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Precocious Silvering of Farmed Eels with Special Reference to Their Evidence for Genotypic Sex Determination

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ABSTRACT

In the present investigation, developmental stages of gonads from the hatchery specimens of European yellow and silver eels (*Anguilla anguilla* L.) were examined. Based on histological observations of gonads, the total collected specimens of farmed eel was classified into four groups i.e., undifferentiated sex, intersex, males and females, respectively. The obtained results revealed that the total catch specimens of yellow eels (N=36)contain 44.44% males, 41.67% females,2.78% undifferentiated sex, and 11.11% intersex; whereas, silver eels (N=36) includes 83.33% male, 0.0% females, 11.11% intersex and 5.56% undifferentiated sex in decreasing order, respectively. The abundance of males in hatcheries and greenhouses had concluded that Environmental Sex Determination (ESD) mechanism was found to have predominateon Genotypic Sex Determination (GSD) at future stages. The skewed sex ratio's of silver eel in hatcheries and greenhouses can possibly be explained by differences in growth and silvering strategies between the sexes, which result in an earlier culling of the females by the farmer. Therefore, while studying the sex differentiation mechanism of eel fishes, only yellow eel population was seems to be considered. Thence, based on the observation that the ratio of males *vs.* females in the yellow eel group was 50:50, which revealed that Genotypic Sex Determination (GSD) is prevailing at this stage.

KEY WORDS: *Anguillaanguilla*, Yellow and silver eels, gonad differentiation, Environmental Sex Determination (ESD), Genotypic Sex Determination (GSD).

INTRODUCTION

The geographical distribution of eels have been found to have some kind of relation to sex of fish. Males are mostly occur near the coastline in littoral estuaries and lagoons. This might be because characteristics of all these waters holdhigh population densities and less abundance of food. While in contrast, female fishes are usually found more near land inward in fresh water bodies with low population densities and great abundance of food [1]. However, still it is not clear that whether such sexual distribution is determined by a preference of the selection of a specific type of habitat by the migrating of glass eel of a certain sex. The Genotypic Sex determination (GSD) theory assumes that the sex of the glass eel is determined at the moment of fusion of the gametes at the spawning grounds in the Sargasso Sea. Whereas, the selection and preference for a certain water body have been proved to have some kind of sex-linked [2]. However, the alternative theory assumes that there is a random distribution of the glass eel over the several water bodies and unknown environment factors e.g. temperature, food supply, social interaction, stocking density determines the final phenotypic sex or gender of eel fish. This theory is called Environmental Sex Determination (ESD) [2]. More information regarding to the mechanism of sex determination can also be gather from aqua culturists or fish culturists throughout the world particularly in Japanese greenhouse culture or ponds stocked with eels contain 75 to 90% males [3-4]. Similar results of skewed sex ratio were observed in intensive eel culture systems in the Netherlands (Pers. comm. Ir. J.van Rijsingen, Royaal BV Helmond). In

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comparison to the natural water bodies with high stocking densities and large abundance of males [5], present study was conducted to determine the hypothesise that whether the ESD (Environmental Sex Determination) is the sex-determining mechanism? Or whether all those farms that are stocking with high population densities will lead to a majority of males? Therefore, based on these hypothesis, the basic objective of our present study was to describe the microscopic observation of the different developmental stages of gonads i.e., undifferentiated, male, female, intersex for both yellow and silver eels, respectively. As no information was available to clarify that how the sex ratios are distributed among silver and yellow eels that are culturing in a farm or hatchery, therefore, our present study was also use to describe the distribution of sex ratios among yellow and silver eel groups in a hatchery.

MATERIAL AND METHODS

1. *Fish Samplings*

Eels were obtained from a commercial eel farm (Royaal BV, Helmond, the Netherlands) during the three years period from 2010 to 2012. The glass eel, at the moment of stocking in the hatchery, originated from France. From glass eel stage until the time of sampling, the eels were kept in a recirculation system, with fish tanks, upflow filters for sedimentation, and trickling filters for nitrification.

Temperature in the hatchery was kept between 21 and 26°C, the lowest temperatures during winter. Water pH was between 5.0 and 6.0. NH_{4+}, NO_{2-} and NO_{3-} were below 5-10, 50-80 and 1000 mg per litre, respectively. Denitrification and regular fresh water supply (10-20% per week) guaranteed a constant water quality. Eels were kept (6 grams to > 100 grams) in 4000-liter tanks, with increasing density at larger body size (density of 60-300 kg/m^3). The relative food supply was decreasing with increasing body weight. The feeding level for 6-gram animals was 2 % of the body weight per day, while for 100-150 gram animals this was 0.6 % of the body weight per day.

The sampling of the eels was performed in 2012 on groups of eel that entered the hatchery as glass eel in 2010, 2011 and 2012 corresponding to the age groups i.e., 1, 2 & 3 years, respectively. An experienced farm employee subdivided in a selective way the eels per year class into yellow and silver in spring 2012. The criteria for yellow where: lateral side yellow-green shine, no sharp transition from dorsal to ventral side, soft skin. The criteria for silver where: silvery shine, sharp transition to a white abdomen, tough skin and enlarged eyes. In this way we had 3 groups of 12 yellow eels of respectively 1, 2 and 3-years and 3 groups of 12 silver eels of respectively 1,2 and 3 years. In the laboratory, the animals were kept in aquaria for one month with a 14:10 light/dark cycle, in running local tap water at 20°C. They were fed daily with Provimipelletted food (Provimi, Rotterdam, and The Netherlands).

In order to compare the histological structure of the female silver gonad with the yellow stage, we obtained 24 silver female eels from local fisherman with weight ranging from 1.0 to 1.5 kg and total length 80 to 90 cm from the Grevelingen water-bodies during their seaward migration in autumn 2012.

2. *Experimental protocol and sampling procedure*

The fish were quickly anaesthetised with 300 PPM MS222 (3-aminobenzoic-acid-ethyl-ester methanesul-fonate salt, Sigma, St. Louis, USA). After three minutes, the anaesthetised fish were taken out of the aquarium. Thereafter, the mean weight and body length of the eel was measured prior to sampling. The animal was dissected, the gonads were removed, weighed to determine the Gonado Somatic Index (G.S.I.) and fixed in Bouin solution.

3. *Histology gonads*

The gonads were fixated with Bouin fixative until they were completely saturated with Bouin. Next, they were taken through a series of accumulating alcohol percentage (60%, 70%, 80%, 90%, 96% and 100%) to wash out the Bouin fixative and to prepare them for embedding in paraffin. Slices of 5 µm thick were cut, using a Leica microtome and stained with Gill's Hemotoxiline-eosine. The gonads were classified qualitatively by the experimentator and divided in the following classes: undifferentiated, male, female, intersex based on gonad characteristics such as thickness of the layer of connective tissue, type, stage and number of germ-cells and formation of tubules in the testis.

For 22% of the yellow eels and 8% of the silver eels the gender could not be determined based on the Gill's Hemtoxiline-eosine staining. Therefore in subsequent studies of these animals new histological coupes were prepared and stained with the Trichroom-Masson-staining method. This method clearly can distinguish between connective tissue (bright blue-green colour) and germinal tissue (bright red-purple colour). This facilitates for the experimentator the distinction between undifferentiated *vs.* male animals and male and female animals *vs.* intersex animals. In this way we were able to determine the gender of all examined animals.

4. *Statistics analysis of data*

In order to test for a silver (silver *vs.* yellow) and/or years (2010, 2011, 2012) effect, statistics were performed using a two-way ANOVA with interaction term (Proc. GLM, SAS version 6.12; SAS Institute 2010).

$Y_{ijk} = \mu + Stage_i + Year_j + (Stage*Year)_{ij} + e_{ijk}$

Where Y_{ijk} corresponds to G.S.I., $Stage_i$ relates to a yellow- or silver-effect; $Year_j$ relates to a year-effect; $(Stage*Year)_{ij}$ relates to the stage-year interaction effect while e_{ijk} represents the error term. Comparisons of mean squares of the ANOVA were tested using F-tests. $P \leq 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov and F_{max} tests, respectively.
Testing the G.S.I with this two-way ANOVA with interaction term, only the G.S.I. data of hatchery animals were used. The G.S.I. data of the silver Grevelingen females were only used for comparative reasons to describe in a qualitative way the gonad and for clarity used in the two sample T-test pairwise comparison between relevant groups (see below).
With a two-sample t-test the mean value of the G.S.I. was compared pairwise between group of animals: 1) split up to gender between years (2010*vs.*2011, 2011*vs.* 2012, 2010*vs.* 2012; 2) split up to gender between yellow and silver animals; 3) split up to development stage (yellow vs. silver) between gender (male *vs.* female, male *vs.* intersex, female *vs.* intersex).

RESULTS

1. *Qualitative description of the gonad*

Gonads were examined microscopically and were classified on the basis of criteria including i.e., thickness of the layer of connective tissue, type, stage and number of germ-cells and formation of tubuli in the testis.

A. *Undifferentiated Gonads*

The undifferentiated gonad can be characterised by thick layers of connective tissue with a low amount of germinal tissue (Figure 1). With increasing maturation, the amount of germinal tissue was also increases, respectively.

B. *Testes*

Microscopically observations of the testis from the cultivated young males revealed that it has more germinal tissue. But still ratherit contain some thick layers of connective tissue as shown in Figures 2 & 3, respectively. In testis, the germinal tissue was arranged into chord-like testicular lobules containing spermatogonia. In incidental cases, sertoli cells were visible, while tubuli were clearly visible in yellow as well as in silver eel (see Figures 2 & 3), respectively. The spermatogonia have a round shape nuclei with prominent nucleoli. No spermatocytes or spermatids were observed for either yellow or silver eels. The interlobular connective tissue contains some interstitial and blood cells indicative for an increased blood supply.

C. *Ovaries*

In Ovaries of yellow eels, oogonia and primary oocytes are found intermingled. The oocytes are located in nests, while their nuclei were reaching to the early and late prophase stage of meiosis (see Figure 4). For silver female Grevelingen eels (N=64), the cells develop up to the yolk vesicle stage. We observed that the cortical alveolus stage oocytes were arranged in long chains with cytoplasm containing lipid vesicles as shown in Figure 5, respectively. Those early vitellogenic oocytes contain nuclei that reached the late perinuclear stage of meiosis.

D. *Intersex gonads*

Intersex gonads were always predominantly male with some female germ cells in meiosis as shown in Figure 7, respectively. Incidentally 'female' intersex gonads were found (see Figure6). The structure and orientation of connective tissue andtubuli of the intersex gonad was more irregularly and disorderly orientated in comparison to the male testis (see Figure 7 *vs.* 2 & 3). Also the oocytes and spermatogonia of the intersex gonad were located more disorderly in nests that were arranged in an irregular pattern among connective tissue (see Figures 6 & 7).

Results of microscopically interpretation of the histological coupes of the farmed eels were presented in the Table 1. In the present study, yellow eel population was classified into following four stages, i.e., undifferentiated sex, male, female, and intersex. Sex ratio of approximately 50:50 was noted for males *vs*. females in yellow eels of three-year classes, as shown in Table 1, respectively. In both yellow and silver groups, 11.1% specimens had an intersex gonad with predominantly male characteristics (see Figure 7). While in a few cases, an intersex gonad with female characteristics was also observed in the present study (see Figure 6). In Figure 1, it was can be observed that in silver groups originating from three consecutive years, almost all observed samples were mostly males (83.3%),few were incidentally intersex or undifferentiated sex. This results was in contrast to the sex ratios of yellow groups, where 44.44 % were male and 41.67 % were female, respectively.

Table 1. Shows the number of eels classified after sex based on microscopically observation of Haemaluin-Eosine histological coupes of the gonad. Remark that the ratio yellow *vs*. silver in the initial population was 1:10.

SEX	YELLOW EEL		SILVER EEL	
	Number of samples (N)	% frequency	Number of samples (N)	% frequency
Undifferentiated Sex	1	2.78	2	5.56
Male	16	44.44	30	83.33
Female	15	41.67	0	0
Intersex	4	11.11	4	11.11
TOTAL	**36**	**100**	**36**	**100**

The results of two-way ANOVA revealed that Gonadosomatic index (GSI) ofeel fish shows insignificant ($P \leq 0.073$) impact of stage (yellow or silver eel) or year of sampling ($P \leq 0.052$) i.e., 2010, 2011, 2012, or evenno relationshipwas noted for yellow or silver stagewithsampling year ($P \leq 0.41$). This might be because Gonadosomatic index (GSI) of fish mostly increases with the maturation of the fish, and will reach maximum at peak spawming period and will decline soon after spawning [6]. In comparison between years, the two sample T-test (split up to gender of hatchery animals) hadshown no significant differences, as shown in Table 2, respectively.Therefore the G.S.I. data of the three different year-classes were pooled, split up to gender, and tested for significant differences between yellow and silver eels. Also based on two-sample T-test statistics, split up to stage (yellow *vs*. silver), again no significant differences were observed between the years, 2010-2011, 2011-2012 and 2010-2012 for the different three genders. In general, the G.S.I. for yellow *vs.* silver males was low, around 0.15 to 0.16, as shown in Table 3. Also for yellow intersex animals, the G.S.I. was 0.15 and increase in the silver population until it reached to 0.44 with a large variation (see Table 3). The G.S.I. of yellow females was around 0.44, whereas the G.S.I. of silver Grevelingen females was around 1.45 (see Table 3). Only the G.S.I. between those yellow and silver female groups was highly significantly different ($P \leq 0.0001$), as shown in Table 2, respectively. The comparison between the G.S.I of the different genders, split up to development stage (yellow *vs.* silver) showed only a strong significant difference between yellow males *vs.* yellow female's ($P \leq 0.002$).

Table 2: P-values of a two sample T-test for G.S.I of hatchery eels split up to gender (male, female intersex), and stage (yellow, silver).

YELLOW EEL	2010-2011	2011-2012	2010-2012
MALE	$P \leq 0.316$	$P \leq 0.679$	$P \leq 0.552$
FEMALE	$P \leq 0.218$	$P \leq 0.290$	$P \leq 0.419$
INTERSEX	$P \leq 0.887$	$P \leq 0.406$	&
SILVER EEL	**2010-2011**	**2011-2012**	**2010-2012**
MALE	$P \leq 0.105$	$P \leq 0.293$	$P \leq 0.424$
FEMALE	N.D.	N.D.	N.D.
INTERSEX	&	&	&

Note: N.D. = not determined because comparison between yellow hatchery females and silver Grevelingen females is due to different background not relevant.**&:** Too low number of animals to compare between years.

Table 3: Mean G.S.I ± SD of hatchery eels split up to gender (undifferentiated, male, female and intersex), and stage (yellow, silver).

G.S.I	YELLOW EEL	SILVER EEL	P-Value
UNDIFFERENTIATED	0.9252 (n=1)	0.183263 (n=2)	-
MALE	0.1461 ± 0.007 (n=16)	0.1570 ± 0.048 (n=30)	$P \leq 0.556$
FEMALE	0.4382 ± 0.3324 (n=15)	1.4545 ± 0.2496 (n=24, $)	$P \leq 0.0001$ **
INTERSEX	0.1510 ± 0.055 (n=4)	0.4421 ± 0.5439 (n=4)	$P \leq 0.328$

Note: $ = 24 adult silver Grevelingen females; **: Denotes highly significant different.

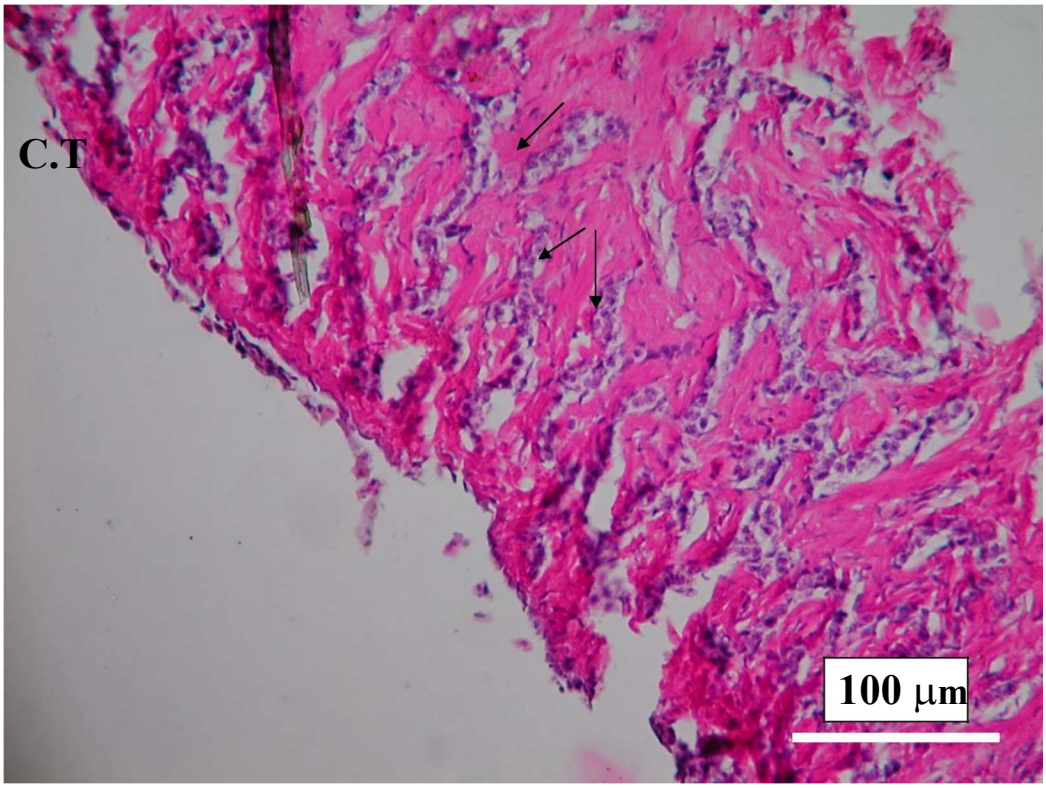

Figure 1: Undifferentiated gonad of yellow eel (W=155 g, L=42.5 g, G.S.I.=0.294, E.I.=16.01). Clusters of mitotic germ cells (**M.G.C.**) are visibly separated by fibrous connective tissue (**C.T.**). No spermatogonia or oogonia are visible.

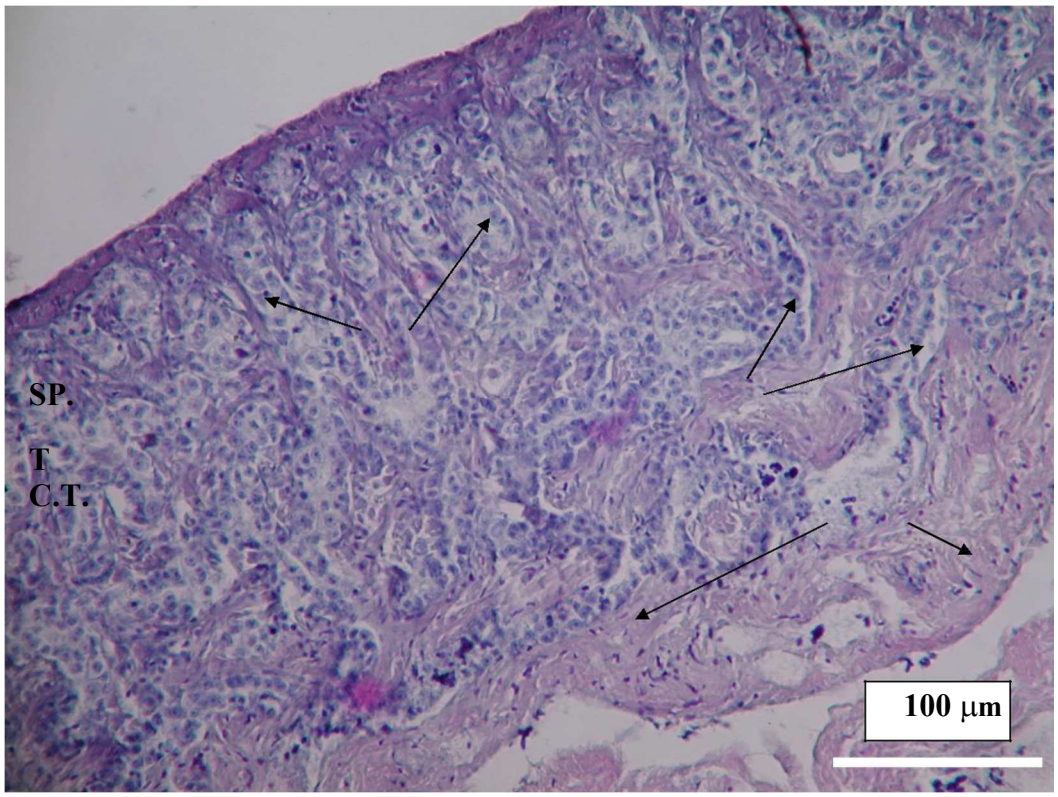

Figure 2: Testis gonad of yellow eel (W=148.9 g, L=43.8 g, G.S.I.=0.106, E.I.=7.47). Tubulair stage with clusters of spermatogonia (**SP**.), separated by connective tissue (**C.T.**) and tubuli (**T.**).

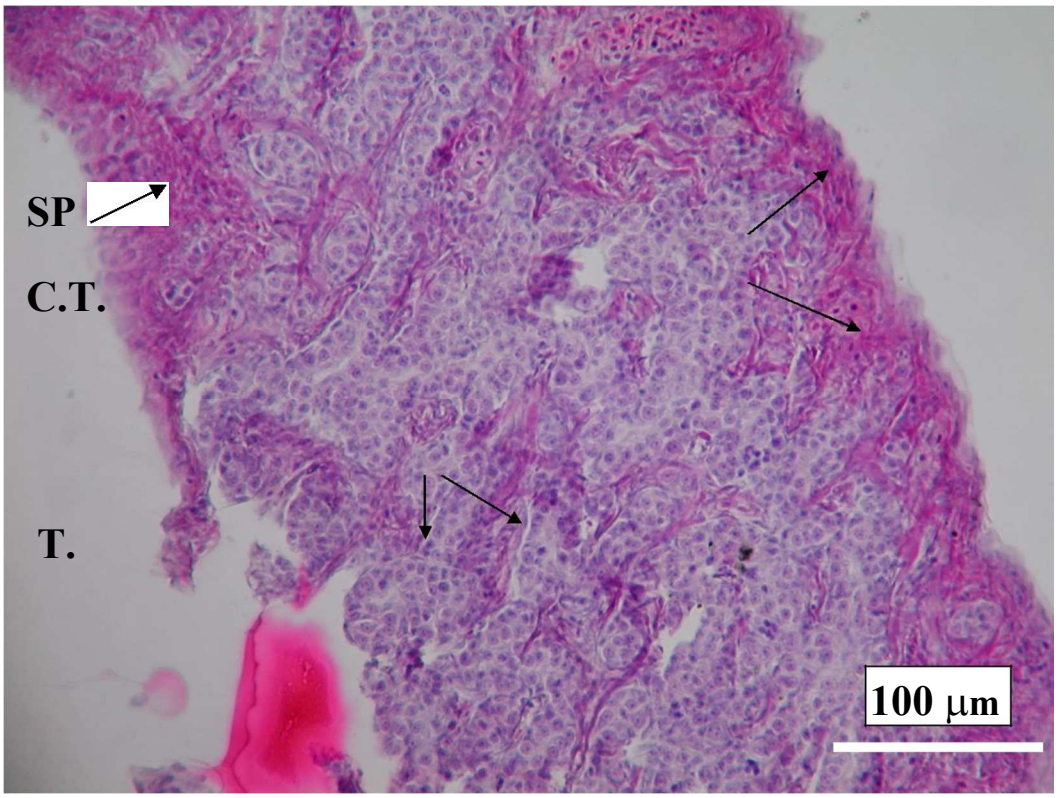

Figure 3: Testis gonad of silver eel (W=129.2 g, L=40.0 g, G.S.I.=0.169, E.I.=7.97). Tubulair stage with clusters of spermatogonia (**SP**.) separated by connective tissue (**C.T.**) and tubuli (**T.**).

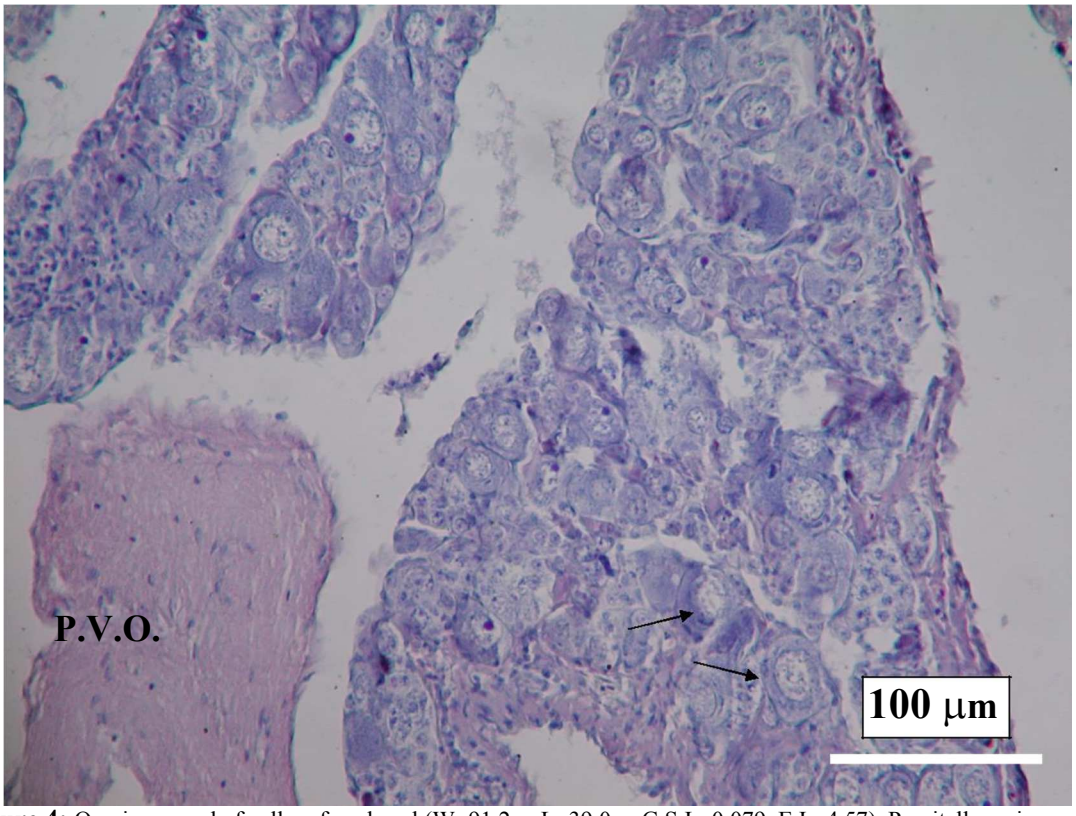

Figure 4: Ovarian gonad of yellow female eel (W=91.2 g, L=39.0 g, G.S.I.=0.079, E.I.=4.57). Previtellogenic oocyte (**P.V.O**.)-stage.

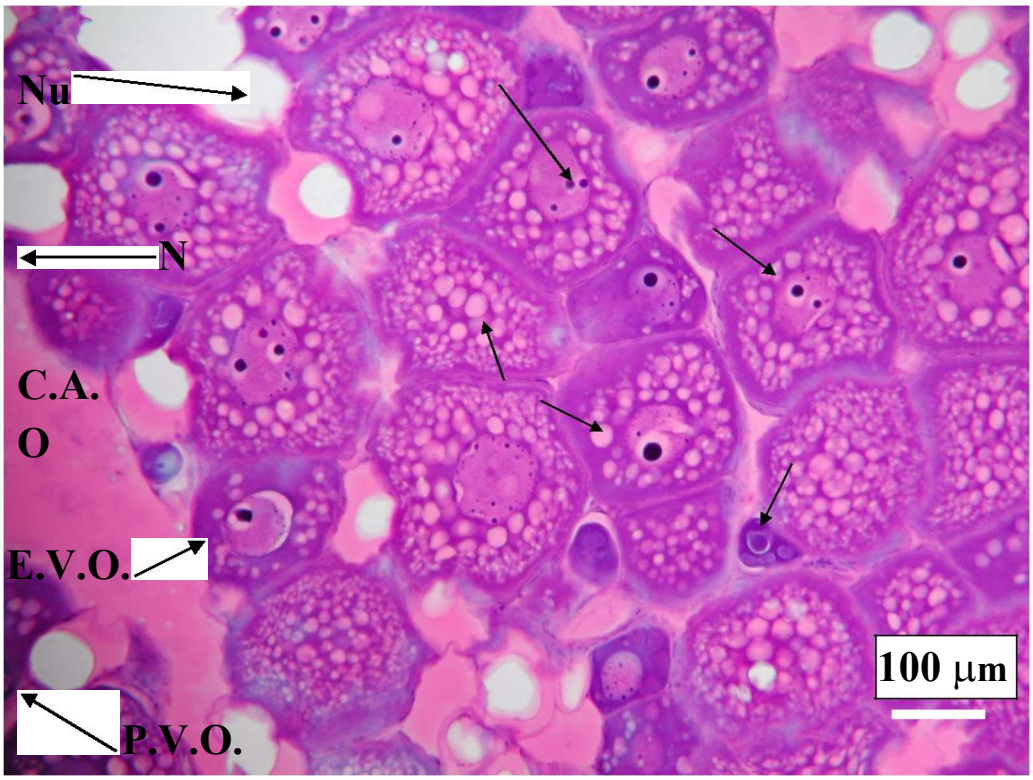

Figure 5: Ovarian gonad of silver female eel (W=1516 g, L=88.0 g, G.S.I.=1.5, E.I.=unknown). Lipid vesicle stage or cortical aleveolair stage. This stage is characterised by oocytes with oil droplets in the cytoplasm but not strongly basophilic cytoplasm. ***Abbreviations:*** Previtellogenic stage (**P.V.O**.) stage. Early vitellogenic oocyte (**E.V.O**.)-stage containg peripheral yolk granules; Cortical Alveoli (**C.A.**); Nucleoli (**Nu**); Nucleus (**N**); Oogonia (**O**).

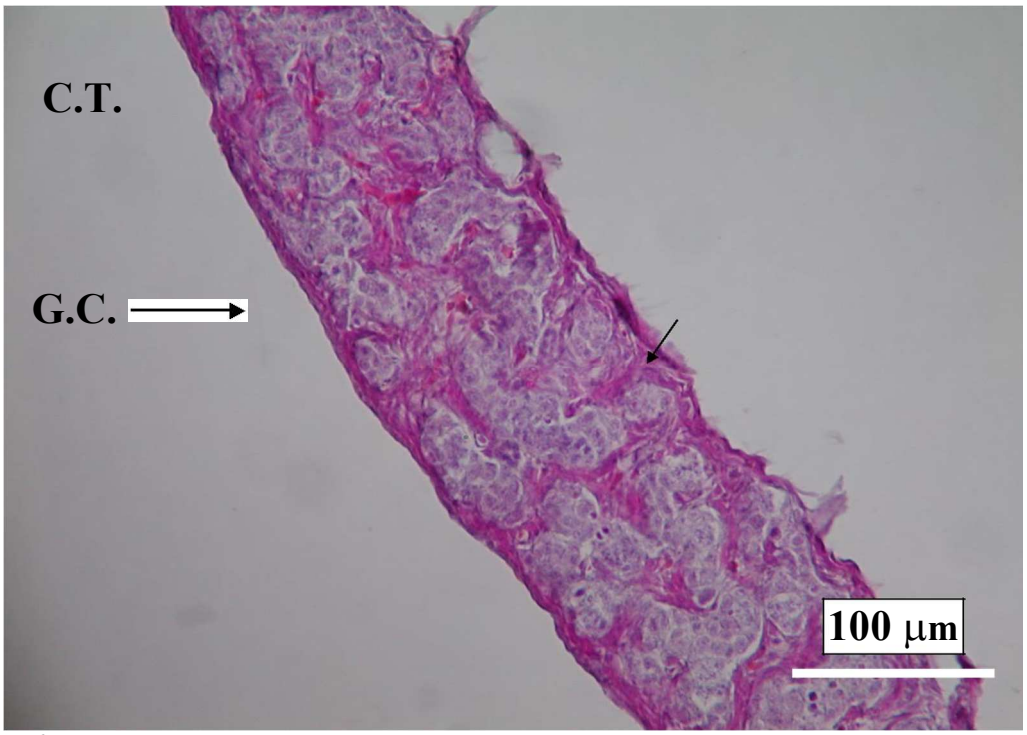

Figure 6: Example of a 'female' Intersex gonad of silver eel (W=98.6 g, L=36.5 g, G.S.I.=0.151, E.I.=14.74). Clusters of germ cells (**G.C.**) in the early prophase of the first meiotic division are visible separated by fibrous connective tissue (**C.T.**). This Intersex contains some female germ cells in meiosis.

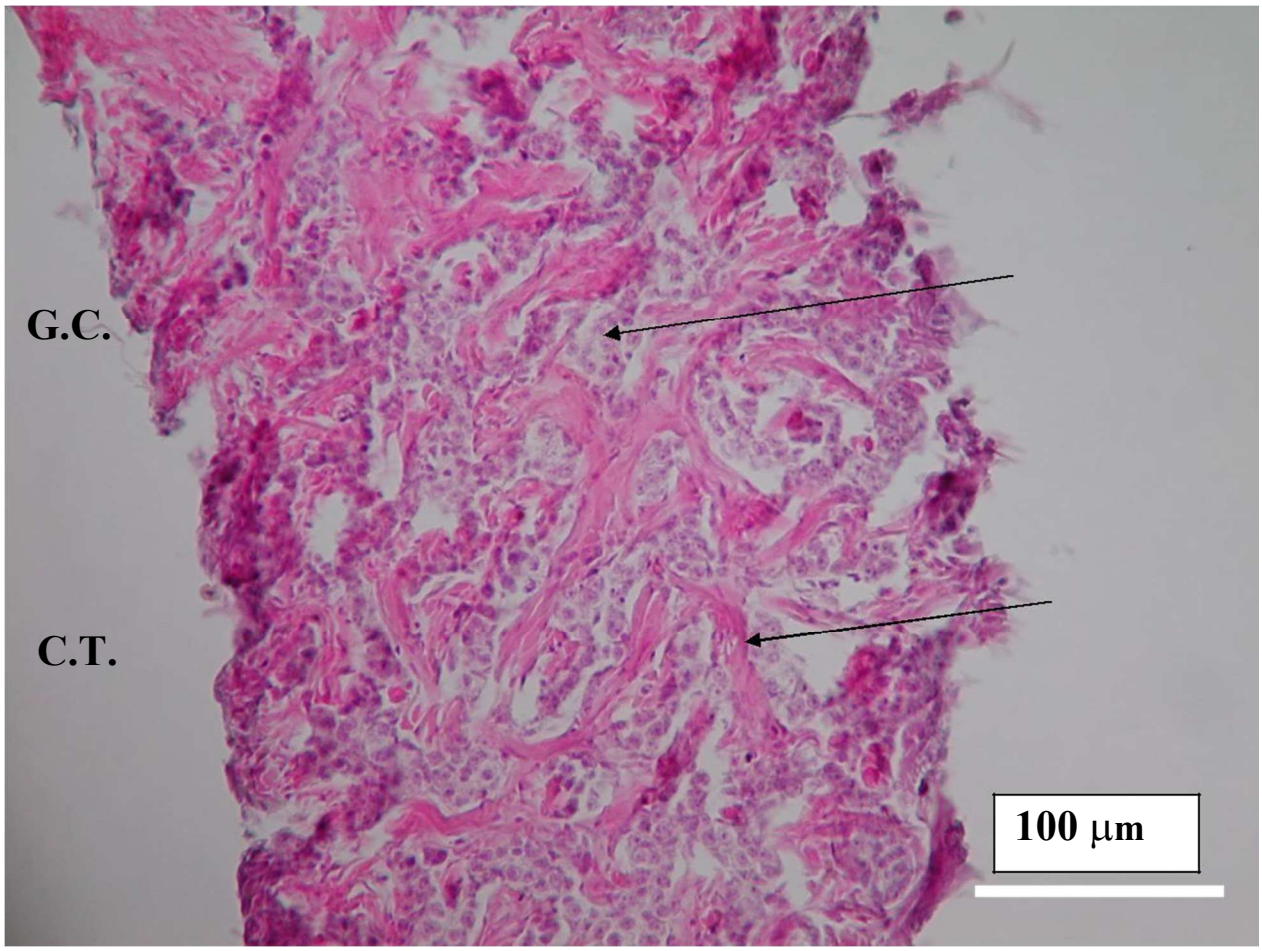

Figure 7: Example of a 'male' Intersex gonad of silver eel (W=109.8 g, L=41.5 g, G.S.I.=0.163, E.I.=9.98). Clusters of germ cells (**G.C.**) in the early prophase of the first meiotic division are visible separated by fibrous connective tissue (**C.T.**).

DISCUSSION

The main hypothesis of the present study was whether ESD or GSD determines the ultimate sex of eel fish or not. Male of eel grow faster and mature early than females. In many studies, there were clues for ESD in *Anguilla* species that certain environmental factors like temperature or stocking density and salinity can determine the sex of eel [7]. High temperature and salinity favours the development of males, while low stocking density and unfavourable condition of growth in habitat favours the growth of females [7]. Whereas, low temperature incubation did not affect sex ratio in *Anguilla rostrata* [8]. In contrast, elevated incubation temperatures for glasseel appear to enhance slightly male-biased sex ratios [9]. In *Anguilla anguilla,* high-stocking densitiesmay also gave a slightly more predominance for the appearance of more males in a given population [5, 10].

The description about ESD or GSD for eel the existence of sex chromosomes was also significant in the present study. However, there was a controversy in literature. Some workers report a ZW-system for three eel species including, *Anguilla anguilla*, *Anguilla japonica* and *Anguilla rostrata* [11-13]. In contrast, other researchers from karyological investigations had reported no such sex chromosomes in eel fish [14-18].However, it has been observed that the occurrence of sex chromosomes is not a prerequisite for GSD. This might be because sex determination is very complex depending on many factors that regulate cellular and hormonal signals. The net effect for the individual is arising from the summated total influence of all genetic factors involved in sex determination in the genome [19]. Still, within the pathways, some components may become dominant in influencing the direction of sex determination in such a way that environmental factors can influence might be reduced on sex determination. Therefore, in all such conditions, the sex of a particular

individual will ultimately be determined by the strength of these genetic factors [19]. Thus, it can be explained that fish species without clear sex chromosomes like eel still can have GSD [14-17].

The basic problem with sex differentiation studies was that they must take place over several years, and selective mortality and fluctuating husbandry conditions may also affect and bias the end result. A new aspect which became clear from this our present study was that a distinction has to be made between yellow and silver eel stages. Sex ratios were differ between the developmental stages. Only yellow eel fraction developed from the sex labile glasseel stage was only suitable for conclusions about ESD or GSD. In the Japanese studies with 75-90 males on ponds and greenhouses, no clear distinction was made between silver or yellow eels [3-4]. In addition to these Japanese studies, we also found that silvery eels were nearly all males. The metamorphosis from yellow to silver stage can bias the sex ratios to great extent.

In theory, three mechanisms can be possible. **First,** during the transition from yellow to silver stage, there was a change of the sex in the direction of male. This interpretation was at first sight unlikely, because studies with oral administration of estradiol indicated that the sex labile phase was appear at the glass eel stage [18-20]. Anobservation must be made that sex reversal can also occur at older age [21]. Estradiol treatment of gonadally undifferentiated European eels causes feminisation. However, when treatment was delayed until post-morphological differentiation of testis had occurred, sex reversal could still occur and resulted in 44% females (normally 95% male in the original glass-eel population) [22]. This indicates that the sex is still to some extent labile in eels that are already differentiated. This topic needs to be studied and elucidated in future studies. **Secondly,** the differences in growth rate between males and females lead at an earlier stage to a sampling preference of the farmer for females. This can be explain while no females were found to be observed in silver groups. Both males and females have different growth strategies. In the study of Holmgren & Mosegaard [23],individual growth of eel was determined by implanting microchips and their study also clearly demonstrates the different growth strategies among male and female sexes. During early developmental stage when fish body weight was less than 40-60 grams, males grow faster than females, however, the growth of male's stops prematurely at 150 grams weight, while females continuously grow faster above this weight. It means females grow continuously for a longer period of time with increase in length and weight, whereas, males grow in such a way to increase its whole body weight but such increment was not shown in its body length [22]. In biological terms, males are 'time minimisers' means shorter in length as they mature at the earliest stages of life, while females are 'size minimisers' as they mature in later stages of life, therefore, its body size increase more and attain higher fecundity [24]. Likewise in our present study, the weight group belong to class 100-150 grams of silver eels were found to have no female fish, which might be because of differences in growth strategies between males and females or Females, which are 'size maximisers', when reached at market weight are consequently sampled by the farmer, when still at the yellow stage. Thirdly, the second metamorphosis of silvering resulting in a catadromic migration, is (split up to gender) also age dependent. Male silver eels may start their oceanic migrating after 4 years or more, while females starts migration after 7 years or more. This statement can be illustrated by an ecological study with elvers stocked in 1980 in a Swedish lake. The majority of males (64.5%) when became silver after 4 to 5 years old, start their seaward migration, while the majority of females (86.3%) started their seaward migration when they reached at the ages between 10-15 years, respectively [25]. The age related to silvering of the different sexes might be resulted in the hatchery in an earlier silvering of the males, which can explain by the abundance of males in the 1-3 years old silver fraction in the hatchery.

CONCLUSIONS

From the present study, it was concluded that by using histological techniques, different stages of gonad development can be examined in both yellow and silver European eels. Based on the observation that the ratio of males *vs*. females in the yellow group was 50:50, we conclude that GSD is prevalent at the yellow stage, therefore, we can reject our initial hypothesis about Environmental Sex Determination (ESD), however, this result of our present study was also in contrast to earliest authors who observed ESD as sex determining mechanism [5-9].The observation that in the silvery group, most fishes were males can be probably explained by differences in growth and silvering strategy. This will result in an earlier culling of the females by the farmer.

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