สหสัมพันธ์ระหว่างการปนเปื้อนสารฆ่าวัชพืชกับผลต่อระบบสืบพันธุ์ของปลากะมัง *Puntioplites proctozysron* ในแม่น้ำน่าน อำเภอเวียงสา จังหวัดน่าน

นายศิลปชัย เสนารัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CORRELATION BETWEEN HERBICIDE CONTAMINATION AND EFFECTS ON REPRODUCTIVE SYSTEM OF CYPRINID FISH *Puntioplites proctozysron* IN NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE

Mr. Sinlapachai Senarat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
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Thesis Title	CORRELATION BETWEEN HERBICIDE CONTAMINATION AND EFFECTS ON REPRODUCTIVE SYSTEM OF CYPRINID FISH *Puntioplites proctozysron* IN NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE
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Thesis Co-advisor	Noppadon Kitana, Ph.D.
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การใช้สา รฆ่าวัชพืชในจังห วัดน่านทางภาคเหนือของประเทศไทยมีปริมาณเพิ่มขึ้นอย่างต่อเนื่อง โดยเฉพาะอย่างยิ่ ง พาราควอต ไกลโฟเสต และอะทราซีน แม่น้ำน่านบริเวณใกล้กับแหล่งเกษตรกรรมจึงมีความเสี่ยงสูงต่อการปนเปื้อนของสา รฆ่าวัชพืชจากน้ำไหลบ่าผิวดิน สัตว์น้ำที่ดำรงชีวิตในบริเวณดังกล่าวอาจได้รับผลกระทบจากการปนเปื้อน การวิจัยครั้งนี้ได้เลือกใช้ ปลากะมัง *Puntioplites proctozysron* เป็นสัตว์เฝ้าระวังผลกระทบของการปนเปื้อนสารฆ่าวัชพืชในแม่น้ำน่าน โดยเก็บตัวอย่างปลาจากแม่น้ำน่านบริเวณใกล้พื้นที่เกษตรกรรม ตำบลส้าน อำเภอเวียงสา จังหวัดน่าน ในฤดูฝน (กรกฎาคม และตุลาคม พ.ศ. 2553) และฤดูแล้ง (มกราคม และเมษายน พ.ศ. 2554) ผลการวิเคราะห์การตกค้างของสารฆ่าวัชพืช พบอะทราซีนในน้ำ (ND-0.15 มิลลิกรัมต่อลิตร) ในดินตะกอน (ND-0.24 มิลลิกรัมต่อกิโลกรัม) และในอวัยวะ สร้างเซลล์ สืบพันธุ์ของปลากะมัง (ND-0.15 ไมโครกรัมต่อกรัม) ในขณะที่ไกลโฟเสตพบ เฉพาะในอวัยวะ สร้างเซลล์สืบพันธุ์ของปลาก ะมัง (1.20-2.01 นาโนกรัมต่อกรัม) การวิเคราะห์ค่าสุขภาวะโดยรวม (CF) และค่าภาว ะการเจริญพันธุ์ (GSI) พบว่า CF ของปลาทั้งสองเพศมีค่าสูง อย่างมีนัยสำคัญทางสถิติในเดือนมกราคม พ.ศ. 2553 ในขณะที่ GSI ของปลาเพศผู้มีค่าสูงอย่างมีนัยสำคัญทางสถิติในเดือนมกราคม พ.ศ. 2554 ส่วนในปลาเพศเมียมีค่าสูง อย่างมีนัยสำคัญในเดือนกรกฎาคม พ.ศ. 2553 เมื่อนำข้อมูล CF และ GSI มาหาความสัมพันธ์กับปริมาณการปนเปื้อนของอะทราซีนในเนื้อเยื่ออวัยวะสร้างเซลล์สืบพันธุ์ โดยใช้ค่าทางสถิติความสัมพันธ์แบบ Spearman ผลการศึกษาแสดงให้เห็นว่า CF และ GSI มีความสัมพันธ์กับปริมาณ อะทราซีนในอัณฑะในทิศทางเดียวกันอย่างมีนัยสำคัญทางสถิติ ส่วนเพศเมีย GSI มีความสัมพันธ์กับปริมาณ อะทราซีนในรังไข่ ในทิศทางตรงกันข้าม อย่างมีนัยสำคัญทางสถิติ แต่อย่างไรก็ตาม CF และ GSI ไม่ได้แสดงความสัมพันธ์อย่างชัดเจนกับปริมาณอะทราซีนในอวัยวะสร้างเซลล์สืบพันธุ์ แต่อาจจะเกี่ยวข้องกับฤดูกาลมากกว่าการปนเปื้อนของสารฆ่าวัชพืชเพียงอย่างเดียว ผลการศึกษาทางพยาธิสภาพเนื้อเยื่อ อวัยวะสร้างเซลล์สืบพันธุ์ของปลากะมังพบความผิดปกติในอัณฑะ เช่น testicular atrophy, testicular degeneration, Leydig cell และ Sertoli cell degeneration, asynchronous development, karyorhexis, karyolysis, germ cell atrophy, germ cell hypertrophy, eosinophilic cytoplasm ส่วนในรังไข่พบความผิดปกติ เช่น ovarian degeneration, oocyte hyperplasia, atresia ในระยะ oogonia, atresia ในระยะ previtellogenic oocytes, vacuolar degeneration และ follicular hyperplasia เมื่อนำพยาธิสภาพที่เกิดขึ้นมาหาความสัมพันธ์กับปริมาณการปนเปื้อนของอะทราซีนในเนื้อเยื่ออวัยวะ สร้างเซลล์สืบพันธุ์ พบว่า testicular atrophy, Leydig cell degeneration ในปลาเพศผู้ ตลอดจน atresia ในระยะ previtellogenic oocyte และ vacuolar degeneration ในปลาเพศเมีย มีความสัมพันธ์กับปริมาณอะทราซีนในอวัยวะสร้างเซลล์สืบพันธุ์ในทิศทางเดียวกันอย่างมีนัยสำคัญทางสถิติ จากผลการศึกษาครั้งนี้สรุปได้ว่าการปนเปื้อนของอะทราซีนในแม่น้ำน่านอาจชักนำให้เกิดพย าธิสภาพในอวัยวะ สร้างเซลล์ สืบพันธุ์ของปลาก ะมัง ซึ่งอาจนำไปสู่ปัญหาในระดับประชากรต่อไป ผลการศึกษายังแสดงให้เห็นว่า Leydig cell และเซลล์ไข่ในระยะต้น เป็ นเซลล์ที่เกิดความเสียหาย ได้มาก พยาธิสภาพที่พบในการศึกษาครั้งนี้ยังพิสูจน์ได้ว่า ปลากะมังสามารถใช้เป็นสัตว์เฝ้าระวังในการประเมินผลกระทบของ สารฆ่าวัชพืชที่ปนเปื้อนในแม่น้ำน่านได้

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 ลายมือชื่อ อที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5272565723 : MAJOR ZOOLOGY
KEYWORDS : HERBICIDE/ NAN RIVER/ *Puntioplites proctozysron*/ REPRODUCTIVE SYSTEM

SINLAPACHAI SENARAT: CORRELATION BETWEEN HERBICIDE CONTAMINATION AND EFFECTS ON REPRODUCTIVE SYSTEM OF CYPRINID FISH *Puntioplites proctozysron* IN NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE. ADVISOR : JIRARACH KITANA, Ph.D. CO-ADVISOR : NOPPADON KITANA, Ph.D., PUTTARUKSA VARANUSUPAKUL, Ph.D., 162 pp.

The use of herbicides, especially paraquat, glyphosate and atrazine in Nan Province, northern part of Thailand is dramatically increased. Nan River nearby agricultural areas is thus susceptible to contamination by the herbicide runoff. Aquatic animals are probably at risk of affecting by the contamination. In this study, *Puntioplites proctozysron* was used as a sentinel species to monitor the effects of herbicide contamination in Nan River. The fish were collected from the river nearby agricultural areas in San Sub-district, Wiang Sa District, Nan Province in rainy season (July and October 2010) and dry season (January and April 2011). The analyses of herbicide residues revealed detectable levels of atrazine in water (ND-0.15 mg/L), sediment (ND-0.24 mg/Kg) and fish gonads (ND-0.15 μg/g), whereas glyphosate was detected only in the fish gonad samples (1.20-2.01 ng/g). The analyses of condition factor (CF) and gonadosomatic index (GSI) showed that, CF of both sexes was significantly higher in January 2011. GSI of male fish was significantly higher in January 2011, while GSI of female fish was significantly higher in July 2011. Spearman's correlation between atrazine concentrations and general health indices revealed the positive correlation in CF and GSI for male fish and negative correlation in GSI for female fish. However, the link between herbicide contamination to CF and GSI of *P. proctozysron* did not clearly exhibited. These trends were alternatively explained by seasonal variation, rather than effect of herbicide contamination alone. The evaluation of herbicide effect in cellular level was done by histopathological observation of gonadal tissue. The testicular atrophy, testicular degeneration, degeneration of Leydig and Sertoli cells, asynchronus development, karyorhexis, karyolysis, germ cell atrophy, germ cell hypertrophy, eosinophilic cytoplasm was observed in testicular tissue of the male fish. Ovarian degeneration, oocyte hyperplasia, atresia in oogonia, atresia in previtellogenic oocytes, vacuolar degeneration and follicular hyperplasia were found in ovarian tissues of the female fish. Atrazine concentrations in gonadal tissues showed significantly positive correlation with testicular atrophy and degeneration of Leydig cells in male fish and atresia in previtellogenic oocyte and vacuolar degeneration in female fish. According to the results, it is concluded that the atrazine contamination in Nan River may involved with several histopathological alterations in the fish gonadal tissue, which may lead to further reproductive problems in the fish population. The results also suggested that Leydig cells in male fish and oocytes at early developmental stage in female fish are most susceptible. Histopathological alterations detected here have proven that *P. proctozysron* could be considered as a good sentinel species in monitoring study of the herbicide contamination in the river.

Department : Biology Student's Signature .....

Field of Study : Zoology Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

Co-advisor's Signature .....

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CHAPTER I

GENERAL INTRODUCTION

Nan River is one of the most important rivers in northern Thailand. It supplies water for not only in general uses, but also in agricultural and industrial purposes for northern and upper central Thai populations. Farmers in Nan Province often apply pesticides, particularly herbicides such as glyphosate, paraquat and atrazine into their agricultural fields in order to increase their agricultural products (Wongwichit et al., 2010). These herbicides are widely used in high quantity for a long period of time. Thus, it is possible that herbicide residues will be found in soil and sediment as well as in surface water through runoff. The agricultural areas of Wiang Sa district in Nan province are located along both sides of Nan River. These areas are therefore vulnerable to deterioration of its environment as a result of the herbicide contamination. There is a high possibility that, in the future, the accumulation of toxic chemicals can lead to a major water pollution crisis. Surveys and monitoring programs are urgently required to provide information relating to the impact of herbicide usage on aquatic environment and aquatic organisms in this area.

Aquatic organisms living in herbicide contaminated environment can be affected both directly via water and indirectly via food chain. Although, these herbicides are believed to be rapidly broken down in the environment, the continuous use in high dosages can produce the adverse effects on aquatic animals, particularly on fish (Pérez et al., 2011).

The biological data obtained from the animal sentinel system are used in complement with the physical and chemical environmental data because it can provides the integration, relevant information, and effects of environmental contaminants (NRC, 1991), and can offers the possibility to expand our understanding and response to the environmental well-being concerns (Van der Schalie et al., 1999). Currently, fish has been widely used as one of the sentinel organisms in environmental monitoring and risk assessment programs because of its characteristic of accumulating toxic chemicals in its tissue without mortality (Heath, 1995; Heath and Claassen, 1999; Kime, 1999; van der Oost et al., 2003). In this study, *Puntioplites*

proctozysron is selected as a sentinel species because it has several properties mentioned in many reviews about good characters of sentinel species (Beeby, 2001; Frame and Dickerson, 2006; NRC, 1991) and it is widely available in Thailand and living in the area of interest.

In order to assess the environmental impact, we choose biomarkers that are appropriated for long-term assessment and monitoring of herbicide contamination. Biomarkers have dramatically been used due to they are an effective tool for environmental monitoring and the early warning in ecological risk assessment (Kammenga et al., 2000; McCarthy and Shugart, 1990; Triebskorn et al., 1997). In general, biomarkers are susceptible and exhibited the first detectable signs of sublethal stress response in an organism exposed to a toxin (Stegeman et al., 1992). Four types of biomarkers were used in the present study: level of herbicide residues, as a biomarker of exposure and condition factor, gonadosomatic index and histopathology as a biomarker of effect, to characterize the health of fish in the herbicide contamination in Nan River. Due to many reports, we believe that the widely used herbicides in agricultural areas of Nan Province can produce reproductive disrupting effects.

This research will provide supportive information about the impact of herbicides uses in agricultural area to the environment and aquatic organisms in Nan River. Meanwhile, we can use a trend data to predict the impact that will influences local people's health and well-being and expand the knowledge for Nan villagers in order for them to gain understanding about how to apply suitable chemicals in their area.

The experimental protocol of this research was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University (Protocol Review No. 1123001).

Objectives

1. To determine types and quantity of herbicide residues in water, sediment and gonadal tissue of the cyprinid fish *Puntioplites proctozysron* living near the agricultural area, San Sub-district, Wiang Sa District, Nan Province.
2. To assess condition factor, gonadosomatic index and histopathological alteration of gonads of the cyprinid fish *Puntioplites proctozysron* living near the agricultural area, San Sub-district, Wiang Sa District, Nan Province.
3. To assess correlation between herbicide contamination and effects on reproductive systems of the cyprinid fish *Puntioplites proctozysron* living near the agricultural area, San Sub-district, Wiang Sa District, Nan Province.

CHAPTER II

LITERATURE REVIEW

2.1 Nan River

The Nan River is one of the most important tributaries of the Chao Phraya River and the biggest river in Nan Province, northern Thailand. It originates from the Luang Prabang mountain range, the border between northwestern Laos and northern Thailand, before flows through several districts in Nan Province (Figure 2.1B). The river exits Nan Province at Na Muen District and then flows southward pass Uttaradit, Pitsanulok and Pichit Provinces, before joins with the Yom and the Chao Phraya rivers at Nakhon Sawan Province (Figure 2.1A). The river extends approximately 1,809 km in length (Nan Province, 2009).

The climate in this area is strongly influenced by the tropical monsoon system including the southwest (May to September), the northeast (October to February) and the southeast (March to April) monsoons. The climatic periods in this region can be characterized into three principle seasons, namely, dry season from March to May, wet season from mid-May to October and winter from November to February (Nan Province, 2009).

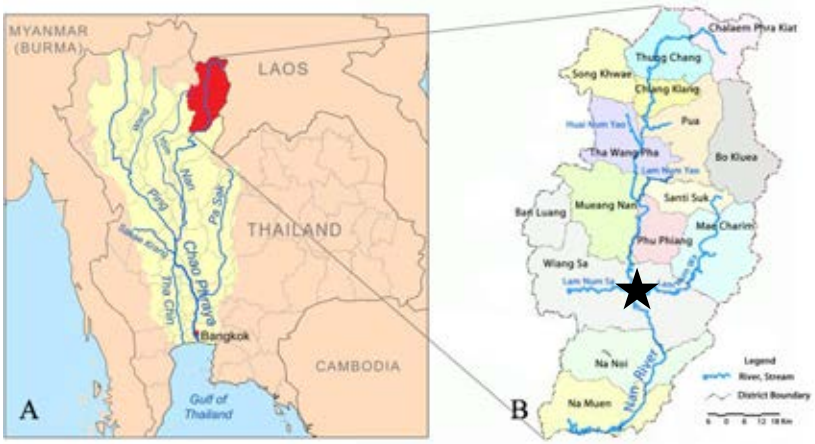

Figure 2.1 Map of the Chao Phraya River drainage basin (A) and map of Nan Province (B) shows the river systems and the sampling locality (★).

2.2 Herbicide usage in Nan Province

Herbicides are a unique group of pesticides used for weed control in agriculture, aquaculture and irrigation/recreational water management activities. Use of herbicides has been increased dramatically over the world since the Second World War (Hopkins, 1994). By 2001, approximately 1.14 billion kg of herbicides were applied worldwide (U. S. EPA, 2004). Twenty-eight percent of herbicide mass was applied in the United States of America (USA), while the balance was applied elsewhere around the world (U. S. EPA, 2004). Some substantial benefits can be gained from the use of herbicides; however, some important environmental effects may occur if herbicides are not used properly. Herbicide may reach to aquatic ecosystem by indirect applications such as agricultural runoff and leaching processes, or direct applications by aquatic weed controls. Once herbicides have been contaminated in the aquatic ecosystems, they may affect to water and aquatic organisms in many aspects, e.g. reduce environmental quality, diminish species diversity and community structures, modify food chains, change the energy flow patterns and nutrient cycling and change the stability and resilience of ecosystems (Pérez et al., 2011).

The Ministry of Agriculture and Co-operatives of Thailand reported the dramatical increase of herbicide usage in Thailand from 33% in 1992 to 49% in 1998. Amongst them, glyphosate, paraquat and atrazine were ranked in the top of common herbicides used in Thailand, accounting for more than 50 million kg in 1995 (Thailand National Statistical Office, 2001; Thapinta and Hudak, 2000).

Agricultural areas in Nan Province have been generally used for cultivation of crop plants such as corn, rice, fruits and vegetables. In general, these plants are developed differently in each climatic season. For example, rice is cultivated once or twice a year. Therefore, to increase annual products and control unwanted weeds in agricultural areas, local agriculturists in Nan Province often apply herbicides throughout a year. In 2008, the amount of herbicide used in Nan Province was 1,274.1 tons (approximately 232.1 million kg) including 1,172.7 tons (92.04 %) of imported herbicides (Office of Agriculture Nan Province, 2011). Wongwichit et al. (2010) reported that glyphosate, paraquat and atrazine were used extensively in this province.

2.2.1 Glyphosate

Glyphosate (N-(phosphonomethy) glycine) is a broad-spectrum, non-selective and post-emergent herbicide. It is designed to use against deep-rooted perennial species, biennial, annual broad-leaved, grass and sedge species in agriculture, forestry and non-agricultural areas, i.e. irrigation and drained water, parks, road and gardens (Grossbard and Atkinson, 1985). It is now being marketed in 130 countries, ranked among the top ten herbicides used in the USA (U. S. EPA, 1999) and in Thailand (Thailand National Statistical Office, 2001).

Application rates of glyphosate depend on the formulations, route of application, season and nature of the site of use. Glyphosate is generally applied as 0.5-5% in water solution by spraying and as a 10-50% solution in water by wiping. The timing of application depends on the use and may be pre or post harvests. For instance, glyphosate may be applied to cereals, potatoes and asparagus instantly before harvest (U. S. EPA, 1993).

Glyphosate has been imported to Thailand around 6,187 metric tons in 1995 (Thapinta and Hudak, 2000).

2.2.1.1 Physical and chemical properties of glyphosate

Glyphosate is a weak organic acid consisting of a glycine and a phosphonomethyl moiety (Knuuttila and Knuuttila, 1979). Its empirical formula is $C_3H_8NO_5P$ and the structural formula is shown in Figure 2.2.

The relative molecular mass of glyphosate is 169.07. The purity of technical grade glyphosate is generally above 90%. Glyphosate is usually formulated as a salt of the deprotonated acid of glyphosate and isopropylamine (Roundup®, CAS registry number 38641-94-0). Surfactants and inserts may be added to the formulations of glyphosate, for example polyxyethylene amine for Roundup® (WHO, 1994).

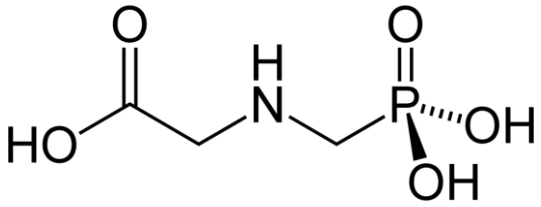

Figure 2.2 The structural formula of glyphosate (WHO, 1994)

2.2.1.2 Degradation of glyphosate

The rate of glyphosate dissipation in soil is related to the microbial composition and the ability of binding to soil particles (Borggaard and Gimsing, 2008; Thailand National Statistical Office, 2001). Glyphosate in soil appears to be degraded by microorganism such as *Pseudomonas* sp. bacteria and filamentous fungi (Castro, 2007; Jacob et al., 1988; Kishore and Jacob, 1987). In the experiment, glyphosate in soil appears to be degradable by microorganism in two ways. One route is via the formation of aminomethyl phosphonic acid (AMPA) and a C_2 fragment that might be glyoxylated. In this route, the splitting of C-N bond is the first step. However, another route of biodegradation is via sarcosine (N-methyl-glycine) and orthophosphate. Sarcosine is degraded to glycine and a one-carbon unit forms CO_2 via formaldehyde (Jacob, 1988; Kishore and Jacob, 1987).

In aquatic environment, glyphosate are degraded through sediment adsorption by enzymatic kinetic model of microbial biodegradation (Zaranyika and Nyandoro, 1993) or by the combination of hydrogen peroxide and UV radiation (Manassero et. al., 2010). The rate of degradation in water is generally slower than in soil, because there are fewer microorganisms in water (Ghassemi, 1981). Glyphosate has relatively long half-lives of about 47 days in soil and 49–70 days in water, making it fairly persistent in the environment (Borggaard and Gimsing, 2008). For example, glyphosate existed in agricultural soil in Finland for 249 days, in three British Columbia forestry sites for 360 days and in eleven Swedish forestry sites for a few years (Cox, 1995).

2.2.1.3 Contamination of glyphosate in the environment

Although glyphosate is relatively immobile in most soil environments as a resulted of its strong adsorption to soil particles, it can contaminate aquatic environments in many ways. Glyphosate can be applied directly to water for aquatic weed control. For indirect ways, glyphosate that bonded to soil particles can be washed and leached through the soil layer via subsurface runoff into drainage and groundwater or by overland flow via surface runoff to open sea such as streams and lakes (Borggaard and Gimsing, 2008; Solomon et al., 2007; WHO, 1994).

The quantity of glyphosate in the environment has been difficult to detect due to its physicochemical properties, e.g., relatively low molecular weight, high polarity,

high water solubility, low organic solvent solubility, amphoteric behavior and forming with metal complexes easily (Sanchís et al., 2012). However, glyphosate contaminations have been reported in several countries (Solomon et al., 2007). For examples, glyphosate was detected in 55 water samples collected from 51 streams in Midwestern States, USA (Scribner et al., 2003). It is noticeable that glyphosate tended to be persisted in streams throughout the growing season rather than other times of the year (Coupe et al., 2011). For groundwater, glyphosate was found in groundwater pipes along railway tracks in Sweden with the concentrations above the European maximum limit of 0.1 μg/L (Torstensson et al., 2005). Groundwater samples collected in Catalonia, Spain are also found contaminated with glyphosate at a concentration as high as 2.5 μg/L and a mean concentration of 200 ng/L (Sanchís et al., 2012). Additionally, the ambient levels of glyphosate and its major degradation product were found in the air and rain samples from USA (Chang et al., 2011).

2.2.1.4 Toxicity of glyphosate

Several reports have described the toxicological effects of glyphosate, glyphosate formulations and surfactants on various non-target aquatic organisms including aquatic bacteria, plants, algae, invertebrates and vertebrates (Giesy et al., 2000; Siepmann, 1995). The studies on acute toxic effects of glyphosate on many species of freshwater fish showed the LC_{50} values ranging from 6.1 to 140 mg/L indicating high acute toxicity of this herbicide on fish (Folmar et al., 1979; Mitchell et al., 1987).

The toxicity of glyphosate to reproductive system of animals was studied in rat, rabbit and freshwater fish. In rat, reduction of epididymal sperm concentrations (20% below control) were observed at 25,000 and 50,000 μg/L levels of glyphosate exposure (Morrissey et al., 1988). Dallegrave et al. (2007) reported that Roundup® did not induce maternal toxicity, but did induce adverse reproductive effects on male offspring rats. These effects included decrease in sperm number per epididymis tail and in daily sperm production during adulthood, increase the percentage of abnormal sperms and decrease in serum testosterone level at puberty and signs of individual spermatid degeneration during both periods. Clair et al. (2012) also showed that within 1 to 48 hours of Roundup® exposure in mature rat, Leydig cells were damaged, within 24–48 hours necrosis were found and at higher doses, apoptosis in germ cells

and in Sertoli/germ cells were induced. In rabbit, male toxic effects were reported including reduction of ejaculate volume and sperm concentration and increase of abnormal and dead sperms (Yousef et al., 1995). In fish, a study on the effects of glyphosate at concentration of 2 µg/L (30 days exposure) in various freshwater fish indicated significantly reduced fecundity and gonadosomatic index (GSI) (Folmar et al., 1979). In addition, Roundup® might inhibited steroidogenesis by disrupting expression of the steroidogenic acute regulatory (StAR) protein, which has potential to disrupt reproductive function in animals (Walsh et al., 2000).

2.2.2 Paraquat

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is one of the most widely used herbicides. It is commercially available in more than 130 countries worldwide. It can control a wide range of weed species, but mostly effective for grasses which has led to a large number of practical usages (Eisler, 1990).

Paraquat can be used as a direct spray to control emerged weeds or as a pre-emergence treatment to emerged weeds in sorghum and corn. It also has been used as a pre-harvest desiccant in cottons, potatoes and soybeans (Watts, 2011). Paraquat has been usually applied as an aerosol for terrestrial weed control (0.28 to 1.12 kg paraquat cation/ha or 0.56 to 2.24 kg paraquat dichloride/ha) and direct apply for aquatic weed control (0.1 to 2.0 mg/L for or 0.019 to 0.372 mg/L for sensitive aquatic plants). Additionally, paraquat has been frequently used in combination with other herbicides (Fletcher, 1974).

Paraquat was imported to Thailand over 21 million kg in 1995 and the importation being continued (Thailand National Statistical Office, 2001).

2.2.2.1 Physical and chemical properties of paraquat

Paraquat is a nonvolatile ionic compound with relative molecular mass of 257.16 g/mol. Its empirical formula is $C_{12}H_{14}C_{l2}N_2$ and the structural formula is shown in Figure 2.3. It is almost completely insoluble in organic solvents (Calderbank, 1975). It is found in variety of many hundreds synthesized forms depends on the introducing of different quaternizing groups on the nitrogen atoms or shifting of nitrogen atoms position. The common paraquat salts are all fully ionized and the anion (e.g., chloride, sulfate, methyl sulfate) do not affect the toxicity of paraquat (Fletcher, 1974).

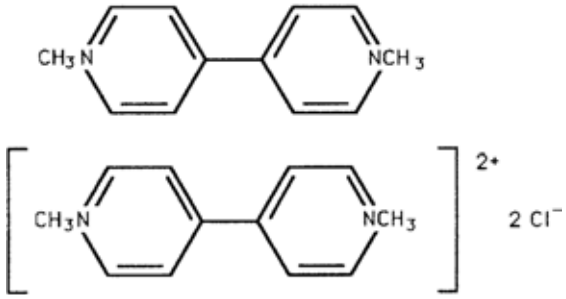

Figure 2.3 The structural formula of paraquat cation (upper) and paraquat dichloride salt (lower) (Fletcher, 1974)

2.2.2.2 Degradation of paraquat

Photochemical decomposition is the predominant mechanism of paraquat degradation in soil (Smith and Mayfield, 1978). In surface soil, paraquat was loss through photodecomposition about 20% to 50% within 3 weeks (Watts, 2011). In laboratory conditions, paraquat in soil slated for disposal can be degraded by ultraviolet (UV) irradiation if oxygen or ozone were presented (Kearney et al., 1985). A numbers of studies shown that paraquat is intrinsically biodegradable by soil microorganisms, including a variety of bacteria and fungus species (Roberts et al., 2002). The biodegradation is strongly related to nitrogen metabolism of the responsible microorganisms and the degradation rate was much higher under aerobic conditions than under anaerobic conditions (Lee et al., 1995).

According to Eisler (1990), paraquat that applied to aquatic systems disappeared from the water rapidly, within 8 to 27 days under field condition, because of adsorption onto sediments and uptake by plants. Paraquat concentration of 0.5 mg/L was completely degraded in 35 weeks when sediment or plants were absented, in 6-8 weeks when sediments were presented, and in 3 - 4 weeks when both sediment and aquatic plants were presented.

2.2.2.3 Contamination of paraquat in environment

Paraquat has been found in surface waters, drinking water and in groundwater in several countries (Watts, 2011), although it is believed to have a strongly binding to soil particles as inactive state for a long time and do not leach to groundwater (U. S.

EPA, 2009). It is likely to enter surface waters from the soil particles, which are resulted of erosion and run-off from uplands (U. S. EPA, 2009). In Spain, paraquat was found in 6.6% of samples from a lagoon with maximum level of 3.95 µg/L, and in 9.35% of samples from a marsh with maximum level of 1.45 µg/L (Fernández et al., 1998). It was found in drinking water sampled from taps in the Caribbean Island of St Lucia and in a number of rivers and dams at a maximum concentration of 1 µg/L (Boodram, 2002). It was also found in one sample from 399 samples of groundwater taken in California in 2006 at a low level of 0.24 ng/L (U. S. EPA, 2009).

In Thailand, water and soil along the main rivers and canals in various agricultural areas has been reported to contaminate by paraquat at the level of 0.01 to 1.37 µg/L and 0.045 to 8.41 mg/L, respectively. Water resources from Fang and Chaiprakarn Districts, Chiangmai Province were also found to contaminate by paraquat with the residue levels ranged from 0.027 to 0.128 mg/L (Department of Agriculture, 1995). It was also has been found in groundwater at the levels up to 18.9 µg/L (Amondham et al., 2006).

2.2.2.4 Toxicity of paraquat

Paraquat is known to be poisonous to various non-target organisms (Eisler, 1990; Watts, 2011). In aquatic environment, most species of crustaceans and invertebrates were relatively unaffected at concentrations below 1000 µg/L of paraquat, although some are significantly affected by 0.9 to 100 µg/L. Aquatic vertebrates were usually adversely affected by paraquat and showed accumulation at 1000 µg/L or lower (Haley, 1979). For fish, the studies on acute toxic effects of paraquat on many freshwater fish species showed the LC_{50} values ranging from 0.2 to 0.38 mg/L, indicating the high acute toxicity of this herbicide on fish (U.S EPA, 2004; 2006). In addition, paraquat has been reported to link with Parkinson's disease in Human (Tanner, 2011; Dinis, 2006).

Although regulatory authorities said that paraquat has no effects on animal reproductive system, a number of evidences still present (Watts, 2011). Zain (2007) reported that, in rats, a medium to high levels of exposure to paraquat (5 and 20 mg/kg) resulted in the decrease of organ weight, the decrease of diameter of seminiferous tubules, the degeneration of epididymal epithelium, the decrease of spermatogonia, spermatocytes, spermatids and Leydig cells, the increase of sperm

mortality and abnormal sperm morphology and the decrease in testosterone, follicle-stimulating hormone, luteinizing hormone and prolactin levels. Quassinti et al. (2009) reported the *in vitro* study that paraquat inhibited testosterone and 17b-estradiol production in the frog *Rana esculenta*. Moreover, Mantecca et al. (2006) reported that at the concentrations of 0.250 mg/L or greater, the cellular vacuolation, lysis and thinness of the germinative epithelia and increase of granulocytes were observed in the digestive gland and testis of the freshwater bivalve, *Dreissena polymorpha*, suggesting the inflammatory capacity of paraquat on these tissues. In fish, Figueiredo-Fernandez et al. (1998) studied the effect of paraquat on tilapia, *Oreochromis niloticus*. They found that no differences were found in the GSI between the males, but the females showed a higher GSI value and a greater increase of late vitellogenic and mature oocytes percentage than those of the control group.

2.2.3 Atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a broad-spectrum herbicide. It is used to control broadleaf weeds by disrupting of photosynthetic mechanisms in weed's leaf and shoot. It has also been widely used in many agricultural crops, such as corn, soy, potato, sorghum sod, sugarcane, pineapple, etc. It is also applied in silviculture, on turf grass and residential lawn (Giddings et al., 2005; Ribaudo and Bouzaher, 1994; Solomon et al., 1996).

Atrazine is available in different formulations including flowable liquids, wettable powder and water-dispersible granular. It can be applied either pre-emergence as a water-dispersed spray or as liquid fertilizer, but pre-plant or post-emergent applications also occasionally in use (Giddings et al., 2005). Application rates of atrazine on corn depend upon climate and soil texture. For example, it should be used in smaller amount when used on sand, loamy sands, and sandy loam soils (Ribaudo and Bouzaher, 1994). Although atrazine can be used alone, it is more common to be used in combination with other herbicides with recommendation rate not more than 0.75 pounds per acre (Johnson et al., 2004).

The usage of atrazine in USA during the period of 1998 through 2002 was accounted totally 35,286 metric tons (Giddings et al., 2005). For Thailand, a total of 1,640 metric tons of atrazine was imported in 1994 (Department of Agriculture, 1995).

2.2.3.1 Physical and chemical properties of atrazine

Atrazine (CAS number 1912-24-9) is a triazine herbicide, usually appeared in colorless powder or crystals, crystalline solid at room temperature with molecular weight of 215.69. It is moderately soluble in water **(**34.7 mg/L at 22°C) and very soluble in more organic solvents, such as acetone, chloroform, diethyl ether and dimethyl sulfoxide. Its empirical formula is $C_8H_{14}C_lN_5$ and the structural formula is shown in Figure 2.5 (U. S. HHS, 2003; Solomon et al., 1996).

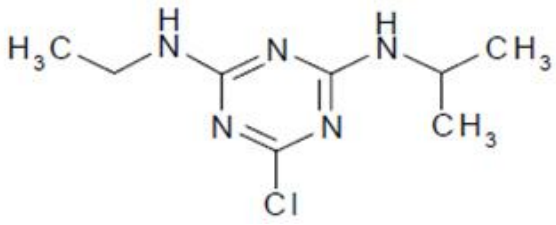

Figure 2.4 The structural formula of atrazine (U. S. HHS, 2003)

2.2.3.2 Degradation of atrazine

The predominant pathway of atrazine degration in soil is through metabolism of microorganisms (bacteria, microbial consortia and fungi), whereas abiotic processes, such as hydrolysis and photolysis are less important (Giddings et al., 2005; Solomon et al., 2008; Sene et al., 2010; Wackett, 2002). But, hydrolysis is rapid in acidic or basic environments, but is slower at neutral pH. Biodegradation of atrazine is slower in subsurface zones than in surface soil because under vadose zone has lower temperature and lack of degrading organisms (Radosevich et al., 1996). Half-lives of atrazine in soil reported from many studies were ranging from 20 to 146 days with a mean value of 44 days (Giddings et al., 2005). In soil, the main metabolites found are 2-hydroxyatrazine (HA), 2-hydroxyterbutylazine (HT), desethylhydroxyatrazine (DEHA), and desisopropylhydroxyatrazine (DIHA). It was also been shown that the hydroxylated degradation products (HA, HT, DEHA and DIHA) are more persistent in soil than DEA since they are absorbed by the organic matter of the soil.

The s-triazine ring makes the atrazine molecule resistant to microbiological degradation in aquatic environment. Thus, biodegradation may be less important than chemical degradation in aquatic environment (Solomon, 1996). Chemical degradation includes hydrolysis, N-dealkylation and splitting of triazine ring as well as photolysis (Chan et al., 1992; Giddings et al., 2005; Gressel et a., 1983; Solomon, 1996; Prosen and Zupančič-Kralj, 2005), although photolysis of atrazine does not occur in water at the wavelengths greater than 300 nm (Pape and Zabik, 1970). Half-lives in water in six studies were ranged from 41 to 237 days with a mean value of 159 day (Giddings et al., 2005). Main degradation products in water are the dealkylated chloro-metabolite, desethyldesisopropylatrazine (DEDIA) and mainly, desethylatrazine (DEA) (Thurman et al., 1994). Its dealkylated metabolites, such as deisopropylatrazine (DIA) and deethylatrazine (DEA) are obtained by means of microbiological transformations. These two metabolites were also found in surface and groundwater (Thurman et al., 1994).

2.2. 3.3 Contamination of atrazine in environment

Most atrazine moves off in solution rather than attaches to soil particles because of its chemical properties make it sensitive to leaching and runoff, especially during heavy rains (Johnson et al., 2004; Ribaudo and Bouzaher, 1994). Therefore, atrazine has been routinely detected in surface and ground waters at above maximum contaminant level of 3 µg/L as suggested by the U.S. EPA (2012) (Solomonet al., 2008). For example, in USA, atrazine was routinely detected in surface and ground water, particularly in mid-western states, at concentrations from 1-25 µg/L (Gilliom et al., 2006). A five-year survey of drinking water wells detected atrazine in an estimated 1.7% of community water systems and 0.7% of rural domestic wells with contaminated level sometimes exceeded the maximum contaminant level (U. S. EPA, 1990). In Thailand, atrazine was found in the water samples from Chao Praya River at concentrations between 58 and 86 ng/L (Kruawal et al., 2005) and from Pa Sak River at concentration of 1.83 µg/L (Chanchek, 2007). It was also detected from ground water samples collected from central and northeastern Thailand at concentrations between 0.5-4.0 µg/L (Sakunthengtong et al., 2001).

2.2.3.4 Toxicity of atrazine

The toxicity of atrazine was reported in variety of organisms for several times (e.g., Giddings et al., 2005; HHS, 2003; Solomon et al., 1996, 2008). The studies on acute toxic effects of atrazine on many species of freshwater fish showed the LC_{50} values ranging from 4.3 to >100 mg/L indicating high acute toxicity of this herbicide on fish (IARC, 1991).

Atrazine is one of the most reported endocrine-disrupting chemicals that are competent of interfering with the synthesis and action of hormones in reproductive system of animals. It has been focused on the potential for adverse effects on reproductive system, reproduction and sexual development in several studies including mammal, fish, reptile and amphibian (Hayes et al., 2011; Rohr and McCoy, 2010; Solomon et al., 2008).

In fish, it appears that atrazine can elicit many effects on the reproductive system in many aspects, such as in the following examples. Kettle et al. (1987) have documented indirect effects of atrazine on fish. They have shown that atrazine has an inhibitory effect on macrophyte communities by inhibited the photosynthesis. This leaded to a decline of essential prey for fish which had a negative effect to the diet and reproduction of fish. Number of young per pond was reduced more than 95% in atrazine treated groups. Bringolf et al. (2004) reported that under laboratory exposures of atrazine to fathead minnow, *Pimephales promelas*, number of late vitellogenic oocytes was increased and sperm maturation was reduced. They also found decreases in egg production (fecundity), reduced fertilization rates, and reduced gonadosomatic index (GSI) in atrazine-exposed fish, but the reductions were not statistically significant. Spanò et al. (2004) studied the exposure of adult goldfish, *Carassius auratus*, to two doses (100 and 1000 µg/L) of atrazine for 21 days. The results showed that atrazine affected on testicular structure (tissue damage), displayed the increase in spaces of the interstitial tissue and induced elevated levels of atresia in ovaries as well as induced suppression in some gonad and plasma sex steroids. Suzawa and Ingraham (2008) have shown that, the zebrafish, *Danio rerio*, exposed to atrazine for 60 days at concentrations of 21.7, 217, 2167 µg/L displayed a dose-dependent increase in the percentage of females and a decrease in the percentage of males. Iwanowicz et al. (2009) observed reproductive health of bass fishes living in up- and downstream of

wastewater treatment plant on the Potomac River in Maryland, USA. They found that a high prevalence of intersex (82–100%) was identified in male *Micropterus dolomieu* and lower prevalence of intersex (23%) was identified in male *Micropterus salmoides*. The authors suggested that the intersex may be the effect of atrazine and its associated metabolites, which were presented in the upstream sites. The study in fathead minnow by Tillitt et al. (2010) also shown that at the level of 0, 0.5, 5.0 and 50 µg/L (between 14 and 30 days), female fish had reduced fecundity and occurrence the atretic follicle (50 µg/L) in ovary. The males showed incidence of intersex in testis after 14 days exposure (5 µg/L). Total egg production was also lower (19–39%) in all atrazine-exposed groups compared to the controls. In addition, gonad abnormalities were observed in both sex in atrazine exposed groups. Hayes et al. (2011) demonstrated that atrazine may induce demasculinization in male gonads by producing testicular lesions associated with reduction of germ cell numbers in teleost fish. It also induced partial and/or complete feminization in fish.

2.3 The concept of sentinel species

Stahl (1997) defined the concept of sentinel species as "any non-human organism that can react to an environmental contaminant before the contaminant impacts humans". Sentinel species has been widely used in the environmental health assessment because it can provide the integration, relevant information, and effects of environmental contaminants (NRC, 1991). This concept also helps expanding our understanding and response to the environmental well-being concerns (Van der Schalie et al., 1999). The use of sentinel species as indicator of human health hazard has various advantages. For example, sentinel species can provide the early warning of potential risks before new emerge diseases can affect to human population. For some toxicants, the toxic effects in both human and the sentinel animal are similar and may be comparable under some condition (Van der Schalie et al., 1999).

The choice of organisms to be used as sentinel species is greatly varied and depends on the type of both pollutant and xenobiotic effects that exert on the species.

Beeby (2001), Frame and Dickerson (2006) and NRC (1991) speculated desirable characteristics for a species to be chosen as an effective sentinel as follows:

(1) The sentinel species should be sensitive to the contaminants

(2) The sentinel species should have a wide geographical distribution.
(3) The sentinel species should have a territory or home range which overlaps the area to be monitored.
(4) The sentinel species should be easily enumerated and captured.
(5) The sentinel species should have a life span that appropriated to the objective of the study. Short-lived species can be used for assessment of acute and sub-chronic effects, while long-lived species is well-suited for chronic effects of contaminant exposure.

Fish have been valued as sentinel species for use in water quality studies for many years. They are easy to identify, their ecology and physiology is relatively well known and as they are at the top of the food chain, they may reflect changes in the community as a whole (Mason, 1981). Many species are widely used in research and aquatic toxicity testing, including trout, carp, suckers, mosquito fish, medaka, flounder, killifish and zebrafish (Adams, 1995; Frame and Dickerson, 2006).

2.4 Smith's barb *Puntioplites proctozysron* Bleeker (1865)

2.4.1 Taxonomy

The Smith's barb, *Puntioplites proctozysron* Bleeker **(**1865**)** is an oviparous fish with taxonomic classification as follows:

Phylum Chordata
Class Pisces
Order Cyprinifromes
Family Cyprinidae
Genus *Puntioplites*
Species *Puntioplites proctozysron*

Common name: Smith' barb
Thai name: Pla Ka-mung

2.4.2 Morphology

The general morphology of *P. proctozysron* is shown in Figure 2.5. The body is flat with general body length about 1.80-2.10 times of head length. The head is small, posed small eyes located superiorly in position. Its body is fairly elongated and moderately deep. The ventral scales range from 22 to 28 scales in both male and

female. The dorsal fin has a short base consisting of 3 spines with the third spine dorsal fin in serrated- shape, followed by 8-9 soft fin rays. The anal fin is relatively short consisting of 3 spines and 6-7 spine soft fin rays. The caudal fin is wide, moderately deep (fork type) and caudal, 18 scales. The medium scales range from 53 to 37. The anal fin consists of 15-17 scales. The body skin is dark, covered by silver scale. The upper posterior margin of fins is also dark in color (Bleeker, 1865; Smith, 1945).

2.4.3 Natural history

P. proctozysron is a tropical freshwater fish. It can survive in a wide range of habitats and different types of water bodies, such as ponds, canals and the main river. Its distribution range covers Laos, Cambodia and northern to northeastern part of Thailand (Smith, 1945). It is omnivorous fish. It feeds on a wide variety of foods such as aquatic plant and plankton (Banyen, 1988).

Maturity of *P. proctozysron* is about 11 cm or greater. In maturation stage, the shape of male's body is slender than that of the female's body. The breeding period of *P. proctozysron* occurs during the rainy season from June to July. In the breeding season, its body size usually increases in both body length and body width (Banyan, 1988; Duangsawasdi, 1988).

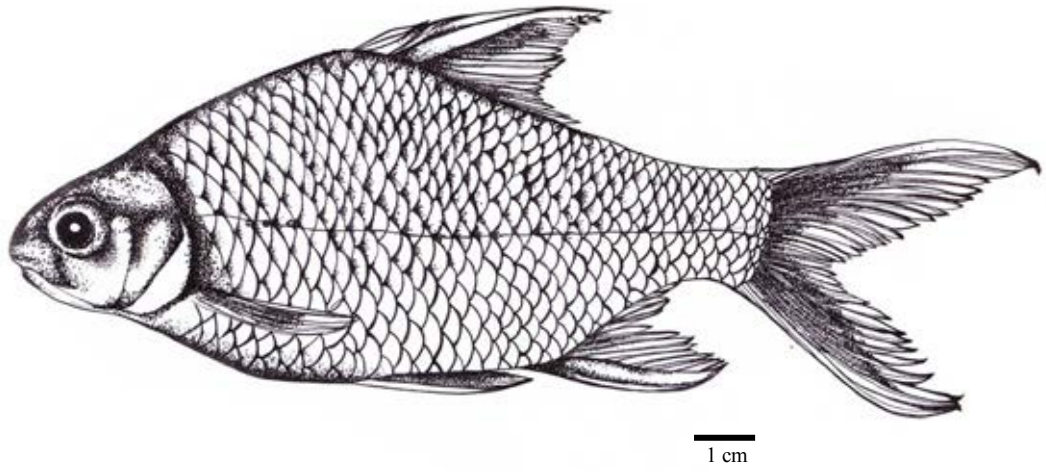

Figure 2.5 Drawing of Smith's barb *Puntioplites proctozysron*, lateral view (Drawing by Sinlapachai Senarat)

2.5 Reproductive system of teleost

2.5.1 Teleost testis

The testes of male fish are typically paired and elongated organs extending along the length of the coelom. It is bound by a thin layer of connective tissue and suspended from the dorsal perineum or mesorchium. The testis is covered by tunica albuginea consisting of dense connective tissue and distinct smooth muscle. A vasa efferentia of each lobule join to form a vasa deferentia before reaching the urogenital papilla. The wall is thickest in the immature testis and thinnest in the fully mature testis (Dietrich and Krieger, 2009; Grier et al., 1980; Nagahama, 1983).

Testicular structures of teleosts are classified based on a gross anatomy into two basic types: the anatomosing tubular and the lobular testis (Figure 2.6). In the anatomosing tubular testis type, the tubule loop at the testis periphery appears as a continuously anastomosing tubular system (Figure 2.6A and 2.8C). This testis type is hypothesized to be typical for lower fish, such as in the coelacantheiform, acipenserifprm, elopiform, salmoniform, siluriform, characiform, clupeiform, lepisoeifprm, esociform and cyprinidiform. Alternatively, in the lobular testis type, the blind lobules terminate at the testis periphery and drain to the main duct (Figure 2.6B, 2.8A and B). The lobules may form an anastomosing system at the midportion of the testis. This testis type is found in perciform, cypriniform, atheriniform, beloniform and possibly in other higher fish taxa (Dietrich and Krieger, 2009; Parenti and Grier, 2004).

Both testis types can be classified further into two types based on the intratubular or intralobular distribution location of spermatogonia: unrestricted and restricted spermatogonial types. The testis which spermatogonia are freely distributed along the length of the tubule or lobule is defined as unrestricted spermatogonial type (Figure 2.7B and 2.8B). This type is common in neoteleostes except for the atherinomorphs. In contrast, the fish testis that whose show the distribution of spermatogonia restricted to the distal end of the lobule is classified as the restricted-spermatogonial type (Figure 2.7A and 2.8A). This type has been so far reported only for lobular testes of atherinomorph fish (Grier et al., 1980; Nagahama, 1983; Parenti and Grier, 2004).

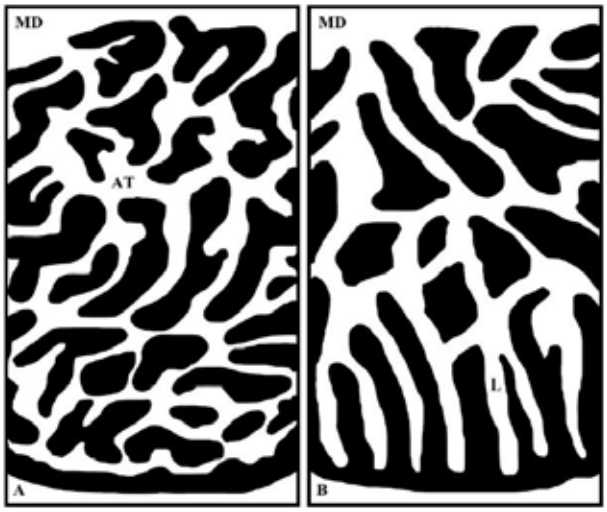

Figure 2.6 Diagram representation of gross structure of testis types in teleosts (Dietrich and Krieger, 2009).

A The anatomosing tubular testis

B The lobular testis

(AT = anastomosing tubular system, L = Lubule, MD = main, longitudinal testis duct)

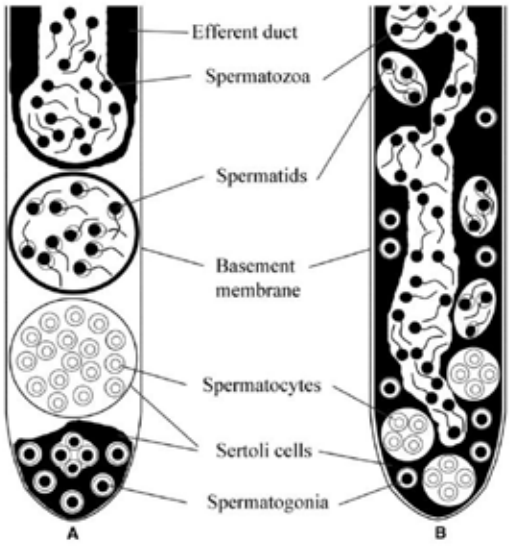

Figure 2.7 Diagram showing two tubular testicular types observed in teleost (Dietrich and Krieger, 2009).

A the restricted spermatogonial testis and

B the unrestricted spermatogonial type

2.5.2 Teleost spermatogenesis

The teleost testis contains numerous convoluted seminiferous lubules. It is organized into two compartments, interstitial compartment and germinal epithelium (Figure 2.9). The interstitial compartment comprises of Leydig cells and blood vessels. The germinal epithelium consists of basement membrane, spermatogenic cells and Sertoli cells. The Sertoli cells extended their thin cytoplasmic processes and form a cyst-liked structure where the same stage of sperm cells are surrounded. The spermatogenesis of teleost is entirely occurred inside this cyst. The formation of Sertoli cell cyst begins when the development of primary spermatogonia started at mitotic division and will be broken down when the germ cell reaching the last stage, then the mature spermatozoa are released into the lumen of seminiferous lobule (Billard, 1992; Dietrich and Krieger, 2009; Nagahama, 1983).

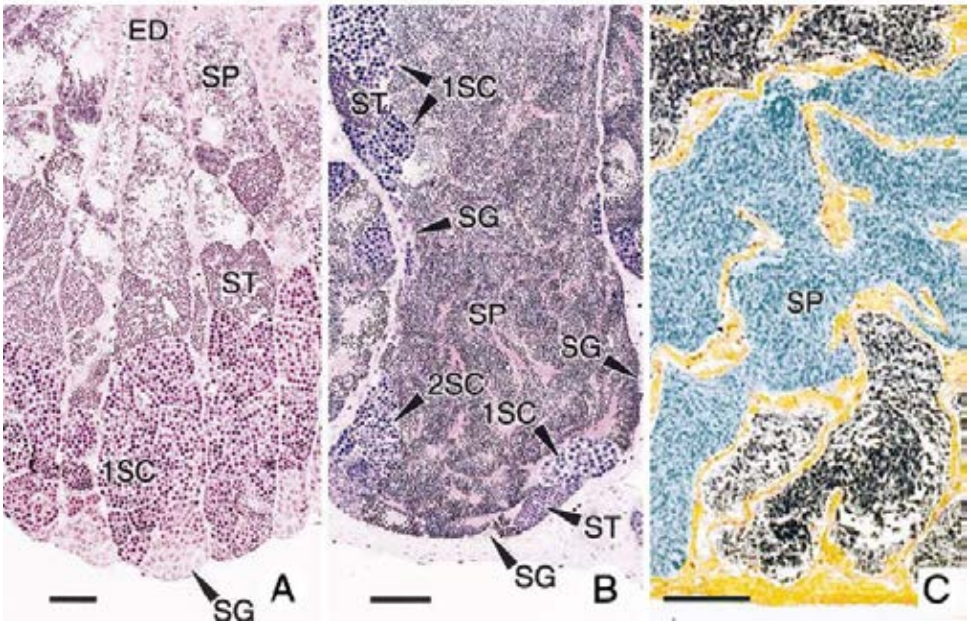

Figure 2.8 Cross sections of the testis illustrate the examples of testis type (modified from Parenti and Grier, 2004).

A the restricted lobular testis of Gulf killifish, *Fundulus grandis*

B the unrestricted lobular testis of striped mullet, *Mugil cephalus*

C the anastomosing tubular testis of tarpon, *Megalops atlanticus*

(ED = efferent ducts; 1SC = primary spermatocytes; 2 SC = secondary spermatocytes; ST = spermatids; SP = sperm; SG = Spermatogonia)

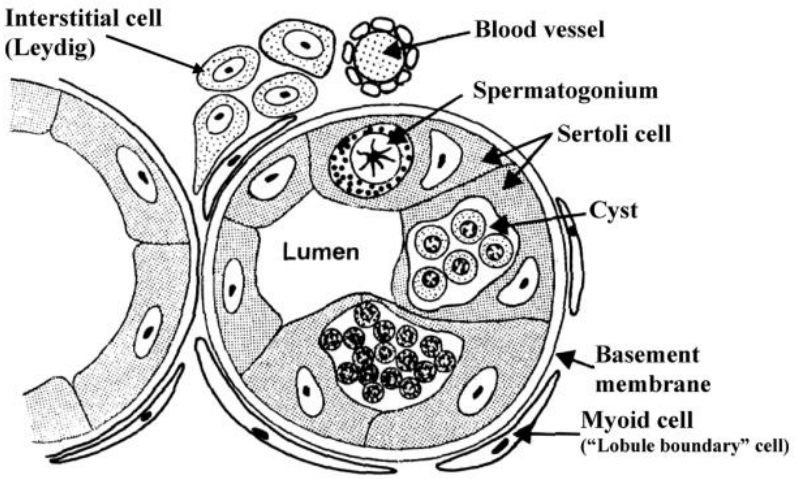

Figure 2.9 Diagram showing the organization of teleost testis divided into interstitial and germinal compartments (Dietrich and Krieger, 2009).

The spermatogenic process of teleost fish is divided into three distinct phase: spermatogonial phase, spermatocyte phase and spermiogenetic phase (Nóbrega et al., 2009; Schulz et al., 2010). The example of spermatogenesis of the zebrafish, *Danio rerio* is shown in Figure 2.10.

The spermatogonial phase consists of the different generations of spermatogonia that undergo mitotic divisions. Spermatogonia can be classified based on morphology into two types: type A and type B spermatogonia. Type A spermatogonia are the largest germ cells in the testis which acted as the stem cell. Type B spermatogonia are slightly smaller and dividing more rapidly than the type A spermatogonia. The number of spermatogonia generations varies among fish species, with approximately 3-14 generations in each cyst before entering meiosis (Schulz et al., 2010).

In the spermatocyte phase, in this phase the genetic material is duplicated, recombined, and segregated by meiotic divisions which consists of two cell cycles.

The primary spermatocyte phase consists of five steps: leptotenic, zygotenic, pachytenic, diplotenic spermatocytes and metaphase I. The general characteristics used to identify these cells are the gradual clumping of nuclear chromatins, the

disappearance or unclear of nucleolus and the cytoplasm is restricted to a narrow rim around the nucleus (Albert et al., 1994; Schulz et al., 2010).

The secondary spermatocytes are smaller than primary spermatocytes usually found together with metaphase II (Schulz et al., 2010). The nuclear characteristics of secondary spermatocytes in some fish such as the ray-finned fish*, Garra gotyla* were identified by either a cup-shaped structure or a clock-faced structure under light microscope. The duration of this stage is short in most of teleosts (Billard, 1992; Nagahama, 1983).

The spermiogenetic phase consists of morphological and functional changes that lead to the differentiation of spermatids into spermatozoa. In fish, three types of spermatids have been proposed based on the orientation of the flagellum to the nucleus and the rotation of the nucleus. These three types are characterized as: type I, a perpendicular flagellum in relation to nucleus with rotation of the nucleus; type II, the flagellum develops in parallel to the nucleus without rotation of the nucleus; type III, flagellum well developed without rotation of nucleus (Schulz et al., 2010).

Spermatozoan is the smallest among spermatogenic cells. It consists of head, neck piece, midpiece, one or two long flagella and generally has no acrosome (Nagahama, 1983).

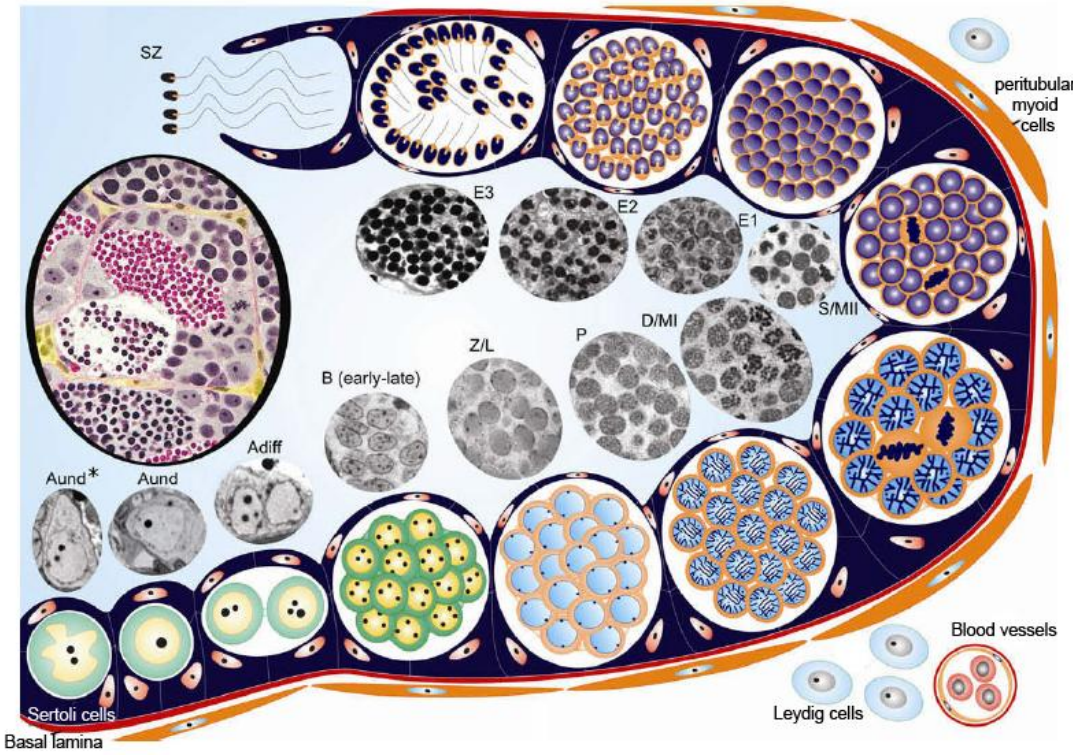

Figure 2.10 The diagram showing spermatogenesis of the zebrafish, *Danio rerio* including spermatogonial phase (modified from Schulz et al., 2010)

2.5.3 Teleost ovary

The ovarian tissue of fish is a hollow bilateral pair of tubular or saccular structure which appeared in most teleost species (Figure 2.11). Ovary is covered by a tunica albuginea (ovarian wall). In adult fish, the numerous ovigerous folds, where oogenesis takes place, are irregular in shape and consist of germinal epithelium and stroma which subjacent to the epithelium. The ovarian ducts of each bilateral ovary are joined and lead to the genital pore (Selman and Wallace, 1989).

Most of teleost fish have a spawning season. The ovary size and stage varies upon reproductive cycle over the year. Fish ovary can be classified in the broadest sense into three basic types (Dietrich and Krieger, 2009; Nagahama, 1983):

First, synchronous ovaries (synchronisme total): the ovary consists of oocytes develop in unison. This typed is found in fish that spawn once in their lifetime and die after spawning, e.g., fish in the genus *Oncorhynchus* and *Catadromous*.

Second, group synchronous ovaries: this type comprises of two different groups of oocyte which are occurred at the same time. The fish that have this ovary type have a single annually and short breeding period, e.g., flounder, *Liopesetta abscura* and rainbow trout, *Salmo gairdneri*.

Third, asynchronus ovaries: this type consists of all stages of oocytes which can be found together in some time. This fish group has a several long breeding period in a year, example as, zeabrafish, *Danio rerio*.

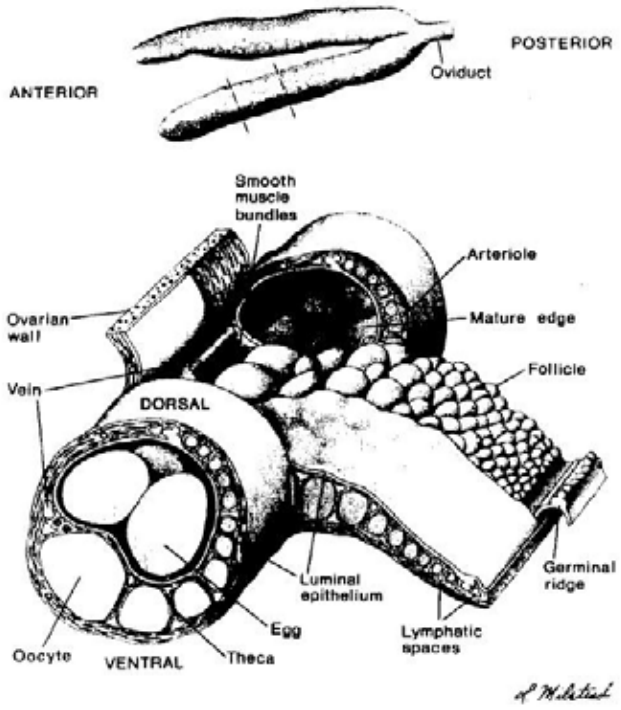

Figure 2.11 Drawing of ovary of the Pipefish *Syngnathus scovelli* (Selman and Wallace, 1989)

General histological structure of oocyte development have been summarized by Wallace and Selman (1981) and Tyler and Sumpter (1996) in many teleost species. The ovarian oogenesis occurs in the ovigerous fold. The first stage of oocyte is oogonia. It is surrounded by the follicle cells that will develop to granulosa cell and thecal cell layers. Therefore, in mature stage or vitellogenic stage, the follicle will consists of granulosa layer and thecal layer which are separated by the basement membrane. The thecal cell layer comprises of fibroblast, blood vessel and collagen

fiber (Figure 2.12). The granulosa cell and thecal cell are steroid-producing cells which can be found in some teleost. Juvenile females are easily identified histologically by the presence of numerous immature oocytes within ovigerous fold (Nagahama, 1983).

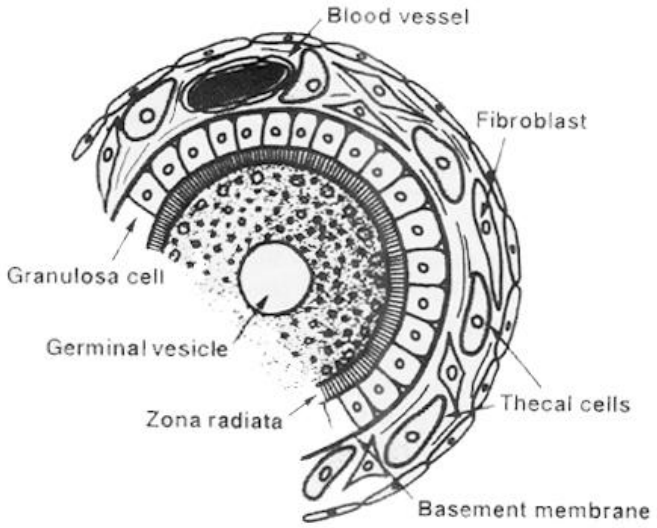

Figure 2.12 Diagram shows the oocyte surrounding by follicle cell layer (Dietrich and Krieger, 2009).

2.5.4 Teleost oogenesis

The classification of oocyte development in teleosts has been investigated by many researchers (Al-Daham and Bhatti, 1979; Gupter, 1975; Mayer et al., 1988). In general, the oocytes of teleosts have been classified into four stages, based on their histological structure: oogonia, immature oocyte maturing oocyte and mature oocyte However, in some fishes, they have additional oocyte stages classified by the uptake of vitellogenin or yolk protein including previtellogenic, vitellogenic and postvitellogenic or mature stage (Gupter, 1975). Abu-Hakima (1987) purposed the division of oocyte stages as follows: oogonia, primary growth phase, early stage of vitellogenesis, mid late vitellogenesis and maturation of yolk granule. While, Mayer et al. (1988) divided the oocyte stages into only two phases: primary growth phase consists of oogonia, chromatin nucleolus stage, early perinucleolus stage and late perinucleolus stage and secondary growth phase which is further divided into lipid

vesicle stage I, lipid vesicle stage II, primary yolk granule stage, secondary yolk granule stage and tertiary yolk granule stage.

2.6 Biomarker

In a modern society, a wide variety of contaminants are released to the environment every day from urban residential, commercial, agricultural as well as industrial sources. Many of these releases cause adverse effects to human society and the environment. In order to monitor those effects, scientists have developed many approaches to use as early warning signal, whereby contaminants will be detected before they exert their effects. One of the biological approaches is "biomarkers", which offer the potential for integrating various interactions within the exposure as a biomarker response, measured at the site of toxicant action of an organism (Lam and Gray, 2003; Shugart et al., 1992; van der Oost et al., 2003).

The National Research Council of USA (NRC, 1987) defined a biomarker as "a xenobiotically induced variation in cellular or biochemical components or processes, structures, or function that is measurable in a biological system or sample". The term is most often used to refer to molecular, physiological, and organismal responses to contaminant exposure that can be quantified in organisms inhabiting or captured from natural systems (Di Giulio and Newman, 2008).

Biomarker can be divided into three main classes (NRC, 1987; WHO, 1993):

I. *Biomarkers of exposure* is an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism in the given environments.

II. *Biomarkers of effect* is a measurable biochemical, physiological, behavioral or other alteration within an organism that can be recognized as associated with an established or possible health impairment or disease.

III. *Biomarkers of susceptibility* is an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance.

It can be said that biomarkers of exposure are used to identify the occurrence of the exposure at lower level of organization. On the other hand, biomarkers of effect are generally changes within the body at higher levels of organization in response to an exposure that can be linked to later health effects, as summarized in Figure 2.13. The dividing line between biomarkers of exposure and biomarkers of effect is not always apparent. So that, many biomarkers fit somewhere between and can be considered as both types (Hanson, 2008; Wallace, 2007).

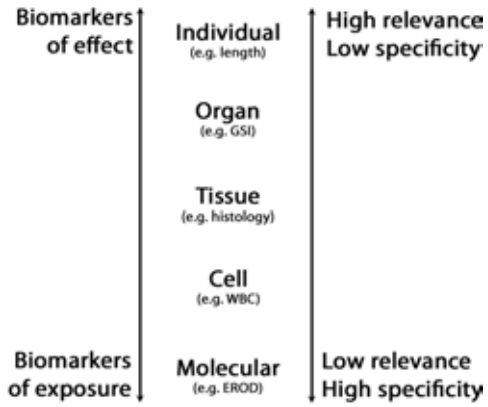

Figure 2.13 Relationship of biomarker types and levels of organization (Hanson, 2008).

Biomarkers in fish have been used in ecotoxicological studies in contaminated aquatic environment for a long time (Hanson, 2009; van der Oost et al., 2003). Because fish can be found in most aquatic environments, they play a major ecological role in aquatic food webs as a top-predator and accumulated pollutants from lower to higher trophic levels. The pollutants that accumulated in fish can be transferred to humans because fish is an important food resource for human. Van der Oost et al. (2003) suggested that the following biological and biochemical parameters may be examined in fish in order to assess the exposure of chemical contaminants in the environments: biotransformation enzymes (phase I and II), oxidative stress parameters, biotransformation products, stress protein, haematological parameters, immunological parameters, reproductive parameters, genotoxic parameters, neuromuscle parameters, physiological parameters and histological parameters.

In this thesis, the accumulation of herbicide in gonads of the fish is selected as a biomarker of exposure and condition factor (CF), gonadosomatic index (GSI) and gonadal histopathology are selected for biomarkers of effects.

2.6.1 Condition Factor

Condition factor (CF) has always been used to assess the well-being of fish species (Goede and Barton, 1990). It can be used to reflect the impact of the environment on the fish growth and provides information on the health status of the fish (Adams et al., 1993). CF is calculated based on a formula by Le Cren (1951) as a relationship between weight (W) and length (L): $W = aL^b$. The equation can be transformed into its logarithmic mode to obtain linear graph as:

$$\text{Log } W = b \log L + \log a$$

While, a is a constant (a is 3 for adult fish, Le Cren, 1951) and b is the slope of the linear regression. Then, CF is calculated from:

$$CF = W/aL^b$$

In general, CF varies directly with nutrition (Tyler and Dunn, 1976), season (Griffiths and Kirkwood, 1995) among fish taxa and various among geographical localities within a species (Doyon et al., 1988; Fisher et al., 1996). CF may vary from normal range in response to pollution exposures (Authman, 2008; Parveen and Javed, 2010). Some physical factor such as an increase in body water may decrease fish weight due to the loss of energy stores (Goede and Barton, 1990).

2.6.2 Organo-somatic indices

Organo-somatic indices (OSI) gain popularity in fish health and population assessment (McDonald et al., 2000). They can be calculated by ratios of the mass of particular organs or tissues relative to total body weight. These indices may reflect the change in size of target organ caused by the environmental factors and stressors more rapidly than the organism weight and length changes (Goede and Barton, 1990). The organosomatic index that will be used in this study is gonadosomatic index (GSI).

Gonadosomatic index (GSI) is routinely used to measure the sexual maturity of animals in correlation to ovarian and testicular development as well as to assess gonadal changes in response to environmental dynamic or contaminant exposure (McDonald et al., 2000). There are several factors that affecting to GSI naturally including age (Patnaik et al., 1994), season, reproductive cycling (McDonald et al., 2000), temperature (Kamanga et al., 2002) and photoperiod cycle (Nakari, 1986) and gender. Male experiencing less gonadal weight gain during recrudesce than female (McDonald et al., 2000),

The GSI is determined by the following formula (McDonald et al., 2000):

$$GSI = \text{gonad weight/body weight} \times 100$$

The measurements should be made on live or freshly killed fish to avoid weight fluctuations induced by storage conditions, such as moisture loss or gain (McDonald et al., 2000).

The changes of GSI according to gonadal alteration after exposure to environmental pollutants, particularly endocrine-disrupting chemicals, have been proved by previous studies (Pait and Nelson, 2002). Bringolf et al. (2004) studied the effects of atrazine on fathead minnow, *Pimephales promelas*. They found that the decrease trends of GSI in both sex were coincided with increased level of the atrazine concentrations, even though these trends were not statistically significant. They argued that the reduction of GSI may resulted by the disruption of steroid hormone by atrazine. On the contrary, Figueiredo-Fernandes et al. (2006) studied gonad health of tilapia, *Oreochromis niloticus* that exposed to a low concentration of paraquat at different temperatures. The results showed that no differences were found for the GSI between males. However, paraquat-treated females showed high GSI values when compared with the control group at 27ºC. This increasing of GSI was related to an increase of the percentage of late-vitellogenic and mature oocytes in treated groups.

2.6.3 Gonadal histopathology

Tissue and organ structure is an integration of many biochemical, cellular and physiological processes occurring within it, as well as any pathological disturbances. Hence, the analysis of ultra-structure of cells and tissues will provides essential information on the pathological changes occurring in those cells and can be related to both biochemical changes at the cellular level and tissue pathological effects resulted from effected form the disturbances, particularly exogenous substances (Lawrence and Hemingway, 2003).

Normal histology and histopathology of fish gonads have been routinely used as a supporting parameter in the study of reproductive health status and to detect possible pathological changes in fishes (Blazer, 2002; OECD, 2009). Gonadal histopathology is often utilized alone or in conjunctions with the morphological studies (e.g., GSI) and measurements of reproductive hormones to indentify gonadal phenotype, tumors, parasites, determine the state of sexual development, investigate reproductive impairment, other abnormalities and quantifying atresia (Blazer, 2002; McDonald et al., 2000). Histopathological investigations of gonads have been proved to be a sensitive tool to detect direct toxic effects of chemical compounds, partially endocrine disrupting chemicals, to the gonads of the fish in both laboratory experiments (e.g., Spanò et al., 2004; Tillitt et al ., 2010) and in field investigations (e.g., Barnhoorn et al., 2004; Iwanowicz et al., 2009).

OECD (2009) recommended the use of histological changes observed in gonadal tissues of fathead minnow, *Pimephales promelas*, the Japanese medaka ,*Oryzias latipes* and zebrafish, *Danio rerio* as potential biomarkers of effect of contaminant exposure. The diagnostic criteria of histopathological changes in the gonads can be divided into two categories, primary and secondary criteria, and consists of:

I. Primary diagnoses
   a. *Male*: increased proportion of spermatogonia, presence of testis-ova, increased testicular degeneration, interstitial cell (Leydig cell) hyperplasia or hypertrophy;

b. *Female*: increased oocyte atresia, perifollicular cell hyperplasia or hypertrophy, decreased yolk formation, changes in gonadal staging;

II. Secondary diagnoses

a. *Male*: decreased proportion of spermatogonia, increased vascular or interstitial proteinaceous fluid, asynchronous gonad development, altered proportions of spermatozoa or spermatocytes, gonadal staging, granulomatous inflammation;

b. *Female*: interstitial fibrosis, egg debris in the oviduct, granulomatous inflammation, decreased post-ovulatory follicles.

OECD (2009) further suggested additional histopathological changes findings that should be observed incorporated with the above diagnostic criteria. They are gender of fish, germ cell neoplasms, germinal epithelium atrophy or hypoplasia, gonadal stromal tumors, increase or decreased of hepatocyte basophilia, histiocytic cells, increased or decreased cells in gonads, macrophage aggregates, mineralization, nephropathy, oocyte membrane folding, ovarian cysts, ovarian spermatogenesis, retained peritoneal attachments/gonadal duct feminization, sertoli cell hypertrophy, sperm necrosis and vitellogenic oocyte.

CHAPTER III

HERBICIDE RESIDUES IN ENVIRONMENT AND GONAD OF *Puntioplites proctozysron* LIVING NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE

3.1 Introduction

Paraquat, glyphosate and atrazine are the most commonly used herbicides in agricultural areas along the Nan River in Nan Province, northern part of Thailand. Thus, using of these herbicides in agriculture may lead to environmental and health problems. The levels of herbicide contamination in environment have certainly increased. High concentration levels of these herbicides in environmental samples have been reported worldwide. For examples, high concentrations of glyphosate were detected from water samples collected near intense cultivation areas in southern Brazil (Da Silva et al., 2003). Atrazine found as high as 40 µg/L in the water near agricultural areas (Rohr et al., 2006; Storrs and Kiesecker, 2004). Moreover, a study in Denmark also reported the concentration of glyphosate in groundwater of about <0.1-4.7 mg/L (Rohr et al., 2006).

Herbicide contamination in environment can cause undesirable effects on non-target aquatic animals, like fish, which directly exposed to the contaminants. Calderbank (1972) studied the rainbow trout, *Salmo gairdneri* that exposed to 1 mg/L of glyphosate. After one week, 0.5-0.6 µg/L of glyphosate was detected in the fish tissue. Earnest (1971) also reported that the freshwater fish exposed to 1.14 mg/L of paraquat for 48 hours had high concentrations of paraquat (0.58-1.06 mg/L) in the tissues. Furthermore, Du Preez and Van Vuren (1992) found bioconcentration of 50.6 µg/g of atrazine in the ovaries of banded tilapia, *Tilapia sparrmanii*, after the exposure of 16.20 mg/L atrazine for 72 hours.

As a few number of research results have been reported for the persistence of herbicides in environmental samples (sediment and water) in Thailand and almost no information on bioaccumulation of residues in the gonad of fish is available. It is imperative to investigate the persistence of herbicides in gonad of fish and the living environment.

3.2 Materials and methods

3.2.1 Study area and sample collection

Water and sediment samples were collected at the same time of field sampling for collecting fish, *Puntioplites proctozysron,* from the Nan River at San Sub-district, Wieng Sa District, Nan Province (Figure 3.1A, B). Water and sediment were kept into acetone-rinsed high-density polyethylene bottles and maintained below 4 ºC during transportation and storage, before quantifying the types and quantitative herbicide concentration. Physical and chemical parameters of dissolved oxygen (DO), pH and temperature (T) were measured by the Waterproof CyberScan PD 300 Multi-parameter meter on the site during sampling.

Mature *P. proctozysron* (approx. >10 cm in total length) were collected by fishing net year round in rainy season (July and October 2010) and dry season (January and April 2011) for 40 individuals per sampling month (20 individuals of each sex: total 160 fishes). All fishes were kept in aerated holding tank and transported to the laboratory at Chulalongkorn University Forest and Research Station, Wiang Sa District, Nan Province. No mortality was found during the transportation. All fish were euthanized by rapid cooling method (Wilson et al., 2009). The posterior abdominal wall was opened. The gonads were removed and kept in aluminum foil. These samples were stored at 0-4 ˚C for further chemical analysis.

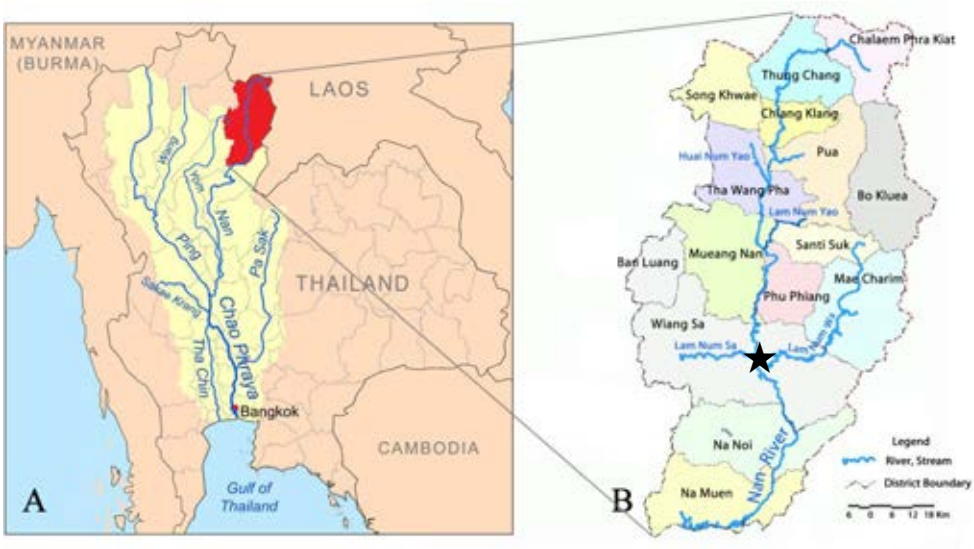

Figure 3.1 **A** Map of the Chao Phraya River drainage basin

B Map of Nan Province shows the river systems and the sampling locality (★) at Nan River, Wiang Sa District, Nan Province.

3.2.2 Determination of herbicide residues in water

Herbicide residues in water was analyzed by Central Laboratory (Thailand) Co., Ltd. (ISO/IEC 17025), an accredited institute for food testing by the Nation Bureau of Laboratory Quality Standards, as listed in Table 3.1.

Table 3.1 Chromatographic methods for paraquat, glyphosate and atrazine analysis.

Compound	Analysis method
Paraquat	HPLC-DAD (Agilent 1100)
Glyphosate	HPLC (Agilent 1100), Post-column derivatizer (Pickering PCX 5200)
Atrazine	GC-MS (Agilent 6890N)

3.2.2.1 Paraquat

A 100 mL of water sample was adjusted to pH 9 and applied on the silica solid phase extraction (SPE). Then, the sample was eluted from the SPE with 10 mL of a mixture of 8 M HCl and methanol (9:1). The eluates were evaporated to dryness and re-dissolved to 2 mL with mobile phase. The sample was filtered through nylon syringe filter (0.22 μm) prior to further analysis by HPLC-DAD.

3.2.2.2 Glyphosate

Sample preparation of glyphosate was followed Borjesson and Torstensson (2000). In brief, a 200 mL of the water sample was adjusted to pH 2 and applied on Chelex 100 column. The column was washed with water and 0.2 M HCl, and eluted with 6 M HCl. The eluates were then clean-up on AG 1-X8 column. Next, the sample was evaporated to dryness under vacuum, adding water and repeated the evaporation. The residue was dissolved in 1 mL of a mixture of water-methanol-HCl (160:40:2.7) Finally, the sample was evaporated under nitrogen and re-dissolved in ethyl acetate prior to further analysis by HPLC with post-column derivatizer.

3.2.2.3 Atrazine

A 500 mL of water sample was added with sodium chloride (NaC1) before extracted with dichloromethane (CH_2Cl_2). The extracted sample was dried up in an evaporator and re-dissolved with ethyl acetate prior to further analysis by GC-MS.

3.2.3 Determination of herbicide residue in sediment

Herbicide residues in sediment was also analyzed by Central Laboratory (Thailand) Co., Ltd. as listed in Table 3.1.

3.2.3.1 Paraquat

An about 25 g of sediment was treated with water, 2-octane, and sulfuric acid, and then refluxed for 5 hours. After cool down, the sample was filtered through filter paper and Celite before cleaned up with silica SPE. Then, the sample was evaporated to dryness and re-dissolved with a mobile phase. The sample was filtered through nylon syringe filter (0.22 μm) prior to further analysis by HPLC-DAD.

3.2.3.2 Glyphosate

A 10 g of sediment was extracted with 1 M NaOH for 30 min twice. The pooled extracts was treated with concentrated HCl before diluted with water and adjusted to pH 2. A 50 mL of a clear extract was then followed the sample preparation of water sample as describe in Section 3.2.2.2.

3.2.3.3 Atrazine

A 10 g of sediment was added with NaCl and water. After well mixing, the sample was extracted with 10 mL of acetonitrile and shaking for 5 hours. The liquid supernatant was separated and evaporated to dryness under stream of nitrogen gas. The residue was re-dissolved to 2 mL with ethyl acetate and treated with anhydrous $MgSO_4$ and primary secondary amine (PSA) sorbent. The sample was filtered through 0.22 μm syringe filter prior to further analysis by GC-MS.

3.2.4 Determination of herbicide residue of whole fish and gonadal tissue

The paraquat and glyphosate were determined by ELISA whereas the atrazine was analyzed by HPLC as listed in Table 3.2. ELISA for paraquat was obtained from Abnova Inc (Paraquat plate kit 96 well) while glyphosate was obtained from Abraxis LLC (Glyphosate plate kit 96 well).

3.2.4.1 Sample preparations

Paraquat

Sample preparations of paraquat in both whole fish and gonadal tissue were modified from the method reported by Brown et al. (1996) and Quick et al. (1990). In brief, 100 mg of lyophilized whole fish or gonadal tissue were added with 200 μL of

MilliQ water and mixed for 1 min. After that, 600 µL of 10% tricarboxylic acid (TCA) was added and mixed for 5 min before the tube was centrifuge at 2,000×g for 15 min. Approximately 550 µL of the liquid supernatant (aqueous extract) was transferred into a new microtube. The remaining pellet was subjected to repeated ~~the~~ extraction step by adding 250 µL of 10% TCA, mixing for 2 min, and centrifuging for 10 min at 2,000 ×g. Approximately 250 µL of the liquid supernatant was pooled into the previous extract. The pooled extract was subjected to lipid removal by adding 400 µL of hexane and centrifuging for 10 min at 2,000 ×g. A 700 µL of liquid supernatant was taken and adjusted to pH 6-8 by adding 300 µL of 2 M Tris buffer. The final samples were stored in microtube at -20 °C until the ELISA analysis.

Glyphosate

Sample preparations of glyphosate in whole fish and gonadal tissue were done by modifying the method of Alferness and Iwata (1994). A 100 mg of lyophilized whole fish or gonadal tissue were added with 200 µL of MilliQ water and mixed for 1 min. After that, 100 µL of chloroform and 400 µL of 0.1 M HCl were added and mixed for 5 min. After addition of 4 mg of sodium sulfate, sample was mixed for 2 min, and then centrifuged at 2,000 ×g for 10 min. The supernatant (400 µL) was added with 400 µL of chloroform and mixed for 2 min, before centrifugation for 10 min at 2,000 ×g. Finally, 350 µL supernatant was transferred to a new tube, evaluated pH value by pH paper and adjusted pH to 6-8 by adding 1 M NaOH or 1 M HCl. The samples were stored in a microtube at -20 °C until the ELISA analysis.

Atrazine

Sample preparations of atrazine in whole fish and gonadal tissue were done by modifying the method reported by Jacomini et al. (2003). A 400 mg of lyophilized whole fish or gonadal tissue were added with 1 mL of MilliQ water and mixed for 30 sec. The sample was added with 400 µL of 1.5 M NaOH and 8 mL of dichloromethane and shaked for 20 min. The sample was centrifuged for 10 min at 2000 ×g. The organic layer (upper layer) was transferred into a clean tube and evaporated to dryness by nitrogen evaporator (Figure 3.2). The residues of all samples were dissolved in 400 µL of mobile phase and filtered through 0.22 µm syringe filter prior to further analysis by HPLC. The samples were stored at 4 °C until the HPLC-UV analysis.

Figure 3.2 **A** High Performance Liquid Chromatography (HPLC),
B-C Evaporator

*3.2.4.1 Analysis of herbicide*s

Paraquat

A 25 µL of standard paraquat (0, 0.375, 0.750, 2.50, 7.50 ng/mL) and sample extracts were loaded into a 96-well plate coated with anti-paraquat antibody. A 100 µL of paraquat-horse radish peroxidase conjugate was added into each well and incubated for 30 min at room temperature. The plate was washed three times with 250 µL of washing buffer, then 100 µL of hydrogen peroxide and stabilized tetramethylbenzidine (TMB) substrate solution were added. The plate was incubated again for 15 min at room temperature. Finally, 100 µL of 3 M HCl was added to stop the reaction. Absorbance was measured at 450 nm using a microplate ELISA reader (Multiskan EX, Thermo Labsystems).

Calibration curve of paraquat standard was obtained by plotting paraquat concentrations (0, 0.375, 0.750, 2.50 and 7.50 ng/mL) on x-axis against the percentage of inhibition, as the following equation.

$$\% \text{ inhibition} = 100 - \frac{\text{Sample absorbance}}{\text{Blank absorbance}} \times 100$$

Paraquat content in dried sample was calculated from ELISA analysis using the following formula;

$$\text{Paraquat in dried sample (ng/g)} = \frac{(8A)(10)}{(6.5)}$$

Where: A is the calculated paraquat concentration in ng/mL from ELISA analysis.

Glyphosate

A 250 µL of sample extracts and standard glyphosate (0, 0.075, 0.20, 0.50, 1.0 and 4.0 ng/mL) was added with 1 mL of assay buffer. After well mixing, 100 µL of derivatization reagent was added and incubated at room temperature for 10 min. A 50 µL of derivatized samples and standard glyphosate were loaded into a 96-well microplate coated with goat anti-rabbit antibody. Then, 50 µL of rabbit anti-

glyphosate antibody solution was added into each well and mixed for 30 sec by rotating on benchtop. The microplate was incubated around 30 min at room temperature. Then, 50 µL of glyphosate-horse radish peroxidase conjugate was added and mixed again for 30 sec before incubation for 60 min at room temperature. The plate was washed three times with 250 µL of washing buffer. After addition of 150 µL of substrate/color solution (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidive), the plate was incubated 20-30 min at room temperature. Then, 100 µL of stop solution was added and the plate was measured for absorbance at 450 nm using a microplate ELISA reader (Multiskan EX, Thermo Labsystems).

Calibration curve of glyphosate was plotted between %B/B0 on y-axis and the corresponding glyphosate concentration in logarithmic scale on x-axis where B and B0 are the absorbance of sample and blank, respectively. A glyphosate concentration in ng/mL could be determined from the standard calibration curve. Recovery of the method was examined by spiking 100 mg of gonadal tissue with 0.8 ng of glyphosate and subjected to extraction and derivatization as in other samples.

Glyphosate in dry sample can be determined by the following formula:

$$\text{Glyphosate in dried sample (ng/g)} = \frac{(1.714275)(A)(10)}{(100)}$$

Where: A is the calculated glyphosate concentration from ELISA analysis.

Atrazine

Atrazine standard solutions were prepared to the concentration of 0.010, 0.050, 0.10, 0.50, 1.0 and 1.5 µg/mL in methanol and analyzed by HPLC as the condition listed in Table 3.2. Calibration curve of atrazine was constructed by plotting atrazine concentrations on x-axis against peak areas on y-axis. The sample extract was analyzed and determined the concentration of atrazine from the standard calibration curve. Recovery of the method was examined by spiking 400 mg of control gonadal tissue with 80 µg of atrazine and subjected to similar extraction process as other sample.

Table 3.2 HPLC condition for atrazine analysis.

Parameter	Description
Instrument	HPLC-DAD (Agilent 1100)
Column	Ultra Aqueous C_{18} (150×4.6 mm), 5 μm
Mobile phase	methanol : water (60:40)
Flow rate	0.80 mL/min
Detector	UV-Vis at wavelength 230 nm

Atrazine content in dried sample was calculated from HPLC analysis by the following formula:

$$\text{Atrazine in dried sample (μg/g)} = \frac{(0.53)(A)}{B}$$

Where: A is the concentration of atrazine in the sample from HPLC analysis in μg/mL,

B is the weight of dried samples in g.

3.2.3 Statistical analysis

The mean concentrations of glyphosate residue were compared between each month using t-test. Spearman's correlation test was used to assess any significant correlation ($p < 0.05$) of herbicide concentration in gonadal tissue with herbicide concentration in water and sediment using statistical package for the social sciences (SPSS) software (version 15.0).

3.3 Results

3.3.1 Herbicide residue in water and sediment

The concentration of herbicide in sediment and water from Nan River measured during July 2010 to April 2011 are shown in Table 3.3. These concentrations were reported as milligram of herbicide per kilogram of dry weight (mg/kg) for sediment samples and as milligram of herbicide per liter (mg/L) for water samples.

For sediment samples, only atrazine was detected, with the lowest concentration at 0.01 mg/kg in July 2010 and the highest concentration at 0.24 mg/kg in January 2011. Glyphosate and paraquat were undetectable or lower than detectable level of the analysis method throughout the study.

For water samples, the atrazine concentration in the water sample was detected 0.15 mg/L in January 2011 and undetectable or lower than detectable level of analysis method for the rest of studied month. Likewise, glyphosate and paraquat were undetectable or lower than detectable level of analysis method throughout the study.

Table 3.3 Levels of herbicide contamination in water and sediment collected from Nan River, Thailand during July 2010 to April 2011.

Month	Herbicide concentration in samples					
	Paraquat		Glyphosate		Atrazine	
	Sediment	Water	Sediment	Water	Sediment	Water
July 2010	ND	ND	ND	ND	0.01 (mg/kg)	ND
October 2010	ND	ND	ND	ND	ND	ND
January 2011	ND	ND	ND	ND	0.24 (mg/kg)	0.15 (mg/L)
April 2011	ND	ND	ND	ND	ND	ND

ND = Non-detectable level or level of contamination was lower than limit of detection (LOD). LOD for paraquat: 0.01 mg/kg for sediment and 0.01 mg/L for water; LOD for glyphosate: 0.01 mg/kg for sediment and 0.005 mg/L for water; LOD for atrazine: 0.01 mg/kg for sediment and 0.01 mg/L for water.

3.3.2 Herbicide residue in fish tissue

3.3.2.1 Paraquat

For paraquat determination, no reaction was detected between the standard or paraquat in the fish gonadal tissue with readily available paraquat reagents in ELISA kit. Therefore, paraquat determination in the fish gonadal tissue was deemed unsuccessfully. Consequently, the experiment was terminated.

3.3.3.2 Glyphosate

Glyphosate concentrations in the tissue of *P. proctozysron*, collected at the Nan River were reported as nanogram of glyphosate per gram of dry weight (ng/g) of whole fish and gonad sample. According to ELISA kit, the estimated detection limit for glyphosate is 0.32 ng/g.

The preliminary study in July 2010, concentration of glyphosate in the whole fish was 22.30 ng/g for male fish and 22.35 ng/g for female fish. Therefore, in October 2010 to April 2011, the concentration of glyphosate was determined from the testes for male fish and the ovaries for female fish as shown in Table 3.4.

Table 3.4 Levels of glyphosate contamination in gonadal tissue collected from Nan River during October 2010 to April 2011.

Month/Sex	Glyphosate (ng/g) in gonadal tissue	
	Male (Mean±SD)	**Female (Mean±SD)**
October 2010	1.55 ± 0.93 (n=3)	1.92 ± 1.46 (n=3)
January 2011	2.01 ± 0.31 (n=3)	1.39 ± 0.12 (n=3)
April 2011	1.20 ± 0.26 (n=3)	1.17 ± 0.26 (n=3)

In male fish, concentrations of glyphosate in testes could be detected in both rainy and dry season which the highest mean concentration was detected in January 2011 (2.01 ng/g) and the lowest in April 2011 (1.20 ng/g).

In female fish, concentrations of glyphosate in ovaries were similar between seasons. Glyphosate concentration was highest in October 2010 (1.92 ng/g) and lowest in April 2011 (1.17 ng/g).

Nevertheless, mean of glyphosate in both sexes was not significantly different between each month (t-test, $p > 0.05$).

3.3.2.3 Atrazine

Atrazine concentration in fish tissues of *P. proctozysron* are shown in Table 3.5 and 3.6. These concentrations were reported as microgram of herbicide per gram dry weight (µg/g) of whole fish and gonadal tissue**.**

The preliminary study in July 2010, concentrations of atrazine in whole fish was found only in one sample of male fish (0.20 µg/g) while undetectable in whole female fish.

In male fish, concentration of atrazine in testes was ND-0.15 µg/g in dry season while the concentration of atrazine could not be detected in rainy season.

In female fish, concentration of atrazine in ovaries was similar between seasons. In addition, the concentration of atrazine in ovaries in dry season (ND-0.12 µg/g) was much higher than that found in rainy season (ND-0.10 µg/g).

Table 3.5 Levels atrazine contamination in whole fish collected from Nan River in July 2010.

Month	**Atrazine (µg/g) in whole fish**					
	Male			**Female**		
	1	**2**	**3**	**1**	**2**	**3**
July 2010 (n=3)	ND	ND	0.20	ND	N/A	N/A

ND = Non-detectable level or level of contamination was lower than limit of detection; N/A = Not Available. Based on this HPLC, the estimated detection limit for atrazine is 0.01 µg/g.

Table 3.6 Levels atrazine contamination in gonadal tissue collected from Nan River during October 2010 to April 2011.

Month	Atrazine (µg/g) in gonadal tissue					
	Male			Female		
	1	2	3	1	2	3
October 2010 (n=3)	ND	ND	ND	ND	0.10	ND
January 2011 (n=3)	0.06	0.15	ND	0.12	ND	ND
April 2011 (n=3)	0.09	0.10	ND	0.09	ND	ND

ND = Non-detectable level or level of contamination was lower than limit of detection. Based on this HPLC, the estimated detection limit for atrazine is 0.01 µg/g.

3.3.3 Correlation between herbicide concentrations in water, sediment and gonadal tissue

The sediment and water were positively significantly correlated, but was not significantly correlated with gonadal tissue (Table 3.7).

Table 3.7 Spearman rank correlations between sediment, water and atrazine concentrations in the gonadal tissue of *P. proctozysron*.

Sex	Atrazine		
	Sediment	Water	Gonadal tissue
Male	1.000**	1.000**	0.866
Female	1.000**	1.000**	0.866

**indicates significant correlation at $p < 0.05$

3.3.4 Physical and chemical parameters of environmental samples

Dissolved oxygen (DO), pH and temperature (T) of water were determined and shown in Table 3.8. The temperature values varied from 27.0 – 35.5 °C with the highest value in April 2011 and the lowest value in January 2011. The DO values varied from 6.6 - 7.9 mg/L with the highest value in July 2010 and the lowest value in April 2011. In addition, the pH values varied from 6.1 - 7.8 with the highest value in April 2011 and the lowest value in July 2010.

The pH and temperature of sediment were determined and presented in Table 3.8. The temperature values varied from 27.0 - 32.0 °C with the highest value in April 2011 and the lowest value in January 2011. In addition, the pH values varied from 6.0 - 7.0 with the highest value in October 2010 and the lowest value in January 2011.

Table 3.8 The Physical and chemical parameters of dissolved oxygen (DO), pH and water temperature (T) collected from this study.

Season	Month	Parameters				
		Water			Sediment	
		T(°C)	DO (mg/L)	pH	T (°C)	pH
Rainy	July 2010	28.3	7.9	6.1	28.0	6.1
	October 2010	28.5	6.8	6.7	27.8	7.0
Dry	January 2011	27.0	6.7	6.5	27.0	6.0
	April 2011	35.5	6.6	7.8	32.0	6.4

3.4 Discussion

This chapter has investigated the persistence of herbicides in environmental samples and the correlation between their persistence and the herbicides residue in gonadal tissues of *P. proctoztsron* living in Nan River during dry and rainy seasons.

3.4.1 The persistence of herbicides in water and sediment in Nan River

According to the results, atrazine was detected only in the water sample collected in January 2011 (dry season) with the concentration of 0.15 mg/L. This field observation confirmed that this area has been contaminated by the high level of

atrazine. The contaminant was also considerably higher than previous reports, such as 0.5-4.0 µg/L in underground waters from the central and northeastern part of Thailand (Sakunthengtong et al., 2001) and 1.83 µg/L in water sample from Pa Sak River, Central Thailand (Chanchek, 2007).

In water, the relatively high amount of atrazine found in January 2011 may reflect intensive agricultural activities in agricultural cities along the Nan River during this time of the year. Atrazine and other herbicides, usually found with relatively high concentration in river after applying the herbicides in nearby agricultural areas along the riverside (Homsby et al., 1995; Meister, 1999). Alternatively, the high level of atrazine in January 2011 may attribute to physical factors such as water body volume and river discharge. According to the Hydrology and Water Management Center for Upper Northern Region (2012; Figures in Appendix A), river discharges of Nan River nearby the collecting site were large during June 2010 to November 2010 (rainy season), but extremely low in other months (dry season). It is logical that if the river discharges decrease, the dispersion of atrazine decrease, resulting in atrazine concentrations increase. This factor was probably emphasized by the low level of Nan River in dry season and the raining in December 2010 (Appendix A, Figure B) that may wash atrazine into the river. On the other hand, in rainy season, the large river discharge and high volume of water body probably diluted atrazine into very low or non-detectable concentration, although atrazine was heavily applied in growing reason. The surface runoff by heavy rain was increased. However, this hypothesis alone cannot explain the case of April 2011, which the absent of atrazine may caused by no agricultural activities in this period. This result is similar to the previous study by Christiansen and Ziegler (1998). They stated that when the annual mean discharges were larger, the annual mean atrazine concentrations were smaller. Nevertheless, it was not comparable between sites because specific physical factors of each site may affect to atrazine concentration, such as crop type, method of application and amount of atrazine applied.

For sediment, atrazine was found in sediment samples collected in July 2010 (rainy season) and January 2011 (dry season) with concentration of 0.01 and 0.24 mg/kg, respectively. The result was considerably higher than those other studies in Thailand. Chanchek (2007) reported the residue of atrazine in sediment from Pasak

River at 0.62 µg/kg during rainy season and 0.53 µg/kg in dry season. Siripat (2009) also reported the residue of atrazine in sediment from Huai Ka Po, Namnao District, Petchabun Province collected in the growing and flood seasons between August to November at average concentrations of 44.9, 26.0, 8.0 and 30.4 µg/kg.

It is noticeably that atrazine concentration in dry season was greater than that in rainy season. Frank et al. (1982) reported atrazine residue in sediment at concentrations between 1.1-1.6 µg/kg. The detected level of atrazine in dry season was higher than that in rainy season. They argued that the sediment in the low discharge in dry season is well suspended than the large discharge in raining season. This pattern is also consistent with Trzedsi and Kowalski (1975). They reported that the quantity and dispersal of rain were the major factors to promote shifting of the herbicide in soil. The increasing movement when the rainfall increased resulting to the less absorbed of atrazine into sediment. In contrast, Chanchek (2006) found the similar concentration of atrazine in sediment between seasons, as mention above. The author argued that because Pasak River is relatively narrow, so the river discharge does not vary between seasons.

Glyphosate and paraquat were not detected from water and sediment samples in the present study, although they have been found in previous reports (Chang et al., 2011; Sanchís et al., 2012). It is possible that the level of glyphosate and paraquat in water are below the detection limit of the analysis method or absent from Nan River due to their physical properties. Glyphosate is relatively immobile in most soil; so it is not easy to reach to the water and its quantity in aquatic environment has been difficult to detect due to its physicochemical properties (Borggaard and Gimsing, 2008; Sanchís et al., 2012). Similarly, paraquat has a strongly binding to soil particles as inactive state for a long time and does not leach to groundwater (Smith and Mayfield, 1978; U.S. EPA, 2009). If it reaches to water, it is disappeared from the water rapidly (8 to 27 days) (Eisler, 1990). Although, the detectable amount of these herbicides were absent in current study, it is undeniable the presence of these herbicides in Nan River, since them have been intensive used in this area (Junpong, 2009; Wongwichit et al., 2010).

3.4.2 The persistence of herbicides in fish gonadal tissue

Atrazine was presented in gonadal tissues in both sexes with its concentration between ND – 0.15 µg/g in testicular tissues and between ND – 0.12 µg/g in ovarian tissues. The factor involving atrazine concentration is the amount of lipid deposit in gonads. Atrazine has moderate lipid solubility, and its uptake efficiency can be correlated to its partitioning (Knusli, 1994; Spacie et al., 1995). Preez and Van Vuren (1992) reported that lipid-rich tissues, such as gonad, accumulated lipophilic xenobiotic greater than leaner tissues, such as muscle, and the variation in bioconcentration related to the amount of lipid content. Lipid deposits in fish gonads are changed during oogenesis and spermatogenesis, by the greater amount are present in breeding season (Fletcher et al., 1974). According to the histological analyses of gonad development in the next two chapters (Chapter V and VI), female *P. proctozysron* was exhibited the late-vitellogenic stage consisting of numerous of mature oocyte in October 2010 which suggested it is in the breeding season. This probably can explain the occurrence of atrazine in both sexes of *P. proctozysron* collected in rainy season, even though no atrazine appeared in water samples. Furthermore, female fish store much more food during breeding season resulting to higher lipid contents in ovaries than that of in male testes. This may be the case of atrazine that was found only in female caught in October 2010.

Physiological mechanism such as metabolism processing within fish body can account for alterations of atrazine concentration prediction. Metabolism is one of the most important factors that govern the bioaccumulation and detoxification in aquatic organisms. However, there are few studies about metabolism of atrazine in fish. Simoneaux (1996) hypothesized that the metabolism of atrazine in fish body is occurred through the same pathway with rat and mice. Atrazine can accumulation in fish tissue within 12-24 hours after the exposure (Gunkel, 1981). When atrazine enters to fish body, it is metabolized by N-dealkylation and then dealkylated product is conjugated with glutathione (GSH) by the glutathione-S-transferease. Therefore, if this metabolism is occurred efficiently and rapidly, the atrazine concentration in fish gonad will low or non-detectable. In addition, the rising of temperature increases the rate of epinephrine release which increases cardiac output and blood flow at fish gill, thus allowing increases atrazine uptake (Karara and Hayton, 1989; Thune, 1994).

For other herbicides, glyphosate was found in both sexes from both seasons with means concentration of 1.49 ng/g in male and 1.61 ng/g in female. These concentrations were considerably very low when compared to other studies. However, it was showed the similar trend to atrazine that the ovary was found herbicide concentration higher than that in the testis.

3.5 Conclusion

Atrazine concentration in water, sediment and fish gonads caught from Nan River can be compared as the following: sediment > fish > water. It seems that the accumulation of atrazine in gonadal tissue is higher than that of glyphosate and paraquat. However, if fish living in this exposure for long term, the adverse affect the fertility and reproductive potential of fish populations may occur. The results also indicated that the accumulation of atrazine in fish body related to its concentration in the environment and nature of target organs. Therefore, *P. proctozysron* can be used as potential sentinel species of herbicide contamination and its gonads can be used as a good biomarker of herbicide exposure.

CHAPTER IV

MORPHOMETRIC AND GRAVIMETRIC STUDIES ON *Puntioplites proctozysron* LIVING IN NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE

4.1 Introduction

Agricultural areas along Nan River in Wiang Sa District, Nan Province are exhibited an extensive use of herbicides, mainly glyphosate, paraquat and atrazine. The aquatic habitats nearby these agricultural areas are thus susceptible to the contamination by these herbicides through runoff. Aquatic animals such as fish living in the river are therefore at risk of affecting by the contamination.

Effects of herbicide on fish have been previously reported for many times, since herbicides have been introduced and applied in agricultural activities for long time ago. For example, the exposure of atrazine was reported to elicit effect on behavior of many fish species (e.g., Davies et al., 1994; Steinberg et al., 1995). The brook trout, *Salvelinus fontinalis*, that received 120 µg/L atrazine had consequently reduced growth (Dewey, 1986), decreased in egg production (fecundity), fertilization rates and gonadsomatic index (GSI) (Moore and Lower, 2001). Some studies on the effects of glyphosate in various freshwater fishes also shown that after the herbicide exposuse at 2 µg/L for 30 days, the reduction of GSI was statistically different between exposed and control groups (Folmar et al., 1979).

The common well-being of fish population can be denoted from its reproductive potential as well as the fitness of its descendant. Any disturbances in usual health can influence the normal physiological condition in fish and induce negative changes (Kime, 1998; Kleinkauf et al., 2004). Fish condition indices, including the growth rate, length-weight relationship and GSI have been widely used as indicators of the fish's health. For examples, low condition factor (CF) and GSI might be caused by the stress from the exposure of contaminant (Adams and Ryon, 1994; Munkittrick, 1992; Goede and Barton, 1990). The previous researches have revealed that a chronic exposure to pollutants may induce a reduction of gonad size, as investigated by GSI deterioration together with anatomical and histological

changes of gonad (Linderoth et al., 2006). According to Adams et al. (1993), GSI value was applied as a biological marker for evaluating fish health. In the same manner, this index can also be used to assess the chronic effects of the pollution (Holm et al., 2006).

Puntioplites proctozysron is a common fish species in Nan River. The fish is directly exposed to potentially contaminated environment through water, sediment and food. So, in this study *P. proctozysron* was used as a sentinel species for the potential effects of herbicide contamination of Nan River. Thus, our research aims to study overall health using CF and reproductive health using GSI in the fish living in Nan River and evaluate its correlation with the level of herbicide contamination.

4.2 Materials and methods

4.2.1 Fish sampling

Mature *P. proctozysron* (approx. >10 cm in total length) were collected by fishing net year round from Nan River, Wieng Sa District, Nan Province. They were collected in rainy season (July and October 2010) and dry season (January and April 2011) for 40 individuals per sampling month (20 individuals of each sex: total 160 fishes). All fishes were kept in aerated holding tank and transported to the laboratory at Chulalongkorn University, Wiang Sa District, Nan Province. No mortality was found during the transportation.

4.2.2 Morphometric and gravimetric studies

All fish samples were euthanized by rapid cooling method (Wilson et al., 2009). For morphological analysis, each fish was measured for total weight and total length. The total length was measured from tip of snout to tail (Figure 4.1). The posterior abdominal wall of the fish was cut and opened. The gonads were removed and measured for the gonad weight.

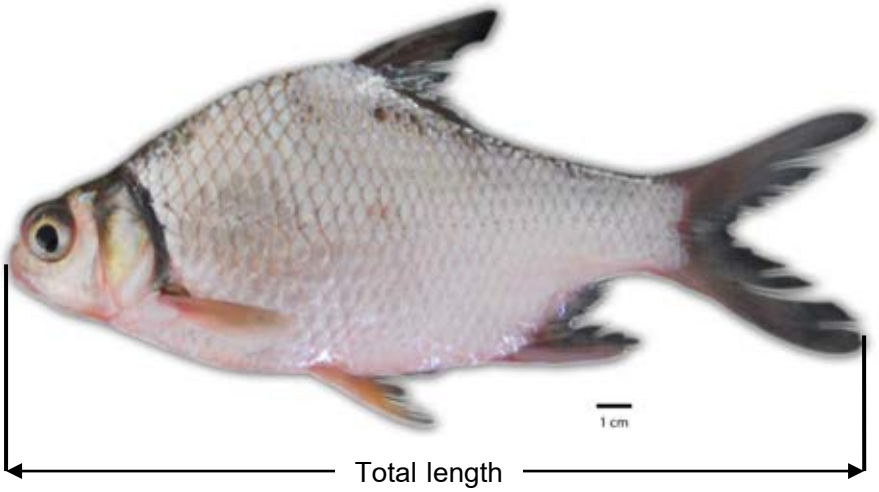

Figure 4.1 The picture showing the measurement of the fish total length.

4.2.2.1 Condition factor

Health of the fish was assessed using condition factor (CF). The CF was calculated from the relation between weight and length (Le Cren, 1951; Knapen et al., 2009) using the following formula:

$$CF = \frac{W}{aL^b}$$

Where *b* was calculated from:

$$\text{Log } W = b \log L + \text{Log } a$$

Where: W = Total weight (g)
L = Total length (cm)
a = constant
b = regression analysis

When: $y = 2.5455x - 1.05$ (*a* = 2.5455 and *b* = - 1.05)
$R^2 = 0.6377$

4.2.2.2 Gonadosomatic index

Gonadosomatic indices of *P. proctozysron* was calculated using the following formula (McDonald et al., 2000):

$$GSI = \frac{\text{gonad weight (g)}}{\text{total weight (g)}} \times 100$$

4.2.3 Data and statistical snalysis

All data of CF and GSI of *P. proctozysron* were checked for normal disturbution by Kolmogorov-Smirnov test and homogeneity of variance before further analysis. One-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison were used to compare the mean of CF and GSI between the sampling months. Spearman's correlation test was used to assess any significant correlation ($p < 0.05$) of herbicide concentration in gonadal tissue with CF and GSI. All of these statistical analyses were calculated using Statistical Package for the Social Sciences (SPSS) software (version 15.0).

4.3 Results

4.3.1 Condition factor

The results of CF of male *P. proctozysron* collected from Nan River in rainy (July and October 2010) and dry (January and April 2011) seasons were presented in Figure 4.2.

Mean CF with standard error of male *P. proctozysron* were range from 0.497 ± 0.033 to 1.173 ± 0.025. The male fish had the highest CF in January 2011 with significant difference ($p < 0.05$, ANOVA) whereas CF in October 2010 and April 2011 were not different.

The results of CF of female *P. proctozysron* collected from Nan River in rainy (July and October 2010) and dry (January and April 2011) seasons were presented in Figure 4.3.

Mean CF with standard error of female *P. proctozysron* were range from 0.485 ± 0.033 to 1.39 ± 0.143. Likewise, in the male fish, the female fish had the highest CF in January 2011. CF in January 2011 was significantly different from other months, (*p*

< 0.05, ANOVA) while, CF from July 2010, October 2010 and April 2011 were not different to each other.

The overall average CF were 0.899 ± 0.028 in males and 0.961 ± 0.057 in females, respectively.

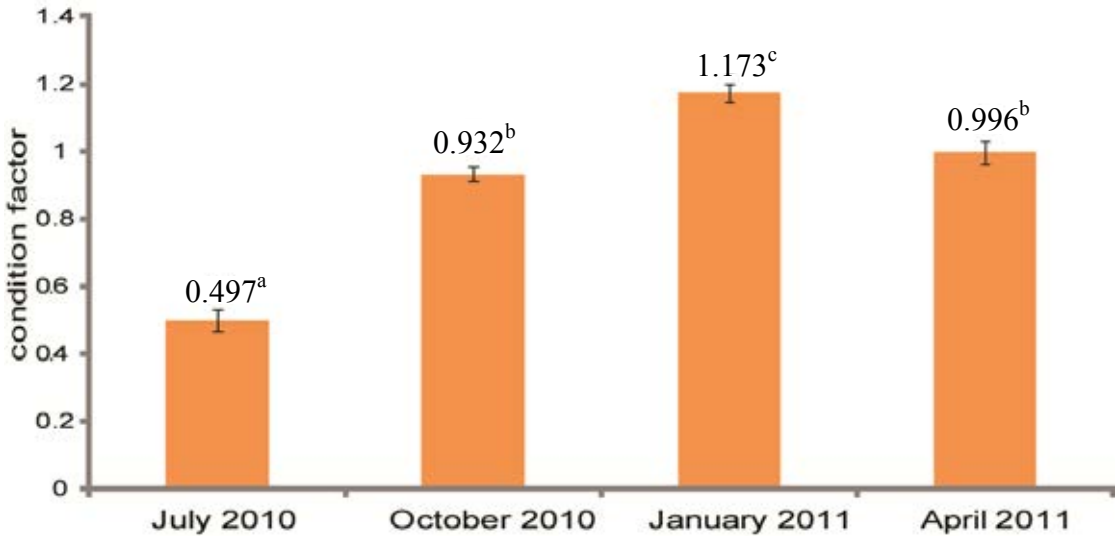

Figure 4.2 Mean±S.E. condition factor (CF) of male *P. proctozysron* caught from Nan River, Nan Province. Different letters indicate significant difference ($p < 0.05$).

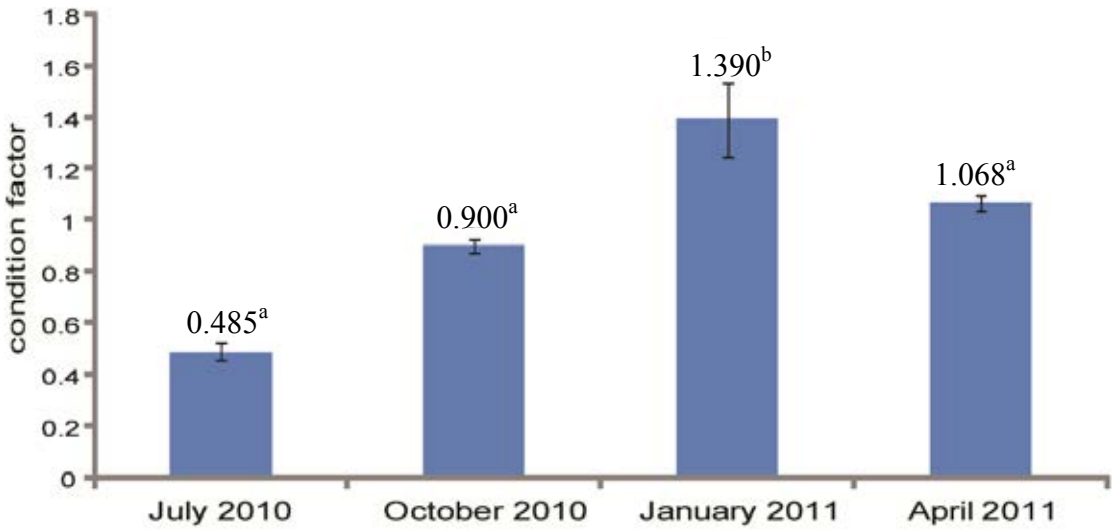

Figure 4.3 Mean±S.E. condition factor (CF) of female *P. proctozysron* caught from Nan River, Nan Province. Different letters indicate significant difference ($p < 0.05$)**.**

4.3.2 Gonadosomatic index

Mean GSI of male and female *P. proctozysron* from Nan River, Nan Province in rainy (July and October 2010) and dry (January and April 2011) seasons were presented in Figure 4.4 and Figure 4.5, respectively.

Mean GSI with standard error of male *P. proctozysron* were ranged from 0.458 ± 0.070 to 1.760 ± 0.270. The male fish had the highest GSI in January 2011. The GSI in July 2010, October 2010 and April 2011 were not different from each other, but were significantly different from the GSI in January 2011 ($p < 0.05$, ANOVA).

Mean with standard error of female GSI was consistently higher than that of the male fish. Mean GSI with standard error of female fish were ranged between 1.899 ± 0.647 to 8.597 ± 0.470. The fish had the highest female GSI in July 2010. Female GSI in July 2010, January 2011 and April 2011 were not different from each other, but was significantly different from GSI in October 2010 ($p < 0.05$, ANOVA).

The overall averages of GSI were 0.939 ± 0.126 in male and 3.848 ± 0.4014 in female, respectively.

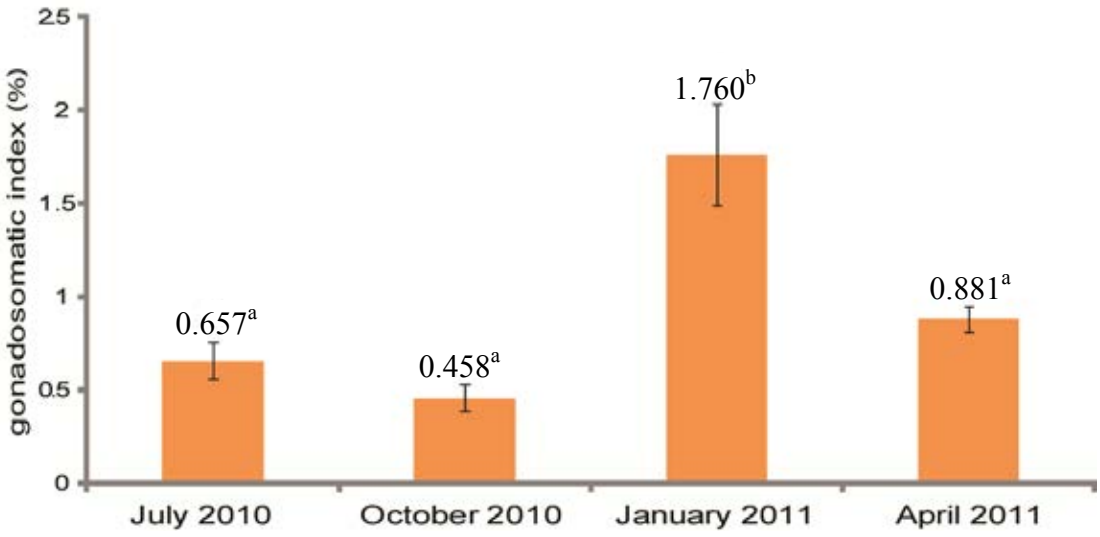

Figure 4.4 Mean±S.E. gonadosomatic index (GSI) of male *P. proctozysron* caught from Nan River, Nan Province. Different letters indicate significant difference ($p < 0.05$)

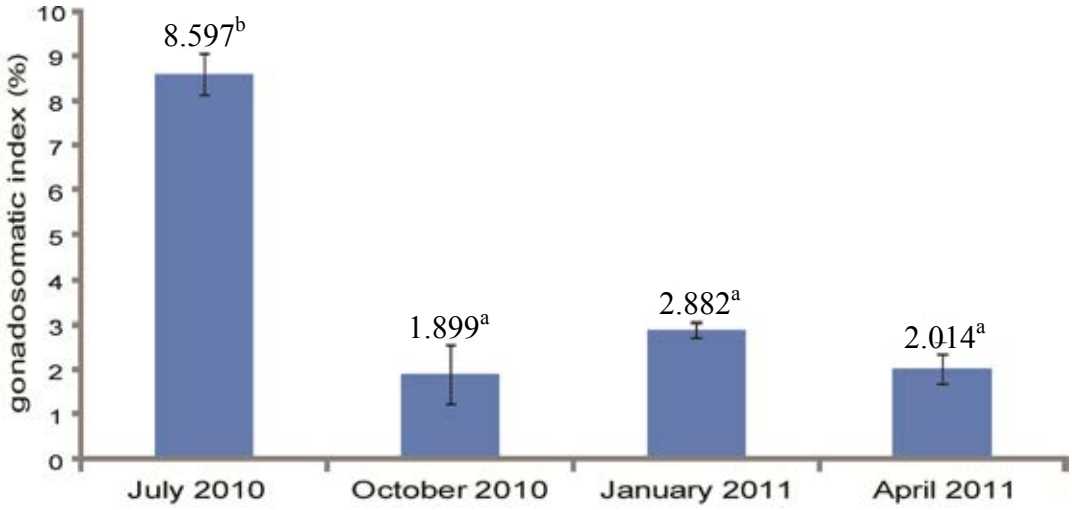

Figure 4.5 Mean±S.E. gonadosomatic index (GSI) of female *P. proctozysron* caught from Nan River, Nan Province. Different letters indicate significant difference ($p < 0.05$)

4.3.3 Correlation between herbicide contamination and general health of *P. proctozysron*

Statistical correlations (Spearman's correlation) between herbicide concentrations (Chapter III) and general health indices (CF and GSI) of *P. proctozysron* were occurred in both positive and negative correlations ($p < 0.05$) (Table 4.6). The atrazine concentration was positively correlated with CF and GSI for male fish and negatively correlated with GSI for female, but was not correlated with CF for female. Glyphosate concentration was not correlated with any indices in both sexes.

Table 4.1 Spearman rank correlations between condition factor and gonadosomatic index and herbicide concentrations in the gonadal tissue of male *P. proctozysron*

Sex	Index	Contaminant concentrations	
		Atrazine	Glyphosate
Male	CF	1.000**	0.500
	GSI	1.000**	0.500
Female	CF	0.500	-0.500
	GSI	-1.000**	-0.500

**indicates significant correlation at $p < 0.05$

4.4 Conclusion and discussion

The objective of this study was to assess overall health in term of CF and reproductive health in term of GSI of the fish living in Nan River. According to the results, the link of herbicide contamination to CF and GSI of *P. puntiolites* showed some correlations. The atrazine concentrations were positively correlated with CF in male fish but was not correlated in the females, whereas the atrazine concentrations was positively correlated with GSI in male fish and negatively correlated with GSI in female fish. The glyphosate concentrations were not correlated with any indices in both sexes. However, it is important to note that this study has been condected using relatively low sample size. The results presented here may not represent the result of the whole fish population.

CF has always been used to assess the well-being of fish species (Goede and Barton, 1990). It can be used to reflect the impact of the environment on the fish growth and provide information on the health status of the fish (Adams et al., 1993). CF of male and female fish from the current study showed the highest value in January 2011. Moreover, there was a significantly positive correlation between CF and atrazine contamination in the fish testis. This correlation means that CF of *P. proctozysron* did not depend on the level of atrazine residue in the fish gonads, although the previous report has claimed that atrazine can cause the reduction of CF under laboratory condition (Fortin et al., 2008). However, Dewey (1986) reported that the lower concentration of atrazine may affected the fish lenght. This finding may be possible to use to support our results. On the other hand, the glyphosate concentration was not correlated with CF in both sexes. This indicated that its low concentration plobably did not effect to CF in both sexes of *P. proctozysron*.

Nevertheless, CF values from this study showed the variation between seasons. It was relatively low in rainy season and extremely high in dry season. Previous reports have stated that the season significantly affected on overall fish well being, because of the changes in food available, metabolism and the changes in gonadal status due to reproductive cycle (Chellappa et al., 1995; Griffiths and Kirkwood, 1995; Saeborowski and Buchholz, 1996). The increase of body water also should be counted as a cause of the reduction in CF. Goede and Barton (1990) stated that a decrease in weight due to loss of energy stores could be offset by an increase in

body water during the rainy season. So that, the high level of river discharge of Nan River during rainy season in 2010 may took a responsibility in the reduction of CF of *P. proctozysron* in this study. Alternatively, the lowest CF in July 2010 may be partly due to a small sample size (11 individuals) because of difficulty to obtain the fish during strong tide in rainy season.

GSI as a percentage of gonadal mass to body mass of the fish has been routinely used to determine reproductive maturity, reproductive status or health of the fish as well as to assess gonadal change in response to environmental conditions (seasonal change) and exposures (McDonald et al., 2000; Schmidt et al., 1999).

From our results, GSI of *P. proctozysron* was significantly highest in January 2011 (dry season) in the males and significantly correlated with the levels of atrazine residue in the fish gonads, with positive correlation. This means that the testicular weight of *P. proctozysron* did not affect by the increasing level of atrazine residue in the organ. However, the highest of male GSI in dry season may caused by the histopathological alterations in testicular tissue rather than the enhanced testicular development, especially from the lesion of testicular atrophy in testicular tissue that we found (Chapter V).

CHAPTER V

TESTICULAR HISTOLOGICAL CHANGES IN *Puntioplites proctozysron* LIVING IN NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE

5.1 Introduction

Nan River is vulnerable to herbicide pollution which is contaminated from agricultural activities of Nan Province. Herbicides such as glyphosate, paraquat and atrazine have been extensively used. Either high or low concentration levels, these herbicides are potential pollutants, which could have negative effects not only on the river system, but also on health of aquatic organisms, especially fish living in the areas.

Glyphosate, paraquat and atrazine were reported as the cause of tissue lesions in male fish in both laboratory or field conditions. Some tissues and organs have been reported to be damaged by these herbicides. Figueiredo-Fernandes et al. (2006) reported the alteration in reproductive activity, enlargement of seminiferous lobule and decreased spermatozoa in male tilapia*, Oreochromis niloticus* after the exposure of paraquat. For atrazine, laboratory exposure to atrazine can cause the reduction of sperm maturation in fathead minnow, *Pimephales promelas* (Bringolf et al., 2004), decreased in plasma testosterone and 11 keto-testosterone concentrations in plasma and increased plasma estradiol concentrations in mature male goldfish, *Carassius auratus* (1000 µg/L for 21 days; Spano et al., 2004) and the incidence of intersex gonad in *Pimephales promelas* (at 5 µg/L, after 14 days exposure; Tillitt et al., 2010).

Puntioplites proctozysron is a common fish in Nan River. The fish is directly exposed to potentially contaminated environment through water, sediment and food. So, in this study, *P. proctozysron* was used as a sentinel species for the potential effects of herbicide contamination of Nan River. At present, very little is known about the effect of herbicides on histology of this fish species. Thus, the objective of this study is to investigate the histopathological changes in the testis of the fish living in Nan River nearby agricultural areas and evaluate its correlation with the level of herbicide contamination.

5.2 Materials and methods

5.2.1 Fish sampling

Mature *P. proctozysron* (approx. >10 cm in total length) were collected by fishing net year round from Nan River, Wieng Sa District, Nan Province. Fishes were collected in rainy season (July and October 2010) and dry season (January and April 2011) for 40 individuals per sampling month (20 individuals of each sex: total 160 fishes). All fishes were kept in aerated holding tank and transported to the laboratory at Chulalongkorn University, Wiang Sa District, Nan Province. No mortality was found during the transportation.

5.2.2 Testicular histology

Sample preparation

The testis of each fish was dissected, weighted and fixed in Davidson's fixative. They were processed using standard histological techniques (Humuson, 1979). The testicular tissues were dehydrated in a series of ethanol and then n-butanol. Then the tissues were cleared in xylene and then embedded in paraplast. The paraffin blocks of testicular tissues were serially sectioned at 5 μm thickness by rotary microtome. The ribbons of these sections were attached to the slide by egg albumin solution and dried at 40°C on warm plate. The sections were deparaffinized with xylene and hydrated through a series of alcohol before stained with Delafield's haematoxylin and eosin (Humason, 1979).

Light microscopy was used to observe the histological structures of the testis. Spermatogenesis of the fish was studied following the criteria by Dietrich and Krieger (2009).

Testicular development

Each histological section of testicular tissue was examined in detail under light microscope in order to determine the stage of testicular development according to Dietrich and Krieger (2009). The testis was classified into different developmental stages (0–4) according to the maturity of germ cells that is a predominant stage in the testis. The developmental stages of testis are as follows: stage 0 (immature), stage 1 (early spermatogenic), stage 2 (mid-spermatogenic), stage 3 (late spermatogenic) and stage 4 (spent).

Histopathology

Each histological section of testicular tissue was investigated in detail under a microscope to determine the histopathological alterations according to Dietrich and Krieger (2009). Each histopathological alteration observed in the testis will be recorded as a mean prevalence.

5.2.3 Statistical analysis

Spearman's correlation test was used to assess any significant correlation ($p < 0.05$) of herbicide concentration in testicular tissue with histolopathlogical changes using Statistical Package for the Social Sciences (SPSS) software (version 15.0).

5.3 Results

5.3.1 Basic morphology and histology of *P. proctozysron* testis

The testes of *P. proctozysron* are elongated-paired organs located on posterior wall of abdominal cavity. They are suspended on the mesochium which run along the length of the testes. The testis is covered by tunica albuginea which composed of connective tissue layer and a smooth muscle layer. Vasa efferentia locates along the dorsal border of the testis extended caudally to join together, forming vasa defferentia. It opens to the external via urogenital papilla (Figure 5.1A-B). Histological study of the testis of *P. proctozysron* revealed that the tunica albuginea consists of a mesothelium and a few layers of connective tissue and blood vessels. The protrusion of tunica albuginea into testicular parenchyma completely divides it into lobular structure consisting of numerous seminiferous lobules. Leydig cells are presented in the interstitial areas between seminiferous lobules. Leydig cell of teleost is a large polygonal cell, found in small groups and considered to be the androgen producing cell of male fish. The seminiferous lobule in this fish contains numerous spermatogonia, distributing along the entire length of the lobule. The result showed that the testis of *P. proctozysron* is an unrestricted spermatogonial type. The seminiferous epithelium consists of spermatogenic cells and Sertoli cells. The Sertoli cells tend to have sharply-defined elongated or triangular nuclei with large nucleoli and ambiguous cytoplasm. In addition, Sertoli cells are usually presented in low numbers. They locate adjacent to lobular septa and express the residual bodies which

formerly known as phagocytosed developing spermatids. The cross section of seminiferous lobule revealed random distribution of various clusters of spermatogenic cells, called spermatocyst, containing a specific stage of spermatogenic cells synchronously proliferating. Each spermatocyst is enclosed by cytoplasmic processes of Sertoli cells (Figure 5.2A-B; 5.3A-B; 5.4)**.**

5.3.2 Spermatogenesis

Spermatogenesis of *P. proctozysron* was observed. The developmental stages of spermatogenic cells were classified into 5 stages based on the cell size, shape, nuclear characteristics, chromatin condensation, amount of cytoplasm and staining properties as follows:

Spermatogonium is the largest of sperm cells generally located close to the basement membrane of seminiferous lobule. It is usually found in single cell or cluster of cells. This stage is characterized by the appearances of lightly basophilic nucleus with prominent nucleolus, distinct nuclear membrane and moderate amount of light granular cytoplasm (Figure 5.5).

Primary spermatocyte is produced by mitotic division of spermatogonia. Its size is smaller than spermatogonia. Primary spermatocyte is surrounded by Sertoli cell process, thus being enclosed within a spermatocyst. It is spherical in shape with basophilic nucleus. In the nucleus, chromatins are condensed and distributed evenly, lead to their dense basophilic characteristic. In addition, the nucleolus is still prominent. Moderate amount of distinct cytoplasm is found in this stage (Figure 5.5).

Secondary spermatocyte is divided from primary spermaocyte by the first meiotic division. This stage of germ cell contributes to the largest spermatocyst. It is smaller than primary spermatocytes. In this stage, dense basophilic nucleus contains perinucleolar chromatin without nucleolus. Amount of cytoplasm is found to decrease compared with the previous stage (Figure 5.5).

Spermatid arises from secondary spermatocyte after second meiotic division. A cluster of spermatid is enclosed within cytoplasmic process of Sertoli cells forming a spermatocyst. Spermatid is small and more condensed with intense basophilic nucleus and small amount of cytoplasm because it loses cytoplasm during spermiogenesis (Figure 5.5).

Spermatozoan is the smallest among spermatogenic cells. It is a mature cell consisting of 2 regions, head and tail. The head of spermatozoan is slightly elongated. Chromatins become completely condense throughout the nucleus. After completing the spermiogenesis, the spermatocyst is ruptured and the spermatozoa are released into the lumen and vasa efferentia, respectively (Figure 5.5).

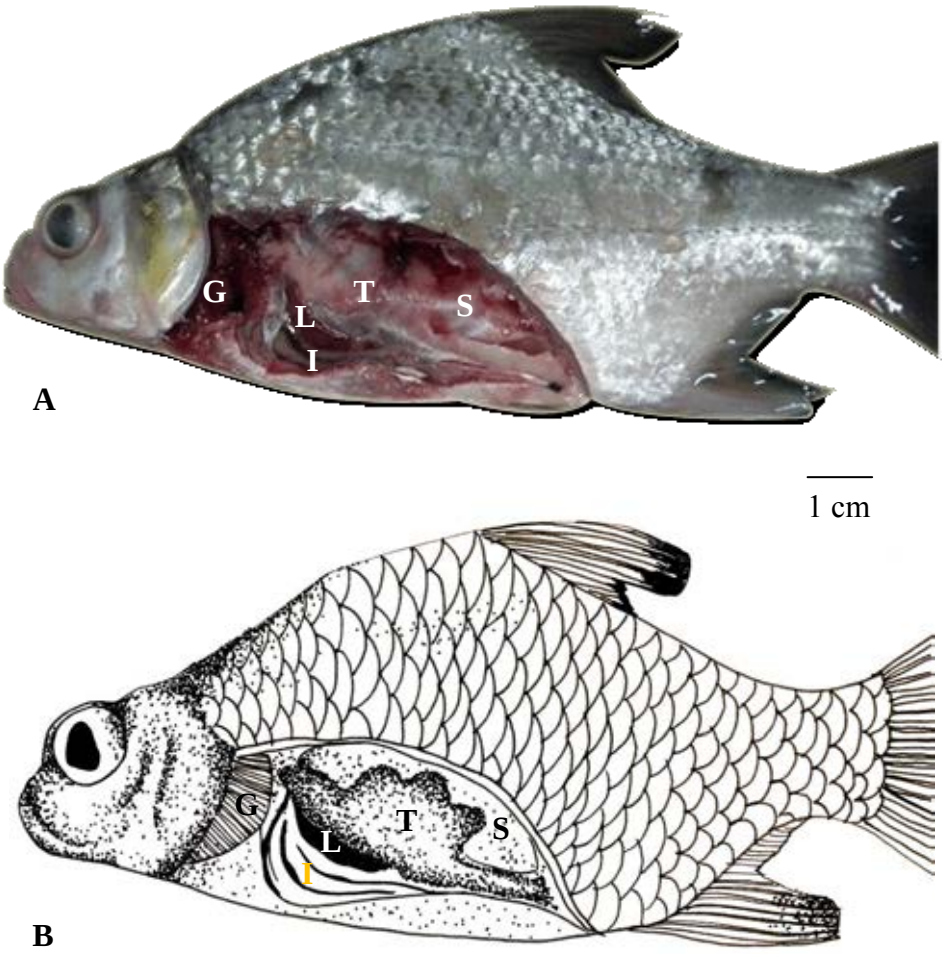

Figure 5.1 Gross morphology of male reproductive system of *P. proctozysron* (Drawing by Sinlapachai Senarat)

A Photograph shows the testis located in abdominal cavity which situated dorsally in the posterior part of the abdomen.

B Drawing shows the internal organs within abdominal cavity.

(G = gill, I = intestine, L = liver, S = swim bladder, T = testis)

Figure 5.2

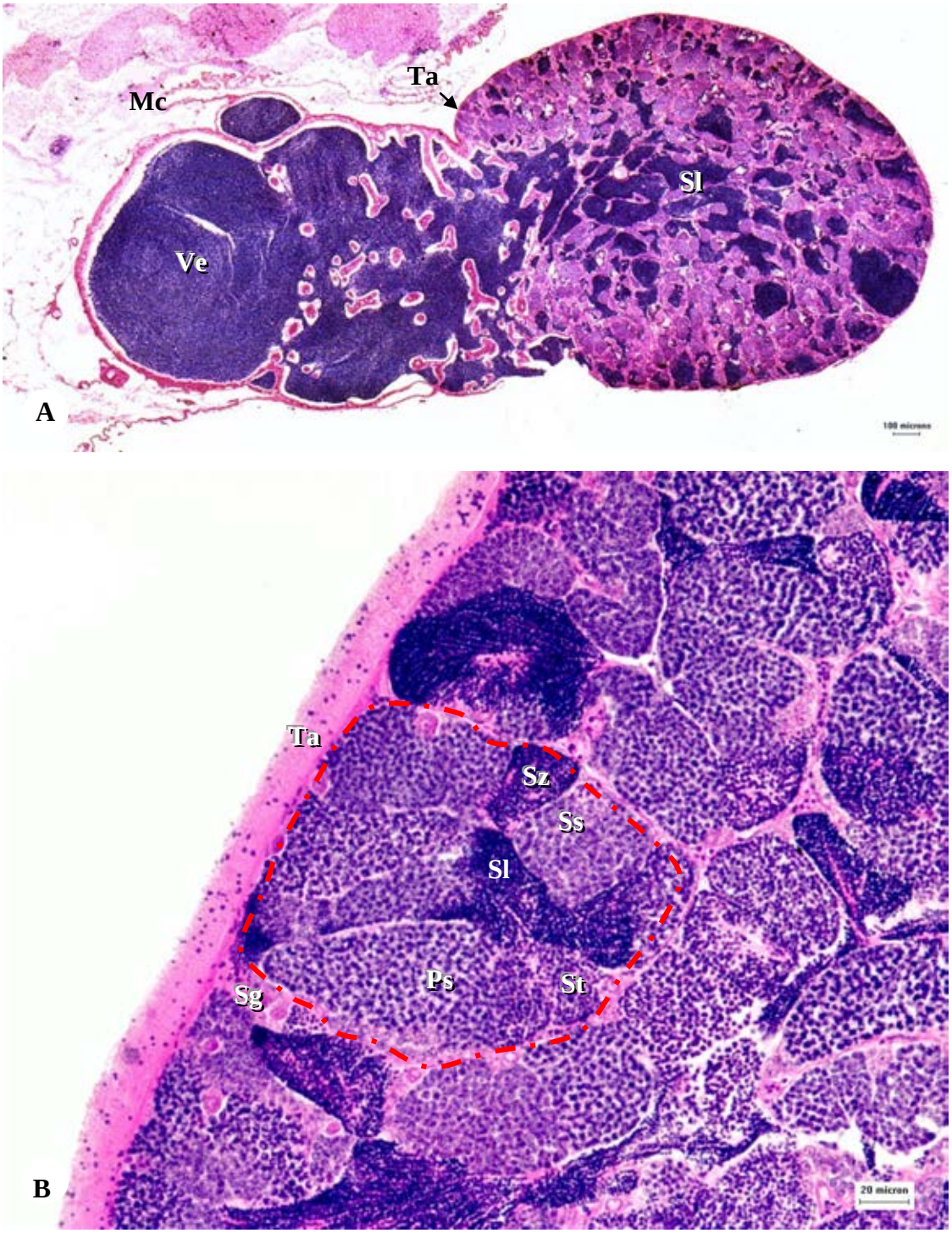

Figure 5.2 Micrograph of *P. proctozysron* testes showing basic testicular histological structure *(H&E stain)*

A Overall structure of the testis containing seminiferous lobules (Sl) and vasa efferentia (Ve). *Scale bar = 100 µm. (Mc = mesochium, Ta = tunica albuginea)*

B Cross section of the testis showing seminiferous lobule (dash line) and thick tunica albuginea (Ta). Spermatogonia (Sg) are found along the wall of each lobule. Different stages of developing spermatogenic cells are present in different spermatocyst including that of primary spermatocytes (Ps), secondary spermatocytes (Ss) and spermatid (St). Spermatozoa (Sz) are evidented in the seminiferous lumen. *Scale bar = 20 µm.*

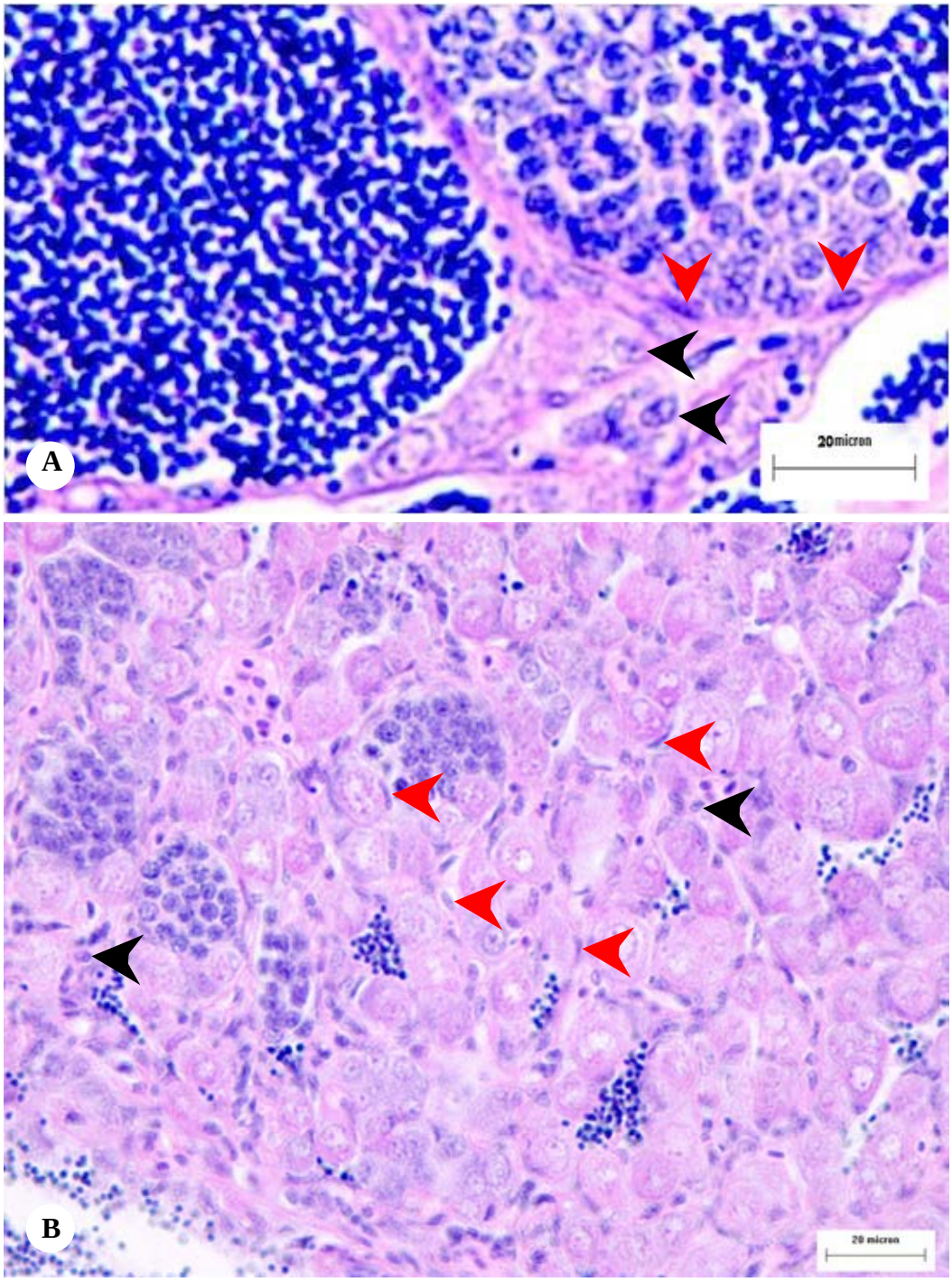

Figure 5.3 Micrograph of *P. proctozysron* testis showing basic testicular histological structure *(H&E stain)*

A-B High magnification showing Leydig cells (black arrowheads) between lobules and Sertoli cells (red arrowheads) within seminiferous lobules. *Scale bar = 20 µm.*

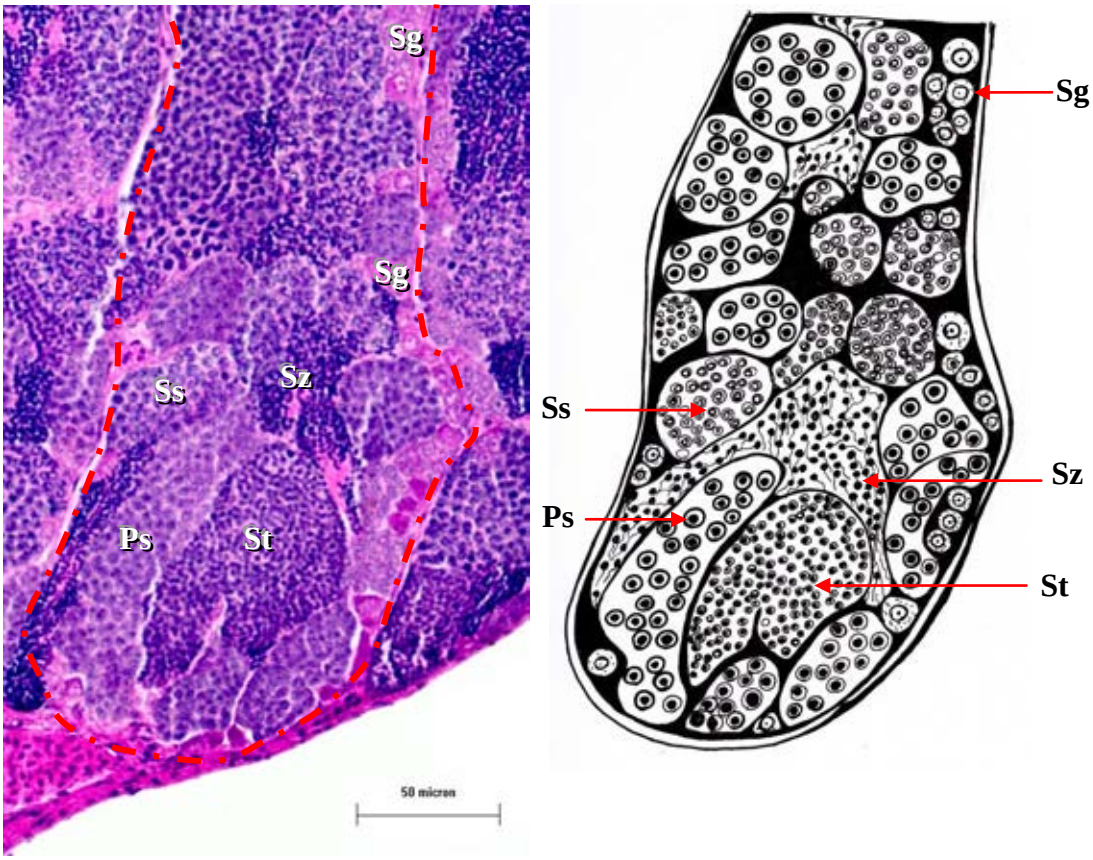

Figure 5.4 Histology and drawing of testis showing unrestricted spermatogonial testis type *(H&E stain). Scale bar = 20 µm.* (Drawing by Sinlapachai Senarat) *(Sg = spermatogonia, Ps = primary spermatocytes, Ss = secondary spermatocytes, St = spermatid, Sz = spermatozoa)*

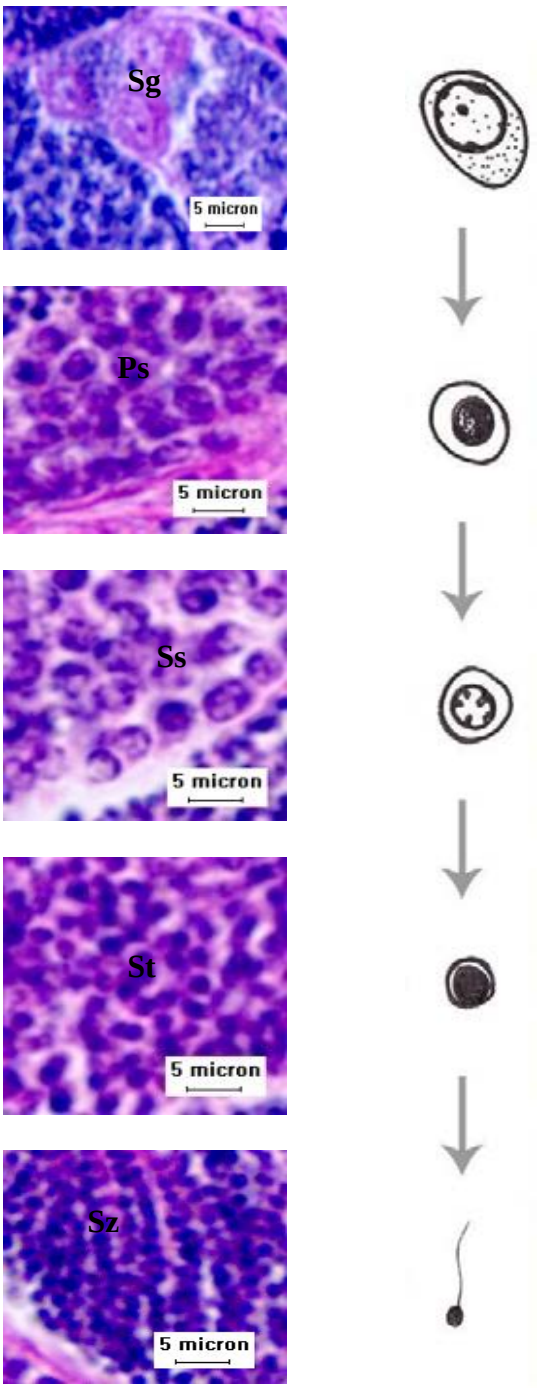

Figure 5.5 Higher magnification histology and diagram shows the change of germ cell morphology during spermatogenesis *(H&E stain). Scale bar = 5 µm.* (Drawing by Sinlapachai Senarat)

(Sg = spermatogonia, Ps = primary spermatocytes, Ss = secondary spermatocytes, St = spermatid, Sz = spermatozoa)

5.3.3 Testicular development

5.3.3.1 Stage of development

The testis of *P. proctozysron* was staged based on the relative abundance of germ cell and histological characteristics in the testis. The testicular cycle was classified into 5 stages as follows:

Undeveloped stage (stage 0)

During this stages, testes are morphologically small and does not have spermatogenic activity. The testis exhibits a large number of spermatogonia with a few primary spermatocytes, secondary spermatocytes and spermatids congregating along the inside wall of the seminiferous lubules, but spermatozoa may also be observed. The lumen is narrow (Figure 5.6A).

Early-spermatogenic stage (stage 1)

Testis in this stage gradually increases in size and the wall of seminiferous lobule is expanded. Spermatogonia are apparently reduced in number, while primary spermatocytes, secondary spermatocytes and spermatids are increased. Spermatozoa maybe observed. The number of spermatids and spermatozoa greatly increase in the late of early spermatogenic stage (Figure 5.6B).

Mid-spermatogenic stage (stage 2)

The testis contains primary spermatocytes, secondary spermatocytes, spermatid and spermatozoa presented in roughly equal proportions. The number of spermatozoa greatly increases in the late step of this stage (Figure 5.6C).

Late-spermatogenic stage (stage 3)

Testis becomes fully mature and physiologically ready to spawn. All stages of spermatogenesis may be observed. A few of spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids is presented on the wall of the seminiferous tubule. The most advanced germ cell stage in this testis stage is spermatozoa. The maximal quantity of spermatozoa is observed in the lumen of the seminiferous tubule. The entire lumen is filled with spermatozoa (Figure 5.6D).

Spent stage (stage 5)

This stage is not presented in this study**.**

5.3.3.2 Seasonal change in testicular development

Percentage distribution of testicular staging of male *P. proctoztsron* is presented in Figure 5.6.

In July 2010, males *P. proctozysron* processed the testes at late-spermatogenic and mid-spermatogenic stages equally. In October 2010, In January and April 2011, the majority of testicular stage found in these months is early spermatogenic stage. It is also note that only in January 2011 that underdeveloped stage of testis was observed at 35% of the fish collected.

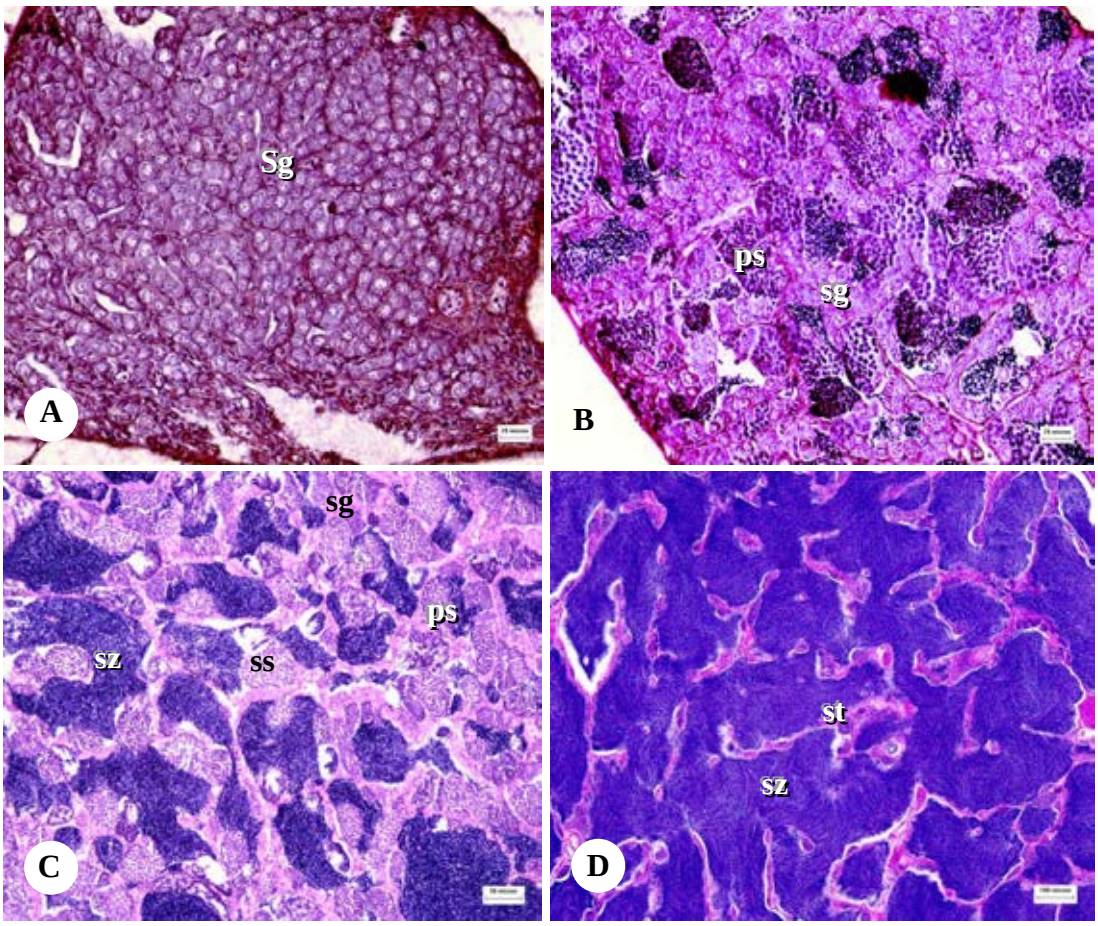

Figure 5.6 Micrographs of *P. proctozysron* testis showing different maturity stages of testicular development during July 2010 to April 2011 *(H&E stain)*

A Undeveloped stage (stage 0) of testicular tissue shows exclusively immature phase (spermatogonia to spermatatid with no spermatozoa). *Scale bar = 20 µm.*

B Early-spermatogenic stage (stage 1) of testicular tissue shows the predominant of immature phases, but spermatozoa maybe observed. *Scale bar = 20 µm*.

C Mid-spermatogenic stage (stage 2) of testicular tissue shows spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. *Scale bar = 50 µm*.

D Late-spermatogenic stage (stage 3) of testicular tissue shows the predominant of spermatozoa, but other stages maybe observed. *Scale bar = 100 µm.*

(Sg = spermatogonia, Ps = Primary spermatocytes, Ss = secondary spermatocytes, St = spermatids, Sz = spermatozoa)

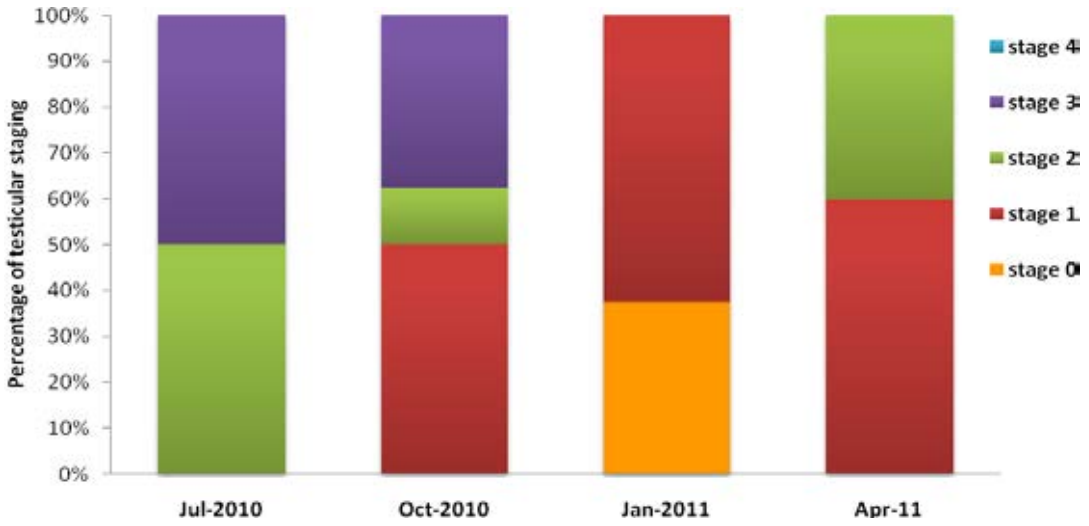

Figure 5.7 Percentage distributions of testicular staging in male *P. proctozysron* from Nan River, Nan Province

stage 0 = undeveloped, stage 1 = early spermatogenic,

stage 2 = mid-spermatogenic, stage 3 = late spermatogenic,

stage 4 = spent)

5.3.4 Histopathology

Several histological changes and their prevalence in testicular tissues of *P. proctozysron* caught from Nan River, Nan Province are shown in Figure 5.8-5.13 and Table 5.1.

5.3.4.1 Histological alterations in the testes of *P. proctozysron* caught in rainy season

In July 2010, the histopathological changes were found in the fish testes. Testicular disorganization (detachment of basal part) was found at 75% prevalence. Pyknosis and dilation of blood vessel were found at 50% prevalence, Degeneration of Leydig cells between the lobules were observed at 25% prevalence. Asynchronous development of germ cells at several stages within seminiferous lobule and apoptosis germ cell was also evidenced at 25% prevalence. In addition, blood congestion, abnormal red blood cell and infiltration of white blood cell (macrophage and other leucocyte aggregation) were found.

In October 2010, the histopathological observation of the fish testis exhibited blood congestion were found at 100% prevalence Germ cell necrosis

(pyknosis, 85.71% prevalence), and abnormal red blood cell (71.42% prevalence) were detected. pyknosis, 85.71% prevalence),. Germ cell adaptation was evidented as hypertrophy at 57.14% prevalence. Eosinophilic cytoplasm and atrophy was found in many gem cells, especially in spermatogonia at 45.85% and 42.85% prevalence. Apoptotic germ cells at were frequently observed at 42.85% prevalence. Testicular degeneration, dilation of blood vessel and karyorhexis were equally at 28.57% prevalence. Moreover, degeneration of Leydig cells, asynchronous development germ cell, dilation of blood vessel were evenly at 28.51% prevalence. In addition, fibrosis and degeneration of Sertoli cells were found.

5.3.4.2 Histological alterations in the testes of *P. proctozysron* caught in dry season

In January 2011, the histopathological observation of the fish testis showed testicular atrophy and degeneration of Leyding cells at 87.5% prevalence, which is noticeably high when compared to other months. Germ cell pyknosis within seminiferous lobules was found at 75% prevalence. Germ cell hypertrophy and blood congestion were found at 62.5% prevalence and degeneration of Sertoli cells were noticed at 50% prevalence. Karyolysis and abnormal red blood cell were equally at 37.5% prevalence. Moreover, karyolysis, germ cell atrophy, eosinophilic cytoplasms of spermatogonia, asynchronous development of germ cells, infiltration of macrophages and other leucocytes and fibrosis were noticed at 25% prevalence. In addition, testicular degeneration and dilation of blood vessel were found.

In April 2011, the histopathological changes were found in the fish testis. Overall testicular alterations were similar to those observed in January 2011. Pyknosis asynchronous development of germ cells within the same spermatocyts and blood congestion were observed in the testes at 80% prevalence. Apoptosis germ cell, karyolysis, germ cell atrophy, Eosinophilic cytoplasm of spermatogonia and dilation of blood vessel were noticed at 60% prevalence. Testicular atrophy, degeneration of Leydig cells and infiltration of white blood cell were detected at 40% prevalence. In addition, karyorhexis, germ cell hypertrophy, abnormal red blood cells and fibrosis were detected.

5.3.4.3 Comparison of testicular alterations of *P. proctozysron* between seasons

Testicular histopathological alterations of *P. proctozysron* living in Nan River between dry and rainy season were clearly different. In dry season, the fish showed greater alteration than in the rainy season. Since dry season, the fish testis had testicular atrophy, asynchronus development, karyorhxis, karyolysis, germ cell atrophy, germ cell hypertrophy, eosinophilic cytoplasm, degeneration of Sertoli cell, degeneration of Leydig cell, and infiltration of white blood cells and dilation of blood vessel. These alterations were found in higher prevalence in the fish collected in dry season than in the fish collected in rainy season.

Table 5.1 Prevalence (%) of different histopathological alterations in the testes of *P. proctozysron* caught from Nan River during July 2010 to April 2011.

Season	Sampling month	Alteration Prevalence (%)															
		Tes. degen	Tes. Atrop.	Apoptosis	Pyknosis	Karyorhexis	Karyolysis	Atrophy	Hypertrophy	Eos. Cyto.	Ser. Degen.	Leydig. Degen.	Asyn. Dev.	Wbc inf.	Bv. Dilat.	Bl. Con.	Ab. rbc	Fibrosis
Rainy	July 2010 (n = 4)	75	0	25	50	25	0	0	0	0	0	25	25	25	50	25	25	0
	Oct 2010 (n = 7)	29	0	43	86	29	29	43	57	46	15	29	29	29	29	100	72	28
Dry	Jan 2011 (n = 8)	13	88	38	75	38	25	25	63	25	50	88	25	25	13	63	38	25
	April 2011 (n = 10)	0	40	60	80	20	60	60	20	60	0	40	80	40	60	80	20	20

Tes. degen. = Testicular degeneration, Tes. atrop. = Testicular atrophy, Apoptosis = Apoptosis germ cell, Atrophy = Germ cell atrophy, Hypertrophy = Germ cell hypertrophy, Eos. cyto. = Eosinophilic cytoplasm, Ser. degen. = Degeneration of Sertoli cell, Leyding degen. = Degeneration of Leydig cell, Asyn. dav. = Asynchronous development germ cell, Wbc inf. = Infiltration of white blood cell, Bv. dilat. = Dilation of blood vessel, Bl. con. = Blood congestion, Ab. rbc. = Abnormal red blood cell.
Highlight indicates the highest value.

Figure 5.8

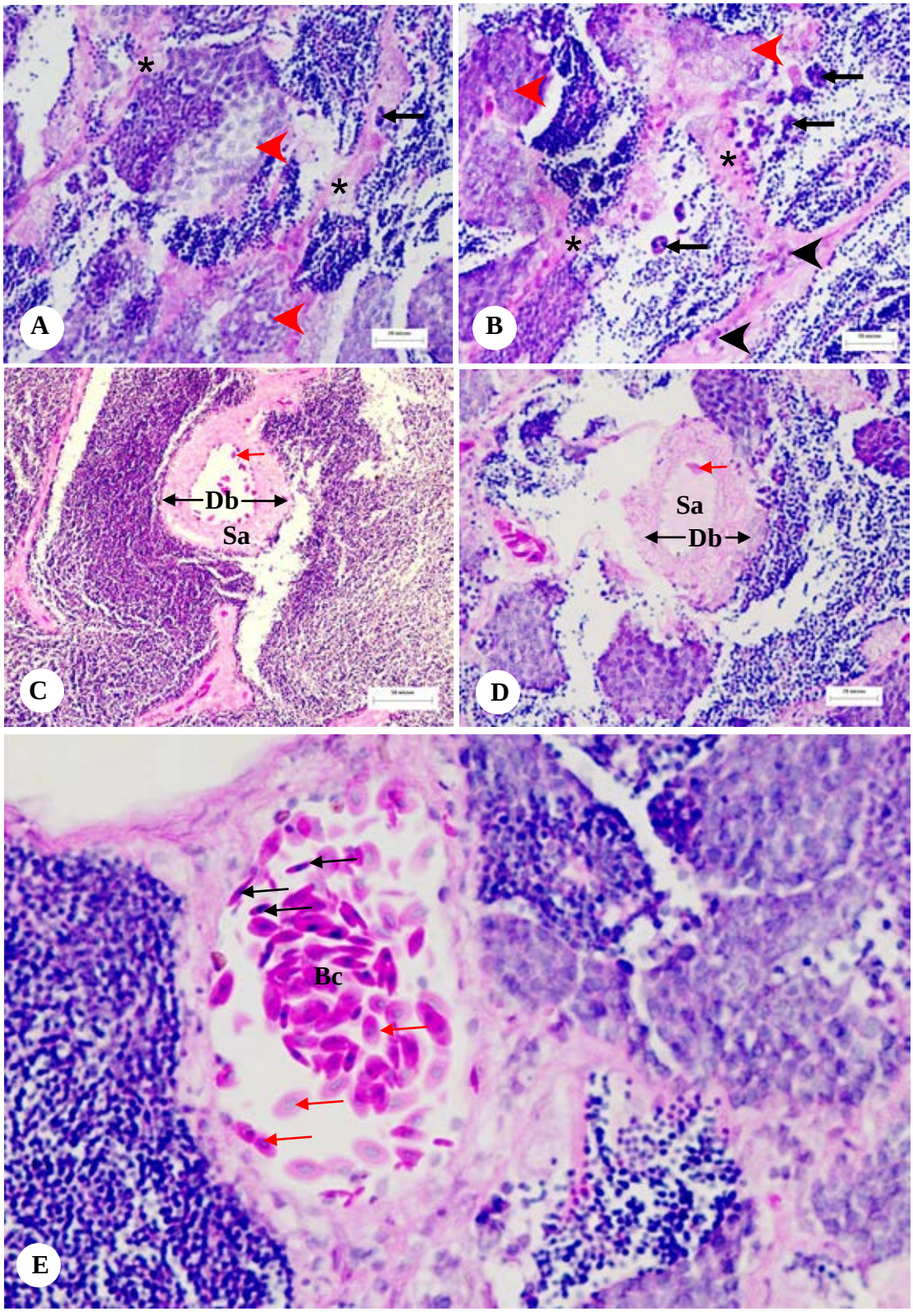

Figure 5.8 Micrograph of the testis of *P. proctozysron* in July 2010 *(H&E stain)*

A-B The seminiferous lobule and interstitial tissue at higher magnification show disorganization of the lobules (*), Leydig cells (black arrowhead), spermatocytes (red arrowhead) and apoptotic germ cell or apoptotic body with the detachment of the basal membrane (arrow). *Scale bar = 20 µm*.

C-D Testicular tissue shows the dilation of blood vessel (Db) and numerous of red blood cells (red arrow) in distended blood vessels. *Scale Bars C = 50 µm, D = 20 µm. (Sa = small artery)*

E Testicular tissue shows blood congestion (Bc), greatly distended and packed with thrombocytes (black arrows). Red blood cells (red arrows) are seen between the lobules. *Scale bar = 20 µm.*

Figure 5.9

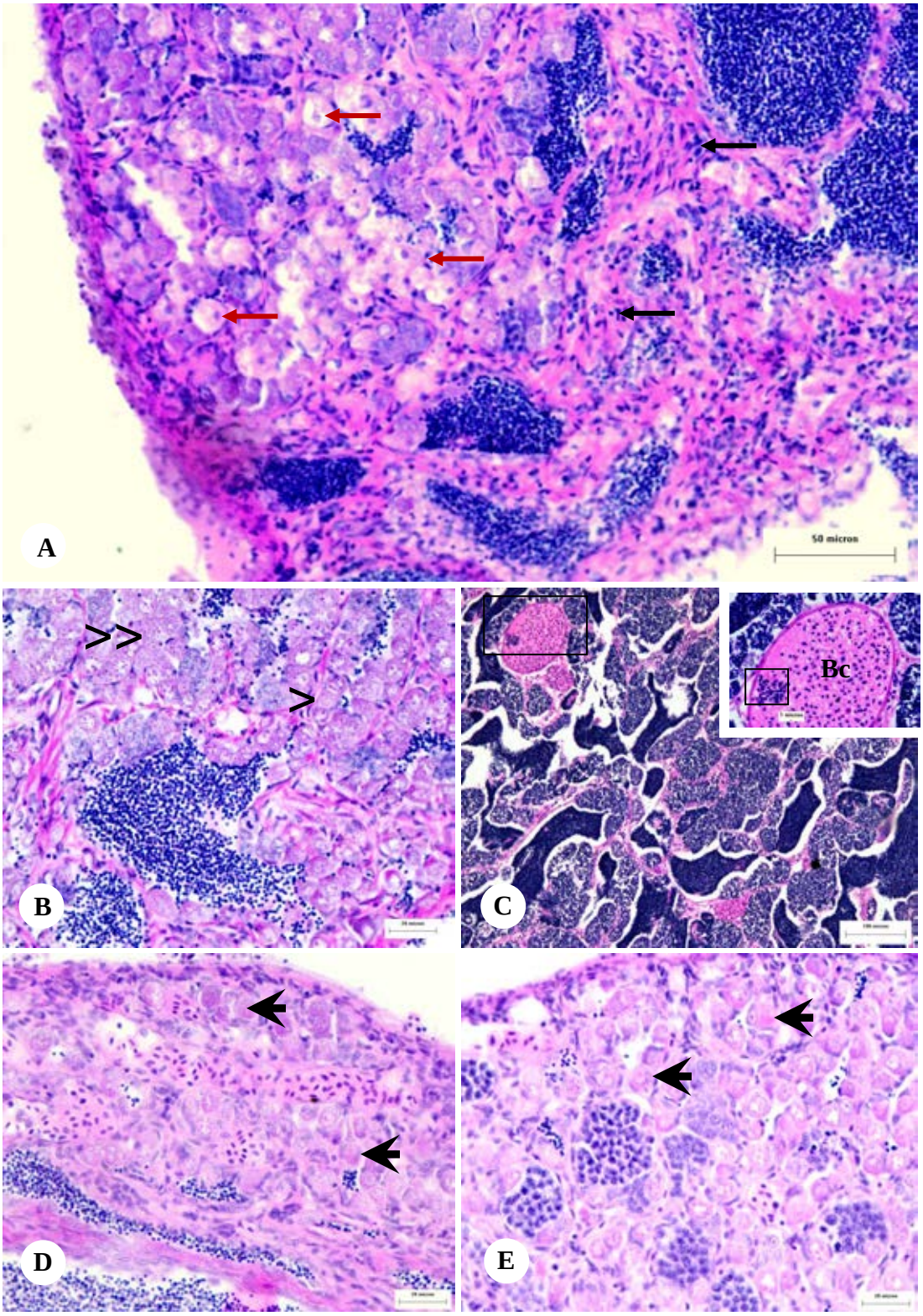

Figure 5.9 Micrograph of testis of *P. proctozysron* in October 2010 *(H&E stain)*

A Testicular tissue shows fibroblasts (black arrows) infiltrated between lobules at a higher magnification. The cells inside the seminiferous lobule show pyknosis (red arrows) characterized by condensation of the nuclei. *Scale bar = 50 µm*.

B Testicular tissue shows the cells inside the seminiferous lobule and eosinophilic cytoplasm in spermatogonia (acidophilic cell). The karyolysis (>>) and karyorhexis (>) are observed in the germ cells. *Scale bar = 20 µm*.

C Blood vessel (Bc) is seen between the lobules as a greatly distended and packed cluster of red blood cells. A group of white blood cells is seen (□). *Scale bar = 100µm*. Insert shows higher magnification. *Scale bar = 1µm*

D-E Inside of the seminiferous lobule shows the group of eosinophilic cytoplasm (arrowheads). *Scale bar = 20 µm.*

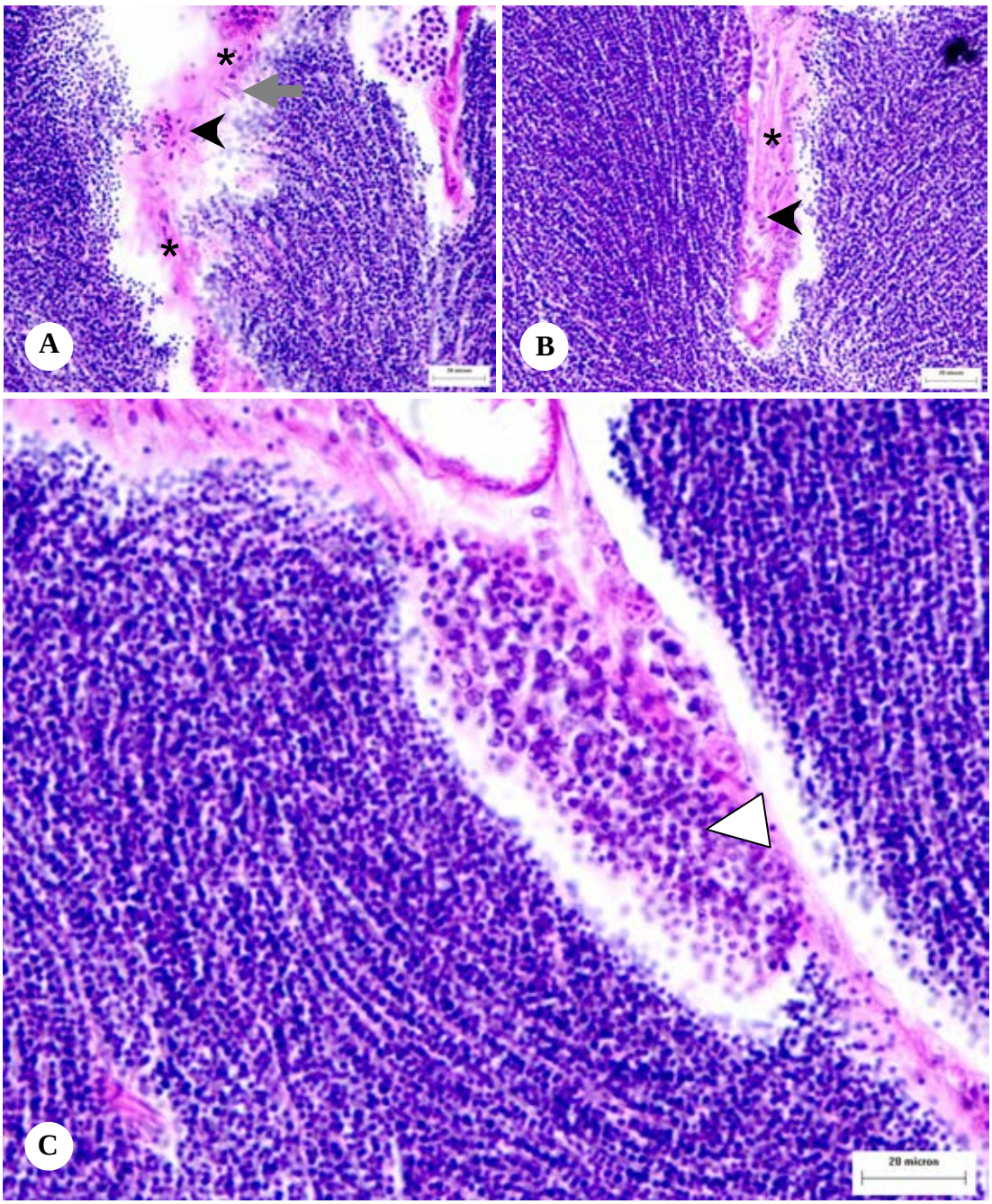

Figure 5.10 Micrograph of testis of *P. proctozysron* in October 2010 *(H&E stain)*

A-B Testicular tissue shows disorganization of the lobules (*), degeneration of Sertoli cell (gray arrow) and Leydig cells (black arrowhead). *Scale bar = 20 µm.*

C Testicular tissue shows spermatocysts inside the lobule with asynchronus development of germ cells (white triangle) during spermatogenesis. *Scale bar = 20 µm.*

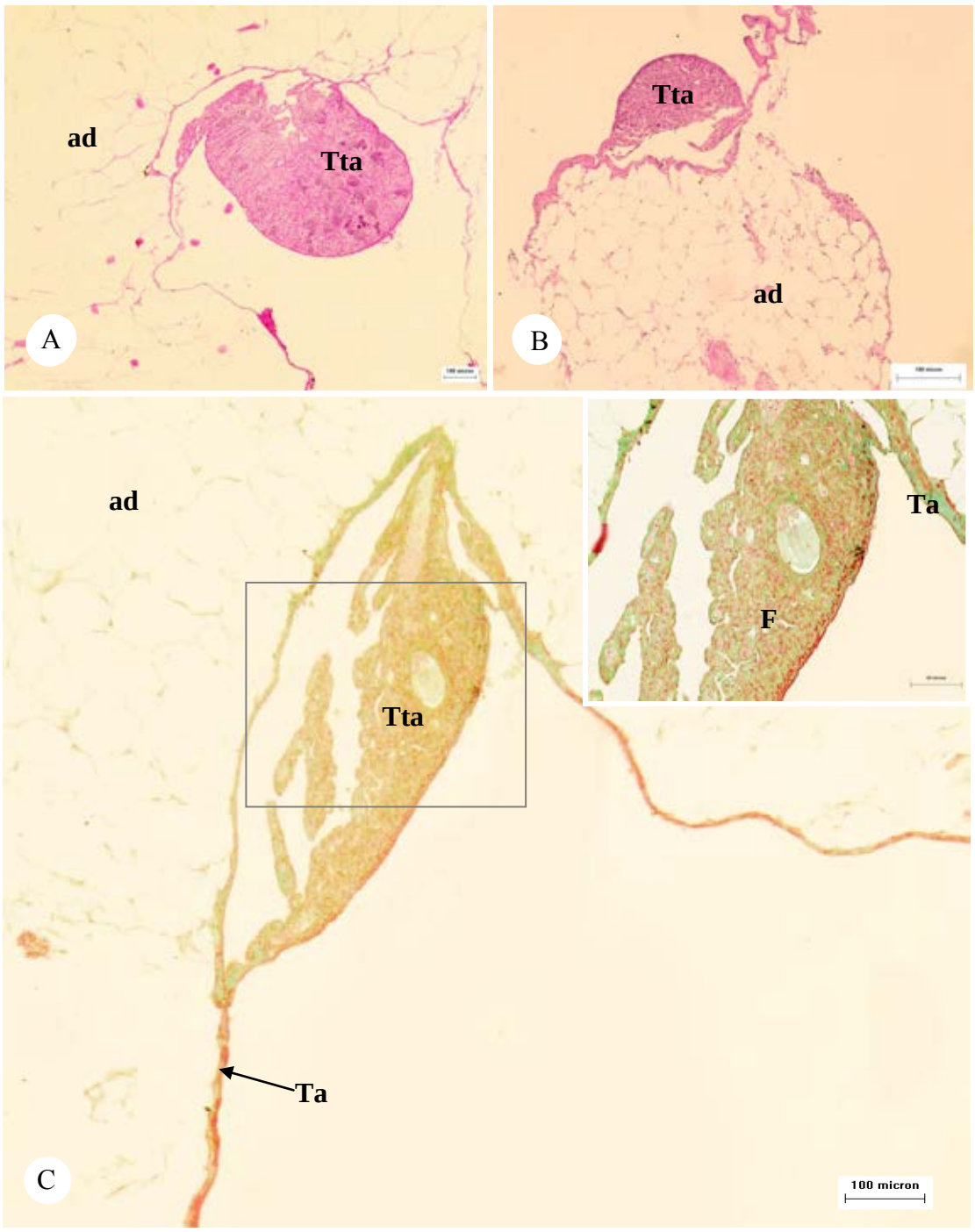

Figure 5.11 Micrograph of testis of *P. proctozysron* in January 2011

A-B Testicular tissue show the testicular atrophy (Tta) substituted with adipose cells (ad) *(H&E stain). Scale bar = 100 µm.*

C Testicular atrophy (Tta) is covered by tunica albuginea (Ta) with adipose tissue (ad) inside. The fibrosis (F) is visible. *(Masson's trichrome stain). Scale bar = 100µm.* Inset shows fibrosis. *Scale bar = 50 µm*

Figure 5.12

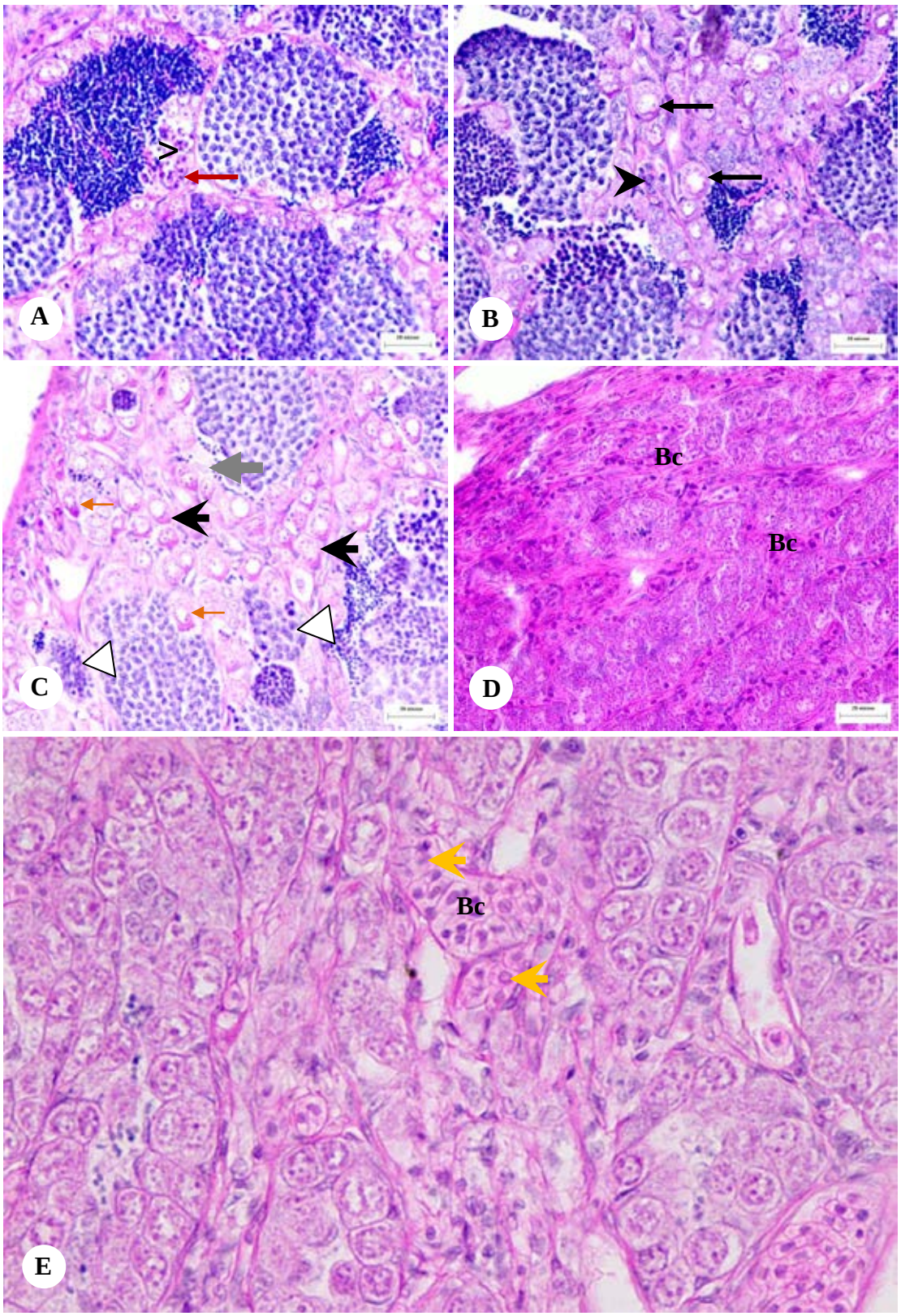

Figure 5.12 Micrograph of testis of *P. proctozysron* in January 2011 *(H&E stain)*

A The pyknosis (red arrow) and karyorhexis (>) of spermatid at a higher magnification *Scale bar = 20 µm.*

B Cells inside seminiferous lobule show hypertrophy (arrows). Degeneration of Leyding cells (black arrowhead) are observed between lobule. *Scale bar = 20 µm.*

C Testicular tissue shows degeneration of Sertoli cell (gray arrow), asynchronous development germ cells (white triangle) Cells inside the cysts show a group of eosinophilic cytoplasm (ec) that are characterized by acidophilic staining. Atrophy (orange arrow) are observed in the germ cell. *Scale bar = 20 µm*.

D-E Testis tissue shows blood congestion (Bc) and abnormal red blood cells (yellow arrows) are seen between the lobules. *Scale bar = 20 µm*.

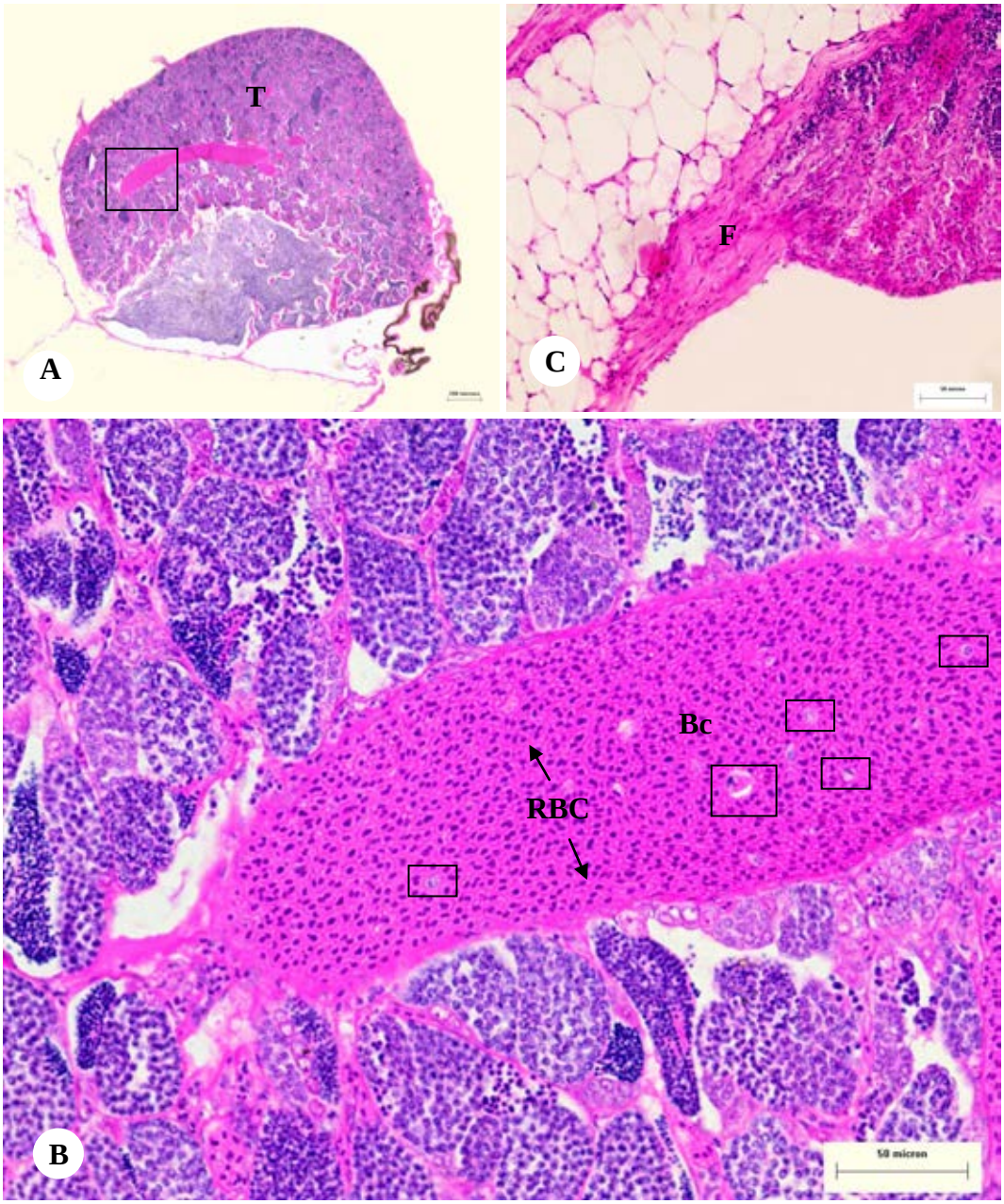

Figure 5.13 Micrograph of testis of *P. proctozysron* in April 2011 *(H&E stain)*

A Micrograph of testis (T) at a lower magnification *Scale bar = 200 µm.*

B Higher magnification (B) shows dilated blood vessel (Bc) between the lobules. It is greatly distended and packed with red blood cells (RBC). Very few white blood cells are visible (□). *Scale bar = 20 µm.*

C Testicular tissue shows disorganization of the testis with abundant of fibrosis (F). *Scale bar = 20 µm.*

Figure 5.14

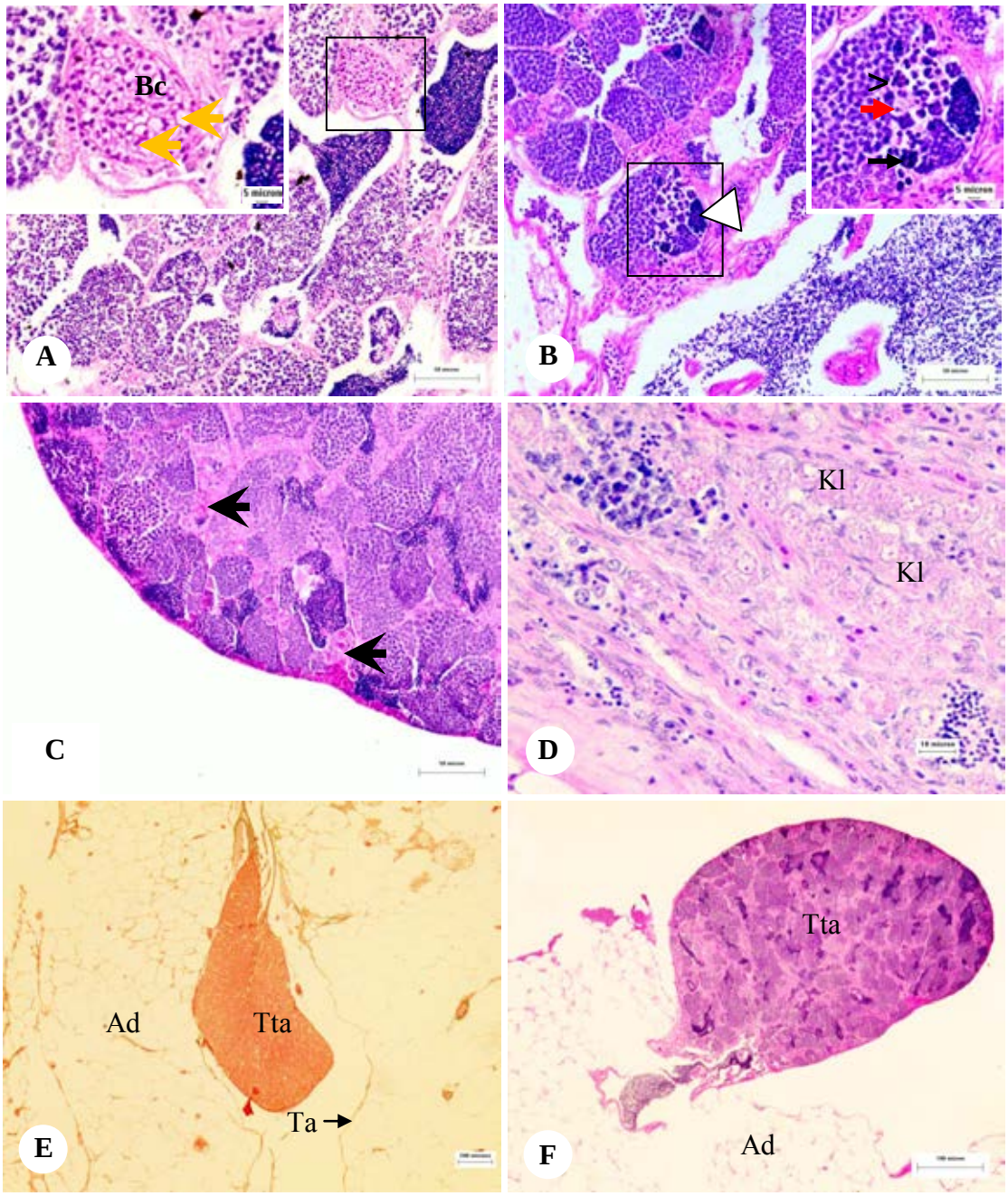

Figure 5.14 Micrograph of testis of *P. proctozysron* in April 2011

A Testicular tissue shows disorganization of the testis and blood congestion (Bc) with marked abnormal red blood cells (orange arrows). *(H&E stain).* *Scale bar = 50µm.* Inset shows abnormal red blood cell. *Scale bar = 5 µm*

B Testicular tissue shows pyknosis (red arrow) and karyorrhexis (>) of spermatid, asynchronous development germ cell (white triaggle) and apoptosis germ cells (black arrow) of sperm cell in the seminiferous lobule. *(H&E stain).* *Scale bar = 50µm.* Inset shows higher magnification. *Scale bar = 100 µm*

C Testicular tissue shows a group of eosinophilic cytoplasm (head arrows) of spermatogonia, which is acidophilic cell. *(H&E stain). Scale bar = 50 µm.*

D Micrograph of testis tissue shows karyolysis (KI) in spermatogonia *(H&E stain). Scale bar = 10 µm.*

E-F Testicular tissue shows testicular atrophy (Tta) covered by tunica albuginea (Ta). Adipose tissues (ad) are visible. *(E = Masson's trichrome stain, F = H&E stain). Scale bar E = 200 µm, F = 100 µm.*

5.4.4 Correlation between herbicides contamination and histopathological alteration in testis of *P. proctozysron*

Herbicide concentrations and histopathological alterations in testicular tissues were significantly correlated in both positive and negative correlations (Spearman's correlation) (Table 5.4).

Atrazine concentration was positively correlated with testicular atrophy and degeneration of Leydig cells and negatively correlated with pyknosis and blood congestion. Glyphosate concentration was positively correlated with germ cell hypertrophy, degeneration of Sertoli cells and negatively correlated with karyolysis, germ cell atrophy, asynchronus development, eosinophilic cytoplasm, infiltration of white blood cells and dilation of blood vessel.

Table 5.4 Spearman rank correlation between histopathology and herbicide concentrations in the testis of male *P. proctozysron*

Histopathology	Spearman's correlation coefficient	
	Atrazine	Glyphosate
Testicular degeneration	-0.500	0.500
Testicular atrophy	1.000**	0.500
Asynchronus development	-0.500	-1.000**
Pyknosis	-1.000**	-0.500
Karyorhexis	-0.500	0.500
Karyolysis	-0.500	-1.000**
Germ cell atrophy	-0.500	-1.000**
Germ cell hypertrophy	0.500	1.000**
Eosinophilic cytoplasm	-0.500	-1.000**
Degeneration of Sertoli cells	0.500	1.000**
Degeneration of Leydig cells	1.000**	0.500
Infiltration of white blood cell	-0.500	-1.000**
Dilation of blood vessel	-0.500	-1.000**
Blood congestion	-1.000**	-0.500
Abnormal red blood cell	-0.500	0.500
Fibrosis	-0.500	0.500

**indicates significant correlation at $p<0.01$

5.5 Discussion

The aims of this chapter were to investigate the histological changes in the testis of *P. proctozysron* living in Nan River nearby agricultural areas and to evaluate its correlation with the level of herbicide contamination.

5.5.1 Basic histology and testicular development

The lobular testis of *P. proctozysron* is classified as an unrestricted spermatogonial type. This type is found in cypriniforms and common in other teleosts (Grier et al., 1980). Spermatogenic cells in each stage are synchronously developed inside each spermatocyst that freely distributed along the lengths of the lobules (Grier et al., 1980; Nagahama, 1983; Parenti and Grier, 2004). Leydig cells and connective tissues are found among the seminiferous lobules, while Sertoli cells are presented in the lobular septa. In general, Leydig and Sertoli cells control the development of sperm cells by producing vital factors for sperm cells and creating the supporting nutrient as well as protecting the sperm cells in the spermatocysts. Leydig cell of teleosts is a large polygonal cell and found in the small groups. It is considered to be the androgen producing cells in male fish (Dietrich and Krieger, 2009; U.S. EPA, 2006).

The presence or absence of spermatogenic stage in a histological section can be used to judge the state of testicular maturity (Leino et al., 2005). From the testicular development results, the highest percentage of late spermatogenic stage was observed in July and October 2010. This suggests that *P. proctozysron* may have breeding season during the rainy season. This finding is concordance with the breeding period reported by Banyen (1988) in June to July.

5.5.2 Histopathological alteration in the testis of *P. proctozysron* living in herbicide contaminated river

Prominent histopathological alterations were seen in the testis of *P. proctozysron* collected from Nan River in this study. In overall, the degree of histopathological alteration in the testis of the fish collected in dry season was more severe than those of rainy season, including testicular atrophy, asynchronus development, karyorrhexis, karyolysis, sperm cell atrophy, hypertrophy of sperm cell, eosinophilic cytoplasm, degeneration of Sertoli cell and Leydig cells, infiltration of white blood cells and dilation of blood vessel. These lesions may correlate to the high

level of atrazine residue in testicular tissue of the fish caught in dry season (Chapter III).

According to the literature review, very few field observations have been reported the possible direct effects of atrazine on testis of the wild fish. Iwanowicz et al. (2009) observed reproductive health of bass fishes living in up- and downstream of the wastewater treatment plant on the Potomac River in Maryland, USA. They found that a high prevalence of testicular oocytes (82–100%) was identified in male *Micropterus dolomieu* and lower prevalence (23%) was identified in male *Micropterus salmoides*. The authors suggested that the testicular oocytes may effected by atrazine and its associated metabolites, which were present in the upstream sites. On the other hand, the effect of atrazine contamination in an environment to amphibianis is relatively well known. Many frog species living in atrazine-contaminated sites across the United States have been reported for the testis abnormality, such as gonadal dysgenesis, presence of testicular oocytes and testicular degeneration, which linked to the potential biological impact of atrazine contamination in the environment (Hayes et al., 2002a, 2002b; Murphy et al., 2006; Reeder et al., 1998). The data from amphibian studies raised a concern about the the potential role of atrazine as well as other endocrine-disrupting pesticides in other aquatic animals, particularly, the fish.

Prominent histopathological alterations were seen in the testis of *P. proctozysron* in this study. One of them was the degeneration of Leydig cells. The degeneration of Leydig cells in the testis of the male fish have been report previously. OECD (2009) have illustrated both hyperplasia and hypertrophy of Leydig cells in the male fathead minnow, *Pimephales promelas*. Shukla and Pandey (1984) studied the effect of arsenic in the Giant gouram, *Colisa fasciatus*. After 15 and 30 days of exposure to 14.0 or 2.0 mg/l arsenic (III) oxide, the degenerative changes in the lobules and Leydig cells and varying degrees of necrosis and pyknosis were observed. In general, Leydig cell involves and produces steroids hormones (11-ketotestosterone and testosterone) that regulate the spermatogenesis (Sharpe and Skakkebaek, 1993). Therefore, if Leydig cell has been damaged, the endocrine regulation of spermatogenesis will be interrupted and consequently the reproductive ability of the fish will be reduced (Trudeau et al., 1993). Several studies have been reported that

endocrine disruptive hormone is related to the reduction of testosterone levels in animals due to the inhibitory effects on the Leydig cell function (Clement, 1985; Rhouma et al., 2001; Goad et al., 2004; Srivastava et al., 2008). Spanó et al. (2004) observed hormone level in mature goldfish, *Carassius auratus*, that exposed to 1000 ng/L of atrazine for 21 days. The results showed that atrazine induced suppression in both testosterone and 11-ketotestosterone after 21 day of exposure. Further, these suppressive effects on plasma androgens were dose- and time-related.

Testis of *P. proctozysron* in the present study also showed the degeneration of Sertoli cells. In teleost, Sertoli cells provide a number of supportive functions in regulation of spermatogenesis (Billard, 1990). Therefore, it is possible that the impaired structure of Sertoli cell may result to its function and might affect to spermatogenesis of the fish. It has been noted that increasing of the sperm necrosis or apoptosis by compounds that cause an arrest of germ cell maturation are related to proliferation of Sertoli cells (Miles-Richardson et al., 1999). A number of pollutant exposures, partially estrogenic chemicals, have been reported for alteration in structure and function of Sertoli cells in fish. For example, Blazer (2002) reported hypertrophy and hyperplasia of Sertoli cells that contained ceroid or lipofuscin pigments in post- spawning of the white perch *Morone americana* and proliferation of Sertoli cells filling much of the lumen of selected lobules in the testes of common carp, *Cyprinus carpio*. Christiansen et al. (1998) studied the male eelpout, *Zoarces viviparous*. After 4-nonylphenol (NP) or 17β-estradiol (E_2) treatment, the reduction in the activity of enzyme γ-glutamyltransferase in Sertoli cells was found and Sertoli cells appeared in very squamous pattern with greater numbers of phagocytozed spermatozoa in the cells.

High prevalence of severe testicular atrophy was also observed. This indicated that the relative testis weight of the fish as the GSI measurement in Chapter IV probably due to the accumulation of the fat in testicular tissue. Similar results of testicular atrophy were obtained in fathead minnow (*Pimephales promelas*) that exposed to 17β-ethinylestradiol (4 ng/L; 176 days) (Laenge et al., 2001) and in other fish from other environment exposures (Colborn and Clement, 1992).

Moreover, evidences of eosinophilic cytoplasm of testis were recorded. This lesion is a dead cell, stained as a bright pink (eosinophilia) and stand out from the

other cell by a degeneration of structural proteins that formed a compact homogenous mass (Yong et al., 2005). Besides, the inflammation was found in the testicular tissue of *P. proctozysron*. Dilation of blood vessel usually occurred after acute inflammation, while the infiltration of white blood cells (macrophage infiltration) was an evidence of chronic inflammation occurred by the long-term injury in the organ. Chronic inflammation involved a diffuse accumulation of macrophages and lymphocytes at the site of injury, resulted in blood congestion (Jaeschke, 2008; Gregus, 2008). Similar results of the number of macrophage infiltration with inflammation have been reported in the testis of adult zebrafish, *Danio rerio* exposed to 17α-ethinylestradiol (9.3 ng/L; 177 days) (Schãfers et al., 2007). In addition, fibrosis also observed. Fibrosis is the formation of excess fibrous connective tissue in an organ or tissue in a reparative or reactive process. Fibrosis has been frequency reported in association with other histhopatological alterations in the testis of several fish species (Blazer, 2002; Kime, 1995; OECD, 2009). The histological changes mentioned above indicate that the testis of *P. proctozysron* probably undergone previous severe injuries, which may also associate with the atrazine found in the environment and testicular tissue.

Atrazine has been noted as an endocrine disrupting chemical. A number of studies have proven the adverse effects to reproductive health of the male fish caused by endocrine disrupting chemicals groups (EDCs) that directly affects to the testicular tissue of the fish (Jobling et al., 1996; Kime, 1998; Pait and Nelson, 2002; Thomas 1999). Testicular structure damages of the fish exposed to EDC have been frequently reported, such as pyknosis germ cells, degeneration and necrosis of sperm cells, inhibited spermatogenesis, degeneration of Leyding and Sertoli cell, fibrosis and inflammation of white blood cells (Islinger et al., 2003; Karels et al., 2003; Miles-Richardson et al., 1999; Weber et al., 2003; Zillioux et al., 2001; Zha et al., 2007).

Statistical correlations between the atrazine concentration in testicular tissue and its histopathology were occurred in both positive and negative correlation. The atrazine concentration was positively correlated with testicular degeneration and degeneration of Leydig cells. The effects of atrazine to the testis of the fish have been previously reported from laboratory experiments. Testicular lesions and reduction of sperm cell numbers have been found in many teleosts after exposed to atrazine (Hayes

et al., 2011). In adult goldfish, *Carassius auratus,* atrazine had affected on testicular structure (tissue damage) displayed the increase in spaces of the interstitium tissue (Spanò et al., 2004). The results from this study and in comparison with those reported before in laboratory condition suggest that the higher up of atrazine concentration might relate with some histopathological changes, especially degeneration of Leydig cell in the testis, which possibly affected to the degeneration of spermatogenesis in *P. proctozysron*. It is important to note that, since the effect of atrazine on the testis of wild fish are not previously available; this hypothesis was generated based on the comparison of histopathology observed in this study with laboratory experimental report. To confirm the hypothesis, laboratory exposure study of atrazine in *P. proctozysron* should be conduct.

For glyphosate, the concentration level of glyphosate was correlated with some histolpathological alterations in the testis. However, no reports have been suggested the possible effects of glyphosate on the testis of the wild fish living in glyphosate-contaminated river or the fish exposed to glyphosate in laboratory. This study is the first report that notes about the possible interfere of glyphosate and testiscular tissue, especially in spermatogenesis, even thought the concentration of glyphosate was found as a very low concentration (1.20-2.01 ng/g).

In addition of herbicide effects, other studies in the wild fish have revealed that testis regressions were linked to other physical factors, such as temperature, photoperiod, pH and DO (de Vlaming, 1971; Lam, 1982; Stacey, 1983; Sundararaj and Vasal, 1976; Singh et al., 2010). In this study, testis regression of *P. proctozysron* may not related with such physical factors, because of temperature, DO and pH collected in the same time with fish were not different between sampling months (Chapter IV).

5.6 Conclusion

Prominent histopathological alterations were seen in the testis of *P. proctozysron*, especially in Leydig and Sertoli cells, during this study. Their impaired structure may result to the function and might affect to spermatogenesis of the fish. Moreover, histopathological alterations have been correlated with atrazine concentration in the testicular tissues. Thus, it is possible to stated that atrazine in the

river may enter to the fish body and disrupt the function of Leydig and Sertoli cells and further damages to testicular tissue and the testis. This finding provides further evidence of the link between herbicide and general health of the fish, especially an endocrine disruption chemical (EDC) to the testicular tissue**.**

CHAPTER VI

OVARIAN HISTOLOGICAL CHANGES IN *Puntioplites proctozysron* LIVING IN NAN RIVER, WIANGSA DISTRICT, NAN PROVINCE

6.1 Introduction

Pollution of aquatic ecosystem caused by herbicides, mainly glyphosate, paraquat and atrazine, is an important problem in Nan Province, northern part of Thailand. A number of herbicides have been used for irrigation and in agricultural activities such as rice and vegetable farming. Water used in the agricultural areas located nearby has been usually discharged directly into the river. The aquatic habitats nearby agricultural areas are thus susceptible to contamination by the herbicide runoff. Aquatic organisms living in the river are at risk of affecting by herbicide contamination.

Fish is a major group of aquatic animals that is naturally suffered from any contamination in water. Numerous organs and tissues of female fish have been reported to be damaged by herbicides, especially the reproductive organs such as ovary. For example, paraquat caused ovarian alteration, increased late-vitellogenic stage oocyte and reduced primary oocytes in *Oreochomis mossambicus* (Figueiredo-Fernandes et al., 2006). The study on the effects of glyphosate exposure (30 days) at concentration of 2 µg/L in various freshwater fish for 30 days indicated significantly reduced fecundity (Folmar et al., 1979). Du Preez and Van Vuren (1992) reported that sublethal exposure of atrazine to *Tilapia sparrmanii* showed a level of atrazine accumulation in ovary. The accumulation of herbicide in ovary is considered as a cause of reduced reproductive capability, as well as tissue abnormalities in fish. Tillitt et al. (2010) studied the effects of atrazine on *Pimephales promelas* at 0, 0.5, 5.0 and 50 µg/l for 14 and 30 days. In 14 day exposure, the results showed reduced egg production and atretic follicle in treated female fish at 50 µg/l. Moreover, intersexuality was found in 5 µg/L treated male fish. Laboratory exposures of *Pimephales promelas* to atrazine increased the number of late stage oocyte (Bringolf et al., 2004).

In this study, *Puntioplites proctozysron* was chosen as a sentinel species for herbicides contamination in Nan River because it has sufficient population size and density. So, enough number of sampling is possible. Furthermore its home range overlaps with the potential affected area. Thus, the objective of this study is to investigate the histological changes in the ovary of the fish living in Nan River nearby agricultural areas and evaluate its correlation with the level of herbicide contamination.

6.2 Materials and methods

6.2.1 Fish sampling

Mature *P. proctozysron* (approx. >10 cm in total length) were collected by fishing net year round from Nan River, Wieng Sa District, Nan Province. Fishes were collected in rainy season (July and October 2010) and dry season (January and April 2011) for 40 individuals per sampling month (20 individuals of each sex: total 160 fishes). All fishes were kept in aerated holding tank and transported to the laboratory at Chulalongkorn University, WiangSa District, Nan Province. No mortality occurred during the transportation.

6.2.2 Ovarian histology

Sample preparation

The ovary of each fish was dissected, weighted and fixed in Davidson's fixative. They were processed using standard histological techniques (Humason, 1979). The ovarian tissues were dehydrated in a series of ethanol and then n-butanol. Then the tissues were cleared in xylene and then embedded in paraplast. The paraffin blocks of ovarian tissues were serially sectioned at 7 μm thickness by rotary microtome. The ribbons of these sections were attached to the slide by egg albumin solution and dried at 40°C on warm plate. The sections were deparaffinized with xylene and hydrated through a series of alcohol before stained with Delafield's haematoxylin and eosin and Masson's trichome (Humason, 1979).

Light microscope was used to observe the histological structures of the ovary. Oogenesis of the fish was studied following the criteria by Dietrich and Krieger (2009).

Ovarian development

Each histological section of ovarian tissue was examined in detail under light microscope in order to determine the stage of ovarian development according to Dietrich and Krieger (2009). The ovary was classified into different developmental stages (0–4), according to the maturity of germ cells that is a predominant stage in the ovary. The developmental stages of ovary are as follows: stage 0 (undeveloped), stage 1 (early-development), stage 2 (mid-development), stage 3 (late-development) and stage 4 (post-ovulatory).

Histopathology

Each histological section of ovarian tissue was examined in detail under a microscope to determine the histopathological alterations according to Dietrich and Krieger (2009). Each histopathological alteration observed in the ovary will be recorded as a mean prevalence.

6.2.3 Statistical analysis

Spearman's correlation test was used to assess the significant correlation ($p < 0.05$) between the herbicide concentration in ovarian tissues and the histopathological changes using the Statistical Package for the Social Sciences (SPSS) software (version 15.0).

6.3 Results

6.3.1 Basic morphology and histology of *P. proctozysron* ovary

The female *P. proctozysron* has a paired ovaries situated in the dorsal part of peritoneal cavity and attached along the dorsal surface by a mesovarium. The 2 ovarian sacs are joined to the genital papillae. Histological study of the fish ovary revealed that the surface of ovary is surrounded by tunica albuginea, which is a thin layer of connective tissue, smooth muscle and numerous blood vessels. Early stage oocytes are developed in the ovigerous fold extending from the tunica albuginea toward the center of the ovary (Figure 6.1A-B; 6.2A). This fish species possesses an asynchronous ovarian development. Developmental stages of oocyte were classified based mainly on the cell size, shape, nuclear characteristics, amount and characteristics of cytoplasm and staining properties. The female germ cells were classified as follows:

Oogonium was found in comparatively low numbers. It is the smallest among female germ cells. Oogonium is characterized by a large nucleus with a small nucleolus and small amount of cytoplasm. Although, it can be found isolately, the oogonia tends to occur in cluster or nest, namely oogonial cyst, inside the ovigerous fold (Figure 6.2B; 6.6).

Chromatin nucleolar stage oocyte is larger than an oogonium. The chromatin nucleolar oocyte has a relatively large nucleus with a large single nucleolus. It contains small amount of strongly basophilic cytoplasm comparing with that of an oogonium. This oocyte is surrounded by prefollicle (Figure 6.2C; 6.6).

Perinucleolar stage oocyte is larger than chromatin nucleolar stage oocyte. The amount of nucleus is increased with multiple nucleoli. The nucleoli are arranged along the nuclear membrane. The cytoplasm is increased in mass and appeared less basophilic than that of the chromatin nucleolar stage oocyte. The follicle is found consisting of a monolayer of simple squamous epithelium (Figure 6.2D; 6.6).

Cortical alveolar stage oocyte is larger than perinucleolar stage oocyte. The nucleus also contains many nucleoli at the periphery near the nuclear membrane. This stage is characterized by the appearance of cortical alveoli or yolk vesicles accumulated in the peripheral region of cytoplasm. In this stage, an acidophilic acellular layer called zona radiata or vitelline envelop is detected for the first time. It is surrounded by a layer of simple squamous follicular cells (Figure 6.2E; 6.6).

Early vitellogenic stage oocyte is also increased in size. The yolk vesicles are enlarged and still occur in the peripheral region of cytoplasm. In this stage, the oocyte is characterized by the appearance of numerous small yolk granules accumulated in the cytoplasm resulting in its acidophilic appearance. It is slightly acidophilic stained. The oocyte in this stage is surrounded by a follicle with a well-developed zona radiata, distinctive granulosa and theca cell layers (Figure 6.3A1,A2; 6.6).

Late vitellogenic stage oocyte is distinctively acidophilic because of yolk deposition. The yolk vesicles (cortical alveoli) are enlarged occupying a discrete zone in the peripheral region of cytoplasm, while the yolk granules are multiplied and increased in size, occupying a broad zone in the inner region of cytoplasm. The follicle consists of a thick zona radiata surrouned by a simple cuboidal layer of well-

developed granulosa cells and a layer of stratified squamous theca cells (Figure 6.3B1; B2; 6.6).

Mature stage oocyte is characterized by an enlargement of yolk granules. Peripheral migration of the nucleus is observed. The follicle at this stage consists of a distinctive thick zona radiata surrounded by granulosa and theca cell layers (Figure 6.3C1: C2; 6.6).

Four major events can be pointed out when Masson's trichome stain was applied for specific staining of connective tissue. These events including formation of yolk vesicle, formation of zona radita, accumulation of yolk granule and early stage oocytes were found developed in the ovigerous fold which stained greenish. Inside the ovary, small artery was observed, consisting of greenish connective tissue. The cytoplasm of oogonia was stained orange-red. The centrally located nucleoli within nucleus became red in the chromatin nucleolar stage oocyte. For perinucleolar stage, the oocyte was recognized by the arrangement of nucleoli around the nuclear membrane. In cortical alveolar stage oocyte, the zona radita was also synthesized and the cortical aveioli were found for the first time staining greenish in the oocyte surface. In early vitellogenic stage oocyte, the zona radiata increased its thickness and all nucleoli became red. In this stage, the oocyte synthesized yolk granules staining reddish. The yolk granules were spread toward the central domain, multiplied and increased in size (stained as reddish) during late vitellogenic stage oocyte. In mature stage, the enlargement of yolk granules (stained as reddish) were observed in the oocyte with zona radiata reached its maximal thickness (Figure 6.4-6.5).

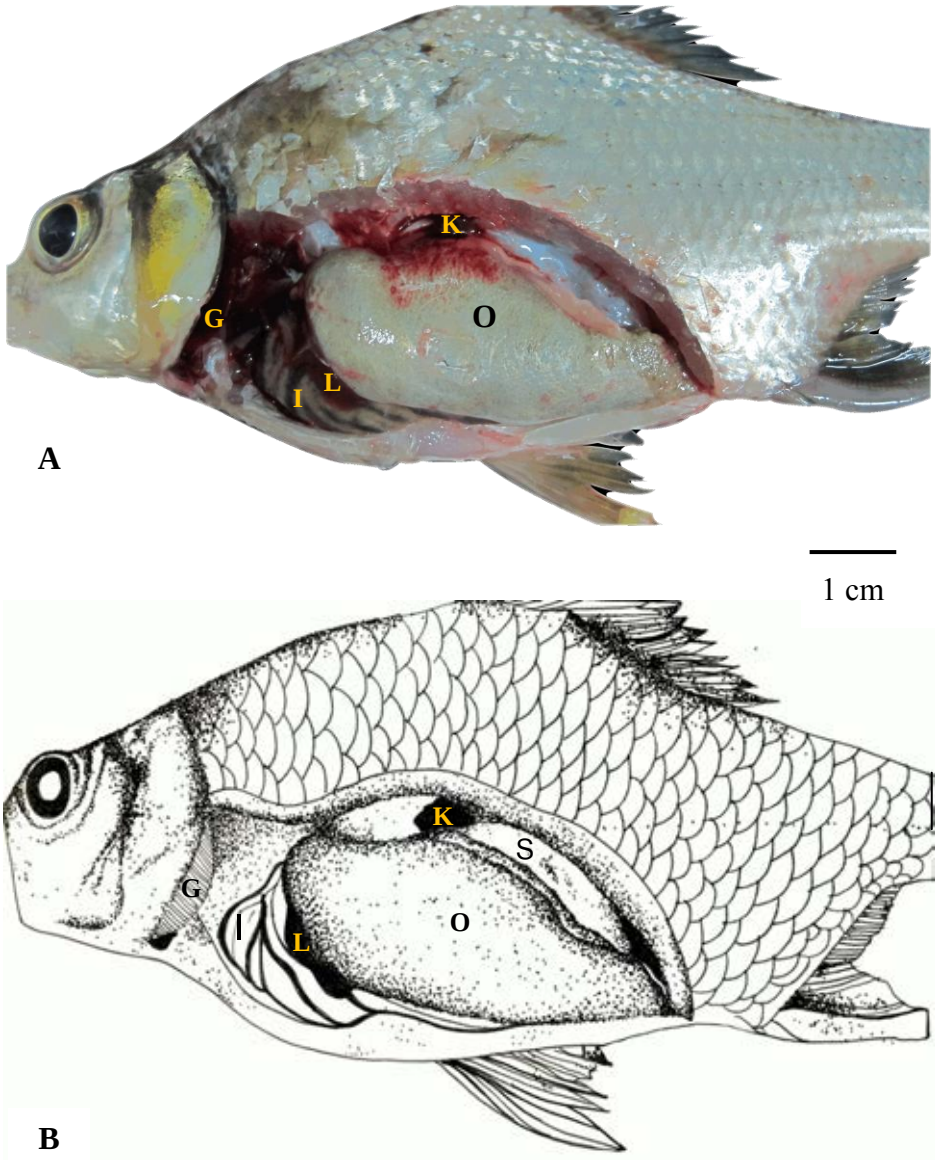

Figure 6.1 Gross morphology of female reproductive system of *P. proctozysron* (Drawing by Sinlapachai Senarat)

A Photograph shows the ovary located in abdominal cavity. It is situated dorsally in the posterior part of the abdomen.

B Drawing shows the internal organs within abdominal cavity.

(G = gill, I = intestine, K = kidney, L = liver, O = ovary, S = swim bladder)

Figure 6.2

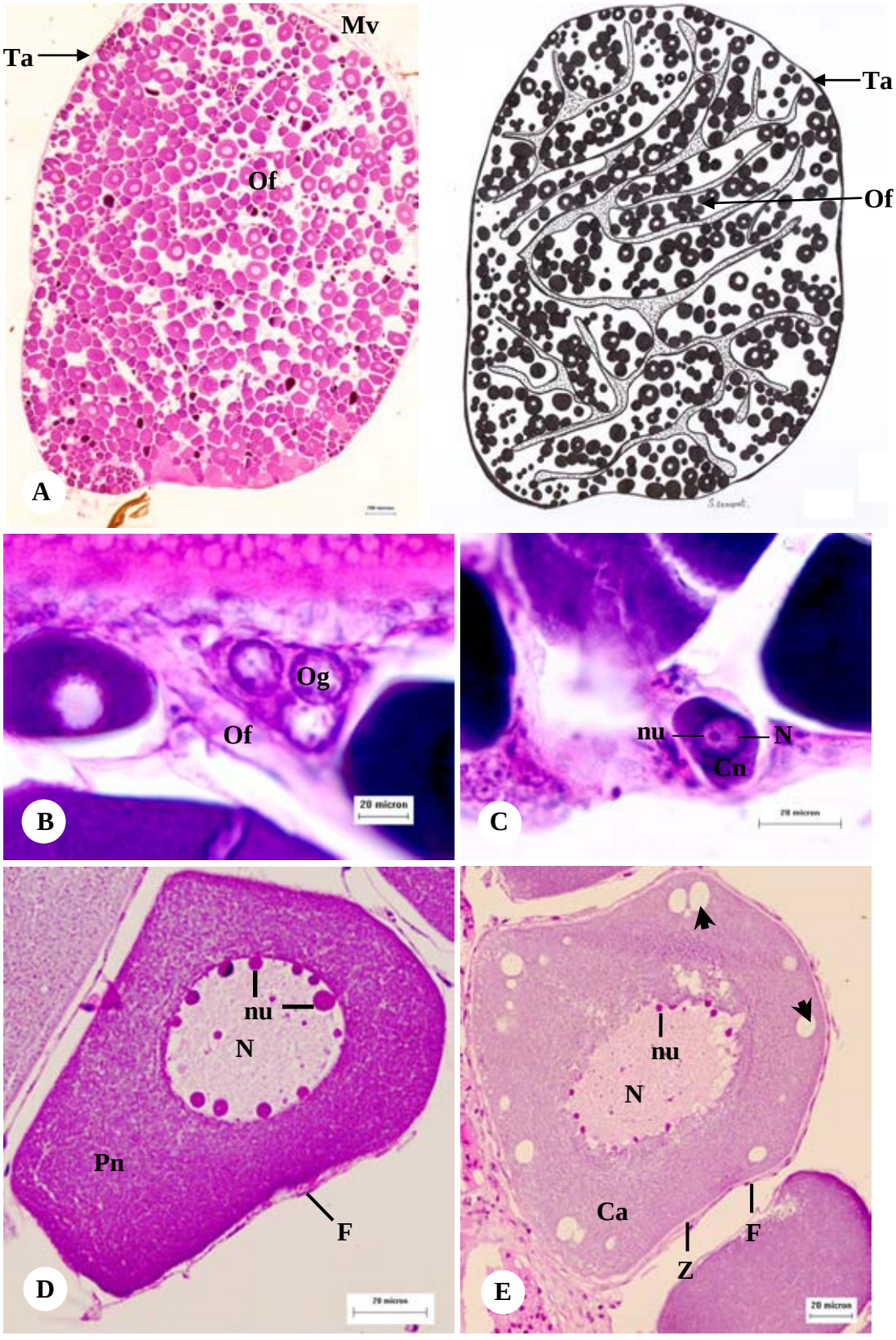

Figure 6.2 Micrograph of *P. proctozysron* ovaries showing basic ovarian histological structure *(H&E stain)*

A Micrograph and diagram of overall structure of the ovary containing oocytes at different developmental stages. The ovary is encapsulated by tunica albuginea (Ta). *Scale bar = 200 µm. (Mv = mesovarium, Of = ovigerous fold)* (Drawing by Sinlapachai Senarat)

B Oogonia (Og) located within the ovigerous fold (Of) and clustered into oogonial cyst. *Scale bar = 20 µm*.

C Oocyte at chromatin nucleolar stage (Cn) containing a nucleus (N) with a single, large nucleolus (nu) and strongly basophilic cytoplasm. It is enveloped by a prefollicular simple squamous epithelium. *Scale bar = 20 µm.*

D Oocyte at perinucleolar stage (Pn) showing a nucleus (N) with numerous nucleoli (nu) appeared near the nuclear membrane. Follicular cell layer (F) is more evidenced as a simple squamous epithelial lining. *Scale bar = 20 µm*.

E Oocyte at cortical alveolar stage (Ca) containing a nucleus (N) with nucleoli (nu) attached to the nuclear membrane. Cortical alveoli or yolk vesicles (arrowheads) are visible in the peripheral cytoplasm. *Scale bar = 20 µm.*

Figure 6.3

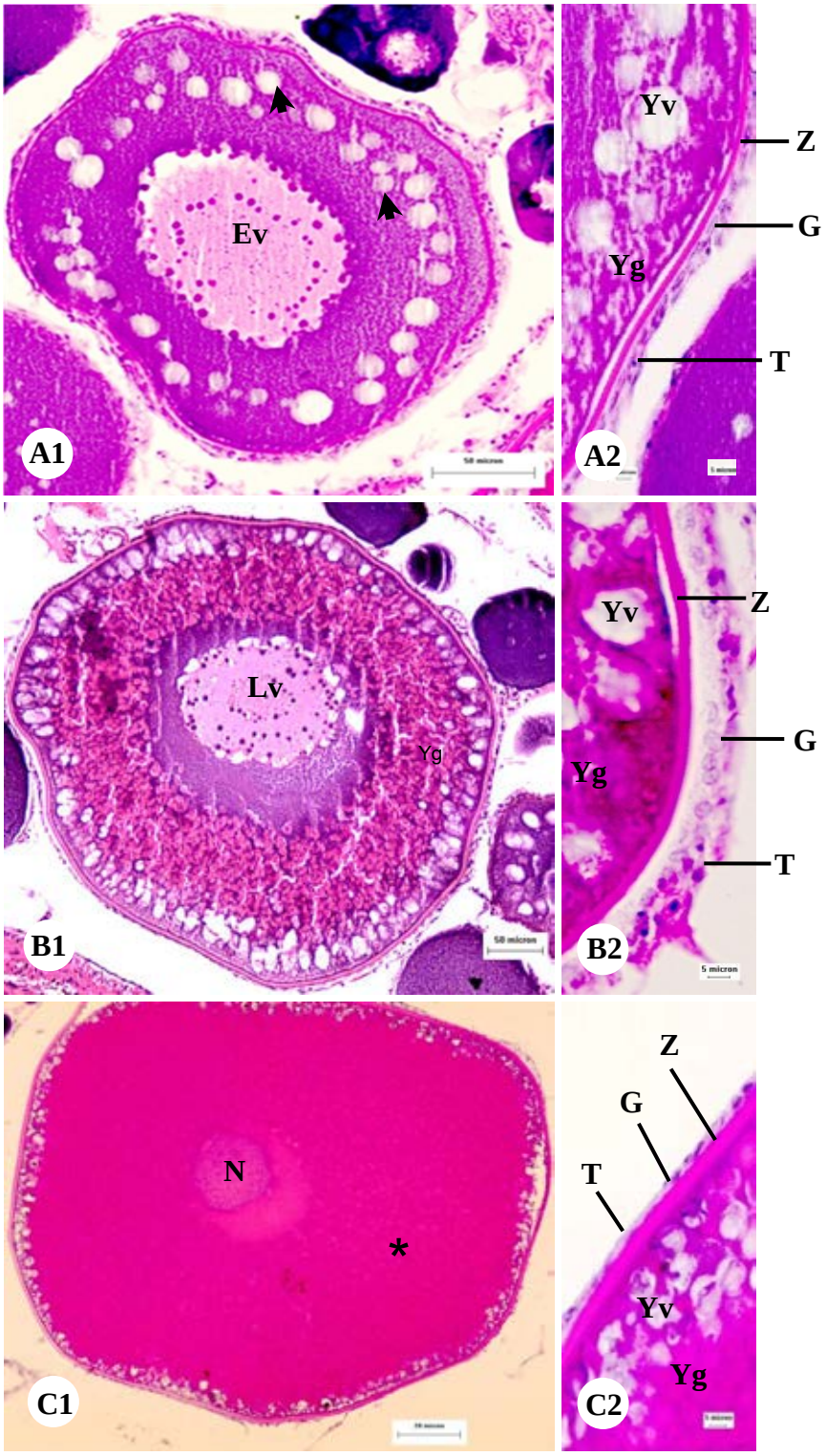

Figure 6.3 Micrograph of *P. proctozysron* ovaries showing basic ovarian histological structure *(H&E stain)*

A1 Oocyte at early vitellogenic stage (Ev) showing acidophilic cytoplasm with yolk vesicles (arrowheads). *Scale Bar = 50 µm.*

A2 The cytoplasm of early vitellogenic oocyte containing numerous acidophilic yolk granules (Yg) and yolk vesicles (Yv). The follicle consists of distinctive zona radiata (Z), a layer of simple squamous granulosa cells (G) and a layer of stratified squamous thecal cells (T). *Scale bar = 5 µm.*

B1 Oocyte at late vitellogenic stage (Lv) containing cytoplasm filled with acidophilic yolk granules (Yg). *Scale bar = 50 µm*.

B2 The Follicle of late vitellogenic oocyte consists of thick zona radiata (Z), a layer of simple cuboidal granulosa cells (G) and a layer of stratified squamous thecal cells (T). *Scale bar = 5 µm. (Yv = yolk vesicles, Yg = yolk granules)*

C1 Oocyte at mature stage showing a migratory nucleus (N) and large amount of yolk-filled acidophilic cytoplasm (asterisk). *Scale bar = 20 µm.*

C2 The follicle of mature oocyte consists of thick acidophilic zona radiata (Z), a granulosa cell layer (G) and a thecal cell layer (T). *Scale bar = 5 µm. (Yg = yolk granules, Yv = yolk vesicles)*

Figure 6.4

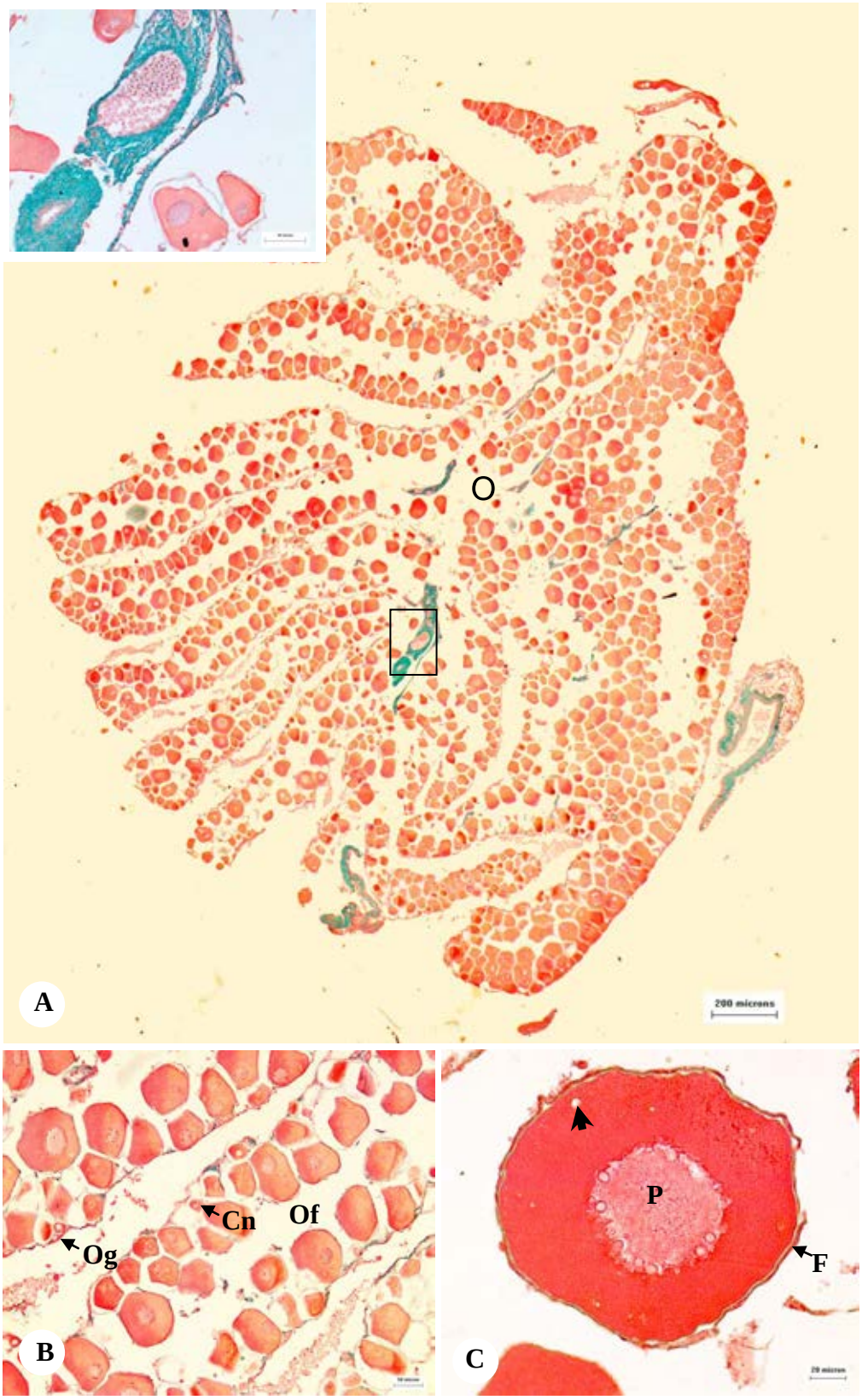

Figure 6.4 Micrograph of *P. proctozysron* ovaries showing basic ovarian histological structure *(Masson's trichrome stain)*

A Overall structure of the ovary (O) shows different stages of oocytes. *Scale bar = 200 µm*. Inset shows small artery (sa) , consisting of connective tissue as greenish. *Scale bar = 50 µm*.

B Ovigerous fold contains different stages of oocyte, including oogonia (og) and chromatin nucleolar stage oocyte (Cn). *Scale bar = 100 µm. (Of = ovigerous fold)*

C Oocyte at cortical alveolar stage (Ca) shows cortical aveoli dispersion in cytoplasm. The oocyte was surrounded by follicular cell layer (F) (greenish stain). The nucleus changed to red. *Scale bar = 20 µm. (N = nucleus; arrowhead = yolk vesicles).*

Figure 6.5

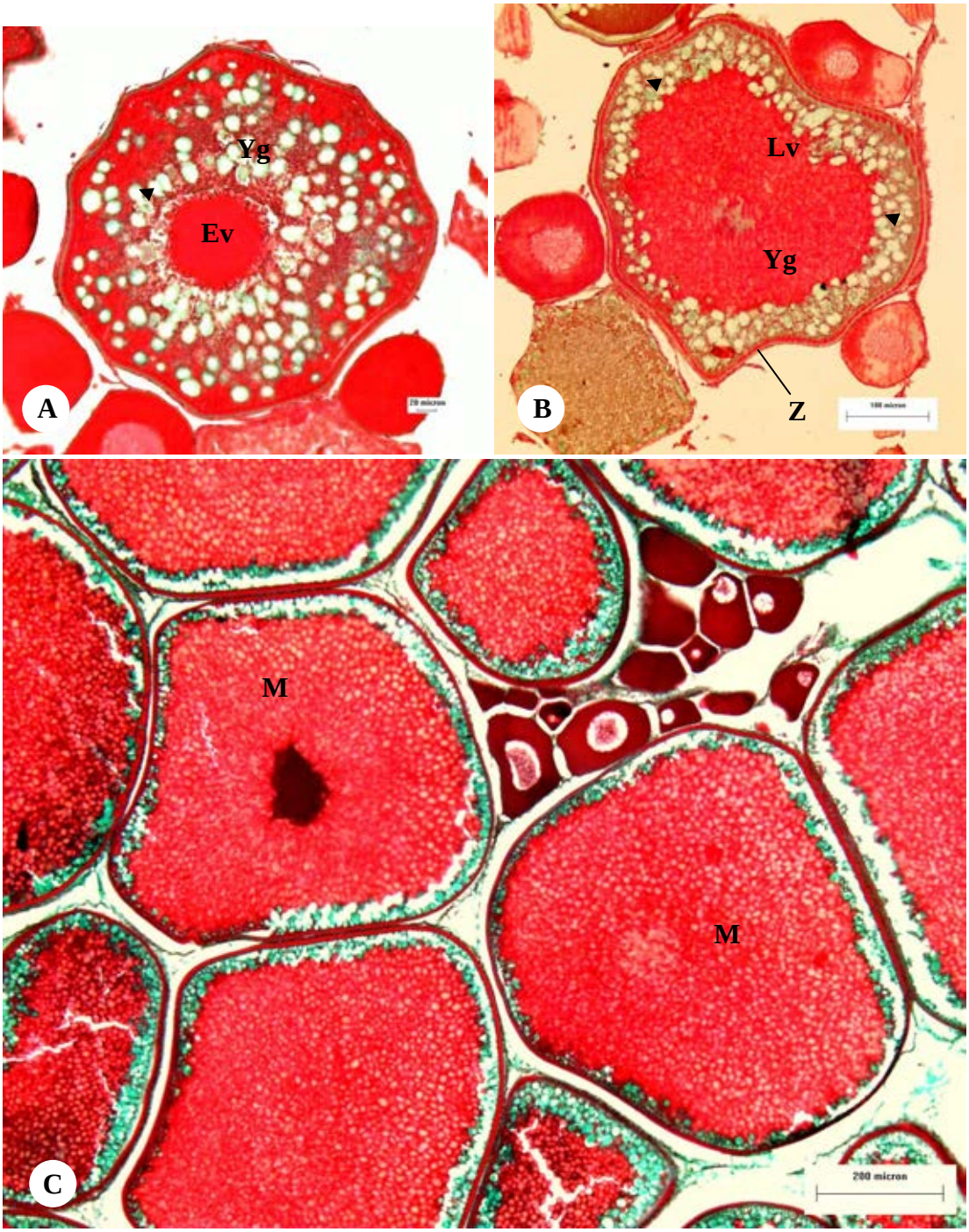

Figure 6.5 Micrograph of *P. proctozysron* ovaries showing basic ovarian histological structure *(Masson's trichrome stain)*

A Oocyte at early vitellogenic stage (Ev) showing an increase of yolk vesicles (arrowhead) dispersed in the greenish cytoplasm and surrounded by follicular cells. *Scale bar = 200 µm.*

B Oocyte at late vitellogenic stage (Lv) with cytoplasm filled with yolk granules (Yg) and the reddish yolk granules dispersed toward the central area, while the yolk vesicles (arrowhead) remain greenish clump. The increase in thickness of zona radiata (Z) is evidented more than that of early vitellogenic stage. *Scale bar = 100 µm.*

C Oocyte at mature stage (M) shows a large amount of yolk granules stained reddish. *Scale bar = 200 µm.*

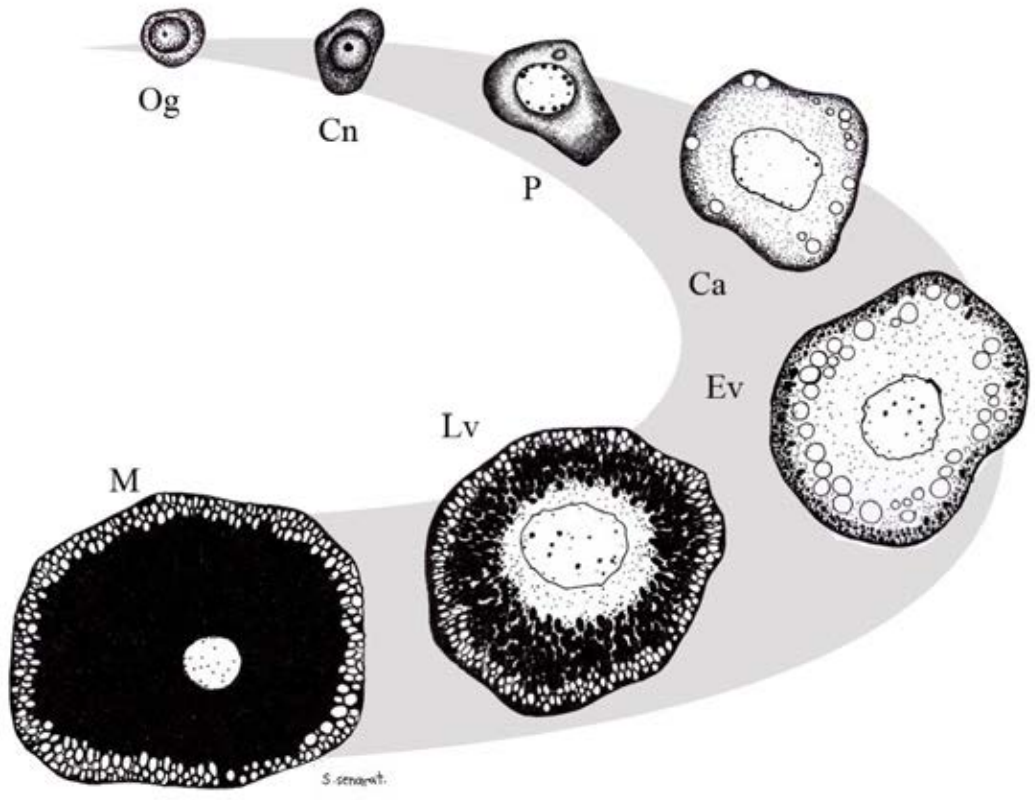

Figure 6.6 Diagram shows female germ cells during oogenesis of *P. proctozysron*. *(Og = oogonium, Cn = chromatin nucleolar oocyte, P = perinucleolar oocyte, Ca = cortical alveolar oocyte, Ev = early vitellogenic oocyte, Lv = late vitellogenic oocyte, M = mature oocyte)* (Drawing by Sinlapachai Senarat)

6.3.2 Ovarian development

6.3.2.1 Stage of development

Based on the development of the most advanced oocytes contained in the ovary and its histological structure, the ovarian stages were classified into 5 developmental stages, including undeveloped, early-development, mid-development, late-development and postovulatory stages.

Undeveloped stage (stage 0)

The ovary is very small, situated inside the abdomen. It contains oogonia, chromatin nucleolar stage oocyte and perinucleolar stage oocyte, but no cortical alveolar oocyte (Figure 6.7A).

Early development stage (stage 1)

The most advanced oocytes contained in the ovary is perinucleolar stage oocyte. Cortical alveolar stage may also found in the ovary (Figure 6.7B).

Mid development stage (stage 2)

Oocytes in several developmental stages may be found together in the ovary. The most advanced oocytes observed in this stage of ovary is early vitellogenic and late vitellogenic oocytes (Figure 6.7C).

Late development stage (stage 3)

The majority of developing oocyte is the mature stage. Oocytes in several developmental stages may exist together in an ovary (Figure 6.7D).

Postovulatory stages (stage 4)

This stage is not presented in this study**.**

6.3.2.2 Seasonal change in ovarian development

Percentage distribution of ovarian staging of female *P. proctoztsron* is presented in Figure 6.8.

The results showed that in July 2010, 100% of the ovaries of *P. proctozysron* were at late development stage. In October 2010, 50% of the fish contained undeveloped stage ovaries. In January 2011, 54.5% of the fish possessed undeveloped ovaries whereas the rest possessed early development ovaries. Finally, in April 2011, the majority of the fish had early development ovaries.

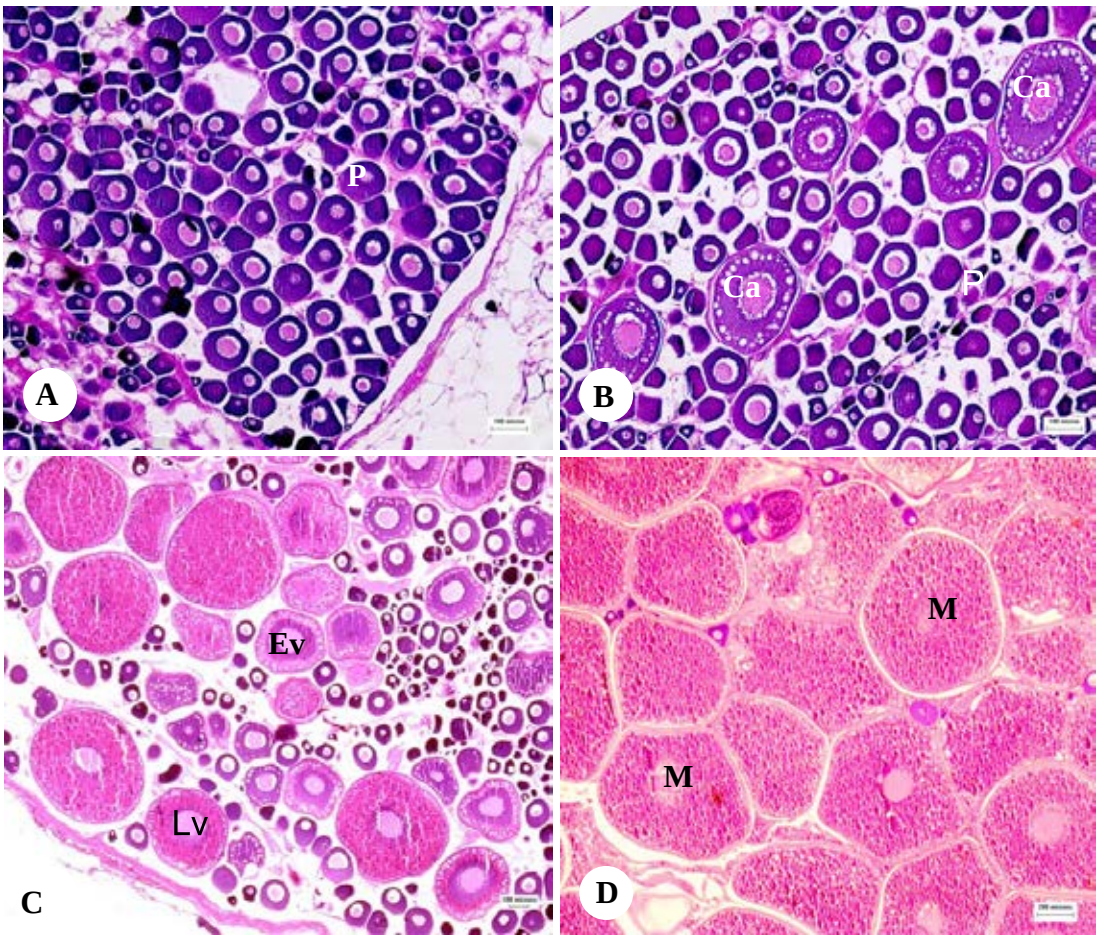

Figure 6.7 Micrographs of ovarian tissues at different maturity stages of *P. proctozysron* caught during July 2010 to April 2011 *(H&E stain)*

A Undeveloped stage (stage 0) of ovarian tissue shows immature oocytes. *Scale bar = 20 µm.*

B Early-development stage (stage 1) of ovarian tissue shows that more than 90% of the ovary contains perinucleolar stage oocyte and cortical alveolar stage oocyte. *Scale bar = 20 µm.*

C Mid-development (stage 2) of ovarian tissue shows the ovary that contains early vitellogenic stage oocyte and late vitellogenic stage. *Scale bar = 50 µm*.

D Late-development (stage 3) of ovarian tissue shows the ovary that contains all oocyte stages. The mature stage is found in high proportion. *Scale bar = 100 µm*.

(P = perinucleolar stage, Ca = cortical alveoli stage, Ev = early vitellogenic stage, Lv = late vitellogenic stage, M = mature stage)

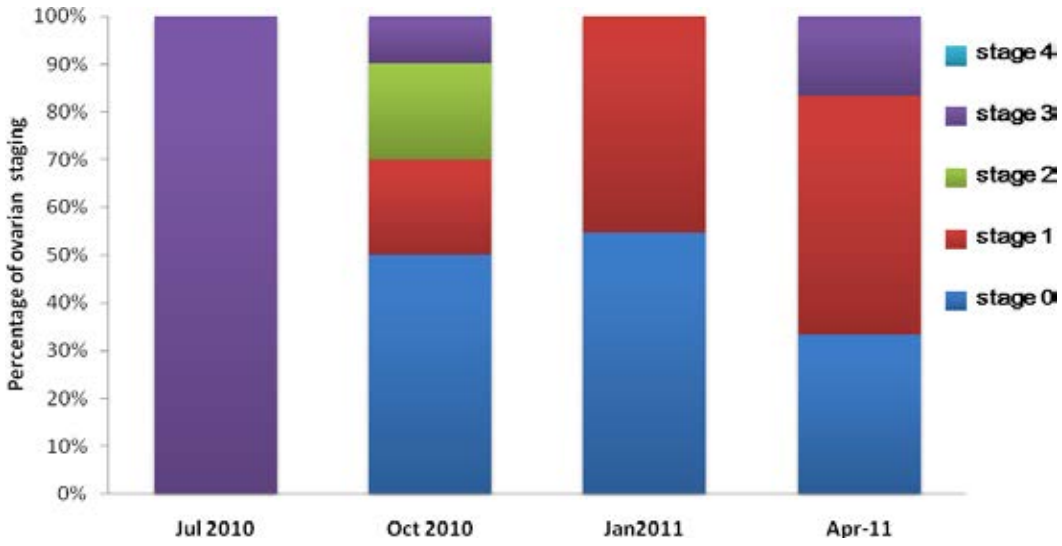

Figure 6.8 Percentage distribution of ovarian staging in female *P. proctozysron* from Nan River, Nan Province

stage 0 = undeveloped, stage 1 = early development,

stage 2 = mid development, stage 3 = late development,

stage 4 = post ovulatory

6.3.3. Histopathology

Several histopathological changes and its prevalence were found in the ovarian tissues of *P. proctozysron* caught from Nan River, Nan Province are show in Figure 6.9-6.16 and Table 6.1.

6.3.3.1 Histological alterations in the ovaries of *P. proctozysron* caught in rainy season

In July 2010, several histopathological changes were found in the fish ovaries. Degenerative and necrotic changes (atresia) were detected in previtellogenic stages, vitellogenic stages and follicular hyperplasia at 100% prevalence and were noted as the highest prevalence when compared to other months. Enlargement of interfollicular space or loss of connective tissues between oocytes, follicle atrophy, dilation of blood vessel blood congestion and abnormal red blood cell were observed at 50% prevalence.

In October 2010, histopathology observation of the fish ovaries exhibited blood congestion at 100% prevalence. Oocytes atresia was found in several oocyte stages, especially in previtellogenic stage at 90% prevalence. Ovarain degeneration, Vacuolar degeneration and abnormal red blood cell was found at 70% prevalence. Oocytes necrosis, infiltration of white blood cells, macrophage aggregations and other leucocytes were observed at 60%. Atresia was found in oogonia, atresia in vitellogenic stage and dilation of blood vessel at 50% prevalence. In some cases, the breakdown of follicular layer and abnormality (hyperplasia, 30% prevalence) of oocyte shape and fibrosis in necrotic areas were found at 30% prevalence. Beside, oocyte hyperplasia was exhibited.

6.3.3.2 Histological alteration in the ovaries of *P. proctozysron* caught in dry season

In January 2011, histopathological examinations of fish ovaries showed high degenerative changes of the oocytes. Atresia were found in many oocyte stages especially in previtellogenic stage at 100% prevalence. Vacuolar degeneration at 90.90% prevalence and was noted as the highest prevalence when compared to other months. Blood congestion was also evidenced at 72.72% prevalence. Oocyte hyperplasia and atresia in oogonia were exhibited at 27.27% prevalence. Oocyte degeneration and abnoemal red blood cell were noticed at 18.18% prevalence. Atresia in vitellogenic, follicular hyperplasia, Infiltration of macrophage aggregations and dilation of blood vessel were observed.

In April 2011, histopathological examinations of fish ovaries showed blood congestion at 100 % prevalence. Atresia in previtellogenic and Infiltration of white blood cell were exhibited at 80% and 60% prevalence, respectively. Breakdown of follicular layer, hyperplasia of oocyte and infiltration of white blood cell were marked at 60% prevalence. Degeneration of connective tissues between oocytes and degenerating ovary were found at 40% prevalence, equally with oocyte hyperplasia, atresia in vitellogenic stage, vacuolar degeneration were observed at 40% prevalence. In addition, atresia in oogonia was noticed.

6.3.3.3 Comparison of ovarian alterations of *P. proctozysron* between seasons

Ovarian histopathological alterations of *P. proctozysron* living in Nan River were clearly different between dry and rainy seasons. The alteration that found only in rainy season were follicular atrophy and fibrosis. The alterations that can be found in both seasons were ovarian degeneration, oocyte hyperplasia, atresia in oogonia, atresia in previtellogenic stage, atresia in vitellogenic stage, follicular hyperplasia, vacuolar degeneration, infiltration of white blood cell, dilation of blood vessel, blood congestion and abnormal red blood cell. For the comparison of prevalence, the results showed that some histopathological alterations were found in higher prevalence in rainy season than in dry season, for example ovarian degeneration, atresia in previtellogenic stage, atresia in vitellogenic stage and follicular hyperplasia.

Table 6.1 Prevalence (%) of different histopathological alterations in the ovaries of *P. proctozysron* caught from Nan River during July 2010 to April 2011.

Season	Sampling month	Alteration Prevalence (%)												
		Ov. degen.	Hyperplasia	Atresia og.	Atresia pv.	Atresia vit.	Foll. atrop.	Foll. Hyper.	Vac. degen.	Wbc inf.	Bv. dilation	Bl. con.	Ab. rbc	Fibrosis
Rainy	July 2010 (n = 2)	50	0	0	100	100	50	100	0	0	50	50	50	0
	Oct 2010 (n = 10)	70	10	50	90	50	0	30	70	60	50	100	70	30
Dry	Jan 2011 (n = 11)	18	27	27	100	9	0	9	90	9	9	73	18	0
	April 2011 (n = 5)	40	40	20	80	40	0	60	40	60	0	100	60	0

Ov. degen. = Ovarian degeneration, Hyperplasia = Oocyte hyperplasia, Atresia og. = Atresia of oogonia, Atresia pv. = atresia in previtellogenic stage, Atresia vit. = Atresia in vitellogenic stage, Foll. atrop. = Follicular atrophy, Foll. hyper. = Follicular hyperplasia, Vac. degen. = Vacuolate degeneration, Wbc inf. = Infiltration of white blood cell, Bv. dilation = Dilation of blood vessel, Bl. con. = Blood congestion, Ab rbc = Abnormal red blood cell
Highlight indicates the highest value

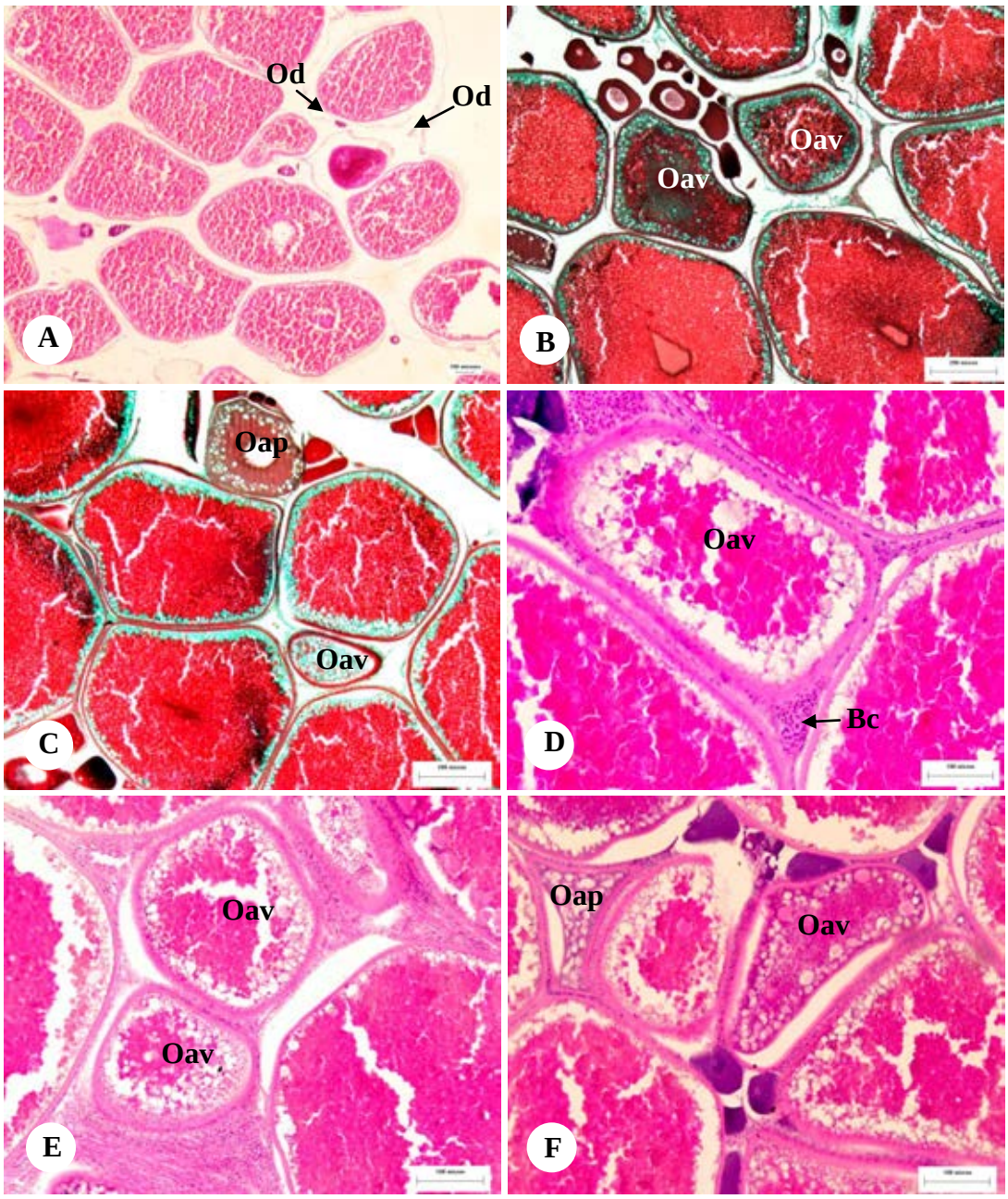

Figure 6.9 Micrograph of ovaries of *P. proctozysron* in July 2010

A Interstitial tissue at low magnification shows a disorganization of the ovary (Od) and loss of connective tissue. *(H&E stain). Scale bar = 100 µm.*

B-C Ovarian tissue shows atresia in previtllogenic stage (Oap) and vitellogenic stage (Oav). *(Masson's trichrome stain). Scale bar = 200 µm.*

D-F Ovarian tissue shows atretic oocyte at different stage of ovarian development. Blood congestions (Bc) are observed. *(H&E stain). Scale bar = 200 µm.*

(Oap = atresia in previtellogenic stage, Oav = atresia in vitellogenic stage)

Figure 6.10

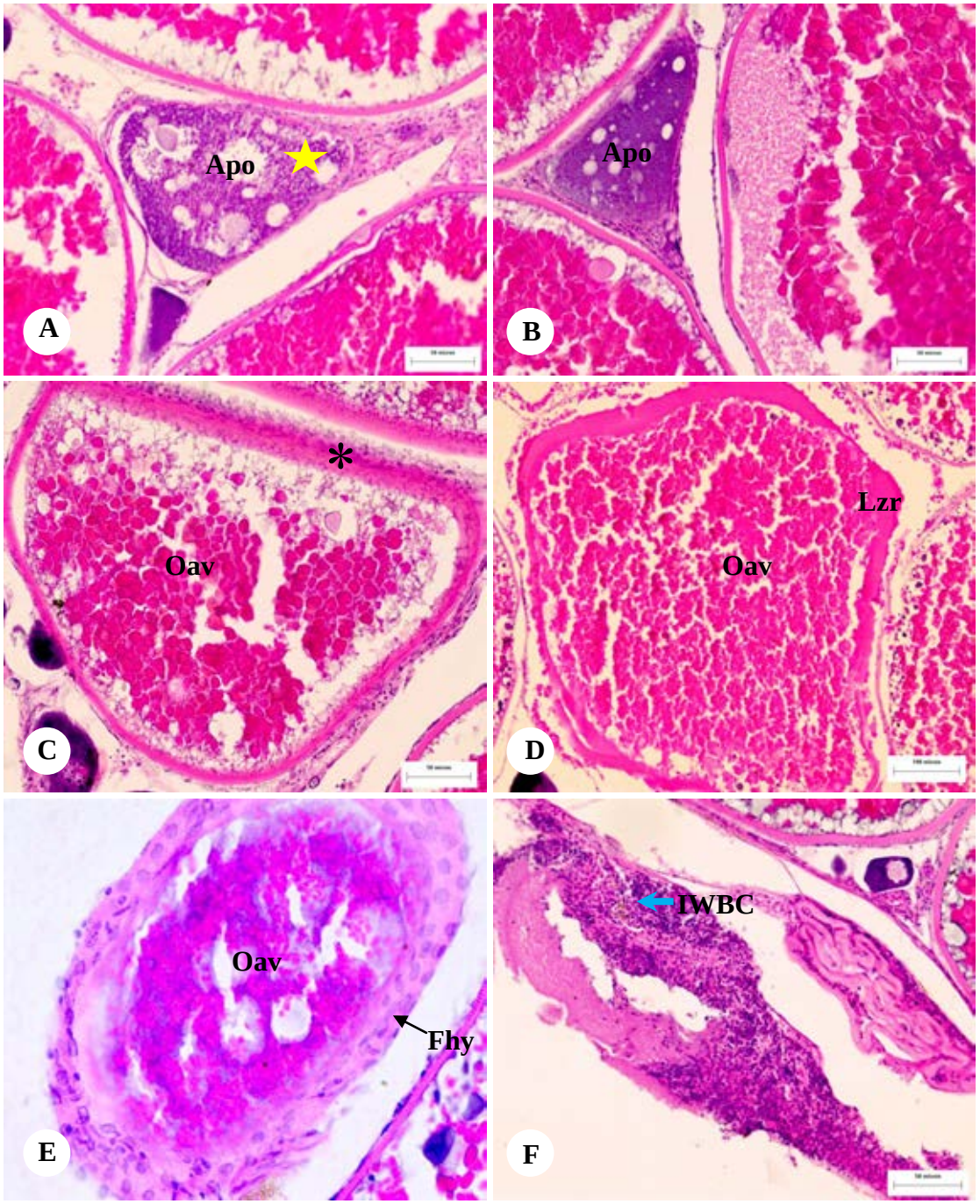

Figure 6.10 Micrograph of ovaries of *P. proctozysron* in July 2010 *(H&E stain)*

A-B Ovarian tissue shows higher magnification of atresia in previtellogenic stages (Apo) and necrosis (yellow star) of oocyte. *Scale bar = 50 µm.*

C-E Ovarian tissue shows higher magnification of atresia in vitellogenic stage (Oav), the major degeneration and resorption (asterisk), hyperplasia (Fhy) of the follicle cell layer and oocyte loss of zona radiata (Lzr)
Scale bar of C, E = 50 µm, D = 100 µm.

F Ovarian tissue shows infiltration of white blood cells (IWBC).
Scale bar = 50 µm.

Figure 6.11

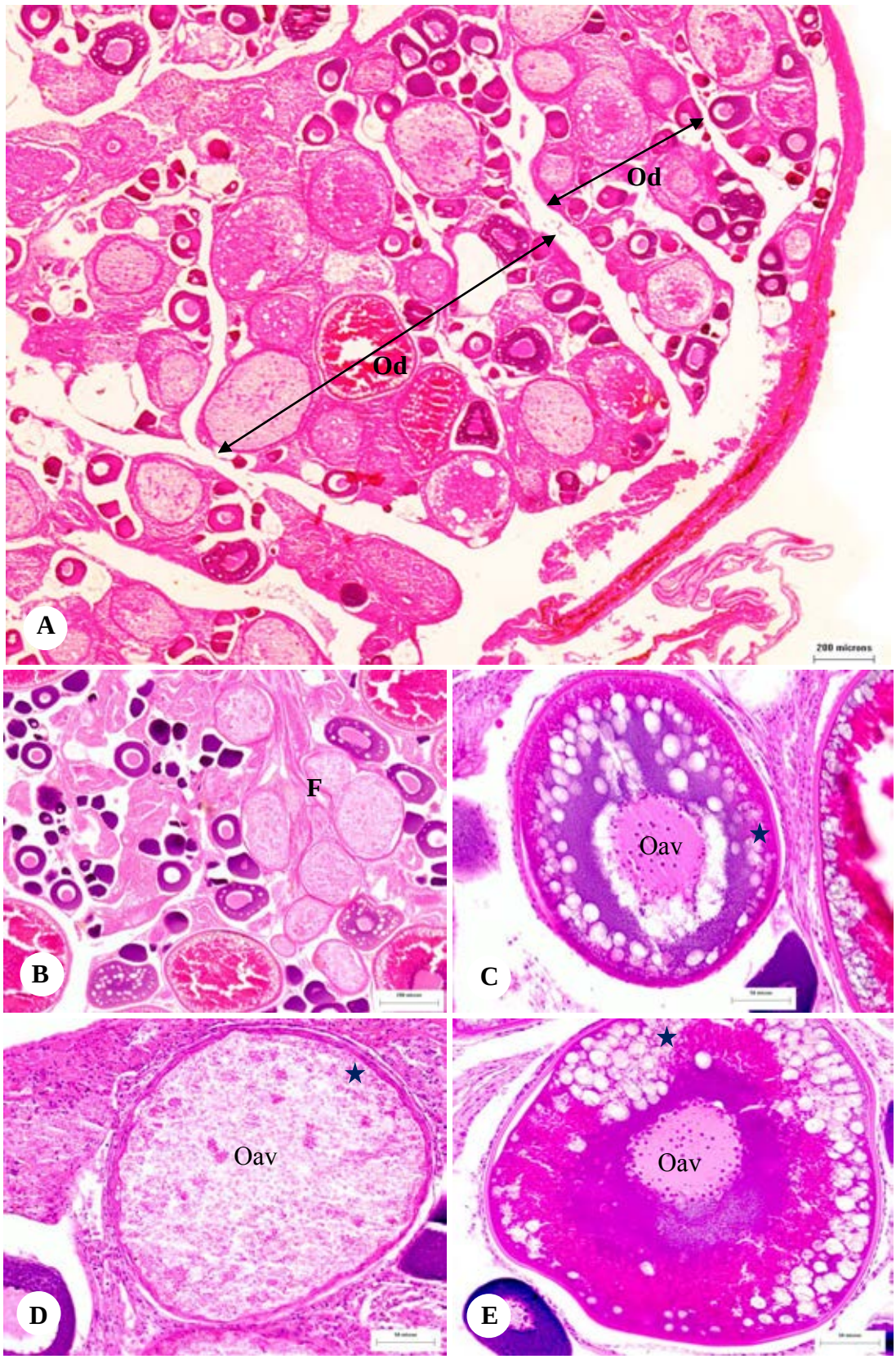

Figure 6.11 Micrograph of ovaries of *P. proctozysron* in October 2011 *(H&E stain)*

A Ovarian tissue shows ovarian disorganization (Od) consisted of atretic oocyte of various ovarian development stages. *Scale bar = 200 µm.*

B Ovarian tissue shows atretic oocyte at different ovarian development stages. Increased of connective tissue between oocytes is seen. *Scale bar = 200 µm. (Oap = atresia in previtellogenic stage, Oap = atresia in vitellogenic stage, F = Fibrosis)*

C-E Ovarian tissue shows higher magnification of atresia in vitellogenic stage (Oav) The major degeneration and resorption (asterisk) are showed. *Scale bar = 50 µm.*

Figure 6.12

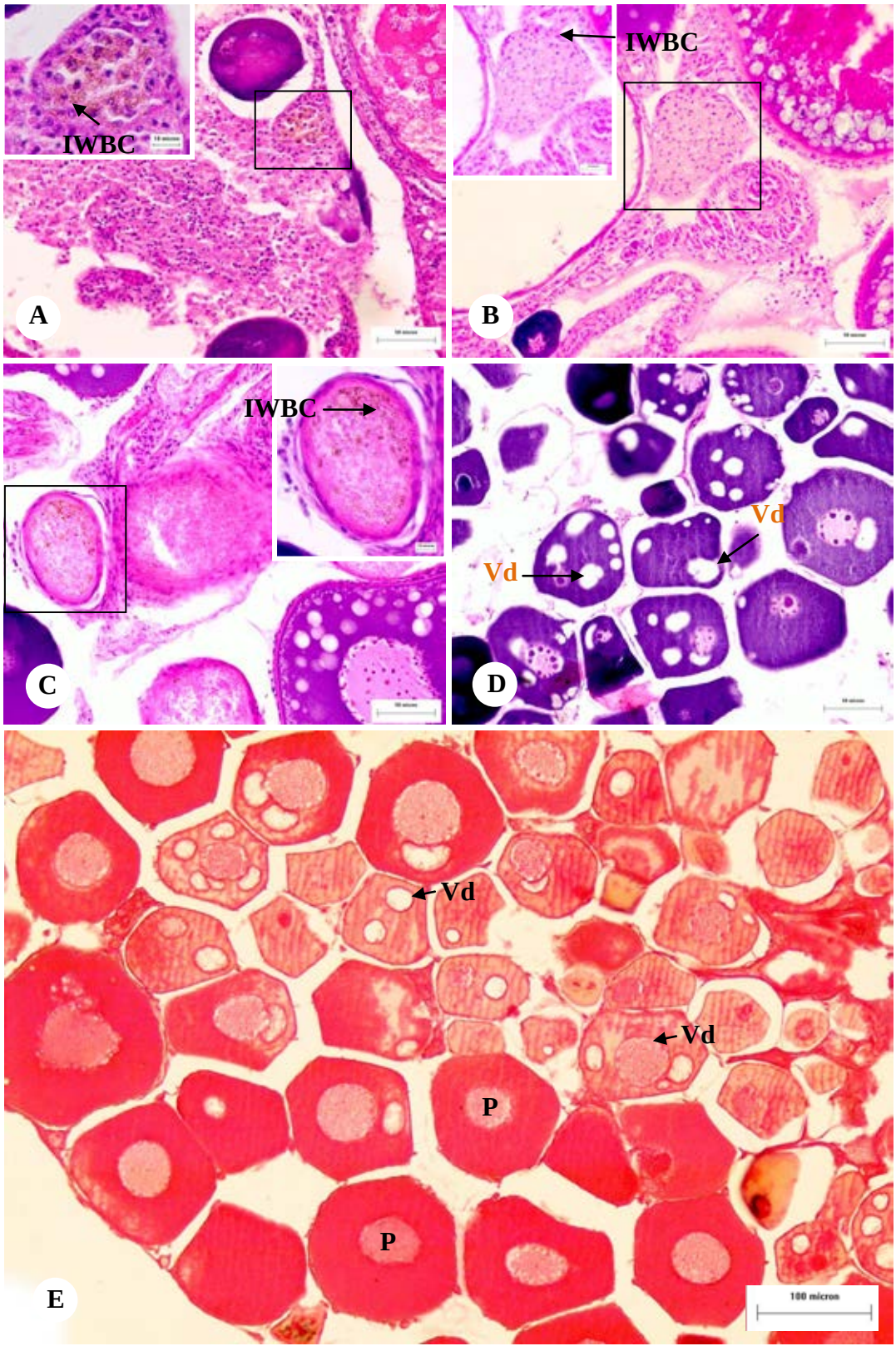

Figure 6.12 Micrograph of ovaries of *P. proctozysron* in October 2011

A-C Ovarian tissue shows infiltration of white blood cells (IWBC). *(H&E stain). Scale bar = 50 µm.*

B Ovarian tissue shows vacuolar degeneration (Vd) at perinucleolar stage oocyte of ovarian development. *(H&E stain). Scale bar = 50 µm.*

D Ovarian tissue shows vacuolar degeneration (Vd) at perinucleolar stage oocyte *(Masson's trichrome stain) Scale bar = 100 µm. (P = perinucleolar stage oocyte)*

Figure 6.13

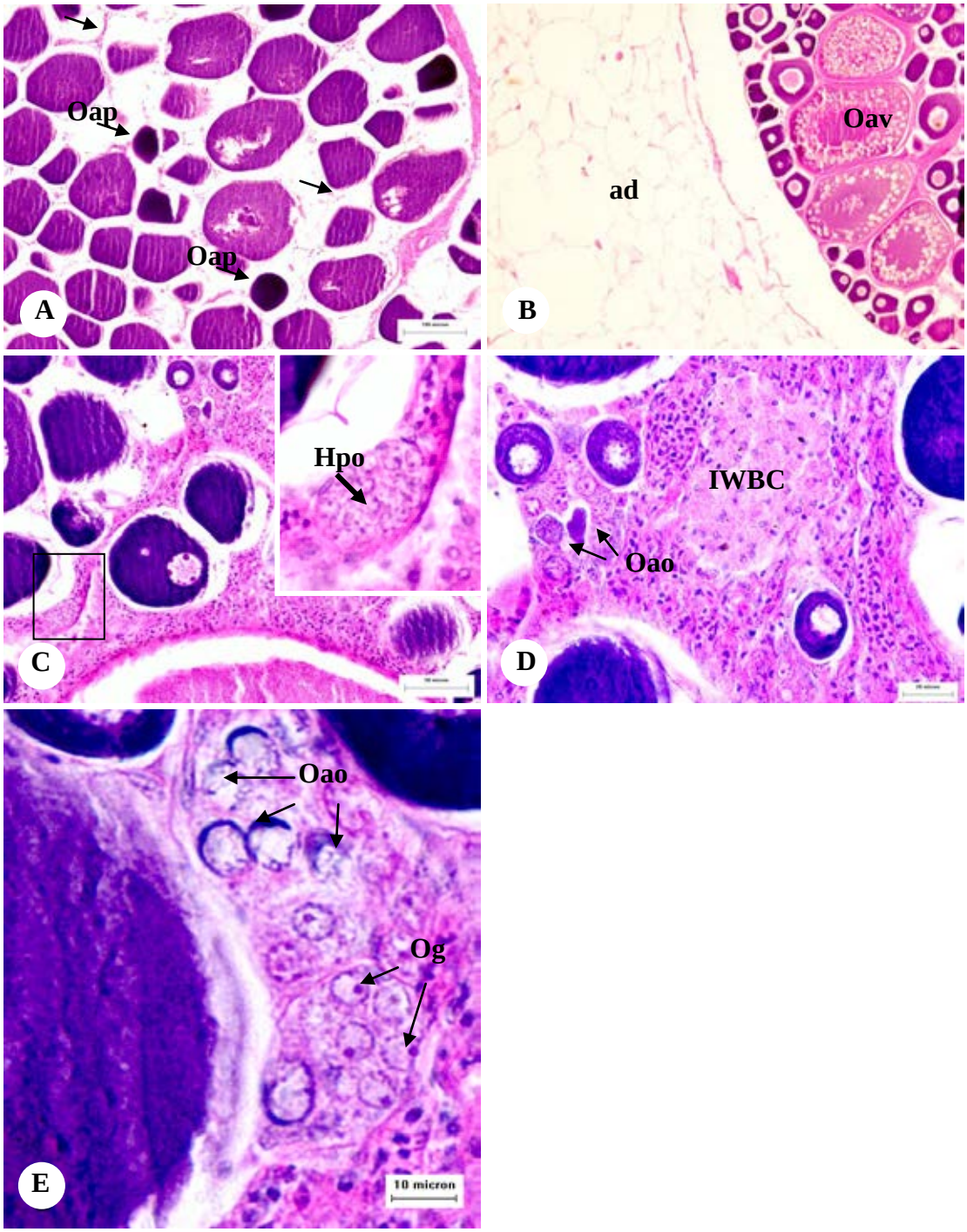

Figure 6.13 .Micrograph of ovaries of *P. proctozysron* in January 2011 *(H&E stain)*

A Ovarian tissue shows atresia in previtellogenic stage (Oap) and the loss of connective tissue between oocytes (arrow). *Scale bar = 100 µm.*

B Ovarian tissue shows atresia in vitellogenic stage (Oav). Scale bar = 100 µm. *(ad = adipose tissue)*

C Ovarian tissue shows hyperplasia of oogonia (Hpo). Scale bar = 50 µm.

D-E Ovarian tissue shows atresia in oogonia (Oao) and infiltration of white blood *cells (IWBC). Scale bar for C = 50 µm, D = 20 µm, E = 10 µm.* *(Og = oogonia).*

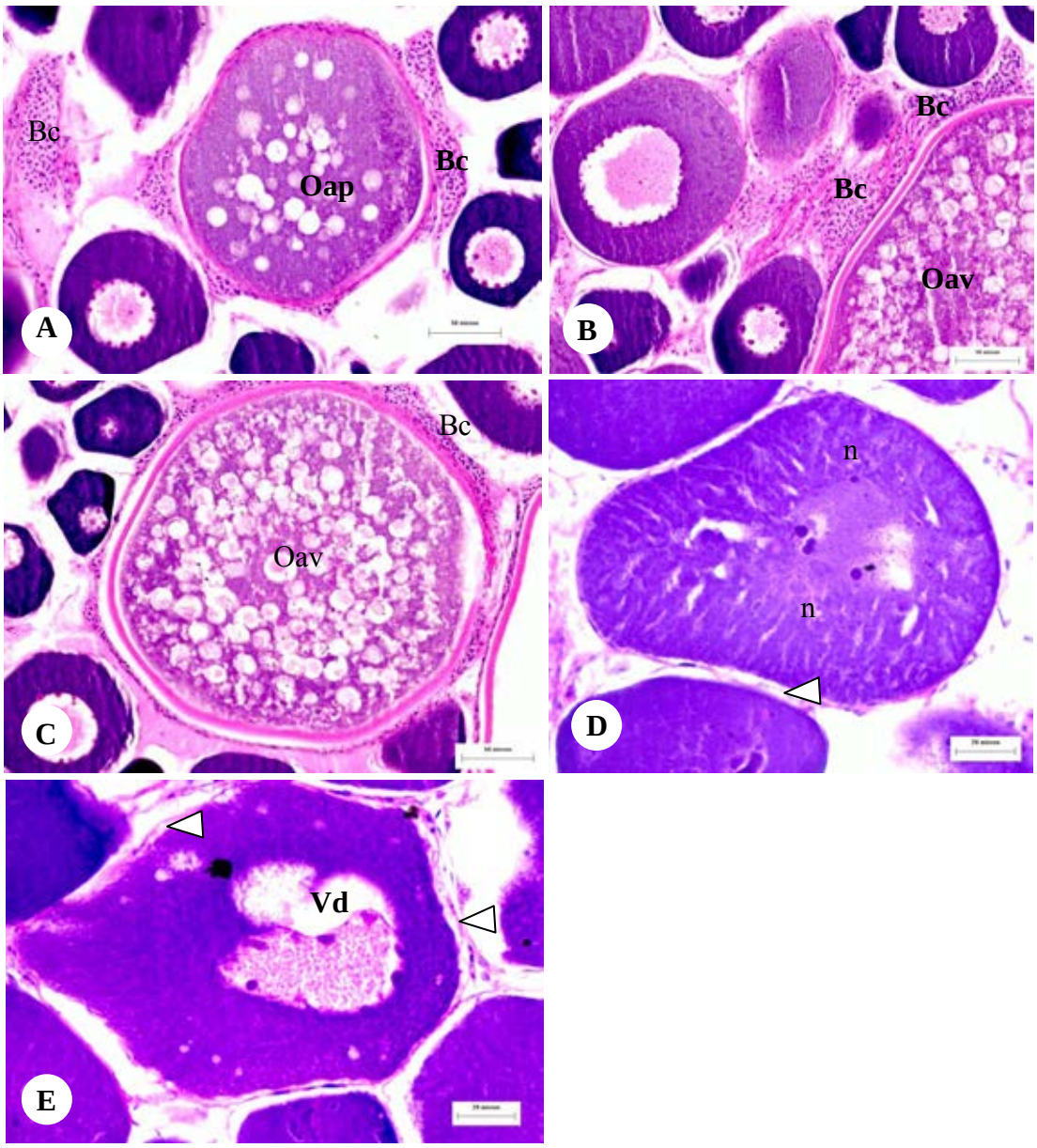

Figure 6.14 Micrograph of ovaries of *P. proctozysron* in January 2011 *(H&E stain)*

A-C Ovarian tissue shows an increasing of atretic follicle and blood congestion (Bc). *Scale bar = 50 µm. (Oap = atresia in previtellogenic stage, Oav = atresia in vitellogenic stage)*

D-E Ovarian tissue shows higher magnification of atresia in previtellogenic stage, breakdown and detachment of follicular layer (white triangle), necrosis (n) and vacuolar degeneration (Vd) within cytoplasm. *Scale bar = 20 µm.*

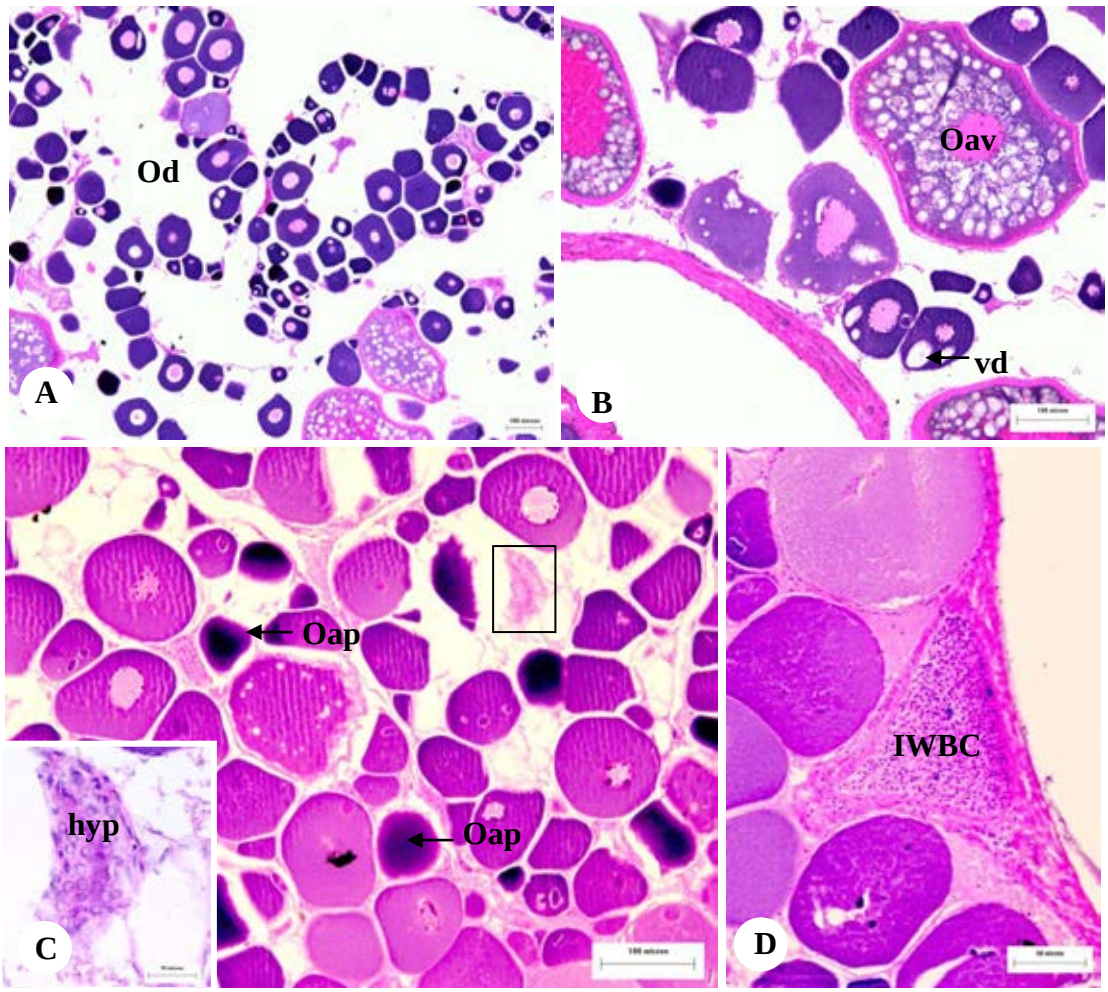

Figure 6.15 Micrograph of ovaries of *P. proctozysron* in April 2011 *(H&E stain)*

A-B Ovarian tissue shows ovary disorganization (Od), atresia and vacuolated degeneration (vd). *Scale bar = 200 µm. (Oav = atresia in vitellogenic stage)*

C Treated ovarian tissue shows oocyte hyperplasia in oogonia (hyp) and atresia in previtellogenic stage (Oap). *Scale bar = 100 µm.*

D Ovarian tissue shows macrophage aggregations and other leucocytes (IWBC). *Scale bar = 50 µm.*

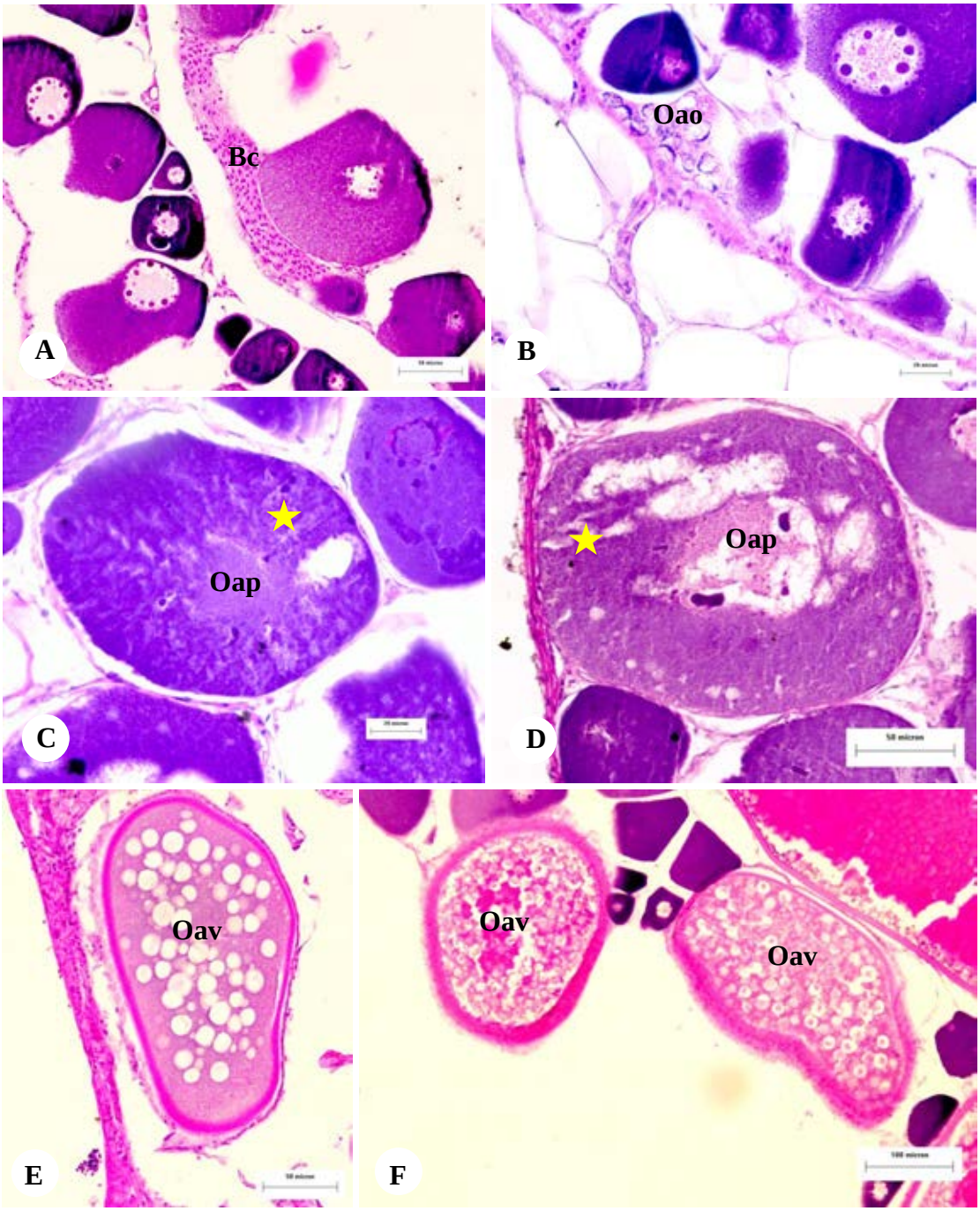

Figure 6.16 Micrograph of ovaries of *P. proctozysron* in April 2011 *(H&E stain)*

A Ovarian tissue shows an increasing of blood congestion (Bc) *Scale bar = 50 µm.*

B Ovarian tissue shows atresia in oogonia (Oao). *Scale bar = 20 µm.*

C-F Higher magnification of atresia in previtellogenic satge (Oap) and vitellogenic stages (Oav). *Scale bar =50 µm.*

6.3.6 Correlation between herbicide contamination and histopathological alterations in ovary of *P. proctozysron*

Herbicide concentrations and histopathological alteration in ovarian tissues were significantly correlated in both positive and negative correlations (sperman's correlation) (Table 6.2).

Atrazine concentration was positively correlated with atresia in previtellogenic stage and vacuolar degeneration and negatively correlated with follicular hyperplasia. Glyphosate concentration was positively correlated with atresia in oogonia and dilation of blood vessel and negatively correlated with oocyte hyperplasia.

Table 6.2 Spearman rank correlation between histopathology and herbicide concentrations in the ovary of female *P. proctozysron*

Histopathology	Spearman's correlation coefficient	
	Atrazine	Glyphosate
Ovarian degeneration	-0.500	0.500
Oocytes hyperplasia	-.0500	-1.000**
Atresia in oogonia	0.500	1.000**
Atresia in previtellogenic stage	1.000**	0.500
Atresia in vitellogenic stage	-0.500	0.500
Follicular atrophy	-	-
Follicular hyperplasia	-1.000**	-0.500
Vacuolar degeneration	1.000**	0.500
Infiltration of white blood cell	-0.866	0.000
Dilation of blood vessel	.0500	1.000**
Blood congestion	-0.866	0.000
Abnormal red blood cell	-0.500	0.500
Fibrosis	0.000	0.866

**indicates significant correlation at $p<0.05$

6.4 Discussion

The aims of this chapter were to investigate the basic histology, testicular development and histopathological changes in the ovary of *P. proctozysron* living in Nan River nearby agricultural areas and to evaluate its correlation with the level of herbicide contamination.

6.4.1 Basic histology and ovarian development

Ovary of *P. proctozysron* shows an asynchronous oocyte development type. The developing oocytes are distributed among the ovigerous fold. The result also showed seven oocyte developmental stages of female germ cells, including oogonia, chromatin nucleolar stage, perinucleolar stage, cortical alveolar stage, early vitellogenic stage, late vitellogenic stage and mature stage. This is accordance with another cyprinids fish, such as the common carp, *Cyprinus carpio* (Smith and Walker, 2004). Oocytes are surrounded by follicular cell layer that have been claimed to control the oogenesis by producing the sex steroid hormone in female fish (Dietrich and Krieger, 2009; U.S. EPA, 2006).

Based on the histological structure, the ovarian developmental stages of *P. proctozysron* are classified into five stages, including undevelopment, early development, mid development, late development and postovulatory stage. The late developmental stage (stage 3- late vitellogenic) was obviously seen in July 2010 with 100% observation. This finding coincides with the high GSI values (Chapter IV) suggested that the breeding season of *P. proctozysron* in Nan River probably occurs during the rainy season around July. This is similar to previous reported (Banyen, 1988). The author suggests that the breeding period of *P. proctozysron* occurs during the rainy season from June to July.

6.4.2 Histopathological alteration in the ovarian tissue of *P. proctozysron* living in herbicide contaminated river

Prominent histopathological alterations were seen in the ovaries of *P. proctozysron* caught from Nan River in this study. Overall, the degree of histopathological alteration in the ovary of the fish collected in rainy season was more severe than that collected in dry season. The histopathological alterations included ovarian degeneration, atresia in oogonia, atresia of previtellogenic stage, atresia in vitellogenic stage, follicular atrophy, follicular hyperplasia, infiltration of white blood

cells, dilation of blood vessel, blood congestion, abnormal red blood cells and fibrosis (Table 6.2). Some of general histological changes were similar to those found in the fish testicular tissue (and have been discussed before in Chapter V), including infiltration of white blood cells, dilation of blood vessel, blood congestion, abnormal of red blood cells and fibrosis. According to literature review, histopathological alterations in ovarian tissue of the female fish living in atrazine-contaminated environment have not been reported before. Only those from laboratory experiments are available (Bringolf et al., 2004, Du Preez and Van Vuren, 1992; OECD, 2009; Spanò et al., 2004; Tillitt et al., 2010).

The histopathological changes of the ovary in rainy season were more severe than in dry season. This might because the ovarian development in rainy season consisted of late-developmental stage that accumulated more lipids. This stage can accumulate more atrazine (and other xenobiotic chemicals that soluble in lipid) by its uptake efficiency that related to its partitioning (Spacie et al., 1995; Knusli, 1974). Du Preez and Van Vuren (1992) reported that lipid-rich tissues were accumulated lipophilic xenobiotic contaminants greater than those in low-lipid tissues. Therefore, high prevalence of the histopathological alteration found in the ovary of *P. proctozysron* caught in rainy season may indicate a susceptibility to be affected by atrazine.

Atresia is a process of degeneration and resorption of ovarian follicles from the ovary (Jamieson, 2009). Atresia is characterized by the disintegration of the nucleus, vitelline envelope breakdown and increase in number and size of follicular (granulosa) cells, liquefaction of yolk globules with follicular cells entering the oocyte to phagocytize degenerating material and degeneration of the follicular cells once yolk resorption is complete and eventually fibroblast-like cells around yellowish-brown material (lipofuscin/ceroid) remain (Blazer, 2002). Although, atresia is a normal physiological event in all fish, it became a pathological condition noted in the fish after exposure to certain environmental contaminants (Blazer, 2002; Cross and Hose, 1988; Johnson et al., 1988; Kirubagaran and Joy, 1988). Atretic oocyte may become the atretic stage at any stage of oogenesis, but more frequently in vitellogenic stage, especailly in previtellogenic follicles (Blazer, 2002; Johnson et al., 1988). In this study, the increase of atresia was found in the ovaries of *P. proctozysron* living in

atrazine-contaminatied environment, particularly in perivitellogtenic stage. This finding is similar to those in previous studies. Spanò et al. (2004), reported the ovarian degeneration and increasing of atresia in the ovary of the goldfish, *Carassius auratus* after the exposure to 100 and 1000 g/L of atrazine. Tillitt et al. (2010) did experiments in the adult fathead minnow, *Pimephales promelas*. The results showed several alterations including in the perinucleolar and vitellogenic oocytes, increased frequency lipid found in ovaries and atretic follicles after the fish were exposed to 5 μg/L and 50 μg/L of atrazine. In present study, atresia was found in several developmental stages of oocytes, including oogonia, previtellogenic and vitellogenic stages. Atresia of preivitellogenic stage showed relatively high prevalence in all sampling months (80-100% prevalences) and was significantly correlated with levels of atrazine concentration in ovarian tissue.

Damaged oocytes and atresia in the fish ovary may cause by a disorder of their controlling hormones. Only little information of the endocrine and other physiological mechanisms that control atresia in teleost ovarian follicle are available (Grier et al., 2009). FSH/GTH-I and LH/GTH-II are glycoprotein hormone in female. They are generated in hypothamus and pituitary gland and play an important role in oocytes development. FSH controls the oogenesis regulation (Campell et al., 2006), while LH is responsible in ovulation (Patińo and Sullivan, 2002; Patińo et al., 2003). Therefore, it is possible that atresia in various stage of oogenecis, follicular atrophy and follicular hyperplasia observed in this study may correlated to the high level of atrazine contamination in Nan River by disruption of oogenesis regulating hormones of the fish. However, the effect of atrazine to the oogenesis regulation hormones in fish never been studied yet. In rats, the death of follicular cells that surrounding the oocytye because of insufficiency numbers of FSH receptor or FSH have been linked to the existence of atresia (Johnson and Everitt, 1995). The reduction of oocyte number may correlate with the low level of FSH, while the increase of atretic follicle may reflect the insufficiency of LH to complete the ovulation. Cooper et al. (2000) studied the Long-Evan rats that ovary was ovariectomized and LH was induced by estrogen treatment. After treated with 300 mg/kg of atrazine, the reduction of LH level in the rat was observed. So, any chemicals (included atrazine) that have an effect to LH surge may have an indirectly effect by interruption of the hormone signal that

regulate the mechanism and function of the ovary and resulted in the abnormality of oocytes. (Baligar and Kaliwal, 2002; Glodman et al., 1994).

In addition, the vacuolar degeneration was occurred associated with the increase of atrazine concentration level. This alteration has been reported in the walking catfish, *Clarias batrachus* exposed to 2,4-D and butachlor (Ateeq et al., 2006). The vacuolar degeneration observed in this study was frequently found in the early stage of germ cell development together with the atresia. Therefore, it could be said that the ovary at early development stage may susceptible to be affected by atrazine. This is consistent with the previous suggestion. Blazer (2002) speculated that the increasing of atresia of oocytes particular in previtellogenic follicles can indicate a pathological condition that associated with exposure to environmental contaminants.

Atrazine is the endocrine disrupting chemical (EDC), which directly affects on the reproductive system, particularly in the ovary. Damage of ovarian tissue caused by EDC exposures has been frequently reported in many fish species, including inhibition of oogenesis, degeneration of oocyte and necrosis of oocytes, increasing in atretic oocytes and follicular cell degeneration (Diniz et al., 2005; Kinnberg and Toft, 2003; Miles- Richardson et al., 1999).

Statistical correlations between atrazine concentration in ovarian tissue and histopathology were occurred in both positive and negative correlations. The atrazine concentration was positively correlated with atresia in perivitellogenic and vacuolar degeneration, while it was negatively correlated with follicular hyperplasia. The effects of atrazine to the ovary of the fish have been proven from laboratory experiments, as discussed above. The results from this study suggest that the higher up of atrazine concentration might correlate with some histopathological changes in ovarian tissue of *P. proctozysron* caught from Nan River, especially a distinctive high prevalence of the atresia in previtellogenic stage. However, this hypothesis was generated based on the comparison of histopathology observed in this study with those from laboratory experimental reports only. To confirm the hypothesis, laboratory exposure study of atrazine in *P. proctozysron* should be conducted.

Glyphosate was correlated with some histolpathological alterations in the ovary, such as ovarian degeneration, oocyte hyperplasia and atresia in oogonia. However, no reports have been suggested the possible effects of glyphosate on the

ovary of fish living in glyphosate contamination or exposed to glyphosate before. So that, this is the first study presented the possible correlation of glyphosate contamination and the fish ovarian alterations, especially in early developmental stage, even thought the concentration of glyphosate detected here was very low.

6.5 Conclusion

Several histopathological alterations in ovarian tissue of *P. proctozysron* in this study have been correlated with atrazine concentration in the environment and its tissues, similar to those in testicular tissue in Chapter V. The results in this chapter also support the potential of using *P. proctozysron* as a sentinel organism in aquatic pollution-monitoring. Furthermore, atrazine concentration in ovarian tissue was positively correlated with atresia in perivitellogenic and vacuolar degeneration of ovarian tissue. It may state that the early development of the ovary may more susceptibility to affect by atrazine than in vitellogenic stage.

CHAPTER VII

GENERAL CONCLUSIONS

Nan River is one of the most important rivers in Thailand. Its tributaries are used for irrigation and agriculture, such as rice field and vegetable farms. Agricultural areas located nearby the tributaries may directly discharge agricultural effluents into the river. Aquatic habitats nearby agricultural areas are thus susceptible to contamination by the herbicide runoff and aquatic animals are also at risk. In this study, the health of the fish *P. proctozysron* living in Nan River was assessed using a set of biomarkers, including biomarker of exposure (herbicide residues in the fish gonads) and biomarkers of effect (condition factor, CF; gonadosomatic index, GSI; and histopathology). This fish species was used as a sentinel species to obtain critical information for ecotoxicological assessment of herbicide usage in this area. Therefore, in this study, multiple biomarker responses in *P. proctozysron* and their correlation with herbicide residue in the environment and gonadal tissue were evaluated.

Atrazine concentrations were found in water and sediment samples in dry season higher than that of rainy season. In sediment, the highest level of atrazine was observed in January 2011 at 0.24 mg/kg and the lowest in July 2010 at 0.01 mg/kg. Atrazine residue was detected at ND-0.15 mg/L from water sample collected in this study. Herbicide residue analysis in fish tissue is one way to monitor the herbicide pollution in aquatic ecosystem. Due to bioaccumulation and less degradable nature of herbicide, these residues are found more easily in fish tissue than in water or sediment. In fish gonadal tissues, atrazine and glyphosate were also detected in both sexes. The concentrations in the ovarian tissues were higher than those in the testicular tissues. However, these herbicide concentrations were considerably lower than those reported in other fish species. Overall, accumulations of atrazine were found in this study in sequential order from the environment to an organismal level. Although, the correlation between herbicide concentration in gonadal tissue and the environment were not correlated, this is the first report of herbicide contamination in fish gonadal tissue in Nan River.

The accumulation of herbicide in gonadal tissue could be associated with various adverse effects on fish. The measurement indices, such as CF and GSI can be used to reflect the impact of an environment to organism (Goede and Barton 1990; McDonald et al., 2000). The results of CF and GSI of fish living in Nan River showed both increasing and decreasing trends varying upon season. However, the link between herbicide contamination to CF and GSI of *P. proctozysron* did not clearly exhibited. These trends were alternatively explained by seasonal variation, rather than effect of herbicide contamination alone. So, CF and GSI are not recommended to use as biomarkers alone, but should be used in supportive with other biomarkers of effects.

On the contrary, the result from histopathological study revealed a strong correlation between histopathological alterations and atrazine concentrations in *P. proctozysron* gonadal tissues. Major correlated alterations are testicular atrophy and degeneration of Leydig cells in male fish and atresia in previtellogenic oocytes, vacuolar degeneration and follicular hyperplasia in female fish. It can be concluded that the atrazine contamination in Nan River has involved with several histopathological alterations in the gonadal tissues of *P. proctozysron.* Furthermore, the results also suggested that Leydig cells in the males and previtellogenic oocytes in the females are the target cell of the contaminant effect.

In summary, the use of biomarkers of effect such as CF and GSI, did not clearly exhibit the effects of herbicide contamination. In contrast, histolopathological alterations in gonadal tissue are a good biomarker for the evaluation of the effects of herbicide contamination in fish. However, the threshold concentration of herbicide in fish should be confirmed in laboratory experiment to see the exact changing of gonadal tissue due to the herbicide effect. The results from the present study indicated that the water in Nan River is under concern of herbicide contamination. Thus, the water is suitable for general uses only and is not recommended for drinking unless suitable water treatments are performed. Although, the herbicide concentrations in fish tissues were relatively low, it may be possible to be transferred through food chain to human who consume the fish. Such problem should be minimized as soon as possible by reducing the use of herbicides in agricultural activities or using them with a well management. Finally, the results presented here may show only a minor

problem affecting on aquatic organisms such as fish. However, if the herbicides usages in this area are continued without any action plan, this may leads to major environmental problem in the future.

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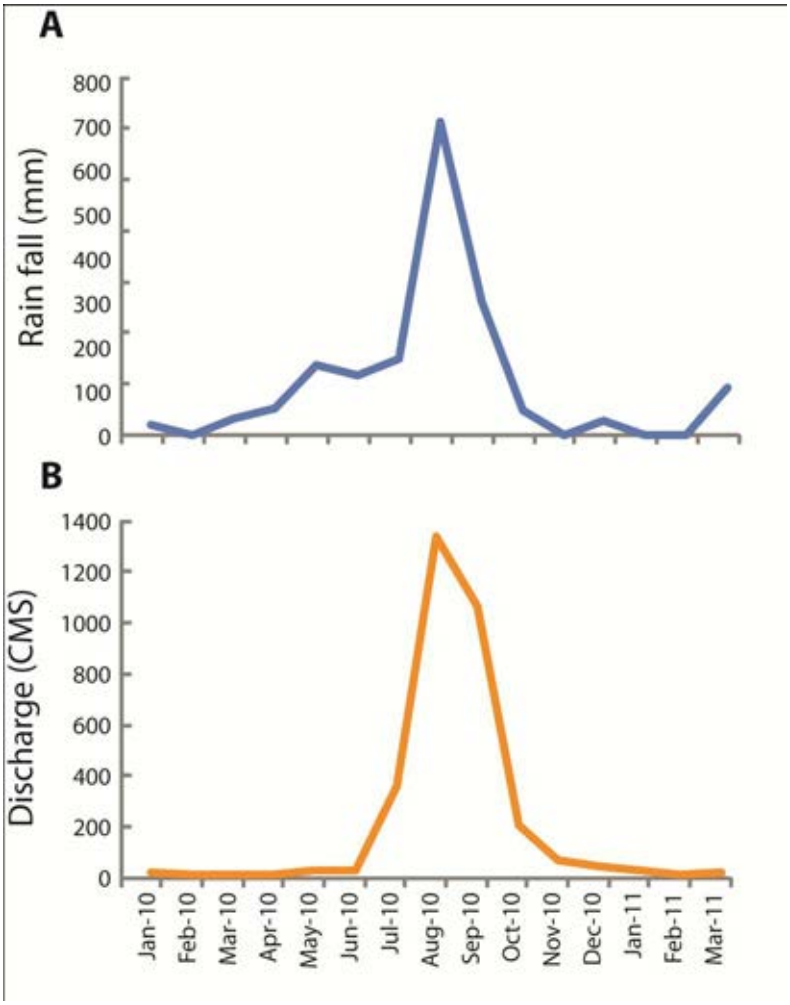
APPENDICES

Appendix A

Rainfall and river discharge in Nan province

Rainfall and river discharge in Nan province during January 2010 to March 2011. Average rainfall in millimeter in Nan Province (A) and discharge of Nan River in cubic meter per second, measured at Ban Bun Nak, San subdistrict, Wiang Sa District, Nan Province (B). Source: Hydrology and water management center for upper northern region, Chiang Mai, Office of Hydrology and water management royal irrigation department Thailand. Available from: http://www.hydro-1.com/index.php [2012, 21 April]



Appendix B

The process of histological study (Humason, 1979)

Embedding procedure

Fix tissue for 24 hours

70% Ethanol in glycerol (infinity)

90% Ethanol (2-6 hours)

95% Ethanol (2-6 hours)

N-butanol (1 hour)

Xylene (1 hour)

Process in the oven

(Temperature 63-65 °C)

Xylene + Wax (1/2 hour)

Wax I (1/2 hour)

Wax II (1/2-1 hour)

Embed with Wax III

BIOGRAPHY

Mr. Sinlapachai Senarat was born December 18, 1985 in Nakhon Sri Thammarat Province. He received a Bachelor of Science degree in Biology from the Faculty of Science, Prince of Songkla University in 2007. Later, he continued his study in Zoology program for Master's degree at Department of Biology, Faculty of Science, Chulalongkorn University in 2008 and he completed the program in 2011.

Proceeding

Sinlapachai Senarat, Noppadon Kitana, Puttaruksa Varanusupakul, Jirarach Kitana. 2011. A histological study of the gonads of Smith's barb *Puntioplites proctozysron* (TELEOSTEI: CYPRINIDAE). 37th Congress on Science and Technology of Thailand.

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Orasa Achayapunwanich, **Sinlapachai Senarat**, Noppadon Kitana, Puttaruksa Varanusupakul and Jirarach Kitana. Health Assessment based on Liver of a Cyprinid Fish *Puntioplites proctozysron* living in a river with potential atrazine contamination in northern part of Thailand. 2011 Abstract, the 5th Internataional Congress of Chemistry and Environment (ICCE), (27-29 May 2011), Port Dickson, Malaysia.