Aquaculture 518 (2020) 734866

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

# Out-of-season artificial reproduction techniques of cultured female tongue sole (*Cynoglossus semilaevis*): Broodstock management, administration methods of hormone therapy and artificial fertilization

Yu Song[a,1], Weiwei Zheng[a,1], Mengqian Zhang[a], Xiangming Cheng[b], Jiayu Cheng[b], Wenbin Wang[c], Jie Zhang[c], Yangzhen Li[a,b,*]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS), Laboratory for Marine Fisheries Science and Food Production Processes, Pilot National Laboratory for Marine Science and Technology, Qingdao, Shandong 266071, China
[b] Engineering and Technology Center for Flatfish Aquaculture of Tangshan, Tangshan Weizhuo Aquaculture Co., Ltd., Tangshan, Hebei 063202, China
[c] Agriculture and Rural Affairs Bureau of Caofeidian District, Tangshan, Hebei 063299, China

ARTICLE INFO

*Keywords:*
Broodstock management
Artificial fertilization
Hormonal induction
Reproduction
Tongue sole
*Cynoglossus semilaevis*

ABSTRACT

The present study aimed at the development of an effective and efficient artificial reproduction technique for cultured female tongue sole (*Cynoglossus semilaevis*) in out-of-season. After about four-month enriching cultivation of broodstocks, a series of experiments were conducted to develop a protocol for the hormonal induction of ovulation and artificial fertilization. The results showed that single injection of combination of luteinizing hormone releasing hormone analogue (LHRH-A3), human chorionic gonadotropin (HCG) and domperidone (DOM) is effective to induce ovulation at different final maturation stages (i.e., stage IV and V). The optimal single injection dosages of combined hormones were (0.15 μg LHRH-A3 + 4 IU HCG + 2 mg DOM) / kg BW and (0.05 μg LHRH-A3 + 2 IU HCG + 1 mg DOM) / kg BW for females at stage IV and V respectively. At the optimized dose of hormones, the fertilization and hatching rates were 44.6% and 80.4%, 30.4% and 63.4% for stage IV and V respectively. The lapsed time to ovulation was very predictable and ranged from 35.5 to 39.5 h post injection. Ovulated eggs in ovarian cavity should be stripped within 15 min in order to obtain the highest fertilization and hatching rates, otherwise the values decreased significantly thereafter. These researches are expected to provide improved implementation of artificial reproduction techniques for the production of fertilized eggs in the commercial aquaculture of tongue sole.

## 1. Introduction

In the last decades, fish aquaculture is the fastest growing food production industry in the world, and in 2014, a total of 49.8 million tonnes of finfish were produced, which is about 70% of the world's aquaculture production (FAO, 2016). One of the prerequisites for this achievement is the development of reproductive technologies (including broodstock management and induced-breeding) of fish in captivity, which allows famers to acquire large number of high-quality seed (i.e., eggs and sperm or fish larvae) for grow-out of the marketable product. In the 80's of the last century, a developed technology, which is known as Linpe method (Lin et al., 1988; Peter et al., 1988), revolutionized fish reproduction in captivity by injecting GnRHa (or analogues) in combination with domperidone or pimozide (a dopamine antagonist). Nowadays, there are plenty of aquaculture fish species that can be induced successfully by hormonal therapies (based on Linpe method) for gamete maturation (i.e., ovulation and spermiation). Hormonal manipulations can be used as very useful management tools to enhance the efficiency of ovum and sperm production, facilitate hatchery operations, and could be enabled for mass spawning or artificial fertilization for genetic selection programs (Mylonas et al., 2010).

Tongue sole (*Cynoglossus semilaevis*) is a commercially important indigenous marine flatfish which is widely cultured in China due to delicacy and rarity (Hu et al., 2014; Li et al., 2019). Recently, the wild resource has reduced gradually due to overfishing. It is now one of the most expensive farmed fish species on the market in China. Because of the high market price, there has been an increasing interest from private companies to invest and establish aquaculture of tongue sole on

* Corresponding author at: Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS), No. 106 Nanjing RD, Qingdao 266071, China.
*E-mail address:* liyz@ysfri.ac.cn (Y. Li).
[1] These authors contribute equally to this work.

https://doi.org/10.1016/j.aquaculture.2019.734866
Received 23 September 2019; Received in revised form 13 December 2019; Accepted 13 December 2019
Available online 14 December 2019
0044-8486/ 

coastal areas of China in the last few years. However, there are several serious problems in tongue sole aquaculture in China. One of the primary problems associated with cultured tongue sole is the reproductive dysfunctions detected in captivity which may due to that the captive environment could not provide appropriate environmental conditions for complete maturation. In many aquaculture fish species, the reproductive dysfunction is an important limiting factor for the successful mass production of high-quality fertilized eggs and juveniles. When fish are reared in captivity, there are many different types of reproductive dysfunctions (reviewed by Mylonas et al., 2010). However, the reasons of underlying the problems of cultured tongue sole are still unclear. In sole species, it has been suggested that these problems could be related to environmental factors, the lack of courtship of cultured broodstock and/or inadequate diet (Howell et al., 2009). Spontaneous spawns of tongue sole in captivity are scarce and mostly unfertilized (Liu et al., 2006). These issues mentioned above are similar to those observed in Senegalese sole (*Solea senegalensis*) (Guzmán et al., 2008; Rasines et al., 2012).

Currently, the hormone inducing reproduction technologies of tongue sole have not been well developed and in most cases the artificial reproduction of fertilized eggs and fingerlings took place in autumn (natural spawning season, mainly September and October). Farmers have to suffer a relative lower water temperature in late autumn and winter which result in higher maintenance cost in hatcheries. Moreover, the nursery of tongue sole may not successful (with high and variable mortality of larvae and juvenile fish at later stages) due to insufficient quality of eggs (average fertilization rate ranging from 10%–30%) and fluctuant water temperatures in late autumn and winter. Thus, large scale production of tongue sole larvae in hatcheries is severely limited. Therefore, for the sake of supplying the juveniles all year round, expanding of commercial tongue sole aquaculture and satisfying the market requirements, methods and techniques of reproduction in out-of-season are needed.

From a business perspective, for obtaining high viability eggs, the immediate priority is to develop the reproductive techniques. One of the most important steps is the determination of administration methods of hormonal therapy for successful fertilization. Hormones such as human chorionic gonadotropin (HCG), gonadotrophin releasing hormone agonist (GnRHa) and its analogues (e.g., luteinizing hormone releasing hormone A3, LHRH-A3) and dopamine antagonist (e.g., domperidone, DOM) have been approved for commercial utilization in commercial aquaculture world widely (Ranjan et al., 2018; Żarski et al., 2017; Mylonas et al., 2015; Mylonas et al., 2013; Rasines et al., 2012, 2013; Black and Black, 2013; Guzmán et al., 2011b; Mylonas et al., 2010; Kahkesh et al., 2010; Robert and James, 2007; Nayak et al., 2001).

Therefore, the objectives of the present work were to 1) develop a practicable broodstock management regime in out-of-season, 2) determine optimized administration methods of hormonal induction of ovulation and artificial fertilization, and 3) investigate the time to ovulation after hormonal treatment and optimal stripping time for obtaining high quality of eggs. This information is expected to provide improved implementation of artificial reproduction techniques for the production of fertilized eggs in the commercial aquaculture of tongue sole throughout the year.

## 2. Materials and methods

### 2.1. Broodstock and maintenance

Cultured tongue sole was obtained and maintained in Tangshan Weizhuo Aquaculture Co., Ltd. In this species, males (6–10 months) mature earlier than females (18–24 months), and in the same population, when females matured males are too old to produce high-quality milt. So, in this study females and males were not from the same batch. 1000 females with top 20% growth rate (produced in June 2017, average body weight, BW, was 1.3 kg) at age of about 18 months and 4000 males (produced in June 2018, average BW was 55.0 g) at age of about 6 months were selected for using in December 2018, before which they were all reared under commercial production aquaculture environment (in nearly total darkness and water temperature was controlled at 19–23 °C). Selected females were tagged with passive integrated transponder tags (PIT-tags, Qingdao Starfish Instruments Co., Ltd) on the dorsal musculature for later monitoring. The pseudo-males in male population were identified by using sex specific AFLP markers and eliminated according to methods as described by Chen et al. (2007). All the fish were maintained in six indoor tanks (6 × 6 × 0.8-m, L × W × H) with flow-through water exchange (500% per day) and constant aeration. The sex ratio was 4♂:1♀. The water temperature was kept at 23.5 ± 0.5 °C, the salinity was 26‰ – 30‰, the dissolved oxygen was 6–8 mg/L, and the photoperiod was controlled artificially at 12 L:12D during the experiment. The luminance was about 80 lx on the water surface. Fish were fed twice daily with a commercial dry pellet feed (51% crude protein, 12% crude lipid and 17% crude ash) (Santong Chubu Feed (Shandong) Co., Ltd., China) five days a week and live polychaete *Nereis succinea* and clam meat two days a week with an amount of about 1.5% of their average live weight.

### 2.2. Ovarian development examination

Because acquisition of ovarian biopsies through intraovarian cannulation in tongue sole is often very difficult and stressful to the fish, in most cases the stage of gonad development of females was determined by the estimation of abdominal swelling and the ratio of gonad length to body length (Liu et al., 2006). In this study, we made some modifications and refined the five different stages of ovarian development: stage 0 corresponded to externally undetectable development; stage I was that when the ovary could only be detected by touching the abdominal region of females, (gonad length, GL) / (body length, BL) < 0.2; stages II and III corresponded to two levels (initial and intermediate) of externally visible abdominal swelling, GL / BL was 0.2–0.3 and 0.3–0.4, respectively; and stage IV was that for optimal ovary development, characterized by high swelling and GL / BL ranging from 0.4 to 0.5; stage V was when the ovary was considerable swelling and GL / BL > 0.5.

Maturation stages were examined regularly by measuring the ratio of gonad length to body length and estimated by external confirmation of abdominal swelling. The ovaries became plump in February 2019 and about 65% individuals reached stage IV in early April. By then the average weight of females was about 2.0 kg, while the average weight of males was about 100 g. Then females with optimal ovary development (stage IV) were selected for spawning induction.

### 2.3. Hormone therapy administration

#### 2.3.1. Optimal single and combined dosage of LHRH-A3 and HCG for females at stage IV

Initially, for determining the effectiveness and optimal dosage of two kinds of hormones, two preliminary trials (T1 and T2) were performed by single injection with LHRH-A3 and HCG (Ningbo Second Hormone Factory, China) respectively by using females with ovarian development at stage IV, each with seven gradients (sub-trials, $n = 10$) of dosage. According to preliminary results (see Section 3.1), subsequent trials (T3, with three sub-trials, $n = 20$) were performed with injection of combination of LHRH-A3 and HCG. The last trial (T4, $n = 20$) was taken for testing the effectiveness of DOM (Ningbo Second Hormone Factory, China) which was added to the optimal combination of LHRH-A3 and HCG. The details of administration of hormones are shown in Table 1.

#### 2.3.2. Alternative administration approach for females at stage V

In reproduction season, some female individuals will undergo stage

**Table 1**
Hormone and dosage in each trial and sub-trial.

| Trial | | Hormone and dosage (/ kg BW) |
|---|---|---|
| T1 | T1–1 | 0 μg LHRH-A3 |
| | T1–2 | 0.2 μg LHRH-A3 |
| | T1–3 | 0.4 μg LHRH-A3 |
| | T1–4 | 0.6 μg LHRH-A3 |
| | T1–5 | 0.8 μg LHRH-A3 |
| | T1–6 | 1.0 μg LHRH-A3 |
| | T1–7 | 1.2 μg LHRH-A3 |
| T2 | T2–1 | 0 IU HCG |
| | T2–2 | 2 IU HCG |
| | T2–3 | 4 IU HCG |
| | T2–4 | 6 IU HCG |
| | T2–5 | 8 IU HCG |
| | T2–6 | 10 IU HCG |
| | T2–7 | 12 IU HCG |
| T3 | T3–1 | 0.15 μg LHRH-A3 + 4 IU HCG |
| | T3–2 | 0.30 μg LHRH-A3 + 3 IU HCG |
| | T3–3 | 0.45 μg LHRH-A3 + 2 IU HCG |
| T4 | | 0.15 μg LHRH-A3 + 4 IU HCG + 2 mg DOM |
| T5 | T5–1 | 0.05 μg LHRH-A3 + 1 IU HCG + 1 mg DOM |
| | T5–2 | 0.05 μg LHRH-A3 + 2 IU HCG + 1 mg DOM |
| | T5–3 | 0.10 μg LHRH-A3 + 1 IU HCG + 1 mg DOM |
| | T5–4 | 0.10 μg LHRH-A3 + 2 IU HCG + 1 mg DOM |
| | T5–5 | 0.15 μg LHRH-A3 + 4 IU HCG + 2 mg DOM |

V inevitably before treatment. Based on our experience, when GL / BL > 0.5 (stage V), dosage of hormones should be reduced or re-adjusted. Therefore, for females with ovarian development at stage V, the dosages of hormones were tested alternatively (T5 involving 5 sub-trials, $n = 10$) (Table 1 and Table 6), the dosage in T4 was set as a control group (T5–5). Hormones were dissolved in saline (0.9% NaCl) and given in a single dose by intramuscular injection (using 5.0 mL disposable syringe) at 17:00. Before injection, BW, BL and GL were measured and recorded individually. For manipulation, the females were anesthetized with MS-222 at a dose of 120 mg/L. The culture conditions (food, light, air, temperature, female-male proportion) were maintained as described above. Females were examined hourly in preliminary trials and 34 h post injection in other trials by gentle abdominal massage. When ovulation was detected, eggs were gently stripped from the ovarian cavity. Female mortality was recorded within 48 h post injection. Elapsed hours between injection and ovulation was defined as time of ovulation.

### 2.4. Protocol of artificial fertilization

Eggs from each female were collected into a dry beaker (1 L) separately, and the sperms of each male were gently stripped and collected in a 1.0 mL dry straw, avoiding contamination by feces, urine and seawater in the both manipulations. Then artificial fertilization was conducted immediately with fresh sperms (approximately 50–100 μL sperms per 100 mL eggs) by adding about 500 mL pond seawater. Considering the limited volume of milt and avoiding quality differences, a pool of sperm from 3 to 5 males was used for fertilization of eggs from each female. Eggs and milt were gently stirred for 1–2 min and let stand for 3 min. The volume of total and floating eggs was measured and recorded individually. Then the sinking eggs (overripe eggs) were discarded and the floating eggs were incubated in a 0.6 $m^3$ hatchery cage (80 mesh size, 165 μm) supplied with constant aeration and flow-through water supply (flow rate of 20 L $min^{-1}$) at 23.5 ± 0.5 °C. During the incubation, sinking eggs were removed by a siphon every 12 h.

### 2.5. Parameters measurement and calculation

To determine the number of eggs per mL, 1.0 mL aliquots (triplicates) were taken from random three batches of eggs using a 1 mL micropipette and placed on a counting plate with sea water; the eggs were then counted under the binocular. Finally, the number of per mL eggs was determined as 865 ± 15. After 60 min of incubation of each batch of fertilized eggs (from each female), 0.1 mL fertilized eggs (with triplicates) were randomly sampled for examining fertilization rate under a binocular. The fertilized eggs were then placed in 6 L plastic incubators with constant aeration. After about 36–38 h of incubation, hatching rates were determined for each incubator. Parameters were calculated as follows:

relative fecundity
= volume of eggs (mL) × 865 $mL^{-1}$/BW of females (kg), $10^3$/kg;

ovulation rate
= (number of ovulated females/total number of induced females) × 100;

egg buoyancy = (mL of floating eggs/total mL of eggs) × 100;

fertilization rate
= (number of eggs with blastomeres/total number of eggs) × 100;

hatching rate = (number of hatched larvae/fertilized eggs) × 100.

### 2.6. Timing of ovulation and optimal stripping

For detecting the accurate time of ovulation and optimizing time of stripping, 20 females were injected with 0.15 μg / kg body weight of LHRH-A3 + 4 IU / kg body weight of HCG + 2 mg / kg body weight of DOM at 17:00 (the same to T4). The water and conditions were the same as described above. 34 h post injection, fishes were checked for ovulation every half an hour by applying abdominal pressure. When ovulation was detected (time 0 min post-ovulation, mpo), a small portion of the ovulated eggs was gently stripped from the ovarian cavity. Then eggs were stripped for another 4 batches, at 15, 30, 45 and 60 mpo respectively. Each batch of eggs, from each female, was immediately fertilized using sperm stripped from 3 to 5 males. Fertilization and hatching rates were determined for each artificial fertilization trial. Only individuals ovulated before 38 h were taken into account.

### 2.7. Statistical analysis

Prior to statistical analyses, all data were checked for normality using a Shapiro-Wilk normality test and homogeneity of variance using a Levene's Test, when necessary, they were transformed to square-root arcsine values. Statistical analysis was performed using one-way analysis of variance (ANOVA) to find significant differences in various parameters between different trials. The possible relationships between the parameters measured were analyzed using a Pearson correlation test. Data are presented as mean ± standard error (SE). Statistical analyses were carried out using R software (Version 3.6.1) with statistical significance set at $P$-value < .05.

## 3. Results

### 3.1. Hormone administration therapy

The preliminary experiment (i.e. T1 and T2 each with 7 sub-trials) results were showed in Table 2 and Table 3. According to these results, LHRH-A3 and HCG are both effective in inducing ovulation, and it seems that the optimal dose was 0.6 μg and 6 IU per kg BW for LHRH-A3 and HCG respectively. To some extent, the HCG treatment showed higher potency than LHRH-A3 protocols, as showed by relative higher buoyancy and lower mortality of parent fish. However, at optimal single injection dosage of LHRH-A3 (i.e. 0.6 μg / kg BW) and HCG (i.e.

**Table 2**
Dose of LHRH-A3 and main production parameters in T1 with 7 sub-trials ($n$ = 10). Values in the same row with different superscript letters are significantly different ($P$ < .05). Body weight of females, egg buoyancy, fertilization rate and time of ovulation was expressed as mean ± standard error.

| | T1–1 | T1–2 | T1–3 | T1–4 | T1–5 | T1–6 | T1–7 |
|---|---|---|---|---|---|---|---|
| Dose of LHRH-A3 (μg / kg BW) | 0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 1.2 |
| Body weight of females (kg) | 2.05 ± 0.06 | 2.05 ± 0.08 | 2.04 ± 0.08 | 1.96 ± 0.06 | 1.93 ± 0.10 | 1.94 ± 0.05 | 2.03 ± 0.03 |
| Egg buoyancy (%) | – | 39.6 ± 2.1$^{c}$ | 54.0 ± 4.1$^{b}$ | 83.2 ± 2.5$^{a}$ | 64.2 ± 4.9$^{b}$ | 57.1 ± 3.3$^{b}$ | 31.0 ± 6.3$^{c}$ |
| Fertilization rate (%) | – | 16.0 ± 4.1$^{b}$ | 17.5 ± 2.8$^{b}$ | 40.0 ± 4.1$^{a}$ | 27.6 ± 3.2$^{b}$ | 24.5 ± 1.8$^{b}$ | 8.0 ± 2.3$^{c}$ |
| Time of ovulation (h) | – | 46.5 ± 0.5$^{a}$ | 43.9 ± 1.1$^{a}$ | 38.3 ± 0.8$^{bc}$ | 40.2 ± 0.5$^{b}$ | 38.0 ± 0.7$^{c}$ | 37.3 ± 0.7$^{c}$ |
| Mortality of spawners (%) | 0 | 0 | 0 | 10 | 20 | 30 | 80 |
| Ovulation rate (%) | 0 | 20 | 40 | 90 | 70 | 80 | 70 |

6 IU / kg BW), the former showed a higher fertilization rate, but they are not significant ($P$ > .05). In view of this, further trials (T3 with 3 sub-trials) with injection of combination of LHRH-A3 and HCG were conducted for investigating the optimal dosage of hormones. The results of T3 were shown in Table 4. Compare to the optimal dosages in T1 and T2, three sub-trials of T3 showed relatively higher fertilization rates (38.6%–44.2%) and very high egg buoyancy (94.6%–98.1%). The optimal combination of hormones and dose is 0.15 μg LHRH-A3 + 4 IU HCG per kg BW (T3–1) which showed a slightly higher relative fecundity, hatching rate and fertilization rate (all not significant, P > .05) than other two sub-trials. Table 5 showed the results of comparison of with or without the addition of DOM. When DOM was added, there was a significant increasing of relative fecundity ($P$ < .05), however, other parameters were increased slightly or nearly identical.

Though females with ovary development at stage V were not recommended for inducing, in this study, the administration methods of hormone therapy for this stage were also investigated (T5, with 5 sub-trials). These results were shown in Table 6. When females with stage V ovary development, the dosage of hormones should be reduced substantially to avoid high mortality of spawners. The dosage of (0.05 μg LHRH-A3 + 2 IU HCG + 1 mg DOM) / kg BW (T5–2) showed a relatively larger relative fecundity and higher egg buoyancy, fertilization rate and hatching rate than other 4 sub-trials. Compare to the results in T4, T5–2 showed a significant higher relative fecundity ($P$ < .05), but with a significant lower fertilization and hatching rate (P < .05). Finally, dosage of hormones in T4 is recommended to induce ovulation of females at maturation stage IV, and dosage in T5–2 as an alternative approach used at stage V.

### 3.2. Timing of ovulation and optimal stripping

According to our results, the time of ovulation is very predictable which is around 37 h, mostly ranging from 35.5 to 39.5 h depending on females. Fertilization rate and hatching rate is negative correlation with time of ovulation. For example, in T4, the Pearson correlation coefficients between time of ovulation and fertilization rate and hatching rate were − 0.78 and − 0.70 respectively, which means shorter time of ovulation with higher fertilization and hatching rate. Once ovulation was detected, the fertilization and hatching rate can be affected by the striping time. At 0 and 15 mpo, fertilization rates (43.7 ± 2.9 % and 47.3 ± 3.9 %, respectively) and hatching rates (74.7 ± 3.2 % and 82.1 ± 2.8 %, respectively) were similar, but at other times post-ovulation they were significantly lower (Fig. 1).

### 3.3. Protocol of hormonal induction to ovulation and mass production of fertilized eggs in out-of-season

At maturation stage IV and V, the optimal dosage of hormones was determined as 0.15 μg LHRH-A3 + 4 IU HCG + 2 mg DOM per kg BW and 0.05 μg LHRH-A3 + 2 IU HCG + 1 mg DOM per kg BW respectively. Using this protocol, from early April to late May, 780 females were used to produce fertilized eggs commercially. The overall ovulation rate and mortality of spawners was 88.5% and 10.3% respectively. Totally, 95.6 L fertilized tongue sole eggs (wet weight 64.8 kg, approximately $8.3 \times 10^{7}$ eggs, overall fertilization rate 41%) were sold to farmers (involving > 30 hatcheries or aquaculture factories) for seeding production and commercial aquaculture in coastal areas of China. After about a 24-h incubation, eggs were packaged in plastic bags filled with seawater (18 °C) and oxygen (volume ratio 1:2, 40–50 mL eggs per L water) and transported by freight logistics (by air or land). These approaches could ensure that fertilized eggs will not hatch before reaching new hatcheries and with a high survival rate (> 90%).

## 4. Discussion

### 4.1. Reproduction techniques: An important method for booming tongue sole aquaculture

As mentioned above, tongue sole in an important mariculture species in China. However, due to the inferior growth performance of males and very high male proportion (70%–90%) in the cultured population (Chen et al., 2008; Hu et al., 2014), the aquaculture production of tongue sole has been severely limited. The genetic mechanism of sex determination has been well revealed and the availability of the sex-specific genetic markers facilitated the sex control breeding and promoted the development of high production of female offspring (Chen et a., 2008; Chen et al., 2012; Song et al., 2012; Chen et al., 2014; Shao et al., 2014; Cui et al., 2017; Chen et al., 2018; Chen and Xu, 2018; Chen and Zhou, 2018). By molecular biology methods, the proportion of females in cultured population can reach to approximately 40%, and

**Table 3**
Dose of HCG and main production parameters in T2 with 7 sub-trials ($n$ = 10). Values in the same row with different superscript letters are significantly different (P < .05). Body weight of females, egg buoyancy, fertilization rate and time of ovulation was expressed as mean ± standard error.

| | T2–1 | T2–2 | T2–3 | T2–4 | T2–5 | T2–6 | T2–7 |
|---|---|---|---|---|---|---|---|
| Dose of HCG (IU / kg BW) | 0 | 2 | 4 | 6 | 8 | 10 | 12 |
| Body weight of females (kg) | 2.01 ± 0.06 | 2.07 ± 0.13 | 2.05 ± 0.06 | 2.07 ± 0.06 | 2.08 ± 0.06 | 1.96 ± 0.06 | 1.95 ± 0.06 |
| Egg buoyancy (%) | – | 91.2 ± 0 | 92.4 ± 2.0$^{a}$ | 96.3 ± 0.6$^{a}$ | 96.3 ± 0.8$^{a}$ | 83.1 ± 2.3$^{b}$ | 70.8 ± 3.8$^{c}$ |
| Fertilization rate (%) | – | 14.0 ± 0 | 26.2 ± 5.9$^{b}$ | 35.3 ± 3.6$^{a}$ | 25.1 ± 3.4$^{b}$ | 18.5 ± 2.5$^{b}$ | 6.5 ± 2.4$^{c}$ |
| Time of ovulation (h) | – | 44.0 ± 0 | 39.8 ± 0.8$^{a}$ | 38.5 ± 0.7$^{a}$ | 38.0 ± 0.6$^{a}$ | 37.3 ± 0.5$^{b}$ | 37.1 ± 0.5$^{b}$ |
| Mortality of spawners (%) | 0 | 0 | 0 | 10 | 20 | 20 | 40 |
| Ovulation rate (%) | 0 | 10 | 50 | 90 | 80 | 80 | 70 |

**Table 4**
Dose of hormones and main production parameters in T3 with three sub-trials ($n$ = 20). Values in the same row with different superscript letters are significantly different ($P$ < .05). Body weight of females, relative fecundity, egg buoyancy, fertilization rate, hatching rate and time of ovulation was expressed as mean ± standard error.

| | T3–1 | T3–2 | T3–3 |
|---|---|---|---|
| Dose of hormones (/ kg BW) | 0.15 μg LHRH-A3 + 4 IU HCG | 0.30 μg LHRH-A3 + 3 IU HCG | 0.45 μg LHRH-A3 + 2 IU HCG |
| Body weight of females (kg) | 2.04 ± 0.08 | 1.97 ± 0.07 | 1.97 ± 0.05 |
| Relative fecundity ($10^3$ / kg BW) | 142.9 ± 7.2$^a$ | 137.7 ± 8.1$^a$ | 133.5 ± 7.2$^a$ |
| Egg buoyancy (%) | 98.1 ± 0.5$^a$ | 95.8 ± 0.3$^a$ | 94.6 ± 1.5$^a$ |
| Fertilization rate (%) | 44.2 ± 3.4$^a$ | 38.6 ± 3.7$^a$ | 39.3 ± 7.3$^a$ |
| Hatching rate (%) | 71.6 ± 5.0$^a$ | 65.3 ± 4.1$^a$ | 69.8 ± 5.3$^a$ |
| Time of ovulation (h) | 38.1 ± 0.6$^a$ | 37.9 ± 0.6$^a$ | 38.3 ± 0.5$^a$ |
| Mortality of spawners (%) | 5 | 10 | 5 |
| Ovulation rate (%) | 90 | 90 | 90 |

**Table 5**
Dose of hormones and main production parameters in T3–1 and T4 ($n$ = 20). Values in the same row with different superscript letters are significantly different (P < .05). Body weight of females, relative fecundity, egg buoyancy, fertilization rate, hatching rate and time of ovulation was expressed as mean ± standard error.

| | T3–1 | T4 |
|---|---|---|
| Dose of hormones (/ kg BW) | 0.15 μg LHRH-A3 + 4 IU HCG | 0.15 μg LHRH-A3 + 4 IU HCG + 2 mg DOM |
| Body weight of females (kg) | 2.04 ± 0.08 | 2.00 ± 0.05 |
| Relative fecundity ($10^3$ / kg BW) | 142.9 ± 7.2$^b$ | 161.1 ± 6.0$^a$ |
| Egg buoyancy (%) | 98.1 ± 0.5$^a$ | 96.0 ± 0.8$^a$ |
| Fertilization rate (%) | 44.2 ± 3.4$^a$ | 44.6 ± 3.9$^a$ |
| Hatching rate (%) | 71.6 ± 5.0$^a$ | 80.4 ± 5.0$^a$ |
| Time of ovulation (h) | 38.1 ± 0.6$^a$ | 37.7 ± 0.5$^a$ |
| Mortality of spawners (%) | 5 | 5 |
| Ovulation rate (%) | 90 | 95 |

in the grow-out period males are usually eliminated within one-year-old for maximizing aquaculture benefits. Therefore, for the purpose of booming the industry of tongue sole, the development of reproduction techniques to satisfy the market demand for juvenile fish is considered as an important approach.

### 4.2. Broodstock management and out-of-season production

In our work, we reported an effective method of broodstock management of tongue sole in out-of-season, which could be used to provide fertilized eggs for hatcheries throughout the year, thereby facilitating the large-scale production of fry. Briefly, we mimicked the natural feeding habits (diet) of tongue sole under controlled culture conditions (mainly temperature and light) for inducing ovary maturation. Evidence showed that wild-caught broodstock population fed with live or fresh polychaete, shellfish and shrimp at optimal water range and controlled photoperiod can spawn spontaneously, with a relative high fertilization and hatching rate (52.5%–68.6% and 64.5%–82.7%, respectively) (Liu et al., 2006). However, as far as we known, in the commercial tongue sole aquaculture conditions (feeding with pellet feed, 21–23 °C), the development of ovary could reach stage IV in autumn but could not ovulate and spawn. In this case, after a treatment of hormones, the fertilization rate and hatching rate is usually very low (20%–30% or even much lower). From this point, therefore, we can deduce that the nutrition of broodstock is an important factor which affects gamete quality. Previous studies revealed that broodstock diets supplemented with arachidonic acid or krill meal could stimulate the gonad development or enhance egg quality in tongue sole (Xu et al., 2017a, 2017b). Broodstock nutrition can significantly impact reproductive performance which is now well accepted (Bobe and Labbé, 2010). In general, nutrition and temperature are of critical importance for the final oocyte maturation of tongue sole, as for photoperiod, however, there has been no definitive conclusion yet, because we learned from other tongue sole breeders that in cases of nearly total darkness aquaculture conditions, females could undergo oocyte maturation but not ovulation. Maybe this can be attributed to the benthic life style of adult fish?

### 4.3. Hormone therapy and artificial fertilization

The results of this study demonstrate that single injection with the combination of LHRH-A3, HCG and DOM is effective for successful ovulation of females. A very small effective dosage of hormones may suggest that tongue is very sensitive to exogenous hormones. The

**Table 6**
Dose of hormones and main production parameters in T5 with 5 sub-trials ($n$ = 10). Values in the same row with different superscript letters are significantly different (P < .05). Body weight of females, relative fecundity, egg buoyancy, fertilization rate, hatching rate and time of ovulation was expressed as mean ± standard error.

| | T5–1 | T5–2 | T5–3 | T5–4 | T5–5 |
|---|---|---|---|---|---|
| Dose of hormones (/ kg BW) | 0.05 μg LHRH-A3 + 1 IU HCG + 1 mg DOM | 0.05 μg LHRH-A3 + 2 IU HCG + 1 mg DOM | 0.10 μg LHRH-A3 + 1 IU HCG + 1 mg DOM | 0.10 μg LHRH-A3 + 2 IU HCG + 1 mg DOM | 0.15 μg LHRH-A3 + 4 IU HCG + 2 mg DOM |
| Body weight of females (kg) | 2.05 ± 0.08 | 1.96 ± 0.06 | 2.03 ± 0.11 | 2.08 ± 0.07 | 2.03 ± 0.06 |
| Relative fecundity ($10^3$/kg BW) | 134.2 ± 9.9$^c$ | 194.6 ± 14.8$^a$ | 133.3 ± 6.9$^c$ | 131.4 ± 5.1$^c$ | 149.5 ± 6.8$^b$ |
| Egg buoyancy (%) | 80.9 ± 3.1$^b$ | 88.4 ± 3.3$^a$ | 80.1 ± 2.0$^b$ | 82.6 ± 2.1$^{ab}$ | 70.4 ± 2.1$^c$ |
| Fertilization rate (%) | 22.6 ± 4.0$^{ab}$ | 30.4 ± 5.1$^a$ | 18.9 ± 3.6$^b$ | 21.9 ± 1.9$^{ab}$ | 25.1 ± 3.4$^b$ |
| Hatching rate (%) | 44.1 ± 8.1$^{ac}$ | 63.4 ± 7.2$^a$ | 54.1 ± 8.1$^{ac}$ | 46.7 ± 8.1$^{ac}$ | 28.8 ± 8.3$^{bc}$ |
| Time of ovulation (h) | 37.1 ± 0.6$^a$ | 36.8 ± 0.4$^a$ | 36.8 ± 0.5$^a$ | 36.6 ± 0.5$^a$ | 36.1 ± 0.7$^a$ |
| Mortality of spawners (%) | 20 | 20 | 30 | 30 | 60 |
| Ovulation rate (%) | 80 | 80 | 70 | 50 | 40 |

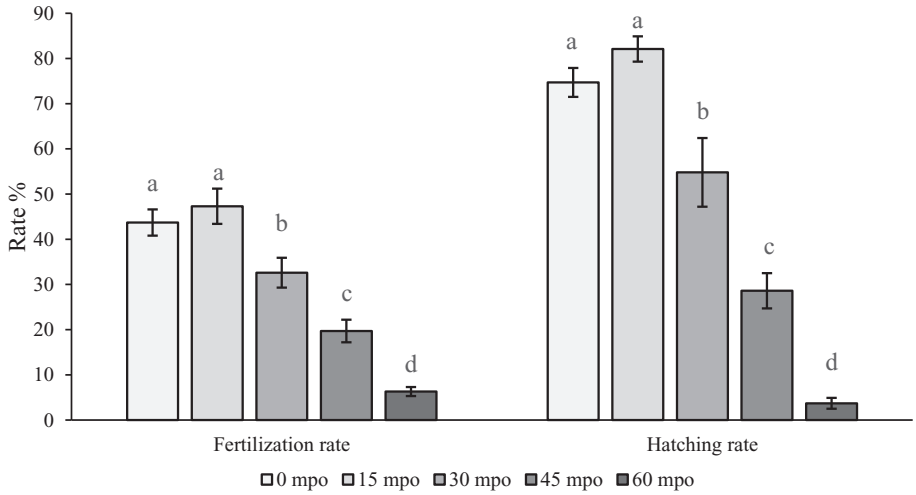


**Fig. 1.** Fertilization and hatching rates at different times after ovulation (mpo = minutes post ovulation). Bars with different letter superscripts were significantly different.

optimal hormonal treatment dosages are usually > 10 times larger in other fish species, such as *Perca fluviatilis* (Żarski et al., 2017), *Perca fluviatilis* (Żarski et al., 2019), *Solea senegalensis* (Rasines et al., 2013), etc. Effective doses of hormones vary widely and are not comparable because of differences in species, temperature, state of maturity, and even hormones themselves (e.g., different manufacturers). Compare T4 with T3–1, DOM did not affect the main reproduction parameters except relative fecundity, which similar to results obtained by Guzmán et al. (2011a) in Senegalese sole. But still, it is worth a try. In tongue sole, a relatively larger dosage can cause mass mortality of spawners and sharply reduced egg quality (over-ripped). Also, the optimal dosage of hormones (T5–2) at developmental maturity stage V is much lower than at stage IV, reducing to about one-third amount (Table 6). However, despite of this, females suffered a higher mortality (20%) and the fertilization and hatching rate reduced significantly. Therefore, from the perspective of controlled reproduction, the best time to start procedures of hormonal therapy is the beginning of final oocyte maturation stage (i.e., stage IV). In our study, the overall fertilization rate of large-scale production is low (41%), which is higher than another sole species, Senegalese sole, to some extent (approximately ranging from 10% to 40%) (Rasines et al., 2012, 2013). The latency between hormone injection and ovulation is very predictable, and once ovulation was detected, eggs should be stripped within 15 min for a higher fertilization and hatching rate. Otherwise, eggs may overripe or lost their vitality with a sharp reduced fertilization and hatching rate. The time between hormonal induction and ovulation and time of eggs remain viable in ovarian cavity varies among species (for example studies in Senegalese sole by Rasines et al. (2012) and their discussion section). According to our results, the fertilization and hatching rate obtained by hormonal induction and stripping for artificial fertilization in out-of-season is lower than previous researches by using wide-caught fishes domesticated in captivity under simulated natural conditions in spawning season (Liu et al., 2006), values are as motioned in Section 4.2). But even so, our efforts had largely alleviated the actual difficulties in the reproduction of tongue sole.

### 4.4. Conclusions and future directions

In this study, the reproduction techniques including broodstock management, hormonal treatment strategy for the induction of ovulation and artificial fertilization are developed for the first time in cultured female tongue sole in out-of-season. We concluded that the thermal and photoperiod stimulation coupled with nutrition enrichment could achieve the finial oocyte maturation, and by applying appropriate hormonal therapies, tongue sole can be successfully reproduced in captive conditions in out-of-season. Based on our researches, it is expected that the reproduction of tongue sole can be realized at any time throughout the year. However, a low to medium fertilization rate (in most cases ranging from 30% to 50%) and not very high hatching rate (70%–80%) implies an enormous scope for improvements. In tongue sole, this maybe a complicated systematic project which has to focus on the following aspects: 1) detecting the optimal environmental conditions (temperature, nutrition, salinity, social factors, etc.) for reproduction in captivity; 2) improving the hormonal therapies (e.g., trying double injection or other times of administration, using other kinds of commercial hormones and evaluating of the accurate stage of reproductive maturation); 3) optimizing the protocols of fertilization (sperm to egg ratio, gamete contact time and effects of time post-stripping); 4) monitoring sperm quality (density, motility and concentration) seasonally or more frequently and enhancing sperm quality by hormones (these information are still not available in tongue sole).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

This work was supported by Key R&D Program of Hebei Province of China (19226704D), and the China Agriculture Research System (CARS-47-G03).

## References

Black, B.J., Black, M., 2013. Efficacy of two exogenous hormones (GnRHa and hCG) for induction of spontaneous spawning in captive yellowfin bream, *Acanthopagrus australis* (Sparidae) and influence of sex ratio on spawning success. Aquaculture 416–417, 105–110.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocr. 165, 535–548.

Chen, S., Xu, W., 2018. Sex identification and control in half-smooth tongue sole. In: Wang, H., Piferrer, F., Chen, S., Shen, Z. (Eds.), Sex Control in Aquaculture, pp. 547–563.

Chen, S.L., Zhou, Q., 2018. Half-smooth tongue sole (*Cynoglossus semilaevis*): whole genome sequencing to molecular sex control. In: Gui, J., Tang, Q., Li, Z., Liu, J., De Silva, S.S. (Eds.), Aquaculture in China, pp. 497–513.

Chen, S., Li, J., Deng, S., Tian, Y., Wang, Q., Zhuang, Z., Sha, Z., Xu, J., 2007. Isolation of female-specific AFLP markers and molecular identification of genetic sex in half-

smooth tongue sole (*Cynoglossus semilaevis*). Mar. Biotechnol. 9, 273–280.
Chen, S.L., Deng, S.P., Ma, H.Y., Tian, Y.S., Xu, J.Y., Yang, J.F., Wang, Q.Y., Ji, X.S., Shao, C.W., Wang, X.L., Wu, P.F., Deng, H., Zhai, J.M., 2008. Molecular marker-assisted sex control in half-smooth tongue sole (*Cynoglossus semilaevis*). Aquaculture 283, 7–12.
Chen, S.L., Ji, X.S., Shao, C.W., Li, W.L., Yang, J.F., Liang, Z., Liao, X.L., Xu, G.B., Xu, Y., Song, W.T., 2012. Induction of mitogynogenetic diploids and identification of WW super-female using sex-specific SSR markers in half-smooth tongue sole (*Cynoglossus semilaevis*). Mar. Biotechnol. 14, 120–128.
Chen, S., Zhang, G., Shao, C., Huang, Q., Liu, G., Zhang, P., Song, W., An, N., Chalopin, D., Volff, J., Hong, Y., Li, Q., Sha, Z., Zhou, H., Xie, M., Yu, Q., Liu, Y., Xiang, H., Wang, N., Wu, K., Yang, C., Zhou, Q., Liao, X., Yang, L., Hu, Q., Zhang, J., Meng, L., Jin, L., Tian, Y., Lian, J., Yang, J., Miao, G., Liu, S., Liang, Z., Yan, F., Li, Y., Sun, B., Zhang, H., Zhang, J., Zhu, Y., Du, M., Zhao, Y., Schartl, M., Tang, Q., Wang, J., 2014. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to benthic life. Nat. Genet. 46, 253–260.
Chen, S., Zhou, Q., Shao, C., 2018. Genomic and epigenetic aspects of sex determination in half-smooth tongue sole. In: Wang, H., Piferrer, F., Chen, S., Shen, Z. (Eds.), Sex Control in Aquaculture, pp. 525–545.
Cui, Z., Liu, Y., Wang, W., Wang, Q., Zhang, N., Lin, F., Wang, N., Shao, C., Dong, Z., Li, Y., Yang, Y., Hu, M., Li, H., Gao, F., Wei, Z., Meng, L., Liu, Y., Wei, M., Zhu, Y., Guo, H., Cheng, C.H.K., Schartl, M., Chen, S., 2017. Genome editing reveals dmrt1 as an essential male sex-determining gene in Chinese tongue sole (*Cynoglossus semilaevis*). Sci. Rep. 7, 42213.
FAO, 2016. The State of World Fisheries and Aquaculture 2016. Contributing to Food Security and Nutrition for all. Rome. 200 pp.
Guzmán, J.M., Norberg, B., Ramos, J., Mylonas, C.C., Mañanós, E., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (Solea *senegalensis*). Gen. Comp. Endocr. 156, 285–297.
Guzmán, J.M., Cal, R., García-López, Á., Chereguini, O., Kight, K., Olmedo, M., Sarasquete, C., Mylonas, C.C., Peleteiro, J.B., Zohar, Y., Mañanós, E.L., 2011a. Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis of Senegalese sole (Solea senegalensis). Comp. Biochem. Physiol. A 158, 235–245.
Guzmán, J.M., Ramos, J., Mylonas, C.C., Mañanós, E.L., 2011b. Comparative effects of human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa) treatments on the stimulation of male Senegalese sole (*Solea senegalensis*) reproduction. Aquaculture 316, 121–128.
Howell, B., Conceição, L., Prickett, R., Cañavate, P., Mañanos, E., 2009. Sole farming: nearly there but not quite?! A report of 4th workshop on the cultivation of soles. Aquacult. Eur. 34, 24–27.
Hu, Q., Chen, S.L., Gao, F., Li, Y., Liu, S., Liu, F., Yang, J., Du, M., 2014. Differences in sex reversion and growth between normal and neomale stock in half-smooth tongue sole, *Cynoglossus semilaevis*. Aquacult. Int. 22, 1437–1449.
Kahkesh, F.B., Feshalami, M.Y., Amiri, F., Nickpey, M., 2010. Effect of Ovaprim, Ovatide, HCG, LHRH-A2, LHRHA2 + CPE and carp pituitary in Benni (*Barbus sharpeyi*) artificial breeding. Global. Veterinaria 5, 209–214.
Li, Y., Wang, L., Yang, Y., Li, X., Dai, H., Chen, S., 2019. Genetic analysis of disease resistance to *Vibrio harveyi* by challenge test in Chinese tongue sole (*Cynoglossus semilaevis*). Aquaculture 503, 430–435.
Lin, H.R., Kraak, G.V.D., Zhou, X.J., Liang, J.Y., Peter, R.E., Rivier, J.E., Vale, W.W., 1988. Effects of [d-Arg$^6$, Trp$^7$, Leu$^8$, Pro$^9$ NEt]-luteinizing hormone-releasing hormone (sGnRH-A) and [d-Ala$^6$, Pro$^9$NEt]-luteinizing hormone-releasing hormone (LHRH-A), in combination with pimozide or domperidone, on gonadotropin release and ovulation in the Chinese loach and common carp. Gen. Comp. Endocrinol. 69, 31–40.
Liu, X.Z., Sun, Z.Z., Ma, A.J., Liang, Y., Zhuang, Z.M., Lan, G.G., 2006. Study on the technology of spawner culture and eggs collection of *Cynoglossus semilaevis* Günther. Mar. Fish. Res. 27, 25–32 (In Chinese).
Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulation of fish reproduction. Gen. Comp. Endocrinol. 165, 516–534.
Mylonas, C.C., Mitrizakis, N., Castaldo, C.A., Cerviño, C.P., Papadaki, M., Sigelaki, I., 2013. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality. Aquaculture (415–415), 318–327.
Mylonas, C.C., Fatira, E., Karkut, P., Papadaki, M., Sigelaki, I., Duncan, N.J., 2015. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity III. Comparison between GnRHa implants and injections on spawning kinetics and egg/larval performance parameters. Aquaculture 448, 44–53.
Nayak, P.K., Mishra, T.K., Singh, B.N., Pandeyand, A.K., Das, R.C., 2001. Induced maturation and ovulation in *Heteropneustes fossilis* by using LHRHa, pimozide and ovaprim for production of quality eggs and larvae. Indian J. Fish. 48, 269–275.
Peter, R.E., Lin, H.R., Van Der Kraak, G., 1988. Induced ovulation and spawning in cultured fresh water fish in China: advanced in application of GnRH analogue and dopamine antagonists. Aquaculture 74, 1–10.
Ranjan, R., Megarajan, S., Xavier, B., Ghosh, S., Santhosh, B., Gopalakrishnan, A., 2018. Broodstock development, induced breeding and larval rearing of Indian pompano, *Trachinotus mookalee*, (Cuvier, 1832) – a new candidate species for aquaculture. Aquaculture 495, 550–557.
Rasines, I., Gómez, M., Martín, I., Rodríguez, R., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326–329, 129–135.
Rasines, I., Gómez, M., Martín, I., Rodríguez, R., Mañanós, E., Chereguini, O., 2013. Artificial fertilisation of cultured Senegalese sole (*Solea senegalensis*): effects of the time of day of hormonal treatment on inducing ovulation. Aquaculture 392–395, 94–97.
Robert, L.B., James, S., 2007. An evaluation of a leuteinizing hormone-releasing hormone analog to induce spawning of channel catfish *Ictalurus punctatus*. J. World Aquacult. Soc. 21, 10–15.
Shao, C., Li, Q., Chen, S., Zhang, P., Lian, J., Hu, Q., Sun, B., Jin, L., Liu, S., Wang, Z., Zhao, H., Jin, Z., Liang, Z., Li, Y., Zheng, Q., Zhang, Y., Wang, J., Zhang, G., 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Res. 24, 604–615.
Song, W., Li, Y., Zhao, Y., Liu, Y., Niu, Y., Pang, R., Miao, G., Liao, X., Shao, C., Gao, F., Chen, S., 2012. Construction of a high-density microsatellite genetic linkage map and mapping of sexual and growth-related traits in half-smooth tongue sole (*Cynoglossus semilaevis*). PLoS One 7, e52097.
Xu, H.G., Zhao, M., Zheng, K.K., Wei, Y.L., Yan, L., Liang, M.Q., 2017a. Antarctc krill (*Euphausia superba*) meal in the diets improved the reproductve performance of tongue sole (*Cynoglossus semilaevis*) broodstock. Aquac. Nutr. 23, 1287–1295.
Xu, H., Cao, L., Zhang, Y., Johnson, R.B., Wei, Y., Zheng, K., Liang, M., 2017b. Dietary arachidonic acid differentially regulates the gonadal steroidogenesis in the marine teleost, tongue sole (*Cynoglossus semilaevis*), depending on fish gender and maturation stage. Aquaculture 468, 378–385.
Żarski, D., Bernath, G., Krol, J., Cejko, B.I., Bokor, Z., Palinska-Zarska, K., Milla, S., Fontaine, P., Krejszeff, S., 2017. Effects of hCG and salmon gonadoliberine analogue on spermiation in the Eurasian perch (*Perca fluviatilis*). Theriogenology 104, 179–185.
Żarski, D., Palińska-Żarska, K., Krejszeff, S., Królb, J., Milla, S., Fontaine, P., Bokor, Z., Urbányi, B., 2019. A novel approach for induced out-of-season spawning of Eurasian perch, *Perca fluviatilis*. Aquaculture 512, 734300.