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Evaluation of Egg Production and Sex Steroid Profiles in the Goldfish *Carassius auratus* During Four Consecutive Seasons

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Abstract: Mature female goldfish (*Carassius auratus*) stripped during the four consecutive seasons (spring, summer, autumn and winter) using simple environmental and hormonal treatments. Plasma levels of sex steroid profiles (17β-estradiol (E_2), 17α-hydroxyprogesterone (17αOHP)), plasma calcium, gonadosomatic index (GSI) as well as a range of indices of ovarian development and potential of eggs production during each season were evaluated. There were no significant differences between absolute fecundity and relative fecundity during the four stripping seasons (P>0.05). Mean oocyte diameter showed a significant increases during the second stripping seasons (summer; 1.32±0.10 mm; P<0.001). Significant higher levels percentage of hatching observed in the spring (96.0±1.82 %) compared with values obtained from other seasons (P<0.001). The maximum one-day embryo survival and five-day larval survival were observed in the summer (94.33±2.94 and 88.80±1.92 % respectively). The changes in E_2, 17α-OH, calcium and GSI were accompanied with the gonadal cycle in goldfish. In the four stripping seasons, plasma E_2 and calcium were elevated during vitellogenesis and decreased steadily one month prior to the initiation of spawning period. The maximum levels of 17α-OH was observed one month before ovulation These findings may be used to available of fry and eggs in freshwater aquarium fish such as goldfish at multiple times of the year without adverse effects on the quality of the eggs and larvae.

Key words: Goldfish · Reproduction · Seasonality · Sex Steroid · Eggs

INTRODUCTION

It is well known that fish reproductive cycle is controlled by the reproductive hormones and environmental factors. Many studies have been performed on the reproductive cycles of seasonal spawning teleosts, particularly in cyprinid and salmonid fishes [1-4]. These seasonal spawners are classified into two categories in terms of the frequency of spawning (multiple and annual spawner). Salmonid fishes are usually annual spawners with an ovary in which oocytes develop synchronously and ovulation occurs only once during the spawning period or as in some cases during their lifetime. In cyprinid fishes, both annual and multiple spawners are observed. Multiple spawning cyprinids ovulate several times within their spawning period and have an ovary of the asynchronous type. The fish reproductive cycle is separated in the growth (gametogenesis) and maturation phase (oocyte maturation and spermiation), both controlled by the reproductive hormones and environmental factors such as season, temperature, water salinity and nutrition quality [5-8]. Environmental factors may be used during gametogenesis to manipulate fish breeding time in order to get viable gametes on a year-round basis [9]. It is also known that environmental factors may modulate steroid conjugation and thus active free steroid concentration [10, 11]. Among environmental factors, temperature is one of the most ubiquitous factors that have various effects on the biological and physiological aspects in many fish species such as the crucial cue in hormonal synthesis, impact directly on steroidogenic structures as a regulating factor, secretion, metabolism, gametogenesis and reproductive fitness [5, 6, 12-14]. Steroid hormones are involved in

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several physiological processes in vertebrates such as growth, digestion, osmoregulation, immune function and reproduction [15]. Gonadal development in female teleosts is known to be dependent on several steroid hormones which are produced by the ovarian follicle in response to stimulation by pituitary gonadotropins (GTHI and GTHII). Among these steroids, estrogens (generally estradiol-17β, E2) have proven a useful indirect indicator of vitellogenesis [16]. Plasma levels of 17-hydroxyprogesterone (17P) (a potential precursor for most other ovarian steroids) are raised in concert with elevated 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) levels around the time of FOM and ovulation [17, 18]. The quality of the produced eggs can be influenced by various parameters which can change during the spawning season, such as water temperature, feed quality, handling stress, endocrine status of females during oogenesis, physicochemical water parameters, broodstock management, etc. [19-23]. Some of the egg quality markers employed in different fishes include fecundity, fertilization success, morphological characteristics (cleavage pattern or distribution of oil globules), eyeing % (in salmonids) and hatching success [19, 22, 24]. Such information will assist commercial facilities in organizing and, if possible, optimizing egg and fry production. The goldfish is one of the multiple spawning cyprinid fishes which mature in the spring and spawn several times under natural conditions. If goldfish kept under 12°C, healthy yolk-laden oocytes can be maintained for several months without ovulation or degeneration. In turn, ovulation can be easily induced by raising the water temperature from 12°C to 20°C [25, 26]. Numerous characteristics of the goldfish (*Carassius auratus*) make this species an excellent model for understanding neuroendocrine signaling, the regulation of reproduction in vertebrates and various experiments such as zoology, cell biology, immunology, toxicology and medicine [27-31]. The alteration of spawning time by environmental manipulation has the potential to meet the demands for all-year-round mass production of eggs and larvae of goldfish especially in commercial aquarium operation. This study was designed to induce spawning female of goldfish by simple environmental and hormonal treatments and evaluation of egg production, sex steroid profiles (17β-estradiol, 17α-hydroxyprogesterone) and plasma calcium during the four consecutive seasons (spring, summer, autumn and winter).

MATERIALS AND METHODS

Fish and Principles for Stimulating Ovulation: Four-hundred and sixty-five sexually mature female (14.80±1.45cm standard length; 45.35 ±1.06 g body weight) and male (9.93±1.11cm standard length; 38.01 ±13.28 g body weight) goldfish *Carassius auratus* were obtained from a commercial dealer in late March, 2010 (the fish had never spawned before). The fish were kept in fiberglass tanks (cylindrical, 1 m in diameter and 0.5 m in depth) with aerated and dechlorinated tap water (pH 7.89±0.9; dissolved oxygen, 7.8±0.5 mg l^{-1}; and total hardness, 295±10 mg l^{-1} as $CaCO_3$) at 16±1°C on a 10-h dark and 14-h light (10D: 14L) natural photoperiod on the campus of Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran, until the start of the experiments. At the beginning of their natural spawning season (April; temperature 22 °C; and 8D: 16L), mature male goldfish were identified by the presence of expressible milt and tubercles on the pectoral fins or the opercula and female with large and soft bellies. Females were selected for induced ovulation based on their ovarian development. The state of the female gonad development was evaluated by biopsies. Biopsies were made on 8 females in order to examine the germinal vesicle position and check for ripeness. Approximately 20 oocytes were taken by cannulation, soaked for 10 min. in a clearing solution of ethanol: formalin: acetic acid, 6:3:1 solution (v/v) [32] and examined using a stereo microscope fitted with a calibrated ocular micrometer. Females with oocytes in which the nucleus was undergoing migration (ca. 0.9-l.0 mm diameter, sampled by cannulation) selected for induction ovulation. Then, female and male received an intramuscular injection of human chorionic gonadotropin (hCG) (Sigma Chemical, St. Louis, MO, 1 IU hCG g^{-1} BW) suspended in Ringer's solution (150 mM NaCl, 3.0 mM KCl, 3.5 mM $MgCl_2$, 5.0 mM $CaCl_2$, 10 mM HEPES, pH 7.4) to induce ovulation and spermiation [33] and were stripped 8-12 h later (at 22 °C). Twenty-five females were used as egg donors, the rest of the females were completely stripped out and used for commercial hatching and then the fish were maintained for the other season. After the first stripping season (spring), fish (mixed-sex) were kept under natural photoperiod and ambient indoor temperature and fed with Commercial "Goldfish food" (Energy, Thailand) pelletized feed, at a rate of 3 % of their total body weight per day until the next seasons. Individual fish marked by fin clipping. Early each season (summer, autumn and winter) male and female goldfish were held separately (as single sex) in groups of 10-20 fish

in each 65 l glass aquarium; then, a chilling period at 10 °C by decreasing water temperature in steps of maximal 2°C per day, was applied. Simultaneously, the photoperiod was changed to 16D: 8L by decreasing 15min. of light per day. These conditions were held constant for a period of 10 days. At the end of the chilling period, to induce spermiation and ovulation, water temperature raised to 22°C (except for summer) in steps of maximal 2 °C per day [25] and photoperiod to 8D: 16L by increasing 15min. light per day. These conditions were held constant for a period of two weeks [18]. Photoperiod was controlled with a 20-W fluorescent lamp connected to an electric timer and water temperature with a commercial aquarium thermostat and heater. To determine whether ovulation had occurred, the fish were checked every morning. When the first external signs of female maturation were observed (females started showing larger and softer abdomens), they randomly selected for more detailed determination of the stage of gonadal development by biopsies as previously mentioned. All males were spermiating, releasing sperm by abdominal pressure and the sperm was considered to be of good quality, having >80 % motile spermatozoa upon activation with fresh water. Then, females and males that appeared ripe received an intramuscular injection of hCG (1 IU hCG g^{-1} BW, above-mentioned) to induce ovulation and spermiation and were stripped 8-12 h later (at 22 °C).

Gamete Collection, the Percentage of Hatching Rate and Fecundity: Eggs and sperm samples were collected by manual stripping. The eggs from each ovulated female were dry fertilized with the milt of 3 males in fertilization solution (tap water containing 0.4 % urea and 0.5 % NaCl), then changed to tap water 1-2 h after fertilization. Hatching was observed after 108 hours at 22°C. Approximately 500 eggs were incubated in plastic pots (in replicates) to determine the percentage of hatching. Later during incubation of eggs, the dead eggs and hatched fry were counted according to method described by Linhart *et al*. [34].

$$H_r = (H_l/E_t) \times 100$$

(H_r): The percentage of hatching.
(H_l) The number of hatched larvae.
(E_t): The total number of eggs placed in the plastic pot.

Relative fecundity (eggs g^{-1} BW) and absolute fecundity per female (eggs $spawn^{-1}$) within the each reproductive season (n=10) were calculated. The absolute fecundity per female was calculated as the sum of the eggs obtained from all the strip spawns within the each reproductive season using the calculation:

Number of eggs = dry weight × 1100.13 eggs per g^{-1}.

Evaluation of Oocyte Diameter and Larval Survival: To estimate oocyte diameter, approximately 100 unfertilized oocytes were measured with the use of a microscope fitted with an ocular micrometer, from samples fixed in 4 % formalin. One-day and five-day larval survival were assessed by counting the number of larvae one and five days after placing 100 newly-hatched goldfish larvae in 1500 ml beakers (in replicates), using the calculation: The number of live larvae at day_i/the number of larvae initially placed in beakers.

Measurement of Plasma Sex Steroid, Calcium (As an Index of Vitellogenin) and Gonadosomatic Index: In the each stripping season, plasma samples were taken from 11 female on day 0 (spawning day), 1, 5, 15, 30, 45, 60, 75 and 85 (day after spawning). Fish were anaesthetized with clove oil (75-115 ppm). For sampling, goldfish were weighed and measured and blood samples were withdrawn by puncture caudal vein into a 1 ml sterile plastic heparinized syringe (27-gauge needle) and centrifuged for 10 min at 3,000 rpm. Plasma was stored at -20 °C until steroid extraction and analysis of levels estradiol-17β and 17-hydroxyprogesterone using a protocol previously validated for goldfish plasma samples [35]. Body and gonad weights were recorded for each animal to assess the variation in GSI [(gonad weight/body weight) × 100 %]. Plasma calcium (mg/dl) was measured by spectrophotometer (WPA-S2000-UV/VIS, Cambridge-UK) using commercial kits (Pars Azmun Co. Ltd, Tehran, Iran).

Data Analysis: All data were represented as mean ± standard error of the mean (S.E.M.) and subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significantly $p<0.05$.

RESULTS

Changes in the absolute fecundity, relative fecundity, oocyte diameter, percentage of hatching, one-day embryo survival and five-day larval survival during the four stripping seasons are illustrated in Fig. 1. As shown in Fig. 1, significant differences were found between

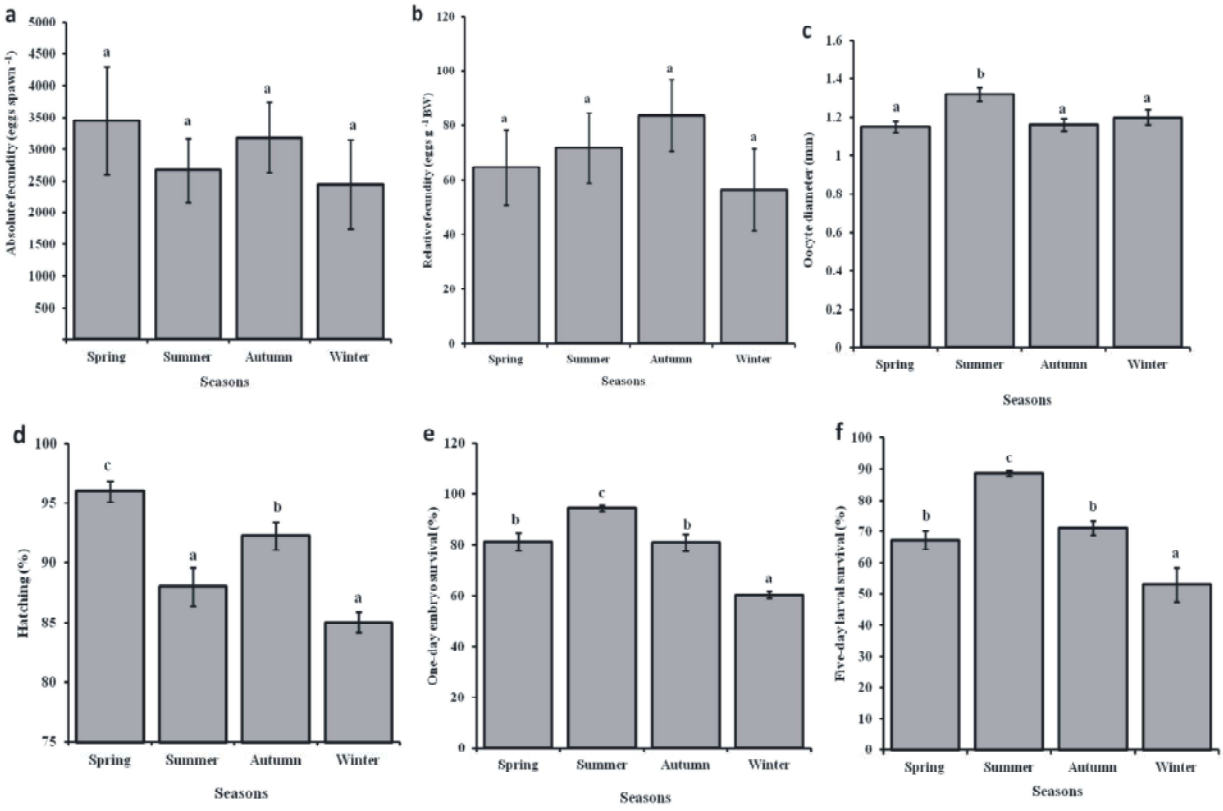

Fig. 1: Mean absolute fecundity (a), relative fecundity (b), egg diameter (c), percentage of hatching (d), one-day embryo survival (e) and five-day larval survival (f) in female goldfish (*Carassius auratus*) stripped during four consecutive seasons (spring, summer, autumn and winter).
Bars are means ± SE.-Differences in means are represented by different letters arrow bars.

one-day embryo survival and five-day larval survival among the four stripping seasons (P<0.001). The maximum one-day embryo survival and five-day larval survival were observed during the second stripping season (summer; 94.33±2.94 and 88.80±1.92% respectively; Fig. 1e and f). Mean oocyte diameter showed significant differences during the four stripping seasons (P<0.001), as the largest oocyte diameter was recorded during the second stripping season (1.32±0.10 mm; Fig. 1c). Although, percentage of hatching was relatively high during the four stripping seasons, but significant higher levels percentage of hatching was observed during the first spawning season (spring, 96.0±1.82; P<0.001; Fig. 1d). No significant differences were observed between absolute fecundity and relative fecundity during the four stripping seasons (P>0.05; Fig. 1a and b).

Changes in plasma concentration of E_2, 17α-OH, calcium and GSI are illustrated in Fig. 2. In all series of experiments different patterns of E_2, 17α-OH, calcium and GSI were observed during the four stripping seasons, but the different patterns in steroids hormones, calcium and GSI were accompanied with the gonadal cycle in goldfish. In the four stripping seasons, plasma E_2 and calcium were elevated during vitellogenesis (forty-five day after ovulation) and decreased steadily one month prior to the initiation of spawning period (Fig. 1b and d). However, the minimum level of plasma E_2 observed one and five day after ovulation. During the first and second stripping seasons (spring and summer) a sudden decrease in plasma calcium was observed five days postspawning. Hormonal concentration of 17α-OH was low after ovulation but it increased rapidly as spawning came approached, reaching its maximum one month before ovulation, however, there were no statically significant differences between the four striping seasons (P>0.05; Fig. 1c). The peak of GSI during ovulation in the spring (14.96±0.39 %) when most gonadal development took place, was statistically significant higher compared with other seasons (P<0.001; Fig. 1a). In the four stripping seasons, values of 17α-OH and GSI declined significantly one day after stripping, where they remained low for further fifteen days.

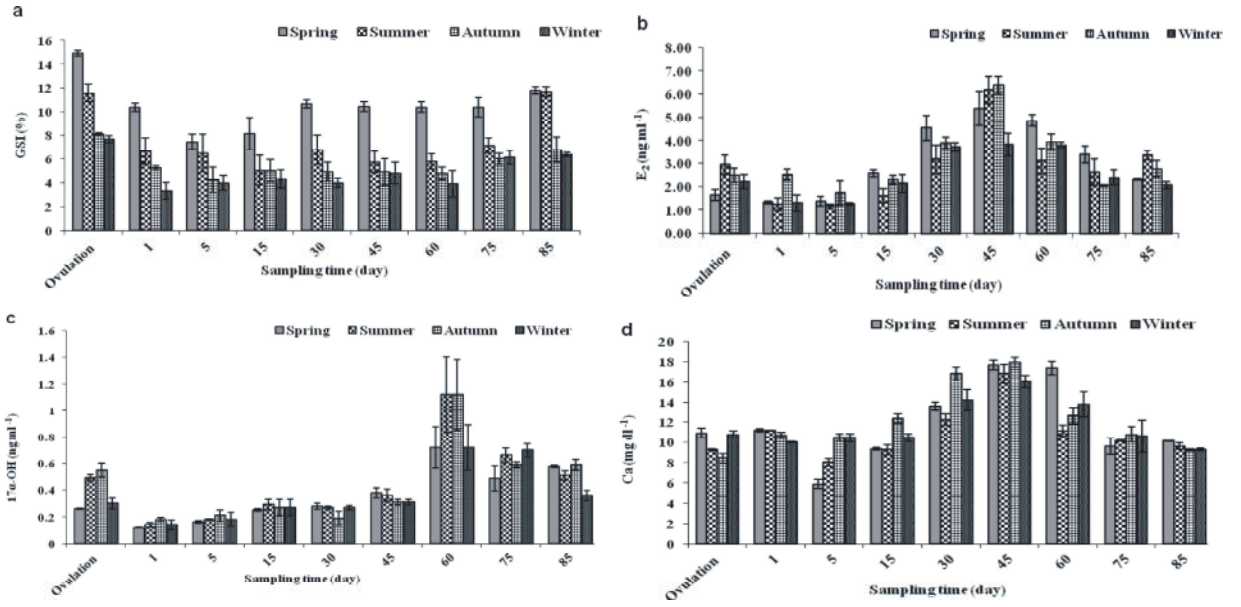

Fig. 2: Mean gonadosomatic index (a), 17β-estradiol (b), 17-hydroxyprogesterone (c) and calcium (d) in female goldfish (*Carassius auratus*) stripped during four consecutive seasons (spring, summer, autumn and winter). Bars are means±SE

DISCUSSION

Our results demonstrate that mature female goldfish could be successfully induced to spawn several times during a year by using relatively simple environmental and hormonal treatments. In this research values of absolute fecundity and relative fecundity were not differences during the four consecutive seasons. It is possible that the number of eggs produced per spawning may relate to the proportion of oocyte developmental stages remaining in the ovary after the previous spawn and may therefore be largely dependent on spawning history [36]. In the present study, oocyte diameter ranged between 1.15 and 1.32 mm during the four stripping seasons, even though the largest oocyte diameter was recorded during the second breeding season (summer). The increased of oocyte diameter during the summer concomitant with increase in the one-day embryo survival and five-day larval survival during this stripping season. A reduction in egg diameter and therefore yolk content in fishes with asynchronous ovarian development, may result in a reduction in the survival of the embryo and larvae [37]. Variations in egg quality parameters between spawning seasons are common [38] and have been attributed to both genetic and environmental factors, such as growth [39], temperature [40], the sex ratio of the broodstocks [41] and the feeding level of females [42]. Although, percentage of hatching was relatively high during the four seasons, but significant higher levels percentage of hatching observed during the first stripping season compared with values obtained from other seasons. In contrast to our result, Watanabe *et al*. [43] reported induced spawning by manipulation of photoperiod and temperature during different seasons of the year was not effect on gamete quality, fertilization and hatching success in southern flounder, *Paralichthys lethostigma*. Also, Bromage *et al*. [44] reported that quality of the gametes under advanced or delayed spawning was no different from that achieved under ambient conditions in rainbow trout. At the population level, observation of female and male goldfish at different seasons of the year showed the absence of sexual synchronization: all ovarian stages could be found all the year round and steroid profiles displayed high variability among individuals in the same ovarian stage. In the present study in all series of experiments the changes in E_2, 17α-OH, calcium and GSI were accompanied with the gonadal cycle in goldfish. During the four stripping seasons, the maximum levels of plasma E_2 and calcium were observed during vitellogenesis with a decrease in plasma E_2 one day after ovulation and lasted for further five days. This suggests that in goldfish, developing oocytes are recruited into vitellogenesis very soon after spawning and that vitellogenesis occurs very rapidly. Profiles of E_2 in goldfish are generally similar to that of annual spawners such as rainbow trout [45], tilapia.

Oreochromis niloticus [36] and Japanese sardine, *Sardinops melanostictus* [46]. In all of these fish, E2 is elevated during vitellogenesis but remains at relatively low levels thereafter until the next spawning phase. Similarly, Aida [18] reported in goldfish Plasma E_2 increased gradually and was maintained at moderate levels during the ovulation process. After ovulation, E_2 values decreased slowly. Plasma calcium, which can be rapidly measured by spectrophotometer, is a practical indicator of ovarian vitellogenesis in fishes. In the present research, during the four stripping seasons, serum calcium decreased one month before spawning with a sudden decrease for the first striping and second stripping seasons, five day postspawning. It is concluded that the total calcium in the serum increases gradually with the advance of ovarian maturation and maintains at moderate levels during the ovulation process. Alteration in serum calcium during the four stripping seasons may be partly due to the difference in the ovarian maturation. Bjornsson and Haux [47] reported that the increase in total plasma calcium is due to the appearance of the calcium-containing yolk protein precursor vitellogenin in plasma. Serum calcium levels fell during the spawning and postspawning in freshwater murrel, *Channa punctatus* [48]. In this research the maximum level of 17α-OH was observed one month before ovulation (at the end of vitellogenesis), however, there were no statically significant differences between the four striping seasons. Similarly, Unal *et al*. [49] observed 17α-OH level increased gradually during vitellogenesis and reached its maximum value at the end of vitellogenesis in *Chalcalburnus tarichi.* In goldfish, 17α-OH may play a role as an indirect inducer on oocyte maturation (as a precursor of 17, 20βP). GSI is a useful index for monitoring the progression of the reproductive cycle of fishes, because the gonadal mass attained during maturation makes up a high percentage of their body biomass, especially in females. In the present study, GSI increased at ovulation time during the four stripping seasons and declined significantly one day after stripping, even though a maximum GSI level at ovulation time was recorded during the first breeding season. The differences observed in the in the sex steroid profiles and egg quality during the four stripping seasons in the present study, may be attributed to the rearing temperature during pre-spawning at the each season, heterogeneity of the maturation stage between fishes, postseasonal stage females and stripping frequency. Temperature modulation of gonadal sex steroid synthesis and secretion may influence the feedback loop to the brain-pituitary axis resulting in decreased secretion of gonadotropins. Temperature has been shown to impact hormone synthesis, secretion, metabolism and the rate of clearance [50]. In many fish species, temperature is considered as the crucial cue in gonadal development and out of season induced spawning factor. Also, it has been proposed that temperature could act directly on steroidogenic structures as a regulating factor [12]. In common carp, Manning and Kime [51] reported that a high variability between individuals, even at the same ovarian stage, concerning steroid production at different temperatures. On the other hand, Kestemont [52] observed under constant temperature and photoperiod conditions (20 °C, 12L: 12D), gonadal development was variable in *Gobio gobio*. Ovarian stimulation in goldfish was reported by long photoperiod and warm temperature during winter and spring [53] and autumn [54]. When the water temperature is raised to about 20°C or higher, or the fish are transferred from cold to warm water, ovulation will occur in sexually mature goldfish within a few days [55].

In summary, this study revealed that under laboratory photo-thermal conditions, goldfish female are able to produce viable eggs during almost all the year. The information obtained from such studies has an important place in applied research, particularly in artificially inducing goldfish in a commercial aquarium operation.

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