72 ± 0.02	0.72 ± 0.00	2.18	1	0.140	0.76	0.00
Head height (mm)	0.22 ± 0.01	0.23 ± 0.00	20.89	1	4.9e-06*	0.66	0.12
Jaw length (mm)	0.09 ± 0.00	0.10 ± 0.00	5.77	1	0.016*	0.47	0.02
Yolk-sac volume (mm^3)	0.03 ± 0.01	0.02 ± 0.00	15.02	1	1.0e-04*	0.69	0.04

Comment [U1]: Editor Comment 2

Abbreviations and symbols: Treatment W, Treatment with parents; Treatment W/O, Treatment without parents; Cond. and Mar., Conditional and Marginal coefficients of determination values (r^2); *, $P < 0.0125$ (aAfter Bonferroni correction). ~~All variables were previously log (x+1) transformed. Conditional (Cond.) and Marginal (Mar.) coefficients of determination values (r^2) are shown. An asterisk denotes a significant difference. After Bonferroni, significance was reached at $P < 0.0125$.~~