SUBSTITUTION OF LIVE FEED BY FORMULATED DIET: EFFECT ON THE GROWTH AND SURVIVAL OF *BETTA SPLENDENS* (REGAN) FRY

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ABSTRACT

The present work evaluates the effectiveness of partial or total replacement of live feed (LF) (*Tubifex*) together with formulated diet (FD) for *Betta splendens*. Three hundred *Betta splendens* fry of uniform size (mean weight 0.19±0.01g) were equally distributed in five treatment groups with three replicates in glass aquaria of 35 litre capacity. Fishes were given diets at different ratio of LF and FD viz. T1(C)100% LF; T275% LF, 25% FD; T350% LF, 50% FD; T425% LF, 75% FD and T5100% FD and the experiment continued for 105 days. T2 group registered highest ($P<0.05$) % body weight gain (125.61±0.26) and specific growth rate (2.34±0.02), which was similar to T1 and T3 groups. Lowest FCR was recorded in T2 (2.40±0.11) group, which was similar to T1, T3 and T4 groups. Highest ($P<0.05$) PER was observed in T4 (1.00±0.03) group, which was similar to T3 and T5 groups. At the end of experiment, highest % survival was recoded in T1, T2 and T3 groups (96.67±1.67), which was similar to T4 group. From the study, it is concluded that LF can be successfully replaced up to 75% by FD without any adverse effect on the growth and survival of *Betta splendens*.

Key words: *Betta splendens*, Formulated diet, Growth, Live feed replacement, Survival.

INTRODUCTION

Ornamental fishes are kept as pets in confined space of an aquarium or garden pool for recreation. Keeping of aquarium fish has emerged as one of the most popular hobbies in recent years. The increasing demand for aquarium fishes gradually paved the avenue towards global trade of ornamental fishes. India is one of the global hotspots of ornamental fishes (Kumar *et al.*, 2005). Approximately 90% of freshwater ornamental fish are captive bred (Tlusty, 2002). They are gaining importance very rapidly due to their enormous commercial value in the export market (Kumar *et al.*, 2005). Ornamental fish farming is recognized as one of the major activities in rural and sub-urban areas, which is playing a promising role in poverty alleviation (Rana, 2009).

Several dry feed formulations have been tried as substitutes for LF for ornamental fishes. A low cost feed is necessary for successful entrepreneurs in ornamental fish production (Chapman and Fitz-Coy, 1997). Nutrition plays an important role in maintaining good health, external appearance, growth and it is largely based on extrapolation of results derived from food fishes under intensive farming conditions (Keshavanath and Patil, 2006). The development of nutritionally balanced feeds could reduce operational costs in ornamental fish farming. Formulation of cost-effective, nutritionally complete larval feeds would be great help to commercial culture operations (Robinson *et al.*, 2005). Majority of the ornamental fish entrepreneurs still depends on the supply of LF. But assured availability along with high cost of the LF is the major bottleneck for successful rearing of ornamental fishes. Hence, substitution of LF with FD is crucial for lowering the production costs and sustaining production.

Betta splendens is one of the most popular fish having great commercial value to the aquarium fish industry. A lot of work has been carried out on *Betta splendens* and mostly on aggression (Baenninger, 1984; Teresa *et al.*, 2006), mate choice and spawning success (Clotfelter, *et al.*, 2006) and bubble nest habitat characteristics (Jaroensutainee and Jaroensutainee, 2001). Little work has been done and reported by James and Sampath (2002) on the use of animal and plant protein, water hardness and feeding schedule. However, no report is available on replacement of LF using FD to evaluate the growth and survival of *Betta splendens*. Keeping this in mind, the present work was planned to formulate a diet, use it alone or in combination with LF (*Tubifex*) to observe their effect on growth and survival of *Betta splendens*.

MATERIALS AND METHODS

Fish, diets and experimental design

Three hundred *Betta splendens* fry (one month old) of uniform size (average weight 0.19±0.01g) were equally distributed in five distinct treatment groups in triplicate in glass aquaria of 35 litre capacity. The experimental tanks were arranged in completely randomized design (CRD). All tanks were filled with fresh chlorine free water from a Hi-Tech Reverse Osmosis Water System (I-MAX India LTD.). The trial examined on various *Tubifex* replacement options are mentioned as below:

1. 100% *Tubifex* (T1)
2. 75% *Tubifex*: 25% FD (T2)
3. 50% *Tubifex*: 50% FD (T3)
4. 25% *Tubifex*: 75% FD (T4)
5. 100% FD (T5)

The total feed was divided in to two split doses and offered at 10:00 and 17:00 hrs. Before feeding, faecal matter was removed from all the experimental tanks every day morning and 20% water was replaced. Continuous aeration was supplied through air blower to maintain the dissolved oxygen at optimum level. Although *Betta splendens* can take atmospheric air directly yet aeration helps in removing ammonia from tank water. At the beginning, feed was given at 5% of the total biomass and gradually adjusted based on the observations of daily feed consumption. The feeding trial was continued for 105 days and the fishes were put on fastened for overnight before sampling to minimize faecal contamination during sampling.

Water quality

Water quality parameters like temperature, dissolved oxygen (DO), pH, free carbon dioxide, hardness, alkalinity, chlorides, ammonia-N, nitrite-N and nitrate-N were estimated every week as described in APHA (1998). Water quality parameters were maintained at desired levels by replacing about 20% fresh chlorine free bore well water using a Hi-Tech Reverse Osmosis Water System every day in the morning.

Formulation of diet and feeding

The experimental diet with 35% crude protein (CP) was formulated (Table 1) using fish meal, squid meal, beef liver meal, soybean meal, corn flour, wheat powder, sunflower oil, cod liver oil, mineral-vitamin mixture, vitamin C, carboxymethyl cellulose (CMC) and butylated hydroxyl toluene (BHT). The dry ingredients were blended to make a homogenous mixture. Then the diet was mixed with boiled water to form dough. BHT was dissolved in oil and mixed thoroughly in the dough and cooked by steam for 15 minutes. The mineral-vitamin mixture and vitamin C were mixed after cooling the dough. Pellets (2 mm size) were prepared with a hand pelletizer and dried in the oven at 60 °C to a moisture content of around 78%. Feeds were packed in a zip-locked polythene pack for feeding fish during the experimental periods. The proximate analysis of both FD and LF (*Tubifex*) (Table 1) was estimated by the method of AOAC (2005).

Growth parameters

The growth parameters studies were assessed by taking their body weight at three weeks interval. All growth parameters were calculated at three weeks sampling intervals keeping in mind the different growing stages and growth pattern of *Betta splendens*. Different samplings also indicated the feed acceptability, palatability and survival rate (%) of *Betta splendens* with respect to different sampling periods. Various growth parameters and nutrient utilization of fish were evaluated as follows:

$$\text{Average body weight (ABW) (g)} = \frac{\text{Total weight of fish in tank (g)}}{\text{Number of animals at sampling in tank}}$$

$$\text{Body Weight Gain (\% BWG)} = \frac{\text{Final body weight - Initial body weight}}{\text{Initial body weight}} \times 100$$

$$\text{Specific Growth Rate (\% SGR)} = \frac{\text{Ln (final weight) - Ln (Initial weight)}}{\text{Number of days}} \times 100$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Feed given (dry weight)}}{\text{Body weight gain (wet weight)}}$$

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{Body weight gain (wet weight)}}{\text{Crude protein fed}}$$

$$\text{Survival rate (\%)} = \frac{\text{Number of surviving fish}}{\text{Total number of fish stocked}} \times 100$$

Statistical analysis

All the statistical analysis was performed by using the software SPSS (Version 16.0). Significant differences among treatment groups were done by one-way analysis of variance (ANOVA) and the comparison of mean values were done by Duncans's multiple range test (DMRT).

RESULTS AND DISCUSSION

Water quality

Water quality parameters like temperature, pH, DO, carbonate hardness, alkalinity, chlorides, free CO_2, ammonia-N, nitrite-N and nitrate-N in all experimental tanks were analyzed at weekly intervals (Table 2). Most of the water quality parameters were found to be within the normal range for fish requirement. Temperature, DO and pH were maintained at 25.5-30.5 C, 6.0-7.2, mg L-1 and 6.2-7.5, respectively. Alkalinity, carbonate hardness, chloride and free CO_2 values were found 14.0-12.0, 4.0-14.0, 2.0-16.0 and 0.2-1.2 mg L, respectively. Nitrate-N, nitrite-N and ammonia-N1 were recorded as 3.8, 0.3 and 0.1-0.7 mg L, respectively. Continuous aeration was maintained round the clock throughout the experiment. So, animals were not affected due to slightly higher levels of ammonia nitrogen.

Table 1: Formulation of the experimental diet and proximate composition (dry matter basis)

Ingredients	**% Inclusions**	***Tubifex***
Fish meal [1]	12	–
Squid meal [1]	10	–
Beef liver meal [1]	10	–
Wheat bran [1]	22	–
Corn flour[1]	20	–
Soybean meal [1]	16	–
Cod liver oil [2]	4	–
Sunflower oil [3]	4	–
Mineral-Vitamin mix (EMIX PLUS)[4]	1.5	–
Vitamin C [5]	0.01	–
Carboxymethyl cellulose (CMC) [6]	0.5	–
Butylated hydroxyl toluene (BHT) [6]	0.01	–
Proximate composition of formulated feed and Tubifex (dry matter basis)		
Nutrients	**Formulated diet**	***Tubifex***
Crude Protein	34.07 ± 0.39	59.70 ± 0.06
Ether Extract	8.84 ± 0.04	17.5 ± 0.50
Ash	8.80 ± 0.09	4.48 ± 0.02
Total carbohydrate	48.30 ± 0.43	18.33 ± 0.47
Moisture	6.40 ± 0.18	82.0 ± 0.60
Digestible energy (Kcal 100^{-1} g)	409.20	467.06

Digestible energy (Kcal 100^{-1}g) = (4 x Crude protein %) + (9xlipid %) + (4xCarbohydrate %)

[1]Procured from local market; [2]Densa Pharma Private Limited, Mumbai

[3]Ruchi Soya Pvt. Ltd., Raigad, India

[4]Composition of vitamin mineral mix (EMIX PLUS) (quantity/ 2.5 kg)

Vitamin A 55,00,000 IU; Vitamin D_3 11,00,000 IU; Vitamin B_2 2,000 mg; Vitamin E 750 mg; Vitamin K 1,000 mg; Vitamin B_6 1,000 mg; Vitamin B_{12} 6 mcg; Calcium Pantothenate 2,500 mg; Nicotinamide 10 g; Choline Chloride 150 g; Mn 27,000 mg; I 1,000 mg; Fe 7,500 mg; Zn 5,000 mg; Cu 2,000 mg; Co 450 mg; Ca 500 g; P 300 g; L- lysine 10 g; DL- Methionine 10 g; Selenium 50 ppm; Satwari 250 ppm; (Lactobacillus 120 million units and Yeast Culture 3000 crore units).

[5]Sd Fines Chemicals Ltd., India; [6]Himedia Laboratories Ltd., Mumbai, India.

Table 2: Water quality parameters in different experimental groups during 105 days of feeding experiment

Water quality parameter	Treatments				
	Control (T1)	T2	T3	T4	T5
Temperature (^0C)	25.5 –30.0	26.0 –30.5	26.0 –30.0	26.0 –30.5	26.0 –30.0
pH	6.2 –7.0	6.2 –7.0	6.4 –7.0	6.5 –7.5	6.4 –7.5
DO (mg L^{-1})	6.0 –7.2	6.0 –6.8	5.6 –6.8	6.0 –6.8	6.0 –6.8
Hardness (mg L^{-1})	4.0 –14.0	4.0 –10.0	4.0 –8.0	4.0 –8.0	6.0 –8.0
Alkalinity (mg L^{-1})	6.0 –12.0	6.0 –12 .0	6.0 –10.0	4.0 – 8.0	5.0 –10.0
Chlorides (mg L^{-1})	2.0 –16.0	2.0 –14.0	2.0 –12.0	2.0 –14.0	2.0 –14.0
Free CO_2 (mg L^{-1})	0.4 –1.1	0.5 –1.2	0.2 –0.9	0.4 –1.0	0.3 –0.8
Ammonia –N (mg L^{-1})	0.2 –0.4	0.3 –0.7	0.3 –0.6	0.3 – 0.6	0.1 –0.4
Nitrite –N (mg L^{-1})	0.03 –0.09	0.04 –0.06	0.03 –0.08	0.03 –0.10	0.03 –0.3
Nitrate –N (mg L^{-1})	1.2 – 3.7	1.0 –2.2	0.9 –3.8	1.0 – 2.2	0.7 –3.6

Growth parameters and survival rate (%)

Average body weight (ABW), percentage body weight gain (% BWG) and specific growth rate (% SGR) is presented in Table 3. Highest ($P<0.05$) ABW (0.43 ± 0.0g), BWG (125.61±0.26%) and SGR (3.88±0.01%) are observed in T2 group during 1st sampling, which was similar to T1 and T3 groups. No significant changes in the above parameters were observed during 2nd sampling. T2 group recorded highest ($P<0.05$) ABW value during 3rd, 4th and 5th sampling, which was similar to T1 and T3 groups. T3 group exhibited highest ($P<0.05$) BWG and SGR values during 3rd and 4th sampling. On the other hand, T4 group registered highest BWG and SGR values in 5th sampling, which was similar to T1, T2 and T3 groups. At the end of experiment, T2 group recorded highest ($P<0.05$) SGR (2.34±0.02) and BWG (67.1±1.03), which was similar to T1 and T3 groups. Both BWG and SGR values showed decreasing trend from 1st to 5th sampling in all the treatment groups. This might be due to the energy spent on gonad development in fish at the later part of experiment. Male *Betta splendens* spends lot of energy on aggressive behaviour towards other male occupants in the tanks. Kaiser *et al.* (2003) found that gold fish fed a mixture of LF and FD grew at the same rate as fish fed only LF, which is in agreement with the present findings. Kim *et al.* (1996) also reported that Coho salmon fry fed with LF grew faster than those fed with FD. Degani (1991) reported that *Trichogaster trichopterus* fry fed with LF grew faster than those fed with FD due to the palatability, high consumption rate and chemical composition of LF.

The values of FCR, PER and survival rate (%) are presented in Table 4. Highest ($P<0.05$) FCR value was recorded in T5 group in all five samplings compared to other groups. Lowest FCR value was registered in T2 group (1.17±0.01) in 1st sampling, which was similar to T1 and T3 groups. T3 group recorded lowest FCR (1.95±0.08) and (3.13±0.24) in 3rd and 5th samplings, respectively, which was similar to T1, T2 and T4 groups. In 4th sampling, lowest FCR was found in T3 (2.04±0.08) group, which was similar to T2 and T4 groups. No significant ($P>0.05$) changes in FCR values was observed in 2nd sampling. At the end of 105 days of experiment, lowest FCR was recorded in T2 (2.40±0.11) group, which was similar to T1, T3 and T4 groups. FCR values in all treatment groups recorded increasing trend from 1st to 5th samplings. Higher FCR recorded was mainly due to attaining maturity in the fishes at the later part of the experiment and bulk of energy might have spent on reproductive growth rather than somatic growth. Soundarapandian *et al.* (2002) reported that *Macrobrachium malcolmsonii* fed with FD showed significantly higher FCR than they fed with LF.

PER recorded highest ($P<0.05$) value in T5 group during 1st (1.85±0.11) and 2nd (1.61±0.09) samplings, which was similar to T4 group. In 3rd sampling, highest ($P<0.05$) PER was recorded in T3 (1.08 ±0.04) group, which was similar to T4 group. T3 group registered highest ($P<0.05$) PER (0.84±0.05) in 4th sampling, which was similar to T2 and T4 groups. However, T4 (0.26±0.02) group recorded highest ($P<0.05$) PER in 5th sampling, which was similar to T1, T2 and T3 groups. At the end of 105 days of experiment, highest ($P<0.05$) PER (1.00±0.03) was recorded in T4 group, which was similar to T3 and T5 groups. From 1st to 5th sampling PER values showed decreasing trend in all the treatment groups. No mortality was observed in any treatment groups till 2nd sampling. T1, T2 and T3 groups recorded highest ($P<0.05$) % survival (96.7±1.7) at the end of 105 days of experiment. A few mortality occurred was due to aggressive fighting among the males, which caused damages to the males and females. The fighting bevaiour of *Betta splendens* has been reported by many authors (Baenninger, 1984; Teresa *et al.*, 2006). Mahmoudzadeh *et al.* (2008) did their work with *Coregonus lavaretus* and found that survival rates of the fry declined from 75% to 61.4%, from 74.3 to 45.8% and from 72.7 to 54.5% for LF, mixed diet and FD, respectively, which supports the present study. In gold fish, the use of a mixture on dry matter basis of 50% *Artemia* (LF) and 50% FD recorded similar growth and survival when compared with LF; fish fed FD grew significantly slower than those fed LF or a mixture of both (Abi-Ayad and Kestemont, 1994). Types of feed influenced significantly on the growth and feed intake in *Betta splendens*. The results observed that fish fed with either only *Tubifex* (LF) or partial replacement of LF gave better results compared to fish fed with only FD. James and Sampath (2002) also reported that *Betta splendens* fed with mixed diet showed higher feed intake than those fed with only FD. Results from the present study conclude that *Tubifex* can be successfully replaced to the extent of 75% by formulated diet without any adverse effect on the growth and survival of *Betta splendens*.

Table 3: Effect of different replacement level of live feed with formulated diet on average body weight, percentage body weight gain and specific growth rate in *Betta splendens*

Rearing period (days)	Treatments					p-value
	T1	T2	T3	T4	T5	
Average body weight (g)						
0	.. ^{A} ± 0.0	0.19 ^{A} ± 0.0	0.19 ^{A} ± 0.0	0.19 ^{A} ± 0.0	0.19 ^{A} ± 0.0	0.873
21	$0.42^{c\,B}$ ± 0.01	$0.43^{c\,B}$ ± 0.0	$0.41^{bc\,B}$ ± 0.01	$0.39^{ab\,B}$ ± 0.0	$0.37^{a\,B}$ ± 0.01	0.005
42	$0.75^{ab\,C}$ ± 0.04	$0.78^{b\,C}$ ± 0.01	$0.72^{ab\,C}$ ± 0.03	$0.69^{ab\,C}$ ± 0.01	$0.65^{a\,C}$ ± 0.05	0.085
63	$1.20^{c\,D}$ ± 0.05	$1.23^{c\,D}$ ± 0.05	$1.17^{c\,D}$ ± 0.02	$1.03^{b\,D}$ ± 0.02	$0.88^{a\,D}$ ± 0.03	<0.001
84	$1.64^{c\,E}$ ± 0.05	$1.84^{c\,E}$ ± 0.11	$1.79^{c\,E}$ ± 0.03	$1.44^{b\,E}$ ± 0.04	$1.07^{a\,E}$ ± 0.04	<0.001
105	$2.02^{c\,F}$ ± 0.10	$2.21^{c\,F}$ ± 0.04	$2.07^{c\,F}$ ± 0.03	$1.83^{b\,F}$ ± 0.07	$1.15^{a\,F}$ ± 0.03	<0.001
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	
Body weight gain (%BWG)						
21	$119.01^{c\,D}$ ± 5.51	$125.61^{c\,D}$ ± 0.26	$113.59^{bc\,D}$ ± 5.22	$103.80^{ab\,E}$ ± 4.14	$97.49^{a\,C}$ ± 5.41	0.009
42	78.06 ^{C} ± 5.12	82.73 ^{C} ± 0.84	76.50 ^{C} ± 5.62	74.96 ^{D} ± 1.93	73.50 ^{C} ± 1.85	0.469
63	$59.89^{d\,B}$ ± 2.62	$56.91^{c\,B}$ ± 6.61	$64.00^{e\,BC}$ ± 5.40	$50.43^{b\,C}$ ± 0.96	$37.34^{a\,B}$ ± 1.12	0.007
84	$37.06^{b\,A}$ ± 2.26	$49.26^{cd\,B}$ ± 4.67	$52.85^{d\,B}$ ± 0.93	$39.80^{bc\,B}$ ± 5.11	$22.62^{a\,AB}$ ± 2.29	0.001
105	$23.77^{b\,A}$ ± 5.42	$21.08^{b\,A}$ ± 5.19	$15.34^{ab\,A}$ ± 0.48	$26.72^{b\,A}$ ± 2.99	$7.37^{a\,A}$ ± 0.40	0.025
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	
Average	63.6^{c} ± 0.89	67.1^{c} ± 1.03	64.5^{c} ± 1.06	59.1^{b} ± 1.78	47.7^{a} ± 1.05	<0.001
Specific growth rate (SGR)						
21	$3.73^{bc\,E}$ ± 0.12	$3.88^{c\,D}$ ± 0.01	$3.61^{bc\,D}$ ± 0.11	$3.39^{ab\,E}$ ± 0.10	$3.24^{a\,C}$ ± 0.13	0.011
42	2.74 ^{D} ± 0.14	2.87^{C} ± 0.02	2.70 ^{C} ± 0.15	2.66 ^{D} ± 0.05	2.62 ^{C} ± 0.18	0.665
63	$2.24^{b\,C}$ ± 0.08	$2.14^{ab\,B}$ ± 0.20	$2.35^{b\,BC}$ ± 0.16	$1.94^{ab\,C}$ ± 0.03	$1.79^{a\,B}$ ± 0.06	0.053
84	$1.50^{b\,B}$ ± 0.08	$1.90^{cd\,B}$ ± 0.15	$2.02^{d\,B}$ ± 0.03	$1.59^{bc\,B}$ ± 0.17	$0.96^{a\,AB}$ ± 0.09	0.001
105	$1.01^{b\,A}$ ± 0.21	$0.90^{b\,A}$ ± 0.20	$0.68^{ab\,A}$ ± 0.02	$1.12^{b\,A}$ ± 0.11	$0.34^{a\,A}$ ± 0.02	0.019
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	
Average	2.24^{bc} ± 0.04	2.34^{c} ± 0.02	2.27^{c} ± 0.02	2.14^{b} ± 0.06	1.72^{a} ± 0.04	<0.001

Mean values with different superscripts (a, b and c) in a row are significantly different (P<0.05). Different superscripts (A, B and C) in a column are significantly different (P<0.05) with respect to different sampling period. Values in means ±S.E. (n=3)

Table 4: Effect of different replacement level of live feed with formulated diet on feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate in *Betta splendens*

Rearing period (days)	Treatments					p-value
	T1	T2	T3	T4	T5	
Feed conversion ratio (FCR)						
21	$1.24^{ab\,A} \pm 0.06$	$1.17^{a\,A} \pm 0.01$	$1.30^{ab\,A} \pm 0.05$	$1.42^{bc\,A} \pm 0.03$	$1.52^{c\,A} \pm 0.10$	0.008
42	$1.64^{B} \pm 0.15$	$1.53^{A} \pm 0.02$	$1.66^{AB} \pm 0.139$	$1.69^{A} \pm 0.04$	$1.76^{A} \pm 0.126$	0.628
63	$2.09^{a\,C} \pm 0.07$	$2.22^{a\,B} \pm 0.22$	$1.95^{a\,B} \pm 0.08$	$2.45^{a\,B} \pm 0.07$	$3.70^{b\,B} \pm 0.28$	<0.001
84	$2.76^{b\,D} \pm 0.11$	$2.20^{ab\,B} \pm 0.22$	$2.04^{a\,B} \pm 0.08$	$2.69^{ab\,B} \pm 0.31$	$4.30^{c\,BC} \pm 0.24$	<0.001
105	$3.25^{a\,E} \pm 0.13$	$3.36^{a\,C} \pm 0.29$	$3.13^{a\,C} \pm 0.24$	$3.89^{a\,C} \pm 0.29$	$4.71^{b\,C} \pm 0.29$	0.008
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	
Average	$2.78^{a} \pm 0.26$	$2.40^{a} \pm 0.11$	$2.87^{a} \pm 0.06$	$2.60^{a} \pm 0.15$	$3.20^{b} \pm 0.21$	0.002
Protein efficiency ratio (PER)						
21	$1.29^{a\,E} \pm 0.06$	$1.51^{b\,D} \pm 0.01$	$1.56^{b\,D} \pm 0.06$	$1.68^{bc\,E} \pm 0.04$	$1.85^{c\,C} \pm 0.11$	0.002
42	$0.99^{a\,D} \pm 0.09$	$1.16^{ab\,C} \pm 0.01$	$1.26^{b\,C} \pm 0.11$	$1.39^{bc\,D} \pm 0.04$	$1.61^{c\,C} \pm 0.09$	0.002
63	$0.73^{a\,C} \pm 0.02$	$0.82^{ab\,B} \pm 0.09$	$1.08^{c\,C} \pm 0.04$	$0.96^{bc\,C} \pm 0.03$	$0.75^{a\,B} \pm 0.07$	0.006
84	$0.46^{a\,B} \pm 0.016$	$0.68^{b\,B} \pm 0.06$	$0.84^{b\,B} \pm 0.05$	$0.74^{b\,B} \pm 0.11$	$0.35^{a\,AB} \pm 0.03$	0.001
105	$0.24^{b\,A} \pm 0.05$	$0.22^{b\,A} \pm 0.05$	$0.18^{b\,A} \pm 0.01$	$0.26^{b\,A} \pm 0.02$	$0.04^{a\,A} \pm 0.00$	0.007
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	
Average	$0.74^{a} \pm 0.03$	$0.88^{b} \pm 0.02$	$0.98^{c} \pm 0.03$	$1.00^{c} \pm 0.03$	$0.92^{bc} \pm 0.03$	<0.001
Survival rate (%)						
21	$100.0^{A} \pm 0.0$	100.0 ± 0.0	100.0 ± 0.0	$100.0^{B} \pm 0.0$	$100.0^{C} \pm 0.0$	–
42	$100.0^{A} \pm 0.0$	100.0 ± 0.0	100.0 ± 0.0	$100.0^{B} \pm 0.0$	$100.0^{C} \pm 0.0$	–
63	$100.0^{b\,A} \pm 0.0$	$96.7^{b} \pm 1.7$	$98.3^{b} \pm 1.7$	$96.7^{b\,AB} \pm 3.3$	$78.3^{a\,B} \pm 4.4$	0.001
84	$100.0^{c\,A} \pm 0.0$	$96.7^{bc} \pm 1.7$	$98.3^{bc} \pm 1.7$	$93.3^{b\,A} \pm 1.7$	$49.0^{a\,A} \pm 2.0$	<0.001
105	$96.7^{b\,B} \pm 1.7$	$96.7^{b} \pm 1.7$	$96.7^{b} \pm 1.7$	$93.3^{b\,A} \pm 1.7$	$49.0^{a\,A} \pm 2.0$	<0.001
p-value	0.034	0.171	0.382	0.056	<0.001	

Mean values with different superscripts (a, b and c) in a row are significantly different (P<0.05). Different superscripts (A, B and C) in a column are significantly different (P<0.05) with respect to different sampling period. Values in means ±S.E. (n=3).

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