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THE DEVELOPMENT OF DOCTOR FISH, *GARRA RUFA* (HECKEL, 1843), LARVAE AND THE EFFECT OF TEMPERATURE ON THEIR GROWTH

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ABSTRACT

The 'doctor fish,' *Garra rufa* (Heckel, 1843), is an endemic Mesopotamian freshwater species that is legally protected in Turkey against over-harvesting and commercial exploitation. The present study investigated the larval development of *G. rufa* and the effect of different temperatures (24, 26, 28, 30, 32, 34ºC) on larval growth and survival. Spawners were collected from Kangal (Turkey) and reared in the laboratory for 12 months. Eggs were obtained under intensive aquarium conditions, and the number of eggs and hatched larvae was observed. The mean total length (TL) of the hatched larvae was 5mm (range 5–5.5mm) initially. The mouth opened 2–3days after hatching (DAH). The yolk sac had been totally absorbed, and the larvae started to swim actively 4–5DAH. Embryonic development was completed within 35–36h at 28±1ºC. The morphological metamorphosis was completed and the larvae transformed into juveniles 27–35DAH. The final total lengths of juveniles at 24, 26, 28, and 30ºC were significantly higher ($P<0.05$) than at 32 and 34ºC, the lengths being 37.93±2.59, 38.41±1.99, 37.84±2.48, 37.97±1.26, 34.938±4.43, and 33.34±2.35 mm, respectively. The maximum total length (38.41±1.99mm) and weight (0.56±0.07g) were attained on the 75thDAH at 26°C. The results indicated that *G. rufa* tolerated a range of different temperatures. Specific growth rates (SGR%) were 3.54±0.39, 3.49±0.05, 3.22±0.25, 3.39±0.22, 2.59±0.19, and 2.70±0.20 g at 24, 26, 28, 30, 32, and 34°C, respectively ($P<0.05$). The survival rates (%) were 100, 100, 100, 100, 90, and 90%, respectively ($P<0.05$). These results may assist in the development of better techniques for larva culture and hence the more successful production of doctor fish.

KEYWORDS:
Garra rufa, doctor fish, reproduction, growth, temperature

INTRODUCTION

Garra rufa (Heckel 1843), commonly known as the doctor fish, is one of around 100 *Garra* species in the Cyprinidae family [1]. *G. rufa* is widely distributed throughout the river basins of the Middle East, notably in Turkey, Syria, Iraq, and Iran [2,3]. It is reported that *G. rufa* grazes on aquatic plants, preferring plant materials that mostly consist of benthic chrysophytes and phytoplankton, with a few rotifers and protozoans [4]. *G. rufa* is a fish species that is used in ichthyotherapy for curing skin diseases such as psoriasis, eczema, and atopic dermatitis [1,3,5,6], as well as in the health and beauty industries. It is estimated that 15,000–20,000 *G. rufa* are imported into the United Kingdom each week from Indonesia and other countries in Asia [7]. Most of the fish are collected from natural habitats. *G. rufa* is legally protected in Turkey from over-harvesting and commercial exploitation. Hence, the captive breeding and larval rearing of *G. rufa* can be considered important conservation measures. Some *Garra* species are currently being commercially produced in countries around the world. Nevertheless, limited information is available on breeding and the early life history of *G. rufa*. Some authors [8,9] have investigated the reproductive biology of *G. rufa* and the relationship between fecundity and fish size. There is some literature on reproduction and embryonic development [10,11], spawning induction and captive rearing [12], diet [4], and the feeding frequency of *G. rufa* [13,14]. Knowledge about larval development is vital for the development of techniques in the seed production of *G. rufa*. Hence, this study is mainly focused on larval development and morphology, and secondarily on the effect on the growth and survival rate of *G. rufa* larvae at different temperatures.

MATERIALS AND METHODS

Garra rufa broodstocks were collected from Kangal Balıklı Çermik in Sivas, Turkey (39° 18' 42.6" N, 37° 28' 16.0" E). Approximately 100 specimens were carefully transferred to the laboratory. It was estimated that the total length of the broodstock was between 5 and 8 cm, although the total length of

individuals was not measured to avoid stress. The fish were acclimatized to laboratory conditions for a period of 2 weeks, in four indoor glass aquaria (~55–60 L per aquarium) under the same environmental conditions. After acclimatization, they were stocked in eight glass aquaria, each measuring 60 cm (L) x 30 cm (W) x 60 cm (H), and with a water level of 31–32 cm (H). The maximum stocking density was 50 individuals per aquarium. They were fed with commercial fish feed (protein: 46%, oil: 12%, fiber: 3%, ash: 11%, moisture: 8%) twice a day. Water temperature, pH, and oxygen were monitored at 28 ± 1°C, 8.0–8.4, and 6.0–6.2 mg l^{-1}, respectively, for broodstock maintenance. The water temperature was controlled via additional submerged heaters (100 watt), and the photoperiod was maintained at 12L/12D by fluorescent lighting (lights were on between 07:00 and 18:00). Eight spawning tanks (each measuring 60 cm (L) x 30 cm (W) x 60 cm (H), with a water level of 31–32 cm (H), 55–60 L), were prepared. The stocking ratio of males to females in each tank was 2:2, selected randomly from broodstock tanks. The data were collected from eight broodstock tanks for a period of two months. The fertilized eggs were removed from the breeding tanks by siphoning, before being counted and measured under an Olympus BX51 research microscope (Tokyo, Japan). The eggs were incubated in 30 L glass tanks, filled with water from the breeding tank, and were aerated with airstones. The temperature of the incubation tanks was kept at 28 ± 1°C. All larvae used in the experiment were obtained from the same batch of eggs. The larvae were reared from the hatching to the postlarval stage (30 DAH). Ten percent of the water in the rearing tank was renewed daily from the 4th to the 30th DAH. The larvae were fed with *Artemia salina* (INVE Aquaculture, Inc., Dendermonde, Belgium) from the 3rd to the 15th DAH. Larvae were fed commercial microparticle feed from the 13th to the 30th DAH (500–800 µm; containing protein: 50%, fat: 7%, fiber: 2%, ash: 12%, moisture: 12%; Bio-Aqua, Turkey). They were fed three times per day. Larvae were randomly sampled (n = 2) daily during the 30th DAH stage. They were then observed under a stereomicroscope (Olympus BX51, Tokyo, Japan) and photographed using a colour video camera (Q Imaging, Micropublisher 3.3 RTV, Burnaby, BC, Canada). During the procedure, the specimens were anesthetized with clove oil solution (0.05 mL^{-1}), prepared by dissolving clove oil in 95% ethanol at a ratio of 1:10 (clove oil:ethanol), as described by Anderson et al. [15].

Larval developmental stages were identified according to Kendall, Ahlstrom, and Moser [16] and differentiated into four periods: I: yolk-sac larva, II: preflexion larva, III: flexion larva, and IV: postflexion larva.

In addition, the growth and survival of *G. rufa* larvae (0–30th DAH) and juveniles (31st–75th DAH) were compared during experimental rearing at temperatures of 24, 26, 28, 30, 32, and 34°C, in line with the temperatures reported in the literature for this species [10,17,18,19]. Larvae were obtained from different spawners and pooled. They were stocked in 12 glass aquaria, each measuring 40 cm (L) x 30 cm (W) x 30 cm (H), and with a water level of 25 cm (H), at a stocking density of 10larvae/tank. Two weeks after hatching, the larvae were randomly selected and stocked at a density of 10 larvae/30 L volume in six different temperature treatments (stocking density = 10 larvae per tank). The total length and weight of the larvae were measured daily using standard methods and the total number was calculated. Larvae were fed with *Artemia* nauplii until the 13th DAH. They were then fed with commercial fish food (500–800 µm) between the 13th DAH and the 50th DAH and with a different commercial food (800–1,200 µm) from the 45th to the 75th DAH. Both types of food had the same content (protein: 50%, fat: 7%, fiber: 2%, ash: 12%, moisture: 12%; Bio-Aqua, Turkey). The growth experiment was replicated twice at different temperatures. Total lengths of larvae were measured with a digital caliper to the nearest 0.01 mm (Mitutoyo model CD-15DCX, Japan), and the weight of larvae measured with an electronic balance to the nearest 0.001 g (Shimadzu Electronic Balance type BL-320H, Japan). No anesthetic agent was used during measurements. The survival rate was checked daily.

Data analysis. The data (total length, wet weight, specific growth rate) were analyzed using a one-way analysis of variance (ANOVA), and the differences were compared using Duncan's multiple range test (Minitab 16.2.3. and the SPSS V 25.0 programs). Pearson's chi-squared test was used to compare differences in survival rates (with the significance level $\alpha = 0.05$) (SPSS V 25.0 IBM Corp, 2017).

RESULTS

The spawning frequency of *G. rufa* ranged between 15 and 30 days. The number of eggs collected from two females per spawning was between 57 and 314 (Figure 1).

The eggs of *G. rufa* were demersal and circular in shape. Their diameters ranged from 2.80 to 2.90 mm, with a mean of 2.83 ± 0.04 mm. The egg capsule was transparent, while the yolk was whitish. The fertilized eggs were non-adhesive. Hatching rates were 85–90% in the aquaria 35 h after spawning (Figure 2). The embryonic development was completed at 35–36 h (Figure 2).

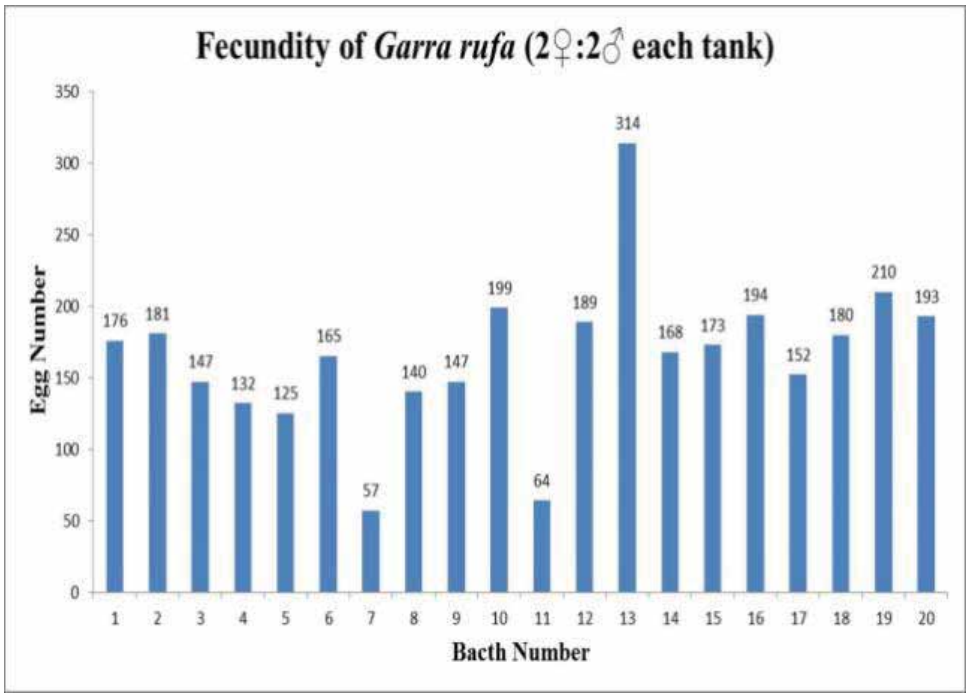

FIGURE 1
Total number of eggs per batch of *G. rufa* (two females per spawning)

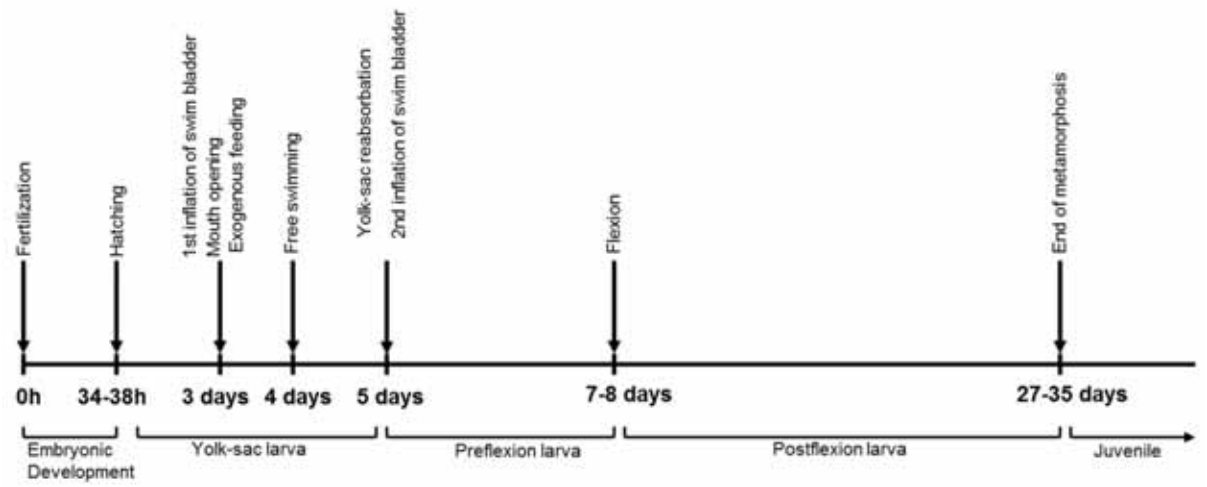

FIGURE 2
Larval development of *Garra rufa*, 1 Dah to 27 DAH

Morphological development. The newly hatched larvae were laterally compressed and elongated. Their mean total length was 5 mm (range 5–5.5 mm) (Figure 4), and the yolk sac was 60% of the total body length on the 1st DAH (Figure 3.1). At this stage, the mouth and the anus were closed, and the body of each larva was transparent (Figure 3.1). The alimentary tract appeared as an undifferentiated short tube, and the eyes were not pigmented. The primordial fin fold was well developed and the pectoral fin was observed (Figure 3.1).

During the 2nd day, the yolk sac became smaller (Figure 3.2). The pigmentation of the eyes and the body increased but remained translucent. The swim bladder was first observed at the 2nd DAH. The primordial fin was slightly differentiated, but not the anal and dorsal fins, and the larvae were not yet able to swim actively. The larvae started to feed exogenously on the 3rd DAH (Figs. 2, 3.3). The swimming activity increased and the first inflation of the swim bladder could be observed at this stage (Figs. 2, 3.3). The larvae started to swim actively on the 4th DAH. The notochord end was slightly flexed. The eyes became very prominent and the number of caudal-fin rays increased (Figure 3). The anal and dorsal fins began to develop (Figure 3). The larvae reached a total length of 8–9 mm on the 5th DAH, by which time the yolk sac had been completely absorbed (Figs. 2, 3, 4). The second inflation of the swim bladder could be observed, and the larvae swam very well (Figure 2). The postflexion stage observation started on the 8th DAH. The number of melanophores

increased. The differentiation of the dorsal and anal fins started at this stage (Figure 3): the caudal fin began to separate from the continuous fin, and the number of rays increased as well. Most of the larvae were still homogeneously distributed in the water column on the 9th DAH. A single barbel bud, located on the ventral side of the lower jaw, appeared between the 9th and 11th DAH (Figs. 3.9, 3.10). At this stage, the pigmentation increased on the head and on the lateral parts of the body. All the fins were more developed, with separated rays, and the caudal fin became forked (Figure 3). The length of the larvae now ranged between 12 and 13 mm (Figure 4).

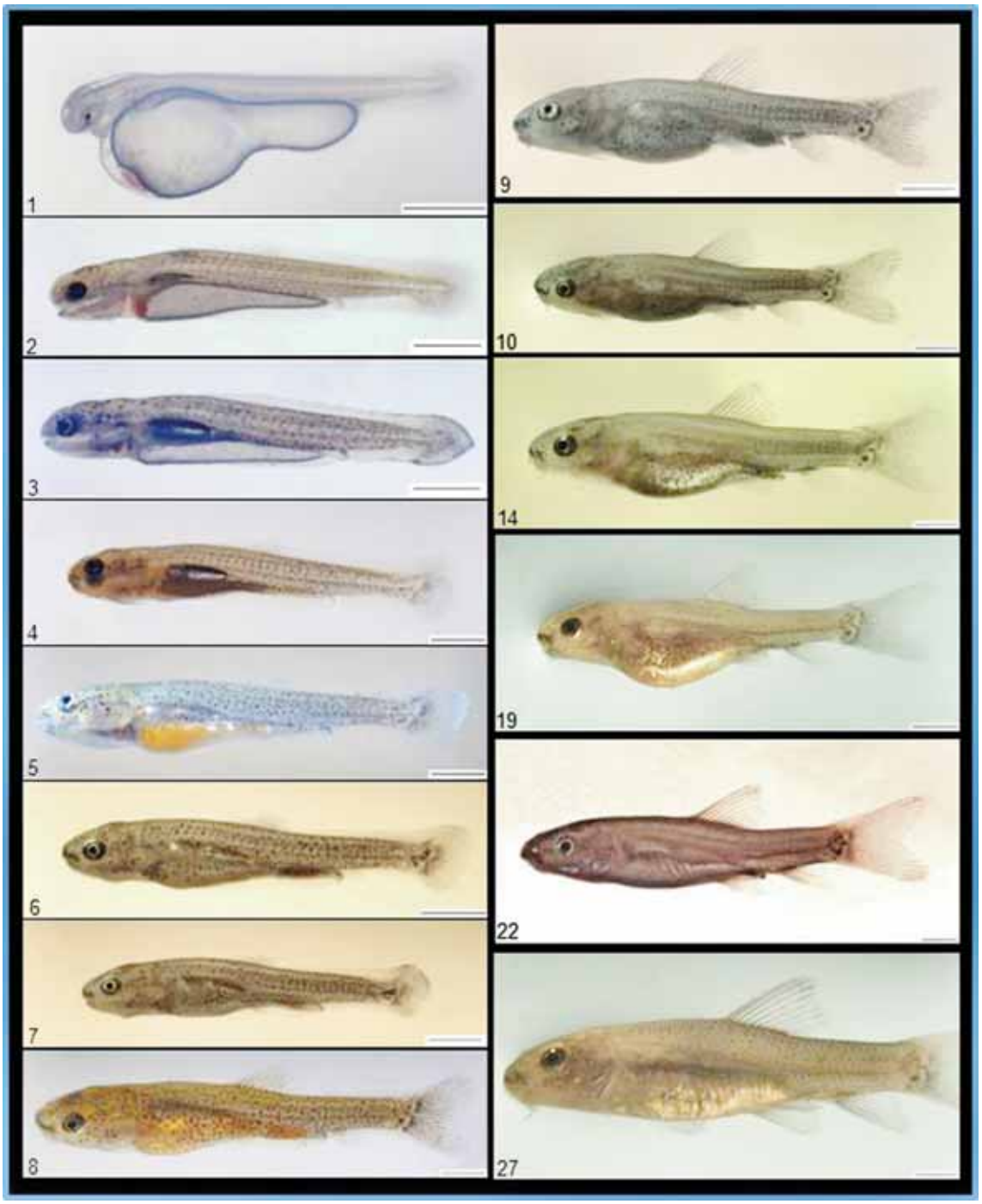

FIGURE 3
Larvae, fry and juvenile development of (*G. rufa*) from age 1 DAH to 27 DAH at 28±1°C. Scale bar = 1 mm.

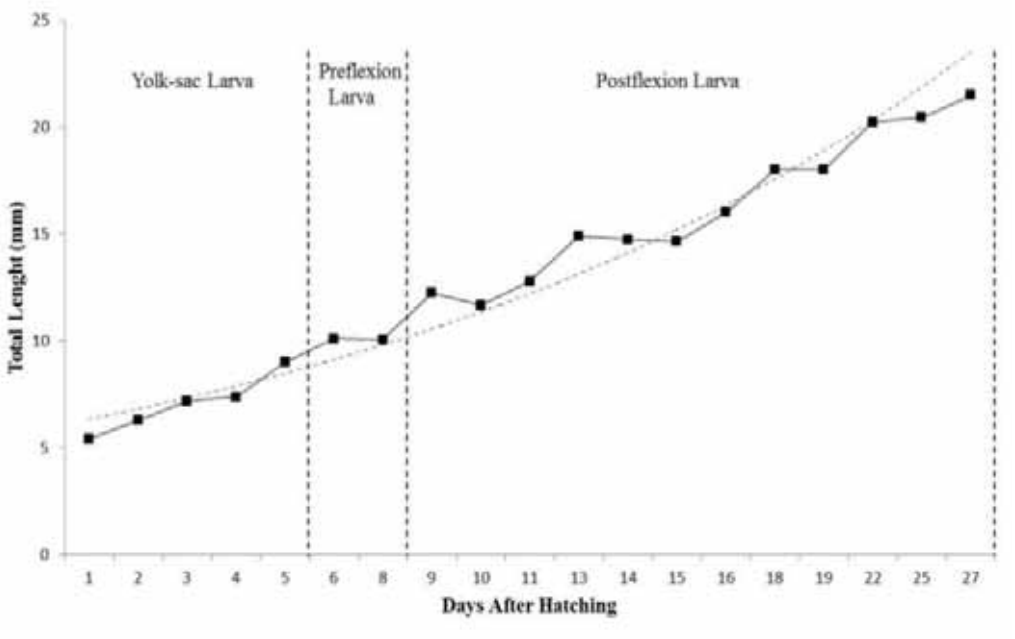

FIGURE 4
Total length of *Garra rufa* from hatching to 27 DAH at 28±1°C.

The body shape and the pigmentation pattern were similar to those of the adult fish between the 14th and the 19th DAH (Figure 3.19), by which time all the fins were well developed and the larvae had grown to a total length between 15 and 18 mm (Figure 5). The body was completely covered by pigments on the 27th DAH (Figure 3.27). On that day, they had reached 21.49 ± 2.43 mm at 28 ± 1°C (Figure 4). The body colour ranged from a dark grey to brown with black spots, the black pigments being dominant. All larvae completed their metamorphosis between the 27th and 35th DAH and transformed into juveniles. The total length of 30-day-old juveniles ranged from 22 to 25 mm (Figure 5).

Early larval development of *G. rufa* was divided into four main periods. The first period was the yolk-sac larva stage (hatching to preflexion) from the 1st to the 5th DAH; the presence of a yolk sac was ventrally in the body. The second period, the preflexion larva stage (from the 6th to the 7th DAH), began with the absorption of the yolk sac and ended with the start of upward flexion of the notochord (between the 4th–6th and 7th–8th DAH). The yolk sac was completely absorbed and the larvae could swim actively. The third period, the flexion larva stage (7th to 8th DAH, the period during notochord flexion), was characterized by the hypural bones assuming a vertical position. The fourth period was the postflexion larva period (9th to 27th DAH), between the completion of flexion and the juvenile stage, namely between the 7th–8th and the 27th–35th DAH (Figure 2).

The final total lengths of *G. rufa* were 37.93 ± 2.59, 38.41 ± 1.99, 37.84 ± 2.48, 37.97 ± 1.26, 34.938 ± 4.43, and 33.34 ± 2.35 mm at 24, 26, 28, 30, 32, and 34°C, respectively (Table 1). Final TL at 24, 26, 28, and 30°C was significantly higher than at 32 and 34°C (one-way ANOVA, $P < 0.05$) (Table 1 and Figure 5). There were no significant differences in final TL between the 24, 26, 28, and 30°C groups (one-way ANOVA, $p > 0.05$). The final weights of *G. rufa* were 0.54 ± 0.12, 0.56 ± 0.07, 0.53 ± 0.09, 0.52 ± 0.06, 0.43 ± 0.17, 0.41 ± 0.08 g, respectively, at 24, 26, 28, 30, 32, and 34°C (Table 1). Final weight at 24, 26, 28, and 30°C was significantly higher than at 34°C (one-way ANOVA, $P < 0.05$) (Table 1 and Figure 5), but there was no difference between the final weights at 24, 26, 28, and 30°C (one-way ANOVA, $P > 0.05$).

Specific growth rates (SGR %) of *G. rufa* were 3.54 ± 0.39, 3.49 ± 0.05, 3.22 ± 0.25, 3.39 ± 0.22, 2.59 ± 0.19, and 2.70 ± 0.20 g at 24, 26, 28, 30, 32, and 34°C, respectively (one-way ANOVA, $P < 0.05$). Overall, the highest specific growth rate was observed at 24°C (3.54% d^{-1}), although it was not statistically different from the growth rate at 24, 26, 28, and 30°C (one- way ANOVA, $P > 0.05$) (Table 1). The lowest growth rate was observed at 32 and 34°C (2.59% d^{-1} and 2.70% d^{-1}) (one-way ANOVA, $P > 0.05$) (Table 1).

The survival rate (%) of *G. rufa* at 24, 26, 28, 30, 32, and 34°C was 100, 100, 100, 100, 90, and 90%, respectively (Pearson's chi-squared test, $P < 0.05$). The survival rate was high in all temperature treatments (> 90%) (Table 1), but the highest survival rate was observed at 24, 26, 28, and 30°C (100%), there being no significant differences in survival rates at these temperatures (Pearson's chi-squared test, $P > 0.05$). The lowest survival rate was observed at 32 and 34°C (90%) (Table 1).

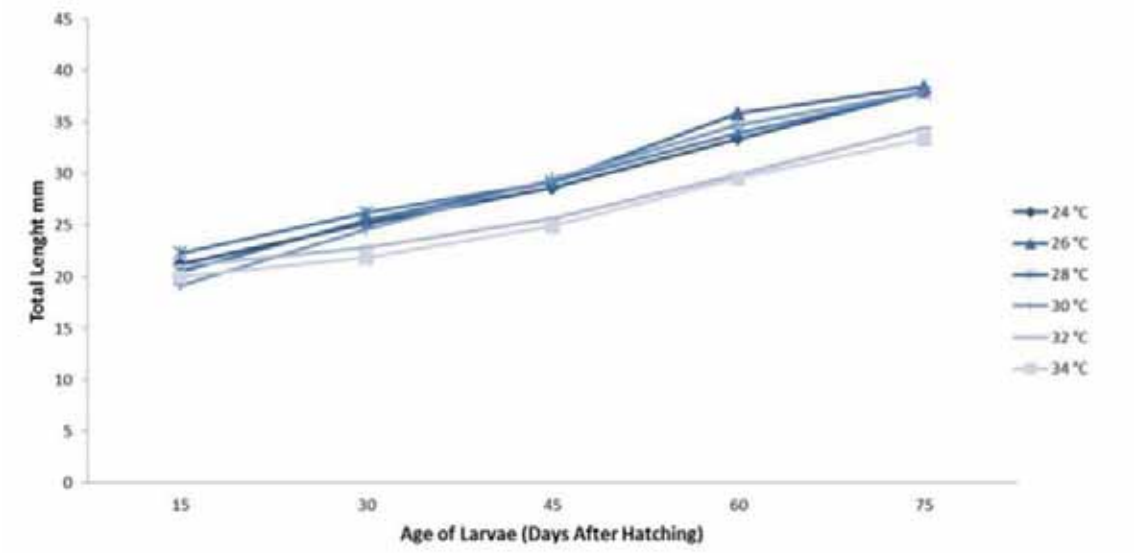

FIGURE 5
Mean total length of *Garra rufa* from 15 DAH to 75 DAH at six different temperature levels (°C).

TABLE 1
Growth performance of *Garra rufa* larvae/juveniles at six different temperatures from 15 DAH to 75 DAH.

	24 °C	26 °C	28 °C	30 °C	32 °C	34 °C
Initial TL	21.18±1.98^{a}	20.51±2.34^{a}	21.69±1.26^{a}	21.18±1.10^{a}	21.04±1.12^{a}	21.71±1.10^{a}
Final TL	37.93±2.59^{a}	38.41±1.99^{a}	37.84±2.48^{a}	37.97±1.26^{a}	34.94±4.43^{b}	33.34±2.35^{b}
Initial W	0.076±0.030^{a}	0.070±0.018^{a}	0.087±0.032^{a}	0.062±0.011^{a}	0.086±0.046^{a}	0.076±0.029^{a}
Final W	0.54±0.12^{a}	0.56±0.07^{a}	0.53±0.09^{a}	0.52±0.06^{a}	0.43±0.17^{ab}	0.41±0.08^{b}
SGR (% day)	3.54±0.39^{a}	3.49±0.05^{a}	3.22±0.25^{ab}	3.39±0.22^{a}	2.59±0.19^{c}	2.70±0.20^{bc}
Survival %	100^{a}	100^{a}	100^{a}	100^{a}	90^{b}	90^{b}

Note: Values marked with different superscripts are significantly different from each other (P<0.05).
TL=Total length (Mean±SD mm), W= Wet weight (Mean±SD g).
SGR = Specific growth rate (% day) = (ln final weight – ln initial weight) x 100 / Time (60 days)
Specific growth rate (SGR) was calculated as $(\ln W_2 - \ln W_1)/\Delta T$ where ΔT is the number of days between times T_1 and T_2.

DISCUSSION

The ovarian development in fish can be classified into three major types: synchronous, group synchronous, and asynchronous [20]. Most of the teleost fishes are classified as group synchronous [21,22]. In the synchronous type, the fish spawn all the eggs and die; in the group synchronous type, the fish spawn seasonally multiple times in a year; and in the asycnhronous type, the fish spawn multiple times in a day. *G. rufa* is an asynchronous spawner, and not all the mature females spawn at once [9,23]. The present study indicates that mature *G. rufa* can spawn a few times during 1–2 months.

The range of fecundity for some *Garra* species is reported as 740–4,390 eggs for *Garra ceylonensis* [21], 562 ± 176 eggs for *Garra ceylonensis* [18], 539–2,968 eggs for *Garra tana* [23], 581–1,800 eggs for *Garra regressus* [24], and 431–5,402 eggs for *Garra surendranathanii* [25]. The findings of the present study indicate that two female *G. rufa* spawned between 57 and 314 eggs per spawning. Abedi et al. [9] reported that the absolute fecundity of *G. rufa* ranged from 283 to 3,794 eggs per female.

The egg size of cyprinids varies widely. The fecundity of *G. rufa* is lower than that of other species but the egg of this species is larger than most of the species in the *Cyprinidae* family. Egg diameters of some cyprinid species are reported as 1.4–1.6 mm for *Carassius auratus* [26], 0.75 mm for *Puntius conchonius* [27], 1.18 ± 0.05 mm for *Capoeta tetrazona* [28], 0.7 mm for *Danio rerio* [29], 1.3–1.5 mm for *Barbus sharpeyi* [30], and 1.8–1.9 mm for *Cyprinion macrostomus* [31]. On the other hand, while the eggs of some species in the *Cyprinidae* family are reportedly adhesive [30], the eggs of *G. rufa* are not.

The diameters of some *Garra species* eggs are reported as 0.77–1.87 mm for *Garra ceylonensis* [18] and 2.46 mm for *Garra surendranathanii* [19].

The mean diameter of fully mature eggs of *Garra regressus* is reportedly 1.81± 0.016 mm, while that of *Garra tana* is 1.22 ± 0.006 mm [24]. On the other hand, in the present study, the eggs of *G. rufa* were transparent, demersal, and non-adhesive, and ranged from 2.80 to 2.90 mm in diameter, with a mean of 2.83 ± 0.04 mm. The study revealed that the eggs hatched in approximately 35–36 h at a temperature of 28 ± 1°C, whereas Gomes et al. [11] reported that the hatching time was between 24 and 48 h at 26°C. Considering other *Garra* species, the hatching time for *Garra ceylonensis* was 36–48 h at 26–28ºC [17], for *Garra surendranathanii* from 35.2 ± 0.3 to 36.9 ± 0.4 h [18], and for *Garra parvifilum* between 24 and 26 h at 25–28°C [17].

Garra rufa larvae absorbed the yolk sac within 4–6 days at 28 ± 1°C and started to feed exogenously before complete absorption of the yolk sac. It has been reported that the larvae of *G. rufa* absorbed 2/3 of their yolk sac in 3 days after hatching at 28°C [12] and in 48 h at 26°C [10,11]. The swim bladder was seen within 2 days after hatching, but larvae could not swim actively because the dorsal and anal fins were undifferentiated and the yolk sac was large. When the yolk sac of the *Garra ceylonensis* larvae was completely absorbed, it was observed that the hatchlings began to swim freely throughout the water column within 3–4 days [17]. Additionally, a study found that the yolk sac was depleted within 5 days after hatching during the larval development of *Garra surendranathanii* [19].

In the present study, the larval development stage of *G. rufa was* completed on the 27th–35th DAH at 28 ± 1°C. Other studies have found that the larval development of *Garra ceylonensis was* completed between the 60th and 64th days at 26–28 °C (hatchling to postlarva stage: the 3rd–4th DAH, postlarva to the juvenile stage: the 5th–6th DAH) [18], whereas the larval development of *Garra surendranathanii* was completed between the 55th and 60th DAH at 28 ± 1°C [19].

Water temperature plays an important role during the embryonic and larval developmental stages. It is also one of the most important parameters affecting the survival and growth rate of fish larvae [32,33,34]. In this study, the water temperature was 28 ± 1°C during embryonic and larval development. The results also confirmed that the temperature affected the larval and the juvenile development of *G. rufa*.

Vazirzadeh et al. [12] examined the effect of the rearing conditions and feeding regime on the survival of *G. rufa* larvae, and reported that a temperature higher than 34ºC or lower than 24ºC was not suitable for larval culture, as the larvae showed the highest rate of mortality at these temperatures. Accordingly, they suggested that a temperature between 28 and 30ºC was the most likely condition for the larval culturing of *G. rufa* [12]. A previous study revealed that *G. rufa* dwells in aquatic habitats with pH ranging between 7.0 and 9.0 and temperatures between 15 and 31.2°C [35], which is in accordance with the conditions observed in the present study. Since *G. rufa* is both a tropical and a subtropical species and normally lives in warm waters, a temperature below 24ºC may decrease its appetite, as well as its digestion rate [12]. Yanar et al. [36] determined the thermal tolerance parameters of *G. rufa* at three acclimatization temperatures, 20, 24, and 28 °C. According to these authors, *G. rufa* was the species that had advantages for aquaculture, thanks to its tolerance of a wide range of temperatures and high acclimation response ratio (ARR) values [36]. *G. rufa* was resistant to both low (3–7°C) and high (38–41°C) temperatures, and thus its thermal tolerance polygon (TTP) values were also high. In addition, it was resistant to temperature fluctuations because of its high ARR values [36]. It was reported that the optimum temperature range, from 24 to 30°C, plays an important role in improving hatchability, growth, food intake, and defence mechanisms during the larval development of *Cyprinus carpio* [37].

In the present study, final weights, total lengths, and specific growth rates (SGR %) were higher at lower temperatures (24–30°C; $P<0.05$) (Table 1). According to Catarino et al. [14], the SGR values of *G. rufa* were lower when the fish were fed twice per day than when the daily dose was distributed three times per day. In this study, the fish were fed three times per day at different temperatures. Catarino et al. [14] examined the optimal frequency of feeding for the production and rearing of *G. rufa*, finding that 90% survival was obtained for the treatment corresponding to a feeding frequency of twice per day, while feeding three times per day meant the rate dropped to 86.7%. In the present study, survival was high in all temperature treatments (> 90%). The survival was more than 90% at all temperatures, but it was 100% when the temperature was between 24 and 30°C. Similarly, it was reported that the larvae of *G. rufa* experienced a lower survival rate when the water temperature was below 24°C or above 34°C [12]. According to this study, the larvae and juveniles of *G. rufa* were able to grow and survive in a wide temperature range (24–34°C) but showed variation in the optimum temperature for better growth.

In conclusion, the present study provided a comprehensive description of the main morphological events that took place during the larval development of *G. rufa*, in order to establish reference data for its normal developmental organogenesis. In addition, it was seen that the larvae and juveniles of *G. rufa* grew better at between 24 and 30°C than at either 32 or 34°C. These results may provide a better understanding of the commercial cultivation techniques of *G. rufa* that would promote the conservation of this species. The results also provided scientific information on captive breeding and larval development, which may also be used as a reference for further studies on *Garra* species. Gomes et al.

[11] reported that *G. rufa* could be marketed in three months and might be potential reproducers in six months. This rapid development, combined with high market demand, makes *G. rufa* a very attractive species for aquaculture [11].

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