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Initial Feeding Behaviour, Eye Structure and Effect of Colours on Prey Capture Rates of *Betta splendens* Larvae

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ABSTRACT

Basic studies on early morphology and behaviour are necessary to support applied research on larval rearing. Thus, the aim of this study was to analyze the initial feeding behaviour, eye structure and the influence of different background colours (red, blue and clear) on prey capture rates (*Artemia* sp.) of *Betta splendens* larvae. The retina was formed by 90 h post-fertilization and consisted of seven layers. The feeding behaviour of the larvae changed with ocular development and thus, prey were captured more efficiently with time. No significant difference ($p>0.05$) was observed between the different background colours on prey capture rates under the experimental conditions tested (10 min of exposure). In conclusion, *B. splendens* larvae displayed a typical feeding behaviour, which changed with larval development and was dependent on visual ontogeny. Even though the different background colours did not significantly affect feeding behaviour, our findings provide important insights for future studies on this field.

Key words: Aquaculture, ornamental fish, morphology, microscopy

INTRODUCTION

The transition of fish larvae from endogenous to exogenous feeding is a critical process in aquaculture due to the high mortality rates observed (Yufera and Darias, 2007). Since, larval development is a very quickly and complex process (Gisbert *et al*., 2002), fish larvae prioritize some structures among your initial development, like head and tail (Osse *et al*., 1997). During this time, the eye is a very important organ for the success in the capture of prey and reception of visual stimuli (Tesser and Portella, 2006), being considered, in some species, a key structural along this period (Chai *et al*., 2006).

Environmental stimuli are known to influence some aspects of fish larvae biology at first feeding such as colour (Pedreira *et al*., 2012; Carvalho *et al*., 2013; Rahnama *et al*., 2015); size (Bailey, 1984) and prey density (Zavala-Leal *et al*., 2013). Specifically, several studies have been indicating the effect of red (Volpato *et al*., 2013) and blue (Volpato and Barreto, 2001; Pedreira *et al*., 2012; Ngugi *et al*., 2014) colour on feeding, growth and stress of fish. According to Pedreira *et al*. (2012) *Prochilodus costatus* larvae appear to see their prey better in blue tanks. Additionally, in a mating choice, mature female of *Betta splendens* (Regan, 1910) preferred a red male than a blue one (Clotfelter *et al*., 2007).

Taking this in to account, we assume that maybe these colours could affect larval feeding behaviour in *B. splendens* larvae, an ornamental fish of great importance in the aquarium hobby. However, basic studies on early morphology and behaviour are necessary to support applied research. Consequently, firstly we analyse the early feeding biology and eye structure in *B. splendens* larvae; afterwards, we look at the effects of different background colours on prey capture rates. The data obtained here will increase the knowledge about the dynamic of initial behaviour and an improvement in the rearing protocols could be future achieved.

MATERIALS AND METHODS

Local and fish: The experiment was conducted at the Ornamental Fish Laboratory of the Aquaculture Centre of the São Paulo State University-UNESP (CAUNESP), Jaboticabal-SP, Brazil. Six pairs sexually mature *Betta splendens* were reared in individually 2 L aquaria and were fed twice daily (to apparent satiety) with commercial pellets (AQUAXCEL 4512 Purina®) composed of 45% crude protein. When the males presented the bubble nest building behaviour, individually female were placed into perforated transparent plastic containers inside the male aquaria for 24 h (with males outside of the container). This enabled the exchange of water and encouraged courtship behaviour. Afterwards, the females were liberated and when spawning occurred, the fishes were removed and the larvae obtained reared in a water recirculation system with controlled temperature (28°C) and photoperiod (12 h light: 12 h dark). Water pH and dissolved-oxygen was 7.2±0.4 and 5.3±1.2 mg L^{-1}, respectively.

Sample collection and preparation for histology: For histological analysis, samples (larvae; n = 6) were collected at the following times: 21 h post-fertilization (hpf, Time zero), then every 1:30 h (h) up to 30 hpf, followed by every 2 h up to 48 hpf (2 days), then every 4 h up to 72 hpf (3 days) and finally every 6 h up to 120 hpf (5 days).

The larvae were euthanized with benzocaine, fixed in 4% formaldehyde solution (in buffer phosphate 0.1 M, pH 7.4), dehydrated in increasing concentrations of ethanol, cleared in Xylene and embedded in Histosec® (Merck, Germany). Samples were serially sectioned (5 µm), mounted onto slides and stained with Haematoxylin and Eosin prior to light microscopy analysis (Tolosa *et al*., 2003).

Development of feeding behaviour: Five larvae were analysed at 108, 120, 132, 144, 156 and 168 hpf. Each larva was transferred to a petri dish (34 mm diameter) containing a clear plastic ring of 0.5 cm in diameter (physical limitation). The petri dish was attached to the base of a stereomicroscope (Leica MZ 8) equipped with a digital camera (Canon PowerShot SX120IS, Japan) that was used to record the videos (Fig. 1). Larvae were deprived of food for 12 h and acclimatized inside the plastic ring for 10 min before being recorded. Each recording lasted for 6 min: 3 min without prey (larvae alone) and 3 min with prey (*Artemia* sp.). If the *Artemia* sp. nauplii were totally consumed before the end of the recording, more was gradually added (All prey consumed were considered for the statistical analysis). The recordings were visualized three times for manual counting (number of occasions) of the categories listed below:

- **Successful attack:** A rapid movement towards the prey that culminates with ingestion
- **Eye movement:** Any eye movement (to the right, left, up or down) with one or both eyes
- **Pursuit:** Movement in direction to the prey which could or not culminate in a successful/unsuccessful attack

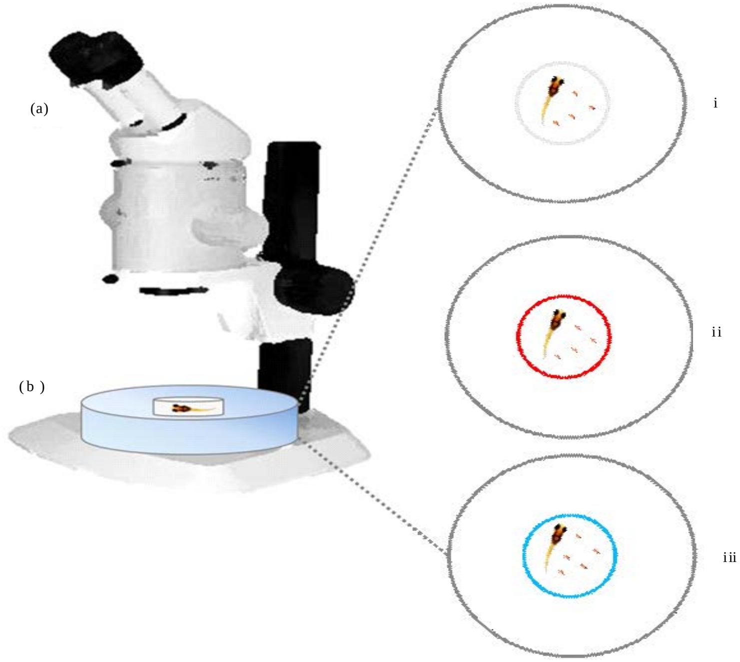

Fig. 1: (a-b): Illustration of the experimental method used to evaluate the feeding behaviour of *B. splendens* larvae and the effects of different background colours on prey capture rates at 10 days post-hatching (a) Stereomicroscope and (b) Petri dish containing (i) a clear, (ii) red and (iii) blue plastic ring surrounding the larva and prey

Effect of different background colours on feeding behaviour: Ten-day old larvae were placed individually on petri dishes containing red, blue or clear plastic rings (Fig. 1) under the same conditions. Larvae were food deprived for 12 h and acclimatized inside the plastic rings for 10 min before having their behaviour recorded. Each recording lasted for 10 min: 3 min without food (larvae alone) and 7 min with prey *Artemia* sp. (approximately 10 nauplii). If the prey was totally consumed before the end of the observation period, more was gradually added. This procedure was repeated ten times for each colour tested and the behaviours analysed were: successful attack, pursuit of prey and eye movement (categories described above). Eye movement during feeding was analysed for 3 min and compared to that of the control (without *Artemia* sp.).

Statistical analysis: The data was tested for normality (Lilliefors test at 5%), subjected to Analysis of Variance (ANOVA) and the means compared by Tukey's test (5%) using the statistical software STATISTICA (Version 10.0, Statsoft, Tulsa, U.S.A.). In the success attack behaviour, we express the dada as percentage (%) because some larvae can ingest more *Artemia* sp. nauplii than others. In addition, this variable were arc sin (√%) transformed to meet the assumptions of normality for parametric analysis.

RESULTS

Eye and retina histology: At 24 hpf, the rudimentary retina consisted of a pseudo-stratified layer of elongated undifferentiated neuroblasts and the lens could be seen as a solid spherical mass

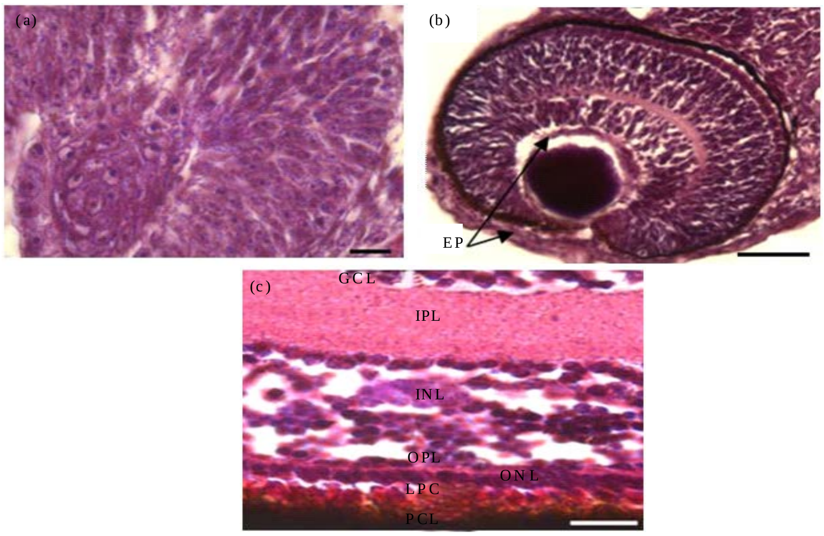

Fig. 2(a-c): Photomicrography of the eye of *B. splendens* at different stages of larval development (h post-fertilization: hpf) (a) 24 hpf: Elongated and undifferentiated cells, scale: 10 μm, (b) 30 hpf: Epithelial cells enveloping the eye and the lens, scale: 50 μm and (c) 90 hpf: Seven retinal layers, scale: 20 μm, EP: Epithelial cells, GCL: Ganglion cells layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer, LPC: Layer of photoreceptor, PCL: Pigmented cells layer

(Fig. 2a). At 30 hpf, epithelial cells enveloped the eye and lens (Fig. 2b) and the inner plexiform layer and the precursor layers of photoreceptors and pigmented cells were also present (Fig. 2b). From this stage onwards the lens gradually separated from the enveloping epithelium. By 90 hpf, seven retinal layers could be seen: Ganglion Cells Layer (GCL), Inner Plexiform Layer (IPL), Inner Nuclear Layer (INL), Outer Plexiform Layer (OPL), Outer Nuclear Layer (ONL), Layer of Photoreceptors Cells (LPC) and Pigmented Epithelium (PE) (Fig. 2c).

Development of feeding behaviour: Recordings were performed from 108-168 hpf. The initial recording time (108 hpf) was chosen based on the first displays of predatory behaviour inside the rings, even though some larvae were seen eating in the tanks within 72 hpf. The process of visual perception of the prey, recognition, pursuit and attack took only a few seconds and are detailed in Fig. 3. Eye convergence was observed during pursuit and ingestion of prey but normal eye orientation was restored once the prey was fully ingested (Fig. 3). Additionally, larval J-turn morphology could be seen concomitantly to eye convergence (Fig. 3).

Pursuit decreased with age (ANOVA, $p = 0.002$; Fig. 4a) while successful attacks gradually increased and unsuccessful attacks decreased (ANOVA, $p<0.0001$, Fig. 4b). In absence of prey, eye movement showed no variation as age progressed (ANOVA, $p = 0.08$, Fig. 5). However, in presence of food, the values increased with development (ANOVA, $p = 0.0006$, Fig. 5). In each time, more values were observed when prey is present (Fig. 5).

Effect of different background colours on feeding behaviour: As observed, with 90 hpf (4 days) the retina has seven layers and with 168 hpf (7 days) the larvae showed an well-developed

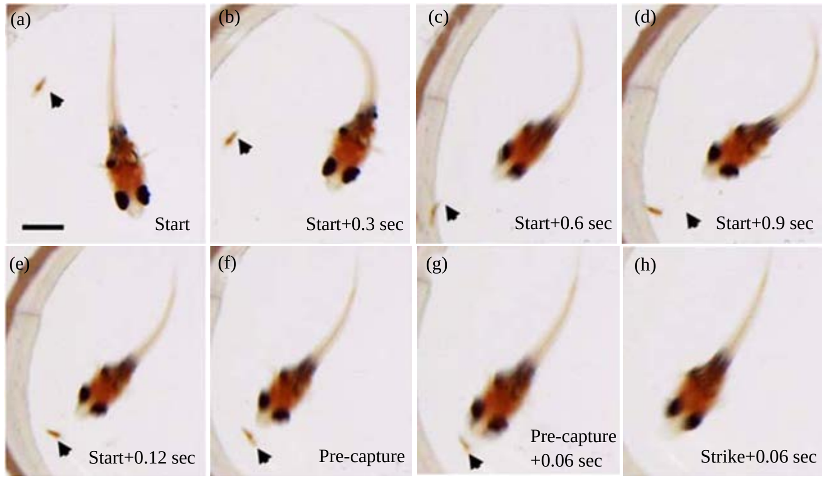

Fig. 3(a-h): Steps of prey-capture behavior in *B. splendens* larvae (168 h post-fertilization) using *Artemia* sp. (black arrow) (a-d): Prey perception and change of body orientation and (e-h): Pursuit of prey after recognition leading to successful attack

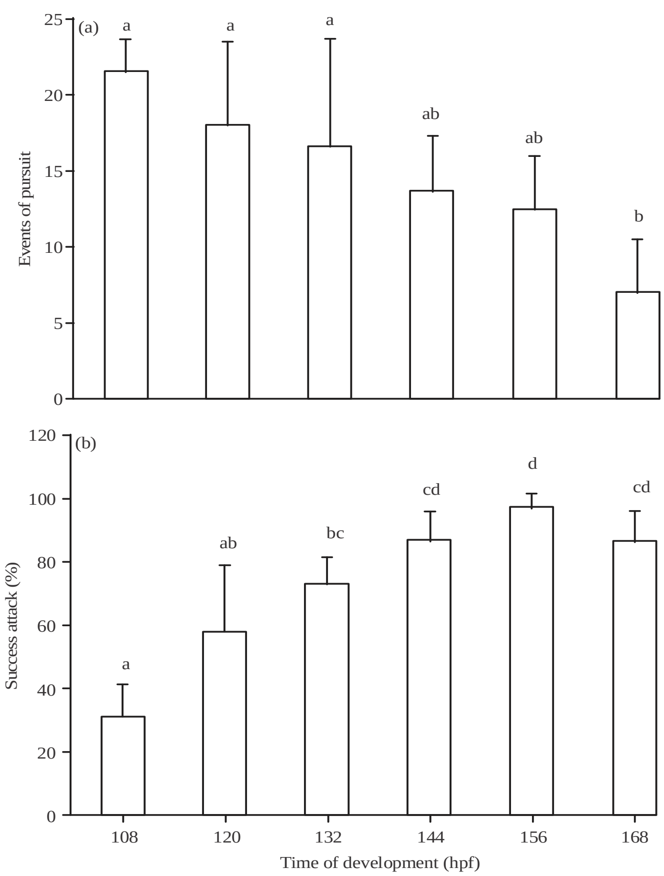

Fig. 4(a-b): Effect of the time (hours post-fertilization-hpf) on prey pursuit events (a) ANOVA, $p = 0.002$, 3 min) and percentage of successful attack and (b) ANOVA, $p<0.0001$, 3 min) by *B. splendens* larvae. Means±SD, Bars that share at least on letter belong to the same non-significant range (ANOVA, $p<0.05$)

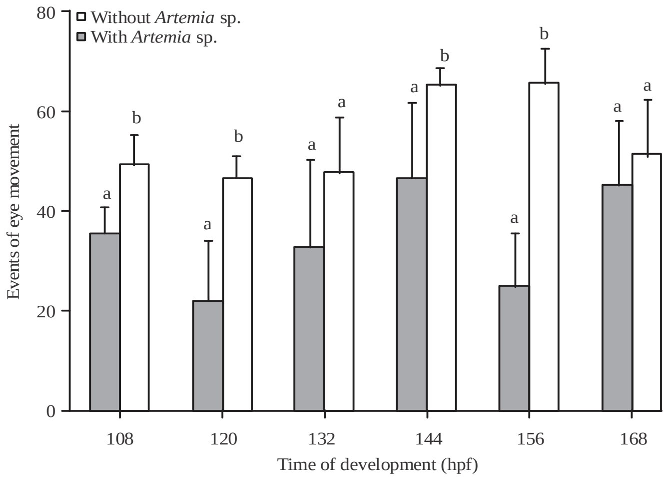

Fig. 5: Effect of the time (hours post-fertilization-hpf) on eye movement of *B. splendens* larvae in the presence (p = 0.0006, 3 min) and absence (p = 0.08, 3 min) of prey (*Artemia* sp.). Means±SD. In time, bars that share one letter belong to the same non-significant range (ANOVA, p<0.05)

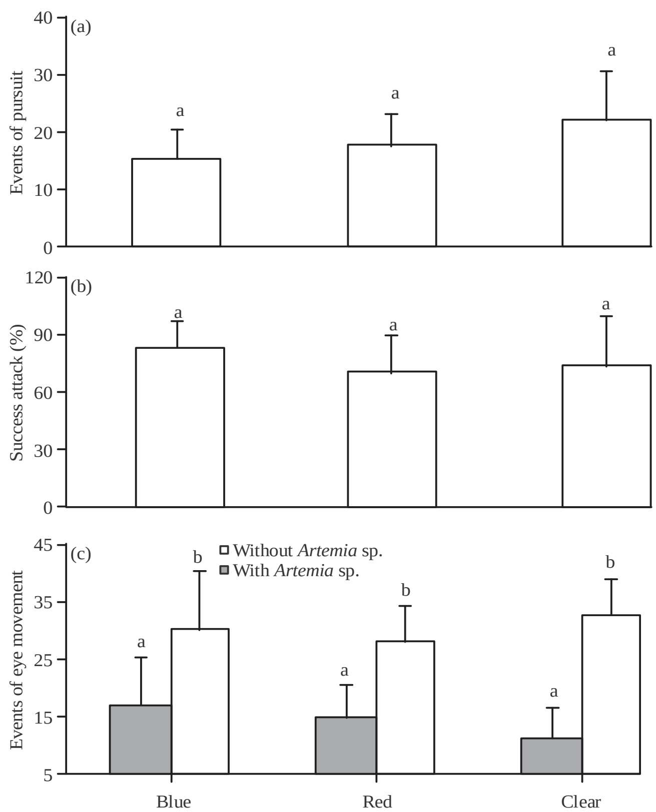

Fig. 6(a-c): Effect of different background colours (blue, red and clear) on prey pursuit rates (a) (p = 0.07, 7 min), percentage of successful attacks, (b) (p = 0.30, 7 min) and eye movement rates and (c) (p = 0.25, 3 min) of ten-day old *B. splendens* larvae. Means±SD, Bars that share at least on letter belong to the same non-significant range (ANOVA, p<0.05) feeding behaviour. Then, in this experiment, we use Ten-day old larvae to guarantee the complete eye development and an active feeding behaviour. However, no significant difference was observed between the different background colours regarding pursuit of prey (ANOVA, p = 0.07, Fig. 6a), successful attack (ANOVA, p = 0.30, Fig. 6b) or eye movement rates (ANOVA, p = 0.25, Fig. 6c). However, the density of prey markedly influenced the ability of larvae to find the food, with lower densities resulting in more time being spent searching for the prey.

DISCUSSION

The retina of *Betta splendens* was formed (with seven layers) within 4 days and similar reports were observed on zebrafish (*Danio rerio*) (Gahtan *et al*., 2005) and catfish (*Pangasianodon hypophthalmus*) (Mukai *et al*., 2010). While, Oliveira and Sa (2012) have reported that the eye of *B. splendens* is strongly pigmented at hatching, other study claim that at this stage the eyes of *B. splendens* are undifferentiated and only slightly pigmented (Valentin *et al*., 2013).

Larval development is directly related to temperature, with higher rates being observed at higher temperatures (Leme dos Santos and Azoubel, 1996) and is also related to the reproductive strategy of the species. Migratory species without parental care show fast larval development rates, which is essential for their survival. Furthermore, these species have highly developed eyes at hatching, as observed in *Brycon cephalus* (Romagosa *et al*., 2001). *B. splendens*, on the other hand, is a species with parental care (Valentin *et al*., 2013) and slow development rates when compared to other ornamental fish (Anjos and Anjos, 2006; Celik *et al*., 2011; Paes *et al*., 2011). Therefore, it is expected to display very different visual development rates to the species mentioned above.

The present study demonstrated that changes in the feeding behaviour occurred throughout the development of *B. splendens* larvae. It was observed that the larvae were able to capture *Artemia* sp. more efficiently as age progressed. Since, prey capture is dependent on the visual system, greater larval eye movement in the presence of food significantly increased the capture success rates. According to Osse *et al*. (1997), fish larvae prioritize the development of certain structures during their first days of life, often showing a positive allometric coefficient for the head and tail. Therefore, the increase of successful attacks by *B. splendens* larvae could be related to the development of feeding and swimming structures. This is in agreement with the reports by Tesser and Portella (2006, 2011) for *Piaractus mesopotamicus* larvae in which the authors observed an increase in the feeding rate with age and that a link exists between these rates and muscle, fin and head development.

Another factor that can influence the success of the attack is experience. According to Croy and Hughes (1991), *Sinachia spinachia* larvae attacked *Artemia* sp. more efficiently as a result of experience. Thus, in time, experience may also improve the feeding behaviour observed in *B. splendens*.

The decrease in pursuit observed in this study corroborates the finding of Wanzenbock (1992), who reported that an increase in attack efficiency by three cyprinid larvae (*Rutilus rutilus*, A*lburnus alburnus* and *Abramis ballerus*) was directly related to a decrease in prey recognition time by the larvae. Wanzenbock (1992) stated that this behaviour was related to the speed of swimming and; thus, as the larvae became faster the chances of the prey escaping were reduced. This is in agreement with the belief that the early development of structures related to swimming and feeding is prioritized (Osse *et al*., 1997). It is important to note that these structures are not only important for prey capture but also for escaping predators.

Eye convergence and J-turn morphology during prey pursuit has also been reported in *Danio rerio* (Bianco *et al*., 2011). Eye convergence increases the overlap between the visual fields of both eyes in preparation for prey tracking and allows the larva to adjust its position prior to striking based on the prey's location (Bianco *et al*., 2011).

The short exposure to different background colours did not have a significant effect on the feeding behaviour of *B. splendens* larvae. These results corroborate those of Zavala-Leal *et al*. (2013), who tested the influence of grey and black backgrounds on prey capture by *Lutjanus peru*. According to these authors, these findings can be explained by the early stage of development of the visual system at first feeding. On the other hand, Pedreira *et al*. (2012) observed that *Prochilodus costatus* larvae appear to see their prey better in blue tanks.

Depending on the biology and ecology of the species analysed, external stimuli could generate a different response, making difficult to compare distinct assays. In this particular study, we believe that the short exposure time to the different colours was not enough to produce a significant response. However, for future experiments, other variables such as different times of exposure and larval ages should be taken in to account.

CONCLUSION

In conclusion, *B. splendens* larvae displayed a typical feeding behaviour, which changed with larval development and was dependent on visual ontogeny. Even though the different background colours did not significantly affect feeding behaviour, our findings provide important insights for future studies on this field.

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