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Short Communication

Embryonic development stages description in *Gambusia puncticulata* (Pisces: Poeciliidae) from Almendares River, Cuba

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ABSTRACT. This research's main goal is the description of the embryonic developmental stages of *Gambusia puncticulata*. It also provides an analysis of the embryonic mean developmental stage, and mean weight in relation to the mother's carcass weight. The fish were caught monthly from July 2006 to June 2007 throughout Almendares River, North of Havana, Cuba. The embryonic mean stage values ($F_{4,600}$ = 14.79, P < 0.001), and mean weight values ($F_{4,395}$ = 7.09, P < 0.001), increased with mother's size increment. This research describes, for the first time, seven embryonic developmental stages in *G. puncticulata*. The applied macroscopic classification of the embryos showed great consistency, due to the large number of examined specimens. The criteria used to define the stages are accurate, and allow to distinguish the stages easily, quickly and with high reliability. These criteria could be applied to other species of the *Gambusia* genus.

Keywords: *Gambusia puncticulata***,** mosquitofish, poeciliids, embryonic stages, reproduction, Almendares River, Cuba.

The species of genus *Gambusia* (Family: Poeciliidae), have been used as a model to study basic process (reproduction, grown and recruitment) on rivers (Downhower *et al.*, 2000; Baber & Babbit, 2004; Dadda *et al.*, 2005). Viviparity is the reproductive strategy of these fishes (Koya *et al.*, 2000). Several authors proposed that the reproductive success of this group is due to the advantages offered by viviparity to these species, as: the protection of the developing embryos (Thibault & Schultz, 1978), the selection of better habitats to have their young (Constantz, 1989), and the transference of nutrients and immunity from the mother to the developing embryo (Lombardi, 1996).

The species of this family are used in different fields of research as genetic, oncology, ecology and developmental biology (Meffe & Snelson, 1993), given the capacity of poeciliids to respond quickly and easily to different environmental conditions with genotypic (Reznick *et al.*, 1990) and phenotypic changes (Rodd *et al.*, 1997). Several studies explain how poeciliids respond to variations in ecological factors, mostly predation (Downhower *et al.,* 2000), environmental stability (Stearns, 1983) and abiotic elements (Brown-Peterson & Peterson, 1990), including temperature and salinity (Meffe *et al.*, 1995; Nordlie & Mirandi, 1996).

In Cuba, these species are very important, because they are a component of the native freshwater ictiofauna, and a natural control of mosquito's larvae (Hernández-Contreras *et al.,* 2004). *Gambusia puncticulata* Poey 1854, is a very abundant species and it is widely distributed (Hernández, 2004). Many authors refer to its taxonomy (Fink, 1971; Alayo, 1973; Lee *et al*., 1983), others use this species as an indicator for anthropogenic impact (Cabrera *et al.*, 2008; 2012). However, there is no knowledge about its embryonic development. This paper describes, for the first time, the study of the development of *G. puncticulata* embryos captured along the Almendares River, Cuba, as a contribution to the knowledge about its embryonic cycle. The main goal was to describe their embryonic developmental stages, and to analyze the embryonic mean developmental stage, and mean weight in relation to the mother's carcass weight.

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The Almendares River (23º07’07”N, 82º24’31”W) is one of the most important watersheds in Cuba. In fact, 52.8% of the watershed is within the City of Havana and 47% of the city’s drinking water comes from groundwater below the basin. Unfortunately, overpopulation, local soil erosion, inadequate water use management, and intensive deforestation, have caused major reduction in the river water quality. Besides, significant and largely uncontrolled contaminant inputs, including heavy metals from industrial and urban sources as well as a large solid waste landfill, have contributed to increased river pollution (Advisory Committee for the Almendares-Vento Basis (ACAVB, 1999); Graham *et al.*, 2011; Cabrera *et al.*, 2012). A research on natural fish populations throughout the Almendares River was done. Using hand-held nylon nets (stretched mesh size 1 mm), every month from July 2006 to June 2007, 4301 *G. puncticulata* were captured in shallow waters near the shore. Fishes were transferred alive to the laboratory, at the Center of Marine Research, University of Havana, Cuba (~60 min), where they were killed by spinal severance before taking measurements and extracting embryos. All female specimens were dissected, but only those females carrying oocytes or embryos in any phase of their development were used. The total carcass wet weight (g) and total length (mm) of each female was recorded. The embryos were grouped according to their embryonic developmental stage. The embryos of each group were weighed separately, using analytical balances (±0.001 g), to determine the mean weight of embryos for each developmental stage. Embryos were classified according to a modified version of a scale proposed by Haynes (1995) for *Gambusia affinis* (Baird & Girard, 1854). A stereomicroscope coupled to a digital camera was used for the classification of the embryos according to their developmental stage. An ocular micrometer, calibrated to measure the embryos’ eggs and eyes at different stages of embryonic development was used.

The following reproductive variables were calculated: a) embryos mean stages, as the sum of the number of embryos multiplied by corresponding stage of development, and divided by total number of embryos for each female specimen; b) embryos mean weight, as the total weight of embryos from the same embryonic developmental stage divided by total number of embryos for each female specimen.

The analysis of variables (embryos mean stages and mean weight), was done on embryos from females that were previously grouped according to their carcass weight ranks (<0.3, 0.3-0.5, 0.5-0.7, 0.7-0.9, >0.9). This provides the advantage of reducing variability in the comparison of variables due to weight rank, and increases the power of the statistical tests used. Besides, this also allows to verify whether the variables remain the same in groups of different weights.

An analysis of variance (one-way, fixed effects ANOVA) was used to determine whether the embryos mean developmental stages and the embryos mean weight value varied among the class of females’ carcass weight. In cases where ANOVA yielded significant *F*-values, the Student-Newman-Keuls (SNK), post-hoc procedure was used to compare all pairs of means. Before performing the statistical analyses, normality of the data, and homogeneity of variances were examined according to the criteria of Zar (1996), and Underwood (1997). The significance level for all tests was 0.05. Statistica version 6.0 software was employed for data processing.

A number of 616 females were analyzed, this allowed to get a representative number of the female population with embryos at different developmental stages. The females examined in this research were mature and according to their average total length (33.4 mm) we suggest that the analyzed females have had offspring more than once because mean total length in this research exceeds the mean total length of sexual maturity described by Ponce de León *et al.* (2013) for this species. These authors determined that the mean total length at sexual maturity for *G. puncticulata* females was 27.20 mm during a study in captivity. As a rule, within the family Poeciliidae females reach larger sizes before being able to reproduce which is very important to guarantee the success of offspring development inside the ovary (Ponce de León *et al.*, 2013). Downhower *et al.* (2000) showed that late mature female’s populations are characterized by large sizes and produce higher offspring than females with early maturation.

In this research females showed a wide variability in terms of the number of embryos carried (from 1 to 112, depending on their developmental stage). This variability could depend on the morphological characteristics of the mother (size, ability to reproduce, age), environmental conditions (temperature, photoperiod, dissolved oxygen concentration, and changes in water levels), and human impact (water pollution). Reznick (1981) stated that the embryo´s size and female’s fecundity depend on the size of the given adult fish; larger females produce big and well-developed embryos (Fernández-Delgado & Rossomanno, 1997). Cabrera *et al.* (2008) found that the number of embryos increased with female’s size. This pattern coincides with that proposed by other authors for several species within the family Poeciliidae (Downhower *et al.*, 2000; Reznick *et al.* 2007; Ponce de León *et al.*, 2011, 2013; Zuñiga-Vega *et al.*, 2011). On the other hand, Vargas

& Sostoa (1996) stated that the fecundity peak of the species *G. holbrooki* Girard, 1859 occurs in July when females are bigger. Studies by Rosen & Bailey (1963) have reported that reproductive performance of females declines in autumn and winter in temperate areas. Burns (1985) found that females living in warm and steady areas can breed throughout the year. This suggests that the reproductive period can vary depending on the water temperature (Nordlie, 2000). However, Cabrera *et al.* (2008), found a decrease in gonadosomatic index, specific fecundity, and number of embryos of the species studied in the most contaminated sites on the Almendares River. It is due to the complex interactions among these factors so that an integrated study to determine the possible cause of this strong variability is required.

In this paper seven stages (from third to ninth) of embryonic development for *Gambusia puncticulata* are described. In addition, the characteristic of three phases that are not part of the embryonic development (the first two phases corresponding to the process of oogenesis and the last phase where the embryo is already a juvenile), are also detailed. These are described below:

Stage 1 (Oogenesis I): Small white eggs (0.2-0.3 mm) distributed throughout the ovary and frequently packed in a membrane (Fig. 1a).

Stage 2 (Oogenesis II): Eggs completely filled with yolk, these have a translucent golden yellow coloration and fat drops evenly disperse on the surface of yolk mass (Fig. 1b).

Stage 3: The first signs of egg fertilization are observed. A small circular spot on the upper surface of the yolk mass is detected, it´s considered as the blastodisc. There are also collapsed fat droplets under it (Fig. 1c).

Stage 4: The first traits of the embryo are observed. A white-lineal structure appears in the center of the yolk mass, it has a length (0.4-0.6 mm) very close to half the diameter of the yolk mass (Fig. 1d).

Stage 5: Signs of optic slit appear in the embryo (Fig. 1e).

Stage 6: Embryo´s eyes are observed but they are not completely pigmented. At first sight, it is detected that the head is bigger in comparison with the trunk. A primordium of the caudal and pectoral fins is observed. In many cases there is a pigmentation (melanophores) in the dorsal region and in the head. (Fig. 1f).

Stage 7: At this phase the eyes have a circular shape and fully pigmented but do not reach their maximum size (0.09-0.2 mm). The region of the head is no longer with respect to the trunk. A dorsal pigmentation and sometimes on both sides of the body is observed. A primordium of the anal and dorsal fins may be observed. Caudal fin has small rays in formation. Generally the mouth is practically inside the yolk sac until the eyes (Fig. 1g).

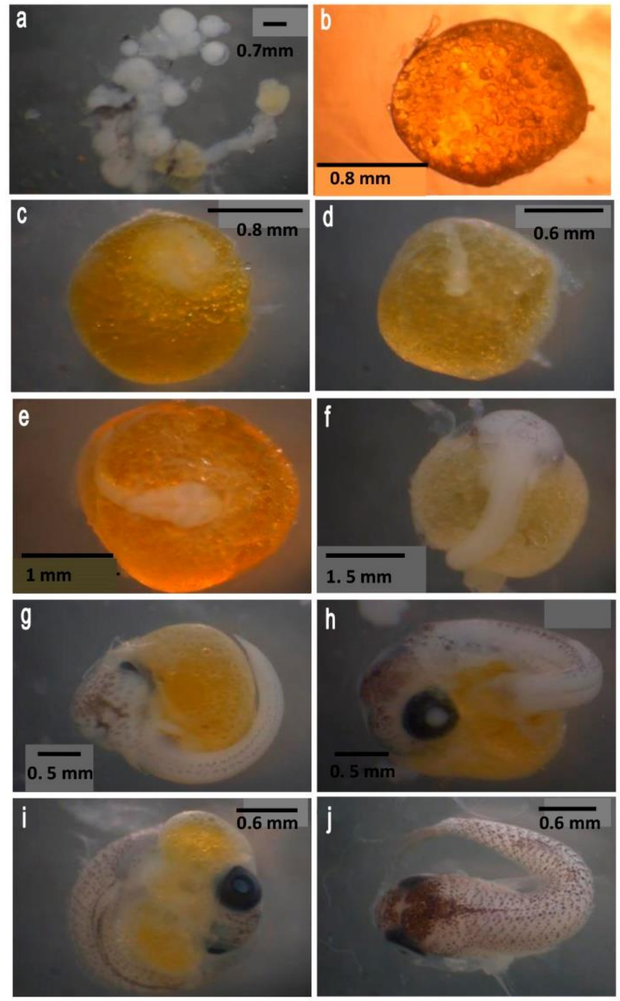

Figure 1. Embryos development stages of species *Gambusia puncticulata* from Almendares River. a) Stage 1 (20x), b) stage 2 (25x), c) stage 3 (25x), d) stage 4 (30x), e) stage 5 (25x), f) stage 6 (30x), g) stage 7 (20x), h) stage 8 (20x), i) stage 9 (25x), j) stage 10 (20x).

Stage 8: The embryo is characterized by having larger eyes (0.23-0.32 mm) but not reaching full size. It has moderate dorsal pigmentation (melanophores) and also along the lateral line. Caudal fin may be bent over the head or around the mouth and the rays of the pectoral fins are visible. A large yolk sac is observed (Fig. 1h).

Stage 9: At this phase the eyes reaches its maximum size (0.37-0.42 mm). Small rays are observed on anal and dorsal fin. The embryo is larger (~2 mm) and the yolk sac, if present, is small and irregular (Fig. 1i).

Stage 10 (juvenile phase): In most cases the yolk sac is completely consumed, although sometimes a remain-

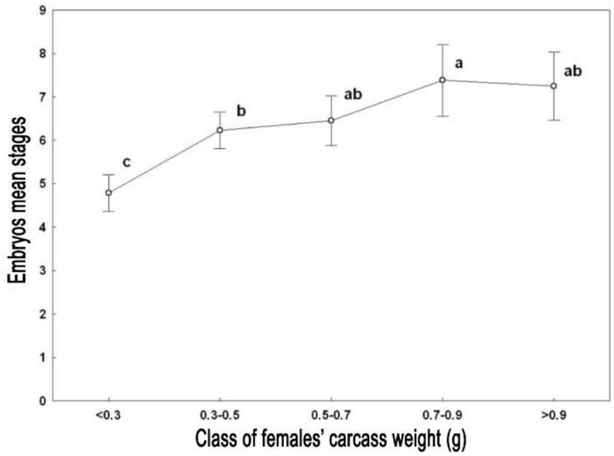

Figure 2. Embryos mean stages and confidence intervals (0.95) for each class of females' carcass weight from Almendares River.

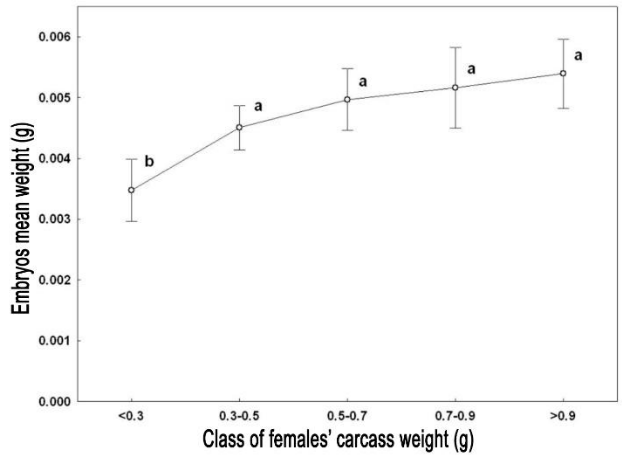

Figure 3. Embryos mean weight and confidence intervals (0.95) for each class of females' carcass weight from Almendares River.

der of it can be observed. Pectoral fins are large and scales appear throughout the body. This embryo is already a juvenile (Fig. 1j).

Haynes (1995) described 11 stages of embryo development for *G. affinis* and considered this classification to be meaningful for other species within the genus. Abney & Rakocinski (2004), used this scale for the study of embryos of *G. puncticulata* in the Cayman Islands. In general terms, this scale is appropriated for the species studied in our research. However, we observed some differences with Haynes' scale that should be taken into consideration. Some of these discrepancies include mean features such as the portal system in stage sixth and the formation of the operculum in stage eighth observed by Haynes (1995), which was not observed in our study. The author mentioned, observed and described an intermediate stage that would be located between first and second stage of our study. This intermediate stage is characterized by yolk eggs which does not reach its maximum size. These yolk eggs have an opaque yellow-orange color and may also have unevenly distributed fat droplets. This phase was not observed in our study. A feature observed in the final stage of the scale, defined in this work, was the presence of a remainder yolk sac in some cases, which was not seen by Haynes (1995).

There are several classifications of embryo's development stages reported in the literature regarding the genus *Gambusia* (Meffe, 1985; Brown-Peterson & Peterson, 1990; Cabral & Marques, 1999). These scales vary in the number of stages proposed, as well as in the features used. However, Oppenheimer (1937) proposed that for the classification of embryos, easily visible features should be used, and each stage should be distinguished without the need of a histological study. This agrees with the classification proposed in this research. Haynes (1995) uses the relative size of the eyes, during the embryonic development, as a main feature to take into account in the classification. However, one feature is not enough to distinguish each stage; this is only possible when several features are observed at the same time, including eye size, presence of fins and pigmentation of the skin, among others.

An analysis of the reproductive variables showed an increase in the mean embryo's stage ($F_{4,600} = 14.79$, $P < 0.001$), and mean embryo weight ($F_{4,395} = 7.09$, $P < 0.001$), corresponding to the increase in female size (Figs. 2-3). In general, as has been pointed out for teleosts, the fecundity in the species of the *Gambusia* genus depends on the female total length (Reznick, 1981; Downhower *et al.*, 2000; Reznick *et al.* 2007; Cabrera *et al.*, 2008; Zuñiga-Vega *et al.*, 2011; Ponce *et al.*, 2013). This agrees with our results. However, no literature has been found to document the increase in mean weight and mean stage of development of embryos with increasing female's weight. A possible reason for this result is that the proportion of embryos reaching the final stage of development is lower in the smaller females or may be due to the natural variability of fecundity in the species studied. However, more detailed research is needed to test this explanation. Marsh-Matthews & Brooks (2005) reported the reabsorption process in developing embryos in the species *G. affinis* and *G. geiseri* Hubbs & Hubbs, 1957, when they were injected with leucina C^{14} substance, during their experiment, which means that some embryos do not reach the final stage of development. These authors suggest that this event rarely occurs, although it has been observed in some occasions for these species.

In summary, the macroscopic classification of the embryos showed great consistency, due to the large number of examined specimens. The criteria used to define the stages are accurate, and allow to distinguish the stages easily, quickly and with high reliability. These criteria could be applied to other species of the *Gambusia* genus.

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