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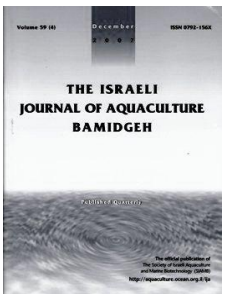
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA:64.2012.674, 6 pages



Induced Reproduction of *Aphanius fasciatus* by Ecophysiological Conditioning and Hormonal Treatment in Fresh and Marine Water

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(Received 24.11.10, Accepted 14.2.11)

Key words: *Aphanius fasciatus*, artificial reproduction, ovary maturation, egg production, larvae production

Abstract

The Mediterranean toothcarp (*Aphanius fasciatus*) can be used to biologically control mosquito larvae. The reproductive performance of 234 Mediterranean toothcarp (180 females and 54 males) was investigated. Reproductive factors were determined in fish kept in fresh or saline water (males and females together), and in fish kept separately by sex in saline water and hormonally-treated with carp pituitary extract (CPE). In the hormonally-treated group, the combined effect of temperature, photoperiod, and hormonal treatment induced the best ovary maturation and larvae production rates. The rate of reproduction was very high (average eggs/female: 5.9-10.5) compared to natural reproduction in the wild (average eggs/female: 2-4). Additionally, the hatching rate was 97-100%. The results of this study show that reproduction of Mediterranean toothcarp can be controlled in an artificial environment: the lack of mortality in adults during acclimation and conditioning in fresh and marine waters indicates good domestication and plasticity in reproductive parameters.

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Introduction

Mediterranean toothcarp *Aphanius fasciatus* (Valencienne, 1821) is a small cyprinodontid that inhabits residual coastal environments around the Mediterranean in France, Italy, Slovenia, Croatia, Albania, Greece, and Montenegro and in North Africa from Egypt to eastern Algeria and Turkey. It reaches a maximum length of 6-7 cm and is characterized by striped coloration and evident sexual dimorphism (Gandolfi et al., 1991). It is a short-lived species that occurs in marsh ponds, ditches, and swamps, and spawns from April to September with good resilience. The population possibly doubles in less than 15 months.

The Mediterranean toothcarp is an endangered species, categorized as a Least Concern species (LC) by the International Union for Conservation of Nature and Natural Resources (IUCN) and for high protection in Annex II of the European Union Habitats Directive due to its small population, special habitat requirements, and high genetic variability (Wildekamp et al., 1999; Cimmaruta et al., 2003; Ferrito et al., 2007). It feeds mainly on small invertebrates such as mosquito larvae, thus, dissemination of this cyprinodontid in affected areas could be an ideal tool for aquatic insect control (Frenkel and Goren, 2000; Caiola and Sostoa, 2005; Yildirim and Karachua, 2007). Introduction of the mosquitofish *Gambusia holbrooki* caused a decrease in the abundance of frogs (Webb and Joss, 1997), markedly reduced the population density of invertebrate predators of mosquitoes (El Safi et al., 1985), and affected *Aphanius* populations (Caiola and Sostoa, 2005). Thus, diffusion of an autocthonous endangered species in brackish and fresh waters could be a welcome replacement for the allocthonous mosquitofish in the Mediterranean area (Caiola and Sostoa, 2005; Alcaraz and Garcia-Berthou, 2007).

Many *Aphanius* species are candidates as biological control agents since they feed on mosquito larvae (Homski et al., 1994) and are very eurythermal and euryhaline (Lotan, 1973). Competition with *Gambusia* and degradation of their natural environments has reduced the distribution of *Aphanius* in many areas, impacting the capacity of *Aphanius* to control Culicidae (mosquitoes). Information exists on the reproduction of *A. fasciatus* in its natural habitat (Gandolfi et al., 1991; Leonardos et al., 1996; Alcaraz and Garcia-Berthou, 2007). The aim of the present study was to develop a mass production protocol in a controlled environment for Mediterranean toothcarp so that small fresh and brackish water basins in public areas (e.g., parks, gardens) could be reinhabited and control of Culicidae improved.

Materials and Methods

Adult Mediterranean toothcarp (*Aphanius fasciatus*) were collected during April 2010 near Cervia (northern Italy) from brackish water channels using a fishing net with a 5.0-mm mesh. Water temperature was 19°C, salinity was 39‰, and the natural photoperiod was 12L:12D. Sex was determined by external characteristics since *A. fasciatus* exhibits sexual dimorphism. Individual total lengths of 30 males and 30 females were measured with a digital micrometer (±0.01 mm), body weights were measured with an electronic balance (±0.01 g), and condition factors (K) were determined as body wt x 100/total length^3. Age was determined by scales taken from the left side of the body, between the end of the thoracic fin and the beginning of the dorsal fin (Garcia-Berthou and Moreno-Amich, 1992).

Twenty females were sacrificed with an overdose of anesthetic 2-phenoxyethanol and gonads were carefully excised and weighed to determine the gonado-somatic index (GSI) according to 100(gonad wt/body wt). A wet mount slide was prepared from the ovaries of each sacrificed fish, examined under a binocular microscope, and the most advanced stage of maturation of the cells was noted. Oocytes from ovaries were placed in Serra's solution for clarification of the cytoplasm. After 5 min, the position of the oocyte nucleus was determined using a 4-stage scale (Frenkel and Goren, 1997): Stage 1 - germinal vesicle in central position (oogonia), Stage 2 - early migration of germinal vesicle (primary oocytes), Stage 3 - late migration of germinal vesicle (secondary oocytes), and Stage 4 - periphery germinal vesicle or germinal vesicle breakdown (mature ova).

During a 14-day acclimation, animals were fed a commercial food daily *ad libitum* and maintained at the same salinity, temperature, and photoperiod as in the wild. After

acclimation, 234 animals (180 females and 54 males) were selected and kept in twelve 50-l aquaria, with a closed recirculation system. The aquaria were equipped with aeration, mechanic and biological filters, and thermo-regulation systems. Illumination was provided by standard fluorescent bulbs in overhead fixtures. No vegetation or refuge was provided to prevent the spontaneous emission of eggs and enable better observation of broodstock behavior. Three groups of 20 females and 6 males/aquarium were gradually adapted during a 10-day period to fresh water. Three groups of 20 females and 6 males/aquarium were kept in the natural salinity of 39±1‰. The applied sex ratio concurred with that existing in the wild where, during the reproductive period, the number of females overwhelms the number of males (Leonardos and Sinis, 1999). An additional three groups of 20 females and three groups of 6 males were kept separated by sex in six aquaria, again in the natural salinity of 39±1‰. Dissolved oxygen, pH, total ammonia, nitrites, nitrates, and total phosphorous were analyzed weekly.

To determine gamete deposition, all groups underwent 21 days of conditioning as follows: during the first 2 weeks, the photoperiod was changed to 15.5L:8.5D and the water temperature increased from 19±0.5°C to 26±0.5°C. The final conditions were similar to natural reproductive conditions (Leonardos and Sinis, 1997), and were maintained throughout the trial (Table 1).

Table 1. Physico-chemical water parameters.

	Treatment		
	Fresh	*Saline (39±1‰)*	*Saline, separated by sex*
pH	7.61±0.12	8.15±0.11	8.16±0.13
Dissolved oxygen (mg/l)	6.52±1.60	6.70±1.20	6.82±1.32
Total ammonia (mg/l)	0.14±0.07	0.12±0.05	0.10±0.08
Nitrites (mg/l)	0.013±0.006	0.012±005	0.016±0.05
Nitrates (mg/l)	2.41±0.71	1.03±0.42	1.12±0.32
Total phosphorous (mg/l)	0.21±0.08	0.18±0.05	0.17±0.06

On day 21, females in the sexually-mixed freshwater and saline groups underwent manual stripping of eggs, and gametes were collected from males. Eggs were manually stripped to prevent cannibalism, a phenomenon that occurs during the reproductive phase (Frenkel and Goren, 1999). In the six sexually-separated groups, fish were hormonally-treated with freeze-dried carp pituitary (CPE) at 10 µg/g body weight for females and 3 µg/g body weight for males (Rothbard, 1981; Zohar and Mylonas, 2001). Twelve females from each treatment (four per aquarium) were used to determine GSI and gonadic maturation stage where females from the hormonally-treated groups were sacrificed 12 h after hormonal treatment for GSI evaluation and stripped 16 h after hormonal treatment to determine gonad maturation stage.

Fertilized eggs were incubated in nine 15-l aquaria (three per treatment) equipped with closed recirculation, aeration, and thermo-regulation systems. Eggs were placed on a suspended PVC 900-µm mesh net and the number of egg-producing females, absolute fertility (no. eggs/female), fertilization and hatching rates, incubation times in degree days (no. days at a given temperature required to achieve hatchlings), and number of larvae were determined. Data concerning spawned eggs/female, larvae/female, and larvae/group were submitted to Mann-Whitney test using an SPSS package.

Results

No captured fish were older than three years and no females were less than two years (Table 2). In general, females were heavier and longer than males in all age classes. Gonads of captured females were in the first three maturation stages: oogonia (1), primary oocytes (2), or secondary oocytes (3); no mature ova (4) were observed.

Captured females had a mean GSI of 4.49±2.21, which increased to 8.58±2.11 in the freshwater treatment, 10.77±2.65 in the saline treatment, and 9.46±1.70 in the sexually-separated treatment by the end of the 21-day conditioning period. The increase in GSI was corroborated by gonad analysis, which revealed a high percent of immature oocytes as well as a variable number of mature oocytes that were detached from the ovary (ripe ovaries) and ranged 19-27 in the saline groups and 7-11 in the freshwater group. No resorbing oocytes were observed.

Table 2. Weights, total lengths, and condition factors of adult Mediterranean toothcarp (*Aphanius fasciatus*) captured in brackish water channels (means±SD).

	Age in years						
	Males				*Females*		
	1 (n = 5)	*2 (n = 16)*	*3 (n = 9)*	*Mean*	*2 (n = 19)*	*3 (n = 11)*	*Mean*
Weight (g)	1.06±0.10	1.34±0.15	1.46±0.13	1.32±0.27	1.63±0.18	2.13±0.17	1.88±0.34
Length (mm)	44.12±1.30	46.90±1.50	48.02±1.10	46.72±3.20	48.57±2.10	52.33±1.30	50.86±4.30
Condition factor (K)	1.24±0.08	1.30±0.10	1.32±0.11	1.29±0.17	1.40±0.18	1.49±0.15	1.43±0.21

The first courtships of males towards females were observed on the 18th and 16th days of conditioning in the freshwater and saline groups, respectively. Ovulated eggs were retained in the abdominal cavity, as desired, because we wanted to control deposition performance and avoid cannibalism of eggs. About 52%, 69%, and 98% of the manually stripped females released eggs in the freshwater, saline, and sexually-separated groups, respectively (Table 3).

Table 3. Reproduction of adult Mediterranean toothcarp (*Aphanius fasciatus*).

	Fresh	*Saline*	*Saline, separated by sex*
Spawned females (no./total)	8/16; 8/16; 9/16	12/16; 11/16; 10/16	16/16; 16/16; 15/16
Spawned eggs (no./female)	5.9±1.4^{b}	9.9±2.1^{a}	10.5±1.7^{a}
Fertilization rate (%)	58.3±2.4^{b}	72.8±2.6^{a}	74.1±2.8^{a}
Incubation time (degree days)	173±4	176±4	182±3
Hatching rate (%)	97±1	100	100
No. larvae/female	3.33±0.3^{b}	7.21±1.1^{a}	7.78±1.2^{a}
No. larvae/group	28.3±2.3^{c}	79.3±10.2^{b}	120.6±5.5^{a}

Different superscripts in a row indicate significant differences (p<0.05; Mann-Whitney test).

Embryos developed normally in all treatments. Eggs were characterized by 1-3 lipidic drops, a diameter of 1.71±0.096 mm, and a chorion covered with long sticky filaments (Fig. 1).

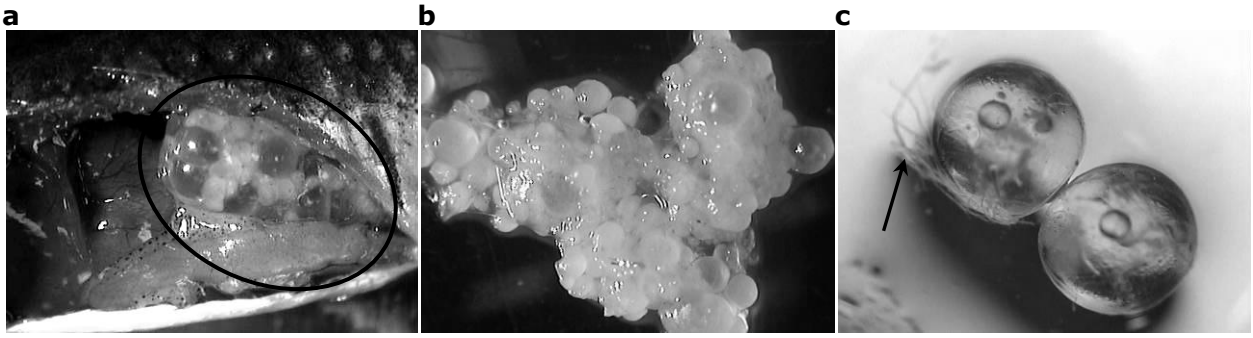

Fig. 1. Mediterranean toothcarp (a,b) ovary with asynchronous maturation and (c) eggs with long sticky filaments.

Discussion

The sizes of the captured animals in the present work conform with previous reports, i.e., 75% of three and four-year-old wild females are 50-52 mm and 60% of over two-year-old wild males are 46-48 mm in length (Leonardos and Sinis, 1999). Gonad analysis showed the presence of more than one maturation stage of oocytes, similar to the typical asynchronous maturation in *Aphanius persicus* (Monsefi et al., 2009). This characteristic is responsible for the lengthy reproductive period in *A. fasciatus* that can last various months (Leonardos and Sinis, 1999). Mature oocytes were also lacking in *A. dispar* where only stage II and III oocytes were observed in females caged in temperatures above

18°C, while mature ova (IV) were observed only in females caged in temperatures above 27°C (Frenkel and Goren, 1999).

Physical factors such as temperature and photoperiod are among the most important parameters for growth and reproduction in fish. At the end of the ecophysiological conditioning period, an increase in GSI as well as the appearance of mature ova were observed in all experimental groups, confirming the efficiency of the applied treatments. The presence of mature oocytes in the freshwater treatment is in discordance with the results of Frenkel and Goren (1999) who observed only primary oocytes in female *A. dispar* maintained in fresh water and a long photoperiod.

The high ovulation response of the females reared separately from the males is probably the consequence of the hormonal treatment (Mordenti et al., 2007), suggesting that induction of ovulation, spermiation, and spawning continue to play an important role in broodstock management (Zohar and Mylonas, 2001).

The lower amount of eggs (absolute fertility) produced by the females raised in fresh water corresponds with the lower number of mature oocytes observed in their gonads. Fertility is reduced in *A. fasciatus* living in low salinity waters (Leonardos and Sinis, 1998). The lower amounts of eggs and mature oocytes obtained from stripping suggest a certain degree of resorption of oocytes in the ovary (atresy) during the reproductive period (Leonardos and Sinis, 1998). Our results are encouraging, in a way, because they were obtained from a single stripping while, similar to other *Aphanius* species, toothcarp females in the wild release a small number of eggs at each spawning. The egg diameter in our study was smaller than the 2.3 mm reported by Leonardos and Sinis (1997) for *A. fasciatus* from Mesolongi lagoon and by Gandolfi et al. (1991) but in agreement with that noted by Monsefi et al. (2009) for *A. persicus*.

Fertilization ratios agree with those reported for cyprinids (Rothbard, 1981). The lower ratio in the freshwater treatment suggests low adaptability of this species to fresh water. The hatching ratios are similar and the required incubation times longer than those (100-125 degree days) obtained by Leonardos and Sinis (1997) for *A. fasciatus* kept in aquaria. Larvae/female values were lower than reported for natural reproduction. The lower reproductive performance of the freshwater females could be ascribed to lower fertility. Our data agree with those obtained in artificial reproduction of *A. iberus* (Oltra and Todolì, 2000). Larvae produced in artificial conditions are not exposed to the dangers of predation and unfavorable environmental variables that may significantly reduce survival rates of deficient, slow-growing, and unstable fish (Frenkel and Goren, 2000).

In conclusion, this study shows that Mediterranean toothcarp reproduction can be controlled in an artificial environment. The absence of mortality in adults during freshwater and marine acclimation and conditioning indicates good domestication and plasticity in reproductive parameters. Further, the validity of the photoperiod, temperature manipulation, and hormonal treatment for induction of maturity and spawning in *A. fasciatus* has been demonstrated. Additionally, marine conditions appear to be favorable for reproduction of this species and the spawning protocol applied here can advance production of seed populations for re-introduction into the wild.

References

Alcaraz C. and E. Garcia-Berthou, 2007. Life history variation of invasive mosquitofish (*Gambusia holbrooki*) along a salinity gradient. *Biol. Conserv.,* 139:83-92.

Caiola N. and A. Sostoa, 2005. Possible reasons for the decline of two native toothcarps in the Iberian Peninsula: evidence of competition with the introduced Eastern mosquitofish. *J. Appl. Ichthyol.*, 21:358-363.

Cimmaruta R., Scialanca F., Luccioli F. and G. Nascetti, 2003. Genetic diversity and environmental stress in Italian populations of the cyprinodont fish *Aphanius fasciatus*. *Ocean. Acta*, 26:101-110.

El Safi S.H., Haridi A.A. and F.M. El Rabaa, 1985. The impact of the exotic fish *Gambusia affinis* (Baird and Girard) on some natural predators of immature mosquitoes. *J. Trop. Med. Hyg.,* 88:175-178.

Ferrito V., Mannino M.C., Pappalardo A.M. and C. Tigano, 2007. Morphological variation among populations of *Aphanius fasciatus* Nardo, 1827 (Teleostei, Cyprinodontidae) from the Mediterranean. *J. Fish Biol.*, 70:1-20.
Frenkel V. and M. Goren, 1997. Some environmental factors affecting the reproduction of *Aphanius dispar* (Rüppell, 1828). *Hydrobiogia*, 347:197-207.
Frenkel V. and M. Goren, 1999. A spawning cage for eliminating predation on larvae of the killifish *Aphanius dispar*. *North Am. J. Aquacult.*, 61:172-174.
Frenkel V. and M. Goren, 2000. Factors affecting growth of killifish, *Aphanius dispar*, a potential biological control of mosquitoes. *Aquaculture,* 184:255-265.
Gandolfi G., Zerunian S., Torricelli P. and A. Marconato, 1991. *I Pesci delle acque interne italiane*. Istituto Poligrafico e Zecca dello Stato, Libreria dello Stato. 617 pp.
Garcia-Berthou E. and R. Moreno-Amich, 1992. Age and growth of an Iberian cyprinodont, *Aphanius iberus* (Cuv. & Val.), in its most northerly population. *J. Fish Biol.*, 40:929-937.
Homski D., Goren M. and A. Gasith, 1994. Comparative evaluation of the larvivorous fish *Gambusia affinis* and *Aphanius dispar* as mosquito control agents. *Hydrobiologia*, 284:137-146.
Leonardos I. and A. Sinis, 1997. Early development of *Aphanius fasciatus* (Nardo, 1827) in the Mesolongi and Etolikon lagoons. pp. 237-240. In: *5th Hellenic Symp. Oceanography Fish*.
Leonardos I. and A. Sinis, 1998. Reproductive strategy of *Aphanius fasciatus* Nardo, 1827 (Pisces: *Cyprinodontidae*) in the Mesolongi and Etolikon lagoons (W. Greece). *Fish Res.*, 35:171-181.
Leonardos I. and A. Sinis, 1999. Population age and sex structure of *Aphanius fasciatus* Nardo, 1827 (Pisces: *Cyprinodontidae*) in the Mesolongi and Etolikon lagoons (W. Greece). *Fish Res.*, 40:227-235.
Leonardos I., Sinis A. and D. Petridis, 1996. Influence of environmental factors on the population dynamics of *Aphanius fasciatus* (Nardo, 1827) (Pisces: *Cyprinodontidae*) in the lagoons Messolongi and Etolikon (W. Greece). *Isr. J. Zool.*, 42:231-249.
Lotan R., 1973. Osmoregulation during adaptation to fresh water in the euryhaline teleost *Aphanius dispar rüppell* (*Cyprinodontidae*, pisces). *J. Comp. Physiol. A: Neurethol. Sens. Neural Behav. Physiol.*, 87:339-349.
Monsefi M., Shiva A.H. and H.R. Esmaeli, 2009. Gonad histology of the Persian tooth-carp *Aphanius persicus* (Jenkis, 1910) (Cyprinodontidae) in Southern Iran. *Turk. J. Zool.*, 33:27-33.
Mordenti O., Scaravelli D., Trentini M., Zaccaroni A. and M. Gamberoni, 2007. Prove di riproduzione artificiale di *Aphanius fasciatus* mediante condizionamento ecofisiologico ed induzione ormonale. pp. 124-125. In: *38° SIBM, S. Margherita Ligure, Italy*.
Oltra R. and R. Todolí, 2000. Reproduction of the endangered killifish *Aphanius iberus* at different salinities. *Environ. Biol. Fishes*, 57:113-115.
Rothbard S., 1981. Induced reproduction in cultivated cyprinids. The common carp and the group of Chinese carps: 1. The technique of induction, spawning and hatching. *Isr. J. Aquacult. - Bamidgeh*, 33:103-121.
Webb C. and J. Joss, 1997. Does predation by the fish *Gambusia holbrooki* (Atheriniformes: Poeciliidae) contribute to declining frog populations? *Austr. Zool.*, 30:316-324.
Wildekamp R.H., Kücük F., Ünlüsayin M. and W.V. Neer, 1999. Species and subspecies of the genus *Aphanius* Nardo 1897 (Pisces: *Cyprinodontidae*) in Turkey. *Turk. J. Zool.*, 23:23-44.
Yildirim O. and A. Karacuha, 2007. A preliminary study on determination of *Aphanius chantrei*'s feeding behaviour on mosquito larvae. *Acta Tropica*, 102:172-175.
Zohar Y. and C.C. Mylonas, 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. *Aquaculture,* 197:99-136.