



Relative Efficiencies of *Artemia nauplii*, Dry Food and Mixed Food Diets in Intensive Rearing of Larval Crucian Carp (*Carassius carassius* L.)

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Abstract

Effective and safe rearing of larvae of endangered fishes can be facilitated by using dry food diets being suitable for intensive culture. Accordingly, we tested the efficiency of some feeding protocols containing significantly decreased portion of live food or no live food at all in intensive rearing of crucian carp larvae *Carassius carassius* (L.) under controlled conditions. Based on 7 days long preliminary experiments, SDS 100 and Classic C22 were selected over Sera micron and Nutra HP 0.3 as potential components of a dry food diet. In accordance with other studies, it was found that neither of the pure dry food and mixed food diets tested proved to be as effective as the *Artemia nauplii* during the first 21 days of the exogenous feeding. Although, the survival rate of larvae could be maintained at high level in some of the protocols tested, a strong decrease in the growth rate was obvious in all diets containing dry food. It was concluded that crucian carp larvae adapt poorly to commercial dry foods, and thus if large larvae of good fitness are needed (i.e. for stockings to natural habitats) then they should be reared on live food diet.

Keywords: Dry food diet, growth rate, intensive rearing, larviculture, survival rate.

Introduction

Crucian carp *Carassius carassius* (L.) has undergone a substantial decline in most of its native range in Europe (e.g. Wheeler, 2000; Skrzypczak and Mamcarz, 2005; Tarkan *et al.*, 2009; Sayer *et al.*, 2011). According to Sayer *et al.* (2011), of the potential factors being responsible for population degradations three factors can be emphasized being of particular concern: climatic variations result extreme water level changes with temporal or eventual desiccation of habitats; changes of land use alter habitat characteristics and connectivity; and the introduction of non-native fishes may result extinction via competition or predation, and diminish species integrity by hybridization (e.g. Wheeler, 2000; Hänfling *et al.*, 2005; Copp *et al.*, 2008). As a result of the above processes, crucian carp is now considered as endangered, threatened or strictly protected fish species in several European countries, for instance in Austria (Wolfram and Mikschie,

2007), Croatia (Mrakovčić *et al.*, 2007), Slovakia (Lusková *et al.*, 2008) and Romania (Bănărescu, 93, 1994), as well crucian carp is now on the IUCN Red List (IUCN, 2011).

Recently, crucian carp has become the subject of habitat conservation actions especially in pond and wetland habitat rehabilitations (Coop *et al.*, 2008), and it has also been rediscovered by anglers. Hence, the need for pre-reared crucian carp larvae and fry of good fitness for stocking is gradually increasing (Müller *et al.*, 2007). Rearing of larvae of endangered fish bred for species conservational proposes must however meet special criteria warranting species integrity and high survival rate of these valuable larvae. Consequently, such larvae preferably should be reared in monospecific, intensive larvicultures, which work effectively if a feasible dry food based feeding protocol is available.

Although artificial propagation of crucian carp is now successful under laboratory conditions (Müller *et al.*, 2007; Targońska *et al.*, 2009), an appropriate dry

food based feeding protocol enabling the intensive rearing of larvae is still missing. One of the most critical points of an intensive larvae rearing is the selection of the starter food. Several cyprinid species, like for example, barbel *Barbus barbus* (L.) (Wolnicki and Górný, 1995c) and rudd *Scardinius erythrophthalmus* (L.) (Wolnicki et al., 2009), can effectively be grown on commercial dry food diet. Other species are more sensitive to commercial food, and thus, they need supplementary live food for an efficient rearing, such for example, tench *Tinca tinca* (L.) (Wolnicki and Górný, 1995b), ide *Leuciscus idus* (L.) (Wolnicki and Górný, 1995a) and crucian carp (Żarski et al., 2011). Although juvenile crucian carp can successfully be reared on dry food diet (Myszkowski et al., 2002), larvae proved to be sensitive to the food provided, especially at the onset of the exogenous feeding, and diets based solely on dry food were found to decrease the fitness of larvae and increase mortality (Żarski et al., 2011). Since the use of live food is laborious and parlous in intensive

cultures, hence, it is important to develop a feasibly dry food based feeding protocol that minimizes the need of live food.

In this study, we investigated the applicability of some not yet tested (Żarski et al., 2011) alternative feeding protocols containing significantly decreased proportion of live food or no live food at all in intensive rearing of crucian carp larvae under controlled conditions.

Materials and Methods

Experimental Design

Five independent experiments (E1, E2, E3, E4, E5) were carried out to test differences among feeding protocols based on either pure live food, dry food or mixed food diets (Table 1). Four commercial dry foods and freshly hatched *Artemia nauplii* were used in the experiments (Table 2). Experiment 1 and E2 were preliminary trials to test which of the four most

Table 1. Applied foods and feeding protocols tested in the experiments

Experiment number	Treatment group	Number of parallels	Feeding protocol (fish were fed six times a day)	Abbreviation of the feeding protocol
1.	1.1	5	Only <i>Artemia</i> nauplii	A
	1.2	5	Only Sera micron	Sera
	1.3	5	Only SDS 100	SDS
2.	2.1	3	Only <i>Artemia</i> nauplii	A
	2.2	4	Only SDS 100	SDS
	2.3	4	Only Nutra	Nutra
	2.4	4	Only Classic	Classic
3.	3.1	3	Only <i>Artemia</i> nauplii	A
	3.2	3	Only SDS 100	SDS
	3.3	3	SDS five times and <i>Artemia</i> nauplii once	SDS 5× - A 1×
	3.4	3	Only Classic	Classic
	3.5	3	Classic five times and <i>Artemia</i> nauplii once	Classic 5× - A 1×
4.	4.1	3	Only <i>Artemia</i> nauplii	A
	4.2	3	SDS five times and <i>Artemia</i> nauplii once	SDS 5× - A 1×
	4.3	3	SDS four times and <i>Artemia</i> nauplii two times	SDS 4× - A 2×
	4.4	3	Classic five times and <i>Artemia</i> nauplii once	Classic 5× - A 1×
	4.5	3	Classic four times and <i>Artemia</i> nauplii two times	Classic 4× - A 2×
5.	5.1	3	Only <i>Artemia</i> nauplii	A
	5.2	3	Only SDS 100	SDS
	5.3	3	For 5 days <i>Artemia</i> nauplii then for 16 days SDS only	A ₅ SDS ₁₆
	5.4	3	For 10 days <i>Artemia</i> nauplii then for 11 days SDS only	A ₁₀ SDS ₁₁
	5.5	3	Only Classic	Classic
	5.6	3	For 5 days <i>Artemia</i> nauplii then for 16 days Classic only	A ₅ Classic ₁₆
	5.7	3	For 10 days <i>Artemia</i> nauplii then for 11 days Classic only	A ₁₀ Classic ₁₁

Table 2. Characteristics of foods tested

Food type	Particle size (µm)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Crude ash (%)	Digestible energy(MJ Kg ⁻¹)
<i>Artemia</i> sp. nauplii*	590	54	11	-	8	-
Sera micron	-	50.2	8.1	4.2	11.9	-
SDS 100 ^a	80-200	55	14	1	12	-
Nutra HP 0.3 ^b	200-600	57	17	0.5	10	19.9
Classic C22	-	28	7	4	6	12.5

* Based on data of SERA *Artemia*; ^a Vit. A 30000 IU kg⁻¹, vit. D3 2500 IU kg⁻¹, vit. E 700 IU kg⁻¹, vit. C 2000 IU kg⁻¹, ω3 + HUFA 30 mg g⁻¹

^b Phosphorus content 1.3%

commonly used starter dry foods may be applied in crucian carp larvae. The tested foods were Sera micron (Sera GmbH), Nutra HP 0.3 (Skretting), Classic C22 (Skretting) and SDS 100 (Special Diets Services Limited International Dietex GB). As a control food, live *Artemia* nauplii (Sera GmbH) were used. These experiments were performed for 7 days. Based on the results of E1 and E2, two dry foods, the SDS 100 and Classic C22 were selected as potential components of a suitable dry food based diet. Three 21 days long experiments were launched to test how dry foods could optimally be incorporated to intensive crucian carp larvae rearing procedure. In E3 and E4, feeding protocols based solely on SDS 100 or Classic C22 dry foods, and feeding protocols based on alternate feeding with dry food and live *Artemia* nauplii in two types of combinations (i.e. five times dry food and once *Artemia* nauplii per day, and four times dry food and twice *Artemia* nauplii per day) were compared with each other and with the feeding protocol based on pure live *Artemia* nauplii. In E5, feeding protocols using *Artemia* nauplii as a starter food and replaced later (i.e. on the 6th or 11th day of the experiment) with SDS 100 or Classic C22 dry foods were compared with protocols based only on dry food or *Artemia* nauplii.

Experimental Stocks

For the experiments, crucian carp larvae were obtained from artificial propagation of wild spawners. In E1 and E2, the spawners were originated from Lake Pötréte (Zala County, Hungary), in E3 from Lake Rákospalota (Pest County, Hungary), and in E4 and E5 from Lake Várpalota (Veszprém County, Hungary). In E1, out of spawning season maturation of fish was induced by gradually increasing water temperature from 5 to 18°C during 10 days followed by a single hormonal treatment with 6 mg carp pituitary (CP) per kg of body mass (BM). In E2-E5, fish were reproduced in the spawning season and were induced with one 6 mg CP per kg of BM hormonal treatment without the manipulation of the water temperature (18-23°C). Males and females got the same treatment, and propagation was possible after a 16 hours latency time. In E1-E3, stripped eggs of three females were mixed and fertilised with sperm obtained from four males. In E4-E5, eggs of one female were fertilised with sperm of two males. Following the activation of the gametes with water, Woynárovich-solution (10 L water, 40 g NaCl, 30g urea) was added and eggs were stirred in this solution for 60-90 minutes allowing them to swell. Afterwards, the eggs were washed in solution of tannic acid (10 L water, 5 g tannin) two times for 20 sec. Then, eggs were incubated in 1.5 L mini Zugar jars, and larvae hatched between 3-6 days after the fertilisation. Free swimming of fish was observed 2-3 days post-hatch (DPH). Experiments were launched 3-5 DPH when larvae had shifted to exogenous feeding. Maturation

and reproduction of crucian carp, and all larvae rearing experiments were carried out at the Department of Aquaculture of Szent István University in Gödöllő.

Rearing Conditions

Experimental stock densities (90 ind. L⁻¹ and 50 ind. l⁻¹ in E1-2 and E3-5, respectively) were set within the optimum range according to Žarski *et al.* (2011). Larvae were fed six times a day (at 06:00, 08:00, 11:00, 14:00, 17:00 and 20:00) manually. Initial daily feeding rates (day 1) with dry food were approximately 40% of the total fish biomass (36 mg 100 ind.⁻¹, according to the 0.9 mg initial body mass (BM) of crucian carp larvae based on Žarski *et al.*, 2011), and then gradually decreased to about 7% of the total fish biomass (23.8-35 mg 100 ind.⁻¹) during the final days of the experiments. Daily feeding rates with *Artemia* nauplii initially exceeded 300% of the total fish biomass (270 mg 100 ind.⁻¹) in wet weight, and then it was gradually decreased to about 40% of the total fish biomass (1592-2756 mg 100 ind.⁻¹) on day 21.

Fish were reared in tanks, operating in a common recirculation system (1,500 L filtration and puffer system). Specifications of the rearing tanks and stocking densities are given in Table 3. Water flow in each tank was maintained at 0.12 l min⁻¹. Fish tanks were cleaned daily after the last feeding. Water temperature was measured twice a day at 08:00 and 17:00. Other water quality parameters were measured daily in E1 and E2, and at days 0, 10 and 21 in E3-5 (Table 3).

Dead larvae were counted and removed from each tank daily during the scheduled cleanings, and mortality was expressed in percentage of the number of stocked fish. Total length (TL) of fish in each experimental group was measured to the nearest 0.1 mm at the start and the end of experiments by Image J software (National Institutes of Health). At the end of the experiments, total biomass of all survived fish by tanks was also measured with a Sartorius balance to the nearest 0.01 g, and the mean individual BM of fish in each experimental group was calculated by dividing total biomass with the number of survived larvae.

Data Analysis

One way ANOVA followed by Tukey HSD *post hoc* test was used to compare final TL and BM values, and Kruskal-Wallis one-way analysis of variance by ranks followed by Mann-Whitney U-test as *post hoc* test was used to compare percentage survival among experimental groups (i.e. different feeding protocols) in each experiment. Statistical analyses were carried out with SPSS 7.5 for Windows software package. To facilitate a wider comparison with previously published data the following growth indices were also calculated. Daily growth in length was expressed as

Table 3. The main settings and the water quality parameters (mean±SD) during the experiments

Experiment	1.	2.	3.	4.	5.
Tank size (L)	3.3	3.3	1.8	1.8	2.4
Fish per tank	300	300	90	90	120
Stocking density fish L ⁻¹	90	90	50	50	50
Number of tanks	15	15	15	15	21
Duration of the experiment (days)	7	7	21	21	21
Water temperature (°C)	23.9±0.2	22.6±0.4	24.5±1.3	25.2±0.7	25.2±0.7
pH	8.1±0.2	8.3±0.1	8.2±0.3	8.3±0.04	8.3±0.04
O ₂ (mg L ⁻¹)	7.1±1.1	6.8±0.3	6.4±0.5	6.9±1.2	6.9±1.2
NO ₂ (mg L ⁻¹)	0.1±0.1	0.1±0.1	0.02±0.00	0.02±0.01	0.02±0.01
NO ₃ (mg L ⁻¹)	3.8±0.4	4.1±0.3	3.6±0.7	3.1±1.7	3.1±1.7
NH ₄ (mg L ⁻¹)	2.4±0.8	1.0±1.1	2.2±0.1	1.1±0.5	1.1±0.5

$DGL=(TL_t-TL_0)t^{-1}$, specific growth rate in length as $SGR=100(\ln TL_t-\ln TL_0)t^{-1}$ and relative individual growth rate in length as $RGR=100(e^{SGR/100}-1)$, where TL_t is the final TL (mm), TL_0 is the initial TL (mm) and t (days) is the duration of the experiments.

Results

Experiment 1 and E2 showed that during the first seven days of their exogenous feeding, crucian carp larvae grew and survived significantly better on *Artemia* nauplii than on any of the four dry foods tested (Table 4). Of the dry foods, SDS 100 and Classic C22 provided the best survival rate (S), and thus, were selected for further investigations (i.e. for E3-5). In 21 days long E3-5 crucian carp larvae utilized live *Artemia* nauplii more efficiently than the tested commercial dry foods or mixed foods (Table 4). Larvae fed with *Artemia* nauplii consistently showed the highest growth rate ($TL_{21}=15.2-18$ mm, $BM_{21}=39.8-68.9$ mg) and survival ($S_{21}=92.2-98.9\%$). Differences in the final BM were huge in favour of the control groups fed on pure *Artemia* nauplii, and these larvae outperformed those kept on pure dry food or mixed diet by 2.3 (E3: SDS 5× - A 1×; E4: SDS 4× - A 2×) to 11.7 (E3: Classic) times. Based on the survival rate, the second and third best feeding strategies were the first 10 days on *Artemia* nauplii and then shifting to dry food ($TL_{21}=12.7-13.7$ mm, $BM_{21}=16.9-23.3$ mg, $S_{21}=89.4-94.7\%$), and the mixed diet with daily four feedings with dry food and two feedings with *Artemia* nauplii ($TL_{21}=12.1-13.7$ mm, $BM_{21}=18.5-29.4$ mg, $S_{21}=88.9-94.8\%$) (Table 4). Relative performances of SDS 100 and Classic C22 varied over mixed diet experiments, but if were used as exclusive food they clearly resulted the lowest growth and survival rates. Cumulative mortality curves show that larvae fed only with dry food started to die at a higher rate from the 4-5th day of the experiments and the mortality rate remained high until the end of the experiments (Figure 1). In contrary, the mortality of larvae kept on mixed diet was high only during the first week, while two stage feeding protocols (i.e. during the first few days *Artemia* nauplii and then dry food) resulted an opposite trend

with a higher mortality rate in the second half of the experiment when only dry food was provided. However, mortality of larvae was constantly low in all *Artemia*-fed control groups. Total length distribution of larvae remained unimodal throughout the experiments in all treatment groups (Figure 2), and cannibalisms did not occur.

Discussion

In accordance with the experiences of previous studies testing other types of dry food than here (Żarski *et al.*, 2011), it was found that either of the four dry foods examined proved to be much less efficient than the control live food, *Artemia* nauplii, during the first 21 days of the exogenous feeding in crucian carp larvae. Although, the survival rate of fish larvae could be maintained at high level in some of the protocols tested, a strong decrease in the growth rate was obvious in all diet combinations containing dry food.

Present results are in accordance with the previous observations on crucian carp and other fish species where the survival rate of larvae fed with *Artemia* nauplii varied within the range of 87.5-99.0% depending on the species investigated and the stock density applied (Wolnicki and Górný, 1995a, 1995b, 1995c; Kaiser *et al.*, 2003; Wolnicki *et al.*, 2003; Kreiszeff *et al.*, 2008, 2010; Demény *et al.*, 2009; Wolnicki *et al.*, 2009; Mamcarz *et al.*, 2011; Żarski *et al.*, 2011). In this study, the growth rate expressed as RGR varied between 4.53-5.13% d⁻¹ in *Artemia* nauplii fed control groups which is similar to those (5.40-5.85% d⁻¹) observed by Żarski *et al.* (2011).

Seven days long preliminary experiments showed that of the four commercial dry foods tested, highest growth and survival rates were revealed with SDS 100, a pellet of high protein and fat content, and Classic C22 having a low protein and fat content (Table 2). Thus, these two dry foods were further tested in more detailed 21 days long experiments.

In 21 days long experiments, crucian carp larvae fed only with dry food revealed far the poorest survival and lowest growth rates. These results are highly congruent with the findings of other

researchers who concluded that many cyprinid larvae are sensitive to feeding with commercial dry food (Wolnicki and Górný, 1995a, 1995b, 1995c; Kaiser *et al.*, 2003; Żarski *et al.*, 2011). When only dry food was provided, crucian carp larvae grew very poorly and their mean body mass was only 7.3-8.8% at the end of 21 days long rearing experiments compared to the control groups fed with *Artemia* nauplii. Other types of dry foods were tested by Żarski *et al.* (2011) and were also found to perform poorly in crucian carp larvae. Low growth rate was found in most of other cyprinid larvae reared only on dry food as well (Wolnicki and Górný, 1995c; Wolnicki *et al.*, 2009; Mamcarz *et al.*, 2011).

Mixed feeding protocols were tested in several cyprinid species and were found to perform better compared to pure dry food diets, but they also never proved to be as efficient as pure live food diet, especially in respect the growth rate (e.g. Abiyad and Kestemont, 1994; Kestemont, 1995; Wolnicki and Górný, 1995a, b, c; Kaiser *et al.*, 2003; Kwiatkowski *et al.*, 2008; Wolnicki *et al.*, 2009; Kujawa *et al.*, 2010; Mamcarz *et al.*, 2011).

Compared to poor dry food diets, both survival and growth rates of larvae could be increased by using different mixed diets, but the observed values were still well below those attained by the control *Artemia*

fed groups. Best results were achieved with feeding protocols including most live food, such as when *Artemia* nauplii were provided twice a day through the whole experiment as supplementary food or when full *Artemia* nauplii diet was provided during the first 10 days of rearing. Nevertheless, decreased growth rate indicates that none of the tested mixed diets could fulfil the requirements of crucian carp larvae. Based on the experiences and considering the limits of the presently available commercial dry foods in rearing cyprinid larvae, probably the best compromise would be a longer, 5-15 days initial rearing on pure live food and then a gradual habituation to dry food (see also Wolnicki, 2005; Wolnicki *et al.*, 2009; Kujawa *et al.*, 2010).

Live food supplemented dry food based feeding protocols may provide high survival rate comparable to pure live food diet in crucian carp larvicultures, and thus may be used when the high number of pre-reared larvae is the only important criteria. However, the small size of these larvae restricts their applicability for stocking to extensive pond cultures for further rearing, and for stocking to natural or revitalized habitats for population enhancement (i.e. for angling) or species conservation proposes. Since most ecological processes are strongly size-dependent, the size at which the larvae are stocked has an utmost

Table 4. Initial and final total length (TL₀ and TL_t, respectively), final body mass (BM_t) and survival rate (S) of crucian carp larvae by treatment groups during 7 days long experiments 1-2 and 21 days long experiments 3-5. As all larvae originated from the same recruitment, initial TL was supposed to be identical in all treatment groups within each experiment

Experiment number	Feeding protocol	TL ₀ (mm)	TL _t (mm)	BM _t (mg)	S (%)
1.	A	5.5±0.3	7.9±0.8 ^a	-	97.9±0.4 ^a
	Sera		5.6±0.7 ^b	-	47.7±10 ^b
	SDS		6.4±0.8 ^c	-	88.9±1.5 ^c
2.	A	5.6±0.3	8.3±0.8 ^a	-	97.4±0.7 ^a
	Classic		6.0±0.6 ^b	-	85.7±3.0 ^b
	Nutra		6.2±0.6 ^c	-	71.1±4.5 ^c
	SDS		6.4±0.6 ^d	-	91.0±3.6 ^d
3.	A	5.6±0.3	15.2±1.3 ^a	39.8±0.1 ^a	92.2±5.6 ^a
	SDS		9.0±0.9 ^b	3.5±0.7 ^b	17.0±6.8 ^b
	SDS 5× - A 1×		12.6±1.9 ^c	17.0±2.1 ^c	63.3±12.8 ^c
	Classic		7.9±1.1 ^d	3.4±0.2 ^b	50.0±0.0 ^d
	Classic 5× - A 1×		11.0±1.7 ^e	9.9±0.9 ^d	75.9±13.3 ^e
4.	A	6.3±0.7	18.0±2.3 ^a	68.9±3.6 ^a	98.9.1±1.6 ^a
	SDS 5× - A 1×		13.0±2.5 ^b	23.5±2.4 ^b	81.9±10.5 ^b
	SDS 4× - A 2×		13.7±2.1 ^c	29.4±2.6 ^c	88.9±6.9 ^c
	Classic 5× - A 1×		11.5±2.9 ^d	15.3±2 ^d	78.0±10.1 ^b
	Classic 4× - A 2×		12.1±2.2 ^d	18.5±0.7 ^e	94.8±2.8 ^d
5.	A	6.9±0.6	17.5±2.1 ^a	64.5±3.3 ^a	98.3±1.7 ^a
	SDS		9.4±0.9 ^b	5.0±1 ^b	28.1±9.9 ^b
	A ₅ SDS ₁₆		11.3±1.3 ^c	12.5±0.5 ^c	67.8±11.0 ^c
	A ₁₀ SDS ₁₁		13.7±1.8 ^d	23.3±2.6 ^d	94.7±3.8 ^d
	Classic		9.0±1.1 ^b	4.7±0.7 ^b	51.4±3.8 ^e
	A ₅ Classic ₁₆		10.6±1.3 ^e	9.7±1 ^e	68.3±4.2 ^c
	A ₁₀ Classic ₁₁		12.7±1.7 ^f	16.9±0.3 ^f	89.4±5.7 ^f

Explanations of the feeding protocols applied are given in Table 1.

Data by experiments marked with different superscript letter are significantly different (P<0.05) according to the Tukey *post hoc* test.

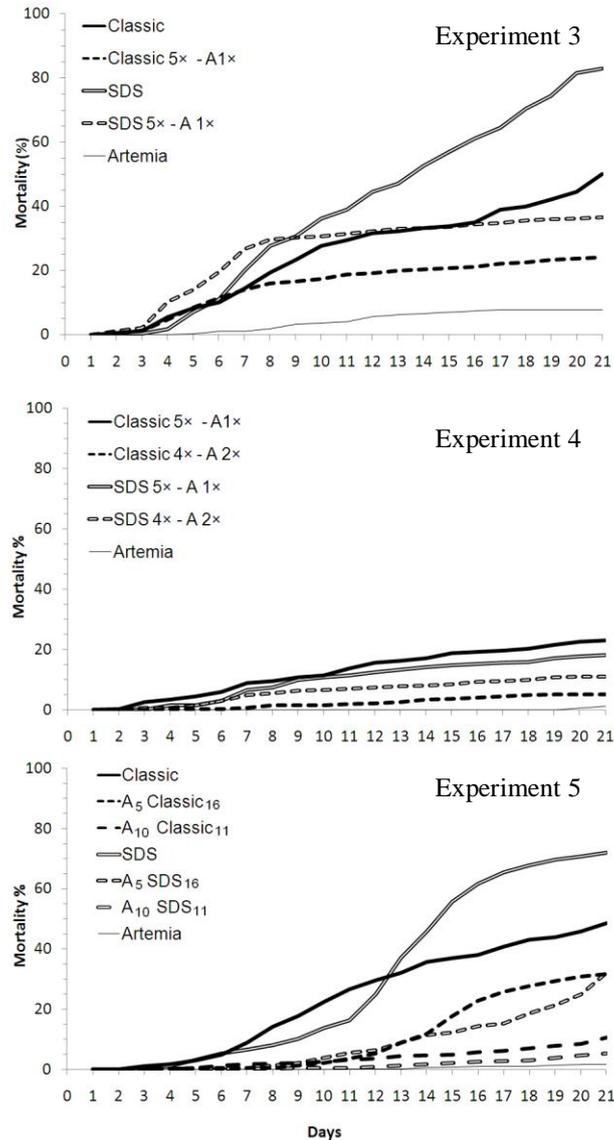


Figure 1. Cumulative mortality rates of crucian carp larvae by treatment groups (feeding protocols) during the 21 days long experiments 3-5. Explanations of the feeding protocols applied are given in Table 1.

influence on their later performance; individuals that are larger or grow faster may shift to larger and more abundant food earlier, may avoid predation and are less sensitive to environmental stressors (reviews: Houde, 1996; Sogard, 1997). Therefore, feeding protocols applied in intensive larvicultures should not only ensure a high survival rate, but also guarantee a high growth rate. As live foods are not preferred in intensive fish cultures, the quality of dry foods needs to be adjusted, and specifically the nutrient requirements of larval fish should be more clearly understood. Further development is required to establish species-specific add-in packages to improve the performance of the common commercial dry foods (Radünz-Neto *et al.*, 1994).

To summarize our findings, crucian carp larvae adapt poorly to available commercial dry foods, and thus it is not recommended to rear them without supplementary live food. Although mixed diets (i.e.

dry food supplemented with a lower portion of live food) provides a satisfactory larval survival they still seem not to be appropriate when pre-reared larvae of large size and good fitness are needed for stocking to natural habitats. It is suggested that (species-)specific dry foods with well-balanced composition should be developed for rearing larvae of crucian carp and other food sensitive cyprinid species.

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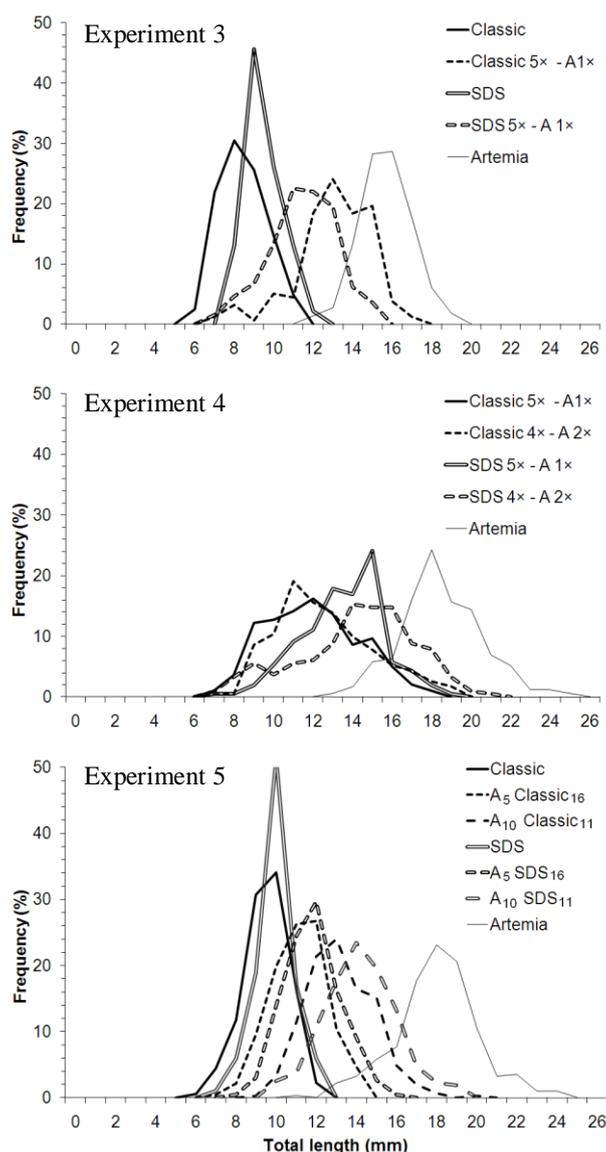


Figure 2. Total length distribution of crucian carp larvae by treatment groups (feeding protocols) during the 21 days long experiments 3-5. Explanations of the feeding protocols applied are given in Table 1.

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