Fish Physiol Biochem
DOI 10.1007/s10695-014-9920-0

The proteolytic digestive activity and growth during ontogeny of *Parachromis dovii* larvae (Pisces: Cichlidae) using two feeding protocols

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Received: 22 October 2013 / Accepted: 10 February 2014
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Abstract The proteolytic digestive activity and growth of *Parachromis dovii* larvae during the ontogeny were evaluated in a recirculation system using two feeding strategies during a 28-day period. Larvae were reared using two feeding protocols (three replicates each): (A) *Artemia* nauplii (at satiation), fed from exogenous feeding [8 days after hatching (DAH)] until 15 DAH followed by nauplii substitution by formulated feed (20 % day^{-1}) until 20 DAH and then formulated feed until 28 DAH; (B) formulated feed (100 % BW daily) from exogenous feeding until 28 DAH. Levels of acid (pepsin type) and alkaline digestive proteases as well as growth and survival of larvae were measured along the feeding period. Survival was high and similar between treatments: 98.9 ± 0.0 for *Artemia*, 97.3 ± 0.0 % for formulated feed. The specific growth rate for length and weight was higher in larvae fed with *Artemia* nauplii than in larvae reared with formulated feed: 3.4 ± 0.1 versus 1.8 ± 0.1 % day^{-1} for body length ($P = 0.009$) and 12.2 ± 0.1 versus 6.5 ± 0.3 % day^{-1} for body weight ($P = 0.002$). The acid and alkaline proteolytic activity was detected, in both treatments, from the beginning of the experiment, at 8 DAH. The total enzymatic activity (U larva^{-1}) for acid and alkaline proteases was higher in larvae reared with *Artemia* after 12 DAH, whereas the specific enzymatic activity was similar for both enzyme types in the two treatments. The results suggest that *P. dovii* larvae were capable to digest formulated diets from the beginning of exogenous feeding and that they could be reared with formulated feeds. However, the formulated feed used should be nutritionally improved because of the poor growth obtained in this research.

Keywords *Parachromis dovii* · Fish larvae · Fish digestive enzymes · Enzymes ontogeny · Larvae feeding

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Introduction

The cichlid *Parachromis dovii* is a freshwater fish that has been exploited for a long time as a resource for sport fishing and food by the Central America population. This autochthonous fish species showed good traits for culture: excellent flesh flavor, easy reproduction in captivity, high resistance to diseases, manipulation and acceptance to formulated feeds (Günther 1996; Bussing 2002).

As a carnivorous species, it requires diets with high protein content along its life cycle, but especially during the first stages of development. The larvae stage is one of the most critical in fish culture, and generally, they need live food for adequate growth during the first days. The first feeding is one of the

Published online: 18 February 2014

biggest problems in larval rearing of many fish species because they do not accept formulated feeds easily or the feed do not meet all the larvae requirements. Further, these feeds must meet several criteria such as easy ingestion, digestion and assimilation of nutrients (Lazo et al. 2007).

In the present time, live food still plays an important role as feed for fish larvae; however, it can represent about 50 % of production costs and possess a variable nutritional quality (Kanazawa 2000; Suzer et al. 2007). For these reasons, formulated diets are now more widely used reducing significantly the feeding costs but resulting in lower growth and, in some cases, in higher mortalities than live foods, especially with marine larvae (Civera-Cerecedo et al. 2004). Further, partial substitution of live feed (co-feeding) is a widely used practice (Hamza et al. 2007).

The use of artificial feeds in larval rearing provides some advantages such as greater availability, easy storage and lower costs. However, their success depends on the digestive capacity of the fish larvae to be fed (Uscanga-Martínez et al. 2011). In this context, studies on the digestive physiology allow a better diet formulation according to fish larvae development, which in turn produce better growth and health, a reduction on feed wastes and an increase in profitability for the producers (Tengjaroenkul et al. 2002; Drossou et al. 2006; González-Félix et al. 2010).

Several works have been focused on the study of digestive enzyme variability during larvae ontogeny and on the digestive system development and its functionality. These studies also demonstrated that the digestive system is not completely developed at first feeding (Yúfera and Darias 2007).

Digestive enzymes ontogeny of larvae has been studied in many freshwater fish groups, such as salmonids (Golchinfar et al. 2011), cyprinids (Chakrabarti et al. 2006; Chakrabarti and Rathore 2010), catfishes (Pradhan et al. 2013), sturgeons (Babaei et al. 2011; Sanz et al. 2011), gars (Comabella et al. 2006), tilapias (Tengjaroenkul et al. 2002; Lo and Weng 2006) and Neotropical cichlids (López-Ramírez et al. 2011; Uscanga-Martínez et al. 2011). These studies have detected digestive enzyme activity in larvae since first feeding. However, the level of enzyme activity, the larvae growth and survival depend on feed quality (Drossou et al. 2006; Aguilera et al. 2012; Kamarudin et al. 2011).

The objective of this research was to evaluate the effect of two feeding protocols on the digestive proteolytic activity, growth and survival during the ontogeny of *P. dovii* larvae.

Materials and methods

Larvae

Parachromis dovii larvae were obtained from spontaneous spawns at the hatchery unit of the Escuela de Ciencias Biológicas of Universidad Nacional, Costa Rica. After hatching, all larvae from one spawn (about 680) were transferred from the spawners' tank to aquaria (11 l) in a recirculation system until beginning of experiment. The water temperature was kept at 25.2–29.4 °C, dissolved oxygen at 6.0–6.8 ppm and NH_3 at 0.05 ppm maximum.

Feeding

Larvae were randomly distributed into six aquaria and reared with two feeding protocols (by triplicates each). Each aquarium contained 113 larvae. In both protocols, larvae were fed in excess at the same feeding frequency. The feeding schedules used were as follows:

(A) *Artemia franciscana* nauplii (Great Salt Lake, Artemia International®) from 8 to 15 days after hatching (DAH), followed by a 20 % daily substitution with formulated feed until 20 DAH and continued with formulated feed until 28 DAH (end of larval development). Larvae were fed four times daily (0900, 1130, 1430 and 1700 hours). *Artemia* nauplii were harvested from 1 l containers (17 ppm) with 3 g cysts after 24 h. The mean nauplii hatching rate was 254 per ml.

(B) Formulated feed (45 % protein and 10 % lipid, Table 1), fed from 8 DAH (at the beginning of exogenous feeding) to the end of experiment (28 DAH). Larvae were fed 100 % of body weight as in protocol A. The feed was prepared by using a pelletizer provided with a 2 mm die. It was oven-dried at 60 °C for 24 h, and then, the spaghetti-like feed was crushed with a laboratory mill. The different feed particles used (powder, 300 µm, 600 µm and 1 mm diameter) were separated using appropriate sieves.

Table 1 Ingredient composition of diet[a] fed *P. dovii* larvae

Ingredients[b]	($g\ kg^{-1}$ as feed)
Tuna meal	480
Soymeal	210
Wheat feed flour	150
Tankage meal	90
Soy oil	20
Cod liver oil	20
Vitamin premix	20
Salt	10

[a] Nutrient content: 45 % protein and 10 % lipid

[b] Tuna meal (IFN: 5-02-023, processing meal, min. 49 % protein), Soybean meal (IFN: 5-20-637, min. 44 % protein), wheat flour (IFN: 4-05-199, 11 % protein), tankage (IFN: 5-00-388, min. 50 % protein)

Before feeding, all feed and feces wastes remaining were removed from the bottom of the aquaria, and during feeding, the water flow was stopped for 30 min to avoid feed losses.

Growth parameters and survival

Total length (mm) and weight (mg) were determined from 10 larvae at 8, 12, 16, 20, 24 and 28 DAH for each aquaria. Previously, larvae were anesthetized with tricainemetansulfonate (MS-222), measured and weighted with a stereoscopy (Olympus 329345 with scale) and an analytical balance (sartorius 2,492 ± 0.1 mg). The growth in length and weight was calculated by the formula:

$$\mathrm{SGR} = (\ln W_\mathrm{f} - \ln W_\mathrm{i} / t_2 - t_1) \times 100 \left(\%\ \text{body length day}^{-1} \text{ or } \%\ \text{body weight day}^{-1}\right)$$

where ln natural logarithm, W_f final length or weight (mm or mg), W_i initial length or weight (mm or mg), t_2 final day, t_1 initial day.

Survival rates were determined by counting remaining larvae at the end of the experiment, and it was corrected for number of sampled larvae.

Enzymatic extract preparation

The proteolytic activity at 8, 12, 16, 20, 24 and 28 DAH was quantified from 10 larvae per aquaria. Larvae were sampled in the morning (at the same hour every period) before feeding and kept 1 h in clear water to allow the emptiness of digestive tract (Gisbert et al. 2009). Viscera samples of about 20 mg larvae^{-1} were removed and homogenized with 1 ml distilled water in an ice bath using a tissue homogenizer (Contes). Samples from the smallest larvae were taken by removing their head and tail. Next, the homogenized sample was centrifuged at 14,000*g*, 4 °C for 30 min (Hettich Mikro 200). The supernatant was kept at −20 °C for further enzymatic analysis.

Soluble protein quantification

Total soluble protein was measured by the Bio-Rad kit, based on Bradford method using bovine serum albumin as the standard (Bradford 1976). Mixtures of 5 μl enzymatic extract, 795 μl distilled water and 200 μl Bradford reagent were incubated for five min at room temperature. The absorbance was read at 595 nm, using a blank without extract, with a Jasco V-630 spectrophotometer.

Proteolytic activity determination

The measurement of alkaline and acid proteolytic activities was done according to Sarath et al. (1989). To determine total alkaline proteolytic activity, enzymatic extract was incubated with azocasein (2 %) pH 9.0 for 30 min at 37 °C in a flux water bath. The reaction was stopped by the addition of trichloroacetic acid (12 %). After 10 min, the precipitated protein was removed by centrifugation. The absorbance of the supernatant was measured at 366 nm, against a blank where the enzymatic extract was previously inactivated. The positive control used was commercial trypsin (SIGMA) at 180 μg ml^{-1}.

To determine total acid proteolytic activity, enzymatic extract was incubated with hemoglobin (2 %) pH 2.0 for 20 min at 37 °C using a flux water bath. The reaction was stopped with trichloroacetic acid (5 %). After 30 min, the precipitated protein was removed by centrifugation. The absorbance of the supernatant was measured at 280 nm, against a blank where the enzymatic extract was previously inactivated. The positive control used was commercial pepsin (SIGMA) at 90 μg ml^{-1}.

The units of enzymatic activity (U), as total activity (U larva^{-1}) and specific activity, (U mg P^{-1}) were measured as follows:

- Total activity:

$$\text{U larva}^{-1} = \left(\frac{\Delta\text{Abs. min}^{-1} \times \text{ DF } \times 1000}{\text{EV}}\right) / \text{No. larva ml}^{-1}$$

- Specific activity:

$$\text{U mg P}^{-1} = \frac{\text{U larva}^{-1}}{\text{mg P larva}^{-1}}$$

where Δ Abs. min^{-1} difference between sample and negative control absorbances/incubation time, DF dilution factor (total reaction volume/extract volume), 1,000 water volume (μl) used to homogenize larvae, P protein and EV extract volume used.

Statistical analysis

The mean values of data were analyzed as follows: to determine differences for (a) growth, survival and enzymatic activity between the two feeding protocols and for (b) the enzymatic activity during sampling periods within each feeding protocol, a one-way ANOVA using the software STATGRAPHICS CENTURION XVI (StatPoint Technologies Inc., 2010) was applied. Additionally, a two-way ANOVA was used to evaluate whether interaction exists between treatments and sampling periods. The homogeneity of variances and the normal distribution were tested according to the Levene and Shapiro–Wilk tests. Comparison among the treatment means was made using the LSD test with 95 % confidence intervals.

Results

Growth performance and survival

The survival was similar for both feeding protocols during the experiment (A: 98.9 ± 0.0 %, B: 97.3 ± 0.0 %, $P = 0.106$).

After 12 DAH, larvae fed initially *Artemia* (protocol A) showed higher total length than those fed only formulated feed (protocol B) ($P = 0.001$, Table 2). This trend was kept until the end of the experiment (A: 18.4 ± 0.2 mm, B: 13.2 ± 0.2 mm). The same results were found for the total length increment (A: 9.1 ± 0.2 mm, B: 3.9 ± 0.1 mm).

The SGR for length showed significantly higher values with larvae fed protocol A than with protocol B (Table 3). At the end of feeding period, final SGR for length was 3.4 ± 0.1 % daily for protocol A and 1.8 ± 0.1 % for protocol B.

After 12 DAH, body weight showed differences between treatments ($P = 0.0004$) (Table 2). The final body weight of larvae fed protocol A (89.0 ± 2.7 mg) was higher than those fed protocol B (28.3 ± 1.0 mg). The weight increment and the SGR for growth were also higher with protocol A (81.3 ± 2.4 mg and 12.2 ± 0.1 % day^{-1}) compared to protocol B (20.6 ± 0.6 mg and 6.5 ± 0.3 % day^{-1}) during all experimental period (Table 3).

Proteolytic activity

For both feeding protocols, the acid proteases activity (pepsin type) was detected from the beginning of exogenous feeding. Temporal variations for acid proteases activity (total and specific) with both protocols showed a similar trend as the activity of alkaline proteases (Figs. 1, 2).

The highest total activity of alkaline digestive proteases (U larva^{-1}) was determined for larvae fed protocol A (Fig. 2a). During trial, two activity peaks were found with larvae fed protocol A: at 16 DAH (1.4) and 24 DAH (4.2). With protocol B, the proteases activity steadily increased and the highest value was obtained at the end of trial (1.2).

Temporal variations with both protocols showed similar specific activity of alkaline proteases (U mg protein^{-1}), except for 24 DAH where larvae fed protocol A showed the highest activity (6.7) (Fig. 2b).

Discussion

As many other freshwater fish, *P. dovii* larvae have a big vitellogenic sac and their stomach develops very early in their life (personal observation by a histology study). Because of this, at the beginning of exogenous feeding, the *P. dovii* larvae are big enough to be capable to feed compound diets, as it has been observed during routine larvae feeding at our hatchery (feed diameter size $\leq$1 mm).

Survival with both protocols was high and not significantly different according with previous observation (Günther and Ulloa 1995). Our results indicate that

Table 2 Total length (mm) and weight (mg) of *P. dovii* larvae during sampling periods according to feeding protocol (A and B)

DAH	Total length (mm)		Weight (mg)	
	Protocol A	Protocol B	Protocol A	Protocol B
8	9.4 ± 0.0[a]	9.3 ± 0.1[a]	7.7 ± 0.1[a]	7.7 ± 0.2[a]
12	12.1 ± 0.2*[b]	9.8 ± 0.0*[b]	17.6 ± 0.3*[b]	8.0 ± 0.2*[a]
16	14.1 ± 0.0*[c]	10.6 ± 0.1*[c]	36.0 ± 0.4*[c]	10.6 ± 0.3*[b]
20	16.1 ± 0.1*[d]	11.4 ± 0.1*[d]	51.3 ± 0.9*[d]	14.8 ± 0.4*[c]
24	17.1 ± 0.1*[e]	12.6 ± 0.1*[e]	65.7 ± 1.5*[e]	20.6 ± 0.6*[d]
28	18.5 ± 0.2*[f]	13.2 ± 0.2*[f]	89.0 ± 2.7*[f]	28.3 ± 1.0*[e]

Mean ± SE ($n = 3$). Different letters, in the same column, indicate significant difference within the same protocol ($P < 0.05$)

* Significant difference between protocols ($P < 0.05$)

Table 3 Specific growth rate (SGR, % BW day^{-1}) for length and weight of *P. dovii* larvae during sampling periods according to feeding protocol (A and B)

DAH	SGR (total length)		SGR (weight)	
	Protocol A	Protocol B	Protocol A	Protocol B
8–12	6.4 ± 0.5*[a]	1.4 ± 0.3*[a]	20.7 ± 1.4*[a]	1.3 ± 1.2*[a]
13–16	3.9 ± 0.6*[a]	1.8 ± 0.3*[a]	18.0 ± 0.9*[a]	6.9 ± 0.9*[b]
17–20	3.3 ± 0.0*[b]	2.0 ± 0.2*[b]	8.8 ± 0.7[a]	8.4 ± 0.5[b]
21–24	1.5 ± 0.2[b]	2.5 ± 0.4[b]	6.2 ± 0.5[b]	8.1 ± 1.8[b]
25–28	1.9 ± 0.3[c]	1.3 ± 0.2[c]	7.6 ± 1.5[b]	8.0 ± 1.3[b]

Mean ± SE ($n = 3$). Different letters indicate significant difference within the same protocol ($P < 0.05$)

* Significant difference between protocols ($P < 0.05$)

P. dovii larvae are able to digest and assimilate both types of feeds (live and formulated) since the time of first feeding. Other freshwater fish species also have shown high survival rates when feed exclusively with compound diets (commercial or practical) during the larval period such as zebra fish: 73 and 84 % (Carvalho et al. 2006) and *Oreochromis niloticus*: 96.4 % (Drossou et al. 2006).

The growth in weight and length was significantly higher from 12 DAH in larvae fed protocol A (*Artemia*), similar to results obtained in our hatchery with *Cichlasoma managuense* (actually *P. managuense*) larvae (unpublished results). Other freshwater fish species have shown variables results. For instance, larvae of *Ctenopharyngodon idella* grew better when fed *Artemia* nauplii but those of *Hypophthalmichthys nobilis* when fed a formulated diet (Rottman et al. 1991); gar (*Atractosteus spatula*) larvae reared with formulated feed also exhibited a better growth than live food (Mendoza et al. 2008). Contrarily, Drossou et al. (2006) found similar growth in *O. niloticus* larvae fed either *Artemia* or a fingerling trout diet.

Live food have several advantages compared with formulated feed: a visual and chemical stimuli, a better digestibility and enzyme profile, a better distribution on water column and an extended nutritional quality (Beux and Zaniboni-Filho 2008). Contrarily, the variable performance of formulated diets for larvae has been mainly attributed to: the lack of attractants to stimulate the feeding behavior and secretion of zymogens to the digestive tract; enzyme inhibitors present in the diet; the presence of essential nutrients with an inappropriate chemical structure; and the imbalance of nutrients due to poor diet formulations (Lazo 2000). Some of the later factors could interfere negatively the growth of *P. dovii* larvae fed our formulated diet. Additionally, we believe that leaching of nutrients from the diet used (pelletized and crumbled) could also have a strong deleterious effect on the larvae growth due to the time larvae lasted to feed. Further, during the weaning time, we observed a deleterious feeding behavior that could contribute with the drastic reduction in growth. It could be

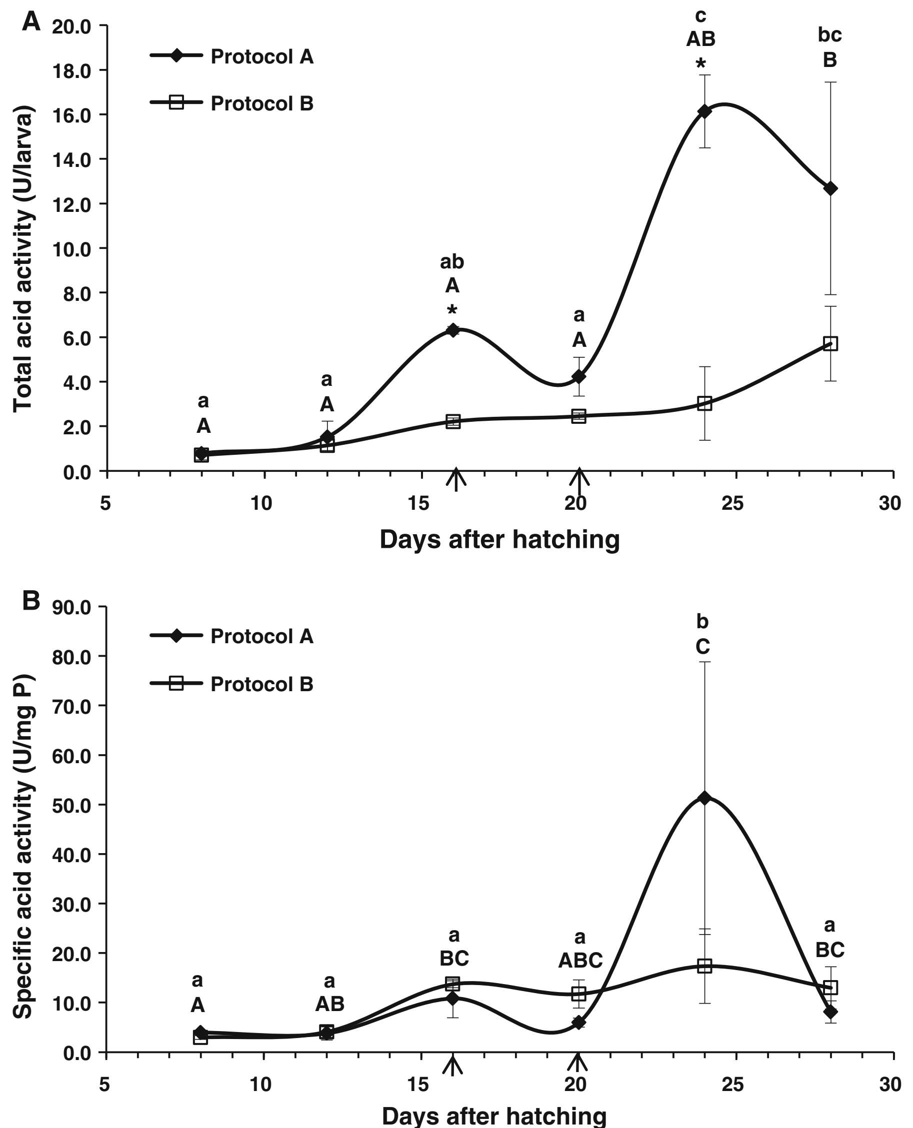

Fig. 1 Proteolytic activity of *P. dovii* larvae: **a** total acid activity, **b** specific acid activity. Mean ± SE ($n = 3$). Means on *curves with different letters* show significant difference within the same protocol (*lowercase letters* for protocol A, *capital letters* for B) ($P < 0.05$). *Significant difference between protocols ($P < 0.05$). *Arrows* show weaning period in protocol A

attributed to a low ingestion rate, because of the delay in the recognition and acceptance process of the formulated feed by larvae as found with tropical gar larvae (Aguilera et al. 2012).

Alkaline and acid proteolytic enzyme activity was determined in *P. dovii* larvae at 8 DAH onward (before exogenous feeding), agreeing with findings with other cichlidae fish species, *O. niloticus* (Tengjaroenkul et al. 2002), *O. mossambicus* (Lo and Weng 2006) and *C. urophthalmus* (López-Ramírez et al. 2011). Zambonino-Infante and Cahu (2001) argued that fish larvae possess a wide spectrum of digestive enzymes before exogenous feeding indicating that initial proteolytic activity is genetically triggered in the larvae.

The finding of acid enzymatic activity is considered an indicator of a functional stomach (Moyano et al. 1996), and this may explain the high survival rate and acceptance of formulated feed by *P. dovii* larvae. A functional stomach has been observed in early developmental stages (before or during yolk absorption) of some freshwater fish species such as *Onchorhynchus mykiss* (Ershova et al. 2004), *A. tristoechus* (Comabella et al. 2006), *O. nilolticus* (Drossou et al. 2006) and *C. urophthalmus* (López-Ramírez et al. 2011).

Temporal variation in proteolytic enzymes activity observed with *P. dovii* larvae accords with that found with other freshwater species as *O. niloticus* (Drossou et al. 2006), *Petenia splendida* (Uscanga-Martínez

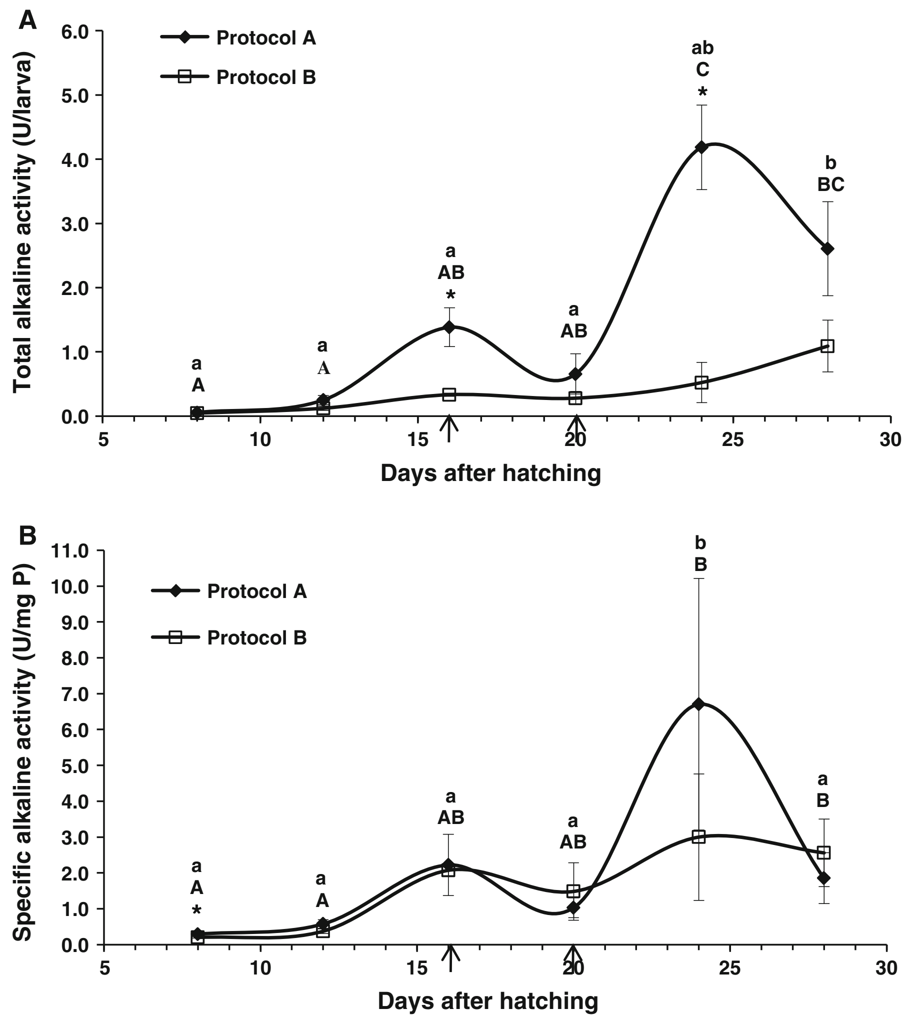

Fig. 2 Proteolytic activity of *P. dovii* larvae: **a** total alkaline activity, **b** specific alkaline activity. Mean ± SE ($n = 3$). Means on *curves with different letters* show significant difference within the same protocol (*lowercase letters* for protocol A, *capital letters* for B) ($P < 0.05$). *Significant difference between protocols ($P < 0.05$). *Arrows* show weaning period in protocol A

et al. 2011), *Cichlasoma urophthalmus* (López-Ramírez et al. 2011) and *Ompok bimaculatus* (Pradhan et al. 2013).

The total acid and alkaline proteolytic activity (U larva^{-1}) after 12 DAH was higher with protocol A than B. It probably may be explained by the larvae bigger sizes and their major associated enzymatic production (Applebaum and Holt 2003; Álvarez-González et al. 2006; Chakrabarti et al. 2006). In addition, the lower buffering capacity of live food results in a lower stomach pH and consequently in a higher acid proteolytic activity. Also, the *Artemia* nauplii might have a stimulatory effect of the enzymatic secretion due to their movement in the gut or their hydrolysis releasing amino acids (Kolkovski 2001).

Zambonino-Infante and Cahu (2001) and Lo and Weng (2006) considered that a higher content of acid and alkaline enzymes reflects a better larvae performance (showing a higher protein hydrolysis and absorption).

The sudden reduction in enzyme activity at 16–20 DAH found with protocol A corresponds to the weaning time. Physiological and particularly feeding changes may cause this fluctuation (Zambonino-Infante and Cahu 2001; Zacarías-Soto et al. 2006). The observed increase in enzyme activity from 20 to 24 DAH, when larvae were shifted to eat only formulated feed (protocol A), may be attributed to adaptive changes in the digestive enzyme levels because of a lower diet digestibility (compared to *Artemia* nauplii) (Zambonino-Infante and Cahu 1994; Aguilera et al. 2012). The

increase in total acid and alkaline proteolytic activity with larvae reared with protocol B may be associated with increment in larvae weight.

A lower amount of larvae protein, as a result of smaller larvae and abdominal fraction, in larvae fed protocol B could probably explain why their specific enzyme activity was similar and, in some periods, higher than larvae fed protocol A. Also, larvae fed initially with *Artemia* nauplii (protocol A) could not require higher digestive enzyme production and secretion than those fed formulated diet. The significant increment in specific activity for both acid and alkaline enzymes that we obtained with larvae fed protocol A from 20 to 24 DAH may be caused by compensation or an adaptation to digest entirely formulated feed as previously explained. In concordance, such increment is not appreciated with larvae fed the same feed all the time (protocol B).

The increment in the specific alkaline proteases activity during the beginning of exogenous feeding may be associated with the spleen and intestine development, whereas the following reduction in activity observed links with the end of larval stage (around 22 DAH) and the beginning of juvenile phase. Some authors believe that this trend is related to physiological changes during larval development such as a reduction in the digestive enzyme synthesis, the production of new enzymes and hormones or that these changes are genetically triggered (Zambonino-Infante and Cahu 2001; Lazo et al. 2007). In conclusion, the proteolytic activity increase found with *P. dovii* at the end of its larval stage may be associated with the complete development of the digestive tract and transition to the juvenile stage.

According to our results, the feed type affected the growth and enzymatic activity found in *P. dovii* larvae. Further and based on the acid enzymatic activity determined with protocol A, the weaning time can be started at 13 DAH. Also, we recommend validating different co-feeding schedules (*Artemia* nauplii and artificial feed) in future research since this technique has been used successfully with other fish species (Nguyen et al. 2011; Liu et al. 2012; Pradhan et al. 2014).

Acknowledgments This research was supported by the project PS Tecnología Acuicultural of the Escuela de Ciencias Biológicas, Universidad Nacional. We acknowledge the help of Dr. Jorge Alfaro M. for reviewing the manuscript.

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