



First feeding of diploid and triploid yellowtail tetra *Astyanax altiparanae*: An initial stage for application in laboratory studies

Rafaela Manchin Bertolini¹ | José Augusto Senhorini¹ | Nivaldo Ferreira do Nascimento²  |
 Matheus Pereira-Santos² | Laura Satiko Okada Nakaghi² | Wellington Adriano
 Moreira Peres¹ | Regiane Cristina da Silva² | George Shigueki Yasui^{1,3} 

¹Laboratory of Fish Biotechnology, National Center for Research and Conservation of Continental Fish, Chico Mendes Institute of Biodiversity Conservation, Pirassununga, SP, Brazil

²Aquaculture Center, São Paulo State University, Jaboticabal, SP, Brazil

³Department of Veterinary Medicine, FZEA, University of São Paulo, Pirassununga, SP, Brazil

Correspondence

George Shigueki Yasui, Department of Veterinary Medicine, FZEA, University of São Paulo, Pirassununga, SP, Brazil.
 Email: yasui@usp.br

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Abstract

In this study, the aim was to establish a protocol for first feeding of diploid and triploid yellowtail tetra *Astyanax altiparanae* in laboratory conditions. The fry were fed with five different diets: (i) *Artemia franciscana* nauplii, (ii) plankton, (iii) dry food, (iv) *Artemia franciscana* nauplii + plankton, and (v) *Artemia* nauplii + plankton + dry food. Additionally, the growth and survival rates of diploid and triploid individuals were also evaluated. On day 10, the length of the fish between the treatments differed significantly ($p = .0001$) and ranged from 4.07 ± 0.06 mm (dry food) to 8.50 ± 0.64 mm (plankton + *Artemia*). The sizes of the fish increased with time, except for the fish fed with dry food. The survival rates were similar for the fish fed with the four diets and ranged from $80.7 \pm 5.4\%$ (dry food + plankton + *Artemia* to $92.0 \pm 1.6\%$ (plankton + *Artemia*), but differed from the fish fed with dry food ($17.7 \pm 5.8\%$, $p = .0017$). Diploids and triploids did not present differences on day 0 ($p = .2252$) and on day 10 ($p = .4844$) when the fish presented 6.77 ± 0.25 mm and 6.54 ± 0.15 mm respectively. Survival of diploids ($87.3 \pm 5.13\%$) and triploids ($74.67 \pm 2.30\%$) were also similar ($p = .0285$). These data are innovative and useful for establishing protocols for this species in both academic and applied sciences.

KEYWORDS

characin, chromosome, feeding, fish, larviculture, polyploid

1 | INTRODUCTION

The yellowtail tetra *Astyanax altiparanae* (Garutti & Britski, 2000) is a small characin species with potential in both aquaculture and academic research. As a r-strategist species, this species is easy to breed and presents high growth rates (Garutti, 2003; Porto-Foresti, Castilho-Almeida, Senhorini & Foresti, 2010). Additionally, this species can survive in limiting environments like decreased water quality and high stocking densities, which

emphasizes its potential in intensive aquaculture and aquarium systems.

In previous studies from this laboratory, a protocol was developed for in vitro fertilization in laboratory conditions (Yasui et al., 2015). In vitro fertilization permitted control of the fertilization timing, and, then, experiments involving chromosome and gamete manipulation were developed. A study regarding gamete ultrastructure, developmental staging, and fertilization was established using such a protocol (Dos Santos et al., 2016). In addition, a protocol for

developing triploid fish was achieved in a recent publication (Adamov et al., 2016), and the newly hatched larvae were used to confirm the ploidy status by flow cytometry. However, in order to evaluate other parameters in adult fish, additional investigations are then necessary to rear the juveniles until they reach the adult stage. Juveniles and adult yellowtail tetras easily accept commercial pellet as a food source if the pellet is of adequate size based on the mouth diameter. Even wild-caught individuals promptly accept such kind of food after a few days of domestication. On the other hand, the best food for first feeding this species is still unknown, which does not allow for development of later works involving such a transition from fry into juvenile and adult stages.

The first feeding is one of the most critical stages in fish because it is a transitory period between endogenous (yolk) into exogenous feeding. In addition, the fish are fragile during this period and, consequently, high mortalities may arise (Kolkovski, Curnow & King, 2004; Yúfera & Darias, 2007). Most fish species require live food during these initial stages, however, some species like the Nile tilapia *Oreochromis niloticus* may be fed exclusively with dry food (El-Sayed, 2002). The main challenge during the first feeding is to raise the fry to an adequate size in which they can be fed *Artemia* sp. nauplii and dry food, since those products may be easily provided in large-scale. In several ornamental fish species where fry are of smaller size, the mouth diameter during the early period presents another bottleneck as it permits capture of only small particles, such as rotifers and protozoans, that are difficult to produce in large-scale. In such cases, "green water," containing a mixture of small organisms, is used to feed small fish until a later stage in which the fish are fed dry food or *Artemia*. However, poor quality of the "green water" may also decrease the survival rates. In the case of yellowtail tetra *A. altiparanae*, juveniles are produced in large-scale aquaculture in fertilized ponds. In such a procedure, newly hatched larvae are released into a fertilized pond, and the fish can select planktonic organisms according to each fish size. However, several aspects when using such a procedure remain unclear, including the optimal type of food and the subsequent survival rates. On the other hand, it is necessary to use adequate feeding in fish development for laboratory studies because exogenous food is not available for those conditions. In a recent work (Adamov et al., 2016), triploid individuals were produced, but decreased hatching and survival rates were found within triploids. However, the performance of the triploids in later stages, including the first feeding, remains unclear.

Therefore, the aim of this study was to evaluate different types of food during the first feeding of yellowtail tetra *A. altiparanae*. Additionally, the performance of diploid and triploid fry was also evaluated, using the best feeding conditions obtained above.

2 | MATERIALS AND METHODS

All the procedures were performed in agreement with the Guide for the Care and Use of Laboratory Animals of CEPTA (CEUA #02031.000033/2015-11).

2.1 | Origin of the broodstock and breeding

Adult yellowtail tetra *Astyanax altiparanae* used in this study were obtained from the Mogi Guassu river (21.925706 S, 47.369496 W) and, then, maintained at Chico Mendes Institute of Biodiversity Conservation, São Paulo State, Pirassununga, Brazil. The fish were maintained in 1,000 m² earthen ponds with 15%–20% of the water surface covered by floating plants (*Eichornia crassipes*), which is the natural substrate for breeding and egg incubation. The fish were fed twice a day with commercial pellets containing 40%–45% of crude protein. Endogenous food (i.e. plankton) was also available in those tanks. As this species breed spontaneously under natural conditions, F1 offspring were obtained, and those juveniles were used for induced reproduction.

Spawning was induced between males (SL ≈ 6 cm) and females (SL ≈ 9 cm) were using the previous protocol (Yasui et al., 2015). Briefly, the fish were anesthetized in menthol (100 mg L⁻¹), then, injected with a single dose of carp pituitary gland (3 mg kg⁻¹ for males and females), and, afterwards, gametes were obtained 8–10 h later. Sperm samples were collected by stripping, using a 1,000 µl micropipette (Eppendorf, Hamburg, Germany) in the papillae, and they were immediately transferred to a tube containing 400 µl of modified Ringer solution (128.3 mM NaCl, 23.6 mM KCl, 3.6 mM CaCl₂, 2.1 mM MgCl₂) to immobilize the sperm. Oocytes were stripped on a 90 mm Petri dish covered by a polyvinylidene chloride film (saran wrap). Subsequently, 100 µl of the diluted sperm was pipetted on the oocytes, and the gametes were activated by 5 ml of water. The fertilized eggs were then incubated in a floating apparatus in which the bottom contained a 100 µm nylon mesh. The mesh was placed in 40-L aquariums with constant aeration until hatching. Dead fish were removed each at 6-h interval.

2.2 | Experiment 1: first feeding of diploids

In this experiment, three batches of fry were used. Each batch was produced from different parental fish and were considered as replicates. After yolk absorption (3 days after hatching), each batch of fry was distributed in circular transparent acrylic containers with a 150 mm diameter and water volume of 1 L. 120 fry were distributed in each container, among a total of 15 containers (three containers per treatment). Each container was placed in a biochemical oxygen demand (BOD) incubator with the temperature set at 26 °C and 12 h light per day. Water quality was maintained by water changes performed twice a day, one hour after feeding.

Five treatments were used: T1 = *Artemia franciscana* nauplii; T2 = plankton; T3: dry food; T4 = plankton + *Artemia franciscana* nauplii; and T5 = plankton + *Artemia franciscana* nauplii + dry food.

Plankton were sampled from a 1,000 m² fertilized earthen pond using a closing plankton mesh (40 µm diameter), and the samples were then passed through a 100-µm nylon mesh in order to keep only small-sized organisms. Such procedure resulted in predominantly rotifers and copepod nauplii forms. In those treatments, dry food was the same pellets used for adults, but it was ground and

passed through a 500 μm mesh (soil sieves). Fish were fed to satiation twice a day.

For evaluation of growth, the length (mm) of 15 individuals collected randomly from each container was measured daily. For measuring the length, fish were placed in a 50 mm-Petri dish and observed under a stereomicroscope (Nikon SMZ-1500, Tokyo, Japan) with a CCD camera (Nikon DS-Fi, Tokyo, Japan). Then, digital images were taken using a Nis-Ar Elements software (Nikon, Tokyo, Japan), and the total length was measured from the digital images (Figure 1). These procedures took less than 8 min for each container, and after image capturing, the fish were immediately returned to the original container.

Growth performance was measured over 10 days, and after this period, the survival rates from each container were also calculated.

2.3 | Experiment 2: performance of diploids and triploids

Previous laboratory protocol (Adamov et al., 2016) were used for creating triploid fry. Three batch of oocytes were fertilized using sperm from different males and considered as triplicates. Then, each batch of fertilized eggs was immediately divided into two groups. One group was heat-shocked at 40 $^{\circ}\text{C}$ for 2 min post-fertilization for 2 min in order to create triploids. The other group was kept intact and served as the control diploids. Diploids and triploids were then incubated in the same conditions described in experiment 1 above and were fed using the best treatment obtained from the same experiment (*Artemia franciscana* nauplii + plankton). Growth and survival rates of diploids and triploids were measured daily as mentioned above in experiment 1.

2.4 | Ploidy analysis

Animals were confirmed as 2n and 3n using flow cytometry by comparing the relative DNA content with control diploids. Hence, 20 larvae from each replicate (total of 60 fish for each treatment) were

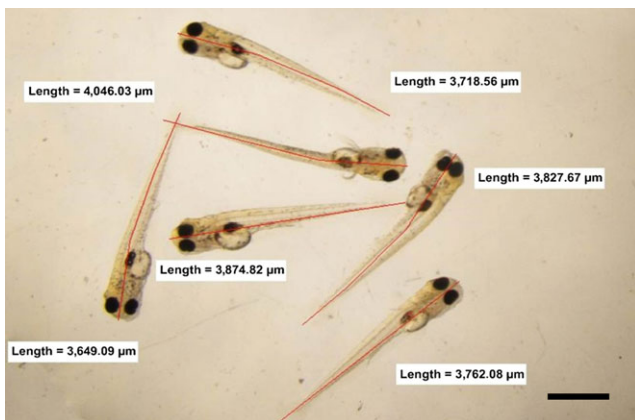


FIGURE 1 Measurement of total length (mm) for *Astyanax altiparanae* larvae from a digital image. Scale: 1,000 μm

randomly sampled at the hatching stage. The larvae were immersed in a solution (9.53 mM $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 47.67 mM KCl, 15 mM Tris, 74 mM Sucrose, 0.8% Triton X-100) for 10 min to remove the membranes. Then, 800 μl of 4.6 Dimidine 2 Phenylidone Di-Hydrochloride—DAPI (0.01% DAPI in Dulbecco's Phosphate Buffer Saline) was added for nuclear staining. The resultant samples were then filtered in a 30 μm -mesh (CellTrics, Partec GmbH, Germany) and analysed in flow cytometer (CyFlow Ploidy Analyzer, Partec, GmbH, Germany).

3 | STATISTICS

Data were taken in triplicates from three different parental fish and are shown as the mean \pm standard error. Data were examined using ANOVA followed by Tukey multiple range test. Comparison of survival rates from diploids and triploids was performed using the *t* test. In all analyses, the software STATISTICA (7.0, StatSoft, USA) was used with the probability set at .05.

4 | RESULTS

4.1 | First feeding of diploids

An isolated analysis within each treatment revealed that only the fish fed with dry food did not increase in length during the whole experimental period ($p = .6745$, Table 1).

A multiple comparison between all treatments indicated that the initial length was not significantly different between treatments ($p = .7804$). The initial length ranged from 3.83 ± 0.08 mm for the fish fed with plankton to 4.07 ± 0.17 mm for the fish fed with dry food + plankton + *Artemia*. Significant differences first arose on day 2 ($p = .0004$), in which the fish fed with dry food were observed as smaller than other treatments.

On day 10, the length was also significantly different between treatments ($p = .0001$). Increased lengths were obtained for the fish fed with plankton + *Artemia* (8.50 ± 0.64 mm), dry food + plankton + *Artemia* (8.21 ± 0.42 mm), and *Artemia* (7.78 ± 0.16 mm). Other treatments presented decreased length, as in the case of the plankton (6.35 ± 0.20 mm) and dry food (4.07 ± 0.06 mm) treatments.

The external morphology of all treatments on days 0, 5, and 10 is shown in Figure 2. In all treatments, fish were capable of eating all types of food, including the dry food (Figure 3). However, the fish fed with dry food presented a fragile external morphology and reduced swim ability (data not shown).

Survival rates were significantly different between treatments ($p = .0017$). In addition, a multiple comparison between treatments indicates that survival rates were similar (Figure 4) for the fish fed with plankton + *Artemia* ($92.0 \pm 1.6\%$), dry food + plankton + *Artemia* ($80.7 \pm 5.4\%$), *Artemia* ($87.7 \pm 2.9\%$), and plankton ($89.0 \pm 7.1\%$). However, the survival rate decreased when the fish were fed with dry food ($17.7 \pm 5.8\%$).

TABLE 1 Growth performance of diploid yellowtail tetra *Astyanax altiparanæ* fed with several diets for 10 days. Fish at 3 days post hatching were maintained in 1-L acrylic tanks (120 fish per tank) at 26 °C. Data are shown as mean ± standard error and each data was generated from triplicates from different spawning. *p*-values shown in the table refers to ANOVA analysis. Identical capital letters within columns and lowercase within rows denote non-significant differences as determined by the Tukey multiple range test ($p = .05$)

Treatments	Days											<i>p</i> -value
	0	1	2	3	4	5	6	7	8	9	10	
Plankton + artemia	3.90 ± 0.05 ^{Aa}	4.29 ± 0.07 ^{Aa}	4.73 ± 0.09 ^{Aa}	5.20 ± 0.23 ^{Aa}	6.04 ± 0.22 ^{Ab}	6.09 ± 0.05 ^{ABb}	6.83 ± 0.22 ^{Ac}	7.17 ± 0.32 ^{Ac}	7.69 ± 0.34 ^{Ad}	8.07 ± 0.31 ^{Ad}	8.50 ± 0.64 ^{Ae}	.0001
Dry food + plankton + artemia	4.07 ± 0.17 ^{Aa}	4.26 ± 0.10 ^{Aa}	4.61 ± 0.08 ^{Aa}	5.08 ± 0.12 ^{Bbcd}	5.64 ± 0.17 ^{ABcde}	6.08 ± 0.19 ^{ABdef}	6.48 ± 0.23 ^{ABdef}	6.89 ± 0.20 ^{ABf}	7.43 ± 0.31 ^{ABg}	7.61 ± 0.35 ^{ABg}	8.21 ± 0.42 ^{Ag}	.0001
Artemia	3.98 ± 0.08 ^{Aa}	4.23 ± 0.14 ^{Aa}	4.53 ± 0.05 ^{Aab}	4.73 ± 0.11 ^{Bab}	5.33 ± 0.18 ^{ABBg}	5.82 ± 0.23 ^{ABBg}	6.20 ± 0.18 ^{ABcd}	6.53 ± 0.19 ^{ABcde}	7.00 ± 0.19 ^{ABdef}	7.33 ± 0.20 ^{Aef}	7.78 ± 0.16 ^{Af}	.0001
Plankton	3.83 ± 0.08 ^{Aa}	4.26 ± 0.11 ^{Aaf}	4.47 ± 0.19 ^{Aab}	4.89 ± 0.13 ^{Bbcf}	5.11 ± 0.19 ^{Bbcd}	5.18 ± 0.19 ^{Bbcd}	5.63 ± 0.17 ^{Bcde}	5.93 ± 0.23 ^{Bde}	6.03 ± 0.20 ^{Be}	6.24 ± 0.15 ^{Be}	6.35 ± 0.20 ^{Be}	.0001
Dry food	4.04 ± 0.14 ^{Aa}	3.96 ± 0.03 ^{Aa}	3.89 ± 0.06 ^{Ba}	3.88 ± 0.01 ^{Ba}	4.02 ± 0.04 ^{Ca}	3.95 ± 0.08 ^{Ca}	4.03 ± 0.12 ^{Ca}	4.00 ± 0.01 ^{Ca}	3.97 ± 0.06 ^{Ca}	4.03 ± 0.03 ^{Ca}	4.07 ± 0.06 ^{Ca}	.6745
<i>p</i> -value	.7804	.2142	.0004	.0002	.0001	.0001	.0001	.0001	.0001	.0001	.0001	–

4.2 | Performance of diploids and triploids

Both diploids ($p = .0001$) and triploids ($p = .0001$) increased in length during the experimental period, as observed in Table 2. A comparison between diploids and triploids revealed non-significant differences over the whole experimental period. The initial length was 4.03 ± 0.04 mm for diploids and 3.96 ± 0.01 mm for triploids. On day 10, the length of the diploids was 6.77 ± 0.25 , and the length of the triploids was 6.54 ± 0.15 mm.

Survival of diploids ($87.3 \pm 5.13\%$) and triploids ($74.67 \pm 2.30\%$) was also similar ($p = .0285$).

4.3 | Flow cytometric analysis

As expected, flow cytometry analysis showed that all intact embryos (control eggs) gave rise to diploids (80/80), and most of the heat-shocked (46/54) embryos gave rise to triploids.

5 | DISCUSSION

In this study, it was observed that the combination of plankton and *Artemia franciscana* nauplii provided better results in the initial feeding of *A. altiparanæ* larvae, and this may be explained by the combination of nutrients and particle sizes provided by both treatments.

Evjemo, Reitan and Olsen (2003) analysed the nutrient content of zooplankton and *Artemia franciscana* and observed different composition, which supports the current proposal. In addition to nutritional content, Kim, Masee and Hardy (1996) emphasize that the absorption of *Artemia* sp. and other live foods is facilitated because the larvae use the prey enzymes for digestion (Kolkovski, 2001). The combination of *Artemia* nauplii and other planktonic organisms provide a wide range of particle sizes with a particular velocity, colour pattern, and other factors that affect the predation ability of the fish fry (Busch, 1996). Live food presents visual or chemical attractants that stimulate the fish fry to recognize such particles as food (Fernando Beux & Zaniboni-Filho, 2008; Tesser & Portella, 2006). Although the size of *Artemia* nauplii is adequate for most *Astyanax altiparanæ* fry based on mouth size, some individuals are smaller and do not accept *Artemia* nauplii as a food source, consequently dying. This emphasizes the importance of providing many options for food during the initial stages.

The fish fed only with *Artemia* nauplii presented better growth and survival performance than the fish fed with plankton. Similar results were observed by Luz and Zaniboni-Filho (2002) for *Pimelodus maculatus*, a Neotropical siluriform species. On the other hand, native plankton is considered the most important food for many freshwater species (Castagnolli, 1992; Woynarovich & Horváth, 1983), and previous studies also stated that the combination of *Artemia* nauplii and plankton improved the survival and growth of fish fry (Hamre et al., 2002). *Artemia* may be critical in freshwater because the *Artemia* nauplii die, and the fish fry may not consider dead nauplii as prey. Thus, dead *Artemia* nauplii have to be removed

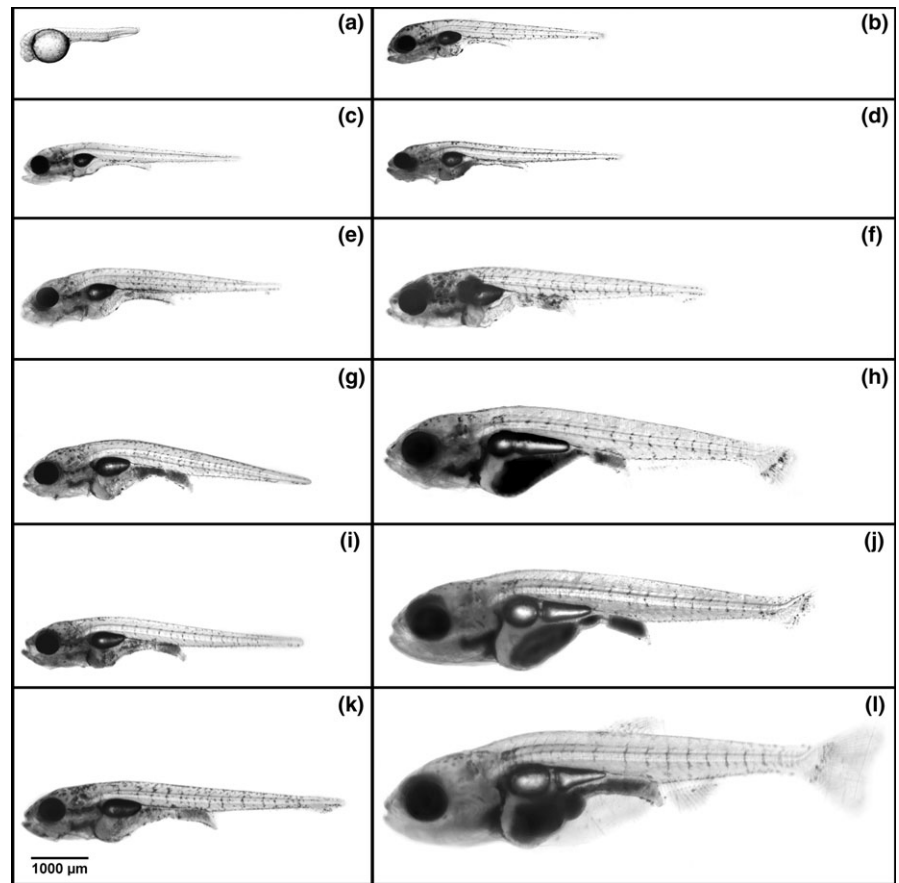


FIGURE 2 External morphology of fish fed with different diets. Newly hatched larvae (a). Fish after yolk absorption on day 0 (b). Dry food on day 5 (c) and on day 10 (d). Plankton on day 5 (e) and day 10 (f). Artemia on day 5 (g) and day 10 (h). Dry food + plankton + Artemia on day 5 (i) and day 10 (j). Plankton + Artemia on day 5 (k) and on day 10 (l)

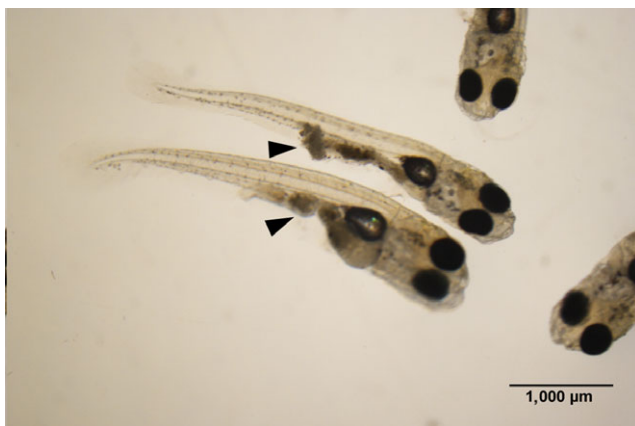


FIGURE 3 Fish fed with dry food on day 5. Arrow indicates the presence of dry food in the digestive tract of the fish

frequently in order to maintain the water quality. Therefore, the use of plankton may be an interesting alternative for fry feeding in freshwater species, given the cost of using *Artemia* sp. for fry rearing (Kolkovski, 2001).

As opposed to other species in which the fry do not capture particles of dry food due to incapacity of capture or recognition of the formulated diet (Aguilera, Mendoza, Iracheta & Marquez, 2012), the fry of the yellowtail tetra recognized the dry diet as a food

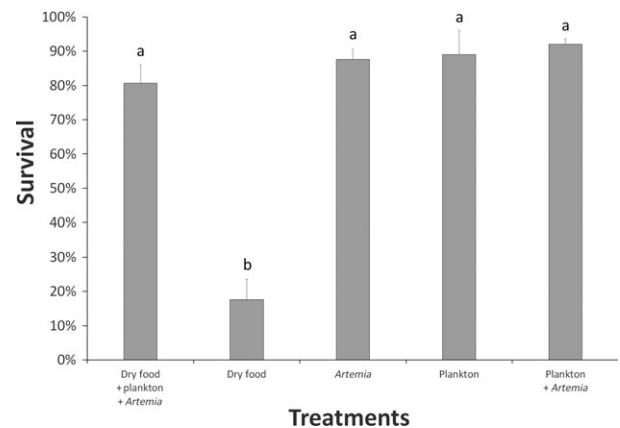


FIGURE 4 Survival (%) of *Astyanax altiparanae* larvae fed with different diets during 10 days of experimentation. Different letters indicate statistically significant differences by the Tukey multiple range test

source, but the fish did not increase the length. However, this fact emphasizes that this species presents potential to accept dry food and other immobile particles, and, as such, other dry food composition may be used in future studies. In addition, frozen or lyophilized plankton may be used for such purpose, and this is attractive because it is readily used and may avoid some fish diseases that arise when fresh plankton is provided. In the case of *Lates calcarifer*, Walford and Lam (1993) emphasize that providing dry diets

TABLE 2 Growth performance of diploid and triploid yellowtail tetra *Astyanax altiparanae* fed with plankton + *Artemia* nauplii for 10 days. Fish at 3 days post hatching were maintained in 1-L acrylic tanks (120 fish per tank) at 26°C. Data are shown as mean ± standard error and each data was generated from triplicates from different spawning. *p*-values shown in the table refers to ANOVA analysis. Identical capital letters within columns and lowercase within rows denote non-significant differences as determined by the *t* test ($p = .05$)

Treatments	0	1	2	3	4	5	6	7	8	9	10	<i>p</i> -value
Diploids	4.03 ± 0.04 ^{Aa}	4.15 ± 0.07 ^{Ab}	4.54 ± 0.09 ^{Aabc}	4.68 ± 0.08 ^{Aabc}	4.85 ± 0.10 ^{Abcd}	5.30 ± 0.05 ^{Acde}	5.42 ± 0.06 ^{Adef}	5.83 ± 0.30 ^{Aefg}	5.96 ± 0.16 ^{Afg}	6.17 ± 0.04 ^{Agh}	6.77 ± 0.25 ^{Ah}	.0001
Triploids	3.96 ± 0.01 ^{Aa}	4.14 ± 0.07 ^{Ab}	4.41 ± 0.05 ^{Aabc}	4.57 ± 0.13 ^{Aabc}	4.77 ± 0.19 ^{Abcd}	5.07 ± 0.12 ^{Acde}	5.32 ± 0.17 ^{Adef}	5.51 ± 0.25 ^{Aefg}	5.82 ± 0.09 ^{Afg}	6.10 ± 0.13 ^{Agh}	6.54 ± 0.15 ^{Ah}	.0001
<i>p</i> -value	.2252	.8659	.2547	.7284	.1507	.6120	.4671	.4861	.6328	.4844	–	–

during the early stages is not suitable because the fish may be not physiologically prepared to assimilate such kind of food, however, in other species, the use of dry food is feasible, as in the case of *Rhamdia quelen*, *Parachromis dovii*, and *Oreochromis niloticus*. Thus, the improvement of larval rearing, using artificial diets for the yellowtail tetra, may depend on utilization of other feeding components.

Regarding the ploidy status, these results showed that during the first feeding of *A. altiparanae*, triploid larvae presented similar growth and survival performance when compared with diploids. Previous studies have showed a decreased performance within triploids during the early stages (Arai, 2001; Piferrer et al., 2009), and those results may be related with the shock treatment during making triploids (Cherfas, Gomelsky, Ben-Dom, Peretz & Hulata, 1994; Piferrer et al., 2009).

In conclusion, the yellowtail tetra *Astyanax altiparanae* may accept several types of food during the first feeding, including immobile and dry food, but better results were found with live plankton + *Artemia* nauplii. Using such a procedure, the growth and survival of diploids and triploids were similar. These results are innovative for the species and open new possibilities for the use the yellowtail tetra as a laboratory and aquaculture fish.

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