



Morphological Development and Allometric Growth in Hatchery-Reared Caspian Shemaya (*Alburnus chalcoides*): from Hatching to The Juvenile Stage

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Abstract

Morphological development and allometric growth were investigated in hatchery-reared Caspian shemaya, *Alburnus chalcoides*, from hatching to the juvenile stage at a temperature of $25.13 \pm 1.61^\circ\text{C}$. The newly hatched larvae were 4.69 ± 0.27 mm in total length (L_T). Mouth opened 3 days after hatching (DAH) and initial swimbladder inflation occurred at 5 DAH. Exogenous feeding started at 6 DAH and the yolk sac was completely exhausted one day later. Squamation was initiated at 46 DAH and completed until 57 DAH. Based on the external morphology, early development of *A. chalcoides* was divided into five periods: yolk-sac stage 0-6 DAH (4.69 - 7.61 mm L_T), preflexion stage 7-9 DAH (7.73 - 7.88 mm L_T), flexion stage 10-18 DAH (8.20 - 9.60 mm), postflexion stage 19-45 DAH (9.30 - 19.97 mm L_T) and juvenile stage 46-57 DAH (21.66 - 27.25 mm L_T). During the first 4 weeks after hatching, there was an evident priority to develop feeding, swimming and respiratory ability by promoting intense growth in head and tail parts. Following this period, there was a clear reduction in growth coefficients. The allometric growth changes in Caspian shemaya during early life reflect the selective organogenesis directed towards survival priorities.

Keywords: Caspian shemaya, *Alburnus chalcoides*, morphological development, allometric growth.

Introduction

The Caspian shemaya (*Alburnus chalcoides*), a benthopelagic and anadromous fish, is widely distributed in the river systems of the Black, Caspian and Aral Seas (Bogutskaya, 1997). Sometimes, this species was described as the species *Chalcalburnus chalcoides* or *Chalcalburnus chalcoides aralensis*, but now it is regarded as a phenotypic variant of *A. chalcoides* (Patimar, et al., 2010). In its native systems, the fish lives in fresh and brackish waters, and the populations living in lakes migrate upstream for spawning from early May to late July (Slastenenko, 1959). Moreover, the fish has high commercial value (Daei et al., 2009) and is caught for consumption in southern regions of the Caspian Sea (Mohaddasi et al., 2013) and in the regions of Lake Toudige (Ünver, 1998). Due to its high tolerance to saline-alkali waters and potential economical value, this species was introduced into China from Uzbekistan in 2001. From then on, aquaculture of the fish has been initiated in many provinces of China. Meanwhile, some studies have been carried out on its physiology (Wang et al., 2007), chromosome karyotype (Geng et al., 2005) and pond culture (Lin et

al., 2005; Ding et al., 2013). Recent work has also focused on ovarium development (Ünver and Saraydın, 2004), life-history (Patimar, et al., 2010), fecundity (Rahbar, et al., 2013a,b), conversation genetics (Geng et al., 2013a, b) of the fish. In contrast, little is known about early development and growth of *A. chalcoides*, except for a brief description on its embryonic and larval development (Lin et al., 2004).

Information about the morphological development and growth patterns of young fish is important for fisheries management and aquaculture (Gisbert et al., 2002; Martínez-Lagos and Gracia-López 2009). A better understanding of early development and allometric growth patterns of Caspian shemaya not only lead to further consideration of its ecology and phylogeny, but also lead to improved the existing rearing protocols. Therefore, the objective of the present study was to (1) describe the larval and juvenile development in detail, and (2) present the allometric growth patterns of body proportions using the hatchery-reared Caspian shemaya from hatching to the juvenile stage.

Materials and Methods

Egg Collection, Incubation and Fry Rearing

This study was carried out in a private hatchery (123°44'16"N, 42°9'38"E) located at Tieling City, Liaoning Province, northeast China. After injection of hormone, 48 females and 54 males were kept in a net-cage (2.40×1.80×1.20 m) for collecting eggs (Figure 1a). Fertilized eggs were transferred into several buckets for incubation (Figure 1b). Larvae hatched on June 17, 2013, and this day was recorded as 0 day after hatching (DAH). About 800 larvae used in this study were reared in 5 plastic cases (0.50×0.35×0.40 m, volume 50 L), and every case contained about 160 individuals. Fry were fed twice daily (05:00 am and 17:00 pm) with the live rotifers at a density of 4 rotifers ml⁻¹ from 4 DAH until 12 DAH, and with cladocera at 8-10 individuals ml⁻¹ and copepoda at 3-5 individuals ml⁻¹ from 8 DAH to 57 DAH (Figure 2). 50% water exchange occurred at least once daily and water supply was well-aerated groundwater. During this experiment, water temperature, dissolved oxygen, and pH maintained 22-28°C, 5.60-6.20mg/L and 8.2-8.7, respectively. Light conditions followed natural light-dark cycle.

Observation and Measurement

During the first 2 weeks larvae were sampled daily (n=20). Sampling frequencies were reduced to 2 days between days 15 to 30, and 3 days between days 31 to 57. After anesthetization with 50 mgL⁻¹ MS-222 (tricaine methanesulphonate), these specimens were observed under a stereomicroscope (Optec SZ66, China) and photographed from their left side using a digital cameral (Canon PowerShot 11). Deformed or

curved fry were not analyzed. According to the study of Kendall *et al.*(1984), the early development of *A. chalcoides* was divided into five phases: yolk-sac stage, preflexion stage, flexion stage, postflexion stage and juvenile stage. Thresholds during early development were considered to be attained when at least 50% of the specimens represented a particular stage.

On each digital photograph, the following morphometric characters associated with vision and locomotion were measured with an image analysis system (Adobe Photoshop 7.0) (to the nearest 0.01mm): total length (L_T), eye diameter (ED), head length (HL), trunk length (TRL), post-anal length (PAL), head depth (HD) and body depth (BD) (Figure 2). In addition, wet weight (W) were recorded by a 1/10,000 electronic balance (Shangping FA2004, China) once in two days.

Data Analysis

The growth patterns for W and L_T were expressed as an exponential function. Allometric growth was modeled by a power function of L_T using non-transformed data of measured characters and following the model $y = aL_T^b$; where y is the measured character, a the intercept, and b the growth coefficient (Fuiman, 1983). The intercept and exponent obtained from liner regression on log-transformed data (Gisbert, 1999; Gisbert *et al.*, 2002). Isometric growth occurred when $b=1$, a positive growth occurred when $b>1$ and a negative one when $b<1$. Inflection points of growth curves were determined using iteration procedure according to van Snik *et al.*(1997) and Gisbert(1999). Growth coefficients were compared



Figure 1. Eggs collection (a) and incubation (b).

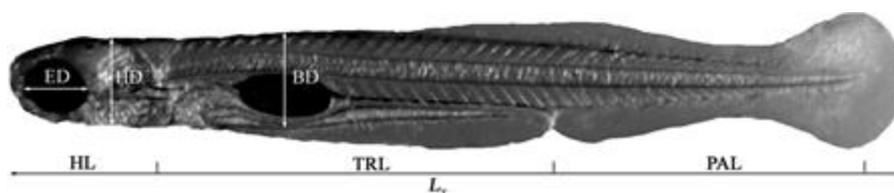


Figure 2. Morphometric characters measured in *A. chalcoides* form hatching up to day 57. BD, body depth; ED, eye diameter; HD, head depth; HL, head length; L_T , total length; PAL, post-anal length; TRL, trunk length.

statistically using *t*-test, and the accepted significance level was set $P < 0.05$ (Gisbert and Doroshov 2006). All statistical procedures and graphics were performed using SPSS 17.0 (SPSS Inc. USA) and Origin 9.0 (OriginLab Corp. USA) respectively.

Results

The eggs of *A. chalcoides* were weakly adhesive and the hatching almost lasted 3 days at a temperature of $25.13 \pm 1.61^\circ\text{C}$ (ranging from 21.6 to 27.8°C). Early development of *A. chalcoides* was divided into five main periods: yolk-sac larvae (0-6 DAH), preflexion larvae (7-9 DAH), flexion larvae (10-18 DAH), postflexion larvae (19-45 DAH) and juvenile (46-57 DAH). Temperature fluctuation and feeding protocol during the experiment were shown as Figure 3.

Morphological Development

Newly hatched larvae (4.69 ± 0.27 mm L_T), partly surrounded by transparent fin, had a large and pear-shaped yolk sac (without oil globule) occupying about 1/2 of total length. The otoliths and unpigmented eyes were easily distinguishable. On this day, the mouth and anus were closed and the larvae often stayed on the bottom without significant movement (Figure 4a). At 2 DAH (6.28 ± 0.22 mm L_T), the head was almost straight and the yolk sac was reduced like a cylindrical body. In addition, pectoral fins without fin rays began to differentiate, coinciding with the appearance of blood circulation and pigmented eyes (Figure 4b). Operculum rudiments and gill arches developed on day 3 (6.83 ± 0.24 mm L_T), when the digestive tract was straight to slightly curved in anterior portion. Meanwhile, the mouth and anus were opened, and the larvae occasionally made rapid movements and immediately reacted to the external stimulation (Figure 4c). When the larvae were 5 days old (7.50 ± 0.16 mm L_T), the elliptic swimbladder appeared, together with stellate melanophores on the dorsal part of trunk and over the digestive tract. Furthermore, the yolk sac shrunk as club-shaped with a tapered posterior part and the

finfold began to narrow at caudal peduncle (Figure 4d). More than 50% of larvae started to swim freely and feed exogenously on 6 DAH (7.61 ± 0.26 mm L_T), when the yolk sac was almost completely exhausted. The xanthophores around the eyes and indigested rotifers present in gut were visible for the first time (Figure 4e). The flexion stage started from day 10 and continued until day 18. At day 10 (7.67 ± 0.40 mm L_T), the notochord tip was slightly flexed and the swimbladder elongated. The xanthophores continued to extend on the dorsal part of body, especially on the head. At the same time, the larvae exhibited voracious feeding behaviors, ventral region of larvae was swollen and pale yellow (Figure 4f). 14 DAH (8.20 ± 0.30 mm L_T), the hypural plate appeared on the base of the caudal fin, as did an 45° upward turn of the notochord tip. The caudal fin, supported by segmented rays, was somewhat concave. The black spots on the head obviously increased in size (Figure 4g). Four days later (9.60 ± 0.88 mm L_T), the anterior swimbladder started to inflate, and the notochord flexion was completed. The silver pigmentation started to appear on the eye, except for the area of lens. In addition, three to four rays were present in the dorsal and anal fins (Figure 4h). A pair of ventral fin buds started to appear on 23 DHA (12.08 ± 1.42 mm L_T), at which time the finfold was almost absorbed, except its preanal segment. Meanwhile, the silvery colour was firstly observed on the ventrolateral region of the body and over the operculum, and the intestine was no longer distinct, but the vertebrae still remained visible. (Figure 4i); At 39 DAH (19.42 ± 3.20 mm L_T), the finfold entirely disappeared and all fins developed well. The numbers of fin rays were: 8-9 dorsal, 22 caudal (segmented rays), 16 anal, 8 ventral. In addition, almost the entire region below the vertebrae showed a silvery shine (Figure 4j). The juvenile stage began at day 46 (21.66 ± 1.52 mm L_T), when scales were first seen on the region close to the operculum. The color of the fry was lemon yellow, black spots spread on the dorsal and caudal fins (Figure 4k). A complete squamation pattern was found in fry 57 DAH (27.25 ± 4.82 mm L_T), which display an entire body covered with scales. Fry were similar to the

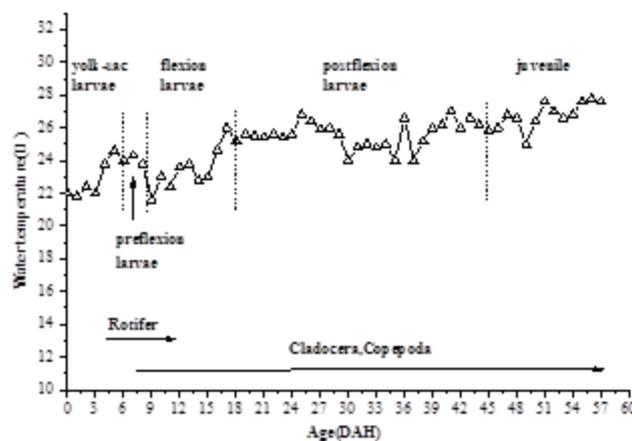


Figure 3. Temperature fluctuation and feeding protocol during early development of *A. chalcoides*.

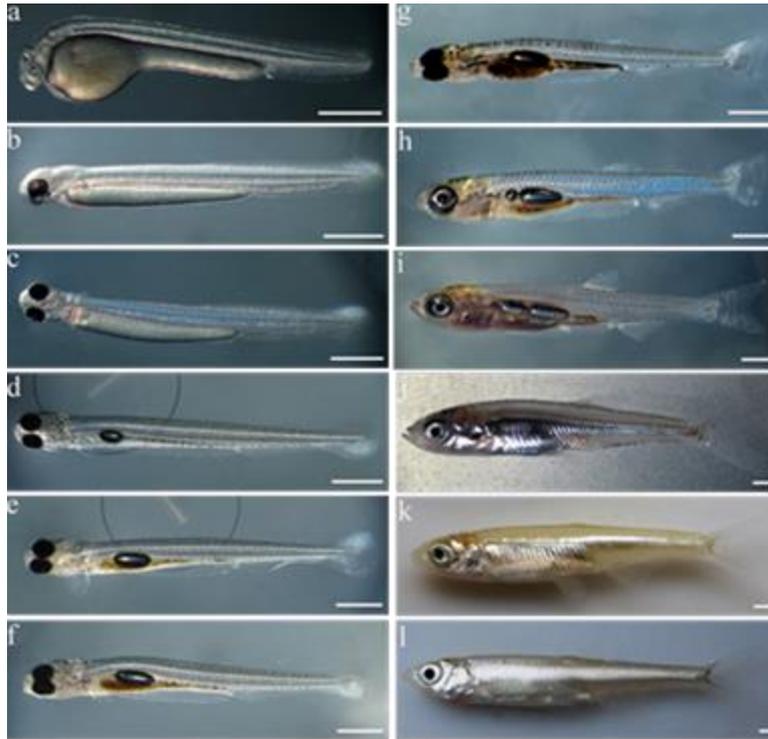


Figure 4. Larval and juvenile development of *A. chalcoides* (a, newly hatched larva, 0 DAH; b, appearance of pigmented eyes and pectoral fin bud, 2 DAH; c, differentiation of operculum rudiments and gill arches, 3 DAH; d, swim bladder inflation, 5 DAH; e, exogenous feeding, 6 DAH; f, notochord slightly flexion, 10 DAH; g, emergence of hypural plate, 14 DAH; h, appearance of anterior swim bladder, 18 DAH; i, formation of ventral fin buds, 23 DAH; j, finfold disappearance, 38 DAH; k, scale differentiation, 46 DAH; l, completion of squamation, 57 DAH.). Scale bars =1mm.

adult in the shape and pigmentation, but no lateral lines were found (Figure 4l). The main events during larval and juvenile development of *A. Chalcoides* were summarized in Figure 5.

Allometric Growth

From hatching up to day 57, the L_T ranged from 4.69 ± 0.27 mm to 27.25 ± 4.82 mm and the W ranged from 0.0007 ± 0.0001 g to 0.1374 ± 0.0505 g. Both L_T and W increased exponentially, and the appropriate growth equations were described as $L_T = 5.9766e^{0.0283d}$, ($R^2=0.95$, $n=323$) and $W = 0.0008e^{0.095d}$ ($R^2=0.94$, $n=208$), respectively (Figure 6).

Body proportions and growth rates changed considerably during early development of *A. chalcoides* (Figure 7). Biphasic growth were found in all measured characters except for BD, which was monophasic and positively allometric ($b=1.42$) throughout the experiment. Overall, five different inflexion points were calculated in this study. The first inflexion point occurred at 8.13 mm L_T , corresponding to an age between 9 and 13 DAH. Further, the second inflexion point was present at 10.74 mm L_T (age interval=21 to 22 DAH), followed by the third inflexion point situated at 11.65 mm L_T (age interval=21 to 25 DAH). After this, the fourth inflexion point, with an age range of 21 to 25 DAH, located at 12.71 mm L_T . Finally, at 13.57 mm L_T , the

growth curve showed the last inflexion point, where the specimens in age ranged from 25 to 29 DAH. In case of HL, HD, ED and PAL, there was a clear reduction in growth coefficient after inflection point from positive ($b=1.99$, 1.54, 1.45 and 1.15, respectively) to near-isometric ($b=0.93$, 0.89, 0.96 and 0.89, respectively). In contrast, TRL showed negatively allometric growth ($b=0.48$) until inflection point and then increased to near-isometric growth ($b=0.94$).

Discussion

Eggs Collection and Incubation

Teleost eggs can be non-adhesive, weakly adhesive or strongly adhesive. Kottelat and Freyhof (2007) reported that *A. chalcoides* deposit sticky eggs. We found, however, that the fertilized eggs are weakly adhesive and can't strongly adhere to the plant or plastic substrate. This phenomenon might be related to the differences in the physicochemical characteristics of incubation water and substrates between the two studies. Since low absolute fecundity (623-38340 eggs) in *A. chalcoides* (Patimar et al., 2010), obtaining enough fertilized eggs with an artificial fertilization method previously described by Golovko et al., (2013) is a complicated work. Conversely, collecting fertilized eggs through a net-

cage as used in this paper, together with incubating them in hatching buckets, might be easier and efficient for large-scale seed production of *A. chalcoides*.

Morphological Development

At hatching, the larvae of *A. chalcoides* measured 4.69 ± 0.27 mm in total length, which is close to that of *A. alburnus* (4.6 mm L_T) (Winnicki and Korzelecka 1997), but is smaller than that of *A. orontis* (5.0 mm L_T) (Özen and Timur 1999). Such difference might be due to the size of eggs because egg diameter is positively relationship with larval length and weight at hatching (Bagrinao and Chua 1986). Furthermore, this difference could be also explained by other factors like intrinsic biological difference, incubation temperature and broodstock size. With transparent finfolds, the larvae of *A. chalcoides* lacked pigments in eyes and skin at hatching. The finfold, a temporary organ during early development, is possibly an adaptation for location in smooth, less turbulent waters and may also function in respiration (Snik et al., 1997; Geerinckx et al., 2008). Moreover, absence of pigment in newly hatched larvae may indicate that the larvae are photonegative during this period (Deng et al., 2012). Vision is fundamental for early development, as it allows spatial orientation and swimming ability (Gisbert 1999; Petereit et al., 2008), as well as foraging and predation avoidance (Evans & Browman 2004). In *A. chalcoides*, eye pigmentation occurred on 2 DAH, prior to the mouth opening (3DAH) and exogenous feeding (6DAH), which might be attributed to the need to develop a function visual system before the first feeding (Hall et al., 2004). In many species such as Dover sole *Solea solea* (Boulhic and Gabaudan 1992), walleye *Stizostedion vitreum* (Marty et al., 1995), striped trumpeter *Latris lineate* (Trotter et al., 2001) and discus *Symphysodon* spp. (Önal et al., 2010), swimbladder inflation coincided with yolk sac depletion and the start of feeding activity. However, swimbladder inflation and exogenous feeding slightly preceded yolk sac depletion in *A. chalcoides*. Similar developmental pattern is also found in zebrafish *Danio rerio* (Kimmel et al., 1995), angelfish *Pterophyllum scalare* (Zilberg et al., 2004), black skirt tetra *Gymnocorymbus ternetzi* (Çelik et al., 2011) and characin *Brycon orbignyanus* (Nogueira et al., 2014). On the other hand, the mixed nutrition period of *A. chalcoides* lasted only one day, which is slightly longer than that of black skirt tetra *Gymnocorymbus ternetzi* (less than 1 day at $24 \pm 1^\circ\text{C}$) (Çelik et al., 2011), but is shorter than that of Chinese sturgeon *Acipenser sinensis* (2 days at $15.5 \pm 0.5^\circ\text{C}$) (Chai et al., 2011), European hake *Merluccius merluccius* (4 days at around 12°C) (Bjelland and Skiftesvik 2006) and grey mullet *Chelon labrosus* (9 days at 16°C) (Khemis et al., 2013). Except for genetic factors (e.g., egg size and yolk sac volume),

rearing conditions (especially temperature) may explain these differences. In addition, larvae must develop feeding ability during the mixed nutrition period, otherwise, they will suffer from starvation and even death (Chen et al., 2006; Ma et al., 2012). Consequently, extending the mixed nutritional period by regulating water temperature (Chai et al., 2011), concomitant with supplying enough and suitable live food such as rotifers at onset of exogenous feeding, may enhance the survival rate in larval culture of *A. chalcoides*. Considering the importance of PNR (the point of no return) in guiding the commercial seed production, further research is needed to determine the appropriate time to introduce the first feed in larval rearing practices.

Allometric Growth

In *A. chalcoides*, the positively allometry of head region was detected during the first 3-4 weeks after hatching, confirming that positive allometric growth of head is a common feature in the early ontogeny of fish (van Snik et al., 1997; Gisbert 1999; Gisbert et al., 2003; Osse and van den Boogart 2004). Indeed, both HL ($b=1.99$) and HD ($b=1.54$) showed strong positive allometric growth patterns during this period, as did ED ($b=1.45$). Ontogenetic events related to head region, such as eye pigmentation, mouth opening and exogenous feeding, were observed as early as yolk-sac stage (0-6 DAH). The rapid growth in head segment appears to be linked to the development of nervous (midbrain and hindbrain), sensory (vision and olfaction), respiratory (gill arches and filaments) and feeding system, as increasing head size matches a more developed nervous system, allowing better oxygen uptake and the uptake of food particles of increasing size (Fuiman 1983; van Snik et al., 1997; Koumoundouros et al., 1999; Gisbert et al., 2002; Kupren et al., 2014a;b). Likewise, the positive allometry of tail region (PAL) occurred within 2 weeks after hatching, corresponding to the start of notochord flexion, the first appearance of caudal fin rays and the resorption of finfold in caudal peduncle. In addition, short period of swimming were observed during this period. Obviously, this behavior must be related to the development of tail region, which would contribute to improving swimming ability and predator avoidance (Fuiman 1983). In contrast, the trunk growth in length (TRL) showed negative allometry ($b=0.48$) during early life stage (from hatching to 25-29 DAH), indicating that trunk segment is completed early in embryogenesis or yolk-sac stage and the number of myotomes does not increase further after that stage (Gisbert and Doroshov 2006; Nogueira et al., 2014). This growth pattern that the anterior and posterior parts of the body grew faster than the middle section was not surprising, as it is common for many fish species (Fuiman 1983; Osse et al., 1997; van Snik et al., 1997; Gisbert 1999; Gisbert et al., 2002; Nogueira et al., 2014; Gisbert et al., 2014;

Kupren *et al.*, 2014a;b). Generally, the positive allometry of head and tail region during early ontogeny reflects the early priority to develop those structures and organs related to vital functions such as feeding and swimming, which are necessary for larvae to increase their survival during early development (Osse and van den Boogaart 2004). On the other hand, this growth pattern not only ensures that the essential organs for primary functions (feeding, respiration and locomotion) are developed first (Gisbert *et al.*, 2002), but also the energy costs of larval transport are optimal (Osse *et al.*, 1997; Osse and van den Boogaart 2004; van Snik *et al.* 1997).

In many teleost larvae, inflexion points of different body proportions lay in a more or less narrow total length interval. For example, in *Cyprinus carpio*, *Paralabrax maculatofasciatus* and *Nannacara anomala*, inflexion points occurred at 6.4-7.8 mm, 5.86-6.45mm and 4.03-4.84 mm respectively (van Snik *et al.*, 1997; Peña and Dumas 2009; Kupren *et al.*, 2014 a), indicating the existence of a shift in swimming model from an anguilliform to subcarangiform (van Snik *et al.*, 1997; Gisbert *et al.*, 2002; Peña and Dumas 2009). In contrast, inflexion points in larval *A. chalcoides* occurred across a wide size interval (8.13-13.57 mm), as was found in *Lota lota* (8.64-27.85mm; Kupren *et al.*, 2014b) and *Leuciscus cephalus* (11.57-25.69 mm; Kupren *et al.*, 2015). These gradual changes in body proportions are probably related to the lack of sharp changes in behavior and habitat and may be also considered to be a gradual adaptation to life in flowing water (Kupren *et al.*, 2015). After inflexion points, nearly isometric growth was characteristic for most measured characters in the present study. A change from allometry to isometry on growth patterns of teleost fish during early development stages has been considered as a natural transition in growth priorities since primary functions has been fulfilled during early development stages (Osse and van den Boogaart 2004; Koumoun-douros *et al.*, 1999; Peña and Dumas 2009). Besides these, BD showed positive allometry throughout the entire experimental period, although trunk length had a negative allometric growth during the first 4 weeks after hatching. This may allow larvae or juveniles to cope with the development of the digestive tract and associated glands. On the other hand, this growth pattern also support the view that negative allometry of trunk sometimes does not adequately reflect the organogenic processes occurring with the abdominal cavity during early ontogeny (Martínez-Montaña *et al.*, 2014).

Generally, the duration of every developmental stage for larvae and juveniles in field conditions was shorter than in captive conditions, and the larvae in pond-cultured conditions often grew faster than those in captive conditions. Therefore, the results presented in this study should be used cautiously in seed production. Nevertheless, the analysis of growth patterns in combination with observed changes in

morphological development provides a basic for further studying the complete early life history of *A. chalcoides*. Similarly, our findings may be helpful for both comparative studies of the ontogeny in other species as well as for aquacultural applications.

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