

**Reproductive Biology and
Maturation Control of Brook Trout
(*Salvelinus fontinalis*, Mitchill) in Tasmania**

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**Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy**

**National Centre for Marine Conservation &
Resource Sustainability
Australian Maritime College
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Declaration

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Bio safety Committees of the University.

Shafaq Fatima

Statement of Co-authorship

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Dedicated

To

My Lord

Allah the Almighty

&

My Family

Abstract

Pilot scale commercial production of brook trout (*Salvelinus fontinalis*) in Tasmania encountered a high incidence of early sexual maturation particularly in males. Attempts by commercial growers to produce all-female populations (by neomale–female crosses) using standard industry techniques were unsuccessful. As maturation has a deleterious effect upon flesh quality, growth and immunocompetency, commercial production was aborted until this issue could be resolved. This study firstly aimed to describe early gonadal development, sex differentiation, developmental and endocrine changes during puberty and throughout the annual reproductive cycle to establish baseline information pertinent to the species reproductive strategies under Tasmanian environmental conditions. Once determined, the results from that study facilitated an experiment investigating the most appropriate timing and approach for the production of neomales. Photoperiod manipulation in mixed populations of brook trout was likewise investigated as a further means of reducing the incidence of early maturation.

Sex differentiation and gonadal development in brook trout was studied from 8 degree days post-hatch (°dph) until the age of 27 months. Gonadal development was histologically studied while plasma profiles of testosterone, estradiol-17 β and 11-KT were measured by RIA and ELISA. Gonadal development began during pre-hatch period and undifferentiated gonads directly developed into testes and ovaries at 3354 °dph. Males had attained puberty by the age of 14 months. However, females did not achieve maturation during their first year. During the second year, maturation was observed during March and May for males and females respectively. This study determined that pre-hatch period might be the appropriate time for sex inversion treatment as development of undifferentiated gonads commenced during this time period. Moreover, variations in gonadal development and profiles of sex steroids were

controlled by seasonal changes in photoperiod during second year thus suggesting the possible inhibition of maturation under manipulated photoperiod conditions.

Neomale production was undertaken by immersion and in-feed treatment (and a combination of both) with 17 α -methyltestosterone (MT). Sex inversion treatment targeted the pre-hatch, hatch and post-hatch period to determine the most sensitive window of time. Treatment given during 4 to 6 days pre-hatch (at 400 μ g/L MT for 4 hours) was found to be the most successful producing a population containing 75% of male fish without the occurrence of any sterile fish. Feeding fish a MT supplemented diet (3 mg/kg) for 60 days from first feeding did not affect the normal sex ratio and MT oral treatment combined with immersion treatments resulted in high percentages of sterile fish. In the present study, 4 to 6 days pre-hatch was found to be the most sensitive window of time to produce the highest male population with only two immersions thus significantly reducing the dose of MT and treatment duration.

Three photoperiod regimes over a 10 month period during the second reproductive year were tested. The regimes were simulated natural photoperiod (NP), advanced photoperiod accelerated by 8 weeks (AP) and continuous illumination (CP). Fish exposed to advanced photoperiod corrected their maturation cycle by an advanced phase shift of their endogenous rhythm. Advanced photoperiod inhibited maturation by 6% and 8% in males and females, respectively, relative to natural photoperiod treated fish in which 100% maturation occurred. However, most of the fish successfully recruited for maturation presumably because the threshold of growth during the “critical phase” of photoperiod treatment was surpassed. Similarly, treatment of continuous photoperiod failed to inhibit the onset of maturation however continuous photoperiod did inhibit the final stage of maturation.

Overall, the present study provided the baseline data about gonadal differentiation, puberty and annual reproductive cycle of brook trout under Tasmanian climate conditions, reported for first time for the southern hemisphere. Furthermore, this PhD project provided the most efficient and commercially applicable sex inversion protocol to masculinize genetic brook

trout females, the most critical step of indirect feminization. Progeny testing by crossing these neomales and normal females can be conducted as a future study to produce all-female population. Furthermore, this protocol may produce 100% male population if applied to gynogenetic females instead of mixed sex culture which needs to be investigated. Moreover, this study suggested the possible success of photoperiod manipulation to control maturation in brook trout but further refinement of photoperiod regimes is required before its commercial trial.

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
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
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
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


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


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


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







treatments. Stage I:  , Stage II:  , Stage III:  Stage IV:  , Stage V:  , Stage VI:  . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment

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APPENDIX I




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
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

Fig. II.2 Monthly variations (Mean \pm SEM) in total body weight (A), fork length (B) and condition factor (C) during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Female:  , Male:  . n = 47 males; 47 females. 246


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List of Abbreviations

AP	Advanced Photoperiod
BPG	Brain-Pituitary-Gonad
CNO	Cortical Nucleolar Oocyte
CP	Continuous Photoperiod
DHEA	Dehydroepiandrosterone
DHP	17 α , 20 β -Dihydroxyprogesterone
dph	Degree Post-hatch
dph	Days Post-hatch
E ₂	Estradiol-17 β
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Early Vitellogenic Oocyte
EVE	Early Vesicular Oocyte
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin Releasing Hormone
GSI	Gonadosomatic Index
GTH	Gonadotrophic Hormone
GVBD	Germinal Vesicle Breakdown
GVM	Germinal Vesicle Migration
HSI	Hepatosomatic Index
LD	Light and Darkness
LH	Leutenizing Hormone
LV	Late Vitellogenic Oocyte
LVE	Late Vesicular Oocyte
MC	Mature Cell
MDHT	17 α -Methyldihydrotestosterone
MIH	Maturation Inducing Hormone
MT	17 α -Methyltestosterone
MV	Mid Vitellogenic Oocyte
NAD	Nicotinamide dinucleotide
NADP	Nicotinamide Adenine dinucleotide Phosphate
NP	Natural Photoperiod
°d	Degree Days
°dpf	Degree Days Post-fertilization
°dph	Degree Days Post-hatch
OO	Ovulated Oocyte
PBPG	Pituitary-Brain-Pineal-Gonad
PGC	Primordial Gonadal Cell
PNO	Peripheral Nucleolar Oocyte
POC	Primary Oocyte
POF	Post Ovulatory Follicle
PVC	Poly Vinyl Chloride

SG	Spermatogonium
SP	Sperm
SSC	Secondary Spermatocyte
ST	Spermatid
StAR	Steroidogenic Actue Regulatory Protein
T	Testosterone
ZR	Zona Radiata
11-KT	11-Ketotestosterone
17 β -HSD	17 β -Hydroxysteroiddehydrogenase
3 β -HSD	3 β -Hydroxysteroiddehydrogenase

Thesis Format

This thesis has been prepared in manuscript form with the exception of chapter 1 (General introduction) and chapter 6 (General discussion) so that each individual chapter could be considered separately for publication. Due to this format, some information may be repetitive.

Chapter 2 is published and its format may slightly vary due to the requirements of journal.

Fatima, S., Adams, M. and Wilkinson, R., 2012. Histological study of gonadal development and sex differentiation in *Salvelinus fontinalis* under Tasmanian climate conditions. Australian Journal of Zoology 59 (5), 321-331.

CHAPTER 1

General Introduction

1.1 Commercial Importance of Brook Trout in Tasmanian Aquaculture

The Tasmanian Aquaculture industry is a major producer of salmonids in Australia contributing over 97% of total Australian salmonid production (ABARES, 2010). Most of the Tasmanian salmonid production consists of rainbow trout (*Onchorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) farming. To ensure ongoing sustainability and commercial diversity, Tasmanian farmers are also looking to alternative species. Among such potential alternatives under review is brook trout (*Salvelinus fontinalis*). Brook trout is native to North America and was introduced to Tasmania from Nova Scotia, Canada in 1962 (MacCrimm and Campbell, 1969; Clements, 1988).

The progeny of these introduced fish were artificially propagated at salmon farms in Tasmania and several waters in the state have been stocked with wild and domestic populations maintained for recreational fishing and supply of brood stock (MacCrimm and Campbell, 1969; S. Chilcott, Inland Fisheries Service, Pers. Comm.). In addition to their commercial purpose, brook trout is emerging as a potential species for fisheries research and development. Work on inter-generic and intra-generic crossbreeding of brook trout has reported better growth and resistance to disease in hybrids and triploids of the genus *Salvelinus* (Gillet et al., 2001). Moreover, good growth, meat quality and food conversion efficiency of brook trout (Rasmussen and Ostensfeld, 2010; M. Amin, NCMCRS, Pers. Comm.) indicate the potential importance of this species in the context of commercial farming.

The major constraint to commercial brook trout culture in Tasmania is the high incidence of early sexual maturation. Precocious maturation negatively affects the growth, flesh quality and immunocompetency of the individual (Galbreath et al., 2003; Haffray et al., 2009). Therefore control of early maturation is a very important step for brook trout Aquaculture in Tasmania and is required to strengthen its status as an alternative species for rainbow trout and Atlantic salmon farming. Two major techniques applied in Aquaculture to control the problem of precocious maturation/maturation are as follows:

1. Production of all-female fish culture
2. Inhibition of maturation by manipulating photoperiod

In order to apply these techniques successfully, a comprehensive background study of early gonadal development, sex differentiation, developmental and endocrine changes during puberty and the annual reproductive cycle is required. This information must be obtained under environmental conditions specific to the growing region with factors such as water temperature and photoperiod playing a major role in influencing the timing of these important developmental events.

1.2 Gonadal Development and Sex Differentiation

Sex determination and gonadal differentiation are crucial aspects of reproductive biology of any species. Sex determination is the genetic or environmental process by which the sex of an individual is established in a simple binary fate decision (Penman and Piferrer, 2008). Initially, Lee and Wright (1981) and Hartley (1987) reported the absence of sex chromosomes in brook trout however Phillips et al. (2002) and Timusk et al. (2011) later respectively identified the sex determining locus and sex linkage groups. Galbreath et al. (2003) also reported the production of all females after crossing sex reversed gynogenetic males with normal females indicating that female homogametic sex determination system

may be present in brook trout. Elaborate and advanced studies are still required to confirm the presence of XX-XY sex determination system in this species.

Sex or gonadal differentiation is the process by which various molecular, genetic and physiological mechanisms produce a male or female from a zygote of a given genotype and parents in a given environment (Bull, 1983). In brook trout, undifferentiated gonads start developing during the pre-hatch period while sex differentiation appears to occur after hatch (Sacobie and Benfey, 2005; Haffray et al., 2009). The hatching time for brook trout has been reported at 523 degree days (°d) (Bascinar et al., 2003), 495 °d (Sacobie and Benfey, 2005) and 420 °d (Haffray et al., 2009). The difference in time of hatch reported in these studies is due to differences in incubation conditions, in particular water temperature (Marten, 1992; Baird et al., 2002).

Similar to time of hatch, time for differentiation of sex varies in different studies on brook trout (Sacobie and Benfey, 2005; Fatima et al., 2012) however the pre-hatch and early hatch period have been reported as the sensitive time period regarding the “decision” about future sex in brook trout. Moreover, activation of genes regulating the activity of aromatase and sertoli cells has been reported before the appearance of morphologically distinguishable characteristics of sex (Cavileer et al., 2009; von Schalburg et al., 2011). This shows that sex is determined before its cellular differentiation and gonads can be genetically identified as presumptive testis or ovary. Initiation of sex differentiation in undifferentiated gonads is histologically identified by migration and exponential proliferation of germ and stromal cells (Billard, 1992; Nakamura et al., 1998; Sacobie and Benfey, 2005). However, Piferrer and Guiguen (2008) and Sandra and Norma (2010) used gene markers of *cyp19a* and *dmrt1* as molecular indicators of sex differentiation instead of histological parameters.

The type of sex differentiation reported in brook trout was one of “direct differentiation” (Sacobie and Benfey, 2005) as undifferentiated gonads develop directly into testes and

ovaries (Yamamoto, 1969). In gonochorist salmonids including brook trout, ovaries differentiate earlier than testes (Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002; Arezo et al., 2007). The first sign of ovarian differentiation is the appearance of pre-meiotic oogonia, followed by occurrence of meiotic oocytes and formation of ovarian lamellae (Lebrun et al., 1982; Dlugosz and Demska Zakes, 1989; Billard, 1992; Chiasson and Benfey, 2008). Testes at this stage are quiescent and contain primordial germ cells and complex cavities (vena comittis) which subsequently disappear by proliferative activity of germ cells (Sacobie and Benfey, 2005; Chiasson and Benfey, 2007; Fatima et al., 2012).

There can be variation in the timing of the occurrence of these developmental changes in different populations of the same species due to different environmental conditions, climate and geography (Nakamura *et al.* 1998; Devlin and Nagahama 2002; Strussmann and Nakamura 2002; Penman and Piferrer 2008; Sandra and Norma 2010). After reviewing an extensive literature about sex differentiation in salmonids, only one study (Sacobie and Benfey, 2005) was found describing all the important histological events in brook trout. As this study was reported from Canada, due to major climatic differences between the two regions, it is therefore important to study the whole process of sex differentiation in brook trout under Tasmanian environmental conditions to investigate the differences caused by temperature. Although development might be similar to that reported by Sacobie and Benfey (2005), however, temporal data of developmental events would be different. This information about the time for sex differentiation was required to study the effect of androgen on masculinization of brook trout (Chapter 4). As this experiment (Chapter 4) was designed to perform sex reversal under controlled conditions (simulated Tasmanian temperature conditions), it was important to find out the time of sex differentiation in brook trout under the same temperature conditions decided for sex reversal experiment. The acquired data will be helpful to decide the appropriate time for oral androgen treatment before sex differentiation.

Although the literature reported for other salmonid species help to assess the basic developmental events during sex differentiation in brook trout but need of an independent study was realized based on the following reasons:

- 1- Different temperature ranges have been reported in various studies which caused the temporal differences in series of developmental events. Due to this reason, data on sex differentiation and early gonadal development for other species could not be recommended as reference for the present study especially for designing the protocol for androgen treatment (Chapter 4).
- 2- Inter-specific developmental differences have been observed during sex differentiation. Chiasson and Benfey (2007) reported the occurrence of a hollow loop in undifferentiated gonads in *Salvelinus alpinus* and considered it as an important event indicating the imminent sex differentiation. On the other hand no such development was observed in brook trout (Sacobie and Benfey, 2005). Moreover, In a few salmonids like masu salmon (*Oncorhynchus masou*), rainbow trout (*Salmo gairdneri*), whitespotted char, (*Salvelinus leucomaenis*) and peled (*Coregonus peled*), germinal tissue was found clustered towards the anterior end of the gonads during early development (Nakamura et al., 1974; Lebrun et al., 1982; Nakamura, 1982; Billard, 1992; Krol et al., 2003). This anterior polarity was considered as an indicator of imminent differentiation of the undifferentiated gonad into ovaries. This anterior polarity was not observed in brook trout in the current study.

Moreover, lack of unequivocal key for identification of sex differentiation in a particular species including brook trout (Table 2.2) is another reason to perform this study. A standardized identification key should be provided for brook trout to be used as a reference for future studies under similar temperature conditions in same species.

1.3 Puberty

Puberty or first maturity is the developmental period during which an individual becomes capable of reproducing sexually for the first time and implies a functional competence of the brain-pituitary-gonad (BPG) axis (Weltzien et al., 2004; Jalabert, 2005; Dufour and Rousseau, 2007). Age at first maturity is considered to be important for commercial stocking and breeding programmes (Brännäs, 2005). In most of the salmonids like chinook salmon (*Oncorhynchus tshawytscha*; Clarke and Blackburn, 1994), sea trout (*Salmo trutta*; Dziewulska and Domagala, 2004) and coho salmon (*Oncorhynchus kisutch*; Silverstein and Hershberger, 1992), males achieve puberty during their first year. Males mature usually one year earlier than females although extent of maturation can vary from 30% to 100% in different species like sea trout (Dziewulska and Domagala, 2004) and between different populations of same species like Atlantic salmon and sea trout (Bailey et al., 1980; Bagliniere and Maisse, 1985; Saunders and Henderson, 1988; Letcher and Terrick, 1998; Utrilla and Lobon-Cervia, 1999; Dziewulska and Domagala, 2004). Considerable phenotypic and genotypic variations have been observed in age and size at puberty within and among strains and families as observed in Atlantic salmon (Gjerde et al., 1994; Wild et al., 1994; Kennedy et al., 2003). Age and size at first maturity are affected by growth conditions and feed availability which are the major factors behind the higher incidence of early maturation in farmed fish due to their higher adiposity and energy stores compared to wild populations (Svåsand et al., 1996). It has been suggested that the onset of puberty is linked to absolute levels or a rate of accumulation of lipid stores (Chinook salmon: Shearer and Swanson, 2000; Shearer et al., 2006) explaining the late occurrence of puberty in females than males because female fecundity and offspring survival gain more by increases in energy reserves (Atlantic salmon: Aubin-Horth et al., 2006). Puberty can be postponed to the next year if an animal fails to exceed genetically determined developmental thresholds, in particular lipid reserves (Atlantic salmon: Taranger et al., 1999; Oppedal et al., 2006).

Puberty is an integrative mechanism involving signals derived from external and internal factors stimulating the BPG axis (Schulz and Goos, 1999; Zanuy et al., 2001; Okuzawa, 2002). Photoperiod is considered as a proximate controlling factor for initiation and completion of puberty in fish species living at moderate and high latitudes (Bromage et al., 2001). It can be successfully delayed and inhibited by manipulating photoperiod while the direction of change in photoperiod is more important than absolute day length in salmonids (Atlantic salmon: Duncan et al., 1998; Oppedal et al., 2006). Different studies have suggested that photoperiod treatments can entrain endogenous circannual rhythms controlling a “gating” mechanisms or “critical time window” during which puberty is allowed to commence and continue depending on the physiological state of the animal or being postponed to the next reproductive season in case of failure to exceed genetically determined thresholds (Arctic charr: Duston and Saunders, 1999; rainbow trout: Randall and Bromage, 1998; Atlantic salmon: Taranger et al., 1999; Oppedal et al., 2006).

Water temperature appears to play a minor role in the proximate control of reproductive cycle in salmonids (Bromage et al., 2001; Davies and Bromage, 2002) and acts as a permissive factor during final stages of maturation (Taranger et al., 2003). However, the onset of puberty can be indirectly affected by an effect of water temperature on somatic growth and lipid reserves (sea bass: Zanuy et al., 1986; rainbow trout: Prat et al., 1996; Atlantic salmon: Taranger et al., 2010). High temperature can arrest or delay maturation whereas cold water can advance and allow spawning (Atlantic salmon: Taranger and Hansen, 1993; rainbow trout: Pankhurst et al., 1996; white sturgeon: Webb et al., 1999; Atlantic salmon: Taranger et al., 2003).

Puberty in fish is finely controlled by stimulation of BPG axis. Its onset is marked by activation of GnRH neurons stimulating pituitary gonadotropins release, triggering pubertal development of the gonad (Taranger et al., 2010). Follicle stimulating hormone (FSH)

signalling is more important in early stages of puberty (Amano et al., 1993; Gomez et al., 1999) at rapid spermatogonial proliferation (Swanson, 1991) and the cortical alveoli stage (Luckenbach et al., 2008). On the other hand, high levels of luteinizing hormone (LH) are observed during late puberty in salmonids (rainbow trout: Prat et al., 1996; Breton et al., 1998; Gomez et al., 1999). Release of pituitary hormones finally stimulates the steroidogenesis in gonads. Early maturation in males is marked with proliferative activity of spermatogonia as a result of FSH mediated growth factors release from sertoli cells (Japanese eel: Nader et al., 1999) and synthesis of 11-ketotestosterone (11-KT) leading to meiosis and later stages of spermatogenesis (Japanese eel: Miura et al., 1991). Estradiol-17 β (E₂) may induce the proliferation of sertoli cells (sea bream: Chaves-Pozo et al., 2007) in males. Leydig's cells proliferate and increase the total testicular androgen output increasing the plasma androgen level accompanying puberty (African catfish: Cavaco et al., 1999). Although E₂ is associated with the growth of pre-vitellogenic oocytes and regulates the accumulation of cortical alveoli (Japanese eel: Endo et al., 2008; Atlantic cod: *Gadus morhua*; Kortner et al., 2009), 11-KT has also been found to be involved in the growth of pre-vitellogenic oocytes (Japanese eel: Matsubara et al., 2003; short finned eel: Lokman et al., 2007).

Keeping in view the role of environmental and internal factors in control of puberty, different techniques can be used to delay or inhibit the early maturation in males. The most commonly applied techniques for farmed salmonids are selective breeding, feeding control, photoperiod control, induced sterility and monosex fish culture (Taranger et al., 2010). These approaches however have not been studied greatly for use in brook trout, especially under Tasmanian environmental conditions.

1.3 Circannual Rhythm of Maturation after Puberty

The annual reproductive cycle of any species provides important background information about its reproductive strategies and dynamics of sex steroids. The annual maturational cycle of brook trout has not been extensively investigated and no study has been reported under Australian environmental conditions. Limited work is available in the literature on the reproductive cycle of females (Tam et al., 1986), the role of 17α , 20β -dihydroxyprogesterone (DHP) in germinal vesicle breakdown (GVBD) and fecundity (Serezli et al., 2010; Bascinar and Okumus, 2004), role of E_2 , DHP and GtH at final stages of oogenesis (Goetz et al., 1987). Moreover, these studies focused to female brook trout and gave no information about males. As these studies presented only a part of gonadal development and have been performed under different temperature and tank conditions with different brood stock, it is difficult to make a standardized comparison and correlate the information. Therefore, there was need to investigate the complete reproductive cycle in both sexes under the persistent husbandry conditions.

During the second reproductive cycle after puberty, BPG axis becomes functionally more active and mature in comparison to the first maturational cycle. Both spermatogenesis and oogenesis are under the fine control of internal endocrine and external environmental factors. Spermatogenesis is a highly organized and complex process which is initiated by self renewal of spermatogonia stimulated by E_2 (Japanese eel: Miura et al., 1999; Japanese huchen: Amer et al., 2001; sea bream: Pinto et al., 2006), testosterone (T) and 11-KT (African catfish: Cavaco et al., 1998). The number of spermatogonial generations passing through this differentiation process of self-renewal and duration of division are species specific and have not been studied for brook trout as yet. These differentiated spermatogonia undergo rapid proliferation due to FSH mediated synthesis of T and 11-KT (Campbell et al., 2003). Elevated level of FSH at this stage may be associated with proliferation of sertoli cells and

mitotic expansion of spermatogonial cysts (Weltzien et al., 2004). Spermatogonial proliferation is switched to meiotic stages of spermeiogenesis by the action of 11-KT mediated by certain growth factors like IGF1 and activin B (Huhtanieni and Themmen, 2005). DHP may also be involved at this stage (Miura et al., 2007) but needs further investigation for its involvement in salmonid males. Final maturation of sperm and spermiation is regulated by LH-induced synthesis of 11-KT and DHP (amago salmon: Ueda et al., 1985; rainbow trout: Milla et al., 2008) which mediates intra tubular changes of sertoli cells and pH control in sperm ducts (Japanese eel: Nader et al., 1999). After spawning, E₂ may induce infiltration of acidophilic granulocytes in males (sea bream: Chaves-Pozo et al., 2007) to remove the cell debris in post-spawned testes.

In females, early stages of oogenesis involve oogonial proliferation mediated by E₂ and DHP. These steroids further initiate the first meiotic division, early primary growth and formation of chromatin nucleolar oocytes (Japanese eel: Miura et al., 2007; rainbow trout: Forsgren and Young, 2012). At progression of oocytes from primary growth to secondary growth, cortical alveoli appear and their synthesis is associated with an increase in plasma and pituitary FSH, E₂ and expression of transcripts encoding ovarian steroidogenic acute regulatory protein (StAR) (Japanese eel: Endo et al., 2008; Atlantic cod: *Gadus morhua*; Kortner et al., 2009). Growth of pre-vitellogenic oocytes and accumulation of oil droplets has been found to be associated with 11-KT in *Gadus morhua*, *Anguilla australis* and *Anguilla japonica* but this role needs to be investigated in salmonids (Matsubara et al., 2003; Lokman et al., 2007; Endo et al., 2008; Kortner et al., 2009). After meiotic arrest following first meiotic division, E₂ continues its regulatory role in hepatic vitellogenin synthesis (Japanese eel: Miura et al., 2007) and uptake of vitellogenin during exogenous vitellogenic stages (coho salmon: Fitzpatrick et al., 1986).

Termination of vitellogenesis and progression to meiosis resumption is associated with an increase in plasma LH switching steroidogenesis from E_2 synthesis to maturation inducing hormone (MIH) (Nagahama and Yamashita, 2008) which is DHP. The final stages of maturation and ovulation in females are regulated by MIH involving a complex series of cellular changes (Nagahama and Yamashita, 2008).

The circannual rhythm of maturation involving the above mentioned developmental and endocrine changes is further integrated with environmental factors like photoperiod and water temperature. In autumn spawning salmonids, gradually increasing photoperiods followed by decreasing photoperiods or short days play a dominant role in the regulation of reproductive cycles (rainbow trout: Bromage et al., 1992). In salmonids, photoperiod and water temperature act as a proximate and ultimate factor, respectively to synchronize the timing of spawning (Bromage et al., 1992). Maturation can be inhibited, accelerated or delayed by manipulating photoperiod and water temperature in salmonids (rainbow trout: Bromage et al., 1992; Atlantic salmon: Andersson et al., 2003; rainbow trout: Duston et al., 2003; Taylor et al., 2008; Atlantic salmon: Leclercq et al., 2011; rainbow trout: Wilkinson et al., 2011). To study the role of these environmental factors in control of maturation in brook trout, a comprehensive study on its reproductive strategies under Tasmanian climate conditions is required which will further reflect the adaptability of this introduced species to Tasmanian climate. Data collected from this study will be required to design the protocol for light treatment in order to control maturation in brook trout (Chapter 5). Particularly information about the time and identification of key histological events at the stage of recruitment in both sexes will be helpful to close the “window of opportunity” to recruit for maturation by light treatment.

A few studies have investigated limited aspects of reproductive physiology of female brook trout only and ignored males (Duffey and Goetz, 1980; Tam et al., 1986, 1990; Goetz, 1987;

Bascinar et al., 2003; Sacobie and Benefy, 2005; Holcombe et al., 2000; Serezli et al., 2010). Tam et al. (1986) described the annual reproductive cycle in female brook trout only and did not measure 11-KT which also plays a role in ovarian development (Lokman et al., 2007). Tam et al. (1990) commenced their study in June, only five months before spawning when females have already recruited for maturation and did not continue the trial further to study the post spawning stage. Therefore the control group in this study did not describe the whole annual cycle of brook trout. Among the important sex steroids they measured only E₂ while GTH was measured by ultrastructural morphometry of secretory cells instead of measuring their plasma profiles which is more reliable technique. Goetz et al. (1987) described variations in the profile of E₂, DHP and GTH only at the final stage of ovarian development. Remaining developmental stages were not studied. Mount et al. (1988) only measured fecundity without any histological study of gonads and hormone analysis. Holcombe et al. (2000) focused to narrow range of time periods (in weeks) to investigate the photoperiodic manipulation. The control in this study was exposed to ambient photoperiod for that specific short time period only. Data given about gonadal development during a short period cannot provide the details about annual variations in gonadal development and hormones profiles. These studies are extensive but focused to their targeted objectives such as effect of photoperiod regimes and pH on gonadal development not the annual reproductive cycle. It can be concluded that previous work available on gonadal development of brook trout is insufficient and limited to female brook trout. Therefore this information could not be considered as a standard reference to study of the annual reproductive development of brook trout in Tasmania. In addition, these studies have potentially been performed on different strains of fish. Moreover, fish were reared under different water temperature and tank conditions. Data given in these different studies cannot be put together for an accurate comparison to the development of gonads during puberty and annual reproductive cycle in brook trout in Tasmania. These research are provide justification to investigate the variation

in gonadal development and sex steroid profiles during puberty and annual reproductive cycle under Tasmanian climate conditions.

1.4 Endocrine Sex Reversal to Control Maturation by Production of all-Female Population

Early maturation in male salmonids before reaching marketable size results in a large size variation in the population, an overall reduction in production (Piferrer, 2001) and increased susceptibility of males to diseases (Haffray et al., 2009). Monosex female stock for finfish Aquaculture is one of the solutions to overcome this problem. In salmonids, female fish show better growth rates than males and usually do not attain maturity during the first year of their life (Clarke and Blackburn, 1994; Dziewulska and Domagala, 2004; McClure et al., 2007). Production of all-female populations can be achieved by applying steroid-induced sex reversal either by direct or indirect feminization.

The method of direct feminization by use of estrogens is considered disadvantageous due to consumer's rejection of steroid treated fish, lack of legislation for steroid handling and utilization in many countries (Piferrer, 2001) and banning of the steroid treated livestock in certain regions (Haffray et al., 2009). The second method of indirect feminization is more applicable and has been successfully applied in salmonids like rainbow trout (*Oncorhynchus mykiss*) (Solar et al., 1984; Schmelzing and Gall, 1991; Demska-Zakes et al., 1999; Amini and Tala, 2003), chinook salmon (*Oncorhynchus tshawytscha*) (Piferrer et al., 1993), coho salmon (*Oncorhynchus kisutch*) (Hunter et al., 1983), Atlantic salmon (*Salmo salar*) (Lee et al., 2004) and brook trout (Sacobie, 2001; Galbreath et al., 2003; Haffray et al., 2009).

Indirect feminization involves the masculinisation of genotypic females from a batch of mixed sex fish and then fertilization of normal ova with sperm produced by neo-males (Piferrer, 2001). The major techniques applied for masculinisation of females are immersion

and dietary treatments with natural or synthetic androgens (Pandian and Sheela, 1995) and aromatase inhibitors (Lee et al. 2003). The most commonly and successfully used technique is masculinization by using androgens. 17 α -methyltestosterone (MT) is the most commonly used androgen for masculinisation of salmonids (Pandian and Sheela, 1995; Devlin and Nagahama, 2002) which causes no negative effect on mortality (Parks and Parks, 1991; Piferrer et al., 1994) and growth (chinook salmon: Baker et al., 1988; coho salmon: Piferrer and Donaldson, 1991) of fish. MT has been used previously in brook trout to vary extent of 60 – 74% successful masculinization (Sacobie, 2001; Haffray et al., 2009). Moreover, MT is routinely used to produce neomales in Tasmania therefore farmers are trained for MT immersion and dietary treatment. Due to the above mentioned reasons, MT was preferred for masculinization in the present study than aromatase inhibitors. Although aromatase inhibitors has been used as an applied solution to produce neomales in Atlantic salmon in Tasmania (Lee et al., 2003) however the proportion of males was only 51%, less than 60 – 74% by using MT (Sacobie, 2001; Haffray et al., 2009) in brook trout.

The sex differentiation period is considered to be the most labile period for sex inversion treatment due to plasticity of gonadal primordia during this time period. In salmonids the occurrence of anatomical sex differentiation has been reported after hatching (white fish: Dlugosz and Demska-Zakes, 1989; coho salmon: Feist et al., 1990; rainbow trout: Billard, 1992; Foyle, 1993; brook trout: Sacobie and Benfey, 2005; Arctic charr: Chiasson and Benfey, 2007) but the most labile period for sex inversion treatment in most of the salmonids was pre-hatch (Pandian and Sheela, 1995; Devlin and Nagaham, 2002). Sensitivity of the pre-hatch period for masculinization may be due to activation of sex determining genes during this period earlier than histological differentiation of gonads (rainbow trout: Cavileer et al., 2009; Atlantic salmon: von Schalburg et al., 2011). Both immersive and dietary techniques are applied for sex inversion and oral treatment with 17 α -methyltestosterone (MT) and 17 α -methyl dihydrotestosterone (MDHT) is commonly used producing 100% male population

in rainbow trout (Solar et al., 1984; Schmelzing and Gall, 1991; Demska-Zakes et al., 1999; Amini and Tala, 2003), chinook salmon (Piferrer et al., 1993) and Atlantic salmon (Lee et al., 2004). However, the success rate of masculinisation by dietary treatment varies between different studies for the same species and on occasion has been found to be ineffective for a few salmonids including brook trout (Solar et al., 1984; Galbreath et al., 2003; Sacobie, 2001; Haffray et al., 2009). In addition the disadvantages and limitations of dietary androgen treatment including non-uniform distribution of androgen in feed, differential feed uptake, endocrine disruption and possible sterility (Pandian and Sheela, 1995; Pandian and Kirankumar, 2003) cannot be ignored. Moreover, molecular studies show the expression of sex regulators earlier than anatomical sex differentiation (Baron et al., 2008; Cavileer et al., 2009; von Schalburg et al., 2011) indicating the need for androgen treatment before hatching.

On the other hand, a few studies have reported successful masculinisation with pre-hatch androgen immersion treatment in chinook salmon and coho salmon (Hunter et al., 1983; Baker et al., 1988; Piferrer and Donaldson, 1991). However, protocols combining pre-hatch immersion followed by oral treatment after swim-up stage resulted in successful masculinisation but with a high incidence of sterility and deformed sperm ducts (Goetz et al., 1979; Hunter et al., 1982; Parks and Parks, 1991; Galbreath et al., 2003; Arslan et al., 2010). The occurrence of sterility in combination treatments is due to super-optimal dose of androgen (Pandian and Sheela, 1995) questioning the real need of dietary treatment in sex reversal protocols (Haffray et al., 2009). Elimination of oral treatment will be helpful in reducing the high incidence of sterility, cost, exposure of farmer to steroid and reduced release of endocrine disruptors into the environment.

1.5 Control of Maturation by Manipulation of Photoperiod

The remarkable reproductive strategy of seasonal spawners is synchronization of endogenous circannual rhythms with environmental factors to ensure that off-spring are produced at the most beneficial time of the year to optimize their survival (Sumpter, 1990). Although a number of environmental factors have been implicated as possible proximate factors, photoperiod is considered to be the major entraining factor for temperate species including salmonids (Bromage et al., 2001).

In salmonids, gonadal development is long and there is some level of gonadal activity occurring in every season. Recruitment for maturation commences after the opening of a “recruitment window” during long days while final maturation is attained during short days which act as “completion window” (Atlantic salmon: Duston and Bromage, 1988). Photoc signals received during the diel cycle of 24h light and darkness (LD) or annual cycle are transformed into nervous or hormonal signals through an autonomous pineal-brain-pituitary-gonad (PBPG) clock machinery driving the rhythmic production of hormones. A major output of this biological clock is melatonin secreted by the pineal gland at night which in turn activates the whole PBPG axis (Falcón et al., 2010).

Considering the commercial interest in inhibiting or reducing the incidence of maturation, successful studies have been reported to achieve this goal by manipulating photoperiod (advancement, acceleration, continuous illumination; discrete photophases) irrespective of thermal regime (turbot: Imsland et al., 1997; sea bass: Zanuy et al., 2001; rainbow trout: Davies and Bromage, 2002; sea bass: Begtashi et al., 2004; Eurasian perch: Migvad et al., 2004; Atlantic cod: Norberg et al., 2004). However, the value of photoperiod-temperature interactions cannot be totally ignored. High water temperature may result in delayed maturation (Taranger and Hansen, 1993; Duncan et al., 1998), retardation in maturation

(Henderson et al., 1963; Wilkinson et al., 2010) and low survival of eggs (Pankhurst et al., 1996).

Similar to other teleosts the direction of change in photoperiod is far more important than critical duration of light treatment in salmonids (Holcombe et al., 2001; Bromage et al., 2001). Different windows of opportunity can be found during the reproductive cycle when a change in the direction of photoperiod may result in delay, advancement or inhibition of maturation. In salmonids, maturation can be advanced by exposing fish to short days (Atlantic salmon: Björnsson et al., 1994; King et al., 2003; rainbow trout: Choi et al., 2010; Atlantic salmon: Skilbrei et al., 2011), accelerated photoperiod (Atlantic salmon: Porter et al., 1999; Andersson et al., 2003; Taranger et al., 2003; rainbow trout: Wilkinson et al., 2010), continuous photoperiod followed by short days (Arctic charr: Frantzen et al., 2004) and long photoperiod followed by short days (Atlantic salmon: Taranger et al., 2003; rainbow trout: Choi et al., 2010). On the other hand, exposure to continuous photoperiod can completely inhibit (Atlantic salmon: Oppedal et al., 1997; Unwin et al., 2005), delay or reduce the probability of maturation to great extent (Atlantic salmon: Taranger et al., 1998; Porter et al., 2000; Peterson and Harmon, 2005; Skilbrei et al., 2011).

As compared to other major salmonid species, less attention has been given to the control of maturation by photoperiod manipulation in brook trout. A few studies have been reported in regards to advancement of maturation by compressing photoperiod (Hoover, 1937; Hoover and Hubbard, 1937; Goddard, 1979) or exposing to long days followed by short days (Hazard and Eddy, 1951; Corson, 1955), partial inhibition by exposure to long days followed by short photoperiod (Carlson and Hale, 1973), delayed maturation (Holcombe et al., 2000) and investigating diel rhythms of melatonin (Zachmann et al., 1992). However, brook trout of only +1 age are affected by altered light regimes whereas fish in their first year did not show marked response to change in photoperiod (Henderson et al., 1963; Goddard, 1979) which

may be due to more control of endogenous factors than environmental signals during puberty (Taranger et al., 2010). Due to limited work reported regarding the role of photoperiod in control of maturation in brook trout and lack of any work from the southern hemisphere on this species, this aspect needs to be investigated further under Tasmanian environmental conditions.

1.6 Aim and Objectives of Research

This research project was designed considering the potential importance of brook trout in the Tasmanian Aquaculture industry focusing on the major problem of precocious maturation in males impeding large scale commercial production. Aim of this study was investigating the reproductive strategies of brook trout under Tasmanian climate conditions and solutions to control precocious maturation in males. Therefore, objectives of this PhD project are as follows:

1. Study of gonadal development from hatch until the sex differentiation.
2. Study of gonadal development and variations in profiles of sex steroids during the first and second maturational year.
3. Development of sex inversion protocol to masculinize the female population and identification of the most sensitive time for MT treatment.
4. Control of maturation by advancement of 8 weeks in photoperiod and continuous illumination.

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CHAPTER 2

Histological Study of Gonadal Development and Sex Differentiation in *Salvelinus fontinalis* under Tasmanian Climate Conditions

2.1 Abstract

This study describes the developmental process of gonads in brook trout from 8 degree days post-hatch (°dph) until completion of sex differentiation (3354 °dph). Gonadal development was divided into undifferentiated (8-2013 °dph) and differentiated phases (2769-3354 °dph). Fertilized eggs (n = 1000) were incubated at 9.5-10 °C until hatching at 66 days post fertilization (dpf). A total of 20% of alevins sampled on 8 °dph were found with unpaired and undifferentiated gonads indicating that gonadal development commenced prior to hatch. Initially, undifferentiated gonads contained stromal tissue and few primordial gonadal cells (PGC) (n = 2-5). During the undifferentiated phase, gonads increased in size and proliferative activity of the PGC increased their number (n = 15-22). The differentiated phase commenced with the appearance of sex differentiation at 2769 °dph where gonads could be clearly differentiated as ovaries and presumptive testes. Ovaries were identified by the presence of oogonia while presumptive testes contained spermatogonia, vena comittis and a proximal network of cavities. Both ovaries and testes underwent further differentiation until the end of this phase (3354 °dph). Oogonia were transformed into primary oocytes while spermatogonial cysts were observed in testes. However, differentiation of steroidogenic cells could not be observed. Direct sex differentiation was found in this study as undifferentiated gonads directly developed into testes and ovaries with anatomical differentiation preceding cytological differentiation. This study confirms previous studies that the pre-hatch period

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should be targeted when attempting to produce future monosex populations via indirect sex reversal using dietary androgen treatment or aromatase inhibitors.

2.2 Introduction

The Tasmanian aquaculture industry, fulfilling 97% of total Australian salmonid production during 2009-2010, relies on Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Australian Fisheries Statistics, 2010). To ensure ongoing sustainability and commercial diversity, Tasmanian farmers are also looking to alternative species. Among such alternatives under review for commercial culture is brook trout (*Salvelinus fontinalis*). Tasmanian brook trout used for commercial culture trials are descendant from stock first introduced from Nova Scotia, Canada in 1962 (Clements, 1988). Since the introduction of this species, the Tasmanian ‘Inland Fisheries Service’ has maintained populations of brook trout (Salmon Ponds site, Plenty, Tasmania) for recreational fishing stocking purposes and for supply of brood stock to commercial producers as required (S. Chilcott, Inland Fisheries Service, Pers. Comm.)

One significant constraint to commercial salmonid culture, including that of brook trout, is the high incidence of early sexual maturation in males which affects flesh quality (Galbreath et al., 2003; Chiasson and Benfey, 2007) and results in high male mortality (Haffray et al., 2009). One solution to this problem is monosex fish culture which can be achieved by applying steroid induced sex reversal. Sex reversal protocols currently used by the Atlantic salmon and rainbow trout industries rely on dietary treatment while steroid immersion during the pre-hatch period has been found to be easier, safer and more effective (Haffray et al., 2009). In order for this to be optimised for this species, a comprehensive background study of gonadal differentiation is required to determine the most labile period for hormonal treatment and timing of sex differentiation. Accurate information about the most labile period before sex differentiation helps to reduce the dose and duration of steroid treatment (Piferrer, 2001).

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Initiation of early development of undifferentiated gonads in brook trout has been reported before hatch period followed by sex differentiation during the post-hatch period (Sacobie and Benfey, 2005; Haffray et al., 2009). Sex differentiation is the process by which the various molecular, genetic and physiological mechanisms produce a male or female from a zygote of a given genotype and parents in a given environment (Bull, 1983). At this developmental stage, gonads can be clearly differentiated as ovaries and testes histologically if not morphologically identified. Sex differentiation in gonochorist fish species can be indirect or direct. In indirect sex differentiation, undifferentiated gonads firstly develop into ovaries and subsequently into testes or ovaries. On the other hand, in direct sex differentiation, undifferentiated gonads develop directly into ovaries or testes (Yamamoto, 1969). Sacobie and Benfey (2005) have reported a differentiated type of sex differentiation in brook trout similar to most of the salmonids (Robertson, 1953; Dlugosz and Demska Zakes, 1989; Billard, 1992; Foyle, 1993; Bogdanova, 2004; Dziejulska and Domagala, 2004; Chiasson and Benfey, 2007). Nakamura et al. (1998), Devlin and Nagahama (2002), Strussmann and Nakamura (2002), Penman and Piferrer (2008) and Sandra and Norma (2010) have extensively reviewed the factors affecting the sex differentiation, with genetic, endocrine, environmental and social factors all playing an important role.

The time of hatching and early post-hatching appears to be an important milestone at which sex differentiation occurs (Sacobie and Benfey, 2005; Haffray et al., 2009). The hatching period varies in different salmonid species and is effectively controlled by water temperature. The hatching time reported for brook trout has been reported to occur 523 °d (Bascinar et al., 2003), 495 °d (Sacobie and Benfey, 2005) and 420 °d (Haffray et al., 2009). Baird et al. (2002) studied the effect of low and high water temperature on four different strains of brook

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trout and found that embryonic development slowed down at high temperature leading to an increase in the incubation period. Similarly, Marten (1992) described the incubation period as a function of the mean temperature from fertilization to 50% hatch in brook trout. The time of sex differentiation also varies amongst different salmonid species although water temperature conditions might be different for each study (Table 2.1).

There is no unequivocal indicator of sex differentiation for salmonids (Dziewulska and Domagala, 2004) and similarly this cannot be clearly defined for brook trout. Different studies have reported various histological criteria to identify the time of sex differentiation in salmonids (Table 2.2). A lack of a standardized identification key in the literature can be suggested as another reason for reported differences in timing of sex differentiation within the same species.

Undifferentiated gonads in teleosts contain primordial germ cells (PGC) and associated somatic cells. The commencement of differentiation of these PGC and somatic cells is identified by migration and exponential division of PGC and stromal cells (Billard, 1992; Nakamura et al., 1998; Sacobie and Benfey, 2005). The role of *cyp19a* as a molecular indicator of sex differentiation by regulating the activity of aromatase has been proposed in teleosts (Piferrer and Guiguen, 2008). A similar role has been suggested for gene *dmrt1* expressed by Sertoli cells (Sandra and Norma, 2010).

In gonochorist salmonids, ovaries differentiate earlier than testes (Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002; Arezo et al., 2007). The first detectable histological criterion in ovarian differentiation is the appearance of pre-meiotic oogonia (Nakamura, 1982; Foyle, 1993; Sacobie and Benfey, 2005) subsequent to meiotic oocytes and formation

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of ovarian lamellae (Lebrun et al., 1982; Dlugosz and Demska Zakes, 1989; Billard, 1992; Chiasson and Benfey, 2007). At this stage, presumptive testes typically remain quiescent and their germ cells resemble PGC, dispersed singly amongst stromal tissue. Subsequently, complex cavities appear around the distinct artery and vein (vena comittis), which are considered as the efferent duct of future testis (Nakamura et al., 1974; Nakamura, 1978; Takashima et al., 1980; Nakamura, 1982; Foyle, 1993; Dziewulska and Domagala, 2004; Sacobie and Benfey, 2005).

Unlike germ cell differentiation, detailed studies about histological differentiation of somatic cells in salmonids are not available in the literature as these events are not easily discernible by routine histology. This technique limited the observation of cell divisions and interference of histology process in the measurement of cell diameters in present study. A few ultrastructural studies have reported the presence of differentiated steroidogenic cells in differentiated gonads in *Onchorhynchus mykiss* (van den Hurk et al., 1982) and *Onchorhynchus rhodurus* (Nakamura and Nagahama, 1993). In present study, certain limitations were faced due to routine histology

According to Nakamura et al. (1998), Devlin and Nagahama (2002), Strussmann and Nakamura (2002), Penman and Piferrer (2008) and Sandra and Norma (2010), environmental conditions, in particular temperature, play a vital role in gonadal development and subsequent differentiation. Although sex differentiation has been studied in many salmonid species (Table 2.1) there are inter-specific differences observed even between the related species of *Salvelinus fontinalis* (Sacobie and Benfey, 2005) and *Salvelinus alpinus* (Chiasson and Benfey, 2007). Moreover, the present study aimed to find out the time of sex differentiation for future androgen dietary treatment (Chapter 4). Due to the inter-specific and intra-specific

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temporal differences in timing of sex differentiation reported in various studies, previous works could not be helpful to calculate the accurate time of sex differentiation in present study. Although sex might have been determined during the pre-hatch period in brook trout as observed in rainbow trout (Vizziano-Canntonet et al. 2008) sex can be reversed in this species before gonads are differentiated into ovaries and testes (Sacobie, 2001; Galbreath et al., 2003; Haffray et al. 2009). It is therefore important to study the whole process of sex differentiation under Tasmanian environmental conditions to investigate the differences caused by temperature from previous studies. Keeping in view the growing commercial importance of brook trout in the Tasmanian aquaculture industry, techniques are needed to develop all-female cultures to avoid problems associated with precocious maturation in males. For this purpose, a comprehensive histological study will help to identify the time of sex differentiation during the post-hatch period which could be the most responsive stage for future endocrine sex reversal treatment. Therefore this study was conducted to determine the time of hatch and characterise gonad differentiation in brook trout during the post-hatch period.

Table 2.1 Time of sex differentiation in different salmonids based on histological study

Species	Time of Sex Differentiation	Technique	Reference
<i>Coregonus lavaretus</i>	108 dph	Histology	Dlugosz and Demska-Zakes, 1989
	82 dph	Histology	Bogdanova, 2004
<i>Coregonus peled</i>	1020 °dph	Histology	Krol et al., 2003
<i>Salmo salar</i>	600 °dph	Histology	Laird et al., 1978
<i>Salvelinus alpinus</i>	510-681 °dph	Histology	Chiasson and Benefy, 2007
<i>Salvelinus fontinalis</i>	393-464 °dph	Histology	Sacobie and Benfey, 2005
<i>Salvelinus leucomaenis</i>	131 dph	Histology	Nakamura, 1982
<i>Salvelinus namaycush</i>	850 dpf	Histology	Wenstrom, 1975
<i>Onchorhynchus kisutch</i>	380 °dph	Histology	Foyle, 1993
	270 °dph	Histology	Piferrer and Donaldson, 1989
	77 dpf	Histology	Feist et al., 1990
<i>Onchorhynchus masou</i>	210 °dph	Histology	Nakamura et al., 1974
	378 °dph	Histology	Lebrun et al., 1982
	410 °dph	Histology	Van den Hurk and Slof, 1981
	18 dph	Histology	Billard, 1992
<i>Onchorhynchus mykiss</i>	65 dpf	Molecular marker of <i>P450arom</i>	Govoroun et al., 2001
	69 dpf	Molecular marker of <i>cyp19a1</i>	Vizziano-Canntonet et al., 2008
<i>Onchorhynchus rhodurus</i>	20-25 dph	Histology	Nakamura and Nagahama, 1993

dpf: days post fertilization; dph: days post-hatch; °dph: degree days post-hatch

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Table 2.2 Key histological changes to identify sex differentiation in different salmonid species

Species	Developmental Changes	References
<i>Salvelinus alpinus</i>	Ovaries contain primary oocytes. Presumptive testes have undifferentiated germ cells and complex cavities.	Chiasson and Benfey, 2007
<i>Salvelinus fontinalis</i>	Ovaries contain pre-meiotic germ cells while testes have undifferentiated gonidia and large cavities.	Sacobie and Benfey, 2005
<i>Salvelinus leucomaenis</i>	Ovaries contain pre-meiotic oocytes while testes have undifferentiated gonidia and large cavities.	Nakamura, 1982
<i>Onchorhynchus kisutch</i>	Ovaries contain ovarian lamellae, oogonia and perinucleolar oocytes. Testes have blood vessel and sperm duct.	Feist et al., 1990
	Meiotic germ cells are observed in ovaries far earlier than testes.	Piferrer and Donaldson, 1989
	Ovaries contain meiotic germ cells while presumptive testes have spermatogonia resembling with gonial cell.	Foyle, 1993
<i>Onchorhynchus masou</i>	Vascular system is formed in males earlier than females.	Nakamura et al., 1974
<i>Onchorhynchus mykiss</i>	Ovaries differentiate with organization of ovarian lamellae and appearance of meiotic oocytes. Testes have undifferentiated germ cells.	Lebrun et al., 1982

2.3 Materials and Methods

This study was conducted according to procedures approved by the University of Tasmania Animal Ethics Committee (Project Approval Number: A0010890). Diploid brook trout eggs at eyed stage (30 days post fertilization) were obtained from Mountain Stream Fishery, Targa, Tasmania (Latitude= 41° 18' 01" S, Longitude= 147° 22' 35" E, Elevation = 425 m) and transferred to the National Centre for Marine Conservation and Resource Sustainability, Aquaculture Centre (University of Tasmania, Launceston, Tasmania; Latitude= 41° 23' 21.17" S, Longitude= 147° 07' 35.49" E, Elevation = ~ 60 m) on June 18, 2010. Prior to transfer, eggs were incubated under ambient farm water temperatures (6-7 °C). Due to the incubation of eggs at two different water temperatures at the commercial farm and University facilities, degree days for the hatching period were calculated on the basis of an average temperature of 7.3 °C throughout this period using the following formula:

Degree days: Days × Water temperature

Post-transfer, eyed eggs (n=1000) were placed into an incubator constructed from poly-vinyl chloride (PVC) pipe (Diameter = 15 cm, Depth = 7 cm), housed in an upwelling Californian tray in 24 h darkness. This PVC incubator was fitted with fibre glass mesh screen at the bottom and provided with water exchange by an 18 mm polyester airlift pipe. Water temperature was maintained at 9.5 - 10 °C by a heat / chill unit (10 KW). Water was supplied from a 4500 L tank, exchanging 10% water per day (using dechlorinated municipal town water), fitted with UV sterilizer (Pentair Aquatics, QL-40) and bio filters. At the swim-up stage (on August 18, 2010; 271 °dph), fish were fed a commercial diet (4% BW/day) (Skretting, Australia) four times a day and maintained at a photoperiod of 12 hours light: 12

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hours darkness. Once fry became more experienced in consuming feed pellets, they were fed twice a day at the same ration. During December, 2010, a total of 100 fry were transferred to a 20 L plastic tank and maintained under identical water temperature, water supply and air supply as previously described. Feed ration was reduced to 2% BW/day at this stage given twice a day. Body weight was checked every week by random (without introducing any size bias into results) sampling to update the weekly ration until December. Monthly weight check was carried out from January until the end of study. A total of 7 sampling points (between July, 2010 – January, 2011) were carried out from this cohort until fish reached an age of 6 months. A total of 100 fish were not sampled to avoid any loss of fish in case of mortality ensuring successful completion of study.

An additional cohort of 4 month old fish ($n= 200$; 1.54 ± 0.14 g) were transferred from the same commercial hatchery to the university Aquaculture Centre during November 2009 in order to study gonadal differentiation from an age of 4 months to 7 months (November, 2009 – February, 2010). Fish were stocked in a 4000 L experimental tank and fed twice daily on a commercial diet (Spectra SS, Skretting, Australia) at 2% BW/day. Photoperiod was ambient and water temperature was maintained between 12-17 °C throughout study period by a heat / chill unit (10 KW) to mimic typical on-farm temperature range typically experienced at hatchery and on-growing locations.

Both the first and second cohort were held to study the sex differentiation and variations in gonadal development during puberty, respectively. Sampling was performed from both cohorts at each sample point. In present study, data collected from the first cohort has been presented as no significant difference was observed between the mean values of all

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parameters collected from both cohorts. Data showing development at 7 month of age was taken from the second cohort as this sampling point was missed from first cohort.

2.3.1 Sampling

From the first cohort, a total of 7 sampling points were conducted (July, 2010 – January, 2011). The first sampling was performed on the day of first hatch (July 23, 2010). 100% hatch was observed at 30 °dph. Next sampling was carried out after one week ensuring that all eggs were hatched. Three weekly samplings were then carried out until August 13, 2010. Following that point a total of 4 samplings was performed on a monthly basis from September 29, 2010 to January 15, 2010. From the second cohort, 4 monthly sampling points were conducted (November, 2009 – February, 2010).

At each sample point, ten eggs/fish per cohort were randomly sampled except for the final two sample points for the first cohort when 5 fish were sampled due to limited fish availability (December – January). At sampling, fish were killed by overdose of anaesthetic by transfer into a 50 L tank containing 30 mg/L iso-eugenol (AQUI-S NZ Ltd.). Whole alevins and fry were preserved in seawater Davidson's fixative (95% ethanol, 40% formaldehyde, glacial acetic acid, distilled water, 3:2:1:3) and stored at room temperature (~ 13 °C). The mid sections of fixed fry (between pectoral and pelvic fin) were used for processing. Data of total body weight, total length and condition factor were given in appendix I.

2.3.2 Histological Study

Fixed samples (n=10 per sampling) were dehydrated through different grades of ethanol (70%, 80%, 95%, 100%; two repeats in each grade; one hour in each) and finally embedded in paraffin to be sectioned at 5 μ m. Serial cross sections of mid gut of collected samples were prepared during 8 – 172 °dph. Cross sections (5 slides per sample) were prepared during 589 – 3354 °dph. Sections were stained using routine haematoxylin and eosin staining (Horobin, 2002). Stained slides were studied at 10-1000 X magnification, using a light microscope (Olympus, Japan) and images captured using Leica DC300F digital camera (Leica Microsystems, Germany). Cross sections were studied dorso-ventrally.

Germ cells were counted in serial cross sections under microscope using the grid. Diameter of cells was measured by using the Enter Magnification plugin of Fovea Pro 4.0. The number of germ cells and their diameter were collected from both gonads and one unpaired gonad (only one gonad throughout the mid gut at initial stage of development). The mean \pm s.e was calculated for both parameters.

Gonadal differentiation in brook trout was classified into 6 stages in the present study. These stages were defined on the basis of distinctive developmental changes occurring during specific time period (Table 2.3).

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Table 2.3 Key to study histological developmental stages during sex differentiation of *Salvelinus fontinalis*

Stage	Developmental Stage
I	Gonads are unpaired and observed in less than 50% of total samples (mid gut sections of each fish). No proliferative activity of primordial gonadal cells (PGC) is observed.
II	Gonads are paired and observed in 50% of total samples. No proliferative activity of PGC is observed.
III	Gonads are observed in all samples. Increase in diameter and condensation of chromatin is noted in PGC indicating imminent occurrence of cell division.
IV	Significant proliferative activity of PGC is observed resulting in increase in number of PGC and decrease in cell diameter.
V	Gonads can be differentiated as ovaries and presumptive testes. (a) Ovaries: have pre-meiotic oogonia and a blood vessel at its proximal end. (b) Presumptive Testis: have spermatogonia apparently resembling undifferentiated gonial cells. A pair of distinct vein and artery (vena comittis; Robertson, 1951) appeared surrounded by complex network of cavities.
VI	(a) Presumptive testes can be differentiated as testes after appearance of spermatogonial cysts which is result of spermatogonial proliferation. (b) Follicular layer is formed around oocytes. Ovarian lamellae appear. Primary growth is commenced by significant increase in size and appearance of chromatin nucleolar oocytes (McMillan, 2007).

2.4 Results

The hatching period for brook trout in this study was found to be a total of 66 dpf or 484 degree days-post fertilization (°dpf). After hatching, larvae took 33 days or 271 °dph for yolk resorption. The process of gonadal differentiation was divided into two phases; the undifferentiated phase (8-2013°dph) and differentiated phase (2769-3354 °dph). Differentiation commenced at 187 dph (2769.50 °dph) and was completed by 215 dph (3354 °dph). On the basis of the histological study, the whole process of gonadal differentiation was classified into 6 stages.

Data of total body weight, total length and condition factor were given in appendix I.

2.4.1 Histological Development of Gonadal Differentiation

The whole process of gonadal differentiation was divided into six phases. These phases were marked on the basis of clearly demarcated sequences of gonadal development (Table 2.3).

2.4.1.1 Histological Development during the Undifferentiated Phase (8 - 2013°dph) (0.04 ± 0.05 - 2.47 ± 0.01 g)

The first four stages (I – IV) of gonadal differentiation are described during the undifferentiated phase (Fig. 2.1).

2.4.1.1.1 Stage I (8 °dph) (0.04 ± 0.05 g)

On hatching (Stage I), 20% of total collected samples contained gonads, however, these were not found in pairs (gonads are present in form of pairs in fish but at this stage only one gonad was observed throughout the mid gut). Small pear shaped gonads were located beside the developing gut, dorsal to the yolk sac and ventral to the mesonephros. Gonads were

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suspended from the dorsal body wall by mesenteries and contained two PGC (n=4), and a few stromal and epithelial cells.

The shape of the other cells by their large size, low nucleo-cytoplasmic ratio, clear nuclear borders and granular chromatin (Fig. 2.1 A).

2.4.1.1.2 Stage II (66 – 115 °dph) (0.04 ± 0.05 - 0.05 ± 0.01 g)

Gonads were found in 40% of total samples at 66 °d which increased up to 60% at the end of this phase (Fig. 2.2 B). Pear shaped gonads without marked epithelium appeared in a pair (number of gonads was two). The position of gonads was the same as observed at 8 °dph. The mean number of PGC was two (n=4) throughout this phase. The remaining gonads contained stromal and epithelial cells. PGC were irregularly dispersed among stromal and epithelial cells. In one sample, gonads contained hollow cavities which were absent in other samples.

2.4.1.1.3 Stage III (172 °dph) (0.05 ± 0.01 g)

This phase was marked with the appearance of gonads in all samples (Fig. 2.1 C). The size of PGC had increased (Fig. 2.3) with large nuclei, containing condensed chromatin. The germinal epithelium (GE) was clearly defined at this stage.

2.4.1.1.4 Stage IV (589 – 2013 °dph) (0.48 ± 0.06 - 2.47 ± 0.01 g)

Initially, PGC were few in number (Fig. 2.1 D). Blood vessels were formed at the proximal end of gonads. Cavities were observed during 589-1282 °dph and gonads markedly increased in width. These cavities were found to be transitory and disappeared during 1453-2013 °dph. At 2013 °dph, gonads were compact and increased in weight. A sudden drop in PGC diameter might indicate the mitotic division (Fig. 2.1). The number of PGC increased (n=15-

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22), however their proportion was less than stromal cells. The GE was more developed and single cell in its thickness.

Throughout differentiation, gonads contained undifferentiated PGC having the same cytological features. There was no bipolarity or distinction of germinal and somatic area during this phase as PGC were observed asymmetrically scattered throughout the gonad. No apparent differences in histological aspects of stromal cells were noticed during the undifferentiated phase. In most of the samples, gonads in same fish were slightly different in size and shape.

2.4.1.2 Histological Development during the Differentiated Phase (2769 – 3354 °dph) (6.20 ± 0.72 – 12.25 ± 0.92 g)

This phase commenced with very clear differentiation of gonads into ovaries and presumptive testes at 2769 °dph (Fig. 2.1 E). Ovaries were identified by the appearance of pre-meiotic oogonia, with a core of stromal cells and fibroblasts covered by epithelium. Presumptive testes contained gonial cells, complex network of anastomosing cavities and vena comittis (complex of large vein and small artery) at the proximal ends of testes.

2.4.1.2.1 Stage V (2769 °dph) (6.20 ± 0.72 g)

This phase showed differentiation of undifferentiated gonads into pear shaped ovaries and presumptive testes (Fig. 2.1 E). Followed by cytological differentiation, ovaries contained oogonia (n= 15) and presumptive testes had spermatogonia scattered throughout the gonads. Spermatogonia had darkly stained nuclei and clear cytoplasm. The diameter of spermatogonia was significantly larger ($P < 0.5$) compared to that of PGC (Fig. 2.2). At this stage no spermatogonial cyst was observed. The pair of distinct artery and vein (vena comittis)

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appeared at the proximal end of presumptive testes, surrounded by anastomosing complex cavities. Blood vessels were also found at the proximal end of ovaries but vena comittis was not observed in any ovary. Ovarian follicular cells could not be identified at this stage

2.4.1.2.2 Stage VI (3354 °dph) (12.25 ± 0.92 g)

Stage VI was marked with anatomical differentiation of presumptive testes into testes after appearance of spermatogonial cysts formed and present throughout the gonad. Chromatin was condensed. This mitotic activity was also indicated by the decrease in spermatogonial diameter compared to that at stage V (Fig. 2.2). Ovaries underwent further differentiation. Oogonia entered into primary growth indicated by an increase in cell diameter from 48.4 μm to 98 μm (Fig. 2.2). Primary oocytes were found at the chromatin nucleolar oocyte stage (CNO) and a few were at peripheral nucleolar stage. Very few oogonia were also present. Ovarian lamellae were observed in only one pair of ovaries at this stage (Fig. 2.1 F). No sign of ovarian cavity formation was noted.

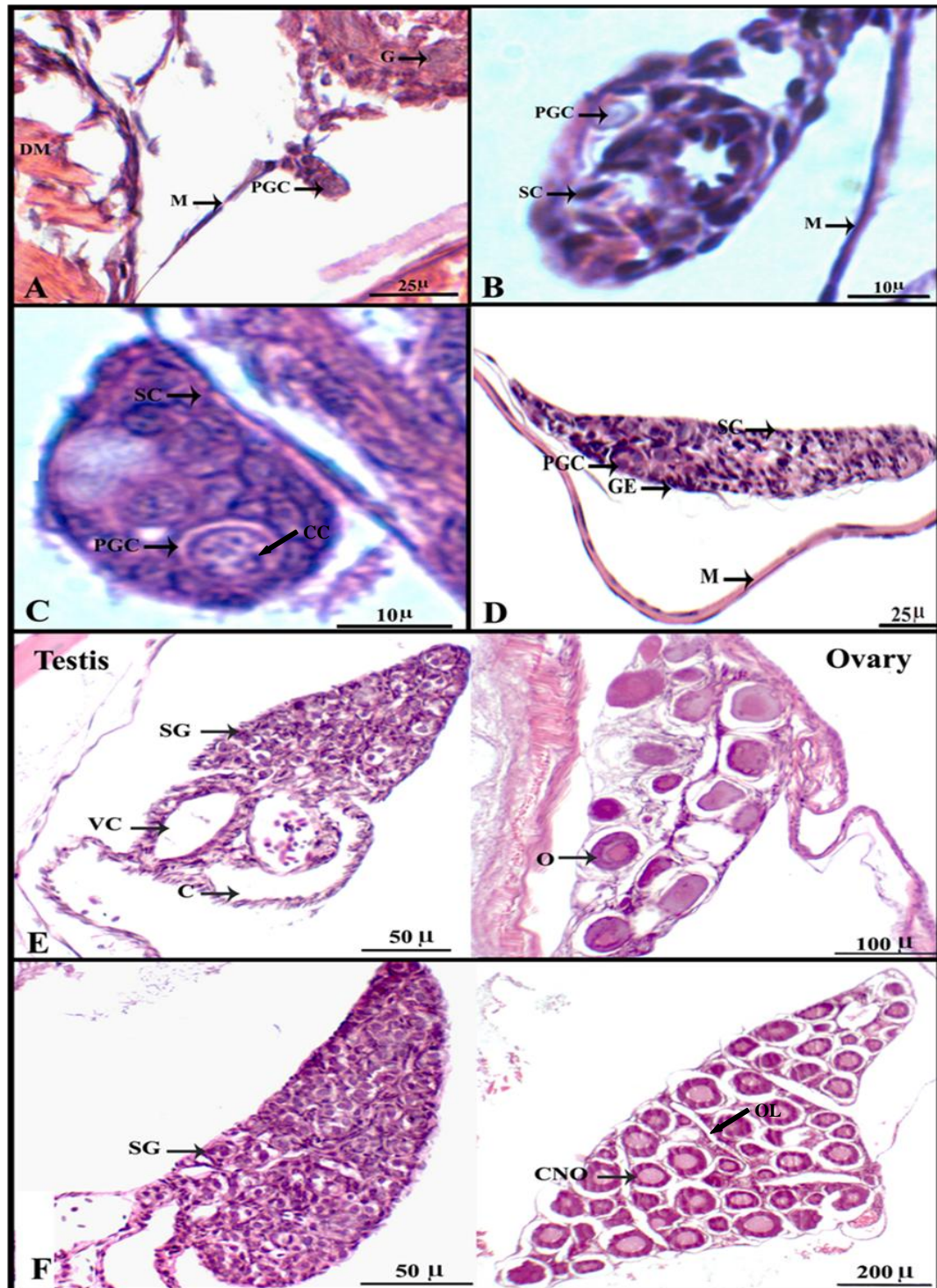


Fig. 2.1 Series of developmental changes during differentiation of gonads from undifferentiated phase (8-2013 °dph) till differentiated phase (2769-3354 °dph). (A) Stage I: 0 °dph; (B) Stage II: 66-115 °dph; (C) Stage III: 172 °dph; (D) Stage IV: 589-2013 °dph; (E) Stage V: 2769 °dph; (F) Stage VI: 3354 °dph. Age of fish in sections: A: 0 °dph, B: 66 °dph, C: 172 °dph, D: 2013 °dph; E: 2769 °dph; F: 3354 °dph. C, cavity; CNO, chromatin nucleolar oocyte; DM, dorsal muscle; G, gut; GE, germinal epithelium; M, mesentery; O, oogonium; OL: ovarian lamellae; PGC, primordial germ cell; SC, stromal cell; SG, spermatogonium; VC, vena comittis. μ : micrometer.

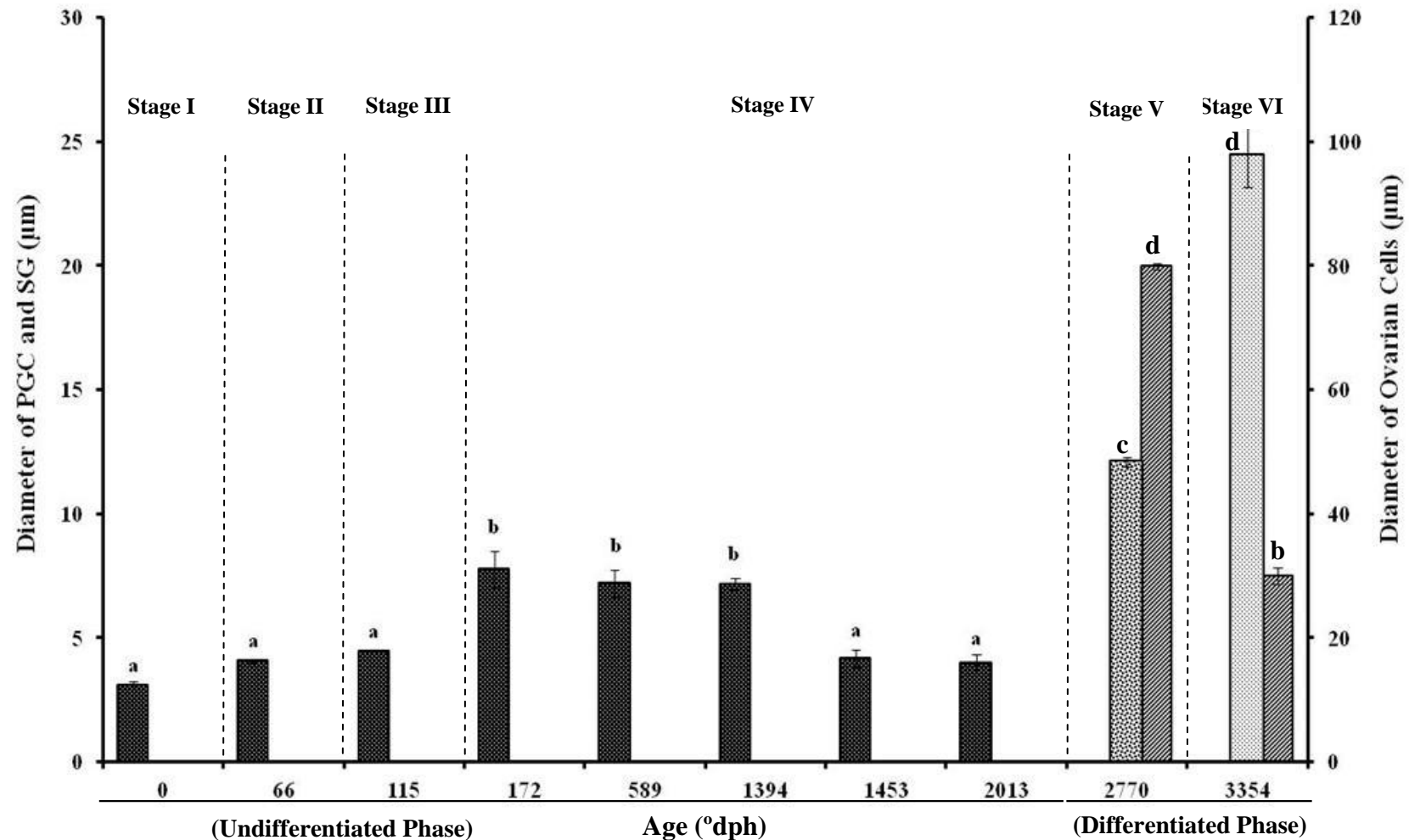


Fig. 2.2 Variations (Mean \pm SE) in diameter of primordial gonadal cells, testicular and ovarian cells during undifferentiated and differentiated phase during gonadal differentiation of *Salvelinus fontinalis*. Age is given in degree days post-hatch (°dph). Stages of undifferentiated phase (I – VI) and of differentiated phase (stage V – VI) are indicated. One-way ANOVA was applied and significantly different subsets ($P < 0.05$) given by Tukey's HSD are indicated by letters. CNO: Chromatin Nucleolar Oocyte; O: Oogonia; PGC: Primordial Gonadal Cell; SG: Spermatogonia (PGC: SG: O: CNO:).

2.5 Discussion

In the present study the development of the reproductive system in brook trout was found to be similar to other salmonids (Nakamura et al., 1998). The hatching period is critically important in gonadal differentiation of brook trout as sex differentiation as defined by histology occurs on hatching or shortly thereafter (Sacobie and Benfey, 2005; Haffray et al., 2009). The incubation period varies in different salmonid species and is effectively controlled by water temperature. More specifically the hatching time reported for brook trout in the literature varies with figures of 589-653 °d (Baird et al., 2002), 523-549 °dpf (Bascinar et al., 2003), 495 °dpf (Sacobie and Benfey, 2005) and 420 °dpf (Haffray et al., 2009). The hatching period for brook trout in present study was found to be 484 °dpf.

Gonadal development started before hatch as 20% of samples were found with unpaired gonads on hatch. Sacobie and Benfey (2005) and Haffray et al. (2009) also reported the initiation of gonadal development during the pre-hatch period. Sacobie and Benfey (2005) observed 40% of samples with gonads at 7 dph (39 °dph) in brook trout but at a different incubation temperature (5.5 °C). This indicates that gonadal development in embryos started prior to hatching in the present study similar to that reported by Sacobie and Benfey (2005).

Higher incubation temperature in present study resulted in hatching and gonadal development one month earlier than reported by Sacobie and Benfey (2005). Sacobie and Benfey (2005) observed 100% of the samples with unpaired gonads at 28 dph (154 °dph) (Fig. 2.1 A) which is very close to that observed 21 dph (172 °dph; 0.05 ± 0.01 g) in current study when paired gonads (Fig. I.1; appendix I) were found in all samples. However, paired gonads appeared earlier in present study (8-14 dph) (66-115 °dph; 0.04 ± 0.05 - 0.05 ± 0.01 g) compared to 35 dph (193 °dph) reported by Sacobie and Benfey (2005). These differences between the

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occurrences of developmental events might be due to different climate (i.e., temperature) and geography as has been suggested previously (Nakamura et al., 1998; Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002; Penman and Piferrer, 2008; Sandra and Norma, 2010).

In a few salmonids, germinal tissue was found clustered towards the anterior end of the gonads during early development (Nakamura et al., 1974; Nakamura, 1978; Lebrun et al., 1982; Nakamura, 1982; Billard, 1992; Krol et al., 2003). This anterior polarity was considered as an indicator of imminent differentiation of the undifferentiated gonad into ovaries. The strong anterior polarity of germinal tissue, noted in other salmonids was not observed in brook trout in the current study (Fig 2.1 D). This is similar to that reported for the ovaries of the same species (Sacobie and Benfey, 2005) and coho salmon (*Oncorhynchus kisutch*) (Foyle, 1993). Thus germ cells were irregularly dispersed across the whole length of indifferent gonads and no pronounced swelling of the gonadal tip with separate generative and somatic parts was observed in presumptive ovaries to differentiate ovaries from testes.

Initially like other salmonids, very few germ cells with large nuclei were found in comparison to stromal cells (Lebrun et al., 1982; Foyle, 1993; Bogdanova, 2004; Sacobie and Benfey, 2005; Chiasson and Benfey, 2007) which showed condensation of chromatin at 172 °dph, indicative of cells entering prophase of mitotic division (Fig 2.1 C) (Foyle, 1993). Developmental stage IV (589-2013 °dph; 0.48 ± 0.06 - 2.47 ± 0.01 g), which was followed by sex differentiation, showed an increase in the number of germ and stromal cells as supported by a decrease in PGC diameter. As a result of this proliferative activity, cavities which appeared (Fig. 2.1 E) at the initial stage of this phase disappeared and gonads became more compact. Exponential division of PGC and stromal cells was found to be a critical point

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regarding the beginning of sex differentiation in many teleosts although not unequivocally observed in all of them (Billard, 1992; Nakamura and Nagahama, 1993; Nakamura et al., 1998; Sacobie and Benfey, 2005). However, in the current study no differential outnumbering of PGC and stromal cells was observed in putative ovaries as has been seen in a few teleosts (Nakamura et al., 1998) and presumed as a component of sex differentiation regulation (Tanaka et al., 2008).

Sex differentiation occurred earlier in genetic females than males, where testicular differentiation is identified later by appearance of spermatogonial cysts, as reported in other gonochorist salmonids (Billard, 1992; Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002; Arezo et al., 2007). In this study the first detectable histological criterion in ovarian differentiation was appearance of pre-meiotic oogonia at 187 dph (2769 °dph; 6.20 ± 0.72). Cellular differentiation in the present study commenced (187 dph or 2769 °dph; 6.20 ± 0.72) later than that reported by Sacobie and Benfey (2005) i.e. between 393 and 464 °dph (~49 – 56 dph). Similarly, the process of sex differentiation was completed at 215 dph (3354 °dph; 12.25 ± 0.92 g) which was later than that reported by Sacobie and Benfey (145 dph or 798 °dph). Although it commenced later, the shorter duration of sex differentiation in the present study might be due to constant higher water temperature as compared to that previous study. On the other hand, no marked developmental difference was observed between the two cohorts of the present study although they were kept at different temperature ranges (9.5-10 °C and 12-17 °C, respectively). A higher temperature has been reported to accelerate the rate of cytological differentiation process (Krol et al., 2003) but this was not observed in the present study. It may therefore be inferred that differences between the present study and that reported by Sacobie and Benfey (2005) might be due to different strains of fish as mitochondrial DNA of brook trout in Tasmania showed diversity from that of Canadian

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strains (Ovenden et al., 1993). This difference may also be due to other factors like brood stock condition, stocking density and growth (Foyle, 1993; Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002; Penman and Piferrer, 2008; Sandra and Norma, 2010). Environmental conditions are anticipated to have variable effects on sex differentiation depending on the genetic background and developmental stability of different strains (Devlin and Nagahama, 2002). Other than environmental factors, genetic differences between species or strain, different criteria by which observers distinguish sex (Foyle, 1993), age and size (Dlugosz and Demska Zakes, 1989) are also involved in the timing of sex differentiation in salmonids. Difference of growth rates in the present study and Sacobie and Benfey (2005) might be a reason of for the difference in timing of sex differentiation. However, Sacobie and Benfey (2005) did not present the data of growth so comparison of growth rates in both studies on brook trout could not be performed. Moreover, a comprehensive study is required to investigate the influence of different ranges of water temperature and growth in conjunction with different strains on sex differentiation in brook trout.

In contrast to ovaries, presumptive testes did not show a clear germinal distinction from undifferentiated gonads at the same time, similar to other salmonids (Devlin and Nagahama, 2002). Appearance of complex cavities present around vena comittis presumed as efferent duct of testes (Fig. 2.1 E) (Foyle, 1993; Nakamura et al., 1998; Sacobie and Benfey, 2005; Chiasson and Benfey, 2007; Chiasson et al., 2008), was the main criterion to identify presumptive testes (Nakamura *et al.* 1998). Direct differentiation of gonads into ovaries and testes indicates that brook trout demonstrates differentiated or a direct type of sex differentiation (Yamamoto, 1969) as observed by Sacobie and Benfey (2005) as well and in agreement with other salmonids (Devlin and Nagahama, 2002).

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At the sex differentiation stage, more stromal cells were observed in presumptive testes than differentiated ovaries, similar to that observed in *Salvelinus leucomaenis* (Nakamura, 1982). The clear differentiation of testes was noted at 3354 °dph with appearance of spermatogonial cysts which is considered as a criterion of testicular differentiation (Nakamura et al., 1998; Dziewulska and Domagala, 2004). A significant decrease in germ cell diameter at this stage also supported the occurrence of spermatogonial proliferation.

As reported by Sacobie and Benfey (2005), cytological differentiation was followed by anatomical differentiation and after differentiation of germ cells into oogonia and spermatogonia, gonads underwent further development forming ovarian lamellae at 3354 °dph. Sex differentiation of steroidogenic cells could not be observed with certainty in indifferent gonads or shortly after gonadal differentiation. Gonadal somatic cell differentiation was microscopically observed after completion of ovarian and testicular differentiation (3643 - 4085 °dph) as reported in other teleosts (Nakamura, 1982; Schreibman et al., 1982; Kanamori et al., 1985; Nakamura and Nagahama, 1985; Nakamura and Nagahama, 1989; Nakamura and Nagahama, 1993; Chiasson and Benfey, 2007). Similarly, Sacobie and Benfey (2005) did not observe differentiation of steroidogenic cells in brook trout until differentiation of ovaries and testes. A bimodal distribution of whole-body androgens had been observed after sex differentiation in salmonids (Fitzpatrick et al., 1987; Feist et al., 1990; Fitzpatrick et al., 1993) which supports the occurrence of differentiation of steroidogenic cells later than gonadal differentiation. However, this question needs further investigation as to whether steroid secretion precedes gonadal differentiation or vice versa keeping in view the role of sex steroids in sex reversal of brook trout (Galbreath et al., 2003; Haffray et al., 2009).

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Based on the current study, rapid mitotic activity in undifferentiated gonads after a long quiescent period may be an indicator of imminent sex differentiation or sex differentiation of gonadal somatic cells (Tanaka et al., 2008) but this needs further comparative study at different incubation temperatures. Changes in whole-body steroid levels may also be indicative of the onset of sex differentiation (Feist et al., 1990). Molecular gene markers like *dmrt1*, *amh*, *cyp19a*, *foxl2*, *P450c17* can best identify the actual point of sex differentiation (Sandra and Norma, 2010) but as yet, these have not been studied for brook trout. von Schalburg et al. (2011) have reported expression of sex differentiation regulators (*dax1*, *dax2*, *foxl2a*, *foxl2b*, *cyp19a*, *cyp19b1*, *mis*, *sfl*, *sox9a* and *sox9b*) in undifferentiated gonads which led to synthesis of target proteins around the time of sex differentiation in Atlantic salmon. Similar results were presented by Baron et al. (2004) and Cavileer et al. (2009) for rainbow trout although the role of maternal contribution and expression of same genes in extra gonadal tissues could not be ignored (von Schalburg et al., 2011). This expression early in development points to sequential activation of pre-sex differentiation events which needs to be investigated for brook trout. The present study used routine histology to identify the different developmental stages instead of using molecular markers. Histological techniques are more economical as compared to the molecular markers which were preferred due to limited budget allocated to this study. Moreover, the aim of this study was to identify the time of sex differentiation during the post-hatch stage (4 months post-hatch). The occurrence of sex determination during pre-hatch phase was not the overall objective. The accurate identification of time of sex determination was not required in the present study. Furthermore, using routine histology for identification of histological gonadal differentiation would provide the fundamental data for comparison for any future study using the molecular markers to identify the time of sex determination and differentiation.

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This study developed a classification key for gonadal differentiation which can be used to identify the distinct developmental changes leading to final sex differentiation in brook trout. Based on these results, it is apparent that gonadal development commences before hatch as reported by Sacobie and Benfey (2005) and Haffray et al. (2009) and sex differentiation occurs four months after yolk sac absorption. This suggests that dietary androgen treatment given at swim up stage might masculinise the genotypic females before irreversible gonadal differentiation at the age of six months. This information will be useful for design of appropriate protocols to skew the sex ratio towards all-male production (i.e. production of neo-males for subsequent use to produce all female progeny) in the Tasmania industry.

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CHAPTER 3

Annual Variations in the Profiles of Sex Steroids and Gonadal Development of Brook Trout (*Salvelinus fontinalis*, Mitchill) during Puberty and the First Reproductive Cycle in Captivity

3.1 Abstract

Puberty and maturation are important reproductive events which require further investigation in brook trout. In particular the reproductive cycle under Tasmanian environmental conditions has not been studied. The present study describes the histological development of gonads and dynamics of estradiol-17 β (E₂), testosterone (T) and 11-ketotestosterone (11-KT) during the first and second reproductive year of male and female brook trout. Males attained puberty at the age of 14 months and 75% of total males were found to be mature however milt could be stripped from only 20% of total mature males. For males, the role of 11-KT in testicular development during puberty was more prominent than T while E₂ presumably play a role in proliferation of spermatogonia and sertoli cells. The peak of GSI in males was observed at 15 months of age (3.0 ± 0.9 %). Females on the other hand did not achieve maturation during their first year and their ovarian development was arrested at the vesicular stage. During the second reproductive year, gonadal development was initiated after the summer solstice in both sexes and both reached full maturity. In males, 11-KT played a more important role in regulating spermatogenesis and spermeiogenesis while final maturation was equally controlled by T and 11-KT. On the other hand, E₂ regulated ovarian development with its peak a month before the end of reproductive cycle. Activity of 3 β -hydroxysteroiddehydrogenase (3 β -HSD) and 17 β -hydroxysteroiddehydrogenase (17 β -HSD)

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was concomitant with the profiles of sex steroids. The highest values of GSI in males (3.4 ± 0.1 %) and females (17.6 ± 1.1 %) were observed at the age of 21 and 22 months, respectively. Between June and mid-July, milt could be manually stripped from all males but only 20% of females (n= 6) could ovulate by manual stripping. This period could be the natural spawning season under wild conditions. After this period, levels of sex steroids remained low in both male and female brook trout relative to that during the developing phase. Total and relative fecundity were observed as 4266 ± 341 oocytes/mature fish and 4 ± 0.24 oocytes/g of mature fish during the second reproductive year. During the second reproductive year variations in gonadal development were concomitant with seasonal changes in photoperiod. However during puberty, endogenous rhythm of maturation through the brain-pituitary-gonadal axis may be less influenced by photoperiod variations.

3.2 Introduction

Since the start of Tasmanian salmonid farming in 1998, production has increased significantly with the Tasmanian Aquacult. industry now producing 97% of Australia's total salmonid production (ABARES, 2010). Rainbow trout (*Onchorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) farming contributes the most to total salmonid production. To ensure sustainability and commercial diversity of these species, brook trout (*Salvelinus fontinalis*, Mitchill, 1814) is a potential alternative for industry. Brook trout was introduced to Tasmania in 1962 from Nova Scotia, Canada (Clements, 1988) and stocked into suitable ponds, lagoons, lakes (MacCrimm and Campbell, 1969) and farms for recreational fishing stocking purposes and supply for brood stock to commercial producers (S. Chilcott, Inland Fisheries Service, Pers. Comm.). Due to its potential importance in Tasmanian Aquacult. and the fact that no information has been reported on the reproductive biology of brook trout under Tasmanian environmental conditions this study aimed at describing the gonadal development during the first two years of life to study puberty and annual reproductive cycle.

Occurrence of early maturation during puberty and every year is a major constraint to its large commercial scale production. The high incidence of early sexual maturation in males affects flesh quality (Galbreath et al., 2003; Chiasson and Benfey, 2007) and results in high male mortality. Control of maturation is a major interest of industry which can be achieved by photoperiod manipulation. To design an efficient light protocol, complete data of gonadal development in relation to photoperiod is required to identify the stages which can open the window of opportunity for recruitment for maturation during puberty and annual reproductive cycle. In the absence of efficient sex reversal procedures for brook trout (investigated in

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chapter 4), farmers have mixed sex production of brook trout. Therefore both males and females were investigated here.

Puberty is the developmental period during which an individual fish becomes capable of reproducing sexually for the first time (Taranger et al., 2010). Major gonadal development leading to puberty commences sometime after sex differentiation during the first year in both sexes (Taranger et al., 2010). There is considerable phenotypic and genotypic variation in both age and size at puberty (Taranger et al., 2010), within and among strains and families (Gjerde et al., 1994; Wild et al., 1994) and between wild and farmed populations (Svasand et al., 1996). A strong relationship was found between body growth rates and age at puberty in salmonids and puberty can be delayed if energy reserves are insufficient (Silverstein and Shimma, 1994; Berglund, 1995; Friedland and Hass, 1996; Duston and Saunders, 1999; Thorpe, 2007). In farmed salmonids, males mostly achieve maturity at an age of 1 year as thresholds of energy reserves required for maturation can be achieved at smaller size and age (L'Abée-Lund, 1989; Silverstein and Hershberger, 1992; Clarke and Blackburn, 1994; Dziewulska and Domagala, 2004; McClure et al., 2007). On the other hand, salmonid females attain complete puberty mostly one year later than males because female fecundity and offspring survival are increased at increased body size (Aubin-Horth et al., 2006).

Puberty is an integrative mechanism of developmental events involving signals from environmental and internal factors (endocrine system) (Taranger et al., 2010). Integration of environmental signals with endocrine system stimulates the brain-pituitary-gonadal axis (BPG). This integration results in the development of gonads and fish achieve the first maturation (maturation is the stage when germ cell development is complete to produce fertilizable and viable gametes which can be released naturally from gonads under the

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hormonal cues or manual pressure. In case of males, sperm is the viable gamete. In females, mature egg contains miscible mixture of yolk and water). Photoperiod and water temperature act as important factors for initiation and completion of puberty. However at moderate and high latitude, photoperiod is regarded as the more important proximate key environmental factor compared to water temperature (Bromage et al., 2001). Variations in photoperiod control the rhythmic production of melatonin from the pineal gland which regulates the BPG circadian system in salmonids (Bromage et al., 2001). Sex steroids are the major product of BPG axis, synthesized by Δ^4 and Δ^5 steroidogenic pathways (Yamamoto, 1969). 3β -HSD and 17β -HSD are the major enzymes in steroidogenesis playing critical role in synthesis of T and E_2 (Kumar et al., 2000).

Onset of puberty after activation of the BPG axis is marked with spermatogonial proliferation in males as a result of follicle stimulating hormone (FSH) mediated growth factor release from sertoli cells. This process is triggered by 11-KT synthesis leading to meiosis and later stages of testicular development (Miura et al., 1991). 11-KT is also involved in the growth of pre-vitellogenic oocytes in *Anguilla japonica*, *Anguilla australis* (short finned eel) and *Gadus morhua* (Matsubara et al., 2003; Lokman et al., 2007; Kortner et al., 2009). Gonadal growth is largely controlled by plasma levels of T (Berglund et al., 1995) while E_2 regulates the growth of pre-vitellogenic oocytes during puberty (*Oncorhynchus kisutch*: Campbell et al., 2003). E_2 may induce proliferation of sertoli cells and infiltration of acidophilic granulocytes in males (Chaves-Pozo et al., 2007). However it is suggested that puberty is more under the control of gonadotropins than sex steroids (Okuzawa, 2002).

In the second reproductive cycle, the BPG axis becomes functionally more active and mature in comparison to the first year. Gonadal development is finely controlled by androgens,

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estradiol-17 β and progestins. Upon GTH stimulation, spermatogonial mitosis switches from slow self renewal to rapid proliferation (Schulz et al., 2010). In males, germ cell renewal is regulated by T and 11-KT (in *Clarias garieppinus*; Cavaco et al., 1998) while E₂ may also play a role at this stage (in *Anguilla japonica*: Miura et al., 1999; *Hucho perryi*: Amer et al., 2001; *Sparus auratus*: Pinto et al., 2006). Further development until formation of spermatids and their transformation into sperm are regulated by the action of 11-KT (*Carassius auratus*: Kobayashi et al., 1991; *Anguilla japonica*: Miura et al., 1991), supported by growth factors (Nader et al., 1999) and 17 α , 20 β -dihydroxyprogesterone (DHP) (Miura et al., 2007). Final maturation of sperm and release of milt is controlled by LH-induced synthesis of 11-KT and DHP as reported in *Oncorhynchus rhodurus* and *Oncorhynchus mykiss* (Ueda et al., 1985; Milla et al., 2008) which mediates intra tubular changes and pH control in the sperm duct to form milt (Schulz et al., 2010).

Oogenesis held at the vesicular stage during the first year is resumed in the second reproductive cycle. E₂ induces proliferation of oocytes while DHP may have a direct role in initiation of the first meiotic division of oogenesis (Miura et al., 2007). After meiotic arrest, E₂ continues its regulatory role in hepatic vitellogenin synthesis (Miura et al., 2007) and uptake of vitellogenin during vitellogenic stages (Tam et al., 1984; Tyler and Sumpter, 1996). 11-KT is involved in oil droplet accumulation and growth of pre-vitellogenic oocytes alongwith E₂ as observed in *Anguilla japonica* (Japanese eel), *Anguilla australis* and *Gadus morhua* (Atlantic cod) (Matsubara et al., 2003; Lokman et al., 2007; Endo et al., 2008; Kortner et al., 2009). However, DHP plays a more significant role in germinal vesicle breakdown (GVBD) (Duffey and Goetz, 1980) and finally ovulation (Goetz et al., 1987; Mayer et al., 1992).

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Precocious maturation in brook trout males during every annual reproductive cycle is a major constraint to its large scale commercial farming in Tasmania. A comprehensive study is required to understand these complex mechanisms to develop different suitable control measures for early maturation in this species. A few studies have investigated limited aspects of brook trout reproductive physiology (Duffey and Goetz, 1980; Tam et al., 1986, 1990; Goetz, 1987; Bascinar et al., 2003; Sacobie and Benefy, 2005; Holcombe et al., 2000; Serezli et al., 2010). Tam et al. (1986) described the annual reproductive cycle but in female brook trout only. They did not measure 11-KT which also plays a role in ovarian development (Lokman et al., 2007). Tam et al. (1990) commenced their study in June, only five months before spawning when females had already recruited for maturation and did not continue further to study the post-spawning stage. Therefore the control group in this study did not describe the whole annual cycle of brook trout. Among the important sex steroids they measured only E₂. GTH was measured by ultrastructural morphometry of secretory cells rather than measuring their plasma profiles by RIA or ELISA which are more reliable technique. Goetz et al. (1987) described variations in profile of E₂, DHP and GTH only at final stage of ovarian development. Stages other than mature were not been studied. Mount et al. (1988) measured only fecundity without any histological study of gonads and hormones analysis. Holcombe et al. (2000) focused to the narrow range of time periods (in weeks) to investigate the effect of photoperiodic manipulation during that period. Their control group was exposed to ambient photoperiod for that specific short time period. Data given about gonadal development during a short period cannot provide the details about variations in development and hormones profiles during reproductive cycle. These studies are extensive but with the reference of their targeted objectives such as effect of photoperiod regimes and pH not the reproductive cycle. It can be concluded that previous work available

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on gonadal development of brook trout is insufficient and focused to female. Their control groups could not be considered as a standard study to be referred for annual gonadal development of brook trout. Moreover, these studies have been performed on different strains and brood stock and fish were reared under different water temperature and tank conditions. Data given in these different studies cannot be put together for a descriptive comparison. That's why there was need to investigate the whole reproductive cycle under Tasmanian climate conditions and on those strains which have been introduced from Canada to study their physiological adjustments according to new environment.

In conclusion, the present study aimed at investigating the gonadal development during puberty (first year of life history) and annual reproductive cycle (second year of life history in present study) of brook trout in Tasmania. Moreover, the reproductive endocrinology and roles of environmental factors in gonadal development were also studied. This study is a pilot work to provide baseline data for any future study regarding control of maturation of brook trout in Australia as has been performed in chapter 5 under manipulated photoperiod.

3.3 Materials and Methods

To study the first and second annual reproductive cycles of brook trout, 4 month ($n = 200$; 1.54 ± 0.14 g) and 16 month old mixed-sex fish ($n = 300$; 356.20 ± 60.20 g) were transferred from a commercial fish farm (Mountain Stream Fishery, Targa, Tasmania; Latitude = $41^{\circ} 18' 01''$ S, Longitude = $147^{\circ} 22' 35''$ E, Elevation = 425 m) to the University of Tasmania, Aquacult. Centre during November 2009 (Launceston, Tasmania; Latitude = $41^{\circ} 23' 21.17''$ S, Longitude = $147^{\circ} 07' 35.49''$ E, Elevation = ~ 60 m). Both age groups were stocked into 4500 L experimental tanks (4 months old fish = 100 fish/4500 L; 16 months old fish = 150 fish/4500 L) and fed on a standard commercial diet (2% body weight/day) (Skretting, Australia). Photoperiod was ambient and the range of water temperature was largely ambient throughout the study with a heat chill unit (10 KW) used to prevent temperature increasing above 18°C during the summer months. Fish were reared for one year (November, 2009 – October, 2010).

3.3.1 Sample Collection

A total of eight sampling points were carried out to study the first year (March, 2010 – October, 2010; age = 8 – 15 months). Sampling points performed during the undifferentiated phase of gonads (November, 2009 – February, 2010) were not included in this study and these results have been presented in chapter 2. To study the second reproductive year a total of 12 sample points were conducted throughout the study period (November, 2009 – October, 2010; age = 16 – 28 months). At each sample point, fish from each age group were randomly selected, anesthetized by transfer into a 50 L tank containing 30 mg/L AQUI-S and killed by cranial blow. The eight month old fish too small to be bled, were frozen whole

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in liquid nitrogen. Frozen fish were stored at -80 °C until processed for whole-body steroid extraction. At this small size, ten fish were collected for whole-body steroid extraction and ten for fixation and histology. When fish grew large enough for blood sampling in April (age = 9 months), fish were then bled instead of whole-body freezing and only 10 fish were sampled (blood sample and histology from the same fish). A total of 10 fish were sampled at each sample point to study the second reproductive year. Blood samples were collected from the caudal sinus of fish using heparinised syringes, centrifuged at 3000 rpm for 15 minutes and plasma was stored at -80 °C until assayed for steroids.

Total body weight (nearest 0.1 g) and fork length (nearest 1.0 mm) were measured before dissection (in appendix II). During the first few months of the first year cycle fish were too small to remove the gonads and livers therefore the mid part of each fish was fixed in Davidson's fixative. At the age of 10 and 12 months the livers and gonads increased in size to be easily removed. Gonads and livers were removed and weighed (nearest 0.1 g). Liver samples from both cohorts were frozen in liquid nitrogen and then stored at -80 °C for crude lipid determination. For fish in their second year, one gonad of each pair per fish was frozen immediately in liquid nitrogen and stored at -80 °C for enzyme activity estimation. The other gonad was fixed in Davidson's fixative for histological study. Gonadosomatic Index (GSI) and hepatosomatic Index (HSI) were calculated by following formulae:

$$\text{Gonadosomatic Index (GSI)} = [\text{Gonad Weight (g)} / \text{Total body weight (g)}] \times 100$$

$$\text{Hepatosomatic Index (HSI)} = [\text{Liver Weight (g)} / \text{Total body weight (g)}] \times 100$$

Total body weight for all indices included the weight of gonads and liver.

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3.3.2 Histology

Tissues were fixed in Davidson's fixative for 24 h and then transferred to 70% ethanol. After fixation, tissues were dehydrated through different grades of ethanol (70%, 80%, 95%, 100%; two repeats in each grade), two repeats of xylene and finally embedded in paraffin to be sectioned at 5 μ m transversally and stained using routine haematoxylin and eosin staining (Horobin, 2002). Histology of each collected sample was performed. A total of 5 stained slides were prepared from each sample and studied at 10-1000 X magnification, using a light microscope (Olympus, Japan) and images captured using Leica DC300F digital camera (Leica Microsystems, Germany). Diameter of cells was measured by using the Enter Magnification plugin of Fovea Pro 4.0. Diameter of a total of 30 cells of each type was measured in each section of testis and ovary. The number of cells was counted by using the grid. Cell count was performed for each section of all samples i.e. five slides for each sample. Mean \pm SE was calculated.

Both testicular and ovarian development was classified into 5 and 6 stages respectively as described by Lone et al. (2009, 2012). Detailed key for classification of testicular and ovarian development has been given in appendix II.

3.3.3 Total Fecundity

Total fecundity was estimated as standing stock of advanced yolk oocytes in the ovary following Almatar et al. (2004). One piece of gonad (1 g) was taken from both the right and left ovary of each mature fish. Oocytes were separated from tissue and then counted in both pieces. The mean of these two values was performed to calculate the total number of ova

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present in entire pair of ovaries. Relative fecundity was also calculated following Serezili et al. (2010) by using the formula:

$$\text{Relative Fecundity} = [\text{Total Fecundity} / \text{Total Body Weight (g)}] \times 100$$

3.3.4 Whole-Body Steroid Extraction

For whole-body steroid extraction, each frozen fish was thawed and homogenized in 1 mL of 0.01 M PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH adjusted to 7.4) in a mortar and pestle. This homogenate was mixed with 8 mL of diethyl ether in 45 mL centrifuge tubes and vortexed for several minutes. The whole mixture was centrifuged at 3000 rpm for 15 minutes, after which time the tissue condensed at the bottom of the tube. Each tube was then incubated at -20°C for 2 hours. The unfrozen portion containing the solvent was decanted into a 10 mL tube and incubated in a water bath at 30 °C. When nearly 500 µL of solvent remained, this extract was transferred into 1 mL eppendorf tubes and evaporated at room temperature. The lipid extract containing steroids was stored at -80 °C until required for assay. Before assay each sample was reconstituted with 1 mL of PBS and vortexed for one minute (Simontacchi et al., 2009; Sink et al., 2007).

3.3.5 Hormone Analysis

Levels of T, E₂ and 11-KT were measured in whole-body lipids extracts (age = 8 months) and plasma (age = 9 – 27 months). At age of 8 – 9 months sex could not be identified by macroscopic study of gonads. Therefore hormone profiles of these unclassified samples at these two sampling points represent the mixed sex. Lipid extracts from whole-body fish were reconstituted with 1mL of PBS and 100 µL were used as per the specific requirement of each assay. The levels of E₂ and T in aliquots of reconstituted whole-body lipid samples and

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thawed plasma were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992). Extraction efficiency (mean recovery of ^3H -labelled steroid from triplicates of a plasma pool) was >90% for E_2 and T and values for each steroid were adjusted accordingly. The lowest detection limit of each assay was 100 pg/mL. Inter-assay variability measured using aliquots of a pooled internal standard was 6.98% and 9.99% for E_2 and T, respectively. Each sample was assayed in duplicates. To measure 11-KT in both plasma and reconstituted extracted whole-body lipid samples an enzyme-linked immunosorbent assay (ELISA) was performed as per manufacturer's instructions (Cayman, USA). This assay typically displays an IC_{50} (50% B/B_0) value of approximately 5 pg/ml and a detection limit (80% B/B_0) of approximately 1.3 pg/ml. Coefficient of variation was 0.9994 and value of cross reactivity with T was < 0.01%. Each collected sample was assayed in duplicates.

3.3.6 Quantitative Estimation of Activity of 3β -HSD and 17β -HSD in Gonads

Enzyme Extraction

The activity of 3β -HSD and 17β -HSD was estimated for fish in their second reproductive cycle only. The method described by Jarabak (1969), Jarabak et al. (1962) and Bhattacharyya et al. (2006) was followed. To estimate the activity of 17β -HSD, T and nicotinamide adenine dinucleotide phosphate (NADP) (Sigma Aldrich, USA, 96%) were used as substrate and cofactor, respectively. For 3β -HSD, dehydroepiandrosterone (DHEA) (Sigma Aldrich, USA, 98%) and nicotinamide dinucleotide (NAD) (Sigma Aldrich, USA, 98%) were used as substrate and cofactor, respectively. Gonads stored at -80°C were thawed and homogenized manually with 10 mL of homogenizing medium (0.25 M Sucrose, 20 mM Tris buffer; pH adjusted to 7.4). During homogenization a cold temperature was maintained by keeping the

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mortar in ice. The homogenate was centrifuged at 3000 rpm for 30 minutes at 4 °C, after which time the supernatant containing enzymes was aspirated.

Estimation of Enzyme Activity

In a spectrophotometer cuvette (Kartell), 600 µL of 0.1 M tetrasodium pyrophosphate buffer (pH 9.0) was added followed by 100 µL of 2.5 % BSA solution and then by the further addition of 100 µL of 1.1 mM NADP. Afterwards, 100 µL of enzyme extract was added and incubated for two minutes at room temperature (16 – 18 °C) until internal reduction of NADP had ceased. To this reaction mixture, 100 µL of 0.3 mMT was added and mixed immediately. Optical density at 340 nm was immediately observed in a UV / VIS spectrophotometer (UNICAM 8625) during the first few seconds for duration of 6 minutes. Samples were run one after another. The same protocol was followed for estimation of 3β-HSD, using DHEA as substrate and NAD as cofactor. Reagent blanks contained all ingredients except substrate.

Determination of Total Tissue Protein

Total tissue protein was determined by following the method of Lowry et al. (1951). A 100 µL aliquot of enzyme extract was taken in a 10 mL tube and diluted up to 1000 µL with distilled water. A total of 5 mL alkaline copper reagent and 500 µL of Folin phenol reagent were added to the extract, mixed very well and incubated for 30 minutes at room temperature for colour development. Optical density was measured at 750 nm against the distilled water as reagent blank. The standard curve of following concentrations was constructed, using BSA as standard.

Activity of each enzyme was estimated according to the following formula:

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One unit of enzyme activity = Change of 0.001 unit in absorbance

Enzyme activity was expressed in unit / mg of proteins / minute.

3.3.7 Determination of Crude Lipids in Liver

Liver samples stored at -80°C were defrosted, weighed and kept in a freeze drier (Dynavac, Australia) to remove the water content. Dried samples were homogenized and 300 mg of each sample was used to determine the total lipids in duplicate. The method described by Bligh and Dyer (1959) was used to extract total lipids with ethanol and chloroform as solvents.

3.3.8 Meteorological Data

Meteorological data for photoperiod from 1999 to 2009 was obtained from the Bureau of Meteorology, Australia (Fig. 3.1A). Water temperature of fish holding tanks was recorded with water temperature loggers (OneTemp, Australia) throughout the study period (November, 2009 – October, 2010).

3.3.9 Statistical Analysis

Monthly variations in all parameters were analysed by one way ANOVA after justifying assumptions by performing Shapiro-Wilk test and test of homogeneity of variance. Tukey's post-hoc test was applied for comparison of means. Linear regression was applied to describe the relationship of fecundity with body length. Log transformed regression equation was used to study the relationship between length and total body weight. Von Bertalanffy growth curve and test of likelihood were applied to study growth of both sexes. Confidence level was set at 95% for all statistical analysis. Means \pm standard error (SEM) are presented.

3.4 Results

3.4.1 Photoperiod and Water Temperature

Over the study period, the maximum value of photoperiod (15.04 ± 0.00 h) was observed in December (summer solstice) while its minimum (9.16 ± 0.00 h) was noted during June (winter solstice) (Fig. 3.1A). On the other hand, the maximum (17.78 ± 0.03 °C) and minimum (11.63 ± 0.03 °C) values of water temperature observed during December and July, respectively (Fig. 3.1B).

3.4.2 First Year (age = 8 – 15 months)

The first year of brook trout commenced after the gonadal differentiation period (0 - 3354 °dph). Details of gonadal differentiation are presented in chapter 2. The first year describes the developmental changes from 8 until 15 months of age.

Gonad Weight and Gonadosomatic Index (GSI) (age = 12 – 15 months)

Significant variance in means of gonadal weight ($F_{3, 18} = 5.90$, $P < 0.05$ in males; $F_{3, 12} = 1.77$, $P < 0.05$ in females) and GSI ($F_{3, 18} = 6.67$, $P < 0.05$ in males; $F_{3, 12} = 10.26$, $P < 0.05$ in females) was observed throughout the study period (Fig. 3.2). Over the first maturational year gonadal weight and GSI in males were observed within the range of 0.03 ± 0.01 - 5.85 ± 0.50 g and 0.03 ± 0.01 - 2.81 ± 0.98 %, respectively (Fig. 3.2A). Ovarian weight and GSI in females were observed within the range of 0.16 ± 0.04 - 0.82 ± 0.10 g and 0.14 ± 0.01 - 0.42 ± 0.17 %, respectively over the study period (Fig. 3.2B). In October (age = 15 months), 75% of the total males were found at mature stage however only 20% of total mature males

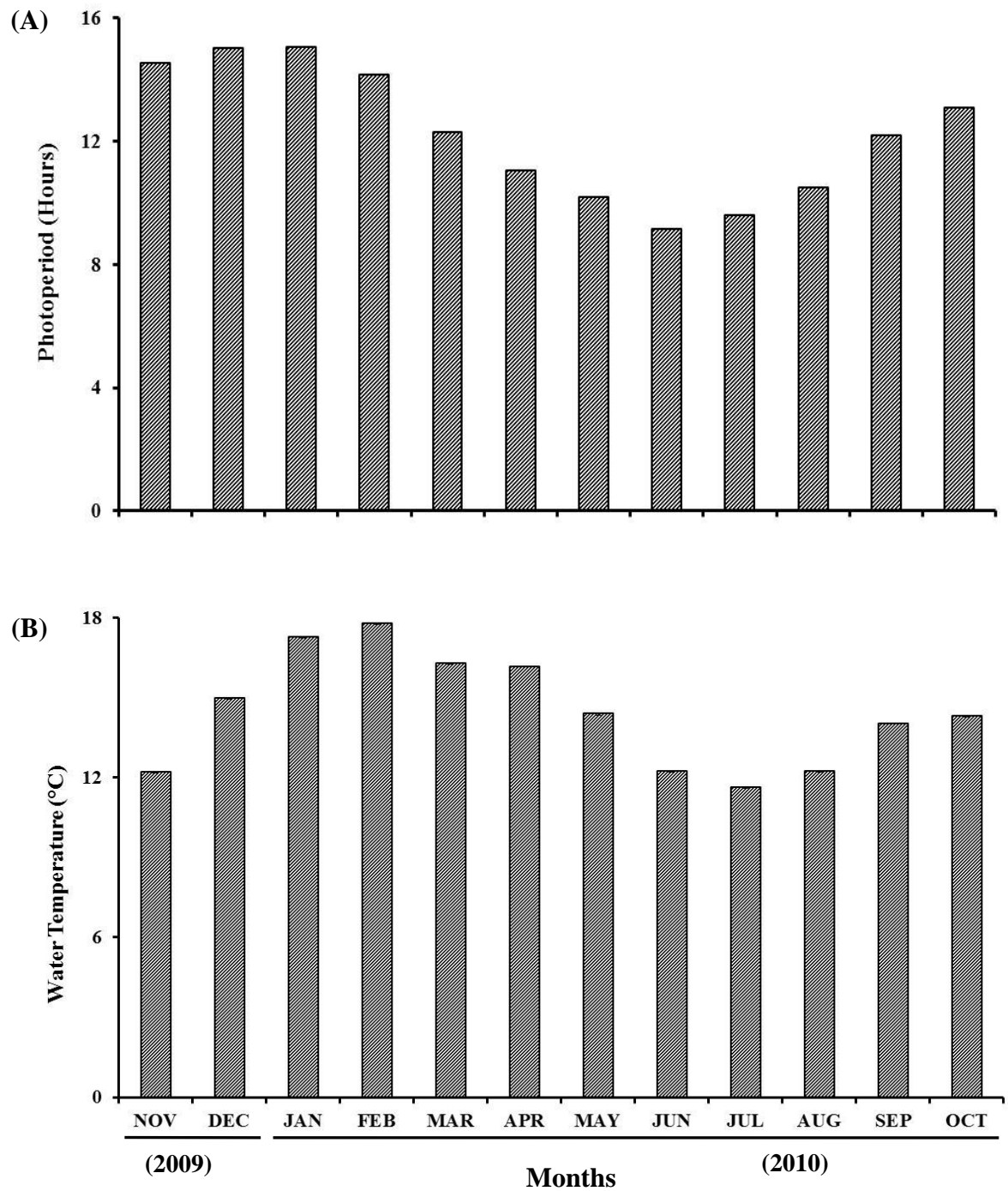


Fig. 3.1 Monthly variations in photoperiod (A) and water temperature (B) during the study period (November, 2009 – October, 2010).

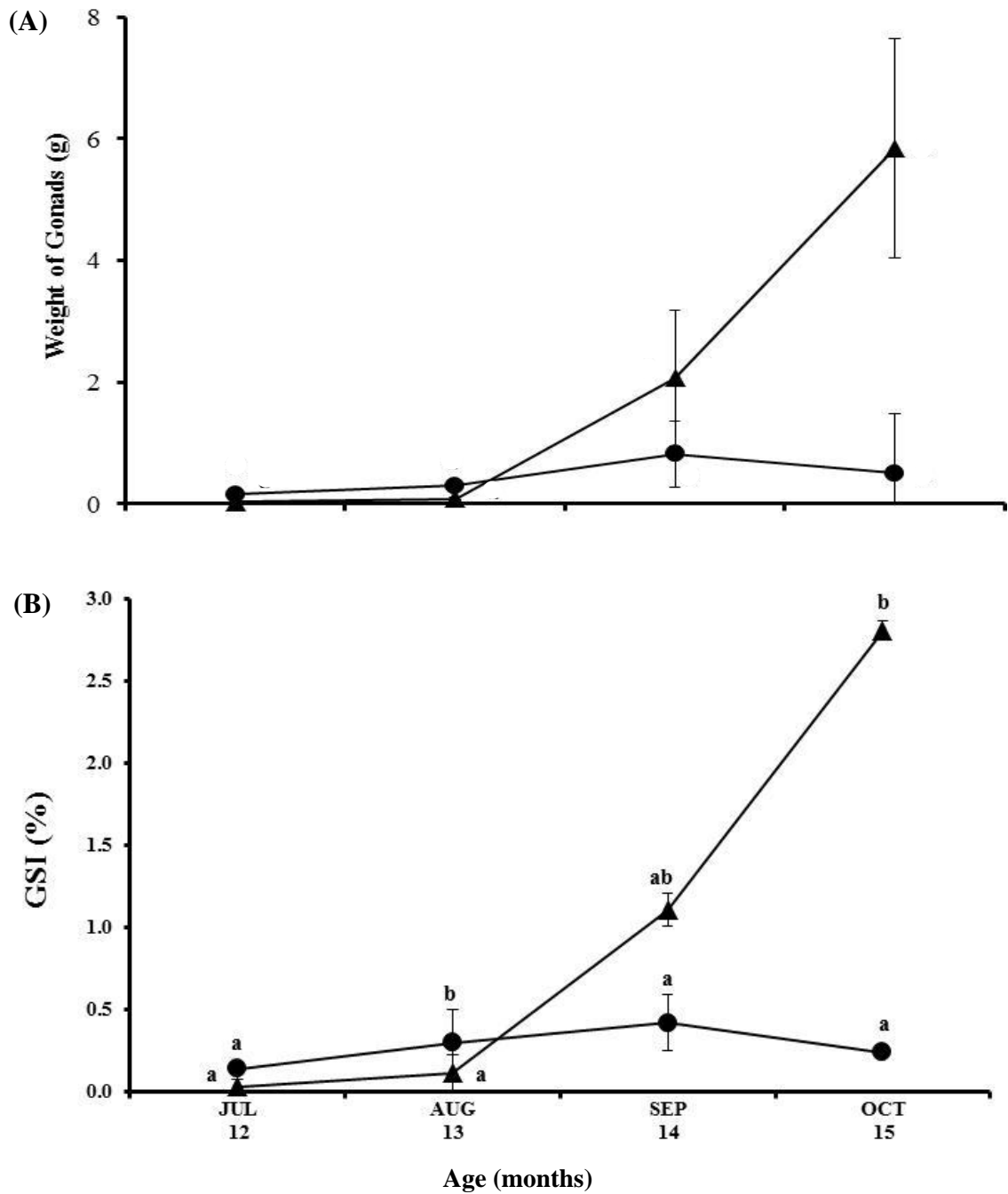


Fig. 3.2 Monthly variations (Mean \pm SEM) in gonadal weight (A) and GSI during the first maturational year of *Salvelinus fontinalis*. Gonadal weight during 8 – 11 months of age could not be obtained due to small size of gonads. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Female: \bullet , Male: \blacktriangle . n = 12 males; 18 females.

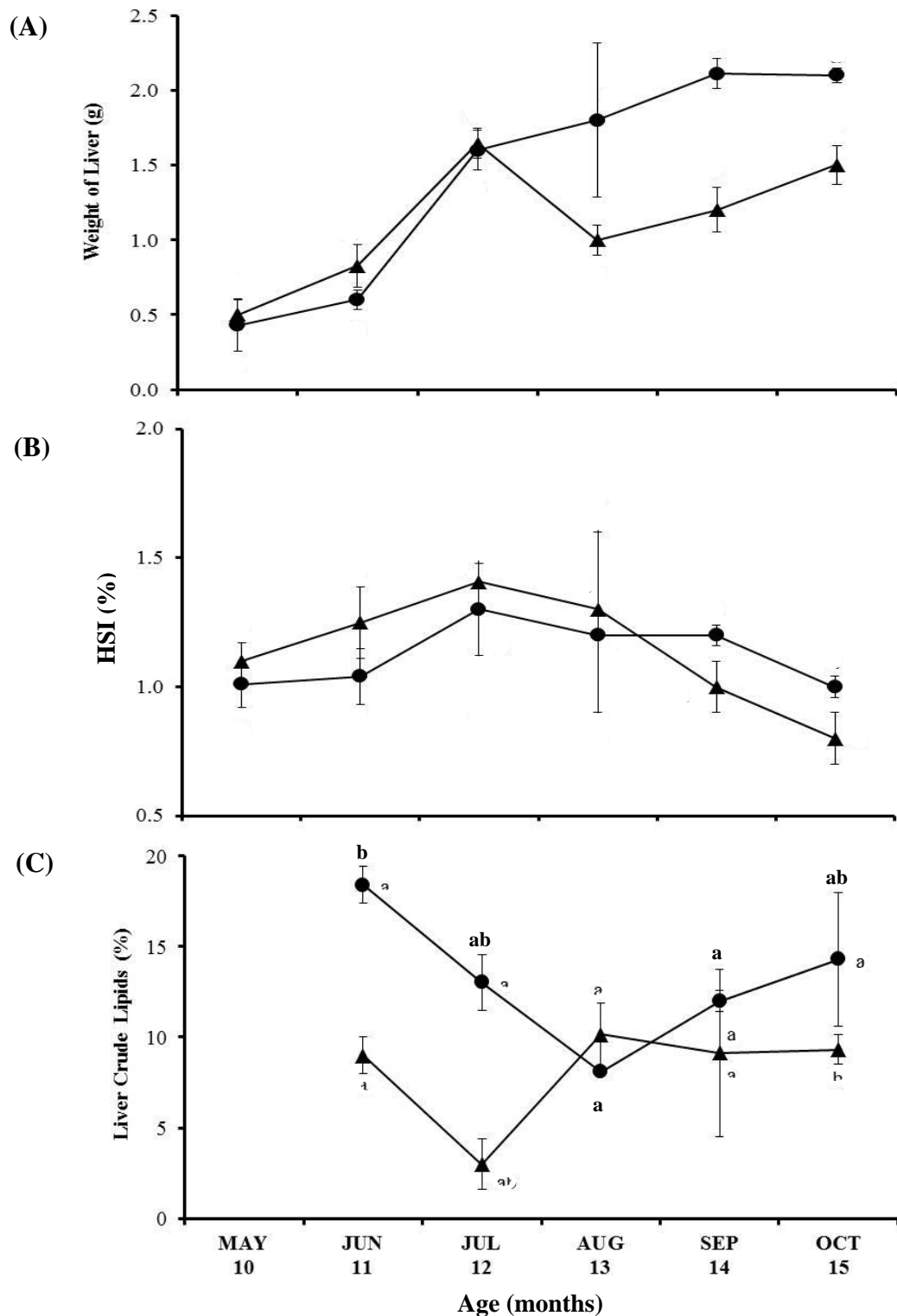


Fig. 3.3 Monthly variations (Mean \pm SEM) in weight of liver (A), HSI (B) and liver crude lipids (C) during the first maturational year of *Salvelinus fontinalis*. Liver weight and liver crude lipids could not be obtained until age of 10 and 11 months respectively due to small size of livers and dried weight. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Female: \bullet , Male: \blacktriangle . Liver weight and HSI: $n = 12$ males; 18 females. Liver crude lipids: $n = 12$ males; 18 females.

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achieved final maturation (hydration of milt and its release on abdominal pressure under tank conditions).

Liver Weight, Hepatosomatic Index (HSI) and Crude Lipids in Liver (age = 10 – 15 months)

Over the study period no significant variability ($P>0.05$) was observed in liver weight and HSI of both sexes (Fig. 3.3). Significant variability ($F_{4, 14} = 5.33$, $P<0.05$) was observed in liver crude lipids in females while liver crude lipids did not show significant variability ($P>0.05$) in males. In males, liver weight, HSI and liver crude lipids were observed between the range of $0.50 \pm 0.10 - 1.64 \pm 0.40$ g, $0.80 \pm 0.05 - 1.41 \pm 0.13$ % and $3.00 \pm 1.42 - 10.15 \pm 1.75$ %, respectively.

In females, liver weight and HSI were observed within the range of 0.43 ± 0.07 g – 2.11 ± 0.30 % and $1.00 \pm 0.04 - 1.30 \pm 0.18$ %, respectively. The highest value of liver crude lipids in females was observed at age of 11 months (18.42 ± 6.10 %) which dropped in the following month and remained variable until the end of the study.

Profiles of Hormones (age = 8 – 15 months)

Plasma profiles of T in males showed a significant variability ($F_{5, 28} = 3.77$, $P<0.05$) over the study period (Fig. 3.4A). Levels of T significantly increased at the age of 12 months (July) and reached its peak at the age of 14 months during September (2.55 ± 0.47 ng/mL). These high levels dropped during October. In females, levels of T remained insignificantly low throughout the study within the range of $1.02 \pm 0.77 - 1.90 \pm 0.48$ ng/mL in females. Plasma profiles of E_2 did not significantly vary ($P>0.05$) in males while significant variability was observed in the profiles of E_2 in females over the study period ($F_{5, 15} = 3.10$, $P<0.05$) (Fig. 3.4B). In males, levels of E_2 ranged between $0.07 \pm 0.01 - 0.65 \pm 0.42$ ng/mL over the

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study period. In females, levels of E₂ reached the highest value (1.80 ± 0.84 ng/mL) at the age of 13 months which decreased later and remained low until the end of the study. Plasma levels of 11-KT significantly increased in males ($F_{5, 28} = 2.68$, $P < 0.05$) at the age of 14 months and its peak (0.86 ± 0.06 ng/ml) was observed at an age of 15 months (September) (Fig. 3.4C). Mean values of 11-KT in females did not show any significant variability ($P > 0.05$) and ranged between 0.03 ± 0.01 – 0.31 ± 0.06 ng/mL in females.

Morphological and Histological Study of Gonads

Ovarian Development (age= 8 – 15 months)

Females remained immature throughout the study period (age= 8 – 15 months) (Fig. 3.5A). During the age of 8 – 9 months, immature ovaries contained peripheral nucleolar oocytes (PNO) which increased in size (Fig. 3.6 A) and number showing the simultaneous development of oogonia into PNO (Fig. 3.5A). Endogenous vitellogenesis (synthesis of lipids in oocytes) began at the age of 9 months and lipid droplets could be seen on the periphery of the oocytes called as early vesicular oocytes (EVE) (Fig. 3.5A). At the age of 10 months ovaries could be macroscopically differentiated from testes. During the age of 10 – 13 months, EVE developed into the late vesicular oocytes (LVE) with a significant increase in cell size (Fig. 3.6 A) and accumulation of oil droplets throughout the cytoplasm (Fig. 3.5B). LVE were surrounded by layers of thecal, granulosa cells and zona radiata. Ovarian development remained arrested at LVE oocytes until the end of study at an age 15 months.

Testicular Development (age= 8 – 15 months)

During the age of 8 – 9 months, both testes and ovaries could not be differentiated macroscopically. Histologically, all males were immature (Fig. 3.5C). Spermatogonial cysts

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were formed. Males remained immature during the age of 10 – 13 months however testes could be morphologically identified. Spermatogonia were found continuously dividing while a few large quiescent spermatogonia were also present in the interstitial spaces. Testis showed a rapid development at the age of 14 months and found to be at the maturing stage. At this stage, spermatogonia developed into the primary spermatocytes (PSC) which underwent further development to form the secondary spermatocytes (SSC). Spermatids and sperm were also observed at the age of 14 months (Fig. 3.5D). In October (age = 15 months), 75% of the total males were found at mature stage however only 20% of total mature males achieved final maturation (hydration of milt and its release on abdominal pressure under tank conditions) (Fig. 3.5E). Secondary sexual characters (hooked jaw, abdominal coloration) appeared in mature males.

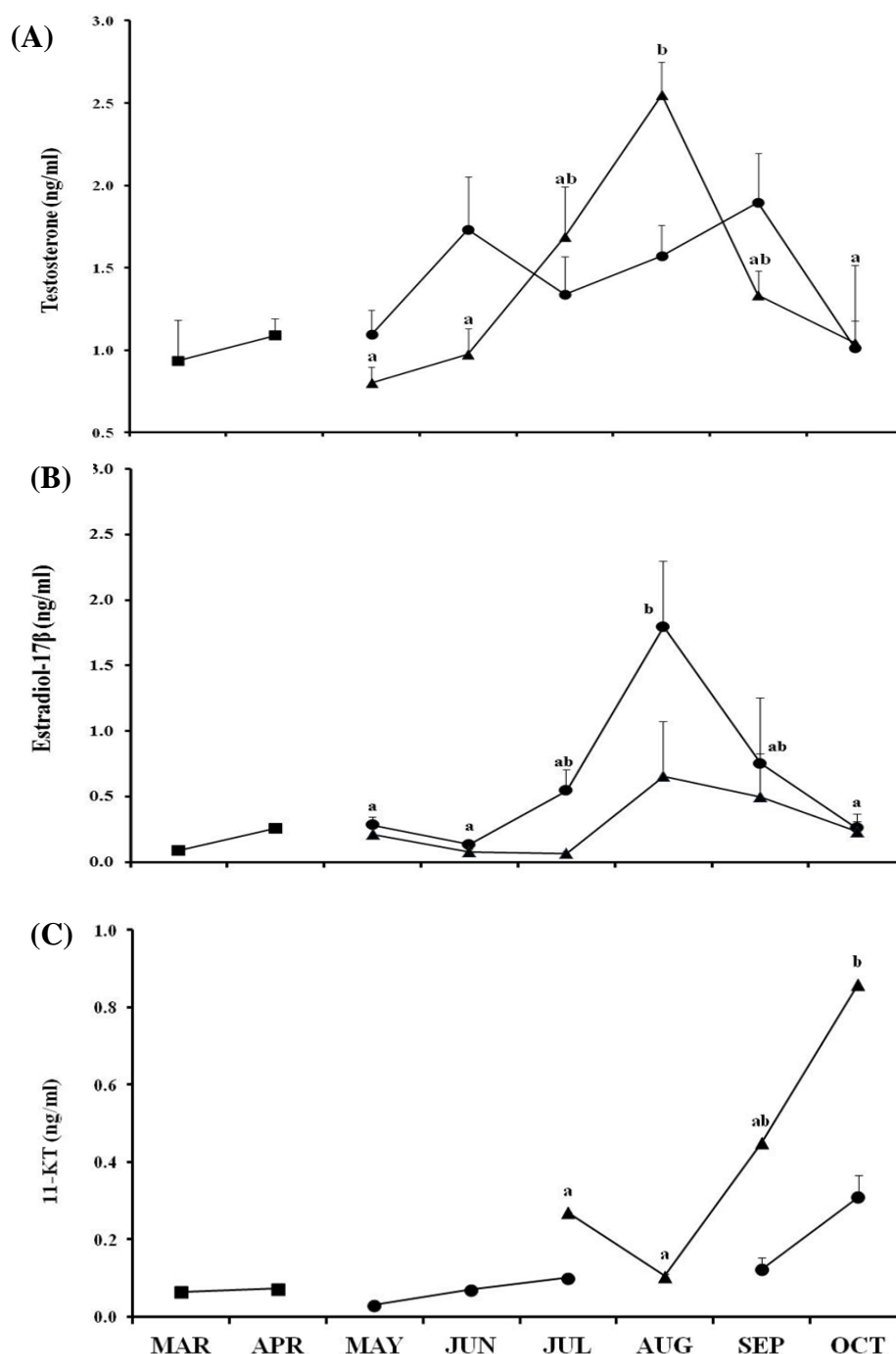


Fig. 3.4 Monthly variations (Mean \pm SEM) in profile of testosterone (A), estradiol-17 β (B) and 11-KT during the first year of *Salvelinus fontinalis*. Hormone profiles were measured from whole-body lipids extracts during March (age = 8 months). Sex could not be macroscopically identified during the age of 8 – 9 months and samples were labelled as mixed. Detection limit (80% B/B₀) of 11-KT assay was approximately 1.3 pg/ml. Values of 11-KT below detection limit are not shown. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Mixed: ■, Female: ●, Male: ▲. n = 15 males; 15 females.

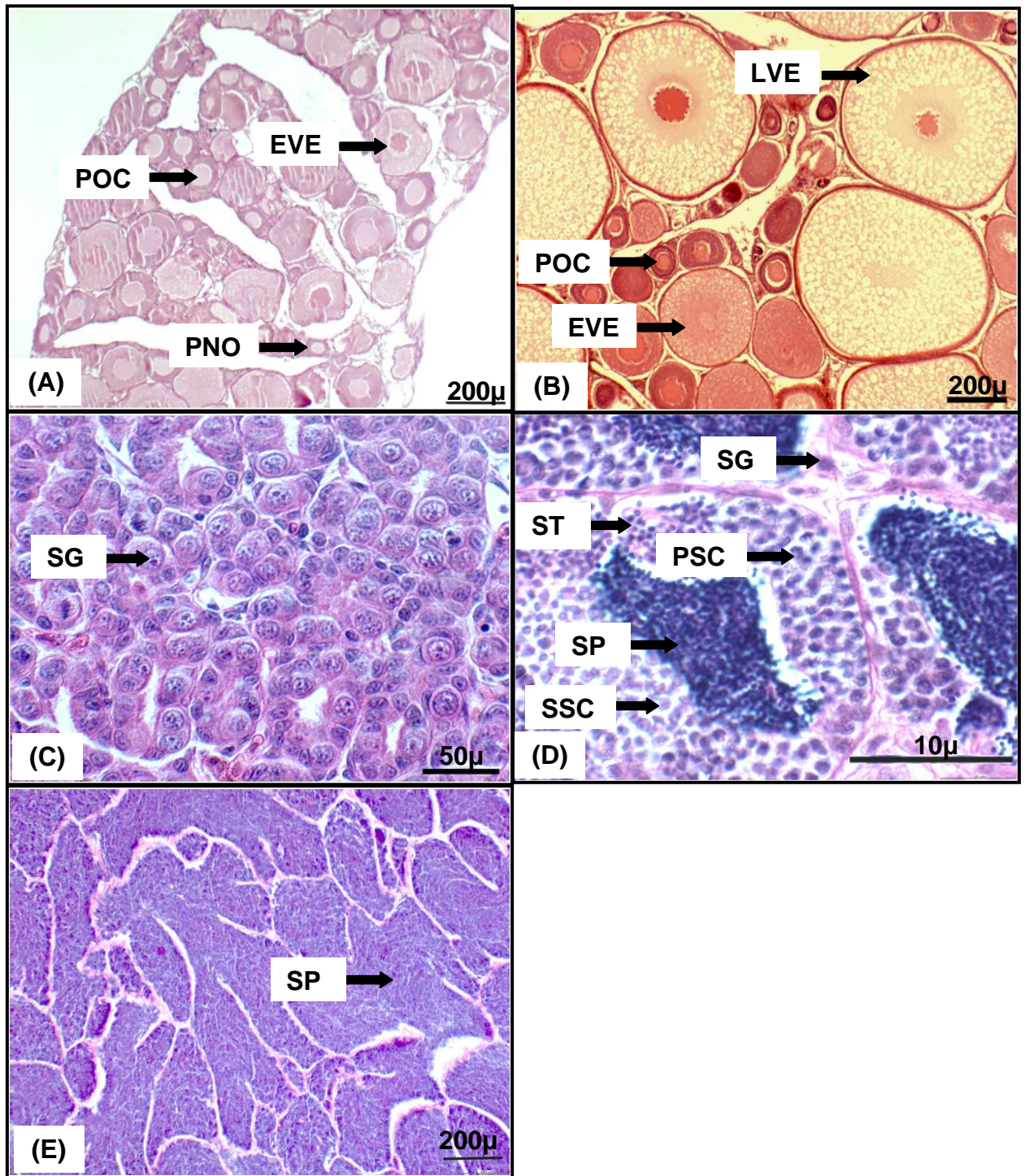


Fig. 3.5 Developmental changes during the first year of *Salvelinus fontinalis* (March, 2010 – October, 2010; age= 8 – 15 months). (A) Immature ovaries at the age of 8 – 9 months, (B) Immature ovaries at the age of 10 – 15 months, (C) Immature testes at the age of 8 – 13 months, (D) Maturing testes at the age of 14 months, (E) Mature testes at the age of 15 months. n= 10 at each sample point but the proportion of males and females varies. EVE: early vesicular oocyte; LVE: late vesicular oocytes; PNO: peripheral nucleolar oocyte; POC: primary oocyte; PSC: primary spermatocyte; SG: spermatogonium; SSC: secondary spermatocyte; SP: sperm; ST: spermatid.

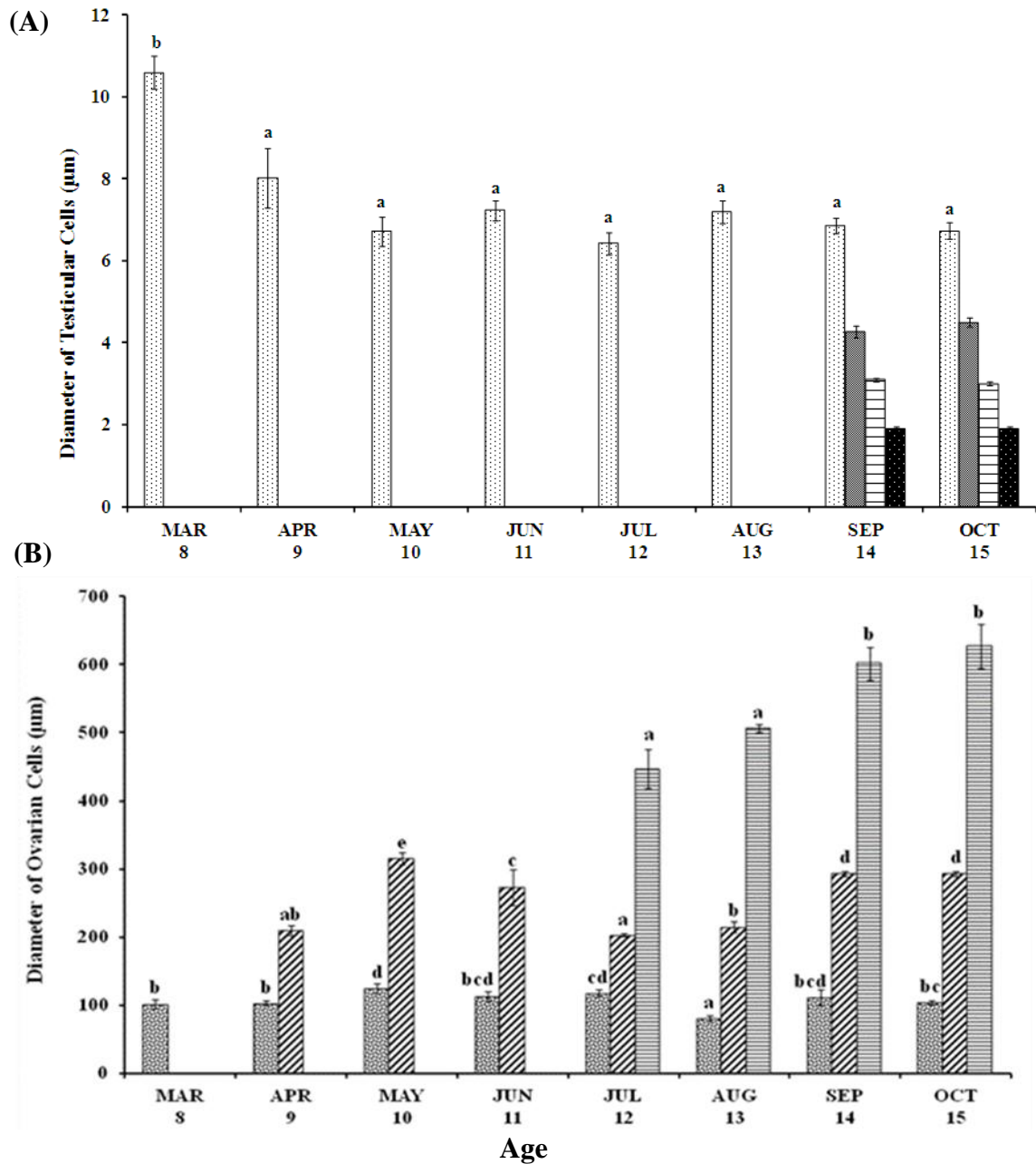









Fig. 3.6 Variations (Mean \pm SEM) in diameter of testicular and ovarian cells during the first maturational year (2010) of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$) or number of groups was less than three. SG: spermatogonia  ; PSC: primary spermatocyte  , SSC: secondary spermatocyte  ; ST: spermatid  ; PNO: peripheral nucleolar oocyte  ; EVE: early vesicular oocyte  ; LVE: late early vesicular oocyte  . n = 15 males; 15 females.

3.4.3 Second Year (age = 16 - 27 months)

Study of the second year began at the age of 16 months and continued until 27 months.

Gonad Weight and Gonadosomatic Index (GSI)

Significant variability was observed among monthly mean values of gonadal weight ($F_{11, 47} = 17.73$, $P < 0.05$ in males; $F_{11, 47} = 49.58$, $P < 0.05$ in females) and GSI ($F_{11, 47} = 25.06$, $P < 0.05$ in males; $F_{11, 47} = 58.46$, $P < 0.05$ in females) in both sexes throughout the study period (Fig. 3.7). Gonad weight and GSI in both sexes started increasing at age of 19 months in February. Following February, these parameters increased in males and their highest values (39.10 ± 5.30 g; 3.44 ± 0.11 %) were observed in April at age of 21 months. These peak values suddenly dropped in July (age = 24 months) and remained low until the end of the study.

Similarly, ovarian weight and GSI gradually increased and their highest values (226.00 ± 28.30 g; 17.00 ± 1.77 %) were observed in June at age of 23 months. These parameters showed a sudden decline in females during July and remained low afterwards.

Liver Weight, Hepatosomatic Index (HSI) and Liver Crude Lipids

Liver weight showed significant variability over the study period ($F_{11, 47} = 4.46$, $P < 0.05$ in males; $F_{11, 47} = 14.81$, $P < 0.05$ in females) while no significant variability ($P > 0.05$) was observed in HSI for both sexes (Fig. 3.8A and B). Liver weight in both sexes remained variable during the study period and the highest values (18.42 ± 3.30 g in males and 26.34 ± 0.40 g in females) were observed at age of 26 and 27 months, respectively. Over the course of

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second maturational year HSI ranged between $0.84 \pm 0.10 - 1.48 \pm 0.75 \%$ and $1.14 \pm 0.14 - 2.56 \pm 0.05 \%$ in males and females, respectively.

Significant variability was observed in liver crude lipids in both sexes ($F_{11, 47} = 11.97$, $P < 0.05$ in males; $F_{11, 47} = 4.68$, $P < 0.05$ in females) over the study period (Fig. 3.8C). Liver crude lipids gradually increased in both males and females and reached their highest levels in May at age of 22 months ($17.85 \pm 2.23 \%$ in males and $26.00 \pm 3.25 \%$ in females). These values dropped in July and remained low until end of the study.

Activity of 3β -HSD and 17β -HSD

Significant variability was observed between mean values of 3β -HSD ($F_{11, 72} = 9.81$, $P < 0.05$ in males; $F_{11, 72} = 8.37$, $P < 0.05$ in females) and 17β -HSD ($F_{11, 72} = 8.99$, $P < 0.05$ in males; $F_{11, 47} = 4.94$, $P < 0.05$ in females) (Fig. 3.9). Profiles of 3β -HSD and 17β -HSD in males reached their highest levels during June (308.06 ± 77.67 units/mg of proteins/min of 3β -HSD; 312.01 ± 54.53 units/mg of proteins/min of 17β -HSD) at age of 23 months. These peak values dropped suddenly in July but showed a rise in August which remained variable until end of the study. In females activity of both enzymes increased after March and the highest activity of 3β -HSD and 17β -HSD was observed in August (240.70 ± 63.63 units/mg of proteins/min) and September (193.72 ± 65.85 units/mg of proteins/min), respectively.

Profiles of Testosterone (T), Estradiol- 17β (E_2) and 11-ketotestosterone (11-KT)

Significant variability was observed between monthly means of T throughout the study period in both sexes ($F_{11, 35} = 6.15$, $P < 0.05$ in males; $F_{10, 39} = 59.32$, $P < 0.05$ in females) (Fig. 3.10A). In males, levels of T started increasing in March and after a drop in May reached its peak in June (48.00 ± 6.58 ng/mL) at age of 23 months. This peak was followed by a

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significant decline in July at age of 24 months which remained low until end of the study. In females, concentration of T increased during March (age = 20 months) and its highest levels was observed in May at age of 22 months (85.52 ± 7.62 ng/mL). This peak level of T dropped during June and remained low until end of the study.

Profiles of E_2 showed significant variability over the study period in females ($F_{11, 44} = 3.27$, $P < 0.05$) while no significant variability ($P > 0.05$) was observed in males (Fig. 3.10B). Levels of E_2 in males ranged between 0.01 ± 0.00 and 0.20 ± 0.15 ng/mL. On the other hand in females, levels of E_2 increased during February and its highest value was observed in March at age of 20 months (23.80 ± 4.62 ng/mL). This peak level dropped in April and remained low until end of the study.

Profiles of 11-KT showed the significant variability over the study period in males ($F_{11, 46} = 6.24$, $P < 0.05$) while insignificant variability ($P > 0.05$) was observed in females (Fig. 3.10C). The first peak of 11-KT in males (70.20 ± 18.70 ng/mL) was observed in April at age of 21 months. A sudden drop was observed in levels of 11-KT in May which again increased and its second peak (26.33 ± 6.47 ng/mL) was observed in June at age of 23 months. Following this second peak, levels of 11-KT dropped again in July (age = 24 months) and remained low until end of the study. Similar to males 11-KT showed two peaks in females during April (16.62 ± 4.11 ng/mL) and June (15.81 ± 5.37 ng/mL) and afterwards remained low until end of the study.

Fecundity

Total and relative fecundity were calculated during April-May (age = 21-22 months). Total fecundity was 4266 ± 341 oocytes per mature fish while relative fecundity was calculated at

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4 ± 0.24 oocytes/g of mature fish. Linear regression revealed a significant relationship between total fecundity and body length ($P < 0.05$).

Fork Length = $512.02 (\text{Total fecundity}) - 15959$

However, no linearity was found between length and relative fecundity ($P > 0.05$).

Morphological and Histological Study of Testicular Development

Cycle of testicular development was classified into five stages based on histological changes.

Stage I (Immature)

(November – January; age = 16 – 18 months)

During the immature stage, testes were very thin and white in colour. Stage I began with mitotic division of spermatogonia resulting in the formation of spermatogonial cysts (Fig. 3.11A). At this stage, spermatogonia could be classified into two types i.e. spermatogonia A (SGA) and spermatogonia B (SGB). SGA were larger in size (Fig. 3.12) and a few in number (Fig. 3.13A). They were present interstitially near the periphery of cysts. SGB were smaller in size and many in number, present within the spermatogonial cysts only. The cysts of SGB were formed by further proliferation of SGA. SGB were changed into primary spermatocytes (PSC) in December (age = 17 months). PSC further developed into secondary spermatocytes (SSC). Cysts contained all three types of cells having SSC in middle while SGB and PSC on their periphery. The population size of SSC was smaller than those of PSC and SGB (Fig. 3.13A). At the end of stage I, all PSC developed into SSC (all types of germ cells at different developmental stage is given in Fig. II.4 in appendix II).

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Stage II (Maturing)

(February – April; age = 19 – 21 months)

Increase in testicular size was observed with the formation of distinct lobes. Testes were creamy white in colour with prominent blood vessels. Due to the increase in testicular length, sperm duct was shortened. Stage II was marked with transformation of SSC into spermatids (ST) (Fig. 3.11B). Walls of cysts ruptured. Ruptured cysts merged to form lobules. Leydig's cells were observed in interstitial spaces. Sperm appeared in February and population size gradually increased over this stage (Fig. 3.13A).

Stage III (Mature)

(May – June; age = 22 – 23 months)

All males were mature and released milt by slightly pressing their abdomen. Blood vessels were very prominent, especially the dorsal vessel. Testes were very soft and creamy white in colour. Lobules were merging and testes were filled with sperm (Fig. 3.11C). A few SG, SSC and ST were seen along the lobular walls. Until the end of the stage III, all SSC and ST developed into sperm (3.13A). A few SGA were also present in interstitium with Leydig's cells. Vasa deferens developed to release milt into sperm duct. Tunica albuginea was very thin due to milt pressure after hydration.

Stage IV (Spent)

(July; age = 23 - 24 months)

Sperm were resorbed during June and mid-July and testes were found very thin as observed at stage I but darker in colour. Sperm duct was again long and thin. Lobules were collapsed

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and empty after resorption (3.11D). Residual cysts containing ST and SP were present. SG were observed in stromal tissue and considered as the most advanced stage at spent phase. No Leydig's cell was observed. Tunica albuginea was extremely folded due to shrinkage after spawning.

Stage V (Regressed)

(August – October; age = 25 – 27 months)

Stage V was marked with rapid mitotic division of SG left after resorption (3.11E; 3.13A). Spermatogonial cysts were formed but not compact until end of stage V. Residual cysts disappeared later and only a few were seen in October.

Morphological and Histological Study of Ovarian Development

The annual ovarian cycle was classified into six stages based on histological and morphological changes.

Stage I (Immature)

(November; age = 16 months)

Ovaries were loose and small in size. Stage I began with endogenous vitellogenesis and initiation of secondary growth. Cortical alveoli were found accumulated in cytoplasm of early vesicular oocytes (EVE) (3.14A). The most advanced oocytes were at late vesicular stage (LVE) with well developed three layers of thecal layer, granulosa layer and zona radiata. A few oocytes were found at α stage of atresia.

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Stage II (Developing)

(December - January; age = 17 – 18 months)

White oocytes could be seen through a very thin tunic. Exogenous vitellogenesis began in December and yolk granules appeared at periphery and interior to form early vitellogenic oocytes (EV) (Fig. 3.14B). At the end of this stage yolk granules coalesced to form yolk globules and oocytes developed into mid vitellogenic oocytes (MV). Cortical alveoli were displaced towards the peripheral ooplasm as yolk globules accumulated centripetally. α stage of atresia was observed in a few oocytes.

Stage III (Maturing)

(February - May; age = 19 – 22 months)

Ovaries were compact containing translucent and orange oocytes. Maturing phase began with termination of vitellogenesis. Yolk globules coalesced to form massive polygonal yolk plates and most of the MV developed into late vitellogenic oocytes (LV) (3.14C). Germinal vesicle breakdown (GVBD) and germinal vesicle migration (GVM) towards animal pole probably occurred in April. Waves of new POC and their further development continued throughout the maturing (Fig. 3.13B). The diameter of LV gradually increased due to accumulation of yolk (Fig. 3.15). At the middle of stage III, interstitial spaces were being filled with interstitial fluid. Cortical alveoli were present along the oocyte periphery. In May, no atretic oocyte was seen and 67% oocytes were at LV stage.

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Stage IV (Mature)

(June; age = 23 months)

A marked increase in ovarian size was observed at this stage. All late vitellogenic oocytes were developed into mature eggs (MC) (late vitellogenic oocytes absorb water and at this stage these hydrated oocytes are considered as mature eggs) by initial uptake of water (3.14D). No oocyte was found at vitellogenic stage. Mature eggs had very thick ZR and cortical alveoli were present along the ZR. Ovulated ova (OO) had very thick chorion (figure of ovulated oocyte has been given in Fig. II.4 in appendix II). There was no significant difference among diameters of MC and OO ($P>0.05$) (Fig. 3.15). Many blood vessels could be seen in fluid filled interstitial spaces.

Stage V (Spent)

(July; age = 24 months)

Spent phase described the histological changes after final maturation. A total of 20% mature females ovulated and released eggs on manual stripping. In ovulated females, tunic was ruptured and eggs were ovulated in the abdomen. Ovaries were loose and blood vessels were broken. A thick tunic was folded enclosing collapsed tissue. All non-ovulated mature eggs were being atretic and were found at α stage of atresia (Fig. 3.14E). Ovaries contained post-ovarian follicles (POF) at stage I. POF contained debris of outer thecal and inner granulosa layer enclosing lumen. Non-atretic EV and MV were observed other than LVE and POC.

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Stage VI (Recruiting)

(August - October; age = 25 - 27 months)

Ovaries contained non-ovulated and atretic oocytes (Fig. 3.14F). In August, atretic follicles were 63% of the total cell population which reduced up to 1% in October. Recrudescence continued during stage VI resulting in increase in population of POC (Fig. 3.13B).

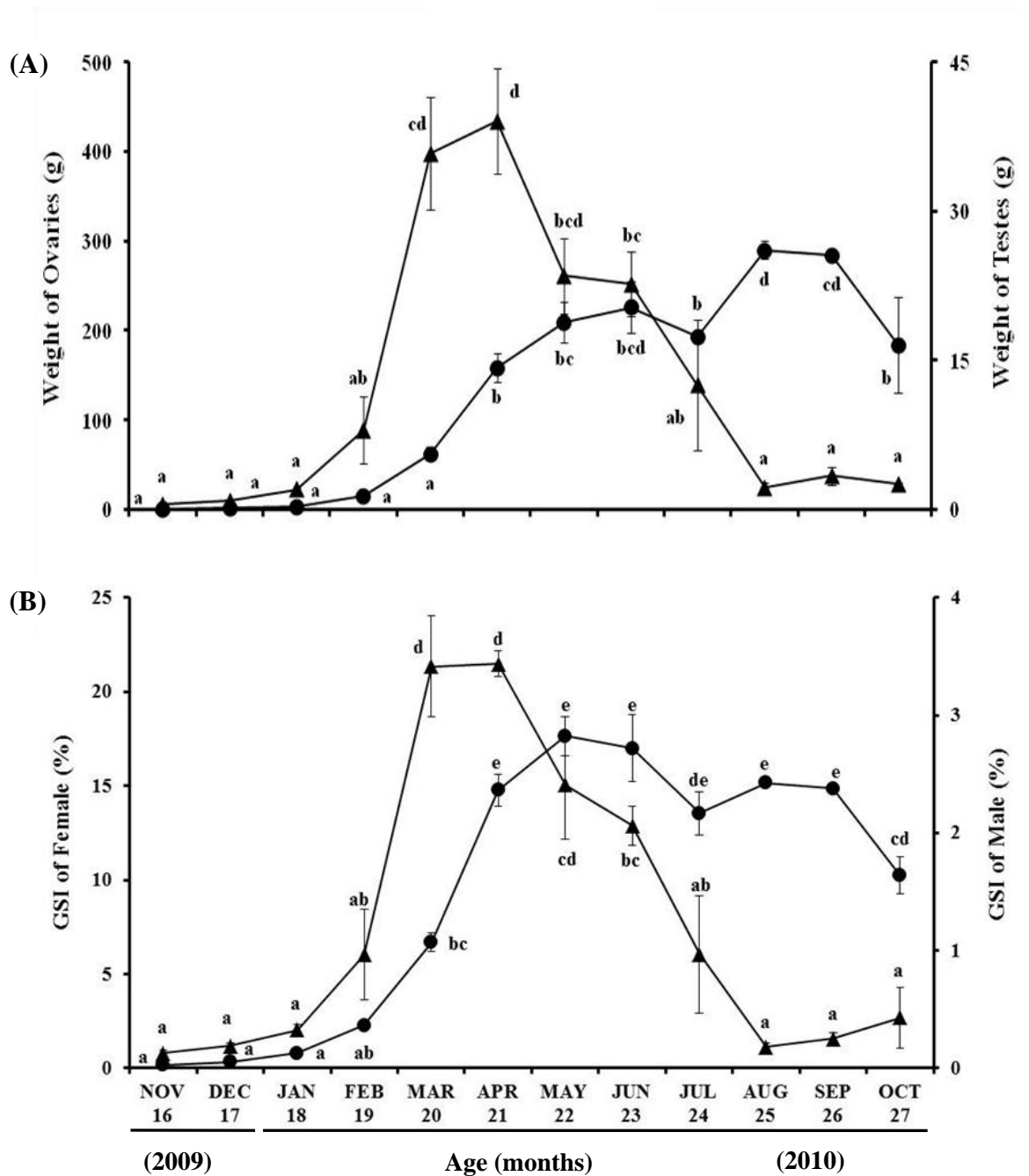


Fig. 3.7 Monthly variations (Mean \pm SEM) in gonad weight (A) and GSI (B) during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. Means of ovarian weight and GSI of females from August until September include values of non ovulated ovaries also. Female: ●, Male: ▲. $n = 47$ males; 47 females.

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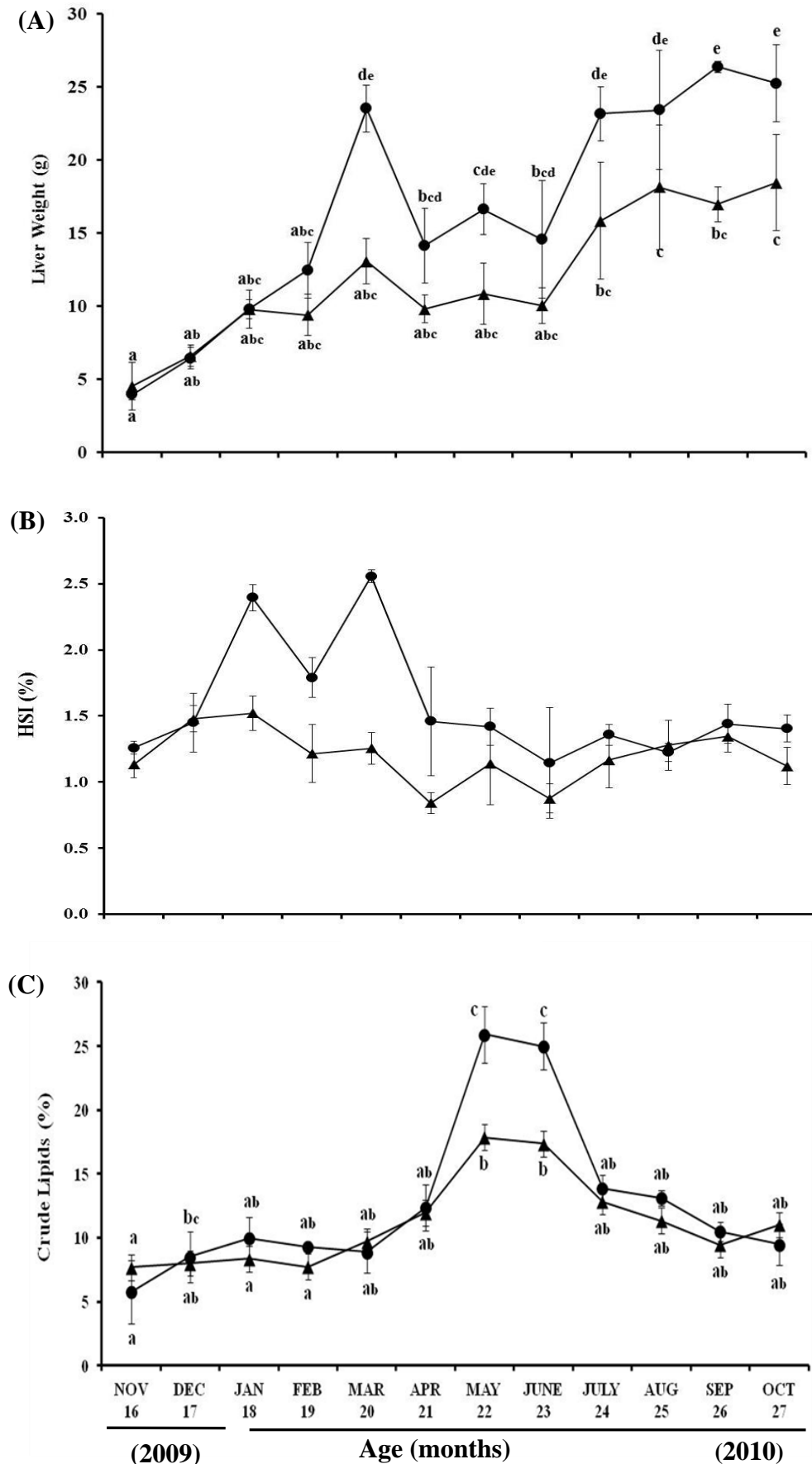


Fig. 3.8 Monthly variations (Mean \pm SEM) in weight of liver (A), HSI (B) and liver crude lipids (C) during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Female: \bullet Male: \blacktriangle . n = 47 males; 47 females.

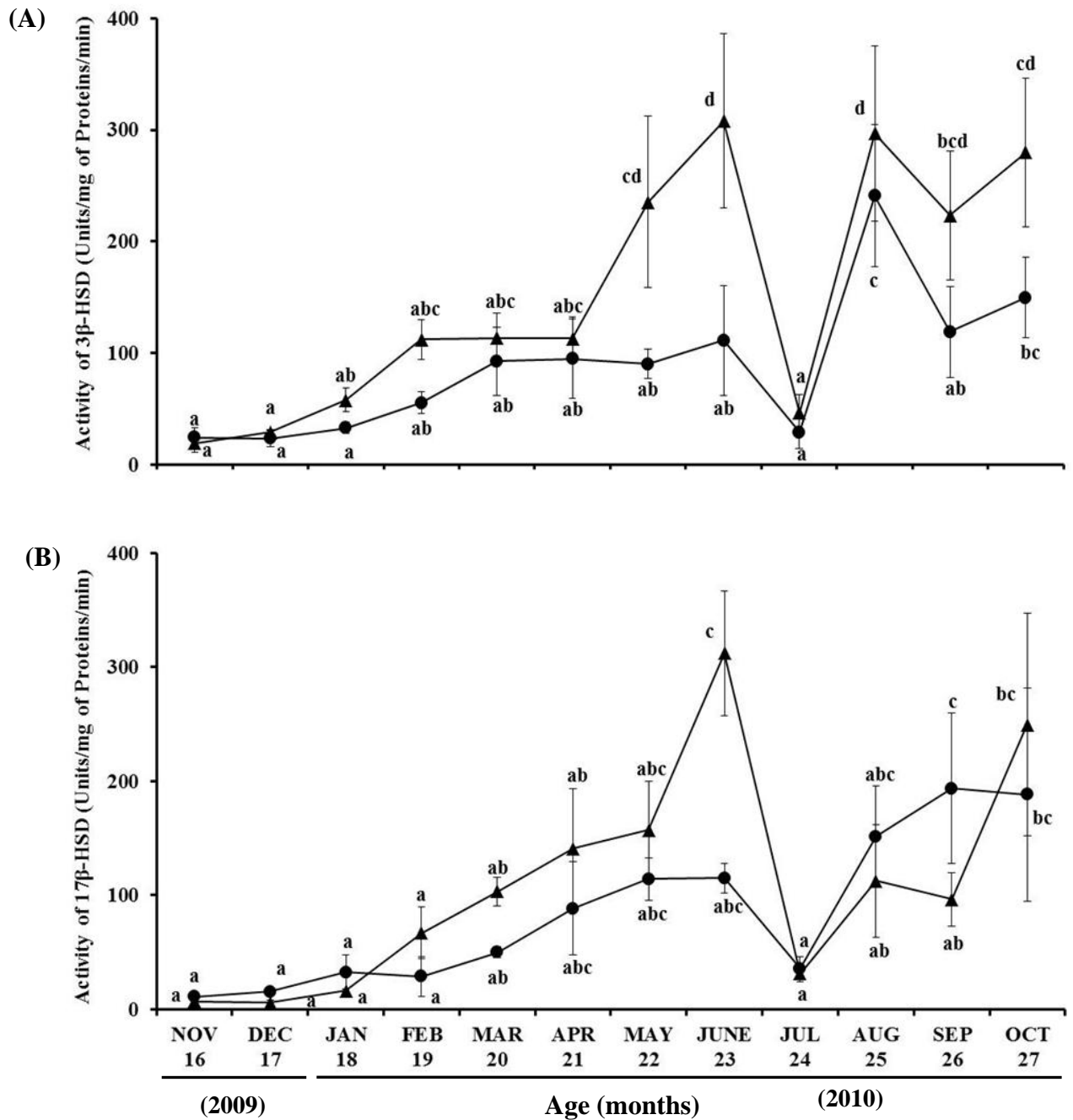


Fig. 3.9 Monthly variations (Mean \pm SEM) in activity of 3 β -Hydroxysteroid dehydrogenase (A) and 17 β -Hydroxysteroid dehydrogenase (B) during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. Female: ●, Male: ▲. n= 72 males; 72 females.

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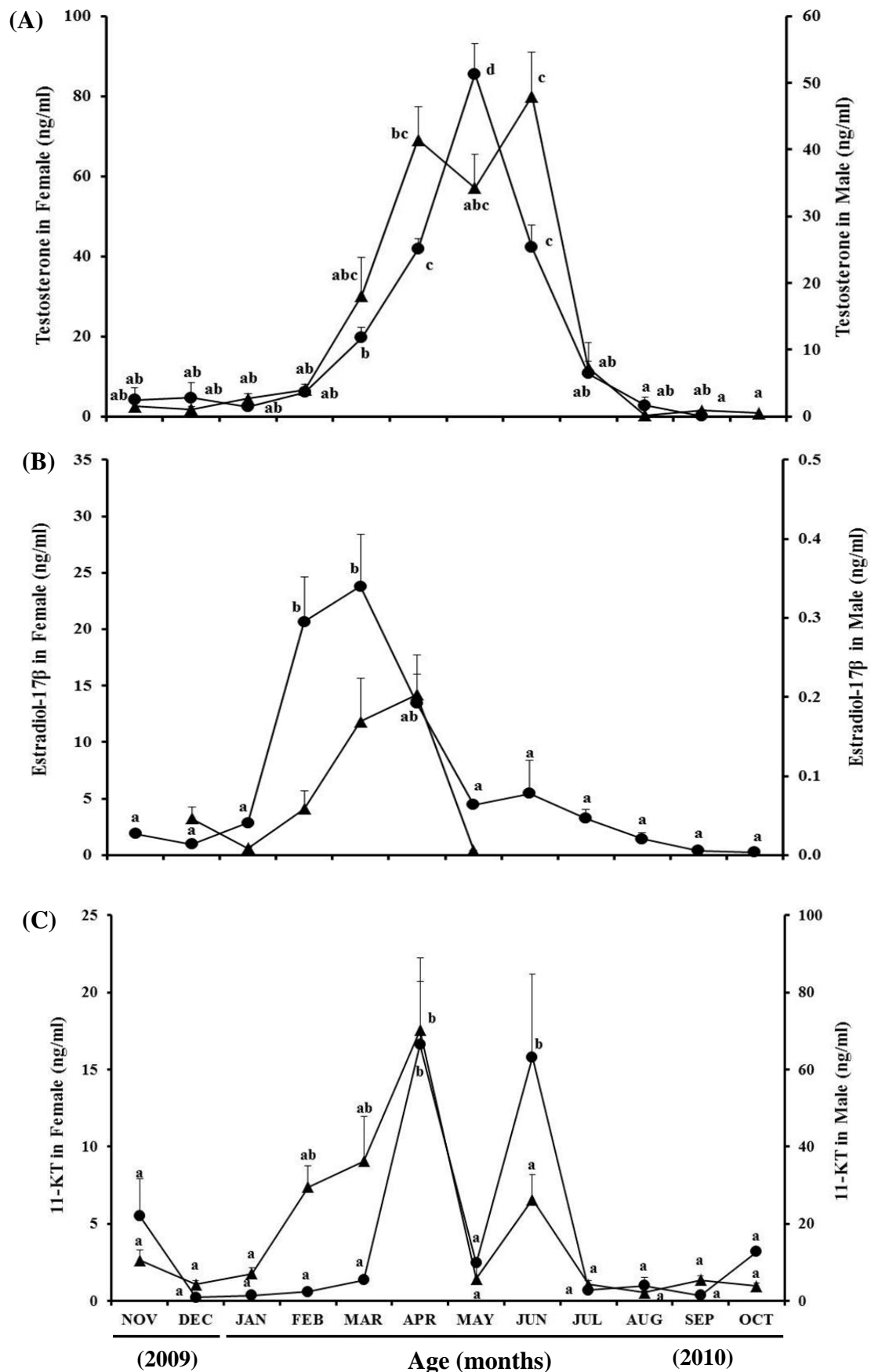


Fig. 3.10 Monthly variations (Mean \pm SEM) in profiles of testosterone (A) and estradiol-17 β (B) and 11-ketotestosterone (C) during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Female: ● Male: ▲.

T: n = 35 males; 39 female. E₂: n = 35 males; 44 females. 11-KT: n = 46 males; 39 females.

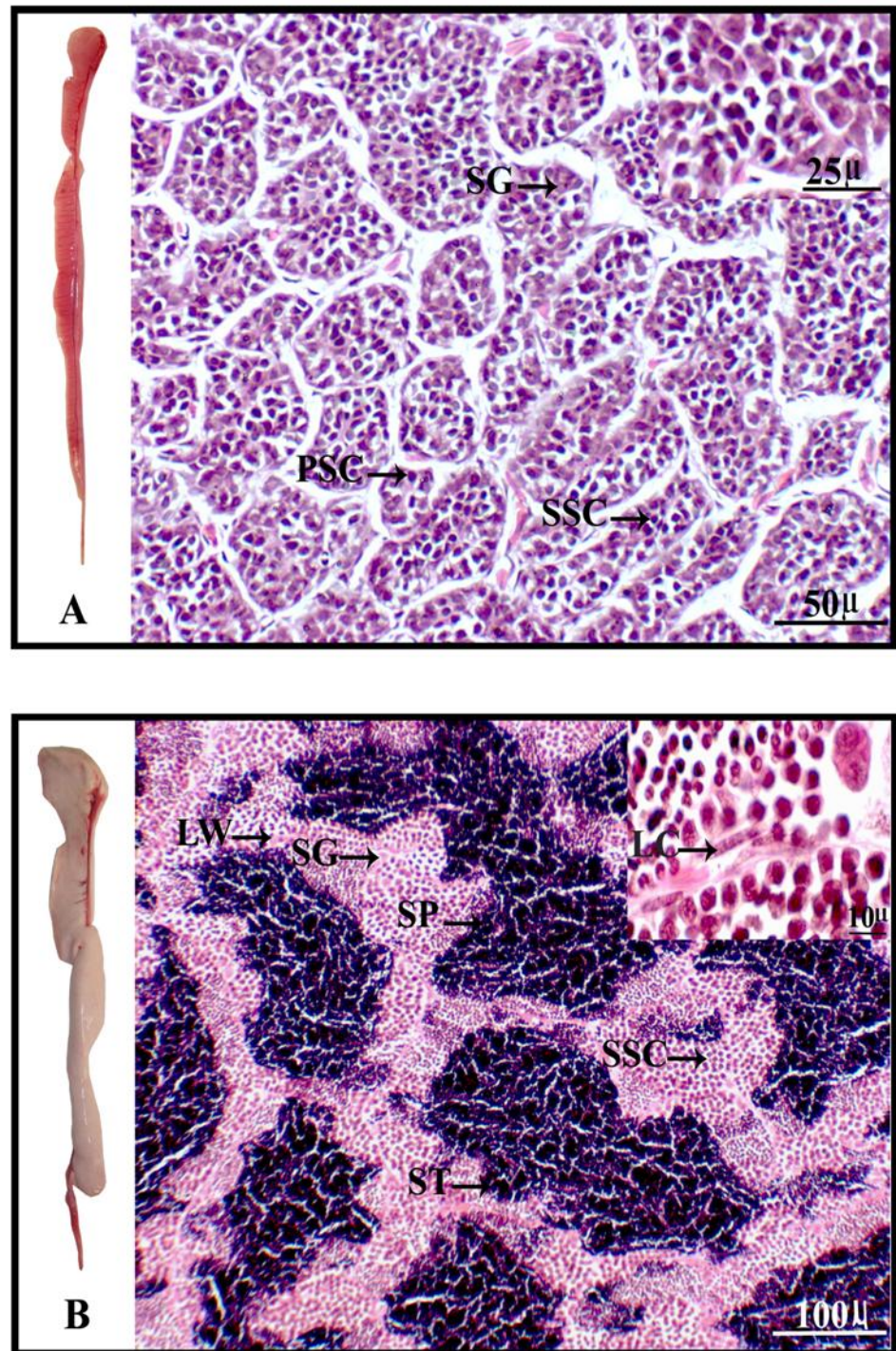


Fig. 3.11 Series of histological changes in testicular development during the second maturational year of *Salvelinus fontinalis*. (A) Stage I - Immature (November - January; 16 - 18 months); (B) Stage II - Maturing (February - April; 19 - 21 months). LC: Leydig's cell; LW: lobular wall; PSC: primary spermatocytes; SG: spermatogonia; SSC: secondary spermatocyte; SP: sperm; ST: spermatid

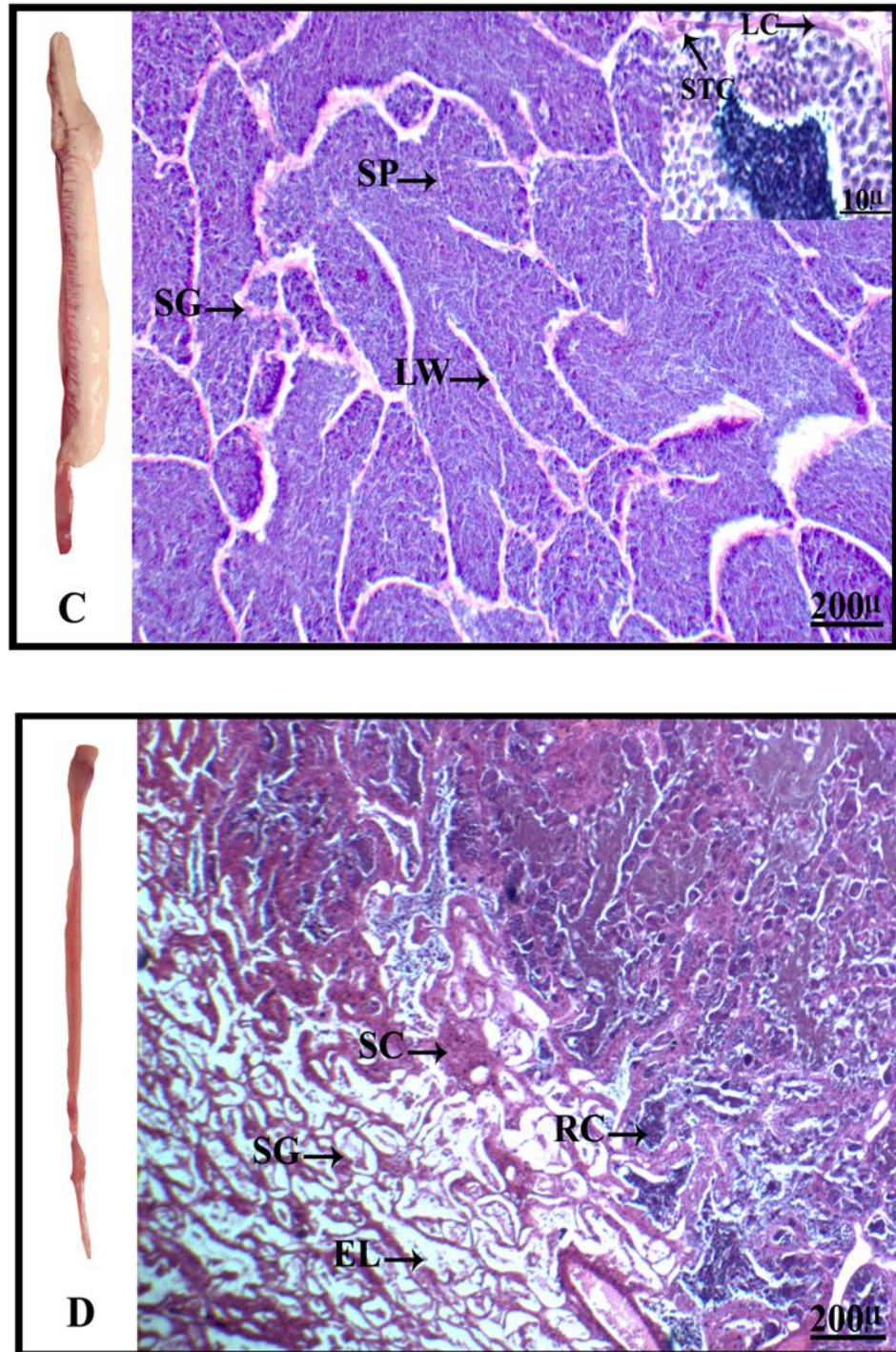


Fig. 3.11 Series of histological changes in testicular development during the second maturational year of *Salvelinus fontinalis*. (C) Stage III – Mature (May - June; 22 - 23 months); (D) Stage IV - Spent (July; 24 months). EL: empty lobule; LC: Leydig's cell; RC: residual cyst; SC: stromal cell; SG: spermatogonia; SP: sperm

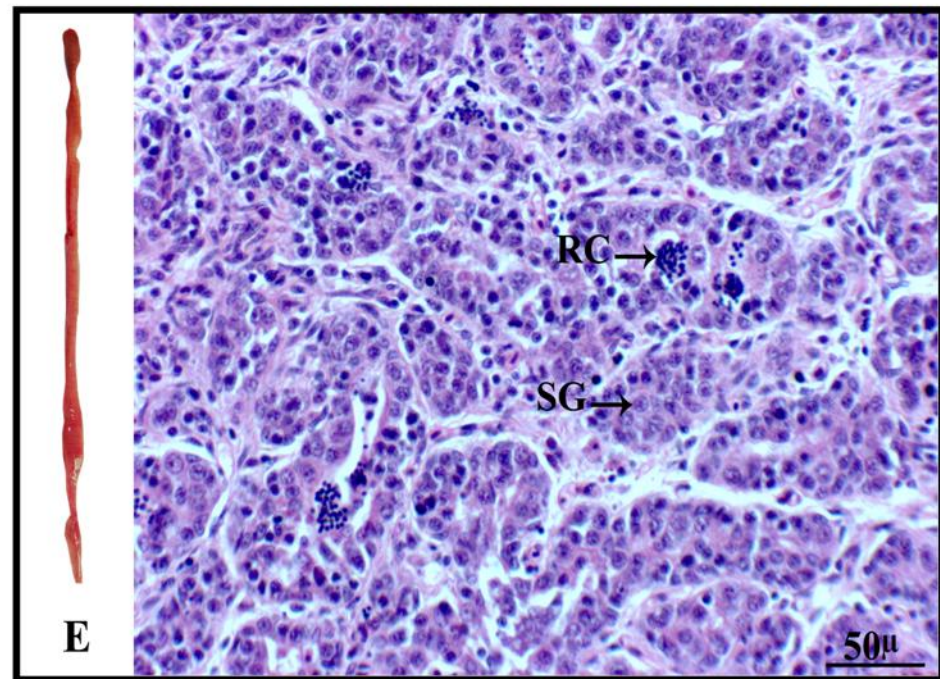


Fig. 3.11 Series of histological changes in testicular development during the second maturational year of *Salvelinus fontinalis*. (E) Stage V – Regressed (August - October; 25 - 27 months). RC: residual cyst; SG: spermatogonia

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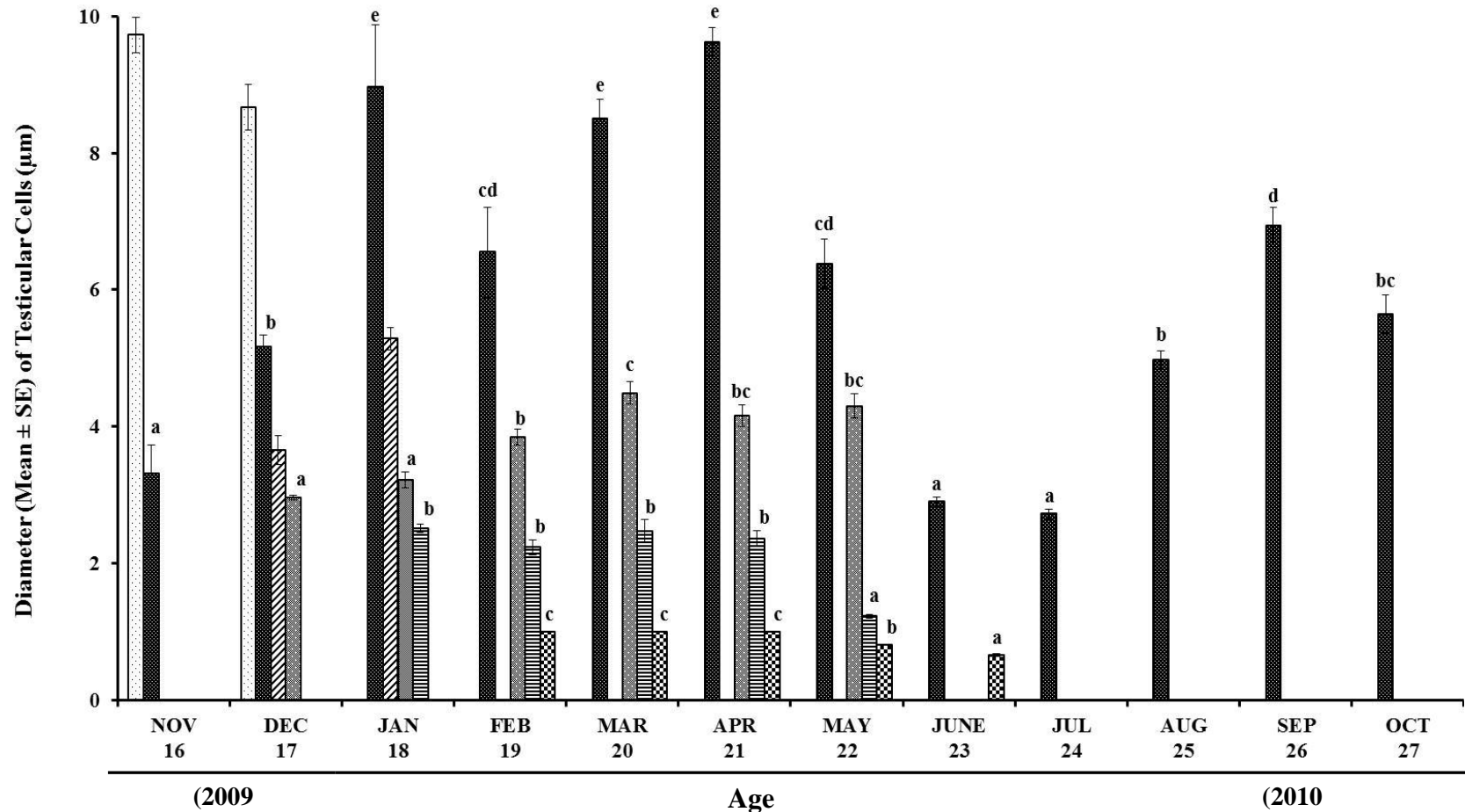








Fig. 3.12 Monthly variations (Mean \pm SEM) in diameter of testicular cells during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) given by Tukey's HSD are indicated by letters. Diameter of SGA is not shown after December due to very small population size. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$) or number of groups was less than three. PSC: primary spermatocyte ; SGA: spermatogonia A ; SGB: spermatogonia B ; SSC: secondary spermatocyte ; SP: sperm;  ST: spermatid . n = 72

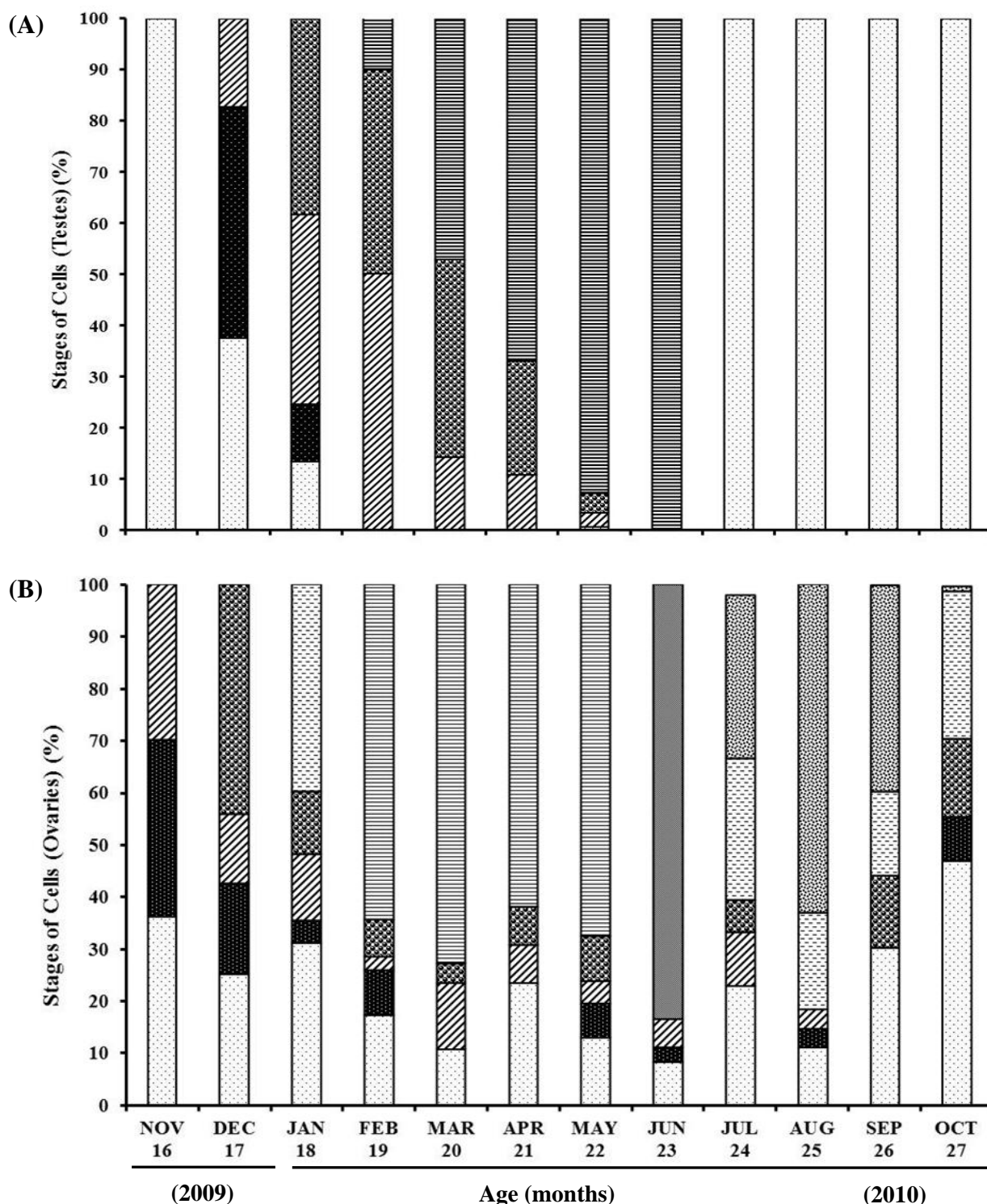


Fig. 3.13 Monthly Variations (Mean \pm SEM) in appearance of testicular and ovarian cells during the second maturational year of *Salvelinus fontinalis*. Population of SGA and SGB were pooled as SG. AF: atretic follicle ; EV: early vitellogenic oocyte ; EVE: early vesicular oocyte ; LV: late vitellogenic oocyte ; LVE: late vesicular oocyte ; MC: mature cell ; MV: mid vitellogenic oocyte ; POC: primary oocyte ; PSC: primary spermatocyte ; SG spermtogonia ; SSC: secondary spermatocyte ; SP: sperm ; ST: spermatid . n = 72 males; 72 females.

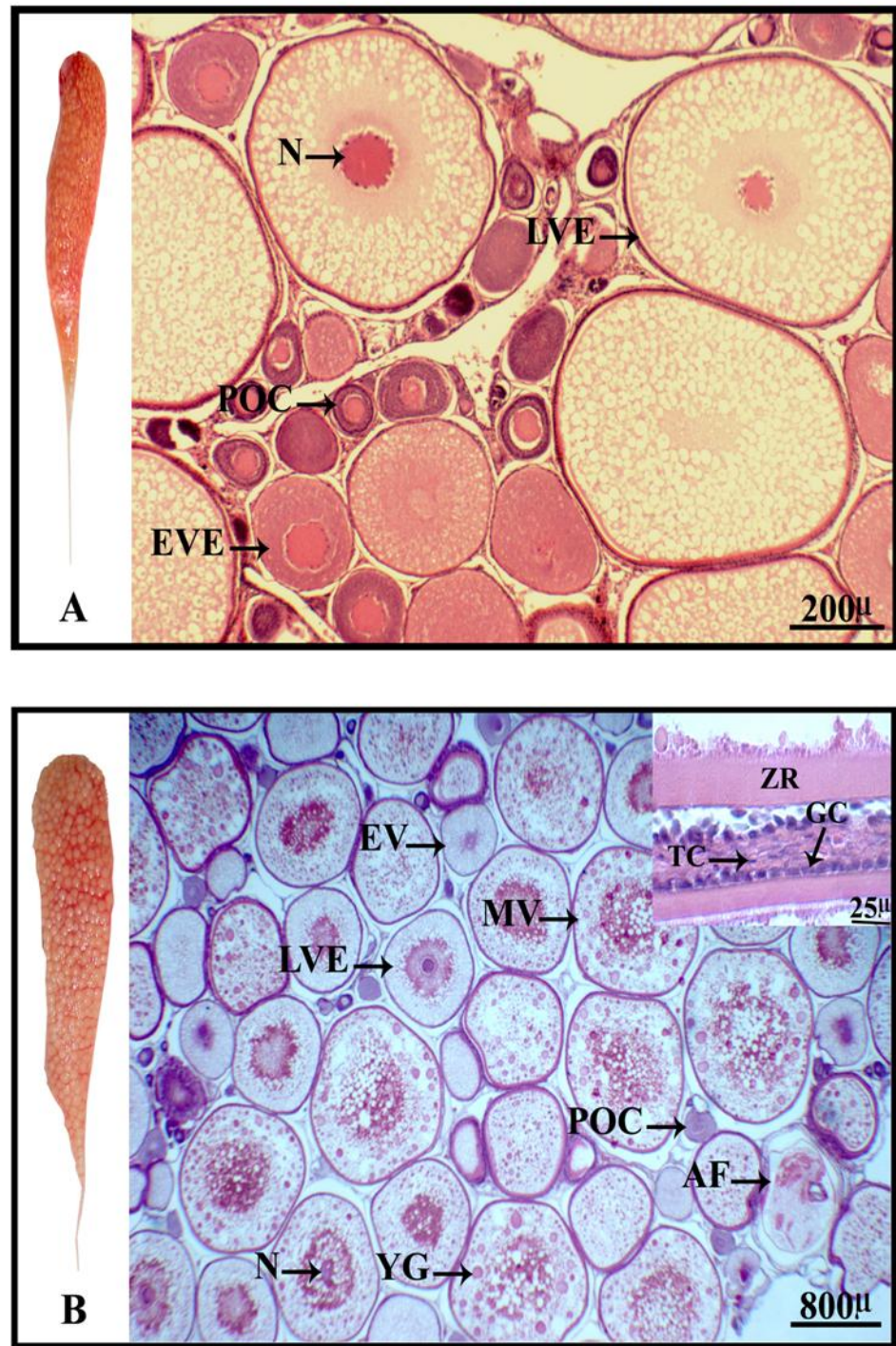


Fig. 3.14 Series of histological changes in ovarian development during the second maturational cycle of *Salvelinus fontinalis*. (A) Stage I – Immature (November; 16 months); (B) Stage II - Developing (December - January; 17 - 18 months). AF: atretic follicle; EV: early vitellogenic oocyte; EVE: early vesicular oocyte; GC: granulosa cell; LVE: late vesicular oocyte; MV: mid vitellogenic oocyte; POC: primary oocyte; N: nucleus; TC: thecal cell; YG: yolk globule; ZR: zona radiata.

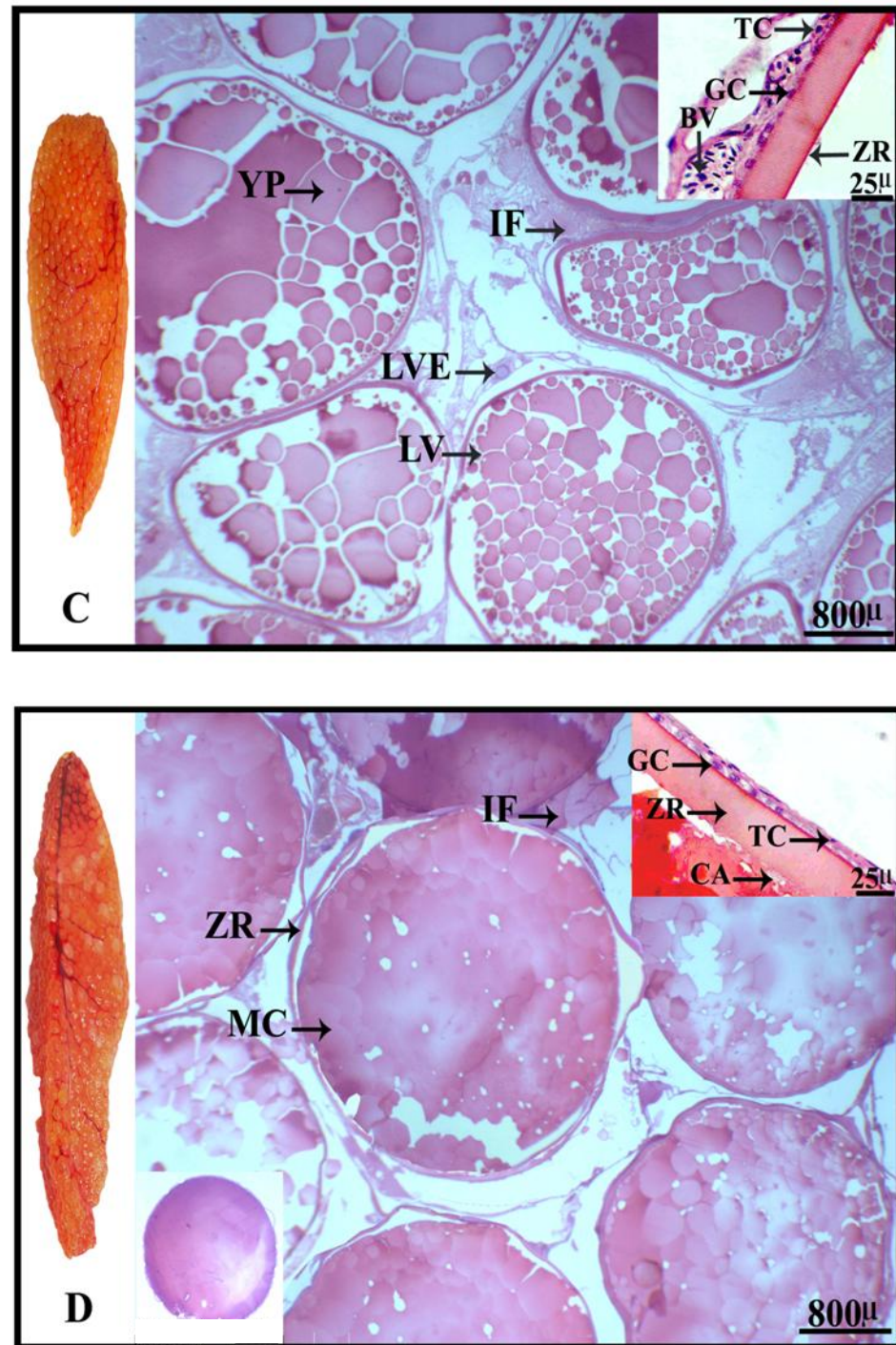


Fig. 3.14 Series of histological changes in ovarian development during the second maturational cycle of *Salvelinus fontinalis*. (C) Stage III - Maturing (February - May; 19 - 22 months); (D) Stage IV - Mature (June - 23 months). BV: blood vessel; CA: cortical alveoli; GC: granulosa cell; IF: interstitial fluid; LV: late vitellogenic oocyte; LVE: late vesicular oocyte; MC: mature cell; YP: yolk plate; ZR: zona radiata; TC: thecal cell

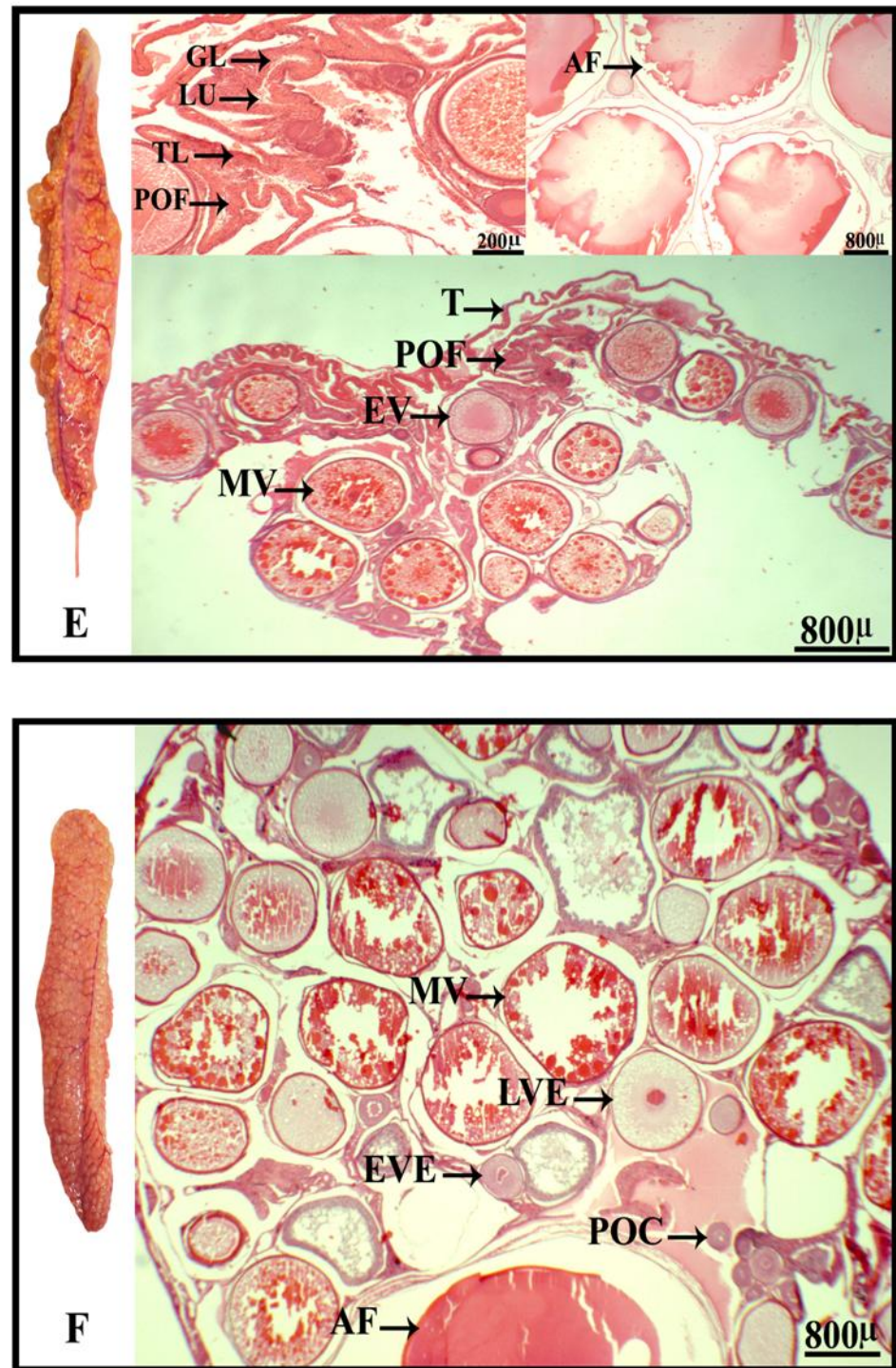


Fig. 3.14 Series of histological changes in ovarian development during second maturational year of *Salvelinus fontinalis*. (E) Stage V - Spent (July; 24 months). (F) Stage VI - Recruiting (August - October: 25 - 27 months). AF: atretic follicle; EV: early vitellogenic oocyte; EVE: early vesicular oocyte; GL: granulosa layer; LU: Lumen; LVE: late vesicular oocyte; MV: mid vitellogenic oocyte; POC: primary oocyte; POF: post ovulatory follicle; T: tunic; TL: thecal layer

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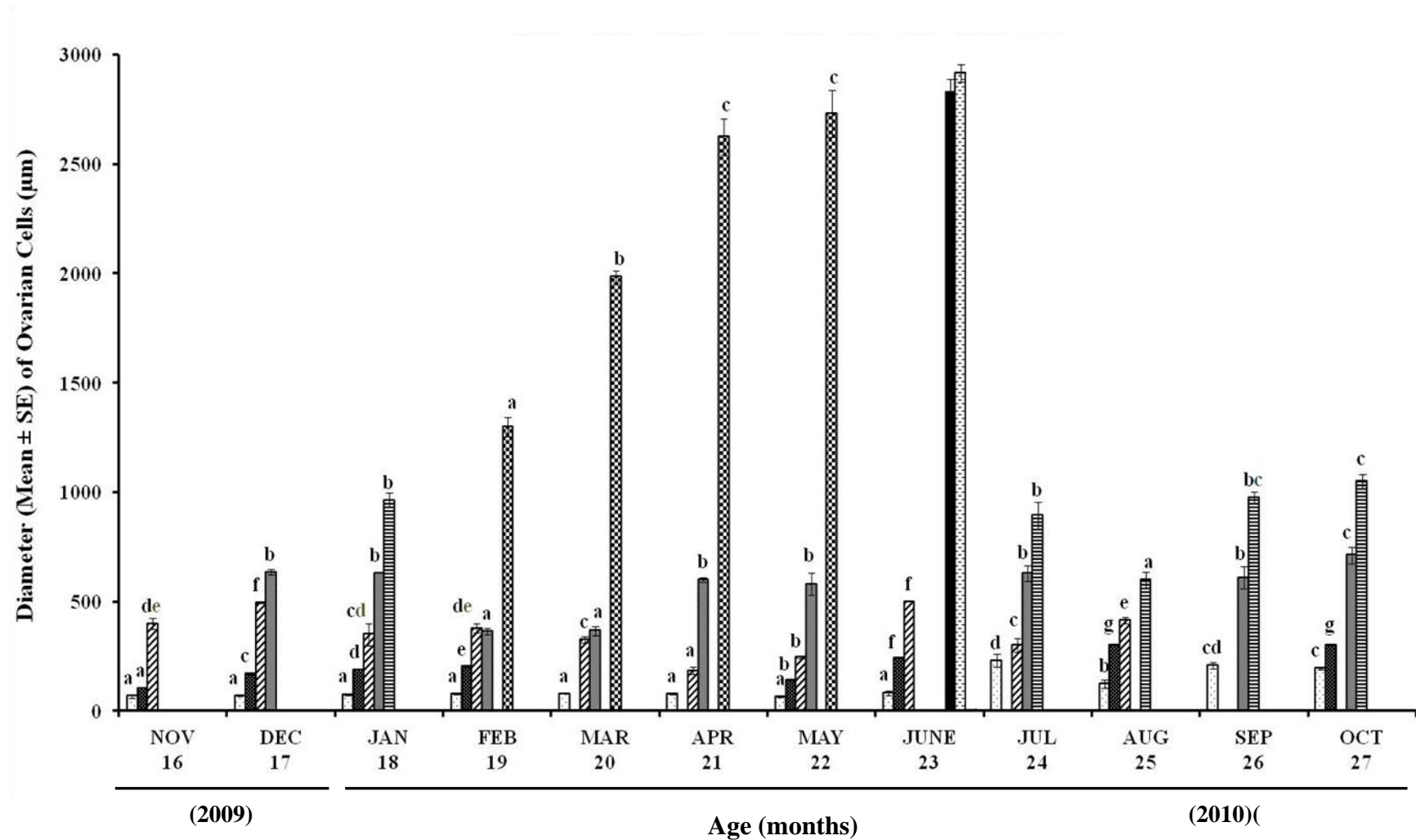


Fig. 3.15 Monthly variations (Mean ± SEM) in diameter of ovarian cells during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$) or number of groups was less than three. EV: early vitellogenic oocyte ■; EVE: early vesicular oocyte ■; LV: late vitellogenic oocyte ■; LVE: late vesicular oocyte ■; MO: mature oocyte ■; MV: mid vitellogenic oocyte ■; POC: primary oocyte □; OO: ovulated oocyte □. n = 72.

3.5 Discussion

The Present study investigated the gonadal development and its endocrine and environmental control during the first and second reproductive year of brook trout under Tasmanian climate conditions. During the first year, maturation in brook trout males was observed between 14-15 months of age which is in agreement with other studies reporting first maturation in brook trout and other salmonid males aged 1 year and older (McCormick and Naiman, 1984; L' Abee-Lund, 1989; Silverstein and Hershberger, 1992; Clarke and Blackburn, 1994; Dziewulska and Domagala, 2004; McClure et al., 2007). However, only 75% of total males could achieve maturation (testicular lobules filled with sperm) and immature males were also present in the population as observed in other salmonids like Atlantic salmon, and sea trout (*Salmo trutta*) (Saunders and Henderson, 1988; Letcher and Terrick, 1998; Dziewulska and Domagala, 2004). Failure of these immature males to attain maturity might be due to low energy reserves and adiposity which would postpone their maturation until the next year similar to that observed in Atlantic salmon and rainbow trout (Taranger et al., 1999; Oppedal et al., 2006).

Interestingly, the time of occurrence of maturation in males during the first and second reproductive cycle was different. During the first year maturation was observed in September (2010) while mature males were observed between March and June (2010) during second year although both cohorts were at similar feeding, water temperature and photoperiod conditions. This difference might be due to the occurrence of gonadal differentiation in February (2010) in first year cohort and subsequent growth to achieve the required energy thresholds for maturation during the first year. Occurrence of maturation in the first year at a different time from that in the second year suggests that puberty in brook trout males might be more under the control of BPG axis than environmental factors (Okuzawa, 2002) or

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delayed until September due to their small size (Piche et al., 2008). Although onset of puberty can be advanced or delayed in salmonids such as masu salmon (*Oncorhynchus masou*), Atlantic salmon and rainbow trout under manipulated photoperiod (Bromage, 1987, Amano et al., 1994, 1995) however Henderson et al. (1963) did not observe such response under manipulated photoperiod in under-yearlings of brook trout. This aspect of puberty in brook trout under yearlings needs further investigation.

During the first reproductive cycle spermatogonial proliferation commenced with the rise in the levels of T and E₂ at the age of 9 months (mixed samples). The increasing plasma levels of T, E₂ and 11-KT resulted in a significant boost in mitotic activity of SG until the end of phase II. The role of E₂ during the first year of males was in agreement with other studies which proposed its involvement in germ cell renewal and spermatogonial proliferation (*Anguilla japonica*: Miura et al., 1999). E₂ might also be involved in marked proliferative activity of sertoli cells during phase II as observed in rainbow trout, zebra fish and Japanese common goby (*Acanthogobius flavimanus*) (Bouma and Nagler, 2001; Menuet et al., 2002; Ito et al., 2007). This proliferation determines the capacity of testes to produce spermatogonia and sperm (Matta et al., 2002). The beginning of phase III at age of 14-15 months (September-October) switched the mitosis to meiosis and thus formation of sperm. These developmental changes were presumably found as a result of 11-KT action mediated by other growth factors produced by sertoli cells as observed in *Carassius auratus* (Kobayashi et al., 1991) and *Anguilla japonica* (Miura et al., 1991). However, only 20% of total mature males could achieve final maturation (hydration of milt and its release on manual abdominal pressure under tank conditions) and found running during puberty. Failure of remaining mature males to reach final maturation might be due to the observed low levels of 11-KT at this final stage as 11-KT is particularly important in milt hydration (Rolland et al., 2009).

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Contrary to males, brook trout females did not mature during their first year which is in agreement with the finding of McCormick and Naiman (1984) for the same species. They found immature females during their first year fed at high rate while high proportion of mature males was observed during this period. McCormick and Naiman (1984) reported that failure of females to achieve puberty during their first year might be due to sexually divergent response in brook trout and limitations imposed by size on egg production relative to sperm production.

Endogenous vitellogenesis in females began at the age of 9 months (April) concomitant with rise in E_2 (mixed samples) which remained high until the next month followed by the development of LVE during phase II. A peak of E_2 at the age of 13 months (August) might result in recruitment of oocytes for maturation and ultimate increase in population of LVE. Other than the role of E_2 at this stage, 11-KT might also be involved in boosting the oil droplet accumulation and increase in cell diameter as reported in Japanese eel, short finned eel, Atlantic cod and coho salmon (Matsubara et al., 2003; Lokman et al., 2007; Endo et al., 2008; Kortner et al., 2009). A high value of HSI and liver crude lipids during this phase further confirmed the improved adiposity and lipid accumulation helping in the accumulation of oil droplets in oocytes. Although yolk granules were not observed at any stage, there is possibility that pre-vitellogenic oocytes started to uptake E_2 mobilized vitellogenin without emergence of yolk granules as observed in rainbow trout (Perazzolo et al., 1999). Recruitment of cortical alveolar oocytes into vesicular oocytes continued until the end of the puberty but LVE did not develop further as observed previously in rainbow trout (*Salmo gairdneri*) (Sumpter et al., 1984). In the present study, this temporary developmental arrest may have been due to a lack of sufficient nutritional reserves to attain (Aubin-Horth et al., 2006) resulting in delay of maturation until the next year as observed in same species previously (McCormick and Naiman, 1984).

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During the second reproductive cycle, in males, SGA were recruited to spermatogonia B after germ cell renewal during stage I. This cell renewal was followed by rapid proliferation of SGB and their immediate subsequent entry into meiotic prophase at an age of 17 months (December; summer solstice). This marked proliferative activity might be due to higher concentration of 11-KT which has been reported to be involved in the initiation of meiosis as observed in *Carassius auratus* (Kobayashi et al., 1991), *Anguilla japonica* (Miura et al., 1991) and *Hucho perryi* (Amer et al., 2001). Transformation of spermatids into sperm began at an age of 19 months in February (Stage II) was concomitant with the marked rise in profiles of both 11-KT and T. Transformation of spermatids into sperm continued for the next five months (February-May) with significant increase in concentrations of T and 11-KT along with a marked rise in sperm population and milt hydration. Both T and 11-KT play equally important roles during milt hydration in brook trout as also suggested by Rolland et al. (2009) in rainbow trout. As compared to only 20% final maturation during first year, all males became mature and could be manually stripped during their second year as observed previously in same species (McCormick and Naiman, 1984).

Ovarian development at the beginning of second reproductive cycle was found arrested at the vesicular stage as observed in a previous study on brook trout (Tam et al., 1986). Following the summer solstice in December, oocyte development was resumed by commencement of exogenous vitellogenesis as reported by Tam et al. (1986). Yolk globules were observed at an age of 18 months (January), six months prior to ovulation, and presumably vitellogenesis continued until the age of 21 months (end of April). Plasma levels of E₂ increased during the stage III regulating the hepatic synthesis of vitellogenin and subsequent vigorous growth of LV due to its uptake as observed previously in rainbow trout and brook trout (Tam et al., 1986; Tyler et al., 1991; Babin et al., 2007). This hepatic vitellogenesis is further supported by high HSI and liver crude lipids during this phase as reported by Patino and Sullivan

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(2002). Exogenous vitellogenesis stopped after GVM as observed previously in brook trout (McCormick and Naiman, 1984). No significant increase in diameter of vitellogenic oocytes was observed until the absorption of water at the age of 23 months (June). Although events of germinal vesicle movement and GVBD were not discernible microscopically but presumably these changes occurred at the age of 21 months (April), two months prior to ovulation. Although these events are very important during ovarian development however, Tam et al. (1984) also did not report the GVM and GVBD in brook trout but observed mature ova similar to present study. Observation of these events might have been missed due to sampling once a month. This presumption was further justified by the observed decline in E_2 levels at age of 21 months which remained very low in subsequent months.

Contrary to drop in levels of E_2 a few weeks earlier than stage IV, the levels of T remained very high until this stage. Similar pattern was observed in activity of 3β -HSD and 17β -HSD during this phase as reported in rainbow trout (Nakamura et al., 2003). This continuous increase in levels of T might be due to drop in aromatase activity (Young et al., 1983; Nakamura et al., 2005; Bobe et al., 2006) which reduced the rate of conversion of T into E_2 as observed in other salmonids including brook trout (Kagawa et al., 1983; Scott and Sumpter, 1983; Tam et al., 1986; Mayer et al., 1992; Pavlidis et al., 1994). A decline in the concentration of E_2 could be due to a shift in steroidogenic pathway towards synthesis of maturation inducing hormone (MIH) prior to spawning (Duffey and Goetz, 1980; Fostier and Jalabert, 1986; Goetz et al., 1987; Frantzen et al., 1997). Final maturation (hydration of mature ova and miscibility of proteins and water) regulated by MIH might be more under the control of GTHs especially surge of LH (Bobe et al., 2003) than sex steroids. A significant drop in activity of 3β -HSD and 17β -HSD at stage V also supports the above statement as due to low activity of steroidogenic enzymes synthesis of T and E_2 from their substrate would be reduced and progesterone would be consumed to synthesize MIH. However, MIH was not

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measured in present study due to the cost of assay. A sudden increase in activity of both enzymes just after stage V could not be explained as the concentration of sex steroid remained very low throughout stage VI as observed in other salmonids (Fitzpatrick et al., 1986; Tam et al., 1986; Frantzen et al., 1997; Tveiten et al., 1998).

Although all females were recruited for maturation during their second year as reported previously for brook trout (Tam et al., 1986) however only 20% females could achieve final maturation and released ovulated eggs from abdomen on manual stripping. This rate was calculated from only six females in present study which was much lower than ovulation rate of ~ 80% on manual stripping at farm. However the growth rates in present study and at farm were found similar during second year (Pers Comm. M. Amin). Therefore, the only reason of the low ovulation rate in present study was small sample size not the growth.

Total fecundity observed in these females was higher than that reported previously in brook trout (723 ± 320 oocytes; Serezli et al., 2010) however eggs were comparatively smaller in size (4.49 ± 0.21 mm; Serezli et al., 2010). This difference between total fecundity and egg size between different populations of same species could be explained by fish size, stocking density, feeding rate, water quality, fish age and environmental stress (Bromage et al., 1992). Large size, relatively old age, small brood size and standardized tank condition can maximize the fecundity while large egg size is a trade-off between number and quality of eggs (Heinimaa and Heinimaa, 2004).

Spawning did not occur in brook trout under tank conditions and gametes were resorbed in both sexes at an age of 24 months in July following the winter solstice (June) as observed in other salmonids (Hunt et al., 1982; Baynes and Scott, 1985; Pottinger, 1988; Scott and Sumpter, 1989; Mayers et al., 1992; Tveiten et al., 1998). However, milt and ova could be released by manual stripping. In captivity, spawning could be induced by GnRH treatment

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but not practiced in present study. Residual sperm and cellular debris were removed by phagocytic activity of sertoli cells in males soon after stage IV as reported by Grier and Taylor (1998) and Almeida et al. (2008) in *Gadus morhua* and *Centropomus undecimalis*. Recrudescence of SGB and POC commenced in males and females, respectively after resorption to start next reproductive cycle as observed in other salmonids (Hunt et al., 1982; Baynes and Scott, 1985; Pottinger, 1988; Scott and Sumpter, 1989; Mayers et al., 1992; Tveiten et al., 1998; Schulz and Miura, 2002). Overall similar to other salmonids like rainbow trout, Atlantic salmon and Chinook salmon gonadal development in both male and female brook trout was found under the fine control of seasonal variations in sex steroids and photoperiod (Amano et al., 2000; Fal  n et al., 2010; Leclercq et al., 2011)

As aforementioned brook trout was introduced to Tasmania 50 years ago from Canada (Clements, 1988), present study also highlighted the adaptations in reproductive cycle of this species according to new climate. In present study brook trout started recruitment for maturation during summer (December – January) which vigorously progressed during autumn (March – April) and its completion was observed during winter (May – June). The timing of occurrence of different developmental stages in reproductive cycle of brook trout under Tasmanian climate has been found different from that observed in study reported from Canada for this species (Tam et al., 1986). Under Canadian climate, recruitment for maturation was observed at the beginning of winter (December) which further progressed over the spring (March – April) and summer (May – August) while final maturation and spawning were observed during autumn (September – October). This seasonal shift in the reproductive cycle of brook trout under Tasmanian climate occurred to ensure the viability of eggs and fishlings at the most suitable water temperature, habitat conditions and availability of food.

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In the present study, maturation in brook trout males was observed during the both first and second year which is the main constraint in its large scale commercial production in Tasmania. Control of maturation in both sexes is a major interest of the Aquacult. industry which can be achieved by exposing fish to manipulated photoperiod as performed for rainbow trout and Atlantic salmon in Tasmania. In the present study, the gonadal development in both sexes was regulated by variations in sex steroids profiles which were entrained with seasonal changes in photoperiod as observed in other salmonids (Hunt et al., 1982; Baynes and Scott, 1985; Pottinger, 1988; Scott and Sumpter, 1989; Mayers et al., 1992; Bromage et al., 1994; Tveiten et al., 1998). The information described in this chapter can be utilized to investigate the effect of manipulated photoperiod on the maturation of brook trout. Based on the results mentioned in this chapter, the “critical period” (growth is at the threshold level to start the recruitment of fish to be mature at the end of cycle) to open the “window of opportunity” for maturation was December – January. If this period is manipulated by delayed, advanced or continuous photoperiod, the “critical period” of December – January may pass without initiating recruitment. Closing of this “window of opportunity” may result into the disruption of the endogenous rhythm and reduction in the proportion of maturing fish (Taranger et al., 2003; Choi et al., 2010). Keeping in view this interaction of both light and reproductive cycle, maturation can be possibly controlled in brook trout by manipulating photoperiod as successfully achieved in Atlantic salmon and rainbow trout (Andersson et al., 2003; Duston et al., 2003; Taylor et al., 2008; Wilkinson et al., 2010; Leclercq et al., 2011).

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CHAPTER 4

Sex Reversal of Brook Trout (*Salvelinus fontinalis*) by 17 α -methyltestosterone Immersion

4.1 Abstract

Commercial culture of Brook trout (*Salvelinus fontinalis*) is impeded by early maturation, particularly in male fish. Body weight and flesh quality at harvest are adversely affected in mature fish, as is immunocompetency during the late stages of the maturation process. Production of all-female populations is one solution to address these problems. Previously reported studies of sex reversal in brook trout proposed laborious protocols using high doses of androgens. The current project aimed to identify the most appropriate timing for exposing brook trout embryos and larvae to 17 α -methyltestosterone (MT) dose that causes female genotypes to become phenotypically male (or neomale) over the considerable period of one week pre-hatch and post-hatch to produce all-male population. The whole treatment period was divided into 11 time windows to find out the most sensitive time for MT treatment. Immersion treatment was given to these 11 groups and subsequently split into two batches at the end of immersion treatment. One of these batches was fed with MT treated diet to study the combined effect of immersion and dietary treatments. The second batch was given untreated diet to study the effect of immersion only treatment.

An immersion dose of MT at 400 μ g/L for 4 h on two alternate days (4 to 6 days pre-hatch) produced a population containing the proportion of 0.75 of males (compared to the proportion males in control C-1 = 0.54) without the occurrence of any sterile fish. Immersions given after the pre-hatch period did not successfully masculinise female fish. Multiple immersions in MT at 400 μ g/L produced a proportion of 0.83 of sterile fish. Feeding fish a MT

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supplemented diet (3 mg/kg) for 60 days from first feeding did not affect the sex proportion compared to that of control. MT oral treatment combined with immersion treatments resulted in variable occurrence of sterile fish (0.04 – 0.30) which was not observed in immersed only groups. Collectively these data indicate that immersions given at 4 – 6 days pre-hatch achieved the largest skew towards maleness without any supplementary dietary treatment. In the present study, 4 – 6 days pre-hatch was found to be the most sensitive window of time for MT immersion treatment at 9°C to masculinize genotypic females in brook trout.

4.2 Introduction

Brook trout (*Salvelinus fontinalis*) is a potential alternative species to the Atlantic salmon and rainbow trout for Tasmanian aquaculture industry. Pilot scale commercial trials in Macquarie harbour (Tasmania) from 2003-2007 encountered a high incidence of early sexual maturation (~ 80%) particularly in males. Maturation has deleterious effects upon flesh quality, growth and immunocompetency (Piferrer, 2001; Galbreath et al., 2003; Chiasson and Benfey, 2007). This led to a cessation of further commercial production for the species until this impediment could be overcome. Production of all-female populations is one solution addressing the problem of early maturation in males. This can be achieved by applying steroid-induced sex reversal either by direct or indirect feminization. Although estrogens can be successfully used to directly induce feminization, steroid treated fish are not acceptable for commercial markets and had been legally banned in certain regions like Europe (Piferrer, 2001). An alternative way to eliminate precocious male maturation is an indirect feminization approach using an androgen or aromatase inhibitors to produce stock which are genotypically female and phenotypically male (known as neo-males). Crossing these masculinised females with normal females ultimately produces an all-female cohort (Galbreath et al., 2003; Lee et al., 2004).

Both immersion (bathing of fish in androgen solution) and dietary techniques (androgen supplemented diet) have been applied for various salmonid species. For sex reversal 17 α -methyltestosterone (MT) is most commonly used and comparatively a more potent androgen than testosterone for most of the salmonids (Pandian and Sheela, 1995), causing no negative effect on growth (chinook salmon [*Oncorhynchus tshawytscha*] Baker et al., 1988), coho salmon [*Oncorhynchus kisutch*], Piferrer and Donaldson, 1991) and survival of fish (brook trout, Parks and Parks, 1991; coho salmon, Piferrer et al., 1994). Dietary treatment with MT has produced 100% successful sex reversal in rainbow trout (*Oncorhynchus mykiss*)

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(Solar et al., 1984; Schmelzing and Gall, 1991; Demska-Zakes et al., 1999; Amini and Tala, 2003) chinook salmon (Piferrer et al., 1993) and Atlantic salmon (*Salmo salar*) (Lee et al., 2004). However, androgen dose, duration of treatment and experimental conditions vary between different studies. Despite previous successes of dietary MT inclusion for neo-male production in other salmonids the technique has been proven difficult for phenotypic sex reversal in brook trout (Galbreath and Stock, 1999; Galbreath et al., 2003; Sacobie, 2001; Haffray et al., 2009).

Identification of the most labile period for sex determination most likely to be influenced by steroid treatment is fundamental for subsequent sex reversal of brook trout. Molecular studies show the expression of sex differentiation genes earlier than anatomical sex differentiation when gonads can be differentiated as ovaries and testes (Baron et al., 2008; Cavileer et al., 2009; von Schalburg et al., 2011) indicating the need for androgen treatment before cytological sex differentiation can be observed.

For previous studies in brook trout, Parks and Parks (1991) fed MT treated diet to brook trout alevins (40 mg/kg) for 77 – 108 days. This treatment resulted in 100% sterility. Galbreath and Stocks (1999) immersed embryos at sac fry stage which failed to produce a significant proportion of males. Galbreath et al. (2003) gave one immersion (1 mg/L) to gynogenetic brook trout fry at ten days post-hatch followed by 60 days MT dietary treatment (1 – 2 mg/kg) which could not skew the sex proportion to males. Post-hatch MT treatment given by Galbreath and Stocks (1999) and Galbreath et al. (2003) failed to produce male skew which showed the insensitivity of this period for sex reversal treatment. However, treatments given during the pre-hatch period, until one week post-hatch did not result in a significant skew of the treated populations towards maleness (Sacobie, 2001; Haffray et al., 2009). However, a combination of four immersions of MT during one week pre and post-hatch followed by oral treatment for approximately 60 days produced a 0.74:0.26 male:female proportions (Haffray

et al., 2009). Although this study suggested that the immediate pre and post-hatch period was a critical time for influencing future phenotypic sexual development it remained unclear whether a more narrowly defined treatment window could be identified. Pinpointing this window may eliminate the need for multiple immersions and possibly the requirement for feeding an androgen treated diet. Indeed, Haffray et al. (2009) questioned whether oral treatment for masculinisation was required suggesting that exclusion of this step would reduce labour, chemical and environmental costs. Despite the success of dietary androgen treatment in other salmonids, the method has additional disadvantages and limitations regarding differential feed uptake and endocrine disruption (Pandian and Sheela, 1995; Pandian and Kirankumar, 2003) and possible high incidence of sterility as observed in coho salmon (Goetz et al., 1979; Hunter et al., 1982) and brook trout (Parks and Parks, 1991; Galbreath et al., 2003).

The objective of present study was to determine the most appropriate timing of an immersion treatment during the week preceding and week following hatch. The requirement for MT supplemented diet either with or without prior MT immersion was likewise evaluated to qualify its necessity for successful sex reversal of brook trout. Successful completion of these objectives will form the basis for development a commercially applied sex reversal protocol for brook trout.

4.3 Materials and Methods

This study was conducted according to procedures approved by the University of Tasmania Animal Ethics Committee (Project Approval Number: A0010890). A total of 14,000 diploid brook trout eggs at eyed stage (30 days post fertilization, incubated at ambient water temperature) were obtained from the Mountain Stream Fishery, Targa, Tasmania and transferred to National Centre for Marine Conservation and Resource Sustainability, Aquaculture centre (University of Tasmania, Launceston, Tasmania) on June 18, 2010. The eggs were divided into 14 circular incubators constructed from PVC pipes (Height = 150 cm, Depth = 7 cm) (1000 eggs per incubator) and placed in upwelling Californian trays in 24 h darkness.

Each PVC incubator was fitted with a fibre glass mesh screen at the bottom and provided with water exchange by an 18 mm polyester airlift pipe. Water temperature was maintained at 9.5 °C - 10 °C by a heat / chill unit (10 KW). Water was supplied from a 4000 L tank fitted with UV sterilizer (Pentair Aquatics, QL-40), solids and biological filtration. Water was exchanged at 10% per day using municipal town water. Chlorine was controlled by the addition of appropriate amounts of sodium thiosulphate sufficient to maintain levels below 0.02 mg/L. Water quality parameters were maintained at pH 6.8- 7.2, total ammonia < 2 mg/L, nitrite < 1 mg/L, nitrate < 80 mg/L. At swim-up stage, the fish were fed on a commercial trout diet (initially at 4% BW/day) (Nutra, Skretting, Australia) and held at a photoperiod of 12h light: 12h dark. Body weight was checked every week by random (without introducing any size bias into results) sampling to update the weekly ration. Weight check was performed regularly to eliminate any chance of slow growth due to less feed which might have an effect on sex ratio or survival rate.

Age of embryos and alevins was not mentioned in degree days due to 30 days post-fertilization incubation at ambient temperature at commercial hatchery before embryos were transferred to research centre and kept at 9.5 -10 °C during treatment and post treatment period.

4.3.1 Methyltestosterone (MT) Immersion Treatment

Immersion treatments consisted of 13 different groups, with approximately 1000 eggs per group. The groups consisted of two negative control groups (C-1: unhandled and C-2: 0.5 mL/L ethanol exposed for 4 hours on each immersion point), one positive control groups (I-1: 400 μ g/L MT exposed for 4 hours on each immersion point) and 10 treatment groups (I-2 – I-11: 400 μ g/L MT) treated for 4 hours on two consecutive immersion points (Table 1). A total dose of 400 μ g/L per immersion and duration of 4 hours was used following Haffray et al., (2009).

Stock solutions of MT at 0.8 mg/mL (MP Biomedicals, France) was prepared in 100% ethanol. A total of 5 mL of each stock solution was added to 10 L of water to prepare the final concentration of 400 μ g/L. During each 4 h immersion procedure each PVC incubator was transferred gently to a plastic tank containing 10 L of water and the MT/EtOH solution at 9.5 – 10 °C. Eggs and larvae were continually aerated during immersion and were rinsed with water afterwards to remove MT/EtOH residues. Immersion treatments were started on July 13, 2010 and continued throughout the pre-hatch, hatching and post-hatch period (Table 4.1).

4.3.2 Methyltestosterone Feeding Treatment

Following hatching, alevins from the 13 incubators were split into two equal batches (by volume) and transferred into a further 13 incubators (400 – 500 alevins/incubator, 26

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incubators in total). During “swim up” fish in incubators designated C-1 – C-2 and I-1 – I-11 were fed with an ethanol treated feed (0.30 mm; Spectra SS, Skretting, Australia). Fish in incubators designated FC-1 – FC-2 and FI-1 – FI-11 were fed with a MT treated diet. Feeding treatment started on September 1st, 2010 and continued until November 1st, 2010 (60 days). Water temperature was maintained at 9.5 – 10 °C throughout dietary treatment.

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Table 4.1 Protocol for 17 α -methyltestosterone immersion treatment for brook trout (*Salvelinus fontinalis*) from July 13 until August 4, 2010. A total of 1000 eggs / alevins in one control (C-2) and 11 treatments (I-1 – I-11) were bathed in MT for 4 hours on each immersion point. An additional control group (C-1) were not handled throughout the period. ✕= no treatment on that date, ✓= immersion on that date.

^a Treatment	Immersion detail	Pre-hatch			Hatch			Post-hatch					
		July 13	July 15	July 17	July 19	July 21	July 23	July 25	July 27	July 29	July 31	Aug 2	Aug 4
					1 st hatch	50% hatch	100% hatch						
C-1	Unhandled control	✕	✕	✕	✕	✕	✕	✕	✕	✕	✕	✕	✕
C-2	Ethanol (0.5 mL/L)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
I-1	MT 400 μ g/L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
I-2	MT 400 μ g/L for each Immersion	✓	✓	✕	✕	✕	✕	✕	✕	✕	✕	✕	✕
I-3		✕	✓	✓	✕	✕	✕	✕	✕	✕	✕	✕	✕
I-4		✕	✕	✓	✓	✕	✕	✕	✕	✕	✕	✕	✕
I-5		✕	✕	✕	✓	✓	✕	✕	✕	✕	✕	✕	✕
I-6		✕	✕	✕	✕	✓	✓	✕	✕	✕	✕	✕	✕
I-7		✕	✕	✕	✕	✕	✓	✓	✕	✕	✕	✕	✕
I-8		✕	✕	✕	✕	✕	✕	✓	✓	✕	✕	✕	✕
I-9		✕	✕	✕	✕	✕	✕	✕	✓	✓	✕	✕	✕
I-10		✕	✕	✕	✕	✕	✕	✕	✕	✓	✓	✕	✕
I-11		✕	✕	✕	✕	✕	✕	✕	✕	✕	✓	✓	✕

^a After immersion, these 13 groups were split into two batches. One batch (C-1F, C-2F and I-1F – I-11F) received MT treated (3mg / kg) feed while other batch (C-1, C-2 and I-1 – I-11) were fed ethanol treated diet during oral treatment (September 1st, 2010 – November 1st, 2010). 100% hatch was observed on 23rd July. Age of embryos and alevins was not mentioned in degree days due to 30 days post-fertilization incubation at ambient temperature before embryos were transferred to research centre and kept at 9.5 -10 °C during treatment and post treatment.

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The MT dose proposed for in-feed treatment was 3 mg/Kg based on Haffray et al. (2009). A stock solution containing MT at 0.075 mg/mL was prepared in 100% ethanol. A total of 20 mL of this stock solution was sprayed onto 500 g of feed (0.5 mm) to deliver a total amount of 1.5 mg of MT/500 g. The control diet was prepared by spraying 20 mL of 100% ethanol onto 500 g of feed following Fesit et al. (1995). Both diets were left overnight in fume hood to dry and then stored at 4°C in sealed bags prior to feeding. After dietary treatment both batches were fed untreated diet until final sampling.

4.3.3 Sampling

Once the feeding treatment was finished, all groups were transferred to 26 tanks (with water capacity of 300 L) within split recirculation systems and fed with a standard commercial diet (0.5 mm, 1% BW/day). Water temperature was maintained at 9 – 10 °C while photoperiod was ambient. At 148 days post-hatch (dph), the number of fish in each tank was randomly reduced (without any size bias into results) to 100 fish. Water temperature was maintained at 14 – 15 °C until completion of the experiment. Monthly weight checks were performed throughout the study period to adjust the feed ration.

Sampling was performed at the age of 11 months post-hatch to classify the sex of fish (sex can be identified at this age in brook trout microscopically [Scobie and Benfey, 2005; Fatima et al., 2012] and macroscopically [Chapter 3]). Sex was identified at similar age in previous studies on sex reversal in brook trout (Sacobie, 2005; Haffray et al., 2009). Sample size of 70 fish from each group (tank) was selected following the previous works on masculinization of brook trout (n = 10, Sacobie, 2005; n = 81 – 120, Galbreath et al., 2003; n = 26 – 100, Haffray et al., 2009). Fish were randomly selected (by dip net) from each group (excluding I-1) and killed by overdose of anaesthetic (30 mg/L iso-eugenol) (Aqui-S® NZ Ltd). Total body weight (nearest 0.1 g) and fork length (nearest 1.0 mm) were measured and

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these values were used to calculate condition factor (appendix III). Fish were dissected to remove gonads and classify sex. Gonads were preserved in seawater Davidson's fixative. Fixed samples were dehydrated through different grades of ethanol (70%, 80%, 95%, 100%; two repeats in each grade) and finally embedded in paraffin to be sectioned at 5 μ m and stained using routine haematoxylin and eosin staining (Horobin, 2002). Stained slides were studied at 10-1000 X magnification, using a light microscope (Olympus, Japan) and images captured using Leica DC300F digital camera (Leica Microsystems, Germany).

Fish from tank I-1 were sampled at 6 months due to a technical failure in aeration system resulting in unexpected morbidity of some fish within that group. These moribund fish were fixed in Davidson's solution and mid-sections of whole body were processed for histology. Sex was clearly identified by microscopic study of whole-body sections.

These histology results were used to develop a key (Fig. 4.1) for the classification of gonads. Sex was classified into following 4 categories:

- (a) Male: Both testes were normal and categorized as immature (Fig. 4.1A), maturing and mature stage (Fig. 4.1B).
- (b) Female: Ovaries were compact and at pre-vitellogenic stage (Fig. 4.1C).
- (c) Sterile: Gonad was a thin streak of stromal tissue containing no germ cell (Fig. 4.1D).
- (d) Intersex: Intersex: Ovaries were loose (smaller and less compact and saggier than normal ovaries) and small. One ovary contained ovarian tissue in the form of a very small anterior lobe terminating into thin streak of stromal tissue. Histologically, intersex gonads contained predominantly pre-vitellogenic oocytes, a few spermatogonia and stromal tissue (Fig. 4.1E).

Mature males having functional sperm ducts and releasing milt on manual stripping were called as functional males (Table III.4, appendix III).

4.3.4 Statistical Analysis

The number of each class of gonads in each treatment was converted to proportions before analysis. Proportions of males and females in the unhandled control (C-1) were used for comparison with those of other groups. The proportions of males and females in C-1 and C-2 were compared to the hypothetically ideal proportion of 0.50 (1:1 sex ratio in natural population according to Mendelian Genetics and literature) by using χ^2 goodness of fit test. The same test was applied to analyse the statistical difference between the proportion of males each treatment and in C-1 (0.54). Similarly, the proportion of females in each treatment was compared with that of C-1 (0.42). The proportions of sterile fish were not analysed statistically as not expected in natural population. χ^2 test of goodness of fit was used to compute determine the difference in proportions of males and females between each immersed group and its MT Fed counterparts. This analysis was performed between proportions of male – male and female – female.

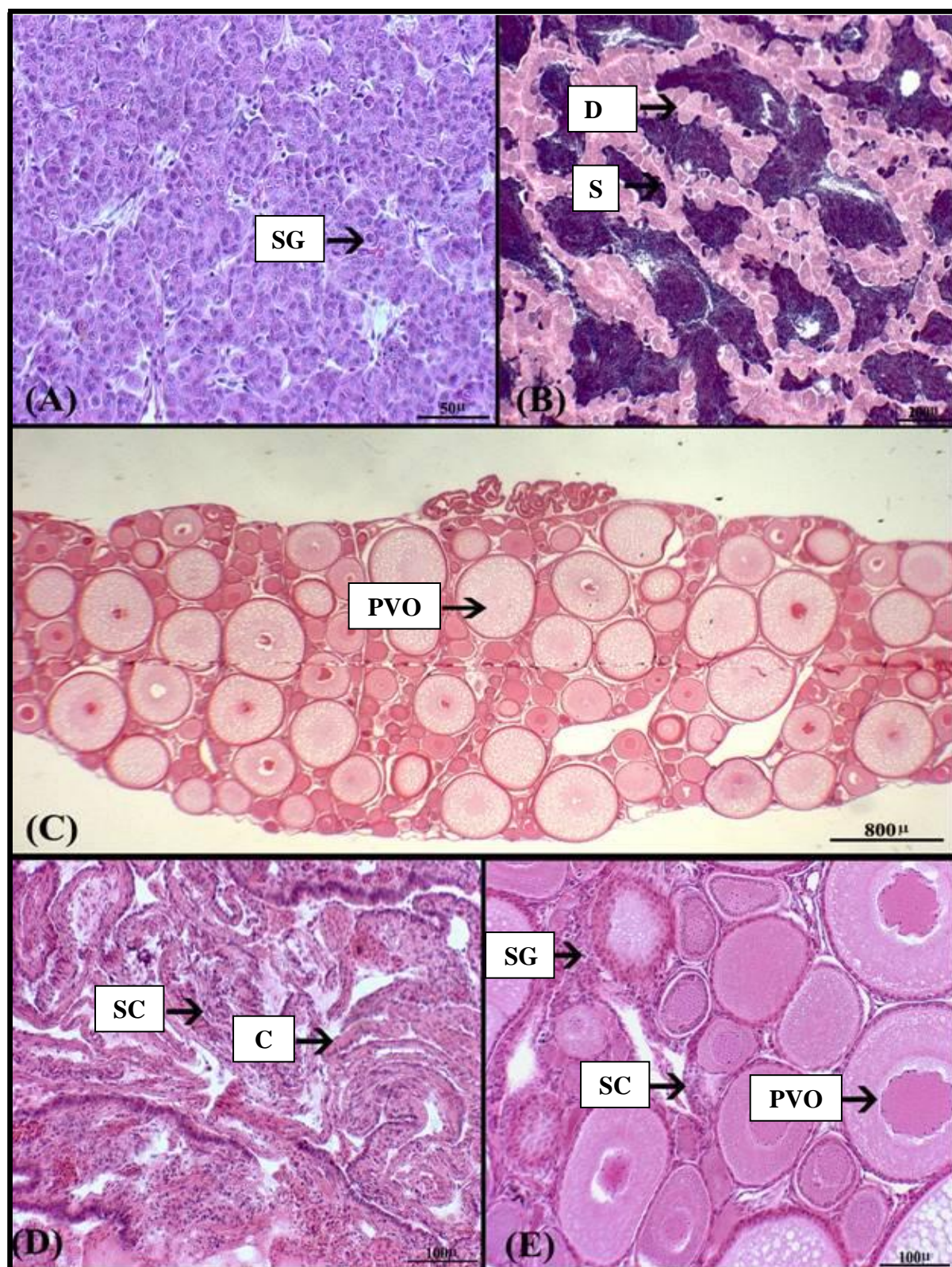


Fig. 4.1 Classification of gonads into 4 classes based on morphological and histological study. Male: Both testes were normal and categorized as immature (A), maturing and mature stage (B). Female: Ovaries were compact and at pre-vitellogenic stage (C). Sterile: Gonad was thin streak of stromal tissue (D). Intersex: Ovaries were loose and small. One ovary contained ovarian tissue in the form of a very small anterior lobe terminating into thin streak of stromal tissue. Histologically, intersex gonads contained pre-vitellogenic oocytes, spermatogonia and stromal tissue (E). C: connective tissue; DC: developing cysts; PVO: pre-vitellogenic oocyte; S: sperm; SC: stromal cells; SG: spermatogonia; μ : micrometre.

4.4 Results

4.4.1 Non-immersed Controls (C-1 and C-2)

The proportion of males and females in C-1 and C-2 showed no significant difference from the expected value (0.5:0.5) ($P < 0.05$) (Fig. 4.2). Sterile fish appeared in small proportions of 0.04 and 0.01 in C-1 and C-2 respectively.

4.4.2 Immersed Treatments (I-1 – I-11)

The minimum proportion of males among all controls and treatments was observed in I-1 (0.17) (Fig. 4.2). I-1 had the highest proportion of sterile fish (0.83) but no female were observed. A significantly high proportion of males were observed in I-2 (0.75) ($P < 0.05$) (Fig. 4.2). I-3 and I-6 showed a high skew to males (0.67) however this was not significantly different to C-1 (0.75) ($P > 0.05$). Among the other treatments, no group showed any significant male skew compared to the expected value (0.54). Intersex fish appeared in the range of 0.22 – 0.33 for all treatments except I-1 which did not show any intersex fish (Fig. 4.2). The proportion of females was within the range of 0.03 – 0.16 for I-2 – I-11 (Fig. 4.2). With the exception of I-2 (0.75)) these proportions were not significantly different from the expected value (0.42). The proportion of functional males ranged between 0.24 – 0.46 in all treatments while the highest proportion of functional males was observed in I-4 (0.46) (Table III.4 in appendix III).

4.4.3 Non-immersed – MT Fed Controls (C-1F and C-2F)

Observed proportions of males and females for both controls (C-1F and C-2F) did not differ significantly from the expected value of males (0.54) and females (0.42) ($P > 0.05$) (Fig. 4.3).

Proportions of sterile fish were very low compared to those of intersex which were observed in the proportions of 0.31 and 0.36 and CF-1 and CF-2, respectively (Fig. 4.3).

4.4.4 Immersed – MT Fed Treatments (I-1F - I-11F)

Proportion of males in I-1F (0.31) was significantly less than the expected value (0.54) ($P < 0.05$) (Fig. 4.3). No females were observed for I-1F however the proportion of sterile fish was very high (0.66). The proportions of males in all remaining treatments (IF-2 – IF-11) were not different from the expected value (0.54) ($P > 0.05$) (Fig. 4.3). The maximum proportion of males among MT fed treatments was noted for I-11F (0.61) however a high proportion of sterile fish (0.14) was also observed in this group. Proportions of females in all MT fed treatments were not significantly ($P > 0.05$) different from the expected value (0.42) with the exception of IF-1 (0.31) and IF-11 (0.61). The proportions of intersex and sterile fish in MT fed treatments were observed within the range of 0.03 – 0.66 and 0.03 – 0.42. The proportion of functional males were observed in the range of 0.01 – 0.11 in all treatments (Table III.4 in appendix III).

A significant difference ($P < 0.05$) was observed in proportions of males between I-2 – IF-2, I-3 – IF-3 and I-6 – IF-6. On the other hand, proportions of females were significantly different ($P < 0.05$) between I-1 – IF-1, I-5 – IF-5 and I-6 – IF-6 only. No negative effect of MT treatment was observed on survival of treated fish except the very low rate of mortality in a few treatments (Table III.4 in appendix III).

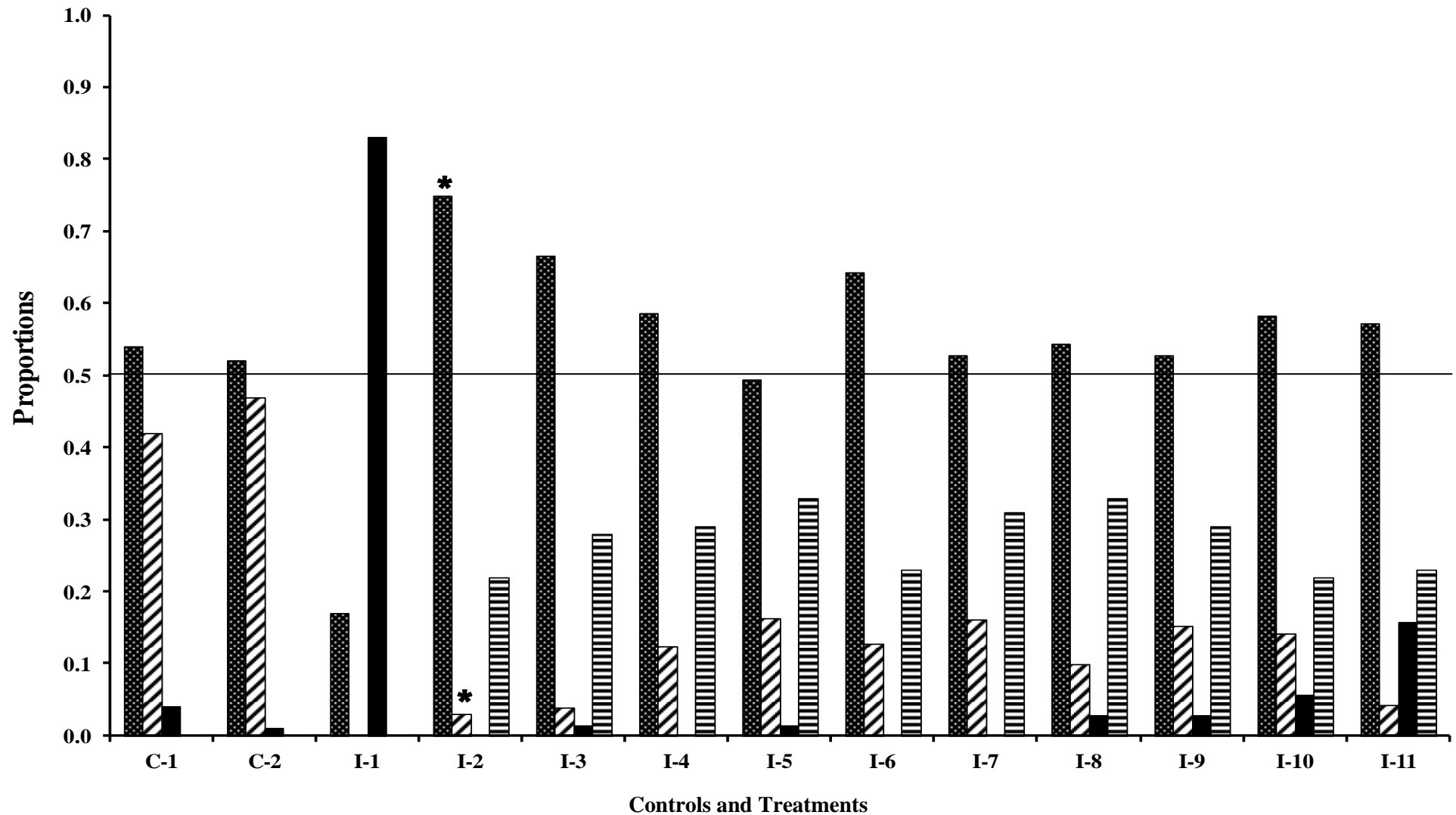


Fig. 4.2 Proportions of male, females, intersex and sterile fish in C-1, C-2, I-1 – I-11. Chi-square test compared the proportions of males and females with those of C-1. Significant difference between these proportions has been shown by asterisk (■=males, ▨= females, ▤ = Intersex, ▩ = Sterile). The median line indicates the ideal sex proportion of 0.5 of males or females in population.

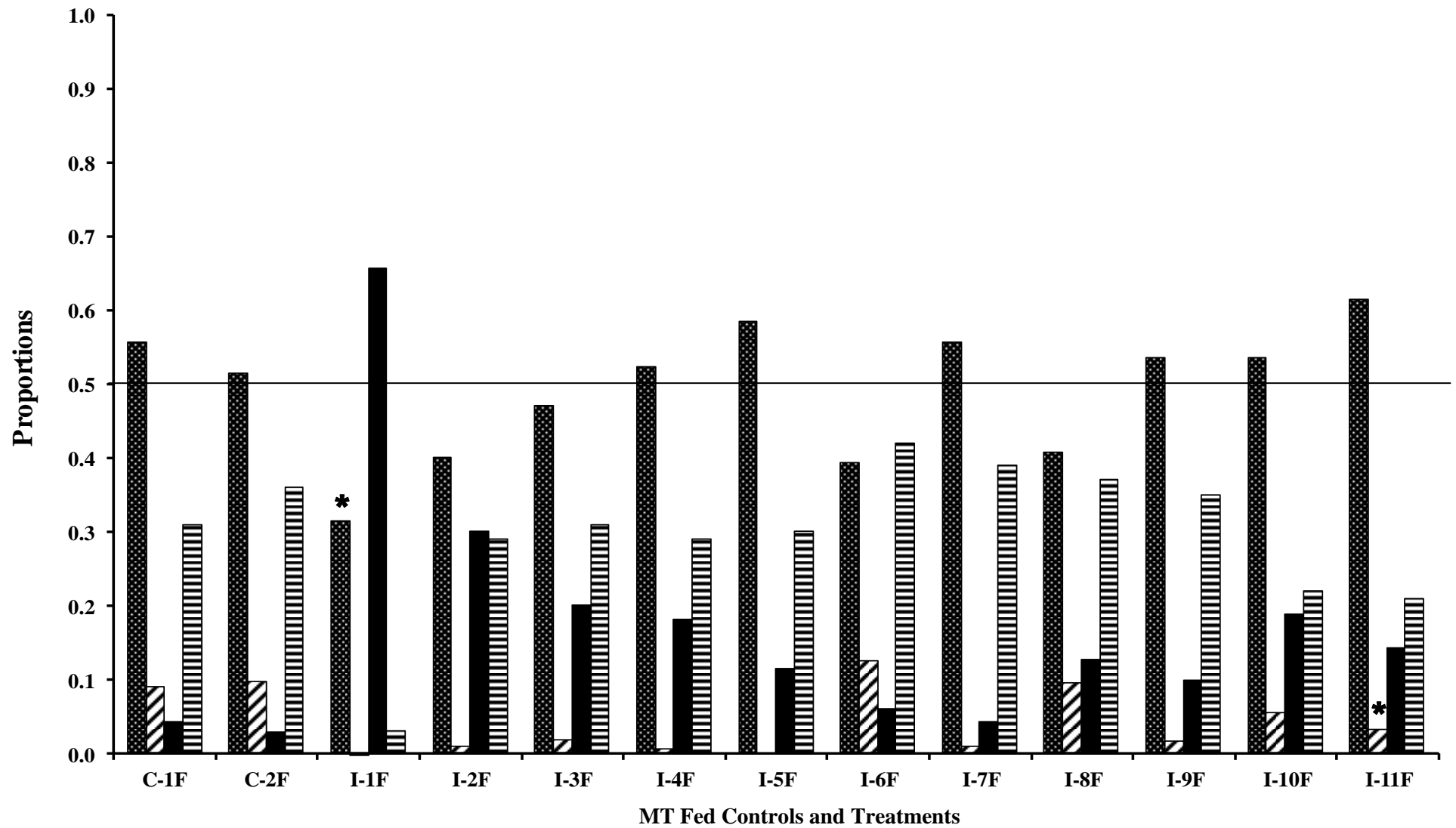


Fig. 4.3 Proportions of male, females, intersex and sterile fish in C-1F, C-2F, I-1F – I-11F. Chi-square test compared the proportions of males and females with those of C-1. Significant difference between these proportions has been shown by asterisk. (■ = males, ▨ = females, ▤ = Intersex, ▩ = Sterile). The median line indicates the ideal sex proportion of 0.5 of males or females in population.

4.5 Discussion

This study has narrowed the treatment window within the pre-hatch period to 4 to 6 days pre-hatch at 9.5 – 10 °C. This is in agreement with other studies (Sacobie, 2001; Galbreath et al., 2003; Haffray et al., 2009) which found the pre-hatch period to be the most responsive time period for phenotypic sex reversal of brook trout. However, these previous studies did not identify the most sensitive time window during the pre-hatch period. MT treatment during this time window resulted in the production of proportion of 0.75 male population with the least number of females (0.03) and without the occurrence of any sterile fish. Although these proportions of masculinisation do not markedly vary from previous sex reversal studies for brook trout (0.74 – 0.80 and 0.60 in Haffray et al., 2009 and Sacobie, 2001, respectively), this masculinisation rate was achieved with fewer immersions and without any dietary manipulation. The success of immersion only treatment in the present study contrasts with Galbreath et al. (2003) and Haffray et al. (2009) who reported complete ineffectiveness of MT immersion only treatment for masculinisation in brook trout. In these studies, comparatively high total dose of MT (0.5 mg/L and 1.2 mg/L; Galbreath et al., 2003 and Haffray et al., 2009, respectively) failed to successfully masculinise the female population. This may have been due to missing the most sensitive time period of pre-hatch (single immersion on 10 days post-hatch; Galbreath et al., 2003) or sub optimal MT dose given during this period (0.4 mg/L given in only one immersion during 1 week pre-hatch; Haffray et al., 2009).

The present study supports previous research findings (Sacobie, 2001; Galbreath et al., 2003; Haffray et al., 2009) suggesting that sex reversal by MT immersion is unachievable during the post-hatch period in brook trout. Successful masculinisation has been reported for Arctic charr (*Salvelinus alpinus*) by application of the same techniques during post-hatch period but

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with a different androgen and a substantially higher dose (Chiasson and Benfey; 2007). The success of post-hatch sex reversal in Arctic charr may be due to inter-specific differences in the occurrence of the labile differentiation period and genetic histories (Pandian and Sheela, 1995).

Other than MT immersion treatment, exclusive dietary MT inclusion is used as a standard protocol for sex reversal in other salmonid species such as Atlantic salmon, chinook salmon and rainbow trout (Johnstone and Youngson, 1984; Solar et al., 1984; Cousin-Gerber et al., 1989; Demska-Zakes, 1999; Amini and Tala, 2003; Lee et al., 2004; Tala et al., 2006; Baron et al., 2008). The observed higher sensitivity of the pre-hatch period for sex reversal observed here and in previous studies, may be attributed to activation of steroidogenic pathways in pre-differentiating gonads. The activation of steroidogenic pathways has been indicated by the expression of genes responsible for steroidogenic enzyme production prior to differentiation as observed in rainbow trout (Govoroun et al., 2001; Baron et al., 2008; Vizziano-Cantonnet et al., 2008). MT treatment at this stage down-regulates those genes which are potentially involved in oogenesis at gonadal differentiation (Baron et al., 2008). It will be difficult to inhibit oogenesis during the post-hatch period when steroidogenic pathways have been irreversibly determined (Govoroun et al., 2001). Similarly, this study found dietary inclusion of MT both exclusively and in conjunction with immersion was either ineffective at increasing the proportion of males or produced a large proportion of sterile fish respectively.

In brook trout, gonadal histological (identification of gonads as ovaries and testes on histological examination when gonads are not morphologically differentiated) and morphological (morphological identification of gonads as ovaries and testes on macroscopic examination) differentiation occurs during the post-hatch period (Sacobie and Benfey, 2005; Fatima et al., 2012) which indicates the possibility for sex reversal immediately post-hatch before sex is irreversibly determined. MT dietary treatment was applied to investigate this

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possibility but was found to be ineffective in trans-differentiating (reversal of sex after cytological sex differentiation) presumptive females; in agreement with previous work (Galbreath et al., 2003; Sacobie, 2001; Haffray et al., 2009). Contrastingly, dietary treatment (with estradiol for direct feminization) was found to be successful for feminization of male brook trout (Johnstone et al., 1979) after feeding an estradiol treated diet for 60 days. This might be due to late differentiation of presumptive testes as compared to ovaries (Sacobie and Benfey, 2005; Fatima et al., 2012).

The occurrence of sterile fish following sex reversal attempts has been reported in brook trout previously where fish were exposed to MT by immersion only (Sacobie, 2001; Galbreath et al., 2003), diet only (Galbreath et al., 2003) or in combination (Parks and Parks, 1991; Galbreath et al., 2003). In the present study no sterility was observed using two immersions during the 4 to 6 day pre-hatch period. The absence of any sterile fish in this treatment further supports the success of this approach. A high incidence of sterility was observed in fish exposed repeatedly over the two week periods either side of hatch. This may be due to the level of exposure to MT (1.6 mg/L) which exceeds the optimum level reported by other studies (Pandian and Sheela, 1995; Galbreath et al., 2003). Sterility was substantially more apparent in fish exposed to combined treatments of MT immersion and dietary inclusion. This result suggests that dietary supplementation of MT can be completely eliminated as an option for sex reversal of female brook trout.

Sterility was also found to be higher in those groups which received MT immersion during pre-hatch period and subsequently fed with a MT treated diet. However the incidence of sterility was comparatively less in fish exposed to post-hatch MT immersions even though they were identically fed MT. This suggests that sterility induced by feed depends upon the time of immersion in combination treatments and that intensity of the same dose of MT varies with the sensitivity of gonads during sex differentiation. These results are in agreement

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with previous studies in other salmonids like coho salmon and brook trout using different doses and durations of androgen treatment (Goetz et al., 1979; Hunter et al., 1982; Parks and Parks, 1991). Due to the lack of the comprehensive work on androgen induced sterility in brook trout, it is difficult to describe the molecular mechanism resulting in the observed sterility outcome and its dependence upon androgen dose and time of treatment.. However, the high incidence of sterility within those treatments is likely to be not due to the potency of MT. MT is the second least potent androgen used in sex reversal protocols typically resulting in fewer incidences of sterility (Pandian and Sheela, 1995). Moreover, the reduced sterility has been reported in brook trout when treated with MT as compared to other androgens like methyl-dihydrotestosterone (Galbreath et al., 2003).

A few sterile fish were observed in non-immersed controls (0.04 and 0.01 in C-1 and C-2, respectively). Occurrence of sterility in controls might be due to contamination of water with MT residues. Both MT fed and non-MT fed treatments and controls were supplied water through same water recirculation system. During oral treatment, MT residues might leech out from uneaten feed and bathed controls. This slight contamination of water might have resulted in occurrence of a few sterile samples ($n = 4$) and a slight skew in the sex proportion of C-1 towards maleness (0.54) as compared to the hypothetically ideal proportion of 0.50 (not statistically significant). However, proportion of sterile fish in C-2 was only 0.01 ($n = 1$). The reason of this slight skew to males in C-1 and occurrence of minor sterility in both controls might be due to the negligible interference of water contamination.. Herman and Kincaid (1991) also reported the occurrence of intersex samples (0.9) in untreated control lake trout (*Salvelinus namaycush*). This may suggest that deviation from the expected sex type of male and female may be possible in controls as a natural incidence instead of an artefact of the experimental design.

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One limitation of present study was the lack of replication of treatments, however, this study aimed to sequentially screen a one week period pre and post-hatch which required a multiple treatment approach. Duplication at the very least would have required 56 separate tanks which was beyond the physical and logistical capacity of the trial facility. Similar experimental approaches have been undertaken in previous sex reversal studies upon rainbow trout (Solar et al., 1984; Cousin-Gerber et al., 1989; Fiest et al., 1995; Demska-Zakes et al., 1999), chinook salmon (Baker et al., 1988), coho salmon (*Oncorhynchus kisutch*) (Piferrer and Donaldson, 1991) and brook trout (Galbreath et al., 2003; Haffray et al., 2009). The present study used a total of 26 treatment groups to identify the most sensitive time window using a complex experimental design without replicates to ensure successful completion of the experiment within available trial facilities. This study provides important information for further optimization of MT dose and duration of immersion in future replicated studies.

The efficiency of sex reversal protocols are determined by the duration and dose of the androgen as well as the proportion of successfully sex reversed fish (Piferrer, 2001). The most successful treatment in the present study significantly reduced the dose and duration of MT treatment by 800 μ g and 8 hours, respectively in comparison to previous studies (Sacobie, 2001; Galbreath et al., 2003; Haffray et al., 2009). Previous research reported longer treatments i.e. a total of 16 h of MT immersion from pre-hatch until post-hatch, followed by 60 days of oral treatment (Haffray et al., 2009) and a total of 120 h of immersion during the post-hatch period (Sacobie, 2001). Synonymous with a longer duration, immersion baths have contained higher concentrations of MT (a total of 1.6 mg, Haffray et al., 2009; a total of 4 mg, Sacobie, 2001) as well as the additional exposure to subsequent dietary treatment (Haffray et al., 2009). Although the current study suggests improved efficiency, there is still an opportunity to improve the male proportion after amendments to MT dose and possibly by further variation to number and duration of immersions. The presence of

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testicular tissue in ovaries of partially reversed females (histology described in Fig. 4.1 E) showed their partial responsiveness to MT treatment. These females might not be completely reversed due to a sub-optimal dose of MT to inhibit the genes responsible for oogenesis at gonadal differentiation (Govoroun et al., 2001; Baron et al., 2008). Further refinement of immersion treatment in terms of dose and duration could result in complete masculinisation of these females.

Production of all-female stock eliminates the problem of precocious maturation in male brook trout which has proven to be major obstacle toward its expanded production in Tasmania. The present study narrowed the treatment duration by identifying the most sensitive time window. This achievement provides clear direction for further optimization of an indirect feminization protocol for brook trout utilising the simple application of only two MT immersions during the pre-hatch period. This finding proposes a streamlined approach to those reported previously that have required multiple immersions and oral MT supplementation (Galbreath et al., 2003, Haffray et al., 2009). Successful masculinisation can be achieved without any dietary treatment which may benefit growth (chinook salmon [*Oncorhynchus tshawytscha*] Baker et al., 1988), coho salmon [*Oncorhynchus kisutch*], Piferrer and Donaldson. 1991 Dunham, 2004), avoid sterility, reduce stress of handling during multiple immersions, decrease user exposure to steroids and reduce its environmental discharge.

Males produced in I-2 could not be tested to sort out the neomales during next year. It could be performed by crossing these males with normal females during next year. However, progeny testing could not be carried out due to unavailability of the trial facility for another year, commencement of another laborious experiment (Chapter 5) and limited time left to complete this PhD project. Future work to optimise the methods reported here could involve further manipulation of exposure of brook trout differing MT concentrations and durations

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within the window identified herein will maximise the proportion of phenotypically reversed females that is presently achievable for other salmonids. In addition, for future studies masculinization in brook trout could be attempted within the identified time window by using aromatase inhibitors as has successfully been applied for Atlantic salmon (Lee et al., 2003) in Tasmania. This option was not tested in the present study due to long duration of treatment, capacity of trial facility and labour required for a parallel trial other than MT treatment. Moreover, MT is commercially used in Tasmania and farmers are more experienced in handling its immersion and dietary treatment. Lee et al. (2003) produced a proportion of 0.51 males after application of two immersions in 1,4,6-androstatriene-3, 17-dione (5 mg/L in each immersion). The present study produced male proportion of 0.75 by giving two immersions of only 400 μ g/L (in each immersion). Furthermore the objective of present study was to identify the most sensitive time window for sex reversal in brook trout. As this narrow time window of 4-6 days has been identified, both androgens and aromatase inhibitors can be to successfully produce the neomales in this species. However aromatase inhibitors can be considered as a future perspective for sex reversal in brook trout.

4.6 References

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CHAPTER 5

The Effect of Photoperiod Manipulation upon Maturation and Sex Steroids Profiles of Brook Trout (*Salvelinus fontinalis*)

5.1 Abstract

Maturation is the major constraint towards the commercial production of brook trout pre harvest maturation which affects growth, flesh quality and immunocompetency. Photoperiod manipulation is relatively used to inhibit or reduce the incidence of maturation in salmonids, so an experimental adaptation of this approach was trialed for brook trout. Mixed sex fish (age =14 months) were subjected to simulated natural photoperiod (NP), advanced photoperiod (AP) and continuous photoperiod (CP) to investigate the response of this species endogenous circannual rhythm of sexual maturation. Light treatments commenced on August 1st, 2011 until May 25, 2012. Maturation was observed in all females and 96% of total males held under NP. Fish exposed to AP corrected their maturation cycle by advanced phase shift of their endogenous rhythm for 8 weeks. These fish achieved final maturation during March-April and could be manually stripped instead of natural spawning season of June-July. Plasma profiles of testosterone (T) and estradiol-17 β (E₂) were also adjusted according to advancement of photoperiod and were at similar concentration levels as observed in fish exposed to NP during specific developmental phases. Exposure to AP inhibited maturation by 6% and 8% in males and females, respectively, however most of the fish successfully attained maturation. Similarly, treatment of CP for 10 consecutive months failed to inhibit the onset of maturation in brook trout however did inhibit the final stage of maturation i.e. spermiation and ovulation. Although photoperiod regimes applied in present study could not successfully inhibit the maturation in brook trout but data suggest that manipulation of maturation is possible in this species if technical approach or regime is refined.

5.2 Introduction

Brook trout (*Salvelinus fontinalis*) was introduced to Tasmania from Canada in 1962 (Clements 1988). Since the introduction of this species, Tasmania's Inland Fisheries Service as well as some privately owned hatcheries have maintained broodstock populations of brook trout for propagation and subsequent stocking of lakes for recreational fishing and to supply commercial producers as required (S. Chilcott, Inland Fisheries Service, Pers. Comm.). A high incidence of precocious sexual maturation in brook trout males (within the first year) and maturation of both males and females prior to their second (harvest) year was observed during pilot commercial trials during 2003-2004. Early maturation is a significant constraint to commercial salmonid culture due to the loss of condition and immunocompetency in maturing fish (Piferrer, 2001; Cuesta et al., 2007).

Temperate fish species show a distinct seasonality with regards to their reproduction and spawning, with some level of gonadal activity occurring in every season (Miguad et al., 2010). Although this circannual rhythm of maturation is endogenously entrained there are open "recruitment" and "completion" windows which can be closed (Randall et al., 1998), delayed (Porter et al., 2000) or advanced (Holcombe et al., 2000) by photoperiod manipulation. In salmonids, increasing day length may signal initiation of the maturation while diminishing day length initiates completion (Bromage et al., 2001; Miguad et al., 2010). Entrainment of reproductive cycle with seasonal changes in photoperiod is regulated by the pineal-brain-pituitary-gonadal circadian system. Rhythmic production of melatonin from the pineal gland plays a major role in this system and its key importance has been reported for all major salmonids (Amano et al., 2000; Fal  n et al., 2010). Moreover, each species has a light duration and intensity-specific threshold (Leclercq et al., 2011) that together with size, growth rate and energy storage (which are directly affected by food

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availability and environmental circumstances) must be surpassed to commence maturation (Shearer and Swanson, 2000).

The incidence of maturation can be reduced in salmonids by exposing fish to short days (Atlantic salmon: Björnsson et al., 1994; King et al., 2003; Skilbrei and Heino, 2011; rainbow trout: Choi et al., 2010), accelerated photoperiod (Atlantic salmon: Porter et al., 1999; Andersson et al., 2003; Taranger et al., 2003), continuous photoperiod followed by short days (Arctic charr: Frantzen et al., 2004) and continuous long photoperiod followed by continuous short days (Atlantic salmon: Taranger et al., 2003; rainbow trout: Choi et al., 2010). On the other hand, exposure to continuous photoperiod can completely inhibit (Atlantic salmon: Oppedal et al., 1997; Chinook salmon: Unwin et al., 2005), delay or reduce the incidence of maturation (Atlantic salmon: Taranger et al., 1995; Porter et al., 2000; Peterson and Harmon, 2005; Skilbrei and Heino, 2011). However, photoperiodic history, direction of change in photoperiod, duration of light treatment, light intensity, age of fish, strain, husbandry conditions vary between different studies. Most of the protocols for photoperiod manipulation in salmonids, particularly for inhibition of maturation, are available for Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) and less attention has been given to the species of genus *Salvelinus*, particularly the brook trout (*Salvelinus fontinalis*).

The limited work available on brook trout reported that compression of the seasonal light cycle resulted in the advancement of maturation (Hoover and Hubbard, 1937; Hazard and Eddy, 1951; Hoover, 1951; Corson, 1955) or delayed maturation (Holcombe et al., 2000) in brook trout without reducing the proportion of maturing fish. However, exposure to long days followed by short days did not affect the maturation in brook trout but continuous short photoperiod followed by continuous long days, short days and long day treatments all caused a delay in functional maturity (ovulation in females and release of milt in males on manual stripping) but did not inhibit maturation in brook trout (Henderson, 1963).

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Although these studies reported ineffectiveness of advanced photoperiod treatment to reduce the number of maturing fish however there was a major drawback in the light protocols followed. These studies had been reported from North America where brook trout spawns during October – November (fall spawner). The “recruitment window” for this species in N. America opens in January (winter solstice) as reported by Tam et al., (1984). They observed the commencement of exogenous vitellogenesis (indicates recruitment of ovaries for maturation) in January when vitellogenic oocytes contributed 55% of total germ cell population. Although Tam et al. (1984) did not study the gonadal development in males it can be inferred that it would be the same time (January) as observed in females. In chapter 3, both male and female brook trout commenced recruiting for maturation (beginning of exogenous vitellogenesis in ovaries and first meiotic divisions in male germ cells i.e. formation of secondary spermatocytes) in the same month (December). The studies reporting the advancement of maturation by exposing fish to long days followed by short days started their light treatment in January (Hazard and Eddy, 1951; Hoover, 1951; Corson, 1955). At this time fish had achieved required energy threshold and exogenous vitellogenesis in ovaries and first meiotic divisions in testes were already started. Henderson (1963) reported the recovery of developing oocytes from atresia caused by exposure to short days (34 days) – long days (30 days) treatment in brook trout which shows the inability of light treatment to stop further development once germ cells have recruited. As these previous studies (Hazard and Eddy, 1951; Hoover, 1951; Corson, 1955) did not expose the fish to manipulated photoperiod during the immature phase (pre-vitellogenic stage in ovaries and pre-meiotic stage in males), they could not close the “recruitment window” which could be the reason of failure of this treatment to reduce the incidence of maturation. Moreover, duration of the post-spawning period until the recommencement of gonadal development for the next cycle is different between brook trout strains found in N. America and Tasmania. Tam et al. (1984) observed spawning in November and recruitment in January reporting the gap of only one

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month between these two important events in reproductive cycle of brook trout in N. America. In Tasmania, this gap was found to be of about five months (Chapter 3). This short recovery period of one month in N. American strains further supports that “recruitment window” was probably missed in previous studies. If the light treatment is commenced earlier than the time of recruitment when gonads are immature there is a possibility that the “recruitment window” can be closed and maturation can be inhibited or reduced in both sexes. Moreover, Carlson and Hale (1973) observed 100% and 61% inhibition of maturation in brook trout females and males, respectively. They exposed fish to accelerated photoperiod at the age of about 5 months until the age of 11 months. Subsequently fish were held under constant short days until the age of 12 months. This study supports that if brook trout are exposed to AP quite early then incidence of maturation can be reduced. However, it is evident that female brook trout do not achieve puberty during first year of their life history (Chapter 3). Therefore, if Carlson and Hale (1973) observed no mature female it could be due to their delayed maturation until next year. However 61% inhibition in males was an important outcome.

In contrast to above mentioned previous studies reporting the advancement of maturation by compression of annual light cycle in brook trout, Holcombe et al. (2000) exposed the brook trout yearlings to extended photoperiod to delay maturation in N. America. They commenced the long days treatment (five months long) during September and shifted to short days (four months long) during February. As they started light treatment two months earlier than opening of the recruitment window in January, their light protocol did not have that drawback which was found in other studies (Hazard and Eddy, 1951; Hoover, 1951; Corson, 1955). After application of similar photoperiod regime, Holcombe et al. (2000) successfully delayed maturation in brook trout without reducing the proportion of maturing fish. This shows the failure of this treatment in brook trout to reduce the number of maturing fish. However, a

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very small sample size at final sample point (7 females and 6 males) affects the reliability of results reported in this study.

The economic loss due to reduced growth, poor flesh quality, inconsistent size and greater disease susceptibility during maturation is a major obstacle in progressing brook trout culture in Tasmania. The present study was designed to inhibit maturation or reduce the proportion of maturing fish in the population by 8 weeks advancement of the seasonal light cycle. The justification of this approach was to exposure fish to long days and simulated photoperiod of December – January (recruitment window) earlier than the natural light cycle to trigger gonadal development. Due to the energy reserves and profiles of sex steroids below threshold levels, it is anticipated that gonads would fail to recruit for further development. This protocol differs from previous studies in brook trout which failed to reduce maturation due to the late commencement of light treatment. Other than 8 weeks advanced photoperiod, the present study aimed to inhibit / reduce maturation by exposing fish to continuous (24 hours) photoperiod. No study has been reported previously on inhibition of maturation by exposure to continuous photoperiod in any species of genus *Salvelinus* including brook trout. Although success of continuous photoperiod in inhibiting maturation has been observed in two species of salmonids i.e. Atlantic salmon (Oppedal et al., 1997) and chinook salmon (Unwin et al., 2005) however due to lack of any available work on the impact of this paradigm on brook trout, this protocol can be investigated as another possible solution to control maturation in this species. Both inhibition and delay in maturation until the fish have reached their marketable size will be helpful to address the above mentioned problems previously encountered in Tasmanian brook trout culture.

5.3 Materials and Methods

A population of mixed sex brook trout ($n = 1323$, age = 6 months) were transferred from a commercial fish farm (Mountain Stream Fishery, Targa, Tasmania; Latitude = $41^{\circ} 18' 01''$ S, Longitude = $147^{\circ} 22' 35''$ E, Elevation = 425 m) to the National Centre for Marine Conservation and Resource Sustainability, Aquaculture Centre (University of Tasmania, Launceston, Tasmania; Latitude = $41^{\circ} 23' 21.17''$ S, Longitude = $147^{\circ} 07' 35.49''$ E, Elevation = ~ 60 m) during November, 2010. Fish were stocked in 4000 L recirculating experimental tanks and fed twice daily on a commercial diet (Spectra SS, Skretting, Australia) at 2% of body weight/day. Photoperiod was ambient and water temperature was maintained between 13 - 15°C by the heat/chill unit (10 KW) until light treatment was started.

When the stock had reached an age of 14 months (July, 2011) a total of 864 fish (135 ± 27 g) were randomly transferred into nine 1000 L circular experimental tanks (each tank containing 96 fish). Fish were acclimatized for one week before commencement of light treatments on August 1st 2011. The nine tanks were maintained by three separate water recirculation systems which were joined in end of February to maintain same water conditions (Fig. 5.1A) (Water recirculation systems were combined at the end of February when fish had already recruited for maturation. Therefore, any impacts of leeching pheromones from one system to another on maturation would be negligible if any.). Each system was fitted with a light proof cover containing a 20 W tungsten globe. Photoperiod in each system was controlled by manually adjusting timers at two week intervals. Water temperature for each system (three tanks per system) was controlled by a heat/chill unit (7 KW). Dissolved oxygen was maintained at >80% saturation and physico-chemical parameters of water (pH 6.8 - 7.2, total ammonia <2 mg/L, nitrite <1 mg/L, nitrate <80 mg/L) were checked twice a week throughout the study period. Fish were fed twice daily on a commercial diet (Spectra SS, Skretting, Australia) at 1% of body weight/day.

5.3.1 Experimental Design

The experiment commenced on August 1st, 2011, about 40 days after the winter solstice in June. Three light treatments were used (Fig. 5.1B) as follows:

A – Natural photoperiod, simulated to that of Launceston, Tasmania (NP)

B – Advanced photoperiod for 8 weeks (AP)

C – Continuous photoperiod (24 hours). (CP)

Each photoperiod treatment had three replicates. Fish were exposed to light treatments for 10 months (August, 2011 – May, 2012). An average light intensity of 6330 lux was recorded at the water surface while an average of 838 lux and 466 lux were recorded at depths of 30 cm (middle depth of tank) and 60 cm (bottom of tank), respectively.

5.3.2 Sampling

A total of seven sampling points were performed on August 1st, 2011, September 28, 2011, November 25, 2011, January 30, 2012, March 14, 2012, April 30, 2012 and May 25, 2012. At each sample point, a total of 14 fish were randomly sampled from each tank (replicate). These fish were transferred to 50 L tank containing mild anesthetic (iso-eugenol, AQUI-S NZ Ltd.). After recording their body weight (nearest 0.1 g) and fork length (nearest 1.0 mm), these fish were recovered and put back to their experimental tanks. An additional six fish were randomly sampled from each tank (replicate), giving a total number of 18 fish per treatment. Overall, 54 fish were sampled from three systems (AP, CP and NP) to collect blood, liver and gonad samples at each sample point. These fish were killed by overdose of anesthetic by transfer into a 50 L tank containing 30 mg/L iso-eugenol (AQUI-S NZ Ltd.). Blood samples of these fish were collected from the caudal sinus using heparinized syringes, centrifuged at 3000 rpm for 15 minutes and plasma was stored at -80 °C until assayed for

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steroids. Total body weight (nearest 0.1 g) and fork length (nearest 1.0 mm) were also measured before dissection (appendix IV). Gonads and livers were removed and weighed (nearest 0.1 g). Gonads were fixed in Davidson's fixative for histological study while liver samples were frozen at -80 °C to determine total crude lipids.

At the final sampling point (May, 2012), a total of 20 fish were sampled from each tank and killed by overdose of anesthetic prior to recording their body weight and fork length. These fish were dissected and the weight of gonads and liver was noted. An additional 10 fish were randomly collected from each tank and samples taken as described above.

Total body weight, fork length, weight of gonad and liver were used to calculate the following indices and specific growth rate for each fish:

$$\text{Condition Factor (} k \text{)} = [\text{Total Body Weight (g)} / \{\text{Standard Length (cm)}\}^3] \times 100$$

$$\text{Gonadosomatic Index (GSI)} = [\text{Gonad Weight (g)} / \text{Total body weight (g)}] \times 100$$

$$\text{Hepatosomatic Index (HSI)} = [\text{Liver Weight (g)} / \text{Total body weight (g)}] \times 100$$

Total body weight for all indices included the weight of gonads and liver.

5.3.3 Histological Study

Fixed samples were dehydrated through an increasing ethanol gradient (70%, 80%, 95%, 100%; two repeats in each grade), cleared in xylene (two repeats) and embedded in paraffin to be sectioned at 5 µm and stained using routine haematoxylin and eosin (Horobin, 2002). Stained slides were studied at 10-1000 X magnification, using a light microscope (Olympus, Japan) and images captured using Leica DC300F digital camera (Leica Microsystems, Germany). Cell count was performed under the microscope using the grid for each section of all samples i.e. five slides for each sample. Mean ± SE was calculated.

5.3.4 Determination of Crude Lipid in Liver

Liver samples stored at -80°C were defrosted, weighed and kept in a freeze drier (Dynavac, Australia) to remove the water content. Dried samples were homogenized with mortar and pestle and 300 mg of each sample was used to determine the total lipids in duplicate. The method described by Bligh and Dyer (1959) was used to extract total lipids with ethanol and chloroform as solvents.

5.3.5 Total Fecundity

Total fecundity was estimated as standing stock of eggs in the ovary following Almatar et al. (2004). Fecundity was measured in female held advanced photoperiod in April and May, 2012. Females held under natural and continuous photoperiod were assessed for fecundity in May, 2012. One piece of gonad (1 g) was taken from both the right and left ovary of each mature fish. Eggs were separated from the tissues and then counted in both pieces. The mean of these two values was performed to calculate the total number of ova present in entire pair of ovaries.

5.3.6 Hormone Analysis

The levels of E₂ and T in aliquots of thawed plasma were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992). Extraction efficiency (mean recovery of ³H-labelled steroid from triplicates of a plasma pool) was >90% for E₂ and T and values for each steroid were adjusted accordingly. The minimum detection limit of each assay was 100pg/mL. Inter-assay variability measured using aliquots of a pooled internal standard was 6.98% and 9.99% for E₂ and T, respectively. The volume of plasma and extract used were adjusted according to the different stages of development. Plasma sample volumes of 200 µL and 100 µL were extracted for immature and maturing fish, respectively. Extract volumes of 200 µL and 100

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μL were assayed for immature and maturing fish, respectively. Each sample was run in duplicates.

5.3.7 Statistical Analysis

Monthly variations in all parameters were analyzed by one way ANOVA after justifying assumptions by performing Shapiro-Wilk test and test of homogeneity of variance. One way ANOVA was applied to compare variability between mean values of each parameter in each treatment for both males and females over the study period. Similarly, one way ANOVA was used to determine the variability between mean values of three treatments on each sample point. Tukey's post-hoc test was applied for comparison of means. Means \pm standard error (SEM) are presented. All statistical analyses were performed using SPSS 17.0 and differences were considered significant if $P < 0.05$.

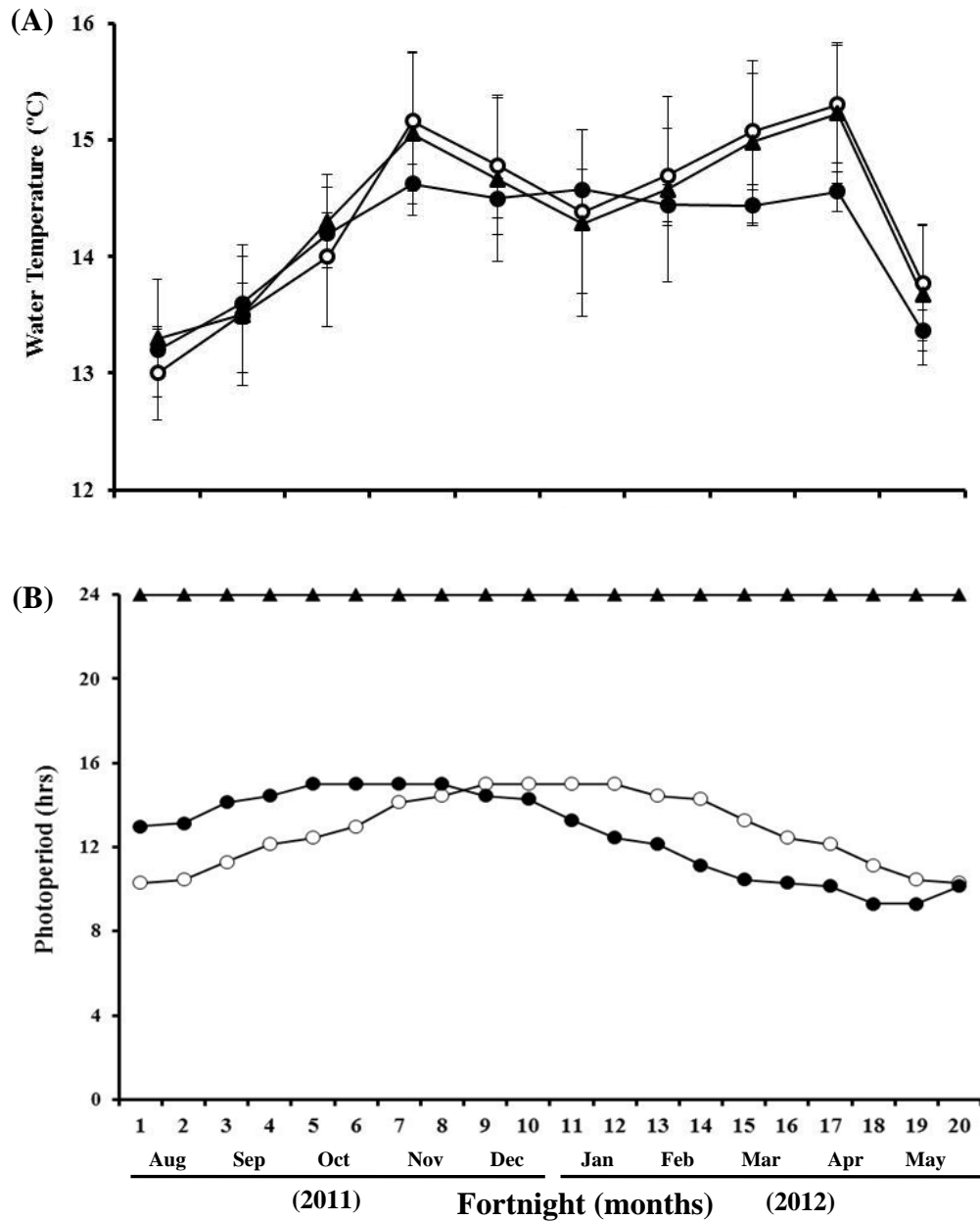


Fig. 5.1 Protocol of water temperature (A) and photoperiod (B). Natural photoperiod (NP) (○), advanced photoperiod (AP) (●) (accelerated for 8 weeks) and continuous photoperiod (CP) L:24 hours (▲). Light treatment commenced on August 1st, 2011 and continued for 10 months until May 25, 2012. A total of 7 sampling points were performed on August 15, 2011; September 28, 2011; November 25, 2011; January 30, 2012; March 14, 2012; April 30, 2012 and May 25, 2012.

5.4 Results

Results of body weight, body length and gonadal weight have been given in appendix IV.

5.4.1 Condition Factor

Condition factor in females exposed to NP reached its highest mean value during April (1.51 ± 0.1) (Fig. 5.2A). On the other hand, highest mean values of condition factor of females in AP (1.62 ± 0.1) and CP (1.56 ± 0.1) were noted during May which were significantly higher than those in NP at this sample point. Significant within treatment variability was noted in females in NP ($F_{6, 97} = 11.23, P < 0.05$), AP ($F_{6, 94} = 26.28, P < 0.05$) and CP ($F_{6, 89} = 5.37, P < 0.05$). Males in all three treatments showed a similar pattern to that observed in females. Condition factor in NP increased and its highest mean value was observed in April (1.47 ± 0.07) (Fig. 5.2 B). Males in AP and CP reached their highest mean values of 1.46 ± 0.02 and 1.51 ± 0.03 during May, respectively. Significant within treatment variability was noted in males exposed to NP ($F_{6, 86} = 1.45, P < 0.05$), AP ($F_{6, 89} = 6.51, P < 0.05$) and CP ($F_{6, 91} = 2.44, P < 0.05$) over the study period.

5.4.2 Gonadosomatic Index (GSI)

Females held under NP showed a significant increase in GSI during April and reached the highest mean value (10.20 ± 0.41 %) during May (Fig. 5.3 A). On the other hand, females exposed to AP showed a significant increase during March (12.25 ± 1.50 %) which remained stable until April but dropped during May (3.32 ± 1.00 %). GSI of females in CP showed a significant increase during March (6.13 ± 1.00 g) which reached its highest mean value during May (9.91 ± 0.52 %), nearly equal to that of females in NP on same sample point ($P > 0.05$). Over the whole study period, significant within treatment variability was noted in GSI of females in NP ($F_{6, 97} = 88.79, P < 0.05$), AP ($F_{6, 92} = 12.35, P < 0.05$) and CP ($F_{6, 89} = 38.82, P < 0.05$).

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GSI of males in NP and AP reached its highest mean value during May (2.60 ± 0.08 %) and April (3.70 ± 0.30 %) which dropped during May in males exposed to AP (Fig. 5.3 B). The highest mean value of GSI of males in CP was noted during May (3.11 ± 0.13 %). Significant within treatment variability was noted in GSI of males in NP ($F_{6, 86} = 54.86$, $P < 0.05$), AP ($F_{6, 89} = 4.89$, $P < 0.05$) and CP ($F_{6, 91} = 29.77$, $P < 0.05$) throughout the study.

5.4.3 Liver Weight, HSI and Liver Crude Lipids

Liver weight of females in NP, AP and CP was found within the range of 2.37 ± 0.12 - 16.41 ± 0.53 g, 2.10 ± 0.12 - 13.00 ± 0.41 g and 1.95 ± 0.13 - 12.23 ± 0.41 g, respectively (Fig. 5.4 A). No significant variability was observed between liver weights in all three treatments with the exception of April and May ($P < 0.05$). Throughout the trial, significant within treatment variability was noted in liver weights of females in NP ($F_{6, 97} = 88.79$, $P < 0.05$), AP ($F_{6, 92} = 19.22$, $P < 0.05$) and CP ($F_{6, 89} = 44.15$, $P < 0.05$).

Liver weight of females in NP, AP and CP was found within the range of 2.01 ± 0.10 - 8.20 ± 0.62 g, 2.03 ± 0.20 - 7.54 ± 0.48 g and 1.17 ± 0.10 - 7.77 ± 0.31 g, respectively over the study period (Fig. 5.4 B). Significant within treatment variability was noted in liver weights of males in NP ($F_{6, 86} = 14.95$, $P < 0.05$), AP ($F_{6, 87} = 11.86$, $P < 0.05$) and CP ($F_{6, 90} = 13.04$, $P < 0.05$).

Over the study period, HSI of females in NP and CP were found within the range of 1.22 ± 0.03 - 2.07 ± 0.04 % and 1.20 ± 0.04 - 1.93 ± 0.06 %, respectively (Fig. 5.5 A). Value of HSI in females exposed to AP increased during March (1.96 ± 0.15 %) which dropped in April and remained low until final sampling point. Significant variability ($P < 0.05$) between means of all three treatments was observed during April and May only. Over the study period, significant within treatment variability was observed in HSI of females in NP ($F_{6, 97} = 16.19$, $P < 0.05$), AP ($F_{6, 93} = 27.16$, $P < 0.05$) and CP ($F_{6, 89} = 44.15$, $P < 0.05$).

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HSI in males exposed to NP, AP and CP ranged between 0.06 ± 0.05 - 1.64 ± 0.04 %, 0.06 ± 0.25 - 1.67 ± 0.90 % and 0.05 ± 0.23 - 1.78 ± 0.05 %, respectively over the study period (Fig. 5.5 B). Throughout the study period, significant within treatment variability was noted in HSI of males in NP ($F_{6, 86} = 31.34$, $P < 0.05$), AP ($F_{6, 89} = 26.69$, $P < 0.05$) and CP ($F_{6, 90} = 76.63$, $P < 0.05$).

Level of crude lipids in males held under NP and CP ranged between 9.32 ± 1.77 – 20.00 ± 1.03 % and 8.00 ± 2.56 – 19.20 ± 1.06 %, respectively (Fig. 5.6 A). Liver crude lipids in females exposed to AP showed a significant increase in March and reached their highest value (27.10 ± 1.86 % during March which dropped in following months. Significant within treatment variability was noted in profiles of crude lipids of females in NP ($F_{6, 27} = 21.86$, $P < 0.05$), AP ($F_{6, 22} = 14.77$, $P < 0.05$) and CP ($F_{6, 21} = 16.25$, $P < 0.05$).

In males exposed to NP, AP and CP, liver crude lipids ranged between 15.11 ± 0.52 – 19.30 ± 0.50 %, 13.23 ± 0.77 – 19.30 ± 1.45 % and 10.00 ± 2.71 – 24.00 ± 1.36 %, respectively (Fig. 5.6 B). Significant within treatment variability was noted in crude lipid profile of males in NP ($F_{6, 25} = 3.80$, $P < 0.05$), AP ($F_{6, 20} = 4.42$, $P < 0.05$) and CP ($F_{6, 24} = 10.40$, $P < 0.05$).

5.4.4 Profile of Testosterone

Levels of T in females of NP group significantly increased during April and the highest value of 26.42 ± 2.89 ng/ml was noted in May (Fig. 5.7 A). Plasma T in females held under AP significantly increased during January (17.60 ± 4.92 ng/ml) which reached its highest level during March (34.00 ± 7.44 ng/ml). This peak level significantly dropped in April and remained low until the final sample point. Levels of T in females in CP ranged between 1.37 ± 0.32 - 23.11 ± 15.00 ng/ml. Over the whole study period, significant within treatment variability was noted in the T profile of females in NP ($F_{6, 37} = 19.55$, $P < 0.05$), AP ($F_{6, 31} = 6.36$, $P < 0.05$) and CP ($F_{6, 36} = 1.97$, $P < 0.05$).

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Plasma T levels in males of NP significantly increased during April and reached its highest value (38.71 ± 1.00 ng/ml) in May (Fig. 5.7 B). Levels of T in males held under AP showed a significantly high value during March which reached its highest (33.72 ± 5.00 ng/ml) during May. Profile of T in males exposed to CP ranged between 1.11 ± 0.30 – 30.22 ± 4.00 ng/ml while its peak value was observed during April. Throughout the study period, significant within treatment variability was noted in profile of T of males in NP ($F_{6, 32} = 0.97$, $P < 0.05$), AP ($F_{6, 36} = 1.45$, $P < 0.05$) and CP ($F_{6, 36} = 2.69$, $P < 0.05$).

5.4.5 Profile of Estradiol-17 β

Levels of E₂ in females of NP group significantly increased during April and reached its highest 38.00 ± 5.00 ng/ml which dropped during May (Fig. 5.8 A). Levels of E₂ in females exposed to AP were similar to that of T in these females and significantly increased during January and the highest mean value of 35.15 ± 5.00 ng/ml was observed during March. This peak of E₂ significantly dropped in April and remained low until the end of the trial in May. Plasma E₂ levels in females of the CP group significantly increased during November and reached its highest value of 11.00 ± 4.00 ng/ml during January which dropped in following months. Over the whole study period, significant within treatment variability was noted in the level of E₂ in females in NP ($F_{6, 37} = 8.67$, $P < 0.05$), AP ($F_{6, 31} = 5.93$, $P < 0.05$) and CP ($F_{6, 37} = 5.07$, $P < 0.05$).

Profile of E₂ in males exposed to NP, AP and CP ranged between 0.05 ± 0.01 - 3.54 ± 0.5 ng/ml, 0.01 ± 0.001 - 0.91 ± 0.10 ng/ml and 0.08 ± 0.01 - 0.73 ± 0.10 ng/ml, respectively (Fig. 5.8 B). Over the whole study period, insignificant variability was noted in level of E₂ in males exposed to NP ($F_{6, 29} = 0.71$, $P > 0.05$) but significant results were computed for AP ($F_{6, 29} = 15.66$, $P < 0.05$) and CP ($F_{6, 31} = 10.21$, $P < 0.05$).

5.4.6 Histological Changes during Ovarian Development

Females exposed to NP remained at the late vesicular stage until September (stage I) (Fig. 5.9 A). Exogenous vitellogenesis was observed during November and 70% of oocytes developed into early and mid vitellogenic oocytes (stage II). Polygonal yolk plates (stage III) appeared during January and 20% of females at stage II developed into stage III. Further development was slow during March and in April a total of 80% females were at late vitellogenic stage. Hydration of eggs was observed in 11% of total females during May (stage IV).

Females held under AP showed similar development as observed in those in NP group until November (Fig. 5.9 B) however atretic oocytes were observed in all females during September. These females showed rapid development during January and 83% of ovaries were found at a late vitellogenic stage. Further development remained slow during March. Ovulation was observed in 79% of females during April which continued until May and 92% of total females ovulated during this month while 8% females were found immature (Table VI.1 in appendix VI). Total fecundity was found to be 4350 ± 254 eggs per mature fish during April. Females exposed to CP exhibited faster development as compared to those in other treatments and exogenous vitellogenesis (stage II) was observed during September which continued until November (Fig. 5.9 C) when 90% of females were found at stage II. January was marked with appearance of 57% females at the late vitellogenic stage which further increased up to 83% during March. Hydration of stage III oocytes was observed during April and 45% of total females were found at this stage during May. No female at a final stage of maturity (stage IV) could ovulate during May however large number of atretic follicles was observed in all females. No immature female was observed at final sampling point.

5.4.7 Histological Changes during Testicular Development

Testicular development in males exposed to NP remained quiescent during August and secondary spermatocytes developed into spermatids (stage II) during September (Fig. 5.10 A). Mature males (stage III) (lobules were filled with sperm and interlobular walls were ruptured to release sperm) were observed in 14% of the population during January which increased up to 87% until May. A total of 65% of total mature males were found running and could be stripped in May. Remaining mature males could not achieve functional maturity (releasing milt on manual stripping) although mature. No immature male was observed at the final sample point in May.

Males exposed to AP showed no further development until September however November was marked with the appearance of 44% of maturing males (stage II) (Fig. 5.10 B). A total of 25% mature males (stage III) were observed during January and fast development was noted during March which resulted in the appearance of 90% running males during April. However during May, 85% males of a total 94% mature population were found running while 6% males were found immature.

Testicular development of males in CP was more similar to those in AP (Fig. 5.10 C). Maturing males (stage II) were observed during September and further development into mature males (40%) was noted during January. During April all immature males developed maturing (5%) and mature (95%) gonads. No hydration of milt occurred during May and no running male was found.

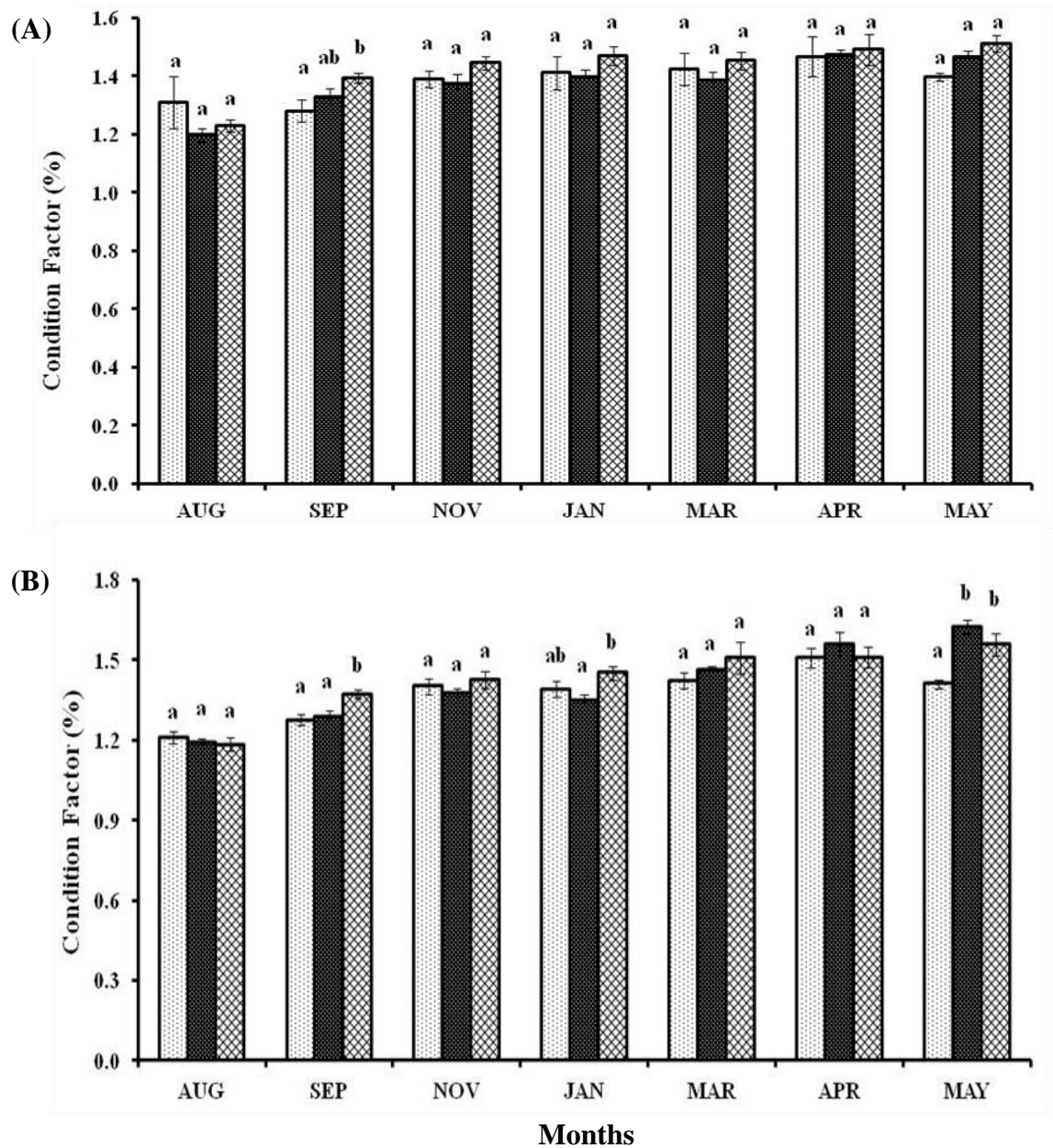





Fig. 5.2 Variations in condition factor of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced:  Continuous:  n at each sample point (AUG – APR) = 60 per treatment. n at final sample point (MAY) = 90 per treatment.

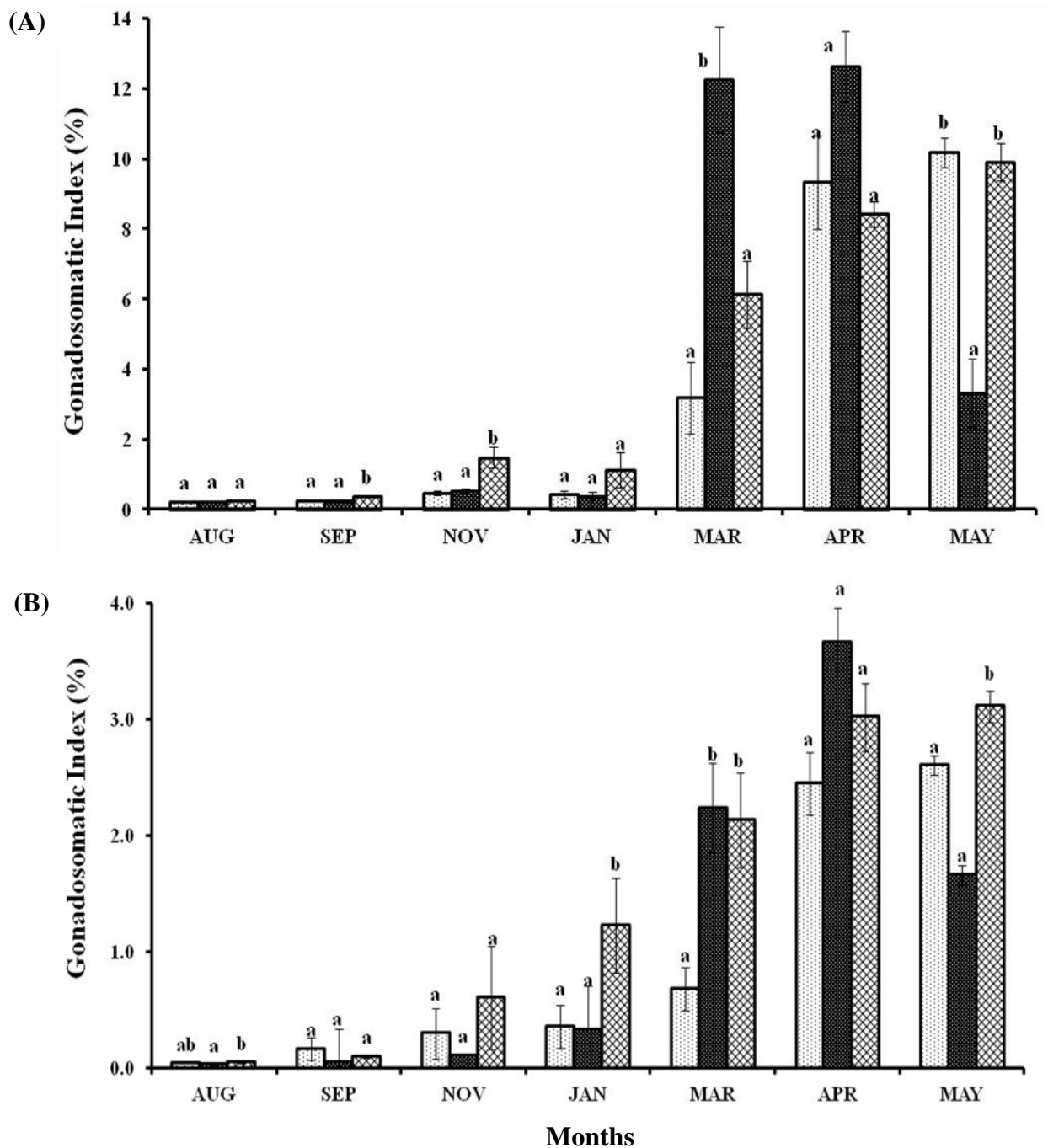





Fig. 5.3 Variations in gonadosomatic index of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced:  Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

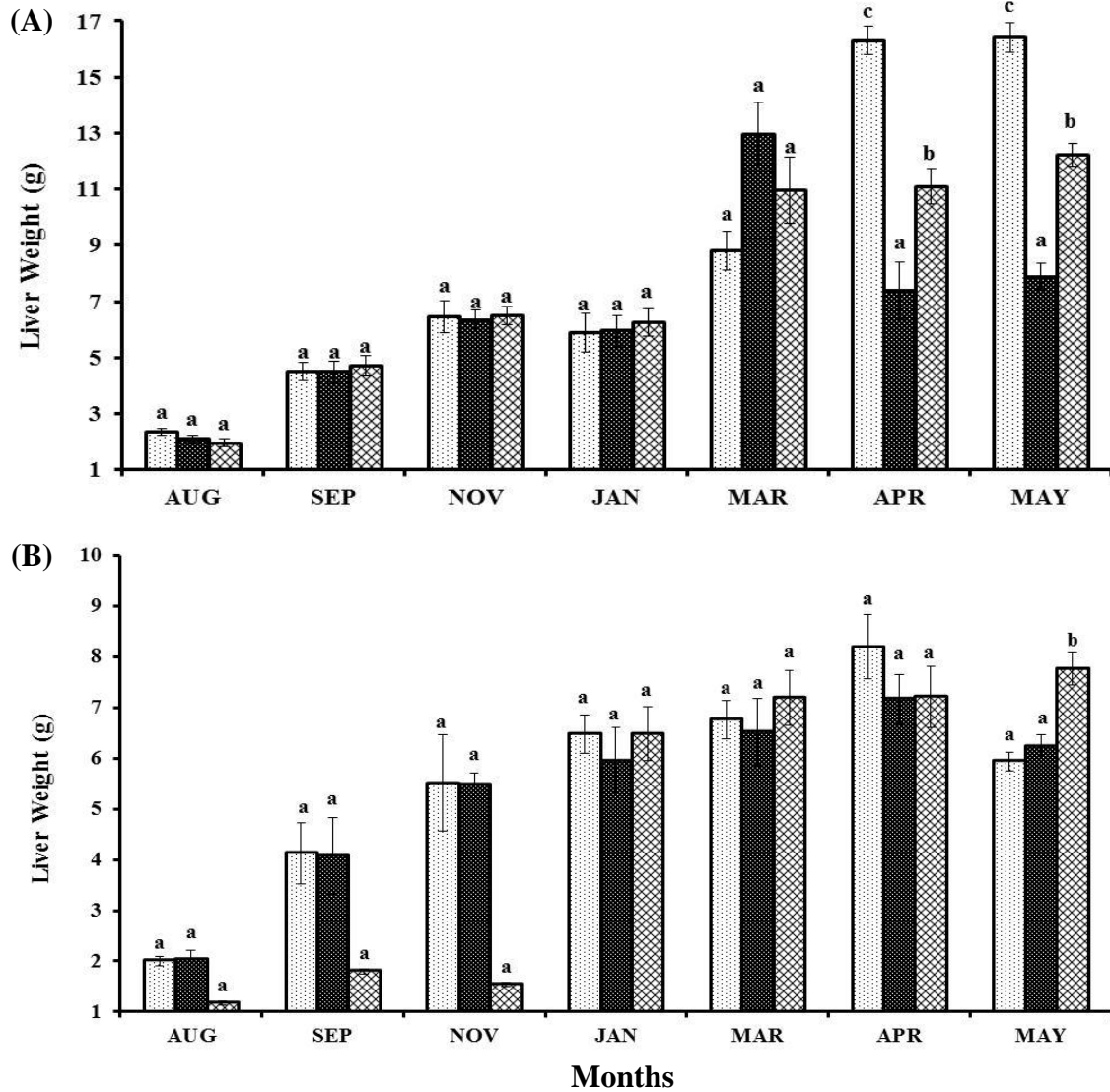





Fig. 5.4 Variations in liver weight of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) photoperiod treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced: , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

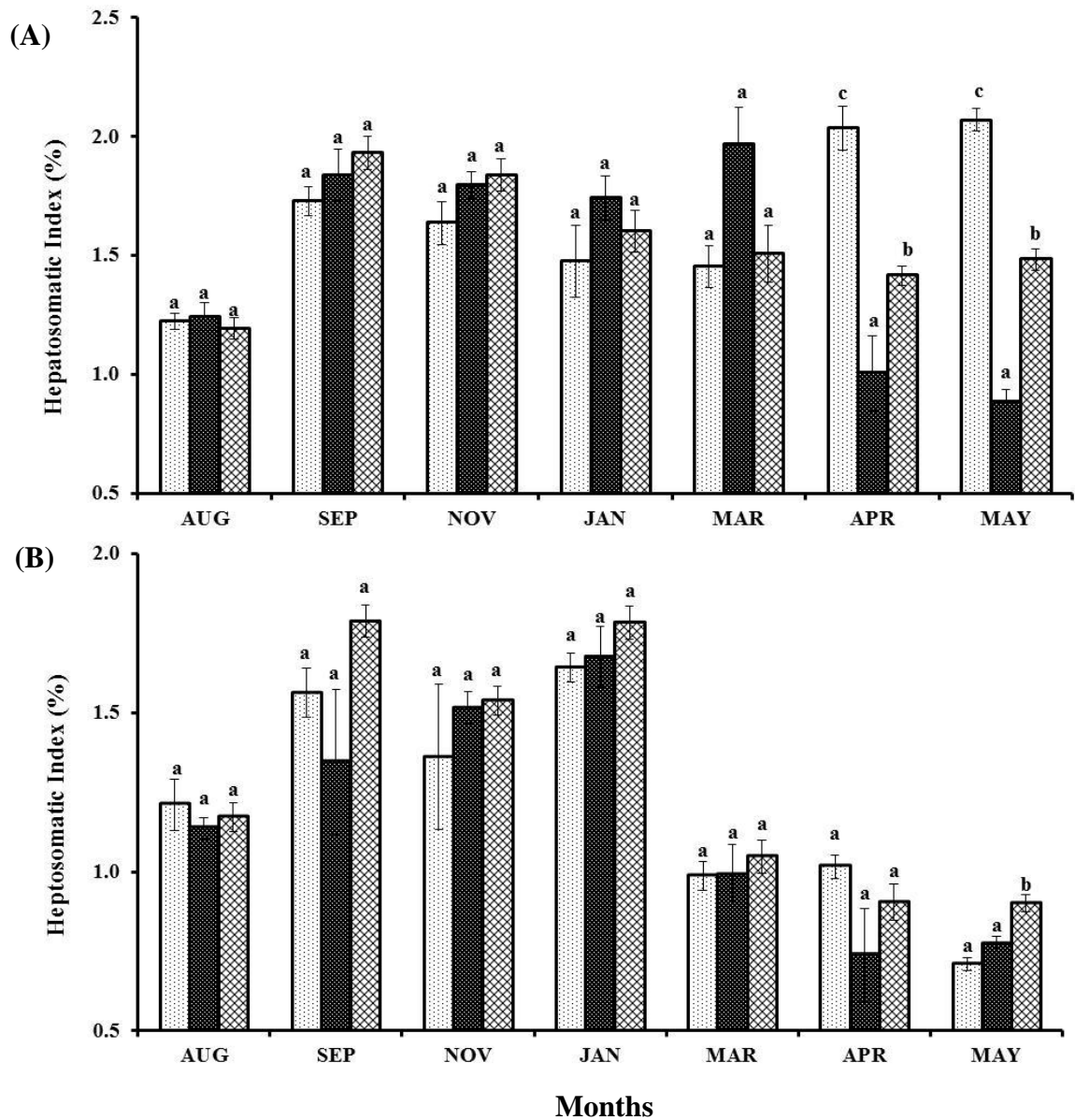





Fig. 5.5 Variations in hepatosomatic index of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) photoperiod treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

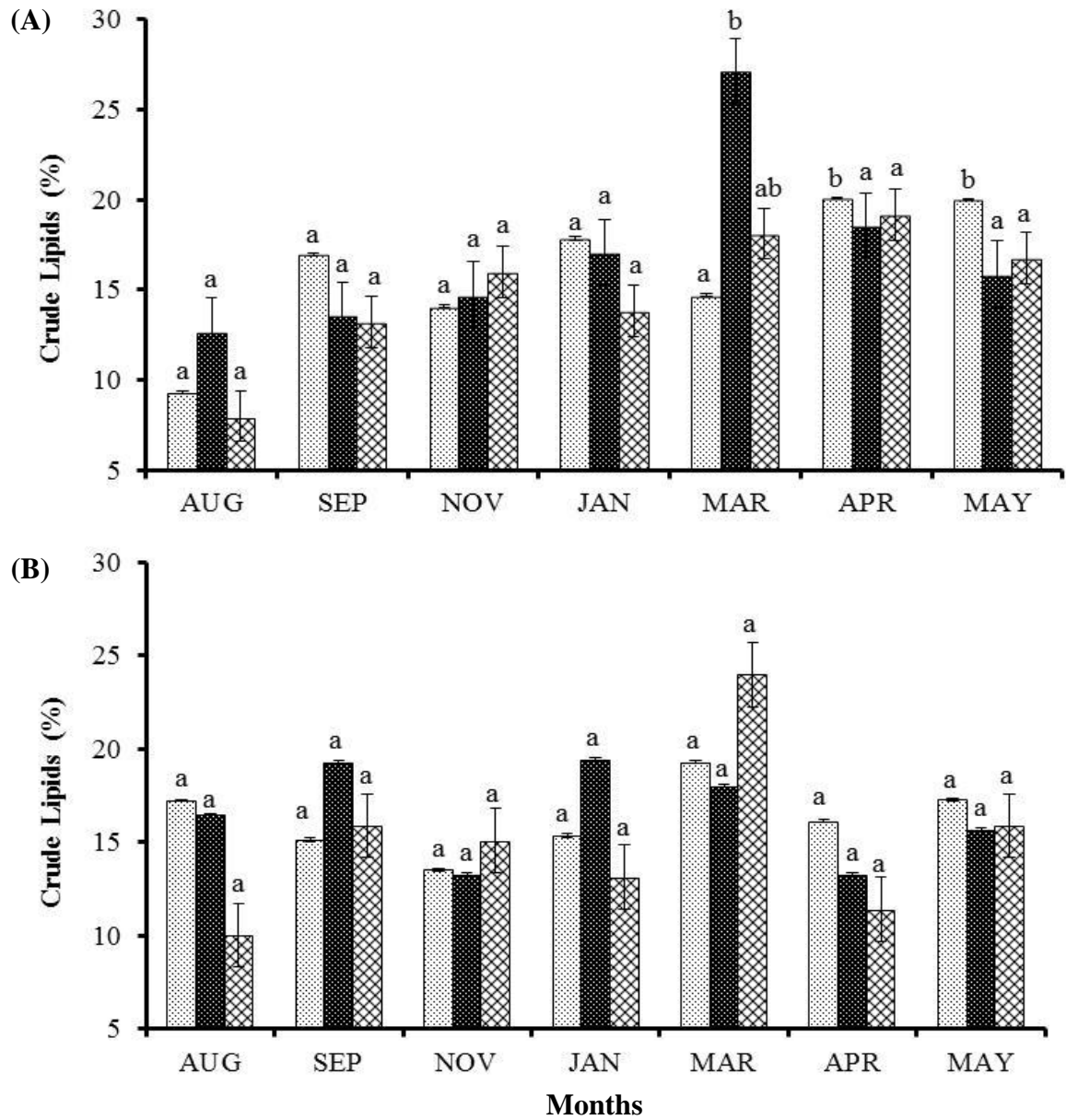


Fig. 5.6 Variations in crude lipids of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) photoperiod treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced: , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

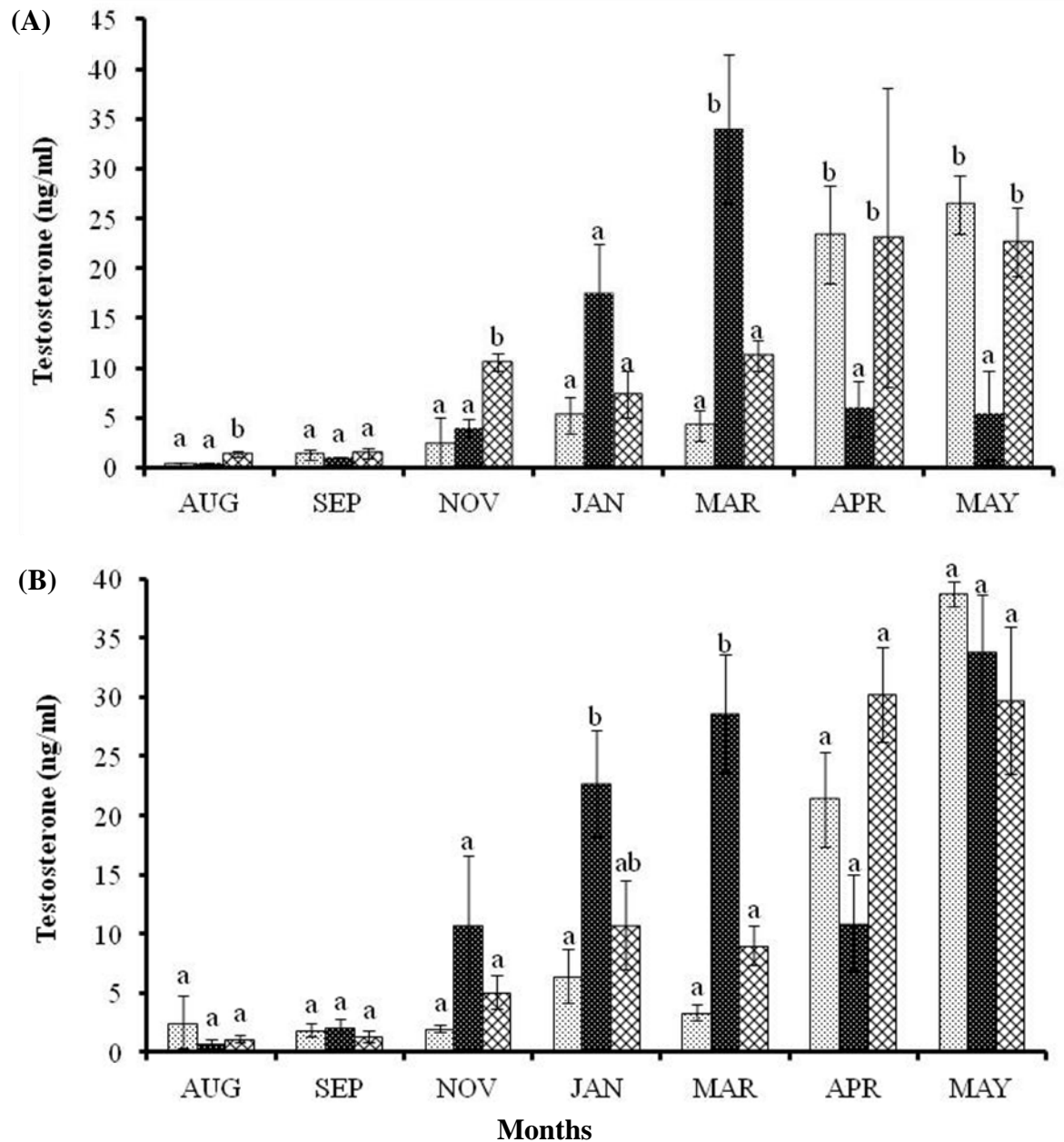





Fig. 5.7 Variations in testosterone profile of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) photoperiod treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced: , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 30 per treatment.

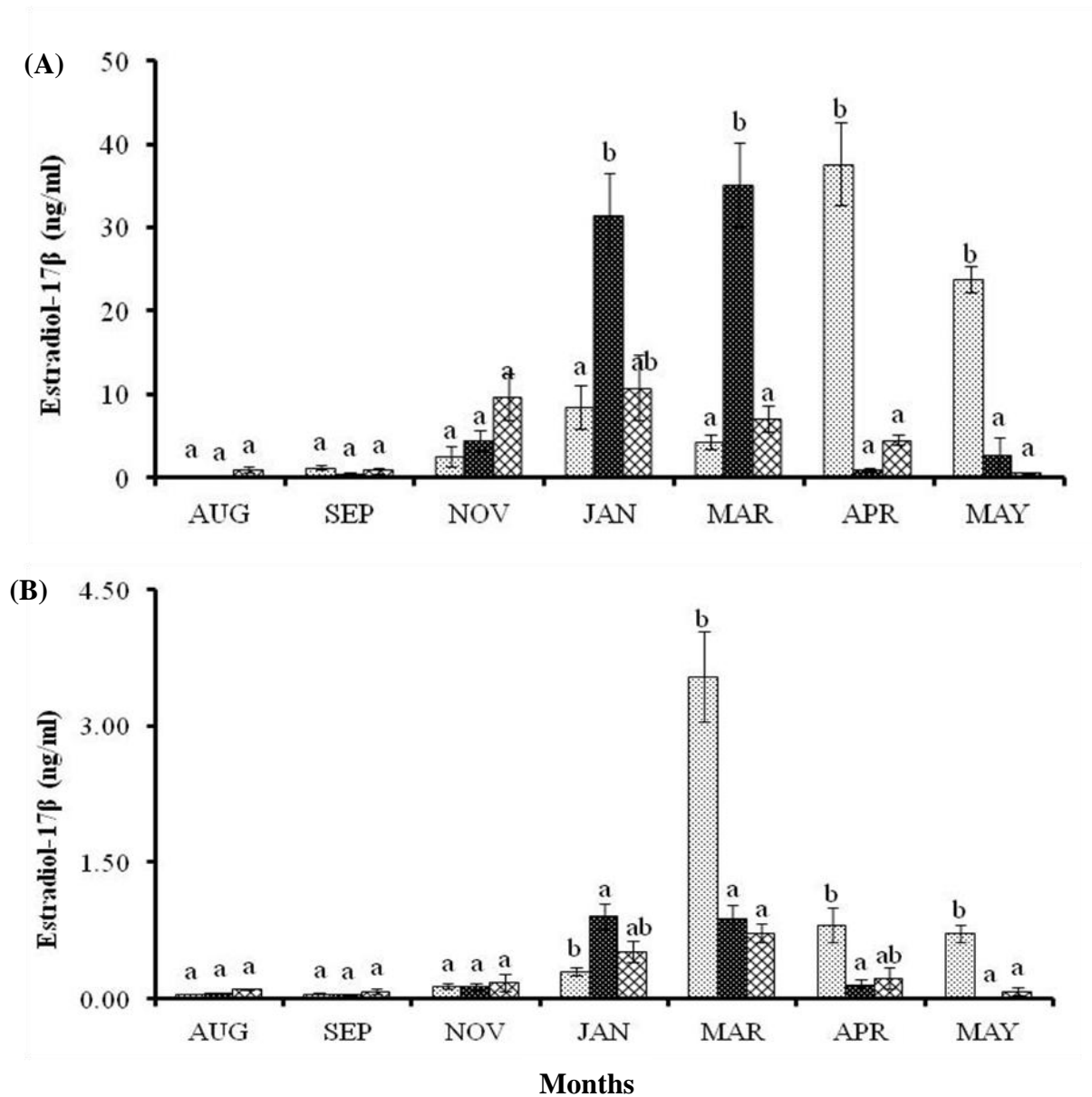





Fig. 5.8 Variations in estradiol-17 β profile of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) photoperiod treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced: , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 30 per treatment.

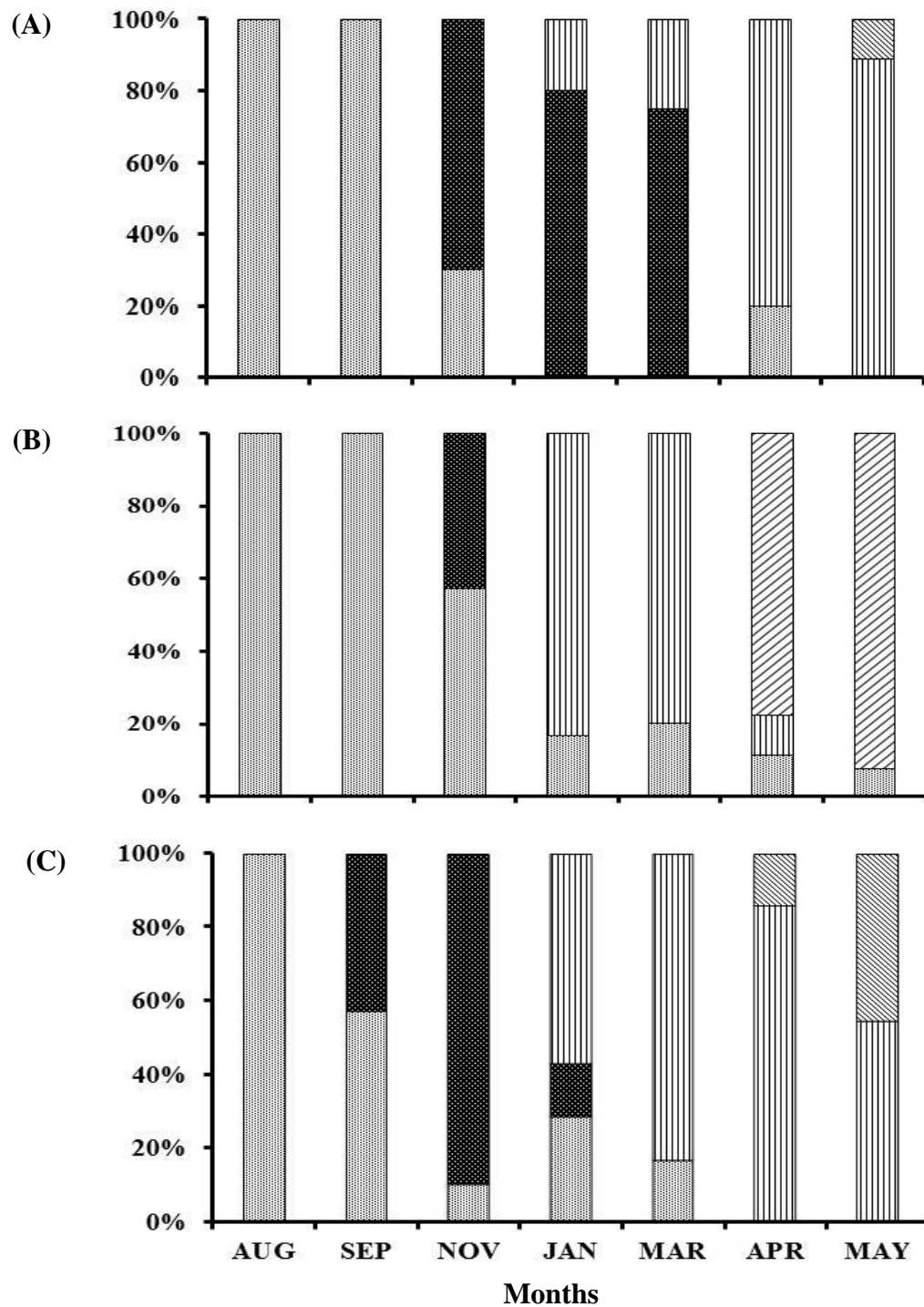








Fig. 5.9 Variations in the percent population of females at different developmental stages exposed to natural (A), advanced (B) and continuous (C) photoperiod treatments. Stage I: , Stage II: , Stage III:  Stage IV:  Stage V:  Stage VI: 
n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment

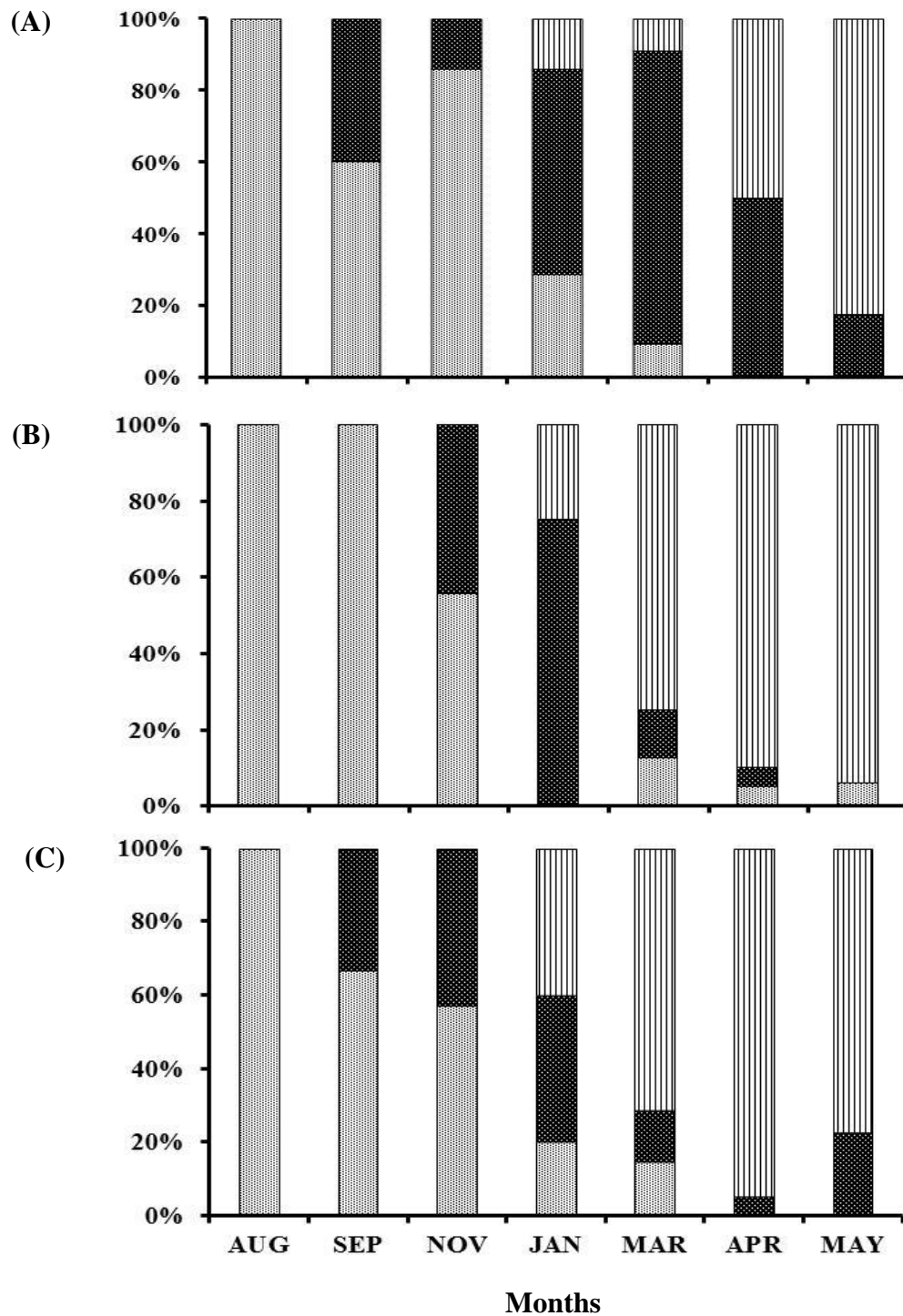


Fig. 5.10 Variations in percent population of males at different developmental stages exposed to natural (A), advanced (B) and continuous (C) photoperiod treatments. Stage I: □, Stage II: ■, Stage III: ▨ Stage IV: ▩ Stage V: ▤. n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

5.5 Discussion

The present study exposed the juvenile brook trout to 8 weeks advanced photoperiod commencing one month after the winter solstice in an attempt to reduce the proportion of maturing fish. Exposure to accelerated photoperiod advanced the maturation in brook trout but failed to reduce the proportion of maturing fish. The fish corrected their endogenous rhythms of maturation and sex steroids (T and E₂) synthesis in response to the change in direction (acceleration in photoperiod) and magnitude of photoperiod (8 weeks). Advancement in photoperiod initially caused atresia in most of the females (when was this observed) and reduced the rate of development in males however this situation was recovered insert when. This shows the strong capacity of brook trout to successfully adjust its endogenous rhythm according to the applied photoperiod shock as observed by Henderson (1963) in the same species under a different manipulated photoperiod. Following this recovery, most of the fish successfully recruited for maturation during the “recruitment window” or “critical period” in regards of environmental conditions (December – January, proposed for brook trout under the ambient conditions in Tasmania, described in Chapter 3). The sufficient energy stores as demonstrated by high condition factor, HSI and liver crude lipids during the critical period might open the “gate” for recruitment during October - November (simulated as photoperiod during December – November under natural photoperiod) under manipulated photoperiod. Good husbandry conditions and food supply further helped these recruited fish to successfully complete their reproductive cycle with normal fecundity in females in a shorter time and resulted in a high proportion of mature fish at the final sampling point. Better nutritional condition and whole body lipids reserves if surpassed the threshold during the “critical period” allow fish to recruit for maturation while these indexes below threshold levels will reduce the rate of maturation and fecundity as observed in rainbow trout under starvation conditions (Bromage et al., 1992).

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Previous studies also reported the advancement of maturation on exposure to accelerated photoperiod in brook trout without any negative impact on rate of maturation (Hoover, 1951; Hazard and Eddy, 1951; Corson, 1955). However in these studies, light treatment commenced after the fish had already begun recruitment for maturation (Even though the present study exposed fish 5 months earlier than the “critical period” during December – January, exposure to advanced photoperiod was still unable to inhibit or reduce the proportion of maturing fish due to entrainment of their endogenous cycle.

Exposure to advanced photoperiod has successfully reduced the proportion of maturing fish in rainbow trout (Randall et al., 1998; Davies et al., 1999; Wilkinson et al., 2010) and Atlantic salmon (Porter et al., 1999; Taranger et al., 1999). However the light paradigms used in these studies were different from each other and that used in present study. Similarly, variations in age of fish, time of commencement and termination of treatment, combining natural and additional light were also observed between different studies. The protocols used to reduce the number of maturing fish in Atlantic salmon culture exposed the fish to continuous photoperiod (duration varies between studies) followed by short days to accelerate the photoperiod (Porter et al., 1999; Taranger et al., 1999). In present study, fish were exposed to 8 weeks advancement at the commencement of treatment and subsequent gradual acceleration to signal as long days instead of continuous photoperiod. Similarly, photoperiod was decreased gradually to signal for short days as compared to the sudden termination of continuous photoperiod the before summer solstice. These differences between protocols used in the previous and present study might be the reason for a different outcome here. However, differences in response and sensitivity to light treatment have also been observed between species and strains (Leclercq et al., 2011; Skilbrei and Heino, 2011). Atlantic salmon has been found to be more responsive to manipulated photoperiod at both parr and grilse stage (Porter et al., 1999; Bromage et al., 2001; Skilbrei and Heino, 2011). The protocol of advanced photoperiod used in present study was more similar to those

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applied for maturation control in rainbow trout (Randall et al., 1998; Davies et al., 1999; Wilkinson et al., 2010). However, advanced photoperiod regimes applied for rainbow trout also vary for each study in terms of time to commence treatment, light intensity and duration. Success of this technique to control maturation in rainbow was due to failure of this species to recruit for maturation because of energy reserves, measured in terms of HSI (Wilkinson et al., 2011) and whole body lipids (Shearer and Swanson, 2000) below threshold under accelerated photoperiod. Feeding fish at low rate ($< 1\%$ of body weight used in the present study) until the closing of “recruitment window” will reduce the rate of growth and condition below a threshold which may result in failure of fish to recruit for maturation. Low feed rate combined with advanced photoperiod may successfully reduce the proportion of maturing fish in brook trout as observed in rainbow trout and chinook salmon (Silverstein et al., 1998; Taranger et al., 1999; Shearer and Swanson, 2000). Moreover, Wilkinson et al. (2011) exposed rainbow trout to advanced photoperiod during May while in present study light treatment was started in August (3 months late as compared to Wilkinson et al., 2011). Although this time was quite earlier than that in previous studies on brook trout however if treatment was started before spawning season (mid June – mid July; Chapter 3) it might inhibit further development of gonads.

The second manipulated photoperiod tested in the current study was exposure of brook trout to continuous photoperiod. The continuous photoperiod was chosen as no work has been reported on control of maturation in brook trout by subjecting fish to continuous light. Success of this method in reducing the number of maturing fish has previously been demonstrated in Atlantic salmon (Taranger et al., 1995; Porter et al., 2000; Harmon et al., 2004) and Chinook salmon (Unwin et al., 2005). However, the above mentioned studies vary in terms of duration of treatment, stage of gonadal development at commencement and termination of treatment, light intensity and age of fish. Exposure to continuous photoperiod in the present study failed to stop fish from becoming mature possibly due to

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desynchronization of the endogenous circannual cycle of maturation from photoperiodic cues and its exhibition as a “free run” which has been observed previously in rainbow trout (Randall et al., 1998; Randall and Bromage, 1998). Under continuous illuminations, fish were exposed to photoperiod shock at the commencement of treatment only. This single abrupt change in photoperiod might not be identified by fish (Randall and Bromage, 1998). This is reinforced by similar desynchronization of synthesis of sex steroids (T and E₂) in both sexes exhibiting variations similar to those in natural photoperiod. Interestingly in females, conversion of T into E₂ was significantly reduced as compared to fish held under natural photoperiod. Due to this reduced conversion, levels of T remained high while those of E₂ were found to be very low compared those observed in natural photoperiod. Although synthesis of aromatase can be adjusted according to compression or extension of photoperiod (suggested by Holcombe et al., 2000; Andersson et al., 2003 in brook trout and Atlantic salmon, respectively) as observed in females held under advanced photoperiod the reason for its reduced synthesis under continuous photoperiod in this study is not known. However, E₂ at low levels still efficiently regulated the ovarian development until the completion of vitellogenesis. Although onset of maturation could not be inhibited under continuous illumination the final stage of maturation i.e. spermiation and ovulation failed to occur. The reason of inhibition of final maturation might be the absence of signal of short days (Fjellidal et al., 2011) or presumably levels of luteinizing hormone, 11-KT and maturation inducing hormone were below threshold at this stage (Davies et al., 1999).

This finding of the present study is in contrast to other studies for Atlantic salmon and chinook salmon which reported inhibition of maturation of varying extent after exposure to continuous photoperiod under different regimes. In these studies, the proportion of mature fish was reduced due to inhibition or retardation of melatonin synthesis and ultimately that of sex steroids (Atlantic salmon, 6 – 21% maturation: Taranger et al., 1995; Porter et al., 2000; Harmon et al., 2004; Skilbrei and Heino, 2011; Chinook salmon: 7% and 0% maturation in

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males and females, respectively: Unwin et al., 2005). However these studies reported the success of continuous photoperiod during the first year of life (Taranger et al., 1995; Porter et al., 2000; Harmon et al., 2004) while the present study investigated older fish which may have already established a mature endogenous rhythm. Inhibition of maturation in 1+ year old brook trout was more important in the current study as maturation had not been observed in female brook trout during the first year of their life (Chapter 3). Moreover, although Unwin et al. (2005) reported the inhibition of maturation in 2 year old Chinook salmon the condition factor of fish in that study was comparatively much less than farmed Chinook salmon of equivalent age. Furthermore, Unwin et al. (2005) exposed fish to ambient light during daytime and artificial lights at night which might have played a role in reducing the incidence of maturation although this aspect is not clear. Exposure to ambient light during day time and additional lights to extend photoperiod was not practiced in the present study to provide the same range of light intensity to all replicates.

In salmonids, melatonin synthesis does not show any endogenous rhythmicity under constant photoperiod (Ekstrom and Meissl, 1997) in contrast to desynchronization of sex steroid synthesis and gonadal development under same conditions (Randall et al., 1998; Randall and Bromage, 1998). It is assumed that that exposure to continuous photoperiod would reduce the melatonin synthesis and ultimately disrupt the pineal-brain-pituitary-gonadal axis (Amano et al., 2000) in the present study. Light intensity of 20 – 200 lux has been sufficient to reduce melatonin synthesis in brook trout (Zachmann et al., 1992). The intensity which was maintained in present study (approximately 466 – 6330 lux), was similar to that applied in previous studies on brook trout (430 lux, Corson, 1955; 86 – 667 lux, Henderson, 1963) and Arctic charr (100 lux, Frantzen et al., 2004). Although profiles of melatonin were not measured in present study previous studies suggest that that melatonin synthesis might have been inhibited under continuous illumination here without apparent disruption to the pineal-brain-pituitary-gonadal circadian system. The parallel involvement of determinants other than

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pineal-brain-pituitary-gonadal axis, particularly the excellent growth (as shown by condition factor of fish) in present study cannot be ignored. Furthermore, the intensity of light maintained in the present study was sufficient to induce changes to the endogenous rhythm of maturation in fish exposed to advanced photoperiod as observed in gonadal development and hormone profiles. Although water quality was well maintained during the trial a short period (not more than a couple of days) was encountered discontinuously (until the end of February) where it was not standardized (increased turbidity due to inefficient solid waste removal, reduced O₂ saturation, and high NH₄). This increase in turbidity may have impacted on the lighting, reducing light intensity at depth. However as disruption to stable water quality and light intensity was for short time period, its impact might be negligible. It can therefore be concluded that the failure of the continuous photoperiod treatment was not due to insufficient light intensity.

In conclusion, maturation could not be inhibited in brook trout by exposure to advanced photoperiod due to short duration of light shock which was adjusted by fish without affecting the recruitment for maturation. Similarly, approach of inhibiting maturation by continuous photoperiod, successful for Atlantic salmon and chinook salmon is not commercially applicable for this species due to its ability to desynchronize its endogenous circadian rhythm under constant photoperiod like rainbow trout. Although gonadal development and endocrine cycle had been found desynchronized under continuous photoperiod however further study is required to measure the profiles of melatonin as well to confirm the desynchronization of endogenous rhythm of maturation in brook trout. Moreover, condition of fish was above the energy thresholds during the “critical period” of recruitment which resulted in a high incidence of maturation. There is a possibility that earlier commencement of treatment when fish condition is relatively low (< 1.3) may increase the chances of success of photoperiod regimes applied in the present study as observed in rainbow trout (Wilkinson et al. 2011). Similarly, restricted food supply (< 1% of body weight used in the present study) combined

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with manipulated photoperiod from the beginning of treatment until the “critical period” might reduce the chances for recruitment as observed in rainbow trout and chinook salmon (Silverstein et al., 1998; Taranger et al., 1999; Shearer and Swanson, 2000).

Further refinement of manipulated photoperiod regimes other than advancing the photoperiod is required to achieve successful results. Exposure to a series of photoperiod shocks e.g. continuous short days after winter solstice (no signal of long days for recruitment during critical period) followed by continuous long days after summer solstice (no signal of short days for maturation) may be more effective in this species to reduce/inhibit maturation than photoperiod advancement. Moreover, brook trout can adjust its endogenous rhythm according to the light shock given at commencement of treatment or during the trial (gradual increase or decrease in photoperiod). Therefore delayed photoperiod might not be successful as well in 1+ year old fish. Moreover, manipulation of water temperature and diet can be combined with light treatment. Henderson (1963) did not report any significant effect of water temperature on inhibition or retardation of maturation in brook trout however the duration of trial was very short in that study (60 days). Wilkinson et al. (2009) observed that elevated water temperature combined with advanced photoperiod reduced the efficiency of photoperiod manipulation and increased the proportion of maturing rainbow trout in Tasmania. A similar effect of different water temperature regimes combined with manipulated photoperiod may be expected for brook trout under Tasmanian climatic conditions which needs to be explored further.

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CHAPTER 6

General Discussion

Pilot scale commercial trials of brook trout (*Salvelinus fontinalis*) in Macquarie Harbour on Tasmania's west coast (from 2003 – 2008) demonstrated that maturation prior to harvest was a significant impediment toward full scale commercial culture of the species. Early maturation in brook trout and other salmonids is a constraining factor in Aquaculture world-wide resulting in deterioration of flesh quality and immunocompetency of fish (Hansen et al., 2000; Haffray et al., 2009). Due to these negative impacts of early maturation, mature stock is unmarketable and the industry has to bear the capital loss (Porter et al., 2000; Peterson and Harmon et al., 2005). To resolve these problems, early maturation can be controlled by the production of all female stock and exposing those fish to a manipulated photoperiod regime. To investigate this further, detailed data about the reproductive biology of brook trout under Tasmanian environmental conditions was required. This project therefore investigated the gonadal development during puberty and annual reproductive cycle of brook trout under Tasmanian culture conditions and further trialed two approaches which could be used to reduce the incidence of pre-harvest maturation of stock.

Chapter 2 describes the important histological events during early gonadal development in brook trout until the point of sex differentiation. Gonadal primordia containing a few germ and stromal cells were observed in embryos before hatch while undifferentiated gonads were observed at hatch as reported previously for this species (Sacobie and Benfey, 2005). The presence of undifferentiated gonads during the pre-hatch period provided fundamental information highlighting the sensitivity of this developmental stage for targeting with hormonal sex inversion treatments to masculinize genotypic females (Chapter 4). Based on this information, the pre-hatch period was included for 17 α -methyltestosterone (MT)

immersion treatment of ova. MT immersion treatment given at 4 to 6 days pre-hatch was found to be successful in producing a male proportion of 0.75 with the least number of females (0.3) and without the occurrence of any sterile fish (Chapter 4). Previous studies identified the pre-hatch period to be a relatively more responsive time period as compared to the post-hatch treatment for phenotypic sex reversal of brook trout (Sacobie, 2001; Galbreath et al., 2003; Haffray et al., 2009). These studies achieved success (74-80% and 60% in Haffray et al., 2009 and Sacobie, 2001, respectively) with extended immersions of ova (in MT) coupled with MT supplemented feeding. For previous studies in brook trout, Parks and Parks fed MT treated diet to brook trout alevins (40 mg/kg) for 77 – 108 days. This treatment resulted in 100% sterility despite of any masculinization. Galbreath and Stocks (1999) immersed embryos at sac fry stage which failed to produce significant proportion of males. Galbreath et al. (2003) gave one immersion (1 mg/L) to gynogenetic brook trout fry at ten days post-hatch followed by 60 days MT dietary treatment (1 – 2 mg/kg) which could not skew the sex proportion to males. Post-hatch MT treatment given by Galbreath and Stocks (1999) and Galbreath et al. (2003) failed to produce male skew which showed the insensitivity of this period for sex reversal treatment.

The present study achieved equivalent results with only two immersions and no requirement for subsequent dietary treatment. In the present study the success of this immersion only treatment given during the pre-hatch period is in contrast to previous works (Galbreath et al., 2003; Haffray et al., 2009). These researchers demonstrated an immersion only treatment approach failed to invert the sex of brook trout and suggested supplementary oral treatment was required. Failure of pre-hatch immersion only treatment in these previous studies might be due to missing the time period during pre-hatch most malleable to manipulation (Galbreath et al., 2003) or the MT dose given during this period was sub-optimal (Haffray et al., 2009).

The differentiation of gonads into ovaries and testes was histologically studied in brook trout and differentiated ovaries and presumptive testes were observed at the age of 2769 °dph (6.20 ± 0.72 g) (Chapter 2). After further development, complete sex differentiation was observed at 3354 °dph (12.25 ± 0.92 g) when gonads could be clearly differentiated as ovaries and testes (Chapter 2). The occurrence of sex differentiation in brook trout during this post-hatch period (age = 6 months) in the present study indicated that post-hatch MT immersion and oral treatments might be successful in inducing sex inversion where the genotypic sex of undifferentiated gonads could be phenotypically reversed before it is irreversibly determined at the age of 6 months. This possibility was investigated in the present study by applying post-hatch immersion and dietary treatment (Chapter 4).

However, immersion treatment given during early post-hatch period (one week post-hatch) failed to masculinize the genotypic females. Similarly, sex reversal using MT dietary treatment was found to be ineffective for sex inversion of brook trout in the present study as reported previously for this species (Galbreath et al., 2003; Sacobie, 2001; Haffray et al., 2009). The reason (s) for the observed failure of post-hatch immersion and dietary treatments for sex reversal of brook trout seen here and in previous studies may be attributed to pre-hatch commencement of gene up-regulation responsible for sex differentiation. Steroidogenic pathways are activated in pre-differentiating gonads as indicated by the expression of genes responsible for steroidogenic enzymes production prior to differentiation as observed in rainbow trout (Govoroun et al., 2001; Baron et al., 2008; Vizziano-Cantonnet et al., 2008). Moreover, androgen masculinization proceeds mainly through an early inhibition of female development through dysregulation of early gonadal gene expression in genetic females (Baron et al., 2008). Pre-hatch activation of steroidogenic pathways and inhibition of oogenesis at time of gonadal differentiation may result in cytological sex differentiation much earlier than the post-hatch histological differentiation of gonads into ovaries and testes. Presumably due to this reason, MT treatment at the post-hatch stage failed

to reverse the sex of genotypic females. It might be late to down-regulate the genes involved in oogenesis (Baron et al., 2008) during the post-hatch period. However, there is need to investigate the pre-hatch activation of genes involved in steroidogenic pathways and expression of oogenesis in brook trout determining the sex cytologically earlier than the histological differentiation as reported in rainbow trout (Govoroun et al., 2001; Baron et al., 2008; Vizziano-Cantonnet et al., 2008).

A high incidence of sterility was observed in group immersed 12 times (and later split into I-1 and IF-1) and those groups that were fed MT following two immersions (IF-2 – IF-11). This observation concurs with previous studies on brook trout sex reversal (Parks and Parks, 1991; Sacobie, 2001; Galbreath et al., 2003; Haffray et al., 2009) where sterility was observed in MT supplemented diet treatment. In present study, the occurrence of sterility is a result of exposure to a total dose of androgen above optimum levels as observed previously in brook trout (Parks and Parks, 1991; Galbreath et al., 2003) which was more prominent in groups subjected to a combination of immersion and oral treatment. However, no sterility was observed using two immersions during the 4 to 6 days pre-hatch period. The absence of any sterile fish in this treatment further supports the success of this approach and eliminates the requirement of dietary treatment when designing a sex reversal protocol for brook trout for commercial application. A few sterile fish were observed in the non-immersed controls. This may be due to contamination of water with MT residues during dietary treatment. However as the number of sterile fish was very small, it can be assumed that the occurrence of this slight sterility in controls may be a natural incidence.

The present study pinpointed the narrow and the most sensitive time window over the period from one week pre-hatch until one week post-hatch pre-hatch. The simple application of only two immersions during this window of time successfully skewed the sex proportion towards maleness without any supplementary oral treatment. Achievement of successful

masculinisation in brook trout without any dietary treatment which may benefit growth (Dunham, 2004), avoid sterility, reduce stress of handling during multiple immersions, decrease user exposure to steroids and reduce its environmental discharge. From an animal welfare perspective the immersion treatment of ova only negates the need for handling of fish post-hatch. The use of a targeted immersion significantly reduces the investment of labor by commercial operators to administer the multiple immersions and long dietary treatment.

Although immersion treatment given on 4 and 6 days pre-hatch (the time window identified as the most sensitive for MT treatment) produced a total proportion of 0.75 of males which may be improved by addition of one more immersions on either of 7, 5 and 3 day pre-hatch. Similarly further optimization of androgen dose may also increase the proportion of sex reversed females by applying a single immersion pre-hatch.

After gonadal differentiation into complete ovaries and testes (Chapter 2), reproductive development was further studied in male and female brook trout (Chapter 3) during their puberty and second reproductive cycle. The present study investigated the monthly variation in gonadal development and profiles of sex steroids (T, 11-KT and E₂) in correlation with seasonal changes in photoperiod and water temperature. During first year of life, 75% of the total male population attained puberty (first maturation in life history) but females did not mature during their first year and delayed maturation until the second year which is in agreement with other salmonids like sea trout, chinook salmon, coho salmon, rainbow trout and Atlantic salmon (Silverstein and Hershberger, 1992; Clarke and Blackburn, 1994; Dziewulska and Domagala, 2004; McClure et al., 2007). During the second year, recruitment for maturation was observed in both male and female brook trout during December (summer solstice) and January, concomitant with the rise in levels of testosterone (T) and 11-ketotestosterone (11-KT) in males and estradiol-17 β (E₂) in females. Commencement of recruitment for maturation was observed during this “recruitment window” (December – January) which was indicated by the start of exogenous vitellogenesis in ovaries

(development of yolk globules) and 1st meiotic division in testes (development of secondary spermatocytes). Most of the fish surpassed the energy thresholds and recruited for maturation by shunting energy from lipid reserves to reproductive activity (Silverstein et al., 1998). This biological activity depending upon the better growth and enough energy reserves in form of lipids were demonstrated by high values of condition factor, hepatosomatic index (HSI) and liver crude lipids during this period. Mature males and maturing females were observed during March and April when the photoperiod was gradually decreasing. This vigorous gonadal activity during autumn was concomitant with the high levels of T and 11-KT in males and E₂ in females along with high activity of steroidogenic enzymes. Males remained functional (releasing milt on manual stripping) until June while females achieved their final maturation (appearance of homogenous yolk after absorption of water) during this month (at winter solstice). Final maturation in females might be regulated by maturation inducing hormone i.e. 17 α , 20 β -dihydroxyprogesterone as suggested for other genus *Salvelinus* (brook trout: Goetz et al., 1987; Arctic charr: Frantzen et al., 1997) which was not measured in this study due to low budget. Following the winter solstice during July, profiles of sex steroids and steroidogenic enzymes were reduced significantly. During this month mature gametes in both sexes were resorbed as spawning was not observed under tank conditions as reported for other salmonids like, rainbow trout, brown trout, Atlantic salmon and Arctic charr (Hunt et al., 1982; Baynes and Scott, 1985; Pottinger, 1988; Scott and Sumpter, 1989; Mayer et al., 1992; Tveiten et al., 1998). Overall in brook trout, photoperiod during the summer (December – January) acted as a cue or “recruitment window for maturation”. Gonadal development significantly progressed during autumn (March – April) and its completion was observed during winter (May – June) which can be stated as “completion window”.

Entrainment of gonadal development and synthesis of sex steroids with the seasonal variations in photoperiod (mentioned in chapter 3) showed the vital role of this exogenous factor in the control of endogenous rhythm of maturation in brook trout (Bromage et al.,

2001). Considering the important role of photoperiod in brook trout (Chapter 3), manipulated photoperiod may be an option to reduce the incidence of maturation in brook trout (Chapter 5) as reported for Atlantic salmon, Arctic charr and rainbow trout (Björnsson et al., 1994; Porter et al., 1999; Andersson et al., 2003; King et al., 2003; Taranger et al., 2003; Frantzen et al., 2004; Choi et al., 2010; Skilbrei and Heino, 2011). This could be performed by closing, delaying or advancing the “recruitment and completion windows” during the reproductive cycle which was trialed in present study (Chapter 5).

The information described in chapter 3 was utilized to investigate the effect of 8 weeks advanced photoperiod and continuous illumination on the maturation of brook trout (Chapter 5). Based on the results mentioned in chapter 3, the “critical period” to open the “recruitment window” for maturation was December – January in brook trout. It was hypothesized that due to advancement of photoperiod for 8 weeks, fish might fail to store energy required to recruit for maturation due to a shortened time period while the “critical period” approaches 8 weeks earlier. Energy reserves below thresholds during this “critical period” might close the “recruitment window” for maturation. Although the previous studies investigating the exposure of brook trout to advanced photoperiod reported the failure of this regime to inhibit maturation however fish were subjected to light treatment after the critical period had passed (in January). In present study, light treatment was commenced in August, three months earlier than opening of “recruitment window” increasing the probability of success of applied photoperiod regime. However, brook trout showed rapid adaption to this 8 week photoperiod advancement and corrected its reproductive cycle. This was indicated by the phase shift of 8 weeks in profiles of sex steroids (i.e. T and E₂) accordingly and the fact that most of the fish (94% males and 92% females) became mature.

The reason for this failure was the strong capacity of brook trout to successfully adjust its endogenous rhythm according to photoperiod changes as compared to other salmonids (Henderson et al., 1963). Moreover, growth and energy reserves in fish subjected to advanced

photoperiod might have exceeded the developmental thresholds during the “critical period” and the “gate opening” developmental stage (exogenous vitellogenesis in ovaries and 1st meiotic division in testes, chapter 3) of circannual rhythm. A high condition factor over the study period (>1.3 in both males and females) and sufficient energy stores in these fish shown by HSI and liver crude lipids during the “critical period” might open the window for recruitment of fish for maturation as observed in rainbow trout (Duston and Bromage, 1988; Silverstein et al., 1998; Shearer and Swanson, 2000). Due to these factors most of the fish (94% males and 92% females) subjected to advanced photoperiod successfully recruited for maturation and proportion of maturing fish could not be inhibited in this study.

Exposure to continuous photoperiod inhibits the synthesis of melatonin and ultimately disrupts the function of brain-pituitary-gonad (BPG) axis resulting in the inhibition of maturation as observed in Atlantic salmon (Taranger et al., 1995; Porter et al., 2000; Harmon et al., 2004) and chinook salmon (Unwin et al., 2005). Continuous photoperiod regime was chosen in present study as no study has been reported on inhibition of maturation by subjection to 24 hours light treatment in this species. Success of this regime in Atlantic salmon and chinook salmon and lack of any work for brook trout supported the hypothesis of reducing the proportion of maturing fish by exposure to continuous photoperiod in this species.

However, exposure to continuous photoperiod in the present study failed to inhibit or reduce the incidence of maturation. On the other hand, ovarian development started in females exposed to continuous photoperiod one month prior to those held under natural photoperiod. In males exposed to continuous photoperiod, testicular development was relatively faster than those kept in natural photoperiod. Failure of continuous light treatment to inhibit maturation in brook trout may be due to desynchronization of the endogenous circannual cycle from environmental cues and an exhibition of the reproductive cycle as “free run” as observed in rainbow trout (Randall et al., 1998; Randall and Bromage, 1998). In the absence of any

variation in photoperiod (constant 24 hours light), gonadal development may be endogenously regulated by endocrine system and freed from environmental control which was observed in present study. Although exposure to continuous photoperiod could not inhibit maturation, the final stage of maturation i.e. spermiation and ovulation failed to occur presumably due to an absence of signal of short days (Fjelldal et al., 2011) or that the levels of luteinizing hormone, 11-KT and maturation inducing hormone were below thresholds at this stage (Henderson et al., 1963; Davies et al., 1999).

Previous studies described the negative impacts of maturation on flesh quality (sampled fish at the final stage of maturation) (Paaver et al., 2004; Roth et al., 2007) and Roth et al. (2010) reported a similar effect on flesh quality before final maturation was achieved. Although exposure to continuous photoperiod inhibited maturation at the final stage in brook trout however any potential positive impact of this inhibition on flesh quality was not investigated in present study. It can be assumed that a lot of energy had been already consumed during maturation in both sexes and inhibition of final maturation in present study might not have a significant positive effect on flesh quality.

As the magnitude of advanced photoperiod shock (8 weeks) given in the present study or exposure to continuous photoperiod did not greatly affect the proportion of maturing fish it is therefore concluded that a different artificial light regime other than advanced photoperiod is required to achieve successful results. Exposure to a series of photoperiod shocks may be more effective to reduce/inhibit maturation. A series of shocks or abrupt changes in photoperiod can be suggested due to the ability of brook trout to adapt its endogenous rhythm according to a single shock as observed in present study under both advanced and continuous photoperiod. A protocol that uses continuous short days during summer will signal the fish to delay recruitment. Due to this delay, the “critical period” of December – January (Chapter 3) may pass without initiating recruitment. Following this period, an abrupt change by exposing to continuous long days during winter may signal for recruitment for maturation in brook

trout. If this continuous long days photoperiod is terminated before the beginning of exogenous vitellogenesis in ovaries and 1st meiotic division in testes, gonads may not further develop. These developmental stages act as the phase which may open the “gate” for recruitment in brook trout (Chapter 3). This long days photoperiod if followed by subsequent exposure to continuous short days, may signal for termination of reproductive cycle (Chapter 3). It may result into the disruption of the endogenous rhythm and reduction in the proportion of maturing fish. The similar protocols have been found successful in reducing the proportion of maturing fish in Atlantic salmon (Porter et al., 1999; Taranger et al., 1999). However due to difference in responses of different species to the same protocol, success of suggested protocol cannot be warranted for brook trout until trialed.

Other than different light regime, early commencement of light treatment may also improve the efficiency of protocol in brook trout. In present study, light treatment was started in August. Its commencement earlier than August i.e. May or June may effectively reduce the proportion of maturing fish in this species under Tasmanian culture conditions. Wilkinson et al. (2011) achieved the successful reduction in proportion of maturing fish by commencement of advanced photoperiod in May in Tasmania but in rainbow trout. Moreover, there is possibility that early commencement of treatment when condition is relatively low (< 1.3) or restricted food supply from the beginning of treatment until the “critical period” might reduce the chances for recruitment as observed in rainbow trout and chinook salmon (Silverstein et al., 1998; Taranger et al., 1999; Shearer and Swanson, 2000).

Manipulation of water temperature could be combined with light treatment. Negative impact of elevated water temperature on the efficiency of photoperiod manipulation had been reported for rainbow trout in Tasmania (Wilkinson et al., 2010). It indicates the possible influence of cold water temperature on control of maturation in brook trout and improving the efficiency of light protocol. To manipulate water temperature, water recirculation systems are

required which are not currently used at a commercial scale in Tasmania for this species. However, the role of manipulated water temperature in control of maturation in brook trout could be investigated at an experimental level prior to any commercial application. Delayed photoperiod could be another regime to be used for inhibition of maturation but Holcombe et al. (2000) reported the failure of this treatment in brook trout to reduce the number of maturing fish. They commenced treatment earlier than opening of “recruitment window” thus eliminating the chance of missing the critical period as occurred in other studies on brook trout (Hazard and Eddy, 1951; Hoover, 1951; Corson, 1955). Due to failure of this regime (Holcombe et al., 2000) although treatment started earlier than those in previous studies, this paradigm was not chosen for present study. However, very small sample size at final sample point (7 females and 6 males) affects the reliability of results reported in this study.

Overall, this thesis provided baseline data describing the gonadal differentiation, puberty and the annual reproductive cycle of brook trout under conditions of ambient photoperiod and mean water temperatures simulating those found on farm in Tasmania. Based on this information, the most likely period for androgen treatment success was identified using a screening approach that staggered treatment timings and differing application regimes. This study identified a narrow time window during pre-hatch period when only two immersions can successfully masculinise genetic females. This information has significant importance for further optimization of sex reversal protocols at commercial scale. Development of a protocol for potential commercial growers of brook trout in Tasmania will help to produce all-female stocks thus eliminating the problem of early maturation of males. Furthermore, exposure to 8 weeks advanced photoperiod and continuous illumination did not reduce the incidence of maturation in brook trout. However, continuous photoperiod inhibited final maturation suggesting that further refinement of light regime, intensity, quality and/or diffusion may improve the efficiency of light treatment.

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APPENDIX I

Chapter 2

Sections of materials and methods and results related to the body parameters are given in appendix I.

I.1 Materials and Methods

Total body weight (BW) (nearest 0.1 g) and total length (BL) (nearest 1.0 mm) were measured before fixation. These values were used to calculate condition factor (CF) according to following formula:

$$CF(k) = \left[BW / (BL)^3 \right] \times 100$$

Where CF is measured in *k*, BL in centimeters and BW in grams. Total body weight of alevins included the weight of yolk sac.

Statistical Analysis

Variability in all parameters was analysed by one way ANOVA after justifying assumptions by performing Shapiro-Wilk test and test of homogeneity of variance. Tukey's post hoc test was applied for comparison of means. The overlapping samples taken from both cohorts between November and January revealed no marked developmental differences. As a consequence, results from the second cohort only were presented for stage V and VI in the differentiated phase. Independent t-tests were applied to compute differences between values of males and females during the differentiated phase. Means \pm standard error (s.e.) are presented. Linear regression was computed to study the relationship between body length and weight. Values of β below three indicated negative allometric growth (Froese, 2006; Arnason

et al., 2009). All statistical analyses were performed using SPSS 17.0 and differences were considered significant if $P < 0.05$.

I.2 Results

I.2.1 Total Body Weight, Total Body Length and Condition Factor

A significant gradual increase in total body weight ($F = 27.63$, $_{7, 53}$; $P < 0.05$) and body length ($F = 45.13$, $_{7, 53}$; $P < 0.05$) was observed during the undifferentiated phase (Fig. I.1A, B). During the initial stages of the undifferentiated phase (8-172 °dph), these two parameters remained relatively constant. However, a significant increase in both body weight and length was noted during 589-1282 °dph which remained relatively constant throughout the remaining sample periods. Condition factor showed a gradual but significant drop ($F = 4.73$, $_{7, 53}$; $P < 0.05$) during 8-115 °dph and remained almost constant until end of undifferentiated phase (Fig. I.1C).

During the differentiated phase, both total body weight and total body length showed significant increase during 2769-3354 °dph ($P < 0.05$) (Fig. I.1 A, B), however, no significant difference was observed between mean values for males and females during the differentiated phase ($P > 0.05$). The condition factor dropped significantly showed a significant drop ($P < 0.05$) from January (2769 °dph) to February (3354 °dph) however no differences between males and females were observed ($P > 0.05$) (Fig. I.1C).

A significant linear relationship was found between total body length and total body weight of fish in both the undifferentiated ($P < 0.05$; d.f. = 60) and differentiated phases ($P < 0.05$; d.f. = 27).

Undifferentiated fish: $BL = 0.59 (BW) - 1.38$ ($R^2 = 0.81$)

Differentiated fish: $BL = 2.16 (BW) - 9.31$ ($R^2 = 0.81$)

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Linear regression equation for both phases showed negative allometric growth during and after differentiation as the value of β was found to be smaller than 3.0.

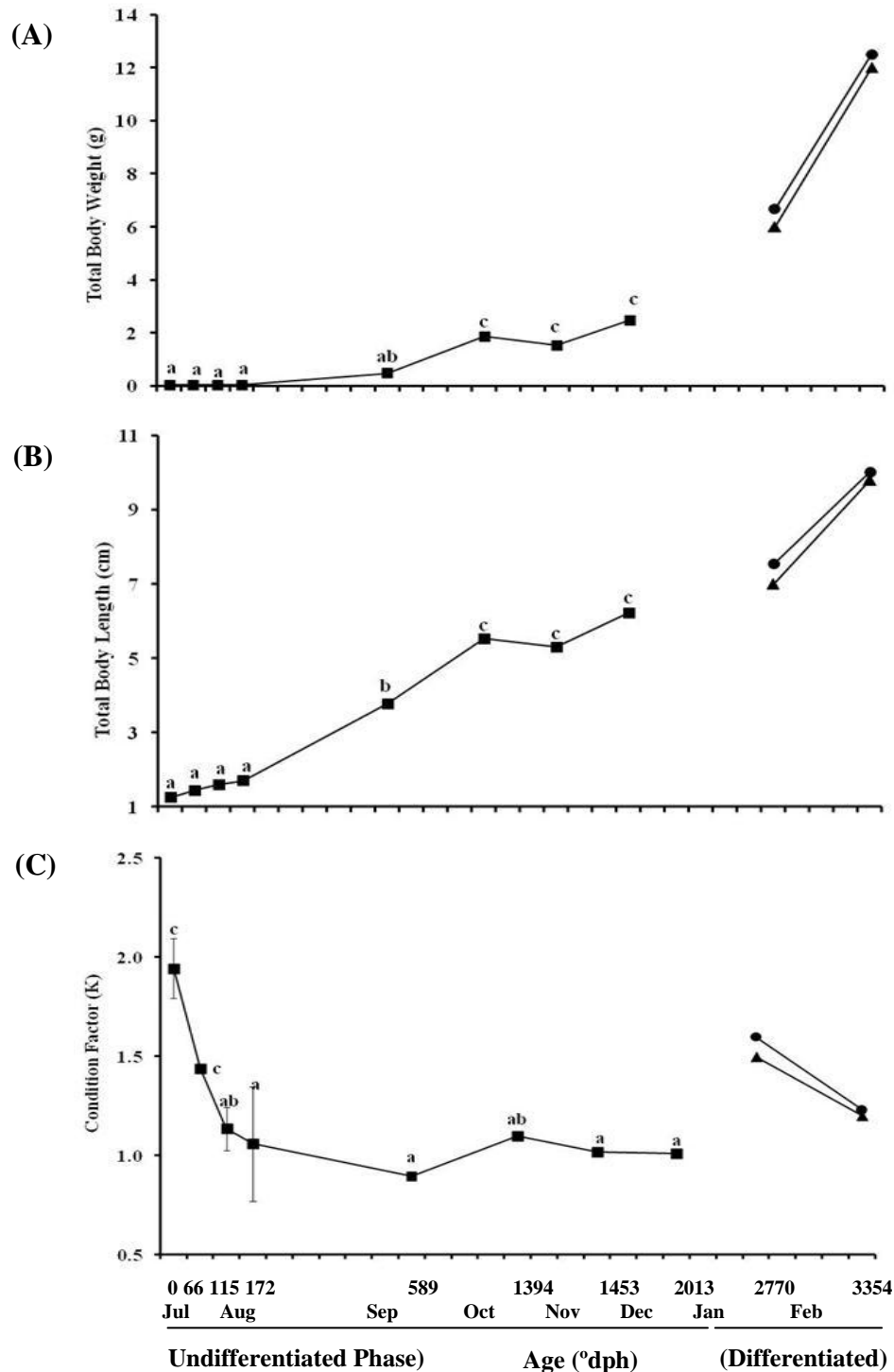


Fig. I.1 Variations (mean \pm s.e) in total body weight (A), total body length (B) and condition factor (C) during undifferentiated (UD) and differentiated phase during gonadal differentiation of *Salvelinus fontinalis*. Months (2010-2011) when sampling was conducted are indicated on the X-axis together with age in degree days post-hatch (°dph). Significantly different subsets ($P < 0.05$) given by Tukey's HSD are indicated by letters. Tukey's HSD could not be run for differentiated phase due to number of means less than 3. T-test did not indicate any significant difference between mean values of male and female. (UD: ■, Female: ●, Male: ▲).

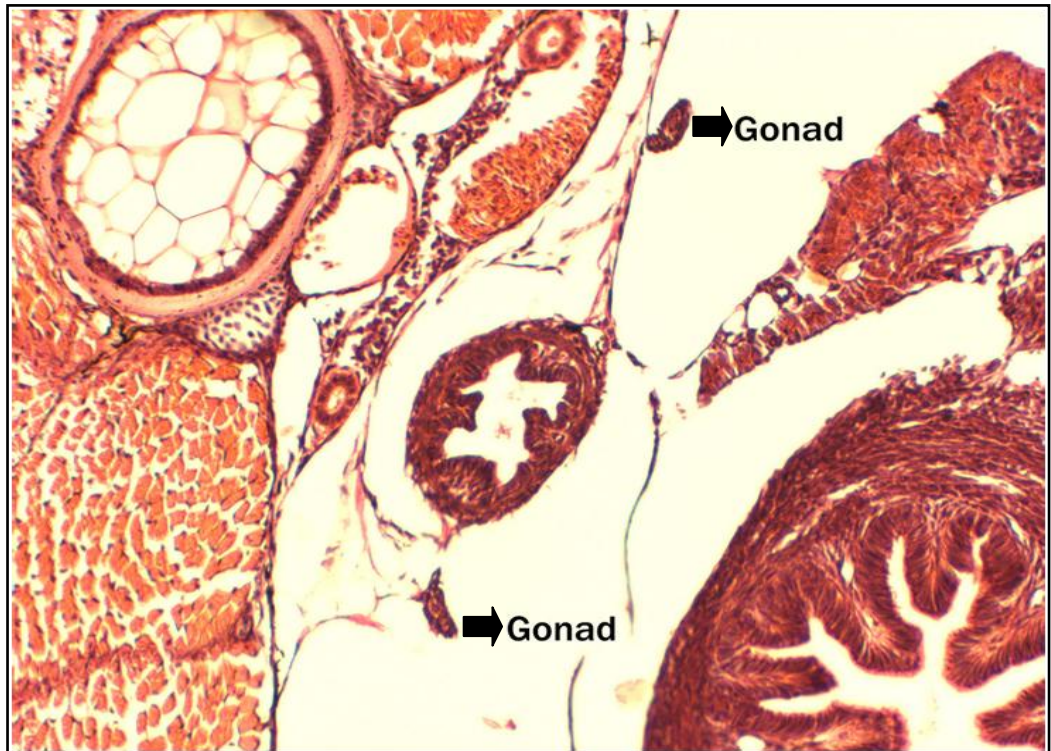


Fig. I.2 Undifferentiated paired gonads at stage II (66 – 115 °dph) in *Salvelinus fontinalis*.

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Early Gonadal Development and Sex Differentiation

Table I.1 Mean values of total body weight, body length and condition factor during the undifferentiated phase of *Salvelinus fontinalis*.

Age (° dph)	Total Body Weight (g)	Fork Length (cm)	Condition Factor (%)
0	0.04 ± 0.05	1.25 ± 0.05	1.94 ± 0.15
66	0.04 ± 0.05	1.45 ± 0.05	1.44 ± 0.02
115	0.05 ± 0.01	1.60 ± 0.01	1.14 ± 0.11
172	0.05 ± 0.01	1.70 ± 0.01	1.06 ± 0.29
589	0.48 ± 0.06	3.76 ± 0.06	0.90 ± 0.04
1394	1.87 ± 0.10	5.53 ± 0.19	1.10 ± 0.08
1453	1.54 ± 0.01	5.31 ± 0.10	1.02 ± 0.06
2013	2.47 ± 0.01	6.23 ± 0.07	1.01 ± 0.03

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Table I.2 Mean values of total body weight, body length and condition factor of male and female brook trout (*Salvelinus fontinalis*) during differentiated phase.

Age	Total Body Weight		Fork Length		Condition Factor	
(°dph)	(g)		(cm)		(%)	
	Male	Female	Male	Female	Male	Female
2769	6.00 ± 0.72	6.70 ± 0.61	7.00 ± 0.36	7.54 ± 0.20	1.5 ± 0.14	1.60 ± 0.14
3354	12.00 ± 0.92	12.50 ± 0.80	9.80 ± 0.26	10.03 ± 0.21	1.2 ± 0.03	1.23 ± 0.03

1.3 References

Froese, R. (2006). Cube law, condition factor and weight length relationships: history, meta analysis and recommendations. *J. App. Ichthyol.* **22**, 241-253.

APPENDIX II

Chapter 3

Sections of materials and methods and results related to the body parameters are mentioned in appendix II.

II.1 Materials and Methods

Total body weight (BW) (nearest 0.1 g) and total length (BL) (nearest 1.0 mm) were measured before fixation. These values were used to calculate condition factor (CF) and specific growth rate according to following formulae:

$$\text{Condition Factor (k)} = [\text{Total Body Weight (g)} / \{\text{Fork Length (cm)}\}^3] \times 100$$

$$\text{Specific Growth Rate (SGR)} = [\{\ln W_f \text{ (g)} - \ln W_i \text{ (g)}\} / d] \times 100$$

[W_f = final weight, W_i = initial weight, d = number of days]

Total body weight for all indices included the weight of gonads and liver.

II.1.1 Classification of the Testicular and Ovarian Development into Stages

Classification of Stages during the Testicular Development

Stage I - Immature

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Spermatogonia A divide to form spermatogonia B (SGB). These spermatogonia divide mitotically and develop into primary spermatocytes (PSC). PSC further undergoes meiosis I and develop into secondary spermatocytes (SSC).

Stage II – Maturing

SSC undergoes meiosis II and develop into spermatids. Spermatids are further transformed into sperms.

Stage III – Mature

Lobules are filled with sperm. After rupturing of lobular walls sperm are collected in the lumen of testes. At this stage milt can be released on manual stripping under tank conditions or naturally in wild.

Stage IV – Spent

Sperms have been released from testis. Empty lobules contain residual sperms which are resorbed. Quiescent spermatogonia (spermatogonia which do not undergo any development during the cycle) can be observed.

Stage V – Regressed

Quiescent spermatogonia divide to form the spermatogonial cysts. Spermatogonia will not undergo meiosis and remain arrested at this stage until the new cycle begins.

Classification of Stages during the Ovarian Development

Stage I – Immature

Cortical alveoli accumulate in cytoplasm of oocytes which are called as early vesicular oocyte (EVE). EVE develop into late vesicular stage (LVE) after formation of lipid globules.

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Well developed three layers of thecal layer, granulosa layer and zona radiata can be seen surrounding the LVE.

Stage II – Developing

Exogenous vitellogenesis begins and yolk granules appear at the periphery and interior of oocyte to form early vitellogenic oocytes (EV). These yolk granules coalesce to form yolk globules and oocytes are called as mid vitellogenic oocytes (MV). Cortical alveoli are displaced towards the peripheral ooplasm as yolk globules accumulate centripetally.

Stage III - Maturing

Yolk globules coalesce to form massive polygonal yolk plates and MV develop into late vitellogenic oocytes (LV). LV increase in size before occurrence of germinal vesicle breakdown (GVBD) and germinal vesicle migration (GVM). Both GVBD and GVM are observed. Cortical alveoli can be seen along the oocyte periphery.

Stage IV – Mature

Late vitellogenic oocytes develop into mature eggs after absorption of water. Appearance of homogenous yolk in eggs can be observed by initial uptake of water. Eggs can ovulate at this stage by manual stripping under tank conditions or naturally in wild.

Stage V – Spent

Thick tunic of ovary is folded enclosing the collapsed tissue after ovulation. All non-ovulated mature eggs (if present) are being atretic. Spent ovaries contain post-ovarian follicles (POF). POF contain debris of outer thecal and inner granulosa layer enclosing lumen. Quiescent oogonia (oogonia which do not undergo any development during the cycle) are present.

Stage VI (Recruiting)

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Quiescent oogonia present throughout the cycle will divide to form POC to commence the cycle again.

II.2 Results**II.2.1 First Year (age = 8 – 15 months)***Total Body Weight, Fork Length and Condition Factor*

Significant variability ($P < 0.05$) was observed among means of body weight ($F_{7, 53} = 27.62$, $P < 0.05$ in males; $F_{7, 44} = 41.16$, $P < 0.05$ in females) and length ($F_{7, 53} = 45.12$, $P < 0.05$ in males; $F_{7, 44} = 79.80$, $P < 0.05$ in females) (Fig. II.1). Total body weight gradually increased throughout the study period and reached the highest at age of 15 months (197 ± 18.81 g in males and 210 ± 27.70 g in females) (Fig. II.1A). A similar pattern was observed in fork length and the highest values of 26 ± 0.63 cm and 27 ± 1.11 cm were observed in males and females, respectively at age of 15 months (Fig. II.1B). The significant differences were observed between body weight and length of mature males (206.60 ± 18.92 g, 26.00 ± 0.64 cm) and immature males (122.20 ± 31.00 g, 23.60 ± 0.80 cm) ($P < 0.05$) at age of 15 months.

Condition factor was highly variable over the study period ($F_{7, 53} = 4.72$, $P < 0.05$ in males; $F_{7, 44} = 4.16$, $P < 0.05$ in females) with the values ranging between 1.10 ± 0.03 – 1.25 ± 0.03 and 1.05 ± 0.02 – 1.33 ± 0.14 in males and females, respectively (Fig. II.1C). Log transformed linear regression showed positive allometric growth in males and females ($P < 0.05$) as value of β was found above 1.

$$\text{Log}_{10} \text{ Fork length of male} = 3.12 (\text{Log}_{10} \text{ Total body weight}) - 2.12$$

$$\text{Log}_{10} \text{ Fork length of female} = 3.05 (\text{Log}_{10} \text{ Total body weight}) - 2.03$$

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II.2.2 Second Year (age = 16 - 27 months)

Total Body Weight, Fork Length, Condition Factor and Growth Rate

Throughout the study period significant variability was observed between mean values of body weight ($F_{11, 47} = 16.43$, $P < 0.05$ in males; $F_{11, 47} = 27.17$, $P < 0.05$ in females) and length ($F_{11, 47} = 29.28$, $P < 0.05$ in males; $F_{11, 47} = 28.12$, $P < 0.05$ in females) in both sexes (Fig. II.2A and B). Body weight and length in males gradually increased and reached their highest (1607.50 ± 297.31 g; 43.00 ± 0.50 cm) at age of 27 months. Females gradually increased in body weight and length and their highest values (1890.65 ± 203.20 g; 43.00 ± 0.91 cm) were observed at age of 26 and 27 months, respectively.

Condition factor in males did not vary ($P > 0.05$) throughout the second maturational year and ranged between 1.40 ± 0.12 and 2.07 ± 0.13 (Fig. II.2C). However, condition factor showed significant variability in females over the study period ($F_{11, 47} = 9.23$, $P < 0.05$). In females, condition factor gradually increased and its highest value (2.64 ± 0.14) was observed at age of 25 months which decreased in following months.

Growth rate was generally higher in females as compared to males (data not shown). Growth rate was higher during summer (December-February) in both sexes. Log transformed regression equation showed positive allometric growth for both male and female fish ($P < 0.05$).

$$\text{Log}_{10} \text{ Standard Length of male} = 3.07 (\text{Log}_{10} \text{ Total body weight}) - 1.90$$

$$\text{Log}_{10} \text{ Standard Length of female} = 4.05 (\text{Log}_{10} \text{ Total body weight}) - 3.35$$

Von Bertalanffy growth curve described the body length as function of age (Fig.II.3). The test of likelihood showed a significant difference between growth of male and female fish ($P < 0.05$).

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Gonadal Development during Puberty and the Annual Reproductive Cycle

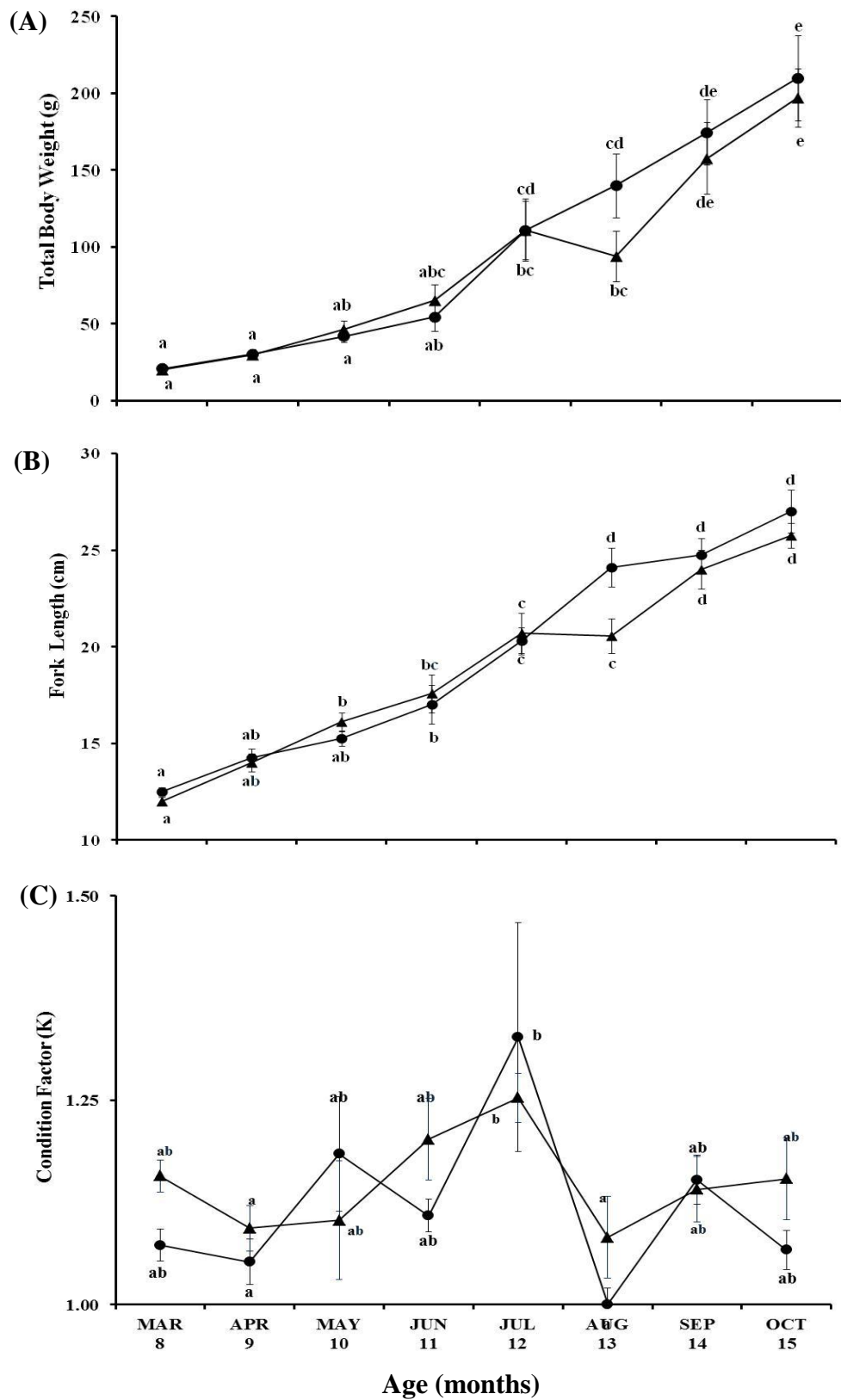


Fig. II.1 Monthly variations (Mean \pm SEM) in total body weight (A), fork length (B) and condition factor (C) during the first maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. Female: \bullet , Male: \blacktriangle . $n = 53$ males; 44 females.

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Gonadal Development during Puberty and the Annual Reproductive Cycle

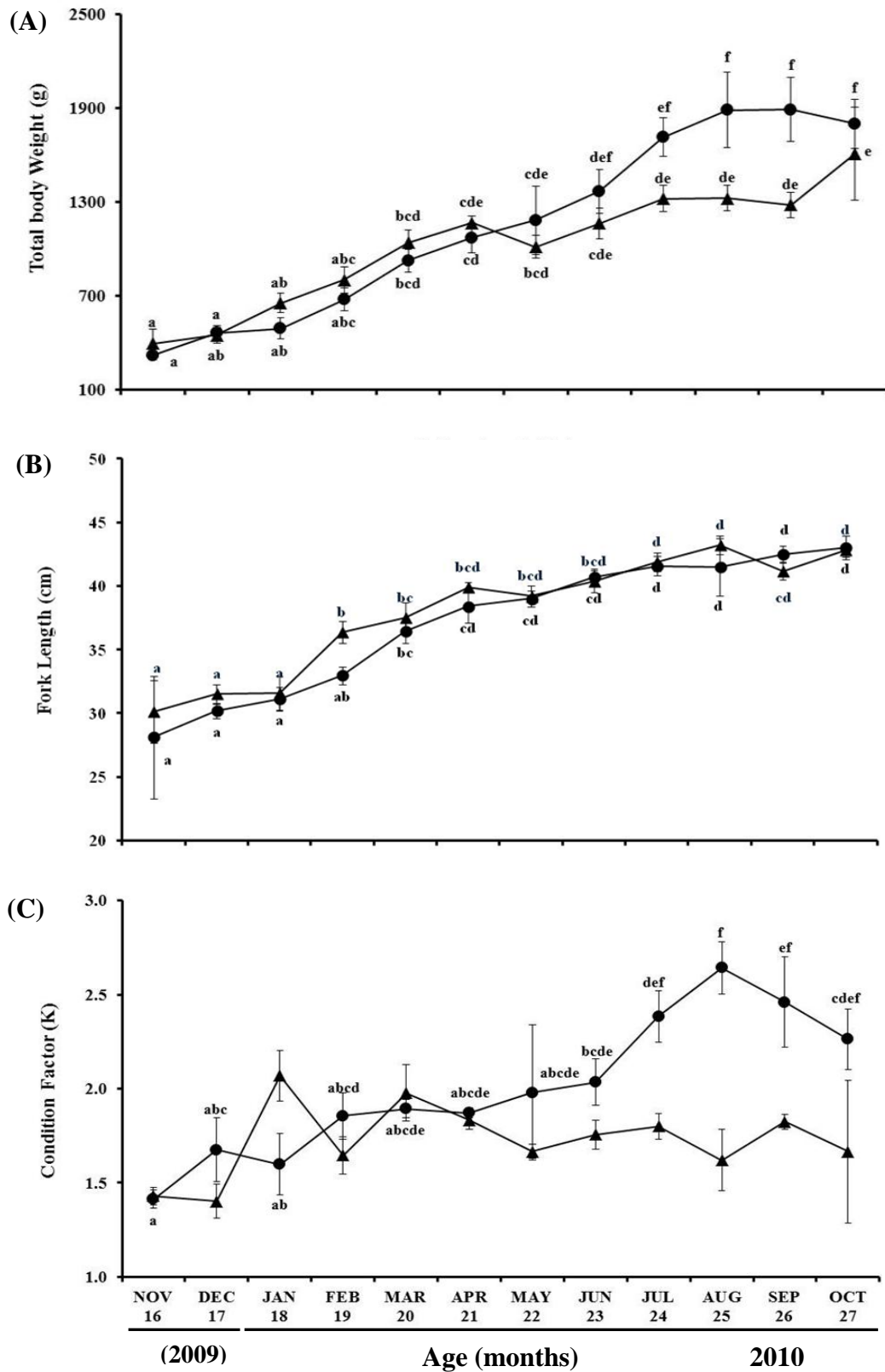


Fig. II.2 Monthly variations (Mean \pm SEM) in total body weight (A), fork length (B) and condition factor (C) during the second maturational year of *Salvelinus fontinalis*. Significantly different subsets ($P < 0.05$) for each sex given by Tukey's HSD are indicated by letters. One way ANOVA was not performed when test of homogeneity calculated insignificant difference ($P > 0.05$). Female: \bullet , Male: \blacktriangle . $n = 47$ males; 47 females.

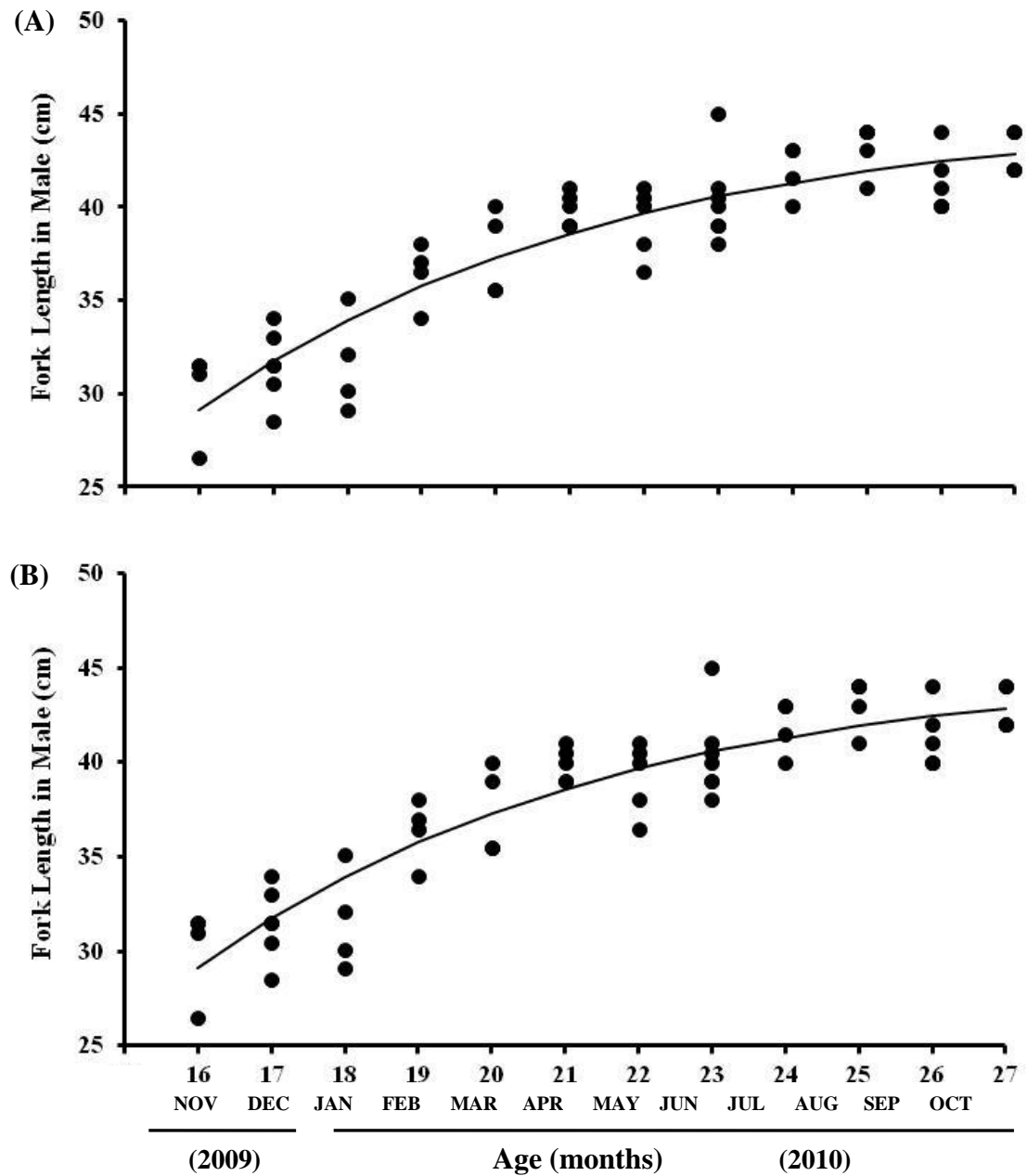


Fig. II.3 Von Bertalanffy growth curve between fork length and age of males (A) and females (B) of *Salvelinus fontinalis* during the second maturational year. Observed value: ●, Predicted value: —.

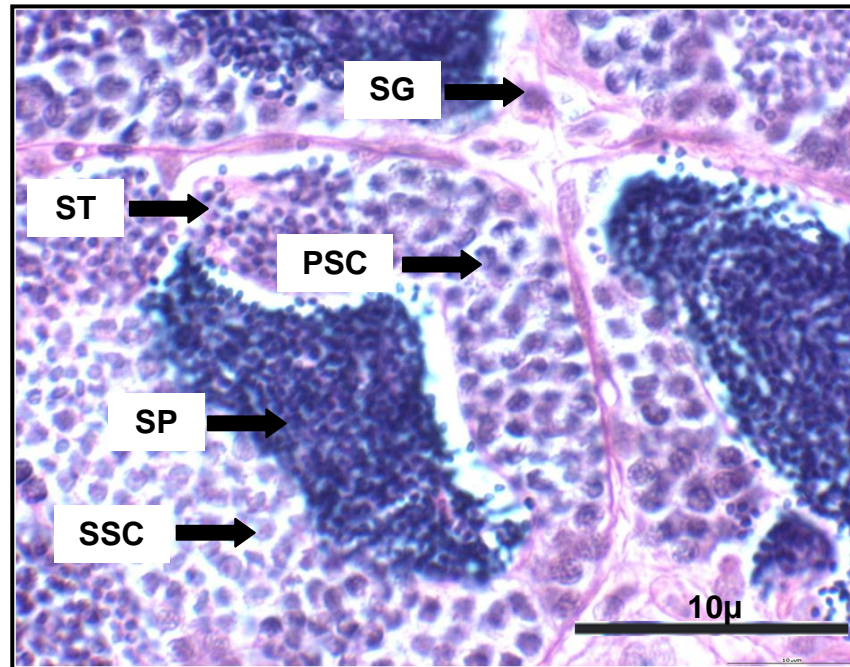


Fig. II.4 Developing germ cells at different stages during testicular development in *Salvelinus fontinalis* at maturing stage. PSC: primary spermatocyte; SG: spermatogonia; SSC: secondary spermatocyte; SP: sperm; ST: spermatid

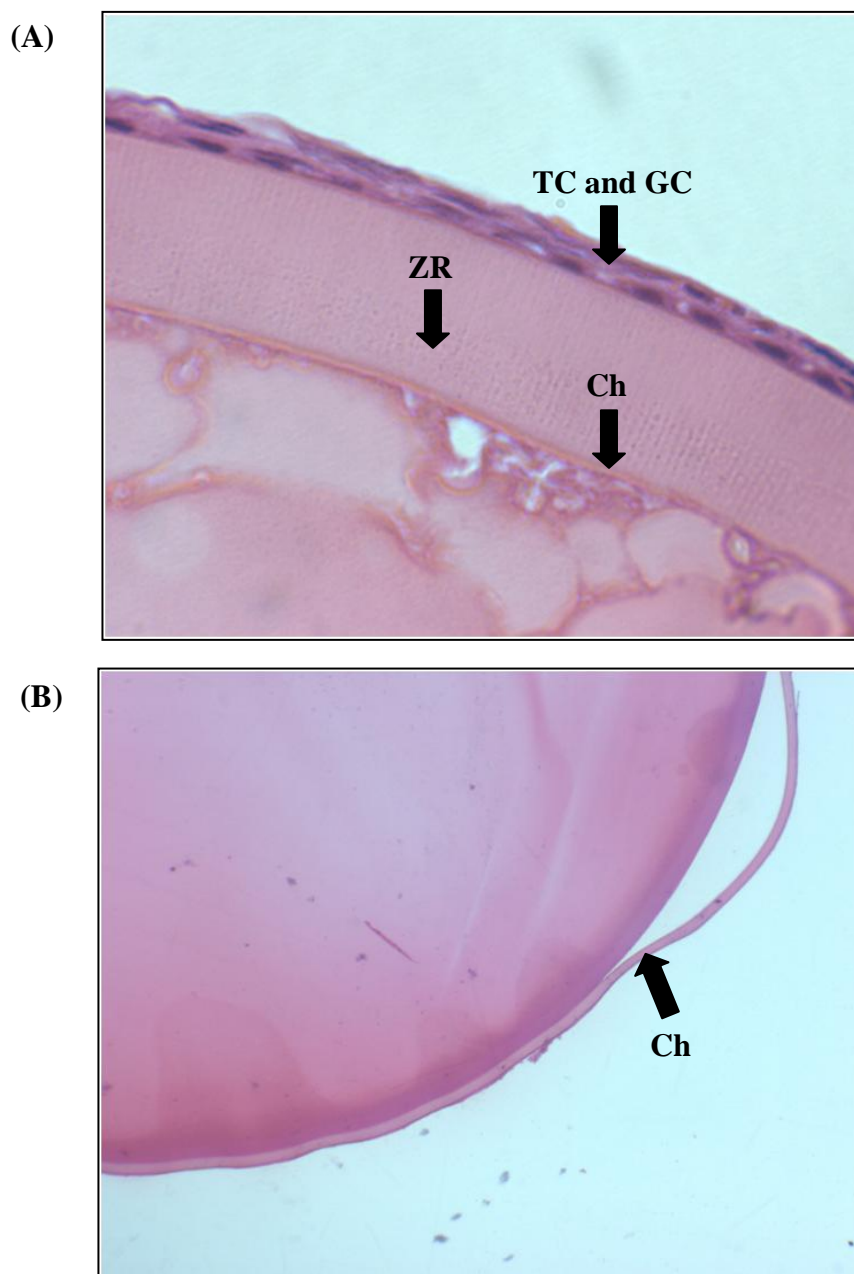


Fig. II.5 Layers of non-ovulated egg (A) at 400X and ovulated egg (B) at 10X in *Salvelinus fontinalis*. Non-ovulated egg contains chorion, zona radiata and layer of granulosa and thecal cells. Ovulated egg has only chorion. Ch: chorion; GC: granulosa cells; TC: thecal cell; ZR: zona radiata.

APPENDIX III

Chapter 4

Sections of materials and methods and results related to body parameters are given in appendix III.

III.1 Materials and Methods

Total body weight (BW) (nearest 0.1 g) and total length (BL) (nearest 1.0 mm) were measured before fixation. These values were used to calculate condition factor (CF) according to following formula:

$$CF(k) = \left[BW / (BL)^3 \right] \times 100$$

Where CF is measured in k , BL in centimeters and BW in grams. Total body weight of alevins included the weight of yolk sac.

III.1.1 Statistical Analysis

Variability in all parameters was analysed by one way ANOVA after justifying assumptions by performing Shapiro-Wilk test and test of homogeneity of variance. Tukey's post hoc test was applied for comparison of means. Means \pm standard error (s.e.) are presented. All statistical analyses were performed using SPSS 17.0 and differences were considered significant if $P < 0.05$.

III.2 Results

III.2.1 Total Body Weight, Fork Length and Condition Factor

Females of control groups (C-1 and C-2) and treatments (I-1 – I-12) showed significant difference in total body weight ($F = 5.97$; $P < 0.05$) (Table III.1) and fork length ($F = 3.30$; $P < 0.05$) (Table III.2) but condition factor did not show any significant difference ($F = 1.03$; $P > 0.05$) (Table III.3). On the other hand, significant difference was noted in body weight ($F = 25.58$; $P < 0.05$) of MT fed females of control groups (C-1F and C-2F) and treatments (I-1F – I-12F) but not in fork length ($F = 1.15$; $P > 0.05$) and condition factor ($F = 1.71$; $P > 0.05$).

Although values of body weight of MT fed females were numerically higher than those of non-immersed and immersed but significant difference was noted between only a few groups (Table III.1). Similarly, females of only a few treatment groups showed significant difference ($P < 0.05$) from those of controls in body length and condition factor between above mentioned group.

Significant differences in body weight ($F = 6.04$; $P < 0.05$) (Table III.1) and length ($F = 4.80$; $P < 0.05$) (Table III.2) were observed for males of control groups (C-1 and C-2) and treatments (I-1 – I-11) but condition factor showed insignificant difference ($F = 1.45$; $P > 0.05$) (Table III.3). Males of C-1F, C-2F and I-1F – I-11F showed significant difference for body weight ($F = 33.47$; $P < 0.05$), body length ($F = 16.70$; $P < 0.05$) and condition factor ($F = 2.44$; $P < 0.05$). Similar to females, body weight and length values of males in most of the MT fed groups were significantly ($P < 0.05$) higher than those of non-MT fed males (Table III.1 and III.2).

Significant difference was not observed in body weight ($F = 2.04$; $P > 0.05$) (Table III.1), body length ($F = 1.52$; $P > 0.05$) (Table III.2) and condition factor ($F = 1.22$; $P > 0.05$) (Table III.3) of sterile fish of control groups (C-1 and C-2) and treatments (I-1F – I-11F). Sterile fish of C-1F, C-2F and I-1F – I-11F showed a significant difference in body weight ($F = 17.17$; $P < 0.05$), body length ($F = 6.51$; $P < 0.05$) but insignificant in condition factor ($F = 0.72$; $P > 0.05$) (Table III.1 – III.3). Significant difference was not observed between values of body weight, length and condition factor of most of the MT fed and non-MT fed sterile fish ($P > 0.05$) (Table III.1 – III.3).

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Sex Reversal by 17 α -methyltestosterone Immersion

Table III.1 Total body weight (g) of male, female and sterile fish in controls (C-1, C-2, C-1F and C-2F) immersed treatments (I-1 – I-11) and immersed – MT fed treatments (I-1F – I-11F).

Trt	Pooled Males	Pooled Females	Sterile	Trt	Pooled Males	Pooled Females	Sterile
C-1	¹¹ 115.23 \pm 3.20	³ 124.49 \pm 5.15	¹ 116.37 \pm 19.23	C-1F	^{*15} 133.77 \pm 4.82	^{*13} 144.27 \pm 6.10	¹ 109.20 \pm 24.60
C-2	¹¹ 114.73 \pm 3.65	⁹ 118.75 \pm 2.83	¹ 88.85 \pm 16.75	C-2F	^{*5} 140.83 \pm 5.02	^{*4} 152.17 \pm 5.23	¹ 118.00 \pm 24.00
I-1	NS	NS	NS	I-1F	⁸ 119.50 \pm 4.54	⁹ 116.00 \pm 14.00	¹ 92.82 \pm 2.80
I-2	¹ 94.56 \pm 2.62	¹ 102.50 \pm 4.32	NA	I-3F	^{*11} 117.20 \pm 3.42	⁸ 109.00 \pm 5.20	¹ 98.53 \pm 3.53
I-3	¹⁰ 115.71 \pm 3.10	⁹ 124.43 \pm 3.51	¹ 132.7 \pm 0.00	I-4F	¹¹ 116.50 \pm 4.00	⁹ 113.90 \pm 4.70	¹ 103.20 \pm 6.85
I-4	^{*11a} 114.73 \pm 4.12	^{*11} 134.51 \pm 4.21	NS	I-5F	¹ 90.92 \pm 2.00	¹ 85.50 \pm 2.60	¹ 77.30 \pm 3.00
I-5	⁸ 111.20 \pm 4.25	⁷ 113.67 \pm 4.05	¹ 92.00 \pm 0.00	I-6F	¹² 121.17 \pm 3.70	⁹ 122.54 \pm 5.85	^{*1} 109.50 \pm 6.65
I-6	¹¹ 115.20 \pm 4.05	⁹ 126.69 \pm 4.73	NS	I-7F	^{*6} 250.70 \pm 24.12	^{*5} 220.77 \pm 16.70	¹ 260.77 \pm 18.30
I-7	^{*13} 125.99 \pm 4.85	^{*4a} 144.87 \pm 5.91	NS	I-8F	⁹ 112.39 \pm 4.61	⁹ 114.20 \pm 4.50	¹ 106.00 \pm 10.30
I-8	¹⁰ 112.43 \pm 4.34	⁷ 115.67 \pm 5.20	¹ 116.95 \pm 12.25	I-9F	¹⁴ 126.60 \pm 5.99	⁹ 119.90 \pm 4.53	¹ 112.34 \pm 9.70
I-9	¹¹ 115.00 \pm 5.32	^{*11a} 130.84 \pm 3.40	¹ 96.65 \pm 2.65	I-10F	⁷ 102.95 \pm 3.53	⁷ 103.73 \pm 3.51	¹ 98.64 \pm 5.55
I-10	⁷ 106.93 \pm 2.60	⁷ 112.97 \pm 6.14	¹ 96.75 \pm 6.52	I-11F	⁸ 107.73 \pm 2.70	⁸ 107.80 \pm 3.80	¹ 86.00 \pm 6.11
I-11	^{*4} 131.53 \pm 3.44	¹³ 143.76 \pm 5.95	¹ 122.02 \pm 6.01	I-12F	¹¹ 115.82 \pm 3.31	¹¹ 126.78 \pm 5.50	¹ 104.50 \pm 7.00

* Shows the significant difference between immersed treatment and its MT fed counterpart. Results of Tukey' s test have been shown by number (1-15). ¹ a, ² b, ³ c, ⁴ d, ⁵ e, ⁶ f, ⁷ ab, ⁸ abc, ⁹ abcd, ¹⁰ bc, ¹¹ bcd, ¹² bcde, ¹³ cd, ¹⁴ cde, ¹⁵ de. ^a shows the significant difference (P < 0.05) between values of male and female of same group. NS: No sample.

Appendix III – Chapter 4
Sex Reversal by 17 α -methyltestosterone Immersion

Table III.2 Fork length (cm) of male, female and sterile fish in controls (C-1, C-2, C-1F and C-2F) immersed treatments (I-1 – I-11) and immersed – MT fed treatments (I-1F – I-11F).

Trt	Pooled Male	Pooled Female	Sterile	Trt	Pooled Male	Pooled Female	Sterile
C-1	¹⁰ 20.12 \pm 0.24	⁷ 21.17 \pm 0.28	¹ 20.70 \pm 1.22	C-1F	* ¹⁵ 21.31 \pm 0.29	¹ 22.00 \pm 0.36	¹ 19.53 \pm 1.83
C-2	¹⁰ 20.10 \pm 0.24	⁷ 21.03 \pm 0.27	¹ 19.15 \pm 1.05	C-2F	* ⁵ 21.70 \pm 0.23	* ¹ 22.35 \pm 0.30	¹ 20.85 \pm 1.15
I-1	NS	NS	NS	I-1F	⁷ 16.55 \pm 0.47	¹ 20.25 \pm 0.25	¹ 19.30 \pm 0.19
I-2	¹ 18.85 \pm 0.18	¹ 20.00 \pm 0.26	NS	I-3F	* ¹² 20.60 \pm 0.25	¹ 28.67 \pm 8.83	¹ 19.34 \pm 0.42
I-3	¹⁰ 20.38 \pm 0.25	⁷ 21.14 \pm 0.22	¹ 21.40 \pm 0.00	I-4F	¹¹ 20.21 \pm 0.38	¹ 20.40 \pm 0.30	¹ 19.81 \pm 0.48
I-4	* ¹⁰ 20.30 \pm 0.30	* ⁷ 21.40 \pm 0.38	NS	I-5F	¹ 18.61 \pm 0.14	¹ 18.30 \pm 0.20	¹ 17.83 \pm 0.26
I-5	¹⁰ 20.10 \pm 0.27	⁷ 20.73 \pm 0.27	¹ 20.00 \pm 0.00	I-6F	¹² 21.00 \pm 0.23	¹ 21.00 \pm 0.40	¹ 20.50 \pm 0.44
I-6	¹⁰ 20.10 \pm 0.27	⁷ 21.40 \pm 0.27	NS	I-7F	* ⁶ 24.67 \pm 0.81	* ¹ 23.70 \pm 0.70	¹ 25.30 \pm 0.80
I-7	¹⁰ 20.61 \pm 0.25	* ¹ 22.00 \pm 0.32	NS	I-8F	¹² 20.40 \pm 0.27	¹ 20.86 \pm 0.29	¹ 20.67 \pm 0.83
I-8	⁷ 19.80 \pm 0.29	¹ 20.12 \pm 0.43	¹ 21.00 \pm 1.20	I-9F	* ¹⁴ 21.14 \pm 0.36	¹ 20.46 \pm 0.32	¹ 20.14 \pm 0.62
I-9	⁸ 20.03 \pm 0.36	* ⁷ 21.10 \pm 0.20	¹ 19.75 \pm 0.25	I-10F	⁸ 19.90 \pm 0.24	¹ 20.15 \pm 0.28	¹ 19.90 \pm 0.45
I-10	⁷ 19.90 \pm 0.25	⁷ 20.70 \pm 0.40	¹ 19.90 \pm 0.66	I-11F	¹² 20.42 \pm 0.23	¹ 20.50 \pm 0.21	¹ 18.43 \pm 0.53
I-11	³ 21.13 \pm 0.23	¹ 22.10 \pm 0.34	¹ 21.25 \pm 0.43	I-12F	¹² 21.02 \pm 0.21	¹ 21.37 \pm 0.35	¹ 20.40 \pm 0.45

* Shows the significant difference between immersed treatment and its MT fed counterpart. Results of Tukey' s test have been shown by number (1-15). ¹ a, ² b, ³ c, ⁴ d, ⁵ e, ⁶ f, ⁷ ab, ⁸ abc, ⁹ abcd, ¹⁰ , bc, ¹¹ bcd, ¹² bcde, ¹³ cd, ¹⁴ cde, ¹⁵ de. NS: No sample.

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Sex Reversal by 17 α -methyltestosterone Immersion

Table III.3 Condition factor of male, female and sterile fish in controls (C-1, C-2, C-1F and C-2F) immersed treatments (I-1 – I-11) and immersed – MT fed treatments (I-1F – I-11F).

Trt	Pooled Male	Pooled Female	Sterile	Trt	Pooled Male	Pooled Female	Sterile
C-1	^{7a} 1.41 \pm 0.32	¹ 1.30 \pm 0.02	¹ 1.30 \pm 0.01	C-1F	⁷ 1.37 \pm 0.03	¹ 1.35 \pm 0.03	¹ 1.44 \pm 0.09
C-2	^{7a} 1.41 \pm 0.02	¹ 1.26 \pm 0.02	¹ 1.25 \pm 0.03	C-2F	⁷ 1.36 \pm 0.02	* ¹ 1.35 \pm 0.03	¹ 1.30 \pm 0.05
I-1	NS	NS	NS	I-1F	⁷ 4.01 \pm 0.24	¹ 1.40 \pm 0.12	¹ 1.30 \pm 0.01
I-2	^{7a} 1.40 \pm 0.02	¹ 1.27 \pm 0.01	NS	I-3F	⁷ 1.34 \pm 0.03	¹ 1.50 \pm 0.26	¹ 1.51 \pm 0.24
I-3	⁷ 1.36 \pm 0.02	¹ 1.31 \pm 0.02	* ¹ 1.35 \pm 0.00	I-4F	⁷ 1.45 \pm 0.08	¹ 1.33 \pm 0.02	¹ 1.30 \pm 0.02
I-4	⁷ 1.37 \pm 0.02	¹ 1.50 \pm 0.19	NS	I-5F	* ⁷ 1.49 \pm 0.05	¹ 1.46 \pm 0.05	¹ 1.21 \pm 0.05
I-5	¹ 1.29 \pm 0.03	¹ 1.27 \pm 0.02	¹ 1.20 \pm 0.00	I-6F	¹ 1.30 \pm 0.02	¹ 1.33 \pm 0.04	¹ 1.26 \pm 0.03
I-6	^{7a} 1.40 \pm 0.02	¹ 1.28 \pm 0.01	NS	I-7F	* ² 1.62 \pm 0.04	* ¹ 1.59 \pm 0.03	¹ 1.61 \pm 0.04
I-7	* ^{7a} 1.41 \pm 0.02	* ¹ 1.35 \pm 0.02	NS	I-8F	¹ 1.30 \pm 0.02	¹ 1.24 \pm 0.02	¹ 1.20 \pm 0.04
I-8	* ² 1.43 \pm 0.02	¹ 1.60 \pm 0.27	¹ 1.31 \pm 0.09	I-9F	¹ 1.31 \pm 0.02	¹ 1.40 \pm 0.03	¹ 1.35 \pm 0.03
I-9	* ⁷ 1.40 \pm 0.03	* ¹ 1.40 \pm 0.02	¹ 1.25 \pm 0.01	I-10F	¹ 1.30 \pm 0.02	¹ 1.26 \pm 0.02	¹ 1.25 \pm 0.04
I-10	⁷ 1.39 \pm 0.06	¹ 1.24 \pm 0.03	¹ 1.23 \pm 0.04	I-11F	¹ 1.27 \pm 0.03	¹ 1.25 \pm 0.02	¹ 1.36 \pm 0.04
I-11	⁷ 1.39 \pm 0.02	¹ 1.32 \pm 0.02	¹ 1.26 \pm 0.02	I-12F	¹ 1.30 \pm 0.07	¹ 1.30 \pm 0.03	¹ 1.23 \pm 0.05

* Shows the significant difference between immersed treatment and its MT fed counterpart. Results of Tukey' s test have been shown by number (1-15). ¹ a, ² b, ³ c, ⁴ d, ⁵ e, ⁶ f, ⁷ ab, ⁸ abc, ⁹ abcd, ¹⁰ bc, ¹¹ bcd, ¹² bcde, ¹³ cd, ¹⁴ cde, ¹⁵ de. ^a shows the significant difference (P < 0.05) between values of male and female of same group. NS: No sample.

Appendix III – Chapter 4
Sex Reversal by 17 α -methyltestosterone Immersion

Table III.4 Ratios of males (M), females (F), sterile (S) and number of functional males (FM) in controls and treatments C-1 – I-11 and C-1F – I-11F. Survival rate for each group was calculated between 148 days post-hatch and final sampling point.

Trt	N	Sex Ratio (M:F:S)	Survival Rate (%)	FM	Trt	N	Sex Ratio (M:F:S)	Survival Rate (%)	FM
C-1	70	41:25:04	100%	19	C-1F	70	39:28:03	100%	10
C-2	71	37:32:02	100%	21	C-2F	70	36:32:02	100%	8
I-1	65	11:00:54	100%	NS	I-1F	70	^a 22:02:46	100%	0
I-2	76	^a 57:19:00	100%	14	I-3F	70	28:21:21	100%	1
I-3	72	^a 48:23:01	100%	17	I-4F	70	33:23:14	99%	6
I-4	70	41:29:00	98%	19	I-5F	105	^a 55:31:19	100%	11
I-5	75	37:37:01	100%	9	I-6F	70	^a 41:21:08	100%	5
I-6	70	^a 45:25:0	99%	17	I-7F	33	13:18:02	98%	1
I-7	70	37:33:00	100%	16	I-8F	70	39:28:03	100%	3
I-8	70	^a 38:30:02	100%	13	I-9F	71	29:33:09	100%	2
I-9	70	37:31:02	100%	15	I-10F	71	38:26:07	100%	2
I-10	72	^a 42:26:04	99%	14	I-11F	69	37:19:13	100%	1
I-11	70	40:19:11	100%	13	I-12F	70	43:17:10	100%	3

^a χ^2 test of independence computed difference of M:F:S ratio of each group of non-immersed controls and immersed treatments (C-1 – I-12) and its counterpart of MT-fed controls and treatments (C-1F – I-12F), shown by letter a. NS: No sample.

APPENDIX IV

Chapter 5

Section of and results related to the body weight, fork length and gonadal weight are mentioned in appendix IV.

IV.1 Results

VI.1.1 Total Body Weight, Fork Length

Body weight of females held under NP, AP and CP was observed within the range of 193.22 ± 8.23 - 811.03 ± 53.43 g, 169.54 ± 6.24 - 874.36 ± 23 g and 164.30 ± 9.57 - 828.40 ± 19.75 g (Fig. VI.1A). Significant within treatment variability was observed throughout the study in females held under NP ($F_{6, 97} = 112.35$, $P < 0.05$), AP ($F_{6, 94} = 86.25$, $P < 0.05$) and CP ($F_{6, 89} = 103.67$, $P < 0.05$). Males in all three treatments showed a similar pattern of increase in their body weight and their mean values were observed within the range of 172.61 ± 19.57 - 838.47 ± 12.77 g in NP, 180.00 ± 18.25 - 806.25 ± 16.94 g in AP and 184.00 ± 4.17 - 861.97 ± 21.76 g in CP during May (Fig. VI.1B). Similar to females, significant within treatment variability was observed in males exposed to NP ($F_{6, 86} = 85.44$, $P < 0.05$), AP ($F_{6, 89} = 80.12$, $P < 0.05$) and CP ($F_{6, 91} = 63.76$, $P < 0.05$).

Fork length of females held under NP, AP and CP increased over the study period and observed within the range of 25.14 ± 0.30 - 37.94 ± 0.21 cm, 24.20 ± 0.30 - 37.64 ± 0.37 cm and 24.00 ± 0.45 - 37.67 ± 0.31 cm, respectively at the final sampling point in May (Fig. VI.2A). Body length showed a significant within treatment variability over the study period in NP ($F_{6, 97}$

= 203.27, $P < 0.05$), AP ($F_{6, 94} = 114.32$, $P < 0.05$) and CP ($F_{6, 89} = 139.08$, $P < 0.05$). Body length in males increased throughout the study period observed between the range 23.50 ± 0.36 - 39.13 ± 0.17 cm, 24.50 ± 0.66 - 38.00 ± 0.26 cm and 24.64 ± 0.18 - 38.82 ± 0.28 cm were observed in NP, AP and CP, respectively during May (Fig. VI.2B). Significant within treatment variability was observed throughout the trial for NP ($F_{6, 86} = 164.10$, $P < 0.05$), AP ($F_{6, 89} = 146.04$, $P < 0.05$) and CP ($F_{6, 91} = 157.50$, $P < 0.05$).

VI.1.2 Gonadal Weight

Significant increase in gonadal weight of females exposed to NP and AP was observed during March and their highest values were noted during April (78.03 ± 14.40 g in NP, 93.21 ± 6.00 g in AP) (Fig. VI.3A). Peak value of gonadal weight in females exposed to NP remained stable until the end of the study while it dropped in females exposed to AP during May. Gonadal weight in females exposed to CP showed a significant increase in during March (44.65 ± 7.35 g) which reached its highest mean value (78.21 ± 5.05 g) during May, equivalent to that of females in the NP group at same sample point. Significant within treatment variability was noted in gonadal weights of females in NP ($F_{6, 97} = 75.33$, $P < 0.05$), AP ($F_{6, 91} = 11.14$, $P < 0.05$) and CP ($F_{6, 89} = 35.53$, $P < 0.05$).

A similar pattern of gonadal weight was noted in males as was observed in females (Fig. VI.3B). Gonadal weight of males in NP increased in April (19.93 ± 2.06 g) and reached its highest mean value during May (21.88 ± 0.80 g). Males exposed to AP showed a significant increase in gonadal weight during March and the highest mean value was observed in April (29.72 ± 3.00 g). Following the peak value during April, gonadal weight of males in AP declined

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Control of Maturation by Photoperiod Manipulation

during May (13.31 ± 0.73 g). On the other hand, gonadal weight of males in CP significantly increased in March and April and their highest mean value was observed during May (26.50 ± 1.31 g). Over the whole study period, significant within treatment variability was noted in gonadal weights of males in NP ($F_{6, 86} = 59.42, P < 0.05$), AP ($F_{6, 89} = 5.11, P < 0.05$) and CP ($F_{6, 91} = 37.88, P < 0.05$).

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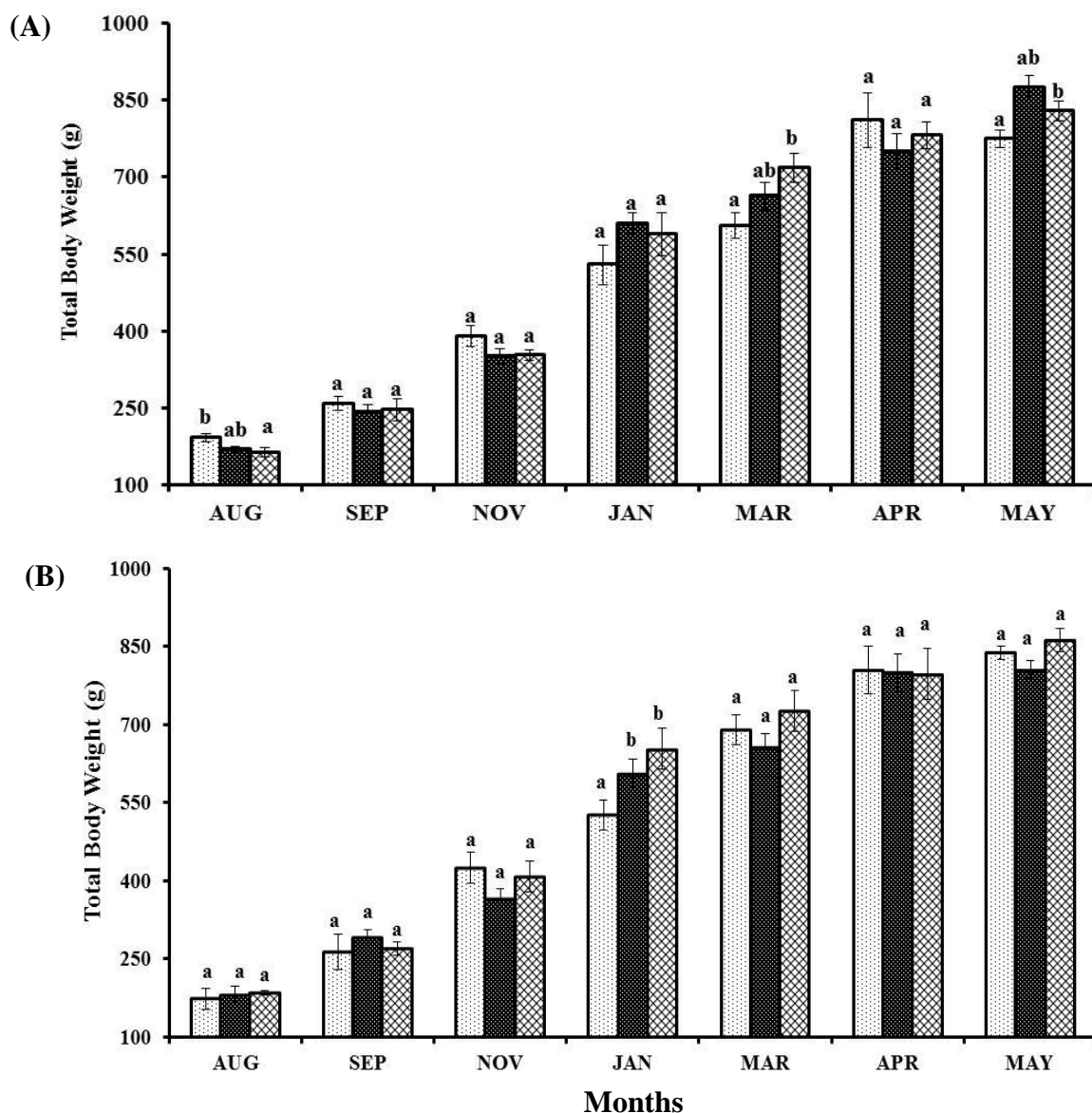


Fig. VI.1 Variations in total body weight of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: [stippled], Advanced: [solid black], Continuous: [cross-hatched]. n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

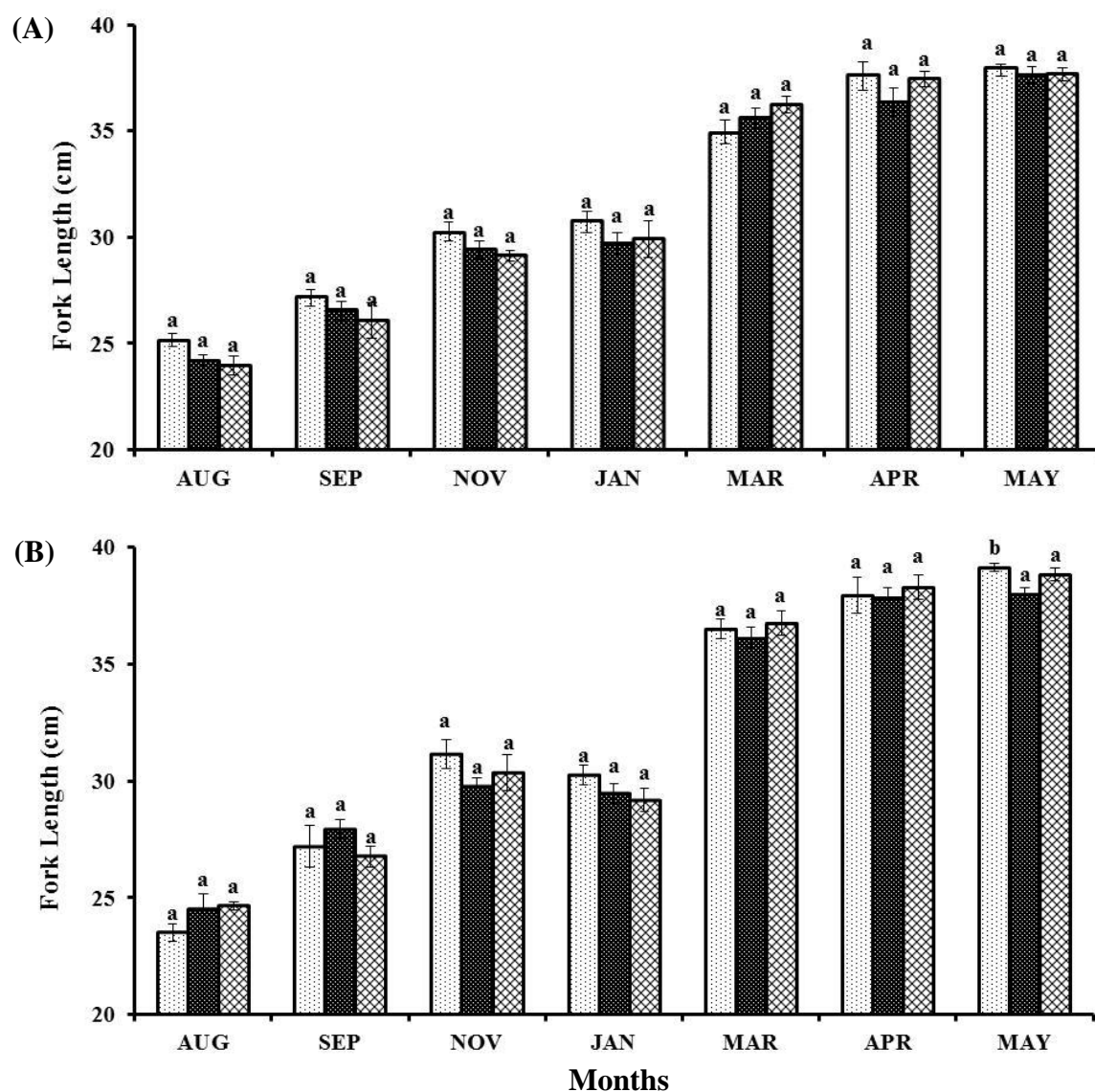





Fig. VI.2 Variations in fork length of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP.) and continuous (CP.) treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced: , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

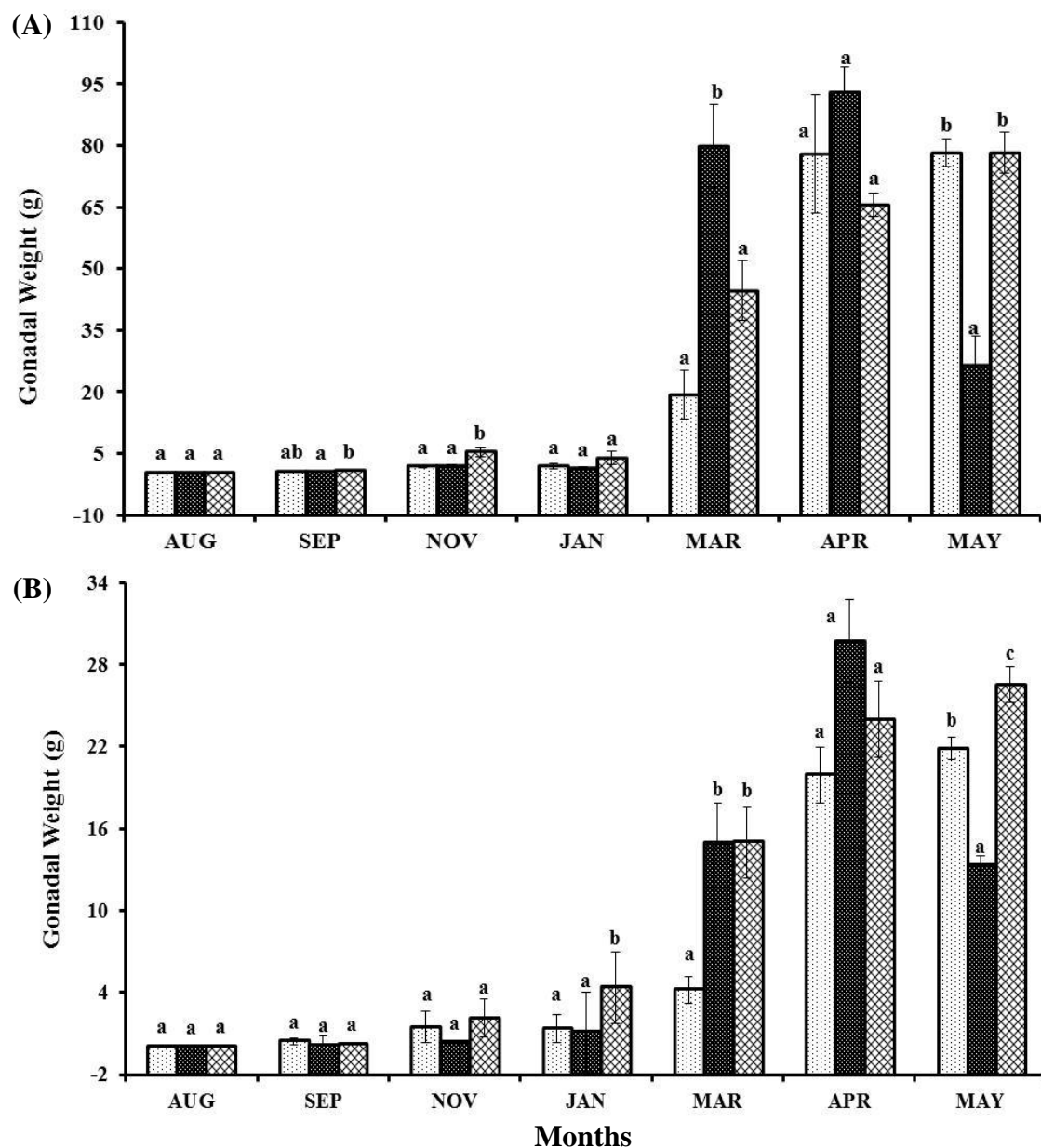





Fig.VI.3 Variations in gonadal weight of females (A) and males (B). One way ANOVA computed difference between values of natural (NP), advanced (AP) and continuous (CP) treatments at level of $P < 0.05$ on same sample point, shown by letters. Natural: , Advanced: , Continuous: . n at each sample point (AUG – APR) = 18 per treatment. n at final sample point (MAY) = 90 per treatment.

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Control of Maturation by Photoperiod Manipulation

Table VI.1 Percent population of fish at different developmental stages exposed to natural, advanced and continuous photoperiod. Data comprised of results collected at final sample point on May 25, 2012 to show the effect of light treatment of 10 months duration commenced on August 1st, 2011.

Trt.	Female			Male		
	Immature	Mature	Atretic	Immature	Mature	
					Running	Non Running
NP	0	100	0	2	65	35
AP	8	92	0	6	85	15
CP	0	0	100	0	0	100