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The ontogenetic development of egg-spots in the haplochromine cichlid fish *Astatotilapia burtoni*

C. Heule and W. Salzburger*

Zoological Institute, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland

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A series of *Astatotilapia burtoni* photographs were used to investigate the ontogenetic development of male egg-spots, a putative evolutionary key innovation of haplochromine cichlids. Four stages of egg-spot development were defined and all males had developed true egg-spots (stage 4) before reaching a standard length of 25 mm. Raising condition only slightly influenced the timing of the first appearance of true egg-spots.

Key words: adaptive radiation; key innovation; ontogeny; egg-dummies; photographic series.

The species flocks of cichlid fishes in the East African Great Lakes Victoria, Malawi and Tanganyika represent the most species-rich and eco-morphologically diverse animal adaptive radiations (Seehausen, 2006; Salzburger, 2009). At least 1500 cichlid species have evolved in East Africa in only the last few thousands to several millions of years (Salzburger, 2009). The species differ greatly in ecologically relevant, *i.e.* naturally selected, structures such as body morphology, jaw and tooth shape, and also in sexually selected traits such as colouration (Salzburger, 2009). The vast majority of East Africa's lacustrine and riverine cichlids and all species of the species flocks of Lakes Victoria and Malawi belong to a single 'tribe' of cichlids, the Haplochromini (Salzburger *et al.,* 2005). Haplochromine cichlids are characterized by a slight modification of the pharyngeal jaw apparatus, pronounced sexual colour dimorphism, derived maternal mouth-brooding behaviour and, in most species, anal fin egg-spots (Fryer & Iles, 1972; Greenwood, 1979; Salzburger *et al*., 2005). Their reproductive system involving polychromatism, female mouth-brooding and egg-spots has been suggested to be one of the evolutionary key innovations responsible for the haplochromines' propensity for explosive speciation (Salzburger *et al.,* 2005, 2007).

Egg-spots or 'egg-dummies' are conspicuous orange, yellow or red spots with an outer transparent circle, which appear on the anal fin of most males of the derived 'modern haplochromines' (Wickler, 1962*a*; Fryer & Iles, 1972; Salzburger *et al*.,

*Author to whom correspondence should be addressed. Tel.: +41 61 267 03 03; email: walter.salzburger@unibas.ch

2005). Note that egg-spots may also occur on females' anal fins, but in a less perceptible manner. Egg-spots are involved in the modern haplochromines' courtship behaviour. Usually, a male initiates courtship by displaying his nuptial colouration (and egg-spots) while quivering in order to attract the female. The female lays a batch of eggs in the pit, which was created by the male in his territory. She then swims a circle and picks the eggs up in her mouth. Seemingly attracted by the colourful egg-spots on the male anal fin, she approaches the male's genital opening where she attempts to take up these putative 'egg-dummies'. In this moment the male releases the sperm, which fertilizes the eggs in the female's buccal cavity (Wickler, 1962*a*, *b*). The female then carries the larvae until fry are fully developed (which may last for several weeks). This leads to fewer but larger eggs that are well protected in the buccal cavity of the mouth-brooding female. Despite the importance of this trait in cichlid evolution (Salzburger *et al.,* 2007), little is known about the developmental basis of egg-spot formation.

In this study, the ontogenetic development of egg-spots was investigated in Burton's haplo *Astatotilapia burtoni* (Günther 1894), a cichlid species living in Lake Tanganyika and its surrounding affluent rivers. It belongs to the modern haplochromines and is, hence, a maternal mouth-brooder (Salzburger *et al.,* 2005). *Astatotilapia burtoni* has been established as a model system to study questions from various fields of biology such as behaviour, ecology, genetics, genomics and development (Salzburger *et al*., 2008). Most importantly, its genome is one of the five cichlid genomes that are currently being sequenced.

Almost 50 years ago, Wickler observed that egg-spots in *A. burtoni* occur only later in ontogeny (Wickler, 1962*b*) and that, in the beginning, the anal fin is transparent. Egg-spot formation starts with a yellowish pattern covering the fin, which then forms blotches that finally become surrounded by an outer transparent circle. The present study focused on the ontogenetic development of egg-spots in *A. burtoni* raised under different conditions. Of main interest were (1) the timing of egg-spot formation with respect to age, body size and mass and (2) the influence of raising condition on egg-spot development.

In order to answer these questions, egg-spot formation was investigated in two main set-ups: a group set-up and an individual series. For the group set-up, *c*. 30 fry of an inbred laboratory strain were taken out of a female's mouth and kept in a net measuring 16·5 × 13 × 13 cm that was placed in a small tank (group 1). In addition, *c*. 30 fish were kept in a small tank measuring 40 × 25 × 25 cm (group 2). They were already 2 months old when starting the experiment. The fish in both tanks were fed with a similar amount of regular flake food. The following procedure was performed every 2–3 days during a period of 12 and 8 weeks, respectively: Six random individuals of each group were anaesthetized with 130 mg l^{-1} MS222 (Sigma-Aldrich; www.sigmaaldrich.com) for 1 min, weighed with a scale (KERN 440-3A, Kern & Sohn Gmbh; www.Kern-Sohn.com) and photographed with a Nikon D40 (www.nikon.com), using a macro lens (Tokina AT-X MACRO 90 mm 1:2.5; www.tokinalens.com) in a specially designed photo-cuvette. This cuvette made it possible to take photographs of the fish in water, so the fish were less stressed. In addition, the fins were well spread in these cuvettes. Usually the photographs were taken in the morning and then used to measure the standard length (L_S) (using the TPSDIG software from Rohlf, 2006) ($n = 158$) and to define the timing of egg-spot development ($n = 1500$). Data were displayed with R (R Developmental Core

Team; www.r-project.org). For the individual series, 14 fish of age 18 dpf (days post fertilization) were kept isolated in nets measuring 16·5 × 13 × 13 cm that were all placed in the same tank. All individuals were photographed and weighed twice a week for a period of 14 weeks (series A) following the above-mentioned procedure. As seven individuals escaped, six fish of about the same size but of a different age (90 dpf) were added in week 7 (series B). These individual fish were also fed with about the same amount of regular flake food. A total of 2854 photographs were taken from the fish of the individual series to define the timing of egg-spot formation; 121 of these were used to measure L_S in males. Additionally, a movie file was created from individual images showing the ontogeny of one male (18–113 dpf) (Movie S1, Supporting information).

The analysis of the group set-up revealed differences in growth rate between group 1 and group 2 [Fig. 1(a)]. The same was true for series A and series B of the individual series [Fig. 1(a)]. The individual series was used to define stages of egg-spot development in *A. burtoni* males, because here fish could be sexed retrospectively. Therefore, only males were used for further analyses. Of the 13 fish raised individually, six were males. The ontogenetic development of egg-spots in one male over 3 months (first 24 photographs) and one additional photograph after a year are shown in Fig. 2. The photographs were inspected by eye and four stages were defined, which are highlighted in different colours. The first stage (green border strip in Fig. 2) was categorized by a transparent anal fin with some yellow pigments only, particularly at the first three fin rays. The second stage (orange) was characterized by the occurrence of orange pigments (arrowhead in Fig. 2). In the third stage (blue) the orange pigments accumulated into amorphic spots. Only in the fourth stage (red), fully developed egg-spots with an outer transparent circle became visible. In all cases, also in the group treatment, there was an initial number

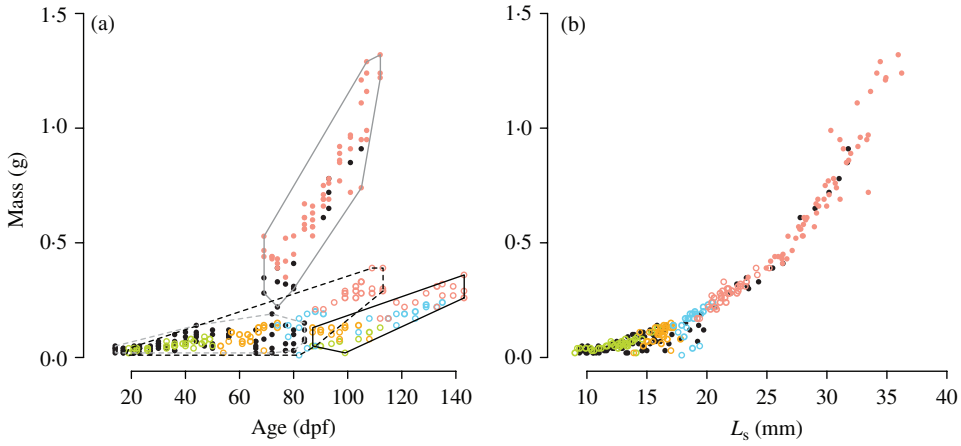

FIG. 1. Ontogenetic development of egg-spots in *Astatotilapia burtoni*, plotted as mass (a) at age in days post fertilization (dpf) and (b) at standard length (L_S). •, group set-up stages 1–3; •, group set-up stage 4; ○, individual series stage 1; ○, individual series stage 2; ○, individual series stage 3; ○, individual series stage 4. The outlines indicate the different set-ups. - - - -, group 1; ——, group 2; - - - -, individual series A; ——, individual series B.

of three egg-spots. Only later in development did this number increase. Females developed egg-spot-like structures that were less conspicuous and occurred much later.

The remaining photographs of the individual series were then inspected by eye and classified into one of the four stages defined above. The same procedure was performed with photographs of the group set-up, although here the main focus was on the identification of the age, size and mass at which true egg-spots in males appeared (*i.e.* stage 4). Note that the classification into stages 1–3 was more problematic in the group set-up, as unlike in the individual series, fish could not be sexed retrospectively. As mentioned above, the distinct egg-spot morphology of females could bias the classification. The data from the group set-up and the individual series were then jointly plotted (Fig. 1). The correlation between age and mass [Fig. 1(a)] was confounded by treatment effects in the different set-ups of groups 1 and 2 and series A and B, whereas treatment did not have an apparent effect on the correlation between L_S and mass [Fig. 1(b)]. As a consequence, L_S was used to describe egg-spot appearance.

This study showed that, in *A. burtoni*, growth rate and the appearance of egg-spots do not depend on age, but rather on the condition in which the fish had been raised. For example, in group 1, where density was higher compared to group 2, fish were smaller at the same age [Fig. 1(a)]. Also, in the individual series, fish of series B were smaller at the same age compared to series A. Note that fish of series B were added 3 months after starting the experiment, but had been raised before in a small tank at higher density than the isolated fish from series A. In the group set-up, growth differences between the groups had an effect on pigmentation, as the orange pigmentation (stage 2) was detectable at a smaller size in group 1 compared to group 2. In contrast, in the individual series, the first detectable orange pigments appeared at the same L_S, but at a much younger age in the fish from series A compared to those of series B. This variation in the timing of the occurrence of the orange pigmentation might be explained by differences in food uptake. Egg-spots are a costly trait, as the orange colour comes from carotenoids imbedded in chromatophores. It is these carotenoids that cannot be synthesized by fishes, so they have to be taken up with nutrition (Endler, 1983; Kodric-Brown, 1985). As the isolated fish did not have to compete for food, they probably had more nutrition available. This would explain why the fish that had been isolated for their whole life (series A) were able to develop their egg-spots at a younger age than those of series B. Alternatively, isolation as such and light condition (it was darker in the small nets than in glass aquaria) might have had an effect on pigmentation. For example, Goldschmidt (1991) has shown that light intensity influenced egg-spot size in Lake Victoria cichlids.

Taken together, four stages of egg-spot formation were defined in *A. burtoni* (Fig. 2). True egg-spots (stage 4) were first observed in an individual male at $L_S = 19{\cdot}2$ mm in the individual series. Although egg-spots appeared at slightly smaller L_S in the individual series, all males showed egg-spots at 25 mm L_S [Fig. 1(b)]. Any fish >25 mm and without egg-spots [black dots in Fig. 1(b)] was most certainly a female. On the basis of the observed differences in pigmentation between group set-up and individual series, it is suggested that individually marked fish are raised in group tanks to rule out condition effects (*e.g.* isolation and light). Future studies on egg-spot development should include other haplochromine species.

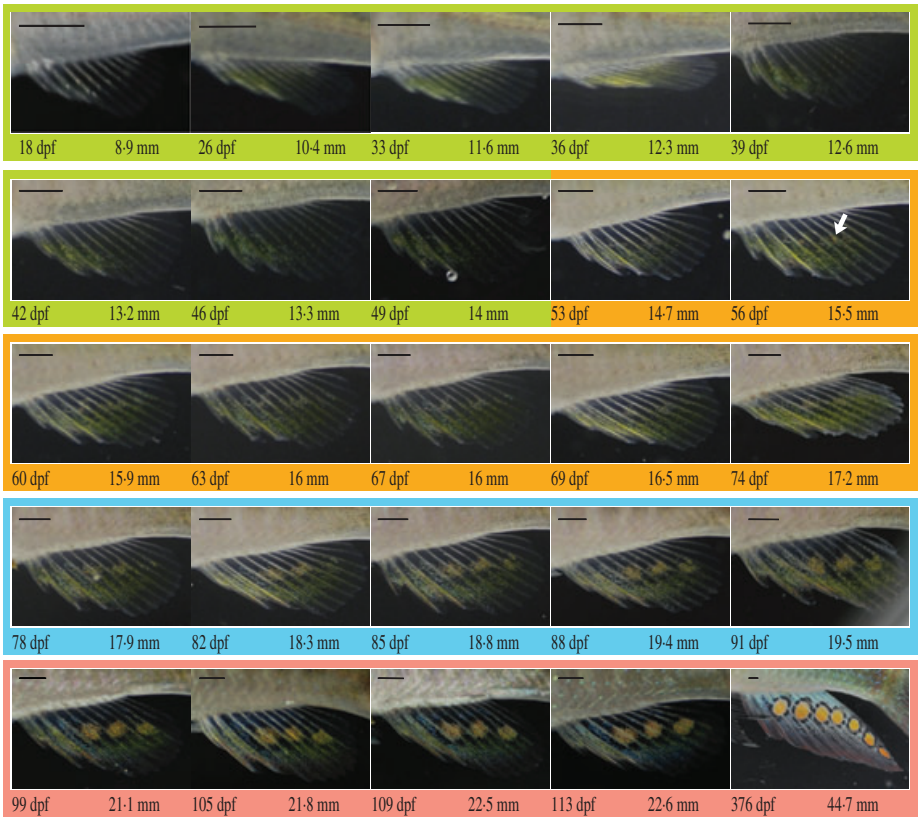

FIG. 2. Pictures series showing the anal fin of an individual male of *Astatotilapia burtoni*. The age (in days post fertilization, dpf) and the standard length, L_S, at every measurement are given below each photograph; scale bars represent 1 mm. Colours indicate the four stages of egg-spot development. ⇨, orange pigments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Movie S1. Egg-spot development in *Astatotilapia burtoni*.

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