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First observations of annualism in *Millerichthys robustus* (Cyprinodontiformes: Rivulidae)

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Millerichthys robustus survives in ephemeral pond habitats from Papaloapan river basin in Mexico by producing drought-tolerant diapausing embryos. Four stages in fertilized embryos were observed: Dispersion/re-aggregation, Diapause I, Diapause II, and Diapause III confirming its annualism. Eggs extracted from soil when the pool was completely dry were found in Diapause I. The chorion surface ornamentation is reported for this species for the first time.

Introduction

The cyprinodontiform family Rivulidae is a diversified group of killifishes, with currently more than 324 valid species occurring between southern Florida and northeastern Argentina (Costa, 2008). A part of the rivulid taxa, known as annual killifishes (Myers, 1942), are uniquely found in swamps and isolated pools that seasonally dry (Nico et al., 1987; Costa, 1995, 1998). The ability to survive in seasonal swamps and pools is due to the thickened chorion eggs, which make eggs resistant to water loss, and a complex embryonic development including diapause (Wourms, 1972a–c).

Three rivulids species are reported in Mexico: *Cynodonichthys tenuis*, *Kryptolebias marmoratus*, and *Millerichthys robustus* (Huber, 1992; Costa, 2008). *Millerichthys robustus* is known from the Papaloapan and Coatzacoalcos River drainages (Miller & Hubbs, 1974), and was included in the subfamily Rivulinae in Costa (1998, 2008) but not in Costa (2011). To date, there is no data available about its lifestyle (Huber, 1992; Costa, 1995, 1998). The primary purpose of the present study is to describe its annual nature.

Material and methods

We have studied a population of *Millerichthys robustus* from a seasonal pond near Papaloapan River in Tlacotalpan, Veracruz, Mexico. The pond (18°37'39.3" N 95°38'53.0" W) was 30 × 20 m, with a maximum depth of 90 cm.

Fish were caught primarily using dip nets and transported to the laboratory in order to establish a laboratory stock. The parameters of water quality were measured with a Hanna® C99 multiparametric spectrophotometer: NH_4, NH_3, NO_2,

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Fig. 1. *Millerichthys robustus*: **a,** male; **b,** female. Scale bars 10 mm.

PO_4, P, carbonate hardness and aluminium. Dissolved oxygen, temperature, pH, electrical conductivity and total dissolved solids were measured with the Hanna® multiparameter model HI 9828.

Adult fishes were housed in 38 l glass aquaria, with a proportion of one male per two females. Tank bottom was left bare in order to simplify cleaning and discourage unwanted spawning. Physical and chemical conditions in which they were maintained simulated the collecting site. Mechanic, biological and chemical filtering was also applied continuously with weekly renewal of ⅕ of the water. Temperature was kept at 26 ± 1 °C, with photoperiod set to 14 light hours and 10 dark hours. Subjects were fed three times a day with *Artemia salina* nauplii. A 12 × 12 × 20 cm glass container was placed in each aquarium with 3 cm of sterilized peat moss as spawning substrate. Substrates were examined twice a week. Eggs were isolated from the peat moss with small brooms and then cleaned and incubated for 24 hours in petri dishes at 26 ± 1 °C in purified water. After this procedure, the eggs were individually placed in 2 × 2 cm hermetic closure plastic bags with a sterilized wet, thick moss. Examinations of the eggs was performed weekly in order to determine development stage. Right after the embryos were examined, they were immediately returned to 26 ± 1 °C and total darkness.

Soil samples were also collected on two different occasions: when the pond was completely dry (June), and when the pond was wet at the beginning of rainy season (July). Soil was sieved with a 1.3 mm gap sieve and searching for eggs was done with a stereoscopic microscope.

The fishes were identified as *M. robustus* following Miller & Hubbs (1974). A microscope was used in the study of developmental stages of fertilized embryos. Definition of embryo stages follow Wourms (1972a–c). Nomenclature for chorion surface ornamentation follows Costa (1998). All measurements are given in millimetres unless stated otherwise and they are all shown as the range followed by the mean ± standard deviation in parentheses.

Results

In February 2010 mature specimens were collected (8 males and 20 females, Fig. 1). Water parameters at the moment of fish harvesting were: dissolved oxygen 7.9 mg · l^{-1}, temperature 18.4 °C, pH 7.6, NH_4 0.7 mg · l^{-1}, NH_3 0.01 mg · l^{-1}, NO_2 1.30 mg · l^{-1}, PO_4 0.05 mg · l^{-1}, P 0.01 mg · l^{-1}, electrical conductivity 0.001 µS · cm^{-1}, total dissolved solids 484.6 mg · l^{-1}.

The rainy season began on the first days of July and from this date onward the pond remained wet until mid-August (pond wet) after the soil was saturated with water and began to fill. Subsequently it was flooded until February (pond water), and remained completely dry from March to June (pond dry) (Table 1).

In June, when pond was completely dry, 23 eggs were collected from substrate in Diapause I (Fig. 2a). They had a diameter of 1.13–1.50 (1.30 ± 0.90). At the end July, about 20 days after the beginning of the rainy season, 10 eggs were collected from the wet substrate, nine of which were in Diapause III and the other one was in Diapause I. The diameter of the Diapause III eggs was 1.30–1.70 (1.50 ± 0.83).

In laboratory the number of eggs collected weekly were 1–115 (49.25 ± 33.6), with a total number of fertilized analyzed eggs being 575.

The fertilized eggs are spherical with a clearly visible perivitelline space. Four stages in fertilized embryos were observed in the laboratory during the study: dispersion / re-aggregation (D/R) stage, Diapause I, Diapause II, and Diapause III (Fig. 2b–f). One oil droplet (or occasionally several smaller ones) was present; they usually fuse within the first 24 hours after fertilization (Fig. 2b). During Diapause I the embryos were in the dispersion phase of the amoeboid blastomeres. When the arrested phase was concluded the re-aggregation occurred. The Diapause

II occurs during embryogenesis; when the embryos have had 38 somites and its optic lobes were without pupil and the heart appeared as a non-contractile tube, like a proto-heart. The heart having contractions is the conclusion of this phase. The embryos of *M. robustus* were observed to be fully developed after 14 days when they entered Diapause III, at which point the organs of the embryos were fully formed and cardiac activity ceased (Fig. 2e–f). Embryos remained in Diapause III for a period of time, namely 47–189 days. Rhythmic heart contractions mark the end of arrest and at this developmental stage the embryo is ready to hatch if the egg is placed into water.

The surface of the chorion is verrucated with mushroom-like projections in an almost regularly distributed pattern (Fig. 3a). Each verruca has one filament on the distal tip and is observed immediately after spawning (Fig. 3b) and measured 6.15–8.62 (7.75 ± 0.80) micrometres of maximal width. The hair-like filaments are not present during Diapause III but instead there is a pore originating at the tip of the verruca (Fig. 3c). These structures are absent around the micropyle area (Fig. 3d).

Discussion

The evidence of the various stages of dispersion-reaggregation and diapauses was clearly recognized in fertilized eggs of *Millerichthys*. *Millerichthys* is clearly an annual fish, term coined by Myers (1942), since their life cycle appears to be completed within a single year (October–May). Even better would be to use the term proposed by Costa (2002) a seasonal fish.

The development of the system of three diapauses seems not an exceptional event in the evolution of killifishes and may have occurred several times during the evolution of this group of fishes as proposed by Hrbek & Larson (1999). At the contrary Costa (1990a) and Murphy & Collier (1997) hypothesized that annualism may correspond to a single event in the evolution of the rivulids. The most parsimonious cladograms obtained by Costa (1998) admit two equally parsimonious interpretations: annualism may correspond to a single event in the evolution of the rivulids or two independent events, in Cynolebiatinae and in Neofundulidae. Indeed simultaneous unique occurrence of apomorphic morphological traits in members of annual aplocheiloid belonging to different lineages may reflect some kind of favoritism associated to annual life style (Costa, 1998). A deep body does not occur in non-annual aplocheiloids, but occurs in distinct annual rivulid lineages including *Millerichthys* (Huber, 1992). Similarly, the somewhat elaborate cephalic neuromast pattern in annual rivulid (Costa 1998) is a condition similar to that in *M. robustus* (Costa, 1995).

The results of all embryo in DI from the eggs extracted from soil when the pool was completely dry were expected because in the natural environment the eggs of annual fish can remain dormant in Diapause I for months during dry season (Scheel, 1990; Bellemans, 2000). After that period the eggs begin to develop further and, at this stage, enter Diapause II. The first rains of the season do usually not, as already indicated, immediately produce bodies of standing water. The early rains will, at least, moisten the substrate or create small shallow pools of water that may evaporate or soak in after a short time. Field evidence suggests that in the natural environment, most eggs will enter Diapause III (Watters, 2009); this was also confirmed in *M. robustus*.

The elaborate chorion structure of aplocheiloid killifishes was first reported by Carvalho (1957) and Siegel (1958a–b), and its potential as a tool for taxonomic studies by Scheel (1968). Costa

Table 1. *Millerichthys robustus* habitat and development egg state in a seasonal pond near Tlacotalpan, Veracruz, Mexico. Darkened areas refer to in-the-field observations over the year 2010.

Month	J	F	M	A	M	J	J	A	S	O	N	D
Pond wet												
Pond water												
Pond dry												
Diapause I												
Development embryo												
Diapause III												

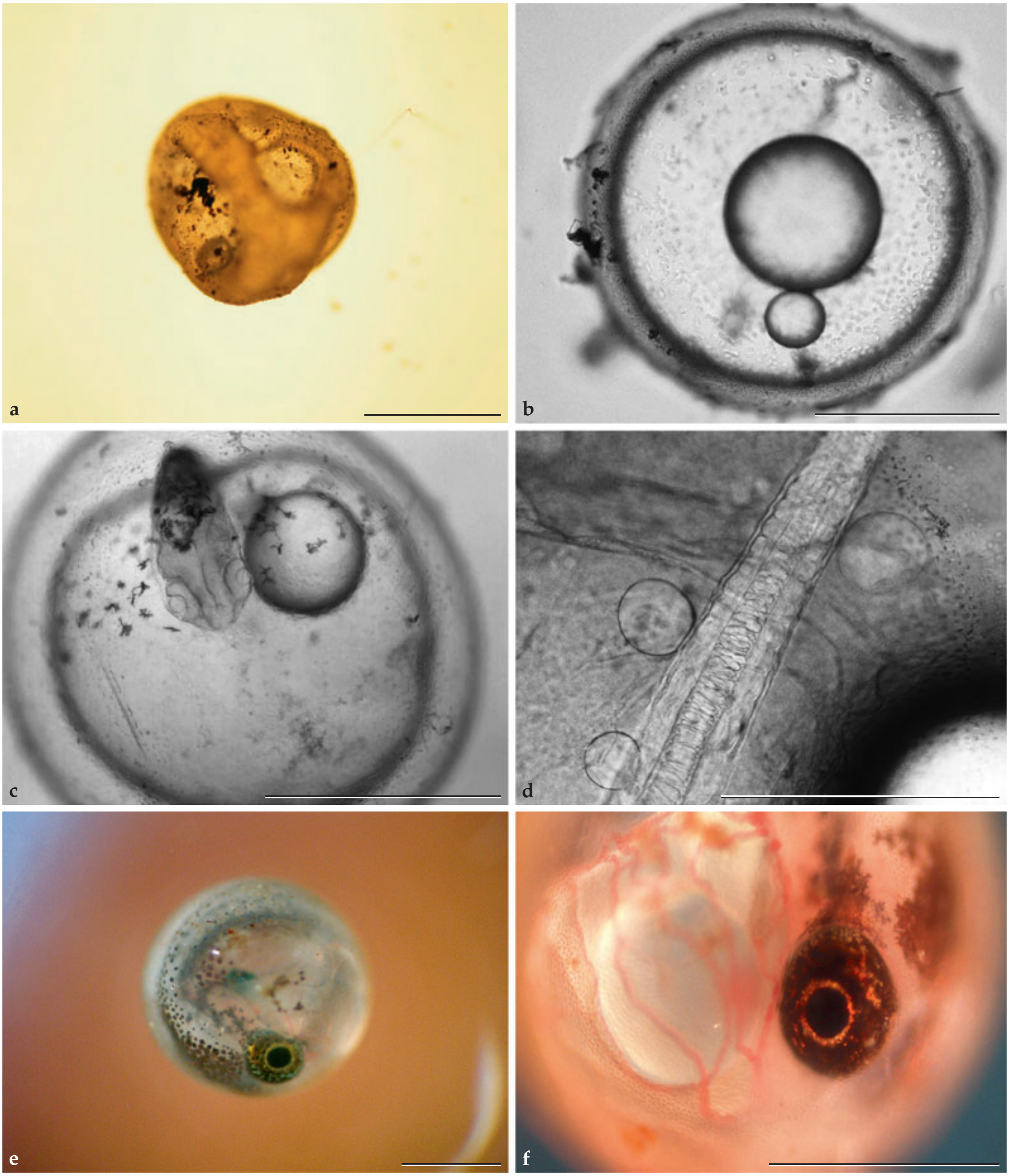

Fig. 2. Eggs of *Millerichthys robustus*. **a–b,** Diapause I (**a,** dehydrated obtained in field; **b,** in laboratory); **c–d,** Diapause II; and **e–f,** Diapause III. Scale bars 0.5 mm.

(1990a–b, 1998, 2009) used features of the chorion structure as phylogenetic characters. The egg surface of *Millerichthys* is similar to that of the annual fish belonging to genera *Cynopoecilus*, *Leptolebias*, *Notholebias* and *Ophtalmolebias* (all belonging to the subfamily Cynolebiatinae) in having mushroom-like projections (called m-projections in Costa, 2009), a synapomorphic condition not occurring in other aplocheiloid killifishes (Costa, 1990a–b; Fava & Toledo-Piza, 2007).

In *M. robustus* spawning occurs within the

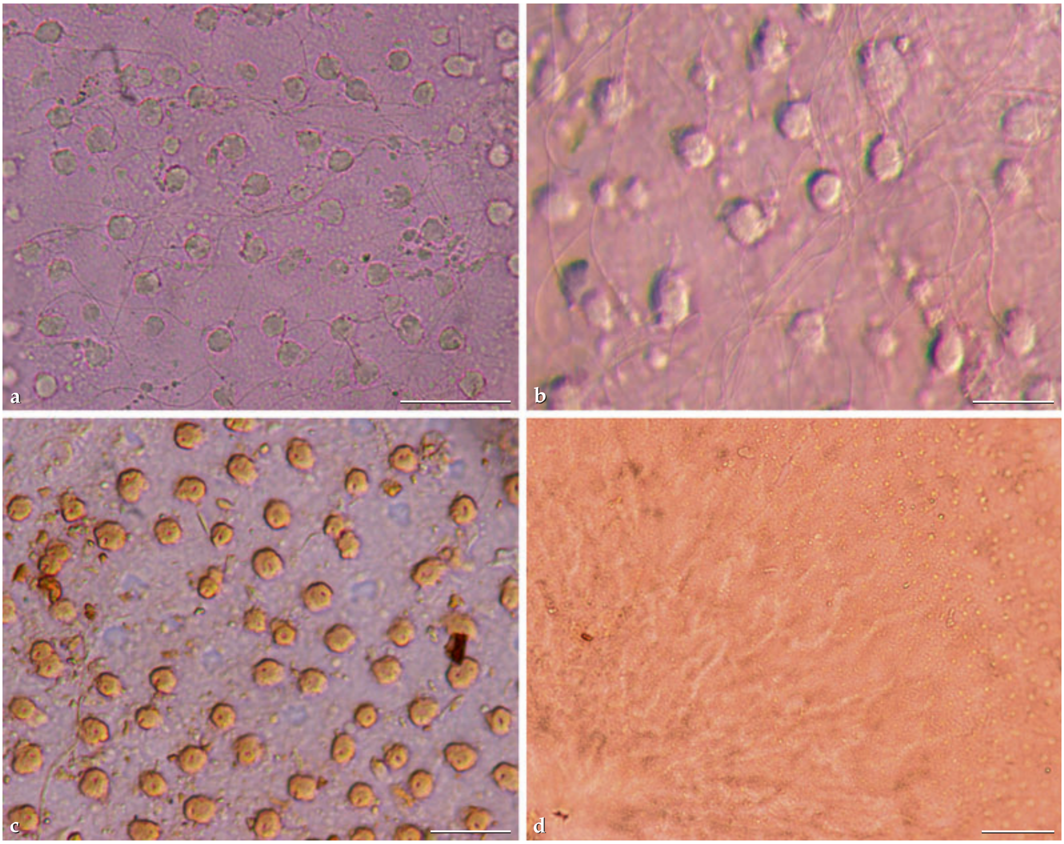

Fig. 3. Egg surface morphology of *Millerichthys robustus*. **a–b,** Projections with hair-like filaments on distal tip of eggs immediately after spawning; **c,** projections during Diapause III; **d,** projections around micropylar region. Scale bars: a–c, 20 µm; d, 100 µm.

substrate. A detailed description of its elaborated reproductive behaviour is in preparation by the authors. Our results about the reproductive nature corroborate Costa's classification, namely that *Millerichthys* is a member of an annual fish clade (Costa, 1998).

Millerichthys robustus is endemic to Mexico and is considered in the ecological norm NOM-059-Ecol-2001 in the list of species at risk in the category "in danger of extinction" (D.O.F., 2010). Efforts to collect this species by several investigators have been unsuccessful, however it was recently found near Tlacotalpan, Veracruz Mexico (Mosqueda-Cabrera & Ocampo-Jaimes, 2009); habitat loss has occurred, and the introduction of the piscivorous cichlid *Petenia splendida* in the Papaloapan river basin has had a negative impact (Miller, 2009). Finally, due to its conservation status and its high vulnerability, studies on the distribution, habitat, and current status of populations are required.

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