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Some Aspect of Reproductive Biology on the Effect of Pollution on the Histopathology of Gonads in *Puntius Javanicus* from Mas River, Surabaya, Indonesia

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Abstract

The present work aimed to study some aspect of the reproductive biology of the *P. javanicus* with emphasis on histopathology abnormalities of gonads and intersex problems. Fish samples of *Puntius javanicus* (local name:*tawes*) were collected from Mas River on the Surabaya downtown whereas highly pollution. Fishes were identified for morphological abnormality and gonad organs were evaluated. Histological preparation was done to observe the gonad condition. This report reveals that 20% male *P. javanicus* were found to be feminized on July 19, 2012 collection from that river. Some histological samples indicated the presence of testis-ova in fish gonad, which indicated the occurrence of intersex of this species. The above finding suggests that greater attention needs to be given to xenobiotic pollutants and endocrine disrupting chemicals problems.

Keywords: Mas River, Pollution, Reproductive biology, Histopathology, Gonads, Intersex

1. Introduction

Mas River is a branching rivers and the lower reaches of the Brantas River Basin (Surabaya River) which located in the city of Surabaya and flowing toward the coast north through the center of Surabaya and empties into the Madura Strait. Mas River has small current relatively to substrate base muddy and rocky generally. This river is approximately 13 km distance from Wonokromo up to estuary, between 25 m to 35 m of wide with a depth ranging from 1.5 cm to 3.5 m (Bayu, 1997). Maximum discharge of water was on March (49.34 m^2/s) and the minimum was on October (1.88 m^2/s). Mas River's flow splitting Surabaya city with a variety of human activities, such as industrial activities, settlements, a boat tour, water sport and other extremely dense human activities. Bayu, (1997) recorded that Mas River's water had 28°C of temperature with 746 NTU of turbidity and pH of 6.6. The water of the river became the main source for the PDAM Surabaya and industries along Surabaya River. Heavy metal content exceeds water quality standards with the highest range for Cd value of 0.007 to 0.039 mg / l, Hg at 0.031-0.056 mg / l and Pb at 0.068 to 0.752 mg / l (Putri *et al*., 2011).

There are about 600 factories spread along the Brantas River but only 60 which having standard level of waste processing system (Ecoton, 2008). Water quality in the Mas River is one of the worst compared to other branching sites at the Brantas River flow. Some sources of waste are originated from household activities, markets, drainage and non-domestic activities around the river. The Mas River main function today is as a sewage and drainage channels in the city of Surabaya, especially those in the middle. Putri *et al*. (2011) has made measurements of some water quality parameters in the Mas River. Several water quality parameters measured including DO, BOD, COD, TSS and NH3-N (Table 1).

Table 1. Water quality parameters in the Mas River (Putri et al., 2011)

Parameter	Value (mg/l)	Water Quality Standards
DO	1.27 - 3.04	4*
BOD	9 – 37	3*
COD	16 – 60	25*
TSS	142 - 182	50*
NH3-N	0.41 - 1.38	≤ 0.02*

*Indicates that the value exceeds the max allowed by Indonesian Government (PP no 82 2001) for fisheries activity

The chemical pollutants distributes through the aquatic environment may give impact to aquatic organisms and found to interact with the vertebrate endocrine system. Xenobiotics thought to act as endocrine disrupters, characterized as the pollutants as a result of anthropogenic impacts on the environment, which is central to the study of research on ecotoxicology (Allner *et al*., 2010). EDC phenomenon has been reported and occurred in some parts of the world. In a small creek downstream of a paper mill discharging bleached kraft mill effluent had been found the presence of masculinised female mosquito fish (*Gambusia affinis*) that induced male secondary sexual characteristics (Howell *et al*., 1980). That study appeared that the effluent contained an androgenic chemical or mixture of chemicals. In the Red River of the North in North Dakota, USA has been reported female channel fish (*Tetalurus punctatus*) exhibiting male secondary sexual characteristics (Hegrenes, 1999). He stated that there are no paper mills discharging effluent into this river, although it does receive waste water from a sewage treatment plant and a sugar beet processing plant. Exposure to treated sewage effluent has also been associated with deleterious effect on gonad differentiation and development in various species of fish and the abnormal development (feminization) of secondary sexual characteristics in male mosquito fish (*Gambusia affinis*) in Australia (Batty and Lim, 1999). Intersex in the cyprinid fish commonly occurred at lowland rivers in the United Kingdom. The fish often have both male and female reproductive ducts and many also have female germ cells (oocytes) within a predominantly male testis. In contrast to the effects observed in female cyprinid fish, there was higher incidence of oocyte atresia and a slight, but statistically significant, lower fecundity in effluent-exposed fish compared with female from the references sites (Jobling *et al.*, 2002). In Taiwan, the intersex tilapia collected from Era-Jiin River were mostly likely caused by endocrine disrupting chemicals, especially when the ratio of intersex was 50% and these fishes were natural inhabitants on this river (Sun and Tsai, 2009).

Our study took place at the Mas River located in the Brantas Deltas area, which received water flow from the Brantas. The past fishes collection from the Brantas River has been done in 1997 by Risjani *et al*. (1998). There were more than 50 fishes species collected from the Brantas River. But in 2011, the number of species decreased. The number of fish species on the Brantas River downstream segments recorded by ECOTON (Ecological Observation and Wetlands Conservation) (2011) found 30 species of freshwater fish, one of which is *Bader putihan* or *Tawes* (*Puntius javanicus*). The causative effect of the decrease of fish species number was pollutant from anthropogenic activities (Risjani *et al*, 2012). In relation with these huge potential impacts on downstream Brantas River basin, especially from domestic waste, household industrial activities, agriculture and the chemical industry, it is necessary to study what extent the effect of pollutants on fish gonad histopathology *P. Javanicus* in the Mas River, which is expected to provide benefits and used as an indicator of ecotoxicology.

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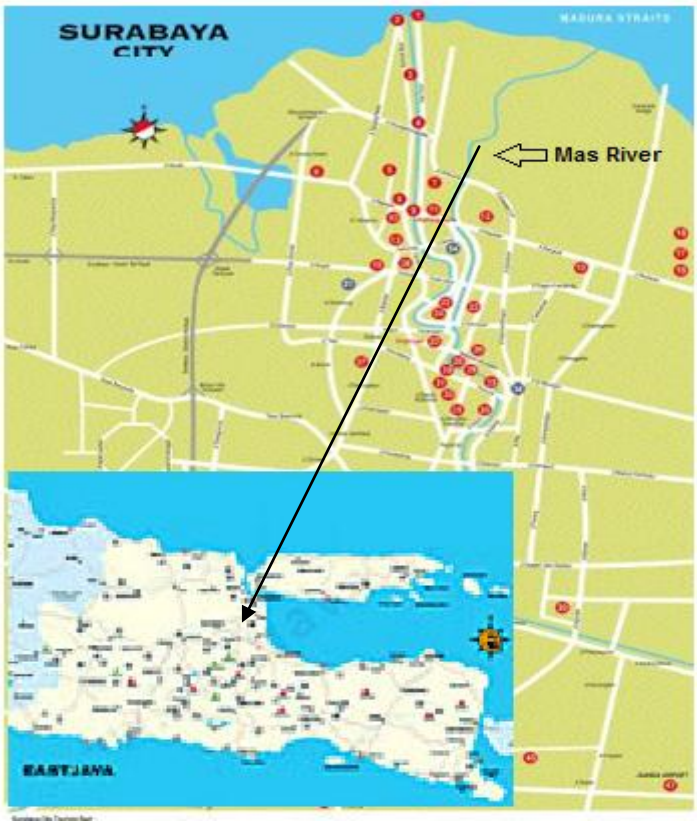

Figure 1. Location of the rivers and sampling station (arrow)

2. Materials and Methods

In order to study the effects of pollution on fish reproduction, samples were collected from along the Mas River (Fig. 1) using gill net (with 3-cm stretch mesh) on July 19, 2012. In total, 10 *P. javanicus* were collected. Fish were identified (length, fish weight, gonad weight) and sexed for morphological evaluation of gonadal maturation. Linear allometric model (LAM) used to calculate the parameters a and b by measuring changes in weight and length. Correction of error in the average weight change from logarithmic unit used to predict weight at length parameter according to the allometric equation: $\mathbf{W = a\ L^b}$ (De Roberts and William, 2008). **W** is the weight of fish (g), **L** is the total length of fish (mm), a and b are parameters. The relative weight (Wr) and coefficient (K) factor conditions was used to evaluate the condition of each individual factor. The relative weight (Wr) was determined by the equation: $\mathbf{W_r = (W/W_s)\ x}$ 100 (Rypel and Richter, 2008). $\mathbf{W_r}$ is the relative weight, **W** weight of each fish, and W_s is the standard weight predicted from the same sample as calculated from the length-weight of regression combined with the distance between species: $\mathbf{W_s = a\ L_b}$. Fulton condition coefficient (K) was determined by formula: $\mathbf{K= WL^{-3}\ x\ 100}$ (Okgerman, 2005). **K** is the condition factor, **W** is the weight (g), **L** is the length (mm) and $^{-3}$ is the coefficient lengths to ensure that the value of **K** tends to 1. The gonadosomatic index was calculated for all the specimens as the percentage of gonad weight to the gutted fish weight. Gonadosomatic Index = [gonad weight (g)/ body weight (g)] x 100 (Mazrouh dan Mahmoud, 2009). Gonads were collected by dissecting the fish and inserted into bottle film containing 10% formalin

solution in order preserving, then stored into the coolbox to be taken to the laboratory for histological preparation.

3. Histological Preparation

3.1 Fixation

Gonad was put into 10% formalin and allowed to stand for at least 24 hours. Cut across and put into tissue tex.

3.2 Dehydration

Gonad was entered consecutive into a solution of Alcohol 70% I, 70% II, 90% I, 90% II, Alcohol Absolute I and II for 45 minutes respectively.

3.3 Clearing

Gonad was entered into the solution of pure xylol I, II and III for 45 minutes respectively.

3.4 Infiltration

Gonad was put into liquid which content of xylol : paraffin (1:1) for 20 minutes, and then enter the liquid paraffin I, II and III for 20 minutes in the oven at 60 ° C respectively.

3.5 Embedding (sample planting) and blocking (blocks making)

Gonad was put into paraffin solution I, II for 45 minutes respectively. Then the samples were taken and placed on a stainless steel mold that was heated on a hot plate. After the sample laid out above the mold, the liquid paraffin was poured over the mold until sample piece immerse all.

3.6 Sectioning (slicing) and laying down on a glass object

Blocks were fitted on the pre-set microtome 5-6 microns thick. The cutting was done to low and constant, when the slices have reached the sample, then slices was transferred into a basin of cold water and placed on glass objects that have been coded, and dipped into a water bath in order to the sample expands. Preparations in glass objects were dried on a hot plate with a temperature of 28 °C.

3.7 Affixing

Gluing by using the albumin and glycerin in the ratio of 1:1 and was stored in a box stock for 1 day

3.8 Deparafinisation

Deparafinisation were done to remove the paraffin by putting down into xylol for 10 minutes.

3.9 Staining (coloring)

Histological preparations that have been dried was moved and processed for haematoxylin and eosin staining:

a) The xylol on histological preparations was sucked by using the filter paper. Then row was inserted into alcohol 96%, 90%, 80%, 70%, 60%, 50%, 40% and 30% respectively for 5 minutes then into distilled water for 5 minutes. And was washed by water flows for 2 minutes approximately.

b) The histological preparation was put into haematoxylin for 4 minutes.

c) The histological preparation was washed by water flows for 10 minutes.

d) The histological preparation was inserted into the distilled water and 50% alcohol, 60%, 70%, 80%, 90% and 96%, respectively some dyes.

e) The histological preparation was inserted in eosin for 1.5 minutes.

f) The histological preparation was inserted on alcohol 70%, 80%, 90% and 95%.

g) The histological preparation was dried and put into xylol for 15 minutes.

h) Canada balsam was poured and covered with a cover glass.

3.10 Mounting (Closure) and Labeling

The histological reparations were closured by using the cover glass and give identity to the preparations.

4. Result and Discussion

4.1 Fish Morphology

Puntius caught at the time of the study were 10 individuals with unbalanced gender ratio, with composition of 4 males and 6 females. There were unequal number of male and female fish caught allegedly due to differences in behavior and catching factors, such as male fish are active, causing the arrest of opportunity is smaller than the female (Fatimah, 2006). Size of fish body length varies between 110-155 mm with the weight range of 30.5 to 53 g. Male fish caught at 130-155 mm in length and weighing 0.35 to 0.54 g. Meanwhile, female fish were caught on the length of 110-150 mm and weight of 30.5 to 52 g (Table 2).

Table 2. Morphological deformities and other abnormalities in *P. javanicus* collected in the Mas River

Item	Description	Percentage (%) of prevalence	Number of fish discovered among these 10 *P. javanicus*
Fin and body surface	Ripped fin, body surface slimy	80	8
Scale	Chipped, necrosis	40	4
Liver	Chocolate color	90	9
Gill	Mucus secretion, necrosis, pale	50	5
Body weight (g)	30.5-53 g		
Gonad weight (g)	0,11-0,54 g		
Body Length (mm)	110-155 mm		

4.2 Reproductive Biology of Fish

a. Length Weight Relationship

Table 3. Length weight relationship and condition factor of *tawes* fish (*P. javanicus*)

Parameter	Value	Average	Standar deviation (SD)
Total length (TL) mm	110-155	135	15.63
Weight (W) g	30.5-53	45.15	7.86
The predicted weight (Ws)	45.15		
Relative weight (Wr)	67.55-117.39	100	17,40
Fulton condition factor (K)	0.67-1.17	0.002	0.00
Index coefficient of determination (R2)	0.876		
Index correlation coefficient (r)	0.936		
b value	1.515		

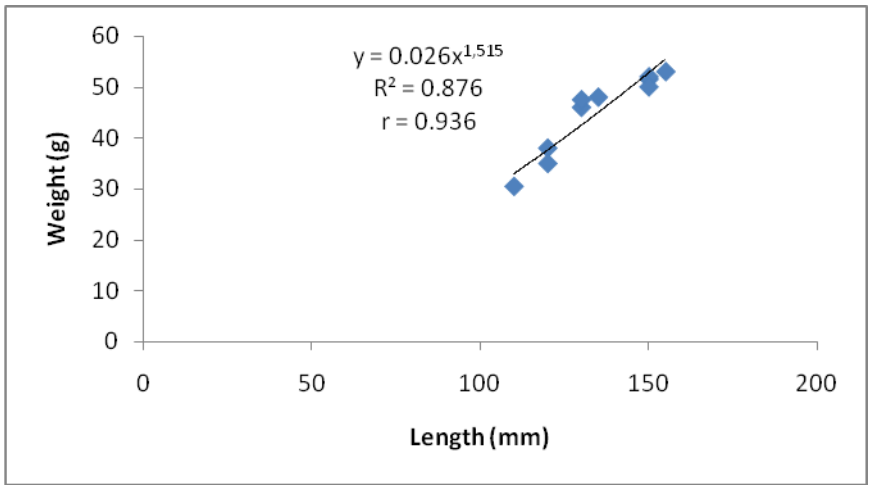

Figure 2. Relation Length -Weight chart of *tawes* (*P. javanicus*)

Length weight relationship of *P. javanicus* can be seen from the value of correction (r) of 0,936 (Tabel 3 and Figure 2). According to Walpole (1995) value of correlation coefficient (r) close to 1, so there is a strong linear relation between body length and weight of fish. Length weight relationship of fish varies depending upon the condition of life in aquatic environment. Ideally, the regression coefficient 'b' of a fish should be very close to 3.0 (Allen, 1938).

2. Stage of Gonad maturity and Gonadosomatic Index

Table 4. Gonadosomatic Index of *P. javanicus* caught from Mas River

Sex	Weight (g)	Gonad Weight (g)	GSI
Male	53	0,54	1,018868
Male	51,5	0,54	1,048544
Female	52	0,5	0,961538
Female	38	0,3	0,789474
Female	48	0,41	0,854167
Female	30,5	0,11	0,360656
Female	35	0,29	0,828571
Male	46	0,35	0,76087
Female	47,5	0,35	0,736842
Female	50	0,51	1,02

Fishes (*P. javanicus*) which were caught having gonadosomatic ranges from 0.360656 to 1.048544 (Table 4). These results suggest that the greater the value weight, the greater the value gonad weight (Effendi, 1997). This results had the same value like Slamet *et al*. (2010) that the heavier the weight of *Plectropoma laevis*, the more severe gonad although there are also some fish that show different results, in which there are fish that have a relatively higher weight but has a smaller gonad weight.

Table 5. Criteria for Fish Gonad Maturity Stage (Puntius schwanefeldi Bleeker) (Siregar, 1993).

Gonad Maturity Stage	Female	Male
I	Ovary like thread length to the front body cavity, clear colors, smooth.	Testis like yarn, shorter (limited) and visible edges in the body cavity, clear colors.
II	Ovarian size larger, darker colors yellow, egg yet to be seen clearly with eyes.	Larger testes size, white as milk, a clearer form than the stage I.

Gonad maturity stage of *P. javanicus* were observed at stage II (Table 5). At stage II, female gonads are larger than stage I, fill a third of the abdominal cavity, light brown. Granules eggs still cannot be seen with eyes. For the male, the gonads have a larger size than stage I, and white (Table 5) (Siregar, 1993).

4.3 Histological Studies

According to histological analyzed from the male gonad, Testis-ova (the presence of oocytes in the testis) were observe in two individuals collected from Mas River (Fig.3a and 3b). The oocytes of the perinucleolus stage that are the characteristic of the testis-ova were scattered in the testis. The testis of *P. javanicus* indicated severe degenerative, collapsing and necrotic changes in both the wall and the cellular elements of the seminiferous tubules with focal areas of fibrosis (Fig.3b and 3c). The deformed testis showed tissue necrosis, congestion of capillaries and vacuolated leyding cells. It also shows disrupted testicular tissue with spacious fibrous interstitial connective tissue with fibroblast and edema. On the female gonad, the fish was at stage II of gonad maturity with atretic oocytes characterized by breaking down zona radiata and proliferation of granulosa layer which invade the dead ova and vacant space in the ovary (Fig.3d).

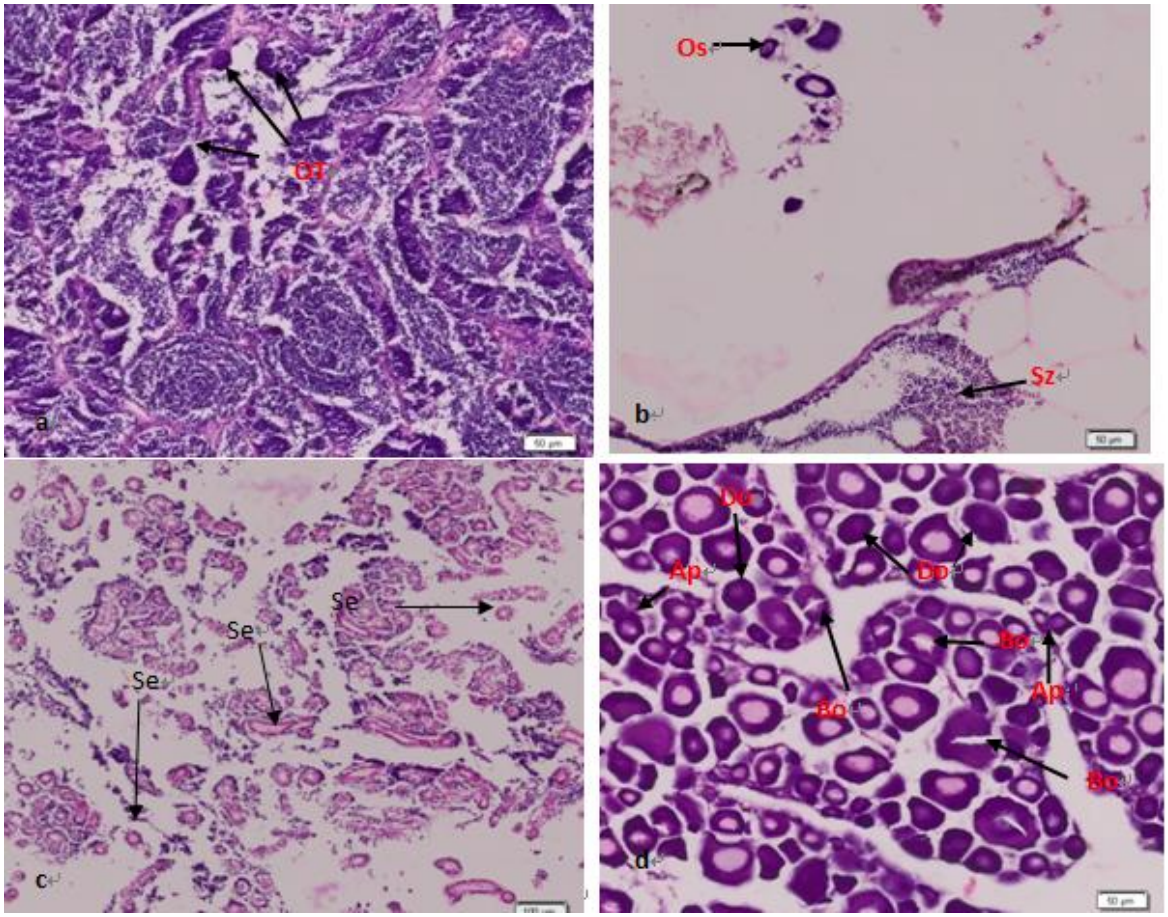

Figure 3. Gonad of Tawes fish from polluted area in Mas, Surabaya. (a) T-O: testis-ova on male gonad (b) T-O: testis-ova in male gonad (c) disrupted testicular tissue with interstitial connective tissue on male gonad (d) destroyed oocyte with atretic perinucleolus oocytes and breaking down oocytes on female gonad. O: ovary, T: testes, Se: seminiferus lobules, Ap: atretic perinucleolus oocytes, Do: destroyed oocytes, Bo: breaking down oocytes.

5. Discussion

5.1 Fish Morphology

The present study recorded the maximum size of *P. javanicus* as 15.5 cm TL, which were lower than the maximum recorded value of 37 cm by ECOTON (2011). However, Rahman *et al.* (2012) reported the maximum TL for *P.sophore* form Chanal *Beel*, North-Central Bangladesh, India as 9.02 cm. In addition, the maximum weight of *P. javanicus* observed in this study (53 g) was lower than the maximum recorded value of 750 g in other sites of the Brantas River flow (ECOTON, 2011). Archarya and Iftekhar (2000) recorded the maximum value of *P.sophore* as 70 g in Maharastra, India.

5.2 Reproductive Biology of Fish

Based on the graph of length (x) and weight (y) obtained $y= 0.026x^{1.515}$ of regression equation. b value in the regression equation is 1.515 ($b<3$) which means that the fish has a negative allometric growth type. It shows the state of the bony fish as in length faster than the increment

weight. In contrast, Rahman *et al.* (2012) recorded the b value of *P. sophore* as 3.322 in Chanal *Beel*, North-Central Bangladesh, India. The coefficient (b) from their studies indicated positive allometric growth with pool barb in Chanal *Beel*, North-Central Bangladesh. Variations in the b values may be atributed to differences in ecological conditions of the habits or variations in the physiology of animals, or both (Le Cren, 1951), sex season (Hossain *et al.*, 2006), feeding rate, gonadal development and growth phase (Tarkan *et al.*, 2006), behavior (active or passive swimmer) and water flow (Muchlisin *et al.*, 2010).

The condition factor (k) is an index reflecting interaction between biotic and abiotic factors in the physiological condition of the fishes (was calculated to assess fish health productivity of fish populations) (Royce, 1972; Effendi, 1997). Commercial, factor conditions have meaning meat quality and quantity of fish available to be eaten (Effendi, 1997). In this study, the condition factor obtained in the ranges of 0.67-1.17 and tends to fluctuate on size of the fish (Table 3). Other studies related to condition factor has been reported by Rahman *et al.* (2012) in Chanal *Beel*, North-Central Bangladesh, India. They found that the condition factor (k) for *P. sophore* ranges between 0.69-2.35. Le Cren (1951) opined that the relative condition factor is an indicator of general well-being of the fish. K was greater than one (1) expressed as indicative of the general well being of fish, whereas its value less than one (1) indicated that fish is not in a good condition. Small size of fish (juvenile) commonly has a higher condition factor and then decrease as the fish grows. It was associated with the changes in the type of food in the moment of growth (Effendi, 1997). In the early period of growth, the formation of cells and tissues in the body of fish required a lot of energy. This situation makes the fish eats as much as possible, so that the condition factor increases.

Other studies related to gonad maturity stage on red side barb (*Puntius bimaculatus*) has been reported by Wijeyaratne and Ponnamperuma (2005) in Sri Lanka. They found that the precentage abundance of different maturity stages I were recorded in June and July. Assessment of gonadal development is not only based on morphological characteristics because only be qualitatively explained thus less informative and subjectively. Yet in gonad development are not only morphological development but there is also the development of eggs and this occurs aligned with the development of gonad weight (Effendi, 1997; Lagler et al., 1977). Description of the development of eggs and gonad weight may provide additional information that can be described quantitatively. Sometimes the value of GSI is associated with gonad maturity which based on morphological characteristics gonad maturity.

5.3 Histological Studies

Pollutants such as industrial and agriculture wastes, pesticides (environmental estrogenic) and also different types of bacteria have histopathological effects on the reproductive tissue of fish gonads. These may disturb the development of germ cells and may reduce the ability of fish to reproduce, while metal accumulation occurring in the testis, affects the process of spermatogenesis and suppressing sperm production. Kumar and Pant (1984) found that 2-4 month exposure of an Indian teleost to lead caused disappearance of oocyes in the ovaries.Blazer (2002) also reported proliferation fibroblast in Cod (*Gadus moruha*) fed Aroclor, polychlorinated biphenyl.

The presence of testis-ova in the gonads is indicating that fish is to be feminizing (Fig.3a and 3b). It can be occured because of disruption of endocrine function in fish which often called Endocrine Disrupting Chemical (EDC). Testis-ova are the most popular abnormal morphological change in fish exposed by estrogenic chemicals (Soyano *et al*., 2010; Jobling and Tyler, 2003). It is well known that testis-ova are induced by these chemicals in the fish that is sex determination phase, which occur during fly and larval stage (Soyano *et al*., 2010). EDCs in organisms was stimulated by the presence of pollution as a result of anthropogenic xenobiotics. Some polluting substances that can cause EDC is chemical waste from factories (Nonyphenol, bisphenol-A, PCBs, PAHs, dioxins, etc.) (Pait and Nelson, 2002; Sun and Tsai, 2009; Soyano *et al*., 2010;) , the compounds of the class of pesticides (Carbofuran, Endosulfan, Lindane, DDT, DDE , etc.) (Pait and Nelson, 2002; Sumpter, 2002) , heavy metals (Hg, Pb, and Cd) and natural products (b-sitosterol, genistein, enterodiol, natural estrogenic) (Pait and Nelson, 2002; Jobling and Tyler, 2003; Sun and Tsai, 2009).

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Living in the aquatic environment, fish can be bathed constantly in a solution of chemical pollutants (Hecker et al., 2006). Furthermore, uptake of chemicals into fish can readily occur via the gills and skin, as well as via diet (the major route of exposure to EDCs in terrestrial animals). Features of the gills including thin epithelial membranes and a large surface area coupled with the relatively high ventilation rates that occur in fish, facilitate the uptake of compounds from the water and their transfer into the blood stream (Marina and Martinez, 2007). Some freshwater fish species are also top-predators and thus, are likely to bioconcentrate EDCs to a greater degree than other organism at lower tropic levels (Bernet *et al*., 2008). Freshwater fish are hypo-osmotic with their surroundings and thus a considerable movement of water into their bodies occurs down an osmotic gradient (taking chemicals with it). A major route of exposure to EDCs in fish during early life is from contaminants that have accumulated in lipid reserves within the egg as a consequence of maternal transfer during ovary development. These contaminants that have accumulated in the egg are mobilized when the lipid reserves are metabolized to fuel embryo development, exposing early life stages to especially high concentration of EDCs at a time of greatest vulnerability to disruptions in their developing endocrine system (Sumpter, 2002; Hecker et al., 2006; Soyano et al., 2010).

Furthermore, early life stages of fish have a limited capacity to metabolize and excrete contaminants, including EDCs (Jobling and Tyler, 2003).

6. Conclusion

Based on these results, we conclude that endocrine disrupting effect on reproductive organ has been occurred in the Mas River, Surabaya. These effects ~~is~~ were indicated by the presence of defects in fish gonads. The defects include atretic oocyte on the ovary, degenerative, collapsing and necrotic changes in both the wall and the cellular elements of the seminiferous tubules with focal areas of fibrosis on the testis and edema. Some fish also showed intersex that characterized by the presence of testis-ova in the gonads.

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