THESES OF DOCTORAL (PhD) DISSERTATION

Tamás Müller

KAPOSVÁR UNIVERSITY

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Kaposvár University
Faculty of Animal Science
Department of Fish and Companion Animal

Director of Doctoral School
Péter Horn (MHAS)

Head of programme and co-supervisor
Péter Horn (MHAS)

Supervisor
Miklós Bercsényi (Ph.D.)

POSSIBLE WAYS OF THE ARTIFICIAL INDUCTION OF SEXUAL MATURATION AND REPRODUCTION OF THE EUROPEAN EEL (*ANGUILLA ANGUILLA* L.).

Written by
Tamás Müller

Kaposvár

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1. RESEARCH OF PRELIMINARIES AND AIMS

The European eel (*Anguilla anguilla*) belongs to the catadromous fish species. This means that after a long-term freshwater living period they migrate to spawn to the Sargasso Sea. Since no one has been able to bring up European eel larvae to glass eel size up to now, we can not speak about eel breeding in the narrow sense of the term, only eel rearing. Eel farms in Europe base their annual production on the capture of glass eels entering in rivers and raise them to market size. The eel populations have been decreasing due to a lot of factors, such as water pollution, overfishing of glass and adult eels *Anguillicola crassus* nematode infection etc. It is not probable that the eels are able to sustain their own stocks without human help.

The subject of my dissertation was to investigate the possibility of the artificial propagation of European eel considering the reproduction feature of eels in Hungarian waters.

The following objectives were formulated:

1st-2nd experimental series

Sexual maturation was induced in different origins of females (Lake Balaton, farmed and Greek eels) and parasite infection stages (stocks were infected by *Anguillicola crassus* or free from it) by using various kinds of hormones, such as carp pituitary, carp pituitary and dopamine receptor antagonist mixture, silver carp pituitary, synthetic GnRH.

3rd experiment

The aim was to investigate the artificial induction of sexual maturation of males and to keep their long-term sperm releasing in freshwater rearing as well as to examine the quantity and quality of stripped sperm. The aim was to try the possibility of the cryopreservation technology, which had been developed for common carp, in the case of European eel as well.

4th experiment

The aim was to investigate the possibility of the multiply use of males as well as to compare the ultrastructure of eel spermatozoa after freshwater rearing with the seawater ones (according to published data).

5th experiment

The aim was to investigate the application of computer tomography for monitoring different physiological changes during maturation in males *in vivo*. This work also aimed to reveal some new information about the location of the fat storage, fat metabolisation and gonad development during the maturation.

2. MATERIALS AND METHODS

The experiments were carried out at the Department of Zoology of Veszprém University (1-4 experiments) and also at the Department of Fish and Companion Animal and Institute of Diagnostic Imaging and Radiation Therapy of Kaposvár University (5th experiment). Complementary experiments were made at the Department of Fish Culture (3rd experiment) and Department of Pathology and Forensic Veterinary Medicine of Szent István University (4th experiment).

1st and 2nd experimental series

The experimental fish originated from different places:

-Eels were selected from catches of commercial electric fishing in Lake Balaton

- Farmed eels from Köröm (Hungary) were given from rearing tanks of eel
- Wild females imported from Ioannina (Greece)

Females were adapted to artificial seawater (30ppt salinity) for 5-7 days and were kept without feeding. The applied doses of different hormones see Table 1. The sexual maturation was determined by using external (eye index) and internal (GSI-gonad somatic index) parameters. The cytological maturation of ovaries was investigated by histological samples. Pieces of the gonads were fixed in 8% formalin, and 7μm thick histological preparations were stained with haematoxylin-eosin. The developmental stages of oocytes were determined according to scientific literature. In the case of fish having ovulated eggs, the number of *Anguillicola crassus* nematodes were counted in the swimming bladder.

3rd-4th experimental series

Males reared in fresh water were treated with human chorion gonadotropin (100 IU hCG and 250 IU hCG/fish/week). After the start of spermiation, sperm was stripped by gentle pressure on abdomen 24 hours after the hormone injection. Changes of body weight, the quantity and quality of sperms were measured. Cryopreservation experiments were carried out with the best quality sperms by using a protocol, which had been developed for common carp.

One fish group, whose spermiation was induced for 15 weeks by using 250 IU hCG/fish/week treatments, was kept for 5 weeks and histological preparations were made from the gonads. These results were compared with others, originated from sexually immatured males and individuals, which were at the top stage of spermiation.

5th experiment

Ten males were induced to sexual maturation for 6 weeks by using weekly 250 IU hCG/fish treatment weekly. Two days after the weekly administration, the eels were scanned by means of a spiral CT scanner. The location of fat stores in the body was observed by image post processing method. Ratio of fat depletion, fat mobilization and volumetric development of the testis were followed up. The fat stores in fillet were checked by using histological preparations.

3. RESULTS

The results are summarised as follows

1st-2nd experimental series

- Ovulated eggs could be obtained in spite of the facts that artificial sea water was used and the experimental fish were infected by *Anguillicola crassus*.
- The application of double amounts of the mixture of carp pituitary, GnRH-A and dopamine receptor antagonist gave contradictory results regarding induced ovulation.
- Silver carp pituitary is effective to induce an advanced phase of sexual maturation in eels.
- GnRH-A in doses of 0.1 and 10µg /fish/ week (OVURELIN) did not induce a remarkable ovarian development during the 81 day experimental period.
- A histological map was assembled following the developmental stages from previtellogenic oocytes to preovulated eggs.

Table 1. Summarised results about the 1st and 2nd experiment series.

n	Groups	Treatment	Stock	Time (day)	Eye index	GSI (%)	Notes
3	1.	Negative control	Balaton	81	7.03±1.47	0.82±0.75	There was no gonad development
3		control 1ml 0.65% NaCl/fish/twice a week			5.17±0.75	1.07±0.32	
4		5 mg SCP/fish/twice a week			8.25±1.82	5.90±4.46	The gonad development was powerful
4		10mg CP+ 2mg D/fish/twice a week		72±8	12.70±1.68	37.33±12.45	egg stripping from two females* spontaneous egg releasing from one eel*
3	2.	Negative control	Balaton	81	11.27± 2.40	0.84±0.04	There was no gonad development
3		0.1µg GnRH-A/fish/ twice a week			7.83±2.24	0.88±0.05	
5		10 µg GnRH-A/fish/ twice a week			7.68±3.26	0.78±0.40	
5	3.	15mg CP/body weight kg /once a week	Farmed	132±16	13.1±1.70	13.93±7.37	egg stripping from 1 female*
3	4.	15mg CP/body weight kg /twice a week	Greek	72±5	15.4±2.09	26.17±5.61	All of them reached the pre-ovulated stage*
5	5.	15mg CP/body weight kg + 2 mg D /body weight kg /twice a week	Balaton	111±6	13.89±1.08	12.15±4.87	The gonad development was powerful

*Abbreviations: SCP-silvercarp pituitary, CP-carp pituitary, D-dopamine receptor antagonist, *20 mg carp pituitary and Ovopel (10µg GnRH-A + 10µg dopamine receptor antagonist) / fish was applied to induce ovulation. Egg stripping was carried out 24-36 h after the final injection.*

3rd-4th experimental series

- Males, reared in freshwater, a long-term (13-15 weeks) sperm production can be induced – in contrast with the most cyprinids - by using weekly hCG administration.
- The males could survive the hormonal induction of sexual maturation similarly to individuals reared in seawater reared ones.
- “Freshwater males” produced morphologically and physiologically (ultrastructure, motility, quantity) similar spermatozoa to "seawater males".
- According to our results, the method originally developed for the cryopreservation of common carp sperm could be applied to the deep freezing of eel sperm using methanol as cryoprotectant.
- A histological map was assembled regarding the different developmental stages from immature testis tissues through mature to regressed form.

5th experiment

- The applied CT scanning method is proven to be suitable to follow the maturation processes *in vivo* (gonad development, fat mobilisation) in males.

4. CONCLUSIONS

Eels from Lake Balaton were heavily infected by *Anguillicola crassus*, however, its real impact on the sexual maturation has not been described exactly so far. Based on our investigations we support the theory that though the nematode infections may cause problems during the migration to Sargasso Sea but probably, it is not the only limiting factor of the artificial propagation under laboratory conditions. The holed PVC tubes and closed the ends by nets were useful and suitable for keeping females with no stress. Among the applied hormones, carp pituitary, carp pituitary with dopamine receptor antagonist and silver carp pituitary were successful in the artificial induction of sexual maturation injected once or twice a week. The application of double amounts of the mixture of carp pituitary, GnRH-A and dopamine receptor antagonist gave contradictory results regarding the induction of ovulation. In the first experimental stage, this hormone combination induced the ovulation, but in the case of the "Greek eels" it caused mortality without egg release.

The ratio of males in the eel stock of Lake Balaton is below 1 % (own observation) therefore males can be obtained exclusively from eel farms in Hungary. In this case the prevention from *dactylogirosis* is an essential technological step in the artificial induction of sexual maturation. Males reared in freshwater can be induced a long-term sperm production by using repeated hCG administration. Histological pictures showed that testis tissues redeveloped naturally and no pathological changes happened under artificial rearing conditions after the occurred spermiation phase. Males reared in freshwater could produce morphologically (ultrastructure) and physiologically (motility and quality) the same spermatozoa as "seawater males". Unfortunately, it is not declared, that fresh water rearing could replace the use of salt water, because the lack of fertilization tests.

The use of an extender (modified Kurokura extender) – the technology was originally developed for the deep freezing of common carp sperm with methanol (as cryoprotectant) and technology (Horváth and Urbányi, 1999) - was found suitable for the cryopreservation of the sperm of European eel.

The applied CT scanning method is proved to be suitable to follow the maturation processes *in vivo* (like gonad development, fat mobilisation) in males. During the artificial maturation experiment, fat stores could be revealed in abdomen and filet. The abdominal fat was covered part of the energy depletion of testes development and mobilised into the fillet increasing its fat content. It can be explained by the special keeping method (without swimming activity).

The CT may be a useful equipment for the selection of experimental fish, because it can show the volumetric fat content in live fish as the development of gonads.

5. NEW SCIENFITIC RESULTS

- Based on my research I can conclude that the artificial induction of sexual maturation in both sexes of the European eel is possible under artificial conditions. For instance, commercial table salt is suitable to simulate seawater salinity.
- The application of double amounts of the mixture of carp pituitary, GnRH-A and dopamine receptor antagonist gave contradictory results regarding inducing ovulation.
- Silver carp pituitary is effective to induce the advanced phase of sexual maturation in eels.
- According to our results the extender originally developed for the cryopreservation of common carp sperm seems suitable for the deep-freezing of eel sperm together with methanol as cryoprotectant.
- The applied CT scanning method proved suitable to follow the maturation process *in vivo* (gonad development, fat mobilisation from abdomen to fillet) in males.

6. PROPOSALS (THEORATICAL AND PRACTICAL APPPLICATION)

There is no technology for the artificial propagation of the European eel. There is little information about the feeding habit of larvae and all this information originated exclusively from wild-caught fry, so practical proposals can be made only in the future.

I suggest collecting fish for maturation experiments from the catches of September and November because the ratio of silver eels increases at this time. Silver eels have well developed gonads and the maturation time can be shorter. It would be important to note, that there is no critical age when eels enter the silvery stage, neither the size and nor the age at the time of maturity are directly related in a relatively old population such as Lake Balaton stock. The age at the onset of maturity in eels inversely related to the growth rate. The start of migration to the spawning place depends principally from the body weight and not on the age. The results of the last years' showed that catching fish suitable for the artificial induction of sexual maturation could become more difficult from Lake Balaton from year to year.

Based on our results, the silver carp pituitary is an other effective hormone extract for maturation. I suggest using longer periods to hormonal treatments with 5mg/fish/treatment or using a double dose (10 mg/fish weekly administration to reach the pre-ovulated stage. There is a huge amount of silver carp caught in Lake

Balaton annually and its pituitary weight is bigger (7-10 mg / fish) than that of the carp pituitary.

I propose to use Ohta protocol (17-hydroxyprogesterone or 1720β dihydroxy-4-pregnen-3-one) for the final injection to induce ovulation. Based on Japanese and European results this MIS hormone induces ovulation with great safety.

According to our result it is in harmony with the results of one of my earlier work and other publications, I do not suggest trying to induce sexual maturation with GnRH-A alone or with dopamine receptor antagonist mixture.

I suggest comparing different types of extenders (Artificial Japanese and European eel seminal plasma, modified Kurokura, glucose etc…) and cryoprotectants (DMSO, ethanol) and these combinations to describe an uniform technical protocol for eel sperm cryopreservation.

I suggest the use of computer tomography as a selection equipment to select the experimental stock for trials of artificial induction of sexual maturation. If females have not enough fat resource at the beginning of the experiment they can not be applied to a long term maturation experiment. Low amount of fat indicates an unsuccessful experiment.

7. PUBLICATIONS IN CONNECTION OF THE THESIS

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Müller, T. (2000): Az európai angolna (*Anguilla anguilla*) hormonálisan indukált ivarérlelésének hagyományos és alternatív utjai. Diploma ((PATE Georgikon MgTud-i Kar, Keszthely), (Kézirat).

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