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SHORT COMMUNICATION

Effect of feeding mosquito larvae on the coloration of Siamese fighting fish (*Betta splendens*) during grow-out

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Abstract Coloration is one of the most valued features in aquaculture or ornamental fish, and the Siamese fighting fish (*Betta splendens*) (Regan 1910) is an excellent model to study coloration. Carotenoids are one of the pigments that express colour in fish. Microalgae synthesize these pigments, which can be transferred through feeding first to mosquito larvae, then to fish when they feed on mosquito larvae. We tested the effect of feeding mosquito larvae on coloration and growth in Siamese fighting fish. Over a 60-day period, 52 individual Siamese fighting fish (32 males and 20 females) were fed with commercial micro pellets (control diet) or mosquito larvae (experimental diet). We expected that fish fed with mosquito larvae would be more colourful and larger than fish fed with commercial micro pellets. Consistent with this prediction, Siamese fighting fish were more colourful when they were fed the mosquito larvae diet than when they were fed a commercial micro pellet diet. We therefore recommend the use of mosquito larvae for Siamese fighting fish production. Additionally, since the Siamese fighting fish is an efficient predator of the mosquito larvae, we suggest the use of this live food as a high quality alternative food and a colour bio-capsule with numerous carotenoid pigments.

Keywords Carotenoid source . Aquarium fish coloration . *Chlorella* sp. . *Culex quinquefasciatus*

Introduction

The Siamese fighting fish, *Betta splendens*, is a popular fish species for ornamental production due to its brilliant colours and large fins (Puello-Cruz et al. 2010; Saekhow et al. 2018). The production of Siamese fighting fish (*Betta splendens*) (Regan 1910) is an important economic activity in countries like Thailand, Indonesia, Singapore, China, Malaysia, Japan, USA, and Mexico (Chuan et al. 2003; Thongprajukaew et al. 2011). Coloration is one of the most valued features in ornamental fish (Barber et al. 2000; Pavlidis et al. 2006). Since fish cannot produce the pigments that give them their colour endogenously, they must acquire them through diet (Sefc et al. 2014). One of the classes of pigments that express colour in fish are carotenoids, and in various studies, the diets of ornamental fish have been enriched with these pigments in order to improve their coloration and increase their market value (Sommer et al. 1992; Pham et al. 2014). The inclusion of pigments in fish food is also necessary for the nutrition of the fish (Gordon et al. 1981). Carotenoid pigments have a variety of physiological roles, for example, as immunostimulants and antioxidants (McGraw and Ardia 2003). Coloration in fish has been evaluated after feeding live food

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including water fleas, mosquito larvae, and *Artemia* nauplii as pigment sources (Torrisen and Naevdal 1988; Thongprajukaew et al. 2014; Arce et al. 2018). Like the fish, these organisms used as food for ornamental fish (*Artemia* and mosquito larvae) cannot produce carotenoid pigments themselves, but rather obtain them from microalgae present in live food cultures (Jones et al. 1993; Nieves et al. 1996; Voltolina et al. 1999). Microalgae are photosynthetic organisms that synthesize different types of pigments and transport them to chloroplasts (Siefermann-Harms 1987; Yong and Lee 1991; Bidigareet et al. 1993); the chloroplasts contain the pigments that transfer the colour to the fish (Siefermann-Harms 1987; Storebakken et al. 1987). Additionally, it has been observed that live food in general stimulates growth and coloration in fish (Torrisen 1984; Arce et al. 2018; Luna-Figueroa et al. 2019).

The goal of this study was to evaluate the effect of feeding mosquito larvae on coloration and growth of Siamese fighting fish. We examined the effect of microalgae present in live food culture on pigment transfer to mosquito larvae and colour expression in fish. We expected that fish fed with mosquito larvae would be more colourful and grow more than fish fed with commercial micro pellets. Although coloration and growth have been assessed before in fish species (Baron et al. 2008; Mat Nawang et al. 2019), this is the first study to assess coloration using mosquito larvae as food.

Materials and methods

Fish acclimation and control and experimental diets

Four-month-old Siamese fighting fish (Blue veiltail-Betta total length: 42.30 ± 3.48 mm; mass: 1.08 ± 0.23 g) were obtained from multiple parents at fish farms (n= 56). The fish were placed into individual circular containers with a volume of 500 mL. The walls of these containers were covered with white plastic film to prevent the fish from seeing each other and to equalize the luminosity because fish use vision to assess their surroundings (Stevens et al. 2007) and because luminosity and wall colour can affect growth and body coloration in ornamental fish (Mat Nawang et al. 2019). The physical and chemical conditions of the water were 24 ± 0.5 °C, pH 7.2 ± 0.1, and the fish were subjected to a 12:12 h light: dark photoperiod using a tube LED lamp connected to a programmed timer (TORK 40382). Prior to beginning the experimental diet treatments, all of the fish to be used in the experiment were acclimated for 20 days to a 1:1 mixture of the control and experimental diets. The control diet, commercial micro pellets, used *Chlorella vulgaris* algae as a source of carotenoids (0.52 ppm). The control diet contained 43% protein, 4.5% lipids, and 5.0% carbohydrates. The experimental diet, mosquito larvae (*Culex quinquefasciatus*, Pham et al. 2014), contained 43.5% protein, 9.5% lipids and 5.2% carbohydrates. Proximate analysis of the control diet was performed in dry matter according to the AOAC (1990) methods. Samples were dried in an oven at 105 °C to constant weight, and ash content was estimated by incineration in a muffle furnace at 600 °C for 6 h. Crude protein was determined by acid digestion using the Kjeldahl method, lipids by petroleum ether extraction, and carbohydrates by subtraction (Yuangsoi et al. 2010). Fish were fed *ad libitum* twice daily at 9:00 h and 16:00 h (Thongprajukaew et al. 2011; Arce et al. 2018). Faeces and uneaten food were removed daily (De la Torre et al. 2018).

Experimental diet protocol and growth measurement

Following the 20-day acclimation period, the 56 fish were randomly divided based on the toss of a coin into two experimental groups of 16 males (n= 32, 1.11 ± 0.04 g, 42.79 ± 0.65 mm) and 12 females (n= 24, 1.03 ± 0.05 g, 42.65 ± 0.69 mm) for the diet experiment (n = 28 per treatment). Each fish was placed in an individual tank with the same physical and chemical water conditions as during the acclimation period. At the beginning of the experimental period, the fish were kept without food for 24 h before biometric analysis to ensure complete gastric evacuation. Fish were weighed with a plate balance (OHAUS; 0.01 g) and measured with callipers (INSIZE; 0.01 mm). For the next 60 days, the control group was fed the commercial micro pellets and the experimental group received two mosquito larvae (*C. quinquefasciatus*). Under both treatments, fish were fed *ad libitum*, daily at 9:00 h and 16:00 h (Thongprajukaew et al. 2011; Arce et al. 2018). Growth of each fish was calculated at the end of the experiment as weight gain (mass,

g) and size gain in total length (mm) by subtracting the initial measurement from the final measurement.

Live food culture

In order to obtain mosquito larvae to use as live food, *Chlorella* sp. microalgae was cultured in eight 590-L fiberglass tanks fertilized with 1000 mg organic matter (chicken manure; Paniagua-Michel et al. 1987; Voltolina et al. 1999; Ekpo et al. 2016). The temperature of the tanks was 26 ± 1 °C, pH 8.5 ± 1.4, and they were kept in natural light. Once fertilized, the tanks were left for 7 days before live food (mosquito larvae) were collected. The tanks remained under observation, and microalgae were counted and identified daily to estimate abundance. The growth of the microalgae and species identification of microalgae was determined by direct counting in a Neubauer chamber using a LEICA ICC50 HD microscope (Avendaño and Riquelme 1999).When the microalgae abundance reached 20000 cells/mL and/or after seven days, we fertilized another eight tanks. This procedure was repeated as many times as necessary to maintain a consistent supply of nutrients for the microalgae from the acclimation period through the end of the experiment. Because *Betta* fish do not discriminate between developmental stages of mosquitos as prey (unpublished data), mosquitoes from 1st instar larvae to pupa were considered "larvae" (Griffith and Turner 1996). Mosquito larvae were collected daily using a net with a mesh size of 0.5-mm. To avoid pathogens, mosquito larvae were provided aeration at 0.5 L/min flow and 10 °C and a 5 µg/L disinfectant solution (Wescodyne) was added for 10 minutes prior to using them as fish food (Kent et al. 2009).

Carotenoid pigments

To determine the concentration of carotenoid pigments in microalgae (mg/m^3), 100 mL of water was filtered daily for the 7 days during the culture in each of the eight microalgae culture tanks (n= 56 samples) using a millipore filter (0.45 µm pore aperture). The membrane was centrifuged with 10 mL of methanol for 10 minutes at 1500 rpm. The carotenoid concentration was determined using a spectrophotometer (HACH DR / 2017; Dere et al. 1998). The microalgae collection started 7 days after fertilization, when microalgae were abundant (630,431 ± 23,922 cell/mL; 8 tanks, 7 days; n= 56) and at the end of the experiment, when algal abundance decreased (226,844 ± 10,003 cell/mL; 8 tanks, 7 days; n= 56). To determine the concentration of carotenoid pigments in mosquito larvae, we collected larvae daily for the 7 days that the culture lasted from each microalgae culture tank (100 mL). Mosquito larvae were rinsed with clean water and macerated in 10 ml of acetone, then carotenoid concentration was determined in the same way as for the microalgae (Dere et al. 1998).

Fish colour measurements

To assess fish coloration, 52 fish (32 males and 20 females) were placed individually into a transparent photographic tank (12 x 12 x 2 cm) at the beginning and end of the experiment with water with the same conditions as those used during maintenance. This photographic tank was placed in a 40 x 40 x 50 cm box with opaque white walls and white light to photograph the fish (Arce et al. 2018). Each fish was photographed in lateral view using a professional digital camera (Panasonic DMC-GH4; Arce et al. 2018). The camera was placed 30 cm from the photographic tank and operated in manual mode to avoid automatic colour modifications. All of the digital images were saved as Tagged Image File Format (TIFF) files (Mat Nawang et al. 2019). A similar area (12.6 mm^2) of the peduncle zone of each fish in each picture was analysed for colour using Image J software (Igathinathane et al. 2009; Schneider et al. 2012; Johansson and Nilsson-Örtman 2013). This software detects the colour intensity of an image on an RGB scale, with hue and chroma values on a scale of 0-255. (Touchon and Warkentin 2008; Touchon and Wojdak 2014).

Statistics

We compared the pigment concentration of the microalgae and mosquito-larvae at the beginning of the microalgae culture and end of the collection period via Student's *t*-test. We compared the colour, weight and size of the individuals assigned to each treatment at the beginning and end of the experiments using linear mixed-effects models (LMM; Bates et al. 2007). Sex and diet were included as fixed factors, and fish

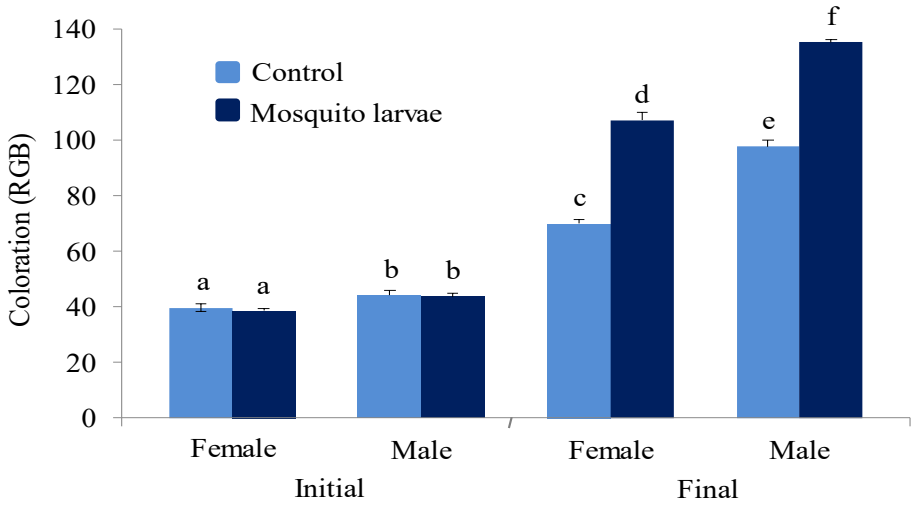

Fig. 1 Coloration in female and male Siamese fighting fish. Different letters indicate significant differences ($p < 0.05$). Mean values and SE are shown.

identity as a random effect to control for the non-independence of data collected from the same individual. We performed Bonferroni-corrected multiple *post hoc* comparisons to reduce type I error. Assumption of data normality and homoscedasticity of variances were verified by residual analysis. An alpha test level of 0.05 was used for all the statistical tests. Data are presented as mean values ± SE. All analyses were performed in R v.3.6.2 (R Core Team 2020).

Results

Carotenoid pigments

Chlorella sp. was the most abundant microalgae in the culture throughout the experiment (99% of total abundance). *Scenedesmus* sp. and *Cosmarium* sp. (1 % abundance) were the only other genera found. The carotenoid pigment concentration of the algae was greater at the beginning (1,634.18 ± 246 mg/m^3) than at the end of the culture period (686.75 ± 240 mg/m^3, $t_{(14)} = 2.75$, $p = 0.01$). The carotenoid content of mosquito larvae was the same at the beginning (581.79 ± 75.47 mg/m^3) and end of the culture period (580.80 ± 128.33 mg/m^3, $t_{(14)} = 0.007$, $p = 0.99$).

Fish coloration

At the beginning of the experiment, neither females nor males differed between the two treatments with respect to coloration intensity (Bonferroni; females: $t_{(22)} = 0.63$, $p = 0.54$; males: $t_{(30)} = 0.17$, $p = 0.88$; Figure 1). Males were more colourful than females at the beginning of the experiments (LMM; $\chi^2 = 40.2$, *d.f.*= 1, $p < 0.001$; Figure 1). The final coloration of *Betta* females and males depended on the diet treatment (LMM; $\chi^2 = 52.40$, *d.f.*= 1, $p < 0.001$). Final coloration intensity was higher in males than females within each treatment (LMM; $\chi^2 = 40.20$, d.f.= 1, $p < 0.001$). Among both females and males, fish fed with mosquito larvae were more colourful than those fed the control diet (Bonferroni; females: $p < 0.001$; males: $p < 0.001$; Figures 1, 2).

Fish growth

At the beginning of the experimental period, neither males nor females differed between the treatments in mass (Bonferroni; females: $p = 0.99$; males: $p = 0.98$; Figure 3), or total length, (Bonferroni; females: $p = 0.99$; males: $p = 0.99$; Figure 4). The final mass of *Betta* females and males depended on the diet treatment (LMM; $\chi^2 = 14.98$, *d.f.*= 1, $p < 0.001$; Figure 3), and final mass was higher in males than females within each treatment (LMM; $\chi^2 = 18.39$, d.f.= 1, $p < 0.001$; Figure 3). Fish fed with mosquito larvae gained more mass

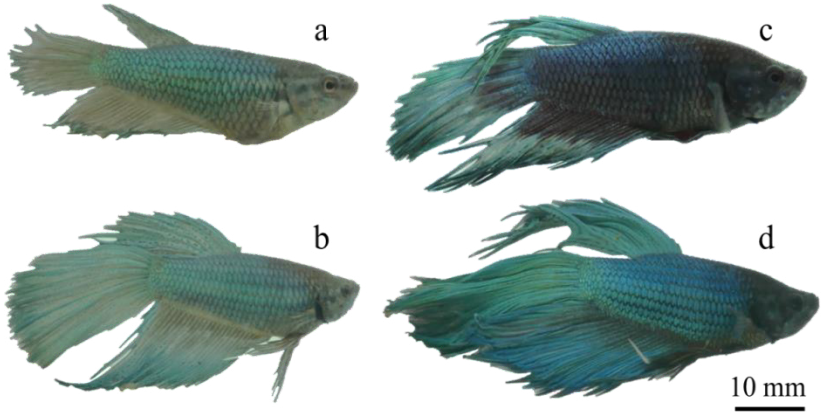

Fig. 2 Final body coloration of Siamese fighting fish. a) female control diet, b) male control diet, c) female mosquito larvae diet, and d) male mosquito larvae diet.

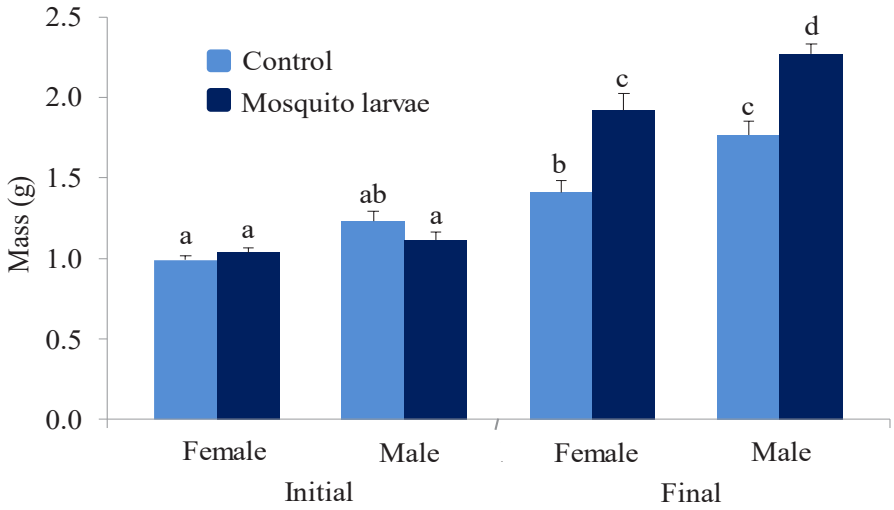

Fig. 3 Mass in female and male Siamese fighting fish. Different letters indicate significant differences ($p < 0.05$). Mean values and SE are shown.

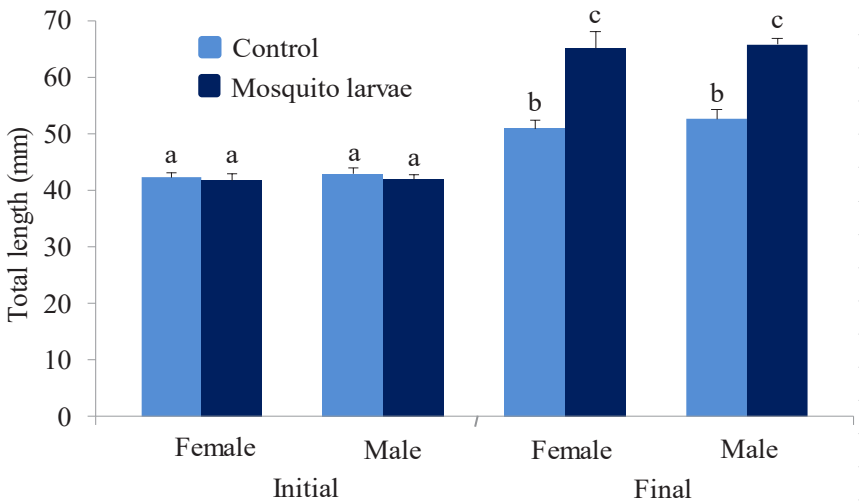

Fig. 4 Total length in female and male Siamese fighting fish. Different letters indicate significant differences ($p < 0.05$). Mean values and SE are shown.

than fish fed with the control diet (Bonferroni; females: $p < 0.001$; males: $p < 0.001$; Figure 3). Final total

length also differed between diets in both sexes (LMM; χ^2= 26.97, d.f.= 1, $p < 0.001$; Figure 4). Final total length did not differ between males and females within each treatment (LMM; χ^2= 0.42, d.f.= 1, p =0.52; Figure 4). Fish fed mosquito larvae were longer than those fed the control diet (Bonferroni; females: $p < 0.001$, males: $p < 0.001$; Figure 4).

Discussion

Live food culture is an important industry for aquaculture (Le Ruyet et al. 1993; Reitan et al. 1993). Microalgae are food for zooplankton, which is the most popular live food for fish, and *Chlorella* sp. is one the most frequently used microalgae in the culture and collection of live food (Reitan et al. 1993; Borowitzka 1999; Agwa and Abu 2014). *Chlorella* was the most abundant microalgae in the culture used in our research, and although there were other species such as *Scenedesmus,* and *Cosmarium*, these accounted for only 1% of the total microalgae population, so their contribution of carotenoids was not significant. *Chlorella, Scenedesmus*, and *Cosmarium* are Chlorophytes and their essential pigments are carotenoids (Priyadarshani and Rath 2012). *Chlorella* is a microorganism with many carotenoid pigments (Herring 1968; Gouveia et al. 1996), so from a consumer's perspective, microalgae improve carotenoid digestion and absorption (Becker and Venkataraman 1984; Sun et al. 2012). Due to the abundance of cultured microalgae, the quantity of carotenoids in water samples at the beginning of the feeding period were greater than at the end of the experimental period. Microalgae are an essential part of the diet of mosquito larvae (Rey et al. 2009). These microalgae are not only fundamental for the nutrition of the mosquito larvae, but mosquito larvae also become a nutritious and colourful capsule for accumulating microalgae and delivering them to fish, even when the microalgae density varies in the water. The carotenoid levels in mosquito larvae that consumed microalgae remained the same throughout the live food culture period, which indicates that mosquito larvae are efficient accumulators of carotenoid pigments, regardless of availability; thus, they offer a high concentration of carotenoid pigments to the next trophic level (fish predator). Carotenoid pigments are used to produce colour in fish and have a variety of physiological functions, for example, as immunostimulants or sexual attractants (Evans and Norris 1996; Maan et al. 2006). Colour in males is a sexually selected trait in many fish (Clotfelter et al. 2007), and it has been demonstrated that carotenoid supplementation significantly increases the immune response (Alonso-Alvarez et al. 2004; Clotfelter et al. 2007). We found that both females and males were more colourful when fed mosquito larvae. The increased colour intensity obtained by using mosquito larvae could be attributed to *C. quinquefasciatus* larvae being filter feeders (microalgae consumers; Agwa and Abu 2014; Manimegalai and Sukanya 2014). They were shown to be excellent consumers of these algae, and we observed a lot of microalgae in the digestive tracts of mosquito larvae, such that mosquito larvae can be considered a colour bio-capsule.

Mosquito larvae have been used as live food, favouring the growth of fish (Barroso et al. 2014; Thongprajukaew et al. 2014; Luna-Figueroa et al. 2019). In this study, both females and males were longer and heavier when they were fed mosquito larvae. Fish growth is affected by food quality. An essential characteristic of mosquito larvae is their high protein content (Luna-Figueroa et al. 2019). The amount of protein contained in *Chlorella* sp. is 50%-60% (Spolaore et al. 2006), whereas in larvae used as live food for fish, it is 43% (Luna-Figueroa et al. 2019). In our research we used living mosquito larvae, since mosquito larvae may filter microalgae from the culture (Marten 2007). In addition to the nutrient quality of the diet, our results may be partly due to the rate of consumption of live versus inert food, since fish fed *ad libitum* may prefer live prey over inert food (Fernández-Díaz et al. 1993).

Conclusion

We demonstrated a positive effect on colour and growth in Siamese fighting fish fed with mosquito larvae and we suggest the use of this live food (with appropriate precautions to prevent pathogen transmission) as a high-quality alternative to pellets and an excellent colour bio-capsule of carotenoid pigments. In future research we suggest using prepared diets with mosquito larvae fed with *Chlorella* sp. (e.g. mosquito larvae prepared in pellet form). Such studies are important, first to test whether mass, size and coloration improvements with the mosquito-based diet fed here can be replicated without the live food component.

Conflict of interest The authors declare that they have no conflict of interest.
Authors' contribution Conceptualization: M.E.M.M., E.A.; Methodology: M.E.M.M., E.A., J.G.R.; Formal analysis: E.A., L.M.B.; Investigation: L.M.B., E.A.; Resources: E.A., J.G.R.

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