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Histological and hormonal changes towards the spawning season of female killifish *Fundulus heteroclitus* from the great bay in New Jersey U.S.A.

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Abstract : Fundulus samples were collected on monthly basis during the period from February 2015 until May 2015 There was a significant increase in the gonadosomatic index started in March and continued in April and May. Spawning seem to have started in March because large proportion of oocytes were at the ova stage and there were also calyxes of ovulated follicles. Thyroid stimulating hormone (TSH) , follicle stimulating hormone (FSH) showed variations that could be attributed to the physiological processes involved during the different stages of oocytes maturation.

Introduction

The reproductive cycle of many fishes is reflected by significant changes in the size of gonads throughout the year [1]. In the majority of teleostean species gonadal weight depends in part on body weight. One of the ways to account for the effect of body size on gonadal size has been to represent gonadal weight as a percentage of body weight which is called thegonadosomatic index (GSI). Although this percentage is used as an indicator of relative gonadal development or activity , it conceals the fact that several physiologically discrete processes take place within the gonad. It is, therefore, more convenient to use the actual changes in oocyte's development to know what is actually occurring in the ovaries[2].

The growth of an oocyte can basically be divided into two phases: the first growth phase which involves an increase in the size of the oocyte, the secondary growth phase in which yolk is accumulated in the oocyte [3]. Weight changes of some visceral organs such as liver may be inversely correlated with oocyte development [4]. Liver changes are generally expressed as a percentage of body weight minus gonad's weight which is called the hepatosomatic index HSI .

Oocyte Maturation

The post-vitellogenic oocyte may remain quiescent for several months but following environmental, social, or pheromonal cues, it begins the process of final maturation. This will commence with a surge in gonadotropin-releasing hormone (GnRH), with or without a decrease in dopaminergic inhibition, followed by a rise in circulating LH. Upon binding of LH to its receptors on the granulosa cells, the ovarian follicle starts the process of maturation, beginning with the production of the maturation-inducing steroid (maturation-inducing steroid (MIS) such as DHP or 17a-20j,21 Trihydroxy-4-pregnen-3one (20j-S)). Binding of the MIS to its receptors on the oocyte plasma membrane is followed by activation of the maturation-promoting factor (MPF), a complex consisting of existing cdc2-kinase and newly synthesized cyclin. The process of oocyte maturation is

reflected morphologically by the migration of the germinal vesicle (GV) toward the animal pole (GV migration) and the disintegration of its membrane, a stage known as GV breakdown (GVBD). The chromosomes then condense, a spindle is formed, and the first polar body is extruded which marks the end of the first meiotic division. At this stage the oocytes absorb water and inflate; this is especially pronounced in marine fish with pelagic eggs. The pressure within the follicle increases, the follicular wall is ruptured, and the oocyte is released (ovulated) into the ovarian lumen or to the coelomic cavity. The meiosis is arrested again at metaphase II. Completion of the second meiotic division and extrusion of the second polar body are further delayed and will proceed only if the egg is fertilized. Vitellogenesis is a process in which yolk proteins are produced in the liver, transported to the ovary and stored in the egg; resulting in tremendous egg enlargement. When conditions are appropriate for final maturation, nuclear development resumes, and the germinal vesicle migrates to one side. Finally, the walls of the germinal vesicle break down and maturity development completes. The association of changes in gonadal development with plasma levels of gonadal steroids has proven to be a valuable tool for understanding the endocrine control of reproduction in teleosts. Moreover, in teleosts, vitellogenesis and final oocyte maturation are regulated by gonadotropins via steroids secreted by the granulosa and theca cells of developing and mature oocytes. The occurrence of steroid production in different cells of the ovary may be related to different phases of oocyte development. Estradiol, (E2) stimulates in fish[5].

The 38 species that currently constitute the genus Fundulus[6] are distributed throughout North and Central America. Fishes of this genus inhabit diverse ecosystems encompassing extremes of abiotic parameters such as dissolved oxygen, pH, temperature, and salinity [7]. Close phylogenetic relationships between species occupying very different physicochemical conditions make Fundulus an opportune genus in which to study the evolution of physiological plasticity in fishes. [8] described the estuarine Fundulus species of eastern North America, mummichog F. heteroclitus and Gulf killifish F. grandis (Actinopterygii, Cyprinodontiformes, Fundulidae), as premier field and laboratory models for understanding how teleost fishes interact with their environment on an individual and population-level basis. This article seeks to improve understanding of the reproductive physiological activities in F. heteoclitus, with the potential to utilize this knowledge to improve understanding the physiology of reproduction of this species and may be other closely related Fundulidae.

In addition to scientific examination, F. heteroclitus and F. grandis are economically and ecologically important in their respective native ranges. In northeastern Florida, geographic distributions of F. grandis and F. heteroclitus overlap [9]; with the latter extending in range up the east coast of North America to the north shore of the Gulf of St. Lawrence, Canada[10].

Materials and Methods

Samples of adult Fundulusheteroclitus were collected from the Great Bay., New Jersey on monthly basis from February 2015- May 2015 .Total length to the nearest 1 mm and total body weight to the nearest 0.1 gm. of each fish were taken. Blood samples were then taken by heart puncture from each fish and were pooled and centrifuged at 3000 g for 15 minutes to obtain serum for hormonal analysis.Sera were stored at minus 80 until analysis. Fishes were then dissected out to expose the ovaries and liver which were weighed to the nearest 0.1 gm.

To assess gonadal development, transverse " steaks" from half-way along the length of the ovary were cut and fixed in 10% formalin [11]. Out of these " steaks" those with gonadosomatic index nearest to the mean were embedded in paraffin, sectioned at 5 uM thickness, stained in haematoxylin and eosin and examined under the microscope and photographed at different magnifications.

Follicle stimulating hormone (FSH) and Thyroid stimulating hormone (TSH) levels were measured usingTSH and FSH Elisa kit supplied by Enzo company (USA) by following the steps according to the kit procedure. Three replicates for each hormone were used. The mean for the replicates was taken to compare the possible variations during the months of study.

Results and Discussion

Morphology of the ovaries

The ovaries were paired elongated organs lying dorsal to the body cavity. The ovaries were suspended by mesovarium which runs along the ovaries .The ovaries were yellowish orange and compact during the early stages of maturation and much less compact in the last stages of maturation.

Stages of maturation

Six stages of oocyte development were distinguished in the maturing ovaries according to [12]. They were:

1. Oogonia were found in the epithelium of the ovaries but were hardly seen due to their very small size (fig.1).
2. Primary oocyte- first growth phase(previtellogenic): They were either early first growth phase or late first growth phase depending to the size , staining affinity and the number of nucleoli. The early growth phase oocytes were smaller , stained darker and had less nucleoli compared to late growth phase primary oocytes The granulosa layer was thin in the early first growth phase and became thicker as the oocyte grew larger.
3. Primary oocyte- secondary growth phase (vitellogenic oocyte): At this stage the zona radiate began to form, and the cytoplasm had less affinity to haematoxylin. This stage was divided into two stages:

a. Endogenous vitellogenic oocyte **:** The cytoplasm lost it's great affinity to haematoxylin stain (fig. 1). This stage is characterized by the presence of vacuoles which first developed at the periphery (fig.1) and spreaded throughout the cytoplasm. Yolk first appeared in the vacuoles at the center of the oocytes and spreaded outwards. Granulosa,theca and zona radiatalayers were moderately developed. The number of nucleoli increased and moved near the periphery of the nucleus (fig.1).

b. Exogenous vitellogenic oocytes: They were characterized by the presence of yolk granules which stained with eosin (fig.3).The smallest yolk granules were present at the periphery of the cytoplasm and the larger ones were usually at the center.No final fusion of individual granules was observed. The zona radiata(oolema or zona pellucida) ,which contains radial canals through which yolk particles are passed to the oocyte cytoplasm [12], were well developed (fig. 1:d)).

4. Secondary oocytes: This stage of oocytes are formed after the first meiotic division. At this stage the oocytes are usually translucent.

5. Ova: They are formed after the second meiotic division is completed. This stage is followed by ovulation, a process in which ova are released from the surrounding follicles.

6. Atretic oocytes: Unspawned ova are degenerated and atretic oocytes are produced. Due to the changes in the follicular layers of the oocyte, the whole follicular structure of the oocyte appeared to be in a state of disorganization as remains of the oocyte and zona radiata layer were gradually being resorbed (fig.2 &3).

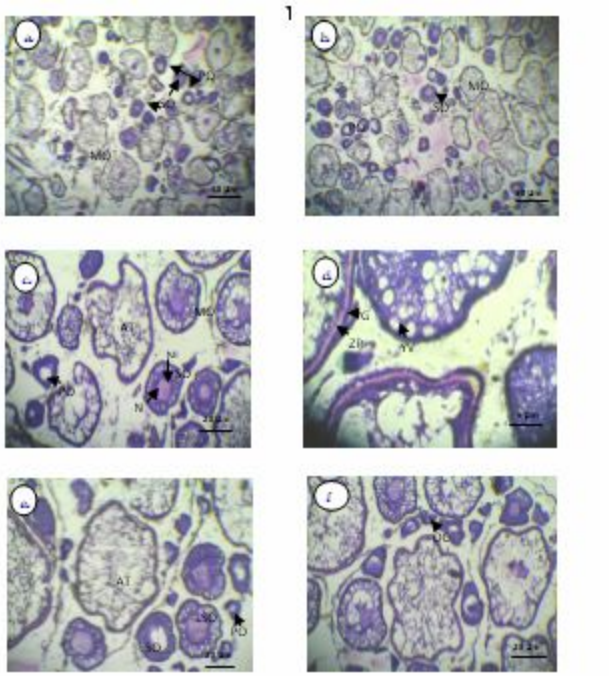

Figure (1) : Transverse section in the ovary of *Fundulusheteroclitus* showing various stages of oocytes (a) and (b) primary oocytes PO, secondary oocytes SO and mature oocytes MO. (H&E) 40x . (c) primary oocytes PO, secondary oocytes SO, mature oocytes MO , nucleus N , nucleolus NL and attractive oocytes AT. (H&E) 100x. (d) zona pellucida ZP, ------ G and yolk vessels YV. (H&E). (e) attractive oocyte AT, primary oocytes PO, late secondary oocytes LSO, and secondary oocytes SO. (H&E) 100x. (f) oogonia OG. (H&E) 100x.

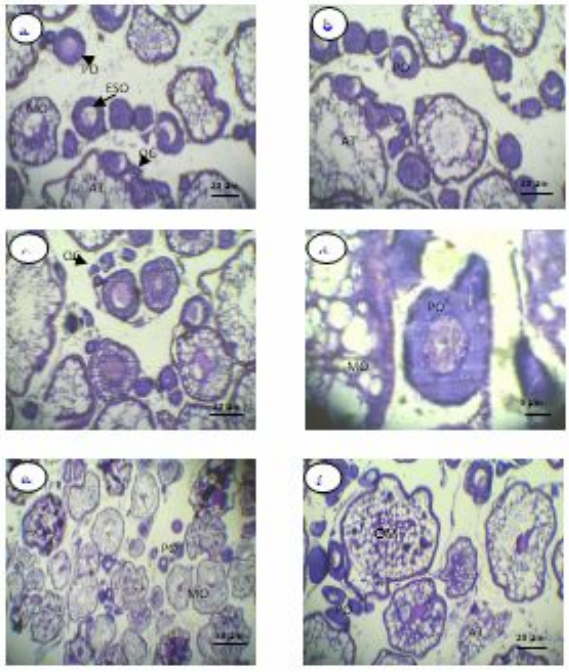

Figure (2) : Transverse section in the ovary of *Fundulusheteroclitus* showing various stages of oocytes. (a) oogonia OG, primary oocyte PO, early secondary oocyte ESO, secondary oocyte SO, mature oocyte MO and attractive oocyte. (H&E) 100x. (b) pimary oocyte PO and attractive oocyte AO. (H&E) 100x. (c) oogonia OG. (H&E) 100x. (d) primary oocyte PO and mature oocyte MO. (H&E) 400x. (e) primary oocyte PO and mature oocyte MO. (H&E) 40x. (f) primary oocyte PO, mature oocyte MO and attractive oocyte AO. (H&E) 100x.

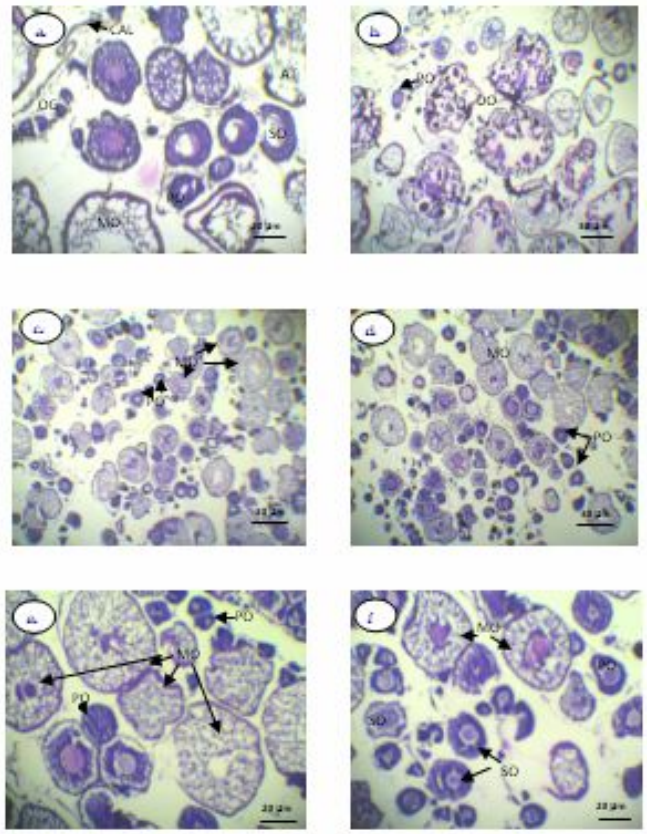

Figure (3) : Transverse section in the ovary of *Fundulusheteroclitus* showing various stages of oocytes. (a) oogonia OG, calyx CAL, primary oocyte PO, secondary oocyte SO, mature oocyte MO and attractive oocyte AO. (H&E) 100x. (b) primary oocyte PO and degenerating oocyte DO. (H&E) 40x. (c)(d) and primary oocyte PO and mature oocyte MO. (H&E) 40x. (e) primary oocyte PO and mature oocyte MO. (H&E) 100x. (f) primary oocyte PO, secondary oocyte SO and mature oocyte MO. (H&E) 100x.

Gonadosomatic changes

The values of the gonadosomatic index (GSI) increased slightly in March and continued to increase in April and May which indicate that more oocytes were continues developing even after the onset of spawning episode of this species in March which explains the long spawning season of this species. This result is in accordance with the results obtained by [13]. The hepatosomatic index followed almost the same pattern of GSI (Fig.4) which may be related to the role it plays in the reproductive cycle during the exogenousvitellogenesis for it's role in vitellin production[14].

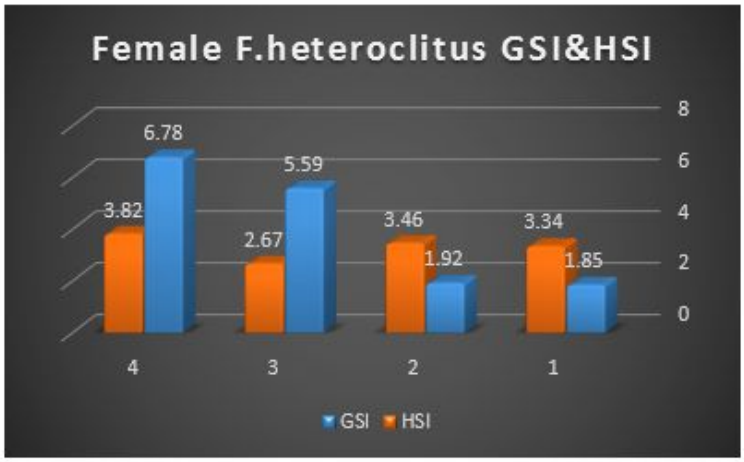

Figure 4: Female *F.heteroclitus GSI* &HSI.

Proportions of oocytes stages

First growth phase primary oocytes were the only cells that were present in the ovaries throughout the period of study which means that more and more oocytes are developed even after spawning had begun which explain the long spawning episode of Fundulusheteroclitus. This was confirmed by the presence of secondary growth phase throughout the period of study. Ova (mature oocytes) started to be found in relatively high proportion in March onwards which means that the ovaries contained large numbers of mature oocytes ready to be ovulated and shed in March onwards. Ovulation and shedding of ova was confirmed by the presence of a fair number of atretic oocytes (fig.3).

Hormonal changes

Follicle stimulating hormone levels showed a significant increase in February and remained high in April and decreased in May in comparison to its levels in February which means that oogenesis had become active in March following the rise in Follicle stimulating hormone levels in February and subsequently the start of the spawning season because FSH plays its role in developing more oocytes during the resumption of the reproductive cycle which seems to have been influenced by the change in temperature and feeding activity in March which represent the beginning of the Spring.

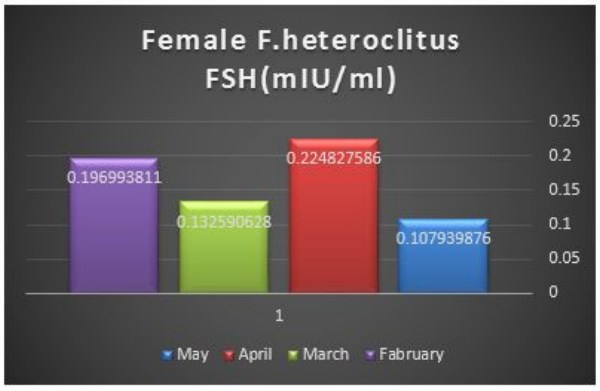

Figure 5: Female *F.heteroclitus FSH (mIU/ml)*

On the other hand, thyroid stimulating hormone (mIU/ml) showed significant changes throughout the period of study (fig. 6). This might lead us to suggest that the thyroid gland might have had a direct or indirect role in the reproductive cycle of this species because it has been suggested that thyroid hormones might play a significant role in storage products deposition and mobilization since and therefore the thyroid hormones may play a direct or indirect effect on the reproductive cycle of fishes especially those known to accumulate energy sources such as lipids in their muscle or viscera to be mobilized and utilized during the reproduction season[11].

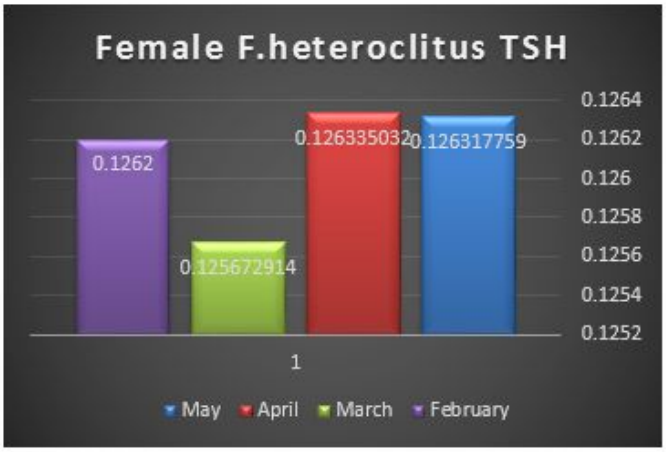

Figure 6: Female *F.heteroclitus TSH.*

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