Reproductive biology of yellowbelly flounder *Rhombosolea leporina* (Günther, 1862) and *Rhombosolea* spp.

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“Evolution is cleverer than you are”

- Leslie Orgel

Abstract

Yellowbelly flounder *Rhombosolea leporina* is a culturally, recreationally, and commercially important flounder species in New Zealand. *R. leporina* contain high-quality flesh and sexually dimorphic growth, where females grow more rapid and reach an overall larger size than males. These biological characteristics make *R. leporina* an enticing opportunity for aquaculture. Aquaculture is becoming an increasingly important capability in global food security and an alternative to wild capture fisheries. Understanding and controlling the reproductive biology of an aquaculture species candidate is required to ensure successful production. The understanding of the reproductive biology also facilitates the effective management of wild stocks. Currently, there is a limited amount of literature on the reproductive cycle of *R. leporina* and no previous literature available on the sexual differentiation in *Rhombosolea* spp. This study provides a histological investigation into the reproductive cycle of adult female *R.* leporina, from November 2015 – October 2017, and sexual differentiation in *Rhombosolea* spp. juveniles. It was found that *R. leporina* spawns over a protracted seven-month season, from June to December. Based on the histological observations it was apparent that *R. leporina* have multiple group synchronous ovarian development with the potential of batch spawning. The investigation into sexual differentiation in the *Rhombosolea* spp. juveniles revealed gonadal cavities in all individuals, making ovarian cavities an inadequate criterion of sex. Therefore, it was found that the juveniles display sexual differentiation at 47 mm TL and 57 mm TL, for females with meiotic oocytes and males with spermatocytes respectively. Furthermore, spermatozoa were present in testes of a 71 mm TL male. These results provide insights into the precise timing of reproductive development and the size at sexual differentiation. The results presented here may facilitate further investigations into the endocrine regulation or manipulation of the reproductive biology of *R. leporina*. The implication of the investigation into *Rhombosolea* spp. juveniles illustrate the necessity of more robust investigations into the timing of sexual differentiation and initial gonadal development alongside insights into gene expression. Nonetheless, these findings provide essential background knowledge on the reproductive biology of *R. leporina* and *Rhombosolea* spp. juveniles for the potential of aquaculture and management of wild stocks.

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General Introduction

1 General introduction

1.1 Background

Yellowbelly flounder *Rhombosolea leporina* is one of the culturally, recreationally, and commercially important flounder species in New Zealand. This species is found in shallow coastal waters around New Zealand, commonly in harbors and estuaries (Ayling & Cox, 1982). This proximity to the shore likely secured its importance culturally within New Zealand. Flounder is not only a delicacy but has provided a reliable food source to Maori (Challenger, 1985). Its reliability as a food source has symbolic meaning in traditional arts, recognizing women's role in collecting flounder to provide for their whānau and iwi (Wallace, Pierce, & Paul, 2003). The current desire and reliability of flounder are further expressed in flounders' high catches by recreational and commercial fishermen. In a panel survey during the 2011 – 2012 fishing year 58.92 tonnes of flounder were collected by 138 recreational fishermen, being the 8th most harvested species (Wynne-Jones, Gray, Hill, & Heinemann, 2014), while the total commercial landings were 2464 tonnes in the 2014 – 2015 fishing season (MPI, 2016). These appear to show the sought-after nature of flounder in New Zealand and its relevance as a food and recreation source.

There are specific characteristics of aquaculture species candidates which make them attractive prospects and *R. leporina* contain some of these desired characteristics. They have high-quality flesh, and although they do not reach a large overall size, less than 50 cm Total Length (Ayling and Cox, 1982), they show rapid growth (Colman, 1974b). Individuals may also reach sexual maturity within two years (Colman, 1972; 1974b), allowing successive generations of selective breeding to be rapidly attained. As a mostly sedentary fish living in a benthic habitat, these fish should not require large water volumes and may suit low-cost infrastructure production systems. These biological characteristics provide the enticing potential for *R. leporina* as an aquaculture candidate species.

Aquaculture provides a valuable opportunity to maintain a food resource and protect natural resources but demands detailed knowledge of the species and its biology for success (Moksness, Kjorsvik, & Olsen, 2004). Currently, with the ever

growing and demanding human population, the ability to control our resources, especially food, becomes increasingly important. While aquaculture already accounts for around half of the global fish production, there is also some precautionary outlooks into the future, where promotion of aquaculture needs to continue to ensure global food sources (FAO, 2018). Thus, in our effort to better manage food resources in the future calls for the development and progression of aquaculture around the globe.

For any fish species, aquaculture requires a detailed understanding of the biology of the species along with an accurate application of the knowledge to successfully produce the desired product. In the goal of offsetting impacts from wild capture fisheries with aquaculture and to maintain food security, a closed-cycle aquaculture system is desired. These sustainable aquaculture operations have an emphasis on maintaining its independence from relying on natural resources for all aspects of production, especially the origin of the new cohorts (Anderson, 2002). Thus, there is significant importance in understanding the reproductive biology of a candidate species. This knowledge could allow for the efficient propagation of new cohorts, through synchrony and reliability in spawning and the potential control of controlling new cohorts sex ratios (Devlin & Nagahama, 2002). Understanding the reproductive biology also provides valuable information for effective management of the species wild stocks (Nakken, Sandberg, & Steinshamn, 1996; Morgan, 2008). The control of reproduction was one of the underpinning factors that allowed for the global spread of trout (Bromage & Cumaranatunga, 1988), which led to pivotal findings about teleost reproductive biology principally from their expansion to new environments. Reproduction is essential for the propagation of a species. However, there are other aspects to reproduction, which are under the umbrella of reproductive biology, that also need sufficient knowledge, to ensure successful production (Moksness *et al*., 2004).

Maintaining successful propagation of new cohorts also relies on the successful initial development of the gonad. This understanding and control of the initial gonadal development is advantageous to the synchrony of spawning in adults and selection of sexually distinct traits (Devlin & Nagahama, 2002). The economically advantageous sexually dimorphic characteristics such as growth can be selected for in early ontogeny through manipulation of sex ratios (Mei & Gui, 2015). The methods to control the sex ratios take advantage of the teleost reproductive

plasticity (Kobayashi, 2013) and include induced triploidy (Utter, Johnson, Thorgaard, & Rabinovitch, 1983; Cassani & Canton, 1985), gynogenesis (Yamamoto, 1999), and hormonal sex reversal (Pandian & Sheela, 1995). These are some of the methods to control sex and require background knowledge into the reproductive development through its genetic controls, endocrine regulation, and how other factors affect initial development. Thus, when trying to maintain a constant production of fish, understanding the adult reproductive biology for successful propagation and the timing of sexual differentiation to control sex ratios is important.

Reproductive development relies on the transduction of environmental variables into endocrine signals which regulate the cascade of events in juvenile and adult gonads (DeVlaming, 1972; Crim, 1982; Bromage, Porter, & Randall, 2001; Devlin & Nagahama, 2002). While the actual mechanism of the transduction of environmental variables is not well understood (Zohar, Muñoz-Cueto, Elizur, & Kah, 2010; Nakane & Yoshimura, 2014), investigating the result of the cascade of events, being gonadal development, can give insights to the related environmental factors and endocrine signals. In iteroparous species adult gonadal development is typically an annual continuum of gonadal quiescence, recrudesce, and spawning but are commonly described through characterized stages of oocyte development (Wallace & Selman, 1981; West, 1990; Brown-Peterson, Wyanski, Saborido-Rey, Macewicz, & Lowerre-Barbieri, 2011). The annual timing of these events are primarily scheduled by environmental conditions to ensure the most advantageous conditions for the future progeny (Bromage *et al*., 2001; Lowerre-Barbieri, Ganias, Sborido-Rey, Murua, & Hunter, 2011). The progeny in gonochoristic species have their sex determined at fertilization or by environmental experiences in their early life, which may also have considerable impact on the former's ultimate phenotypic sex (Nakamura, Kobayashi, Chang, & Nagahama, 1998; Devlin & Nagahama, 2002; Guerrero-Estevez & Moreno-Mendoza, 2010; Kobayashi, 2013). These experiences have effects on the genetic and endocrine regulation on the initial development of the gonad. Both the initial development and the annual continuum of gonadal development have shown susceptibility to manipulation of the seasonal cues and endocrine mechanisms. The plasticity of teleost reproductive biology and susceptibility of the reproductive mechanisms to manipulation have provided the aquaculture industry with enticing opportunities to control their product.

When investigating the reproductive biology of a species, histology allows for the most accurate and detailed observations of gonadal development (Hunter & Macewicz, 1985*b*; West, 1990; Kjesbu, 2009b; Brown-Peterson *et al*., 2011; Lowerre-Barbieri *et al*., 2011). However, histology is the least time-efficient and requires unique skill sets (West, 1990; Hunter & Macewicz, 1985*a*). More time efficient methods are available to assess reproductive development or markers of maturity such as the gonadosomatic index (I_G), oocyte size distributions, and macroscopic staging of fresh ovaries or oocytes (West, 1990). Nevertheless, they lack the detail provided by histology and the accuracy of maturity markers is uncertain until validated through histological analysis (Kjesbu, 2009b). Thus, this investigation aims to histologically describe the reproductive development of female *Rhombosolea leporina* and provide comparisons to other markers of maturity.

Histology is also an essential tool to observe the initial physiological development of the gonads through to their sexual differentiation. The development through sexual determination and differentiation is a series of cellular migrations and transformations principally controlled genetically and by the endocrine system, where the resulting morphological development changes can be microscopically observed (Nakamura *et al*., 1998; Devlin & Nagahama, 2002; Molyneaux & Wylie, 2004; Penman & Piferrer, 2008; Piferrer & Guiguen, 2008; Luckenbach, Borski, Daniels, & Godwin, 2009; Guerrero-Estevez & Moreno-Mendoza, 2010). Thus, the investigation into juvenile *Rhombosolea* spp. sexual differentiation will also utilize the methods of histology.

There are have been many studies focusing on New Zealand's flatfish. However, works focusing within the *Rhombosolea* genus appear to lack detail on the reproductive physiology of *R. leporina*. Studies in the North Island's Haruaki gulf during the 1970s provided insight into *R. leporina* size at sexual maturity (Colman, 1972), macroscopic gonad staging and spawning (Colman, 1973), their movements (Colman, 1974a), and growth of the two species (Colman, 1974b). An investigation complementing the work in the Hauraki Gulf was conducted in the Avon Heathcote estuary, of the South Island which added new information of monthly sex ratios of *R. leporina* but also provided size at sexual maturity, described macroscopic gonad stages with morphometrics, and details of the spawning season (Webb, 1973). Later an investigation into the feeding habits of flatfish in Wellington harbor provided

more detail around the diet of *R. leporina* (Livingston, 1987). The complementary studies provide a satisfactory geographically spaced baseline on the biology of *R. leporina* to which to compare.

More recently, a histopathological focused thesis provided what appears to be the first histological study of *R. leporina* where gonadal tissues were analyzed, however not for gonadal maturity (Nenadic, 1998). Soon after another thesis provided an overview of different aspects of the biology of *R. leporina*, including reproduction (Mutoro, 2001). The investigation on the reproduction focused on the comparison of the reproductive cycles of populations in Manukau and Waitemata estuaries, which supported previous findings by Colman (1973). The investigation also provided histological photomicrographs to accompany the described macroscopic stages of Colman (1973). However, while providing a histological representation of reproductive development, little explanation was made on the histological details observed or analysis across all individuals in the study.

Additional investigations have provided insights into seasonal movements and recruitment of *R. leporina* in Lake Ellesmere of the South Island (Glova & Sagar, 2000; Jellyman, 2011). While some aspects of the biology are studied for *R. leporina* the limited amount of detailed reproductive knowledge, which would give insight to development strategy, leaves doubt into the efficacy of the current management from this background. Further to this, there is no published work on the early gonadal development and sexual differentiation of *Rhombosolea* species.

1.2 Organisation of thesis

This thesis is comprised of two main investigating chapters focusing on the reproductive biology of *R. leporina* and *Rhombosolea* spp. through histological analysis. The first investigating chapter (**Chapter 2)** examines the reproductive progression of sexually mature adult female *R. leporina*. It summarizes two years of ovarian and oocyte development through the reproductive seasons. A collaborative study with Toi Ohomai Institute of Technology (formerly the Bay of Plenty Polytechnic) provided this investigation with ovarian tissue and bodily measurements spanning from November 2015 – October 2017, of wild-caught *R. leporina* from the Firth of Thames, New Zealand. This allowed for the histological

investigation that describes ovarian and oocyte development with links to possible environmental factors.

The second investigating chapter (**Chapter 3**) aims to identify the precise timing of gonadal differentiation in juvenile *Rhombosolea* spp. Wild capture of juvenile *Rhombosolea* spp. took place on the shallow mud flats of the Firth of Thames, New Zealand, during 2017. This investigation used histological cross-sections to provide initial understandings into the timing of sexual differentiation of two closely related species.

1.3 Aims

The combination of the two chapters aims to provide a detailed histological analysis across the entire lifecycle of *R. leporina.* This will provide a platform for a genetic or physiological assay-based studies to overlay and give a more comprehensive understanding of reproduction in *R. leporina* and other closely related species in New Zealand. This will ultimately assist the understanding and management of the species for those who treasure it and want to develop it for aquaculture.

Chapter 2

Reproductive cycle in adult female yellowbelly flounder, *Rhombosolea leporina* (Günther, 1862).

2 Reproductive cycle of adult female yellowbelly flounder, *Rhombosolea leporina* (Günther, 1862).

2.1 Introduction

Reproduction in teleost fish is controlled by an environmentally regulated cascade of hormonal events (DeVlaming, 1972; Crim, 1982; Bromage *et al*., 2001). Day length and temperature are considered to exert the most considerable influence on the timing of reproduction (DeVlaming, 1972; Bromage *et al*., 2001; Pankhurst & Porter, 2003). Seasonal changes of these factors typically cue, key reproductive events, with most species timing reproduction so that progeny receive optimal environmental conditions for survival (Bromage *et al*., 2001; Lowerre-Barbieri *et al*., 2011).

Physiologically, a sequence of key endocrine events occurs between the brain, pituitary gland, and the gonads to drive gamete development (Crim, 1982; Nagahama, 1994; Pankhurst & Porter, 2003). This coordinated endocrine cascade forms the reproductive axis or brain-pituitary-gonad axis (BPG) (Zohar *et al*., 2010). Environmental cues are transduced into the brain (Pankhurst & Porter, 2003) and result in the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus directly into the pituitary. GnRH neurons synapse directly onto the gonadotrope cells in the pituitary (Zohar *et al*., 2010). These are then stimulated by GnRH to produce and secrete the two gonadotropin hormones (GTH), follicle stimulating hormone (FSH) and luteinizing hormone (LH), into the circulatory system (Kawauchi *et al*., 1989; Planas, Athos, Goetz, & Swanson, 2000). The GTHs will then bind to their respective cell surface receptors expressed on the follicle cells surrounding the oocytes in the ovary. The oocyte follicle is a bilayer of steroidogenic cells consisting of the inner granulosa cells and the outer thecal cell layer (Nagahama, 1994; Le Menn, Cerda, & Babin, 2007; Nagahama & Yamashita, 2008). FSH stimulates the thecal cells to take up cholesterol as a substrate for the production of testosterone (T). T then diffuses out of the thecal cells and is bioconverted into 17βestradiol (E_2), in the granulosa cells (Planas & Swanson,

2008). E_2 diffuses into the circulatory system and ultimately stimulates the liver to produce vitellogenin proteins (Nagahama, 1994). These hepatic proteins are transported via the circulatory system to the ovary where they are taken up by the oocytes during vitellogenesis (Tyler, Sumpter, Kawauchi, & Swanson, 1991; Nagahama 1994; Devlin & Nagahama, 2002; Lubzens, Young, Bobe, & Cerda, 2010).

FSH appears to primarily regulate oocyte growth up to the end of the vitellogenic phase of oocyte development immediately prior to final oocyte maturation (Kawauchi *et al*., 1989; Nagahama, 1994; Devlin & Nagahama, 2002; Nagahama & Yamashita, 2008; Lubzens *et al*., 2010). At this point, elevated plasma T concentrations negatively feedback in the pituitary to stop FSH release and stimulate the release of LH (Zohar *et al*., 2010). The binding of LH to ovarian follicle receptors leads to the production of a progestogen, often 17,20β-dihydroxy-4-pregnen-3-one (DHP) termed the maturation-inducing steroid (MIS). This MIS induces the oocyte to complete the first meiotic division and mature after which it will be ovulated to become a fertilizable egg (Nagahama, 1994; Nagahama & Yamashita, 2008; Lubzens *et al*., 2010). Thus, the BPG coordinates reproduction largely through the regulation of the sex steroids that drive gametogenesis. A fine detailed understanding of reproduction can therefore be gained, by measuring key hormones from the BPG in conjunction with the histology of ovarian development over a seasonal time period. While steroid quantification was beyond the scope of this current study the timing of key endocrine events may still be inferred by the presence of characteristically associated markers in the ovarian histology. Histological analysis of ovarian development combined with environmental variables, can, therefore, provide a robust description of seasonal reproduction in a species.

Oogenesis is the hormonally regulated development of immature oocytes to become viable gametes or eggs. Although the pattern of development is a continuous process, it can be described as a series of distinct stages based on the different cellular events that occur throughout development. Immature ovaries should contain oogonia, chromatin nuclear, and perinucleus oocytes in tightly packed lamellae (Wallace & Selman, 1981; Brown-Peterson *et al*., 2011). Oogonia undergo the first meiotic division and develop into primary oocytes, after which the division arrests at the end of the diplotene prophase (Le Menn *et al*., 2007). This

transformation of oogonia into a primary oocyte may be controlled by E_2 and DHP (Lubzens *et al*., 2010). Chromatin nuclear oocytes have a formed follicle layer (Le Menn *et al*., 2007) and during the primary growth phase (chromatin nuclear – perinucleus oocytes) oocytes may increase their volume a hundred-fold (Wallace & Selman, 1981), primarily due to the stimulation of the ovarian follicle by physiological levels of 11-ketotestosterone (11-KT) (Forsgren & Young, 2012).

Cortical alveoli oocytes mark the Secondary growth phase (cortical alveoli – vitellogenesis) which occurs under the regulation of FSH and E2 (Brown-Peterson *et al*., 2011; Forsgren & Young, 2012; Kagawa, 2013). Cortical alveoli oocytes are easily identified histologically with the appearance of the first cytoplasmic inclusions. These consist of cortical alveoli granules (CAG) as well as the possible deposition of oil droplets (OD) (Wallace & Selman, 1981).

Vitellogenesis occurs with increasing concentrations of E2 and continued FSH stimulation of the follicle cells (Nagahama, 1994, Lubzens *et al*., 2010). It has also been suggested that GTHs, likely FSH, is responsible for the regulation of the vitellogenin sequestration (Nagahama, 1994). Plasma levels of FSH and E_2 should continue to increase during early vitellogenesis (Lubzens *et al*., 2010). The inclusion of vitellogenin into the oocyte appear as apparent eosinophilic granules which are also associated with a significant increase of oocyte size (Wallace & Selman, 1981; Lubzens *et al*., 2010; Brown-Peterson *et al*., 2011). Furthermore, as Gonadal-Somatic Index (I_G) is expected to be increasing the Hepatic-Somatic Index (I_H) may also make apparent increases, in part to the metabolic activity, from biochemical processes, in the liver during vitellogenesis (Krosgaard-Emmersen & Emmersen, 1976).

Oocytes entering the final maturation stages (GVM – Hydration) undergo the re-initiation and completion of the first meiotic division under the stimulation of the MIS (Nagahama & Yamashita, 2008). This indicates a switch of FSH to LH regulation in the ovary (Kawauchi *et al*., 1989; Nagahama, 1994; Devlin & Nagahama, 2002; Nagahama & Yamashita, 2008; Lubzens *et al*., 2010). This is evident as the germinal vesicle (nucleus) migrates to the periphery of the oocyte and breaks down to release the first polar body to complete meiosis I (West, 1990; Lubzens *et al*., 2010).

At this time, osmotic pressure is increased in the oocyte through active ion transport (Khan & Thomas, 1999) allowing for the rapid increase in oocyte diameter from

hydration (West, 1990). The now metaphase II oocyte ovulates, rupturing out of the follicle layers, a genomic regulated process (Nagahama & Yamashita, 2008). Depending on the species, the ovulated eggs are either released into the ovarian or gut cavity. This event may be evidenced histologically by the presence of the empty post ovulatory follicles (West, 1990, Brown-Peterson *et al.*, 2011).

Post spawned fish then enter a period of reabsorption where atresia of the un-ovulated oocytes takes place (Lubzens *et al.*, 2010). Atresia may also be induced by unfavorable conditions, such as starvation (Hunter & Macewicz, 1985b), temperature, and stress (Guraya, 1986). Atresia is characterized by hypertrophy of the granulosa cells, which then invade the oocyte to phagocytize its contents (Lang, 1981; Nagahama, 1983; Hunter & Macewicz, 1985b).

Reproduction has been studied in several New Zealand flatfish, such as turbot *Colistium nudipinnis*, brill *C. guntheri* (Poortenaar, Hickman, Tait, & Giambartolomei, 2001), greenback flounder *Rhombosolea tapirina* (Barnett & Pankhurst, 1999), sand flounder *R. plebeia* (Setyono, 2005) and yellowbelly flounder *Rhombosolea leporina* (Colman 1973; Webb 1973; Mutoro 2001). Of these only the greenback flounder *R. tapirina* has included an analysis of sex steroids with the gonadal histology (Barnett & Pankhurst, 1999). Previous studies in the remaining species are largely histologically based. Those that focus on the reproductive biology of *R. leporina* have identified the time of spawning, gonadal condition, size at first maturity as well as a comparison of reproduction in subpopulations (Colman, 1973, Webb, 1973; Mutoro, 2001). However, none of these have provided a comprehensive histological analysis of seasonal reproduction over an extended time period with an account of overlying environmental variables.

This chapter aims to histologically characterize oogenesis in wild caught adult female *R. leporina* using light microscopy and condition indices. This will provide valuable baseline knowledge to inform the management of wild stocks and support future aquaculture and restocking programmes.

2.2 Methods

The investigation in this chapter is part of a collaborative work with Toi Ohomai Institute of Technology (formerly the Bay of Plenty Polytechnic).

2.2.1 Fish collection

In the collaborative portion of this investigation at Toi Ohomai, Adult *R. leporina* were collected, approximately monthly, from November 2015 – October 2017 at two locations in the southern Firth of Thames, New Zealand (**Figure 2.1**). Recreational drag netting was used to collect adults from mud flats at the Miranda site (-37°08’47”S 175°18’18”E) only for November 2015. The remaining adults were collected by a commercial fisherman, on shallow mud flats around the Piako site (-37°10’31”S 175°29’35”E).

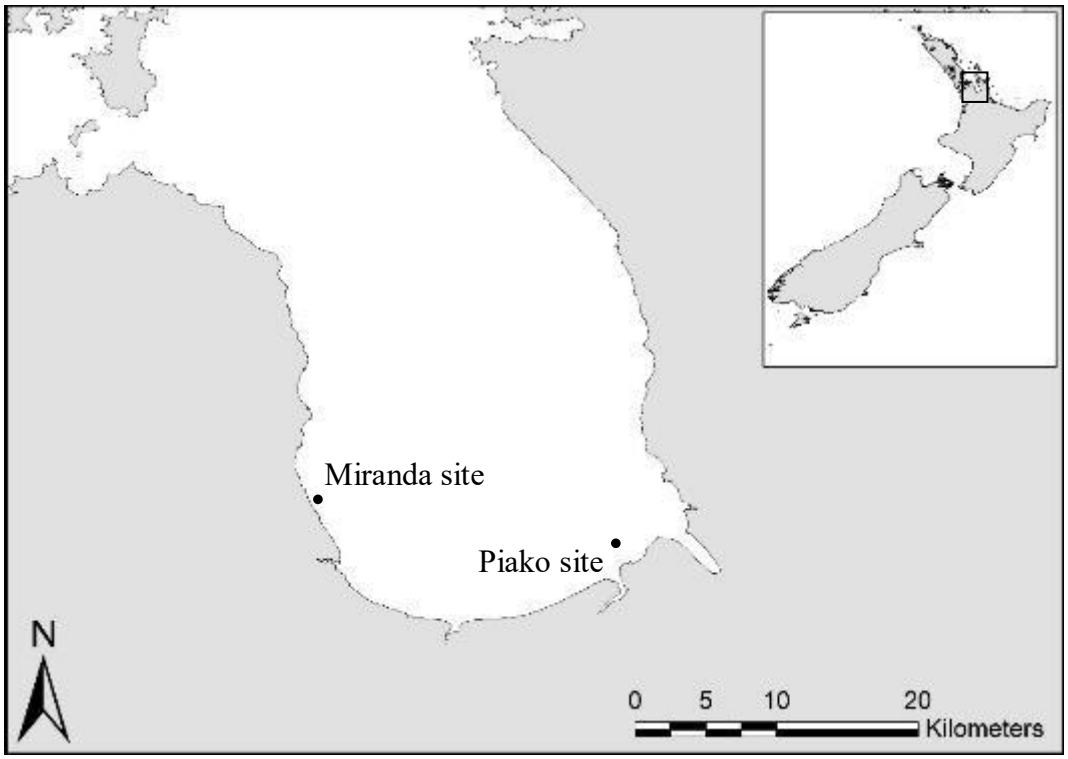

Figure 2.1. Capture sites of *R. leporina* from November 2015 – October 2017. Recreational drag netting was used at the Miranda site (-37°08’47”S 175°18’18”E) in November 2015, while a commercial fisherman collected *R. leporina* at the Piako site (-37°10’31”S 175°29’35”E) for the remaining months.

Captured fish were transported live to aquaculture facilities at Toi Ohomai. Upon arrival, fish were transferred and held in a 1600 L circular tank. These recirculating tanks were maintained between 18 – 20 °C with mechanical and bio filters.

2.2.2 Dissection

Dissection was also completed by the collaborative side of this investigation based at Toi Ohomai. Fish were dissected within 24 hours of arrival. The fish were placed into an aerated 5 – 10 L ice bath, held below 4°C and dosed with 2-phenoxy ethanol at $0.6 ml L^{-1}$, acting as an anesthetic (Pavek, 2016). Fish were held in this dosed ice bath until heavy sedation was observed.

Then, body mass (M_B) was weighed, to the nearest 0.1 g, and measured for total length (L_T), to the nearest 0.5 cm. Weights were taken on either of two scales, Kern FKB 36K0.1 (Max 36000g/d=0.1g; DC 12V/300mA; Ser. No W1609269) or Kern PFB 1200-2 (Max 1200g/d=0.01g; Ser. No: WF1308465; DC 12V 500mA). Fish were then swiftly dispatched and various organs were removed, sampled, weighed, and prepared for later analysis.

At this point, the liver and gonad were weighed, using the Kern PFB 1200-2, to the nearest 0.01 g, and gonadal tissue samples were taken for this investigation. Multiple cross sections from the middle of the ovary were taken, 2 – 5 mm thick, and immediately fixed in 10% neutral buffered formalin (Sigma Aldrich). There was no standardization of which gonad was sampled. However, the eye side gonad was presumed to be the most commonly sampled. Tissues were fixed in 20 – 50 ml of 10% neutral buffered formalin, for 12 – 24 hours. After which the tissues were washed and stored in 70% ethanol for no longer than 19 months.

2.2.3 Histology

A simple tissue processing protocol was used. The protocol is outlined in **Table 2.1**, it follows a standard dehydration, clearing, and infiltration procedure, adapted from Mumford (2004).

Table 2.1: Tissue processing protocol used for *R. leporina*. Adapted from Mumford (2004).

Bath	Duration
1. 70% Ethanol	< 19 Months
2. 80% Ethanol	30 Minutes
3. 90% Ethanol	30 Minutes
4. 100% Ethanol	30 Minutes
5. 100% Ethanol	30 Minutes
6. 100% Ethanol/Clearant (1:1 ratio)	30 Minutes
7. Clearant	30 Minutes
8. Clearant	30 Minutes
9. Clearant/Paraffin (1:1 ratio)	30 Minutes
10. Paraffin	30 Minutes
11. Paraffin	30 Minutes
12. Embedding	

The facilities used during the histological process lacked the specialized equipment needed for high production of blocks and slides. However, provisional replacements mitigated the lack of equipment. Separate heated and cooled granite blocks, 350 mm (L) x 350 mm (W) x 35 mm (H), were used to replace the hot and cold plates found on an embedding station. Paraffin was pre-melted into a beaker in a 60°C oven (Thermo Scientific Heratherm oven Advanced Protocol oven OMH 100), for ease of pouring during blocking out. Other replacements will be identified below with the order of processing.

After the tissues were embedded in paraffin, they were transferred to a -20°C freezer to quickly cool. Blocks did not remain in the freezer for more than 12 hours. At this point, blocks were separated from their molds and stored at room temperature until sectioning.

Histological slides were routinely produced using serial sectioning methods. The serial cross sections were produced on the Lecia Microtome (JUNG RM2025) at 5 – 7 µm. Two types of disposable low-profile microtome blades were used: Lecia DB 80 LS low profile microtome blades for standard applications and Lecia DB 80 LX low profile microtome blades for standard applications – extra durable. The serial sections were floated in a provisional 0.3% alcohol water bath held at 40 –

45°C. Untreated slides were used, Fronine microscope slides, plain ground edge (26 x 76 mm (1 – 1.2 mm)), or iMED® microscope slides, single frosted cut edge (25 x 75 mm (1 – 1.2 mm)), to collect the serial sections from a provisional water bath, within 5 minutes of floating. Slides were subsequently dried in slide racks or boxes for 24 hrs – 2 weeks, at ambient temperature.

Slides were then put through a regressive staining procedure, following protocols from Mumford (2004). Slight modifications were made to this protocol. "Scott's" being replaced with "Scott's tap water substitute" (x5 Scott's tap water substitute (2 g $NaHCO_3$; 20 g $MgSO_4$; $7H_2O$). A range of acid alcohol concentrations were used, from 0.25 – 1% HCL content in 70% ethanol, and a reduction in this bath duration to 15 – 30 seconds. The hematoxylin used was Leica Surgipath Harris Haematoxylin (Ref: 3801560; Produced 2015-10-28: Lot: 102615) and Leica Surgipath Eosin (Ref: 3801600; Produced 2015-10-23; Lot: 102115). Two baths were also added to the staining protocol, both at the end: an absolute Ethanol and a Xylene bath in that order. This allowed for the clearant (Neo-Clear® (xylene substitute) for microscopy) to be washed and replaced with xylene, to improve the compatibility with the mounting media. DPX (Labworks DPX Mounting Media (containing Xylene)) mounting media was used to mount coverslips (LabServ coverslips (22 x 60 mm)) to stained slides.

2.2.4 Oocyte Staging

Staging was completed through a light microscope (Nikon Ni-U DIC light microscope). The staging followed Wallace & Selman (1981), West (1990) and Brown-Peterson *et al*. (2011). Individuals were staged on their Leading Cohort Oocyte (LCO). Fish which displayed signs of spawning activity, the presence of Post Ovulatory Follicles (POF), were classed into the histologically present developing LCO. Descriptions of oocyte and ovarian maturity were only described for which were observed in this investigation. There are known stages of oocyte development which were not observed in this investigation and as a result are missing in the descriptions delivered within this investigation.

Identification of atretic oocytes follows descriptions from Hunter & Macewicz (1985b). Classification of reabsorbing females was based on their ratio of atretic oocytes to healthy oocytes. Two to three regions of a single section of ovarian tissue

were systematically sampled for the presence of atretic or healthy oocytes. Digital images of each region, at x12 magnification, was overlaid using the grid function in Image J (National Institute of Health; http://rsbweb.nih.gov/ij), producing 54 intersections. The presence of atretic or healthy oocytes and the ratio of atretic to healthy oocytes was recorded for each intersection and subsequently, each regions' ratio was averaged, for each individual. The individual's number of analyzable regions depended on the size of ovarian tissue processed and the amount contained in each photo.

The observations of mean ratios of the atretic to healthy oocytes, allowed to set a criterion for the reabsorbing stage at ovaries containing atretic oocytes, > 10%. There was an apparent gap between individuals above and below this criterion, which acts as a simple standard, that appears to correspond with previous work on Greenland halibut *Reinhardtius hippoglossoides* (Junquera, Roman, Paz, & Ramilo, 1999), as a representable selection of females with atresia from the atretic absent females.

Oocyte measurements were taken according to methodology reviewed in West (1990). Only the centered oocytes are measured, taking the minimum and maximum axis for each oocyte. The two measurements are further averaged to produce mean diameter. Measurements were produced from digital images using ImageJ (National Institute of Health; http://rsbweb.nih.gov/ij), taken through a microscope (Nikon Ni-U DIC light microscope) mounted digital camera (Cannon 60D).

Three of the most mature oocytes, in the LCO, of each female were haphazardly selected from varying regions of the observed ovarian tissue and measured, ensuring to not sample the same oocyte twice. All measurements were compiled into their respective LCO and median inclusive quartile minimum and maximums were calculated and presented.

2.2.5 Statistics

Data were analyzed using Microsoft® Excel® 2016 and SPSS v.20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and SPSS v. 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). All values are presented to the third decimal and all significance is set at ($\alpha < 0.05$).

Gonadal-Somatic Index (I_G) and Hepatic-Somatic Index (I_H) was calculated for individual fish. I_G is calculated through the following formula: [$I_G = 100\ M_G\ (M_B - M_G)^{-1}$]. I_H is calculated through the following formula: [$I_H = 100\ M_H\ (M_B - M_H)^{-1}$]. Where M_B = fish weight, M_G = gonad weight, and M_H = liver weight.

Mean I_G of ovarian stages did not meet the assumptions of normality or homogeneity of variances when tested. Thus, nonparametric Kruskal-Wallis H test was used to test differences across the ovarian stages' mean I_G. The nonparametric Mann-Whitney U test was subsequently used to test differences between consecutive ovarian stages' mean I_G. The ovarian stages are not of similar distribution and thus results from both nonparametric tests are interpreted through their mean ranks.

However, I_H met the assumptions of normality and homogeneity of variances when tested, when data were transformed using the inverse square root function ($y = x^{-1/2}$). Allowing a one-way ANOVA test was used to test differences across the ovarian stages' mean I_H. Additionally, a Bonferroni's Correction Post Hoc test, to compare between the consecutive ovarian stages' mean I_H.

Monthly groups of I_G and I_H did not meet the assumptions of normality or homogeneity of variances for one-way ANOVA. Thus, the nonparametric Kruskal-Wallis H test is used to test for difference across the mean monthly I_G and I_H and the Mann-Whitney U test is used to test between each of the consecutive months of I_G and I_H.

The mean monthly I_G and I_H did not display a linear relationship with environmental variables and as previously mentioned did not display normality. Therefore, a nonparametric Kendall's tau-b is used to identify relationships between environmental variables and I_G and I_H.

Table 2.2: Stages of ovarian maturity in *Rhombosolea leporina*, according to the histological observation of leading cohort oocyte. Oocyte diameters presented are the inclusive quartile minimum and maximum, rounded to the nearest 1 µm.

Ovarian stage		Oocyte maturation stage: histological description of the oocyte	Oocyte diameter (µm)	*n*
I	Previtellogenic	The ovary contains primary growth oocytes: 1.Perinuclear (PN): Contains multiple basophilic nucleoli at the periphery of GV. Ooplasm is basophilic. ZR is not yet prevalent, but evidence of the eosinophilic lining available in the most mature. Follicle cells make a thin layer surrounding oocyte. Immature: No atretic evidence within the ovary. Regressed: Reabsorption/regression evident with remnant atretic oocytes or larger and more numerous granulosa cells.	66 – 123	49
II	Cortical Alveolar	The ovary contains PN stage oocytes, possible atretic evidence, and: 2. Cortical alveolar (CA): Significant increase in oocyte size. Now contains CAG and or appearance of OD. CAG form in POOPL and appear opaque. ODs are larger and more translucent, located perinuclearly. Nucleoli are located peripherally in GV still basophilic. Ooplasm remains basophilic. ZR is now visible and eosinophilic.	94 – 233	16
III	Vitellogenic	The ovary contains PN, CA stage oocytes, possible atretic evidence, and: 3. Early vitellogenic (EVO): Small eosinophilic VTG are now visible in the ooplasm, originating at the POOPL and eventually fill inward to the GV. ZR continues to thicken. More CAG and OD may	204 – 260	7

		appear, remaining in their respective locations. Nucleoli may lose peripheral location and be seen throughout the GV.		
		4. Late vitellogenic (LVO): VTG has swelled in size and number, occupying most of the ooplasm. CA if visible are still isolated to the POOPL. OD still visible perinuclearly, potentially coalescing to form less but larger OD. Basophilic ooplasm becoming less visible. Some atretic evidence may be seen.	191 – 342	49
IV	Final Oocyte Maturation	The ovary contains PN, CA, EVO, LVO stage oocytes, possible atretic evidence, and:	271 – 368	7
		5. Germinal Vesicle Migration: GV deviated from the centre. CAG might still be visible at the POOPL. VTG may coalesce into large light eosinophilic masses. Only a few large OD remain.		
		6. Ovulated: Ovaries contained hydrated oocytes which are loose and occupying the ovocoel. There is clear evidence of POFs, however, ovulated oocytes would have been present in the ovary. POFs in this stage are more prominent and thicker.	197 – 249	2
V	Reabsorbing	Ovary may contain PN, CA, EVO, or LVO stage oocytes with:	46 – 172	8
		7. Atretic (AT): Ovaries with widespread atretic oocytes at any identifiable or multiple stages. Atretic oocytes account for > 10% of the oocytes in that ovary. Atretic oocytes were identified with descriptions form Hunter & Macewicz, (1985*b*).		

GV – germinal vesicle, ZR – zona radiata, CAG – cortical alveoli granule, OD – oil droplet, POOPL – peripheral ooplasm, VTG – vitellogenin granules, POF – post ovulatory follicle

2.3 Results

R. leporina possess cystovarian ovaries which fuse anteriorly, positioned one above the other separated by spinal fin rays. The fused portion of the ovary remains in the gut cavity, only protruding slightly out from the caudal muscle wall (**Figure 2.2b,f**). The rest of the paired ovaries extend beyond the body cavity, towards the posterior, through the caudal muscle of the fish. Through the reproductive cycle, the ovaries appear to increase in both length and diameter. The membrane surrounding the ovaries are lightly pigmented, with a marked difference in color respective to the side it originates from. Pigments are a metallic gold or silver (**Figure 2.2a,b**), yet the top gonad can contain darker pigmentation, giving the perception its duller or grey appearance compared to the bottom gonad (**Figure 2.12c,d**).

Histological details of oocyte development stages were described (**Table 2.2**), visually represented (**Figure 2.3**), and classed in relation to leading cohort oocytes (LCO). Oocyte stages are then further grouped into ovarian maturation groups. These ovarian stages are statistically tested to identify differences between them. All developing females showed evidence of oocytes following the LCO in multiple stages of development. The oocytes appear to consistently recruit, displaying the range of development grades behind the LCO. Many fish also displayed evidence of atretic oocytes and POFs, accompanying the developing oocytes.

2.3.1 Field visual examination

The visual examination of any individual *R. leporina* was greatly benefited using a bright light source to observe gonadal silhouette cast through the individual. This allows assessment of the shape and transparency of the gonad to infer sex and condition. Quiescent females will have a weak shadow that is long and thin. The developing females will have a larger and wider profile with a darker shadow. The ovulated females will show almost no shadow, only being observed in two fish which still retained its eggs. Even with the absence of a shadow, they are easily identified through the visual examination of the outline and symmetry of the fish. The flat profile of the fish showed a large lump in the ventral side of the fish (**Figure 2.2c,d,e**). The males will display a very dark triangular shadow, not extending far into the muscle posterior to the abdominal cavity (not shown).

Figure 2.2: Orientations and body profile shapes of different maturation stages of female Yellow-Belly Flounder, *Rhombosolea leporina*. These individuals were collected in the southern Firth of Thames, by commercial set netting methods. (a) perinuclear regressed female; (b) late vitellogenic female; (c) first ovulated female, side on view; (d) first ovulated female, head on view; (e) second ovulated female, head on view; (f) dissected gut cavity of the first ovulated female displayed in (c) and (d) of this figure. The bottom gonad of the female remains in the individual. The gonad and eggs are noticeably transparent.

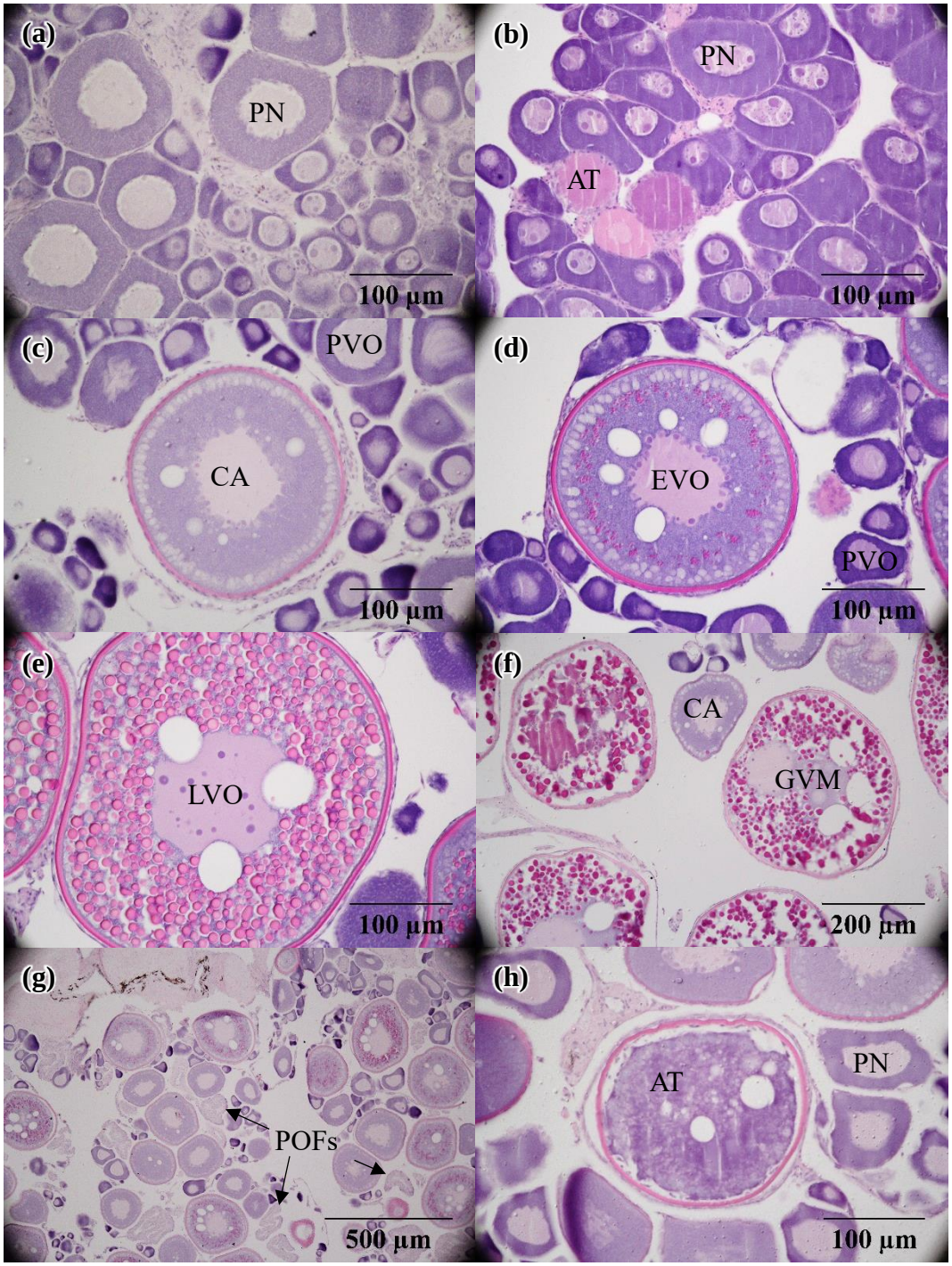

Figure 2.3: Photomicrographs of female yellowbelly flounder, Rhombosolea leporina, oocytes at varying stages. Processed with standard histological protocols, H&E staining, and sectioned at 7 µm. (a) perinuclear immature; (b) perinuclear regressed, minimal atretic evidence is seen; (c) cortical alveolar; (d) early vitellogenic stage, noticeable eosinophilic vitellogenin inclusions to the ooplasm; (e) late vitellogenic; (f) germinal vesicle migration; (g) ovulated ovary; (h) reabsorbing. Atretic oocyte, AT; cortical alveolar oocyte, CA; early vitellogenic oocyte, EVO; germinal vesicle migration oocyte, GVM; late vitellogenic oocyte, LVO; perinucleus oocyte, PN; post ovulatory follicle, POF.

2.3.2 Oocyte diameter

Oogenesis in adult female *R. leporina* appears to progress in a similar manner to that of most teleost. The histological events are characterized in **Table 2.2**. Oocyte diameters appear to increase with the oocyte stage (**Figure 2.4**). There are observed decreases in oocyte diameter in ovulated and reabsorbing females. There are wide ranges in the diameters of cortical alveoli, late vitellogenic, and reabsorbing oocytes. Minimum and maximum values for each oocyte stage are presented in **Table 2.2**.

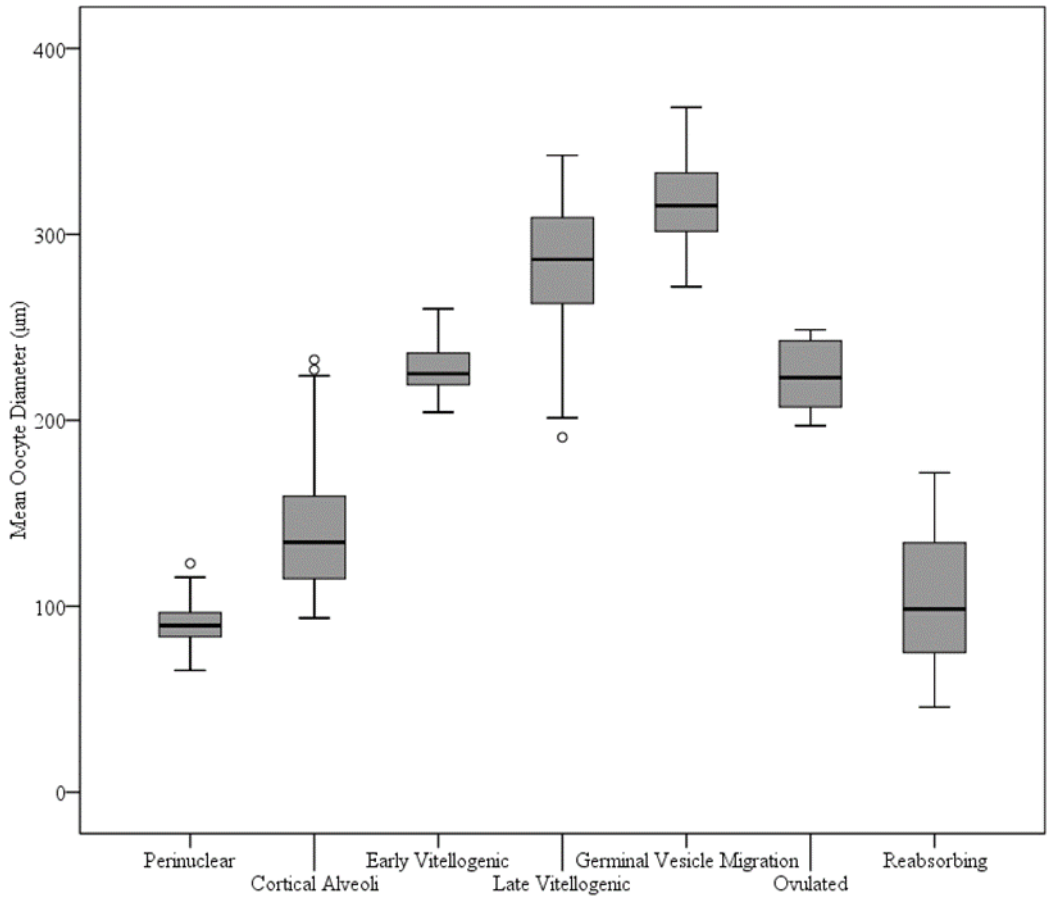

Figure 2.4: Oocyte diameters across oocyte stages. Whiskers represent quartile minimum and maximum, boxes represent the middle 50% quartile, and the cross-box line identifies the inclusive median. Outliers are marked as circles. Ovulated oocyte measurements are of the histologically present oocytes, not of ovulated eggs.

2.3.3 Mean values of organosomatic indices of ovarian maturity stages in female *R. leporina*

The mean I_G for ovarian stages I and II were (mean ± S.E. of 0.827 ± 0.037, 0.928 ± 0.037 respectively. Then the mean I_G substantially increased through stage III and IV females (3.776 ± 0.228 and 7.680 ± 1.729 respectively), followed by a significant drop in stage V females (1.099 ± 0.086) (**Figure 2.5**). Two-stage IV females showed marked I_G values of 16.5 and 15.6%, while the next highest individual clocked in at 9.5%. These two females contained ovulated ovaries.

The I_H varies less and sees a steady incline from stage I – IV (0.741 ± 0.017, 0.844 ± 0.046, 1.264 ± 0.044, and 1.410 ± 0.105 respectively) (**Figure 2.6**). Stage V shows a significant drop in I_H (0.814 ± 0.048) from the peak in stage IV.

Both I_G and I_H showed peaks during ovarian stage IV. The I_H did not differ as much as I_G across the stages but marked changes between the stage's average I_H are observable.

The Kruskal-Wallis H test identified a statistically significant difference in the I_G of the different ovarian stages, $x^2(4) = 99.168$, $p < 0.001$, with mean rank I_G value of 31.81 for stage I, 45.19 for stage II, 100.60 for stage III, 119.11 for stage IV, and 57.63 for stage V. All ovarian stages are seen to be significantly different between stage I and II ($U = 229$, $P = 0.016$), stage II and III ($U = 16$, $P < 0.001$), stage III and IV ($U = 116$, $P = 0.011$), stage IV and V ($U = 0$, $P < 0.001$), and stage V and I ($U = 68$, $P = 0.004$).

A one-way ANOVA displays significant difference across the stages of ovarian maturity mean I_H ($F(4,131) = 47.522$, $P < 0.001$) (**Figure 2.6**). Significant differences between consecutive ovarian stages' mean I_H are seen between stage II – III and stage IV – V.

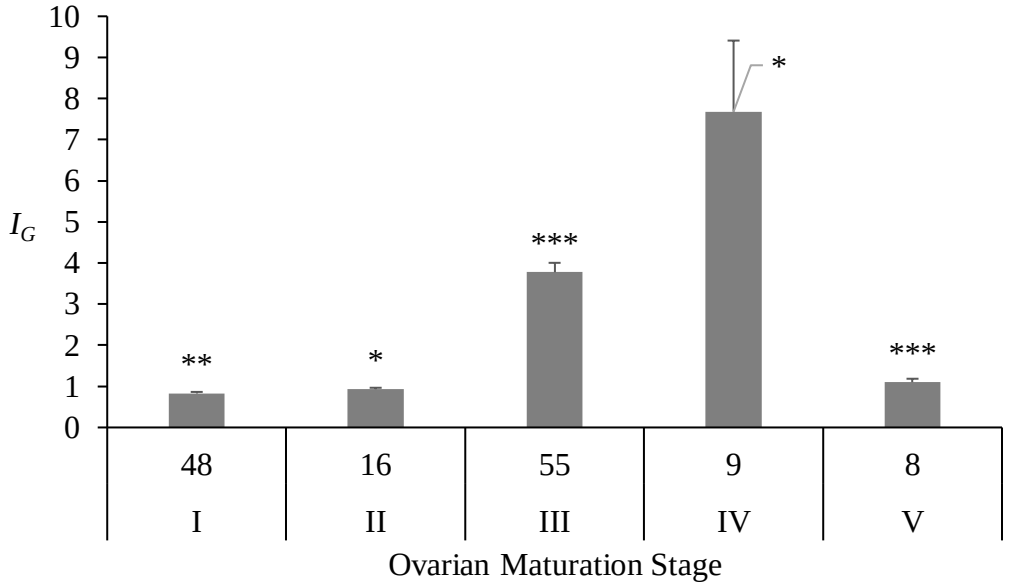

Figure 2.5: Mean ± S.E. I_G at different stages of ovarian development (see Table 2.2) in female *Rhombosolea leporina*. Numbers at the base of the bar indicate sample size. Significant difference is seen across ovarian stages (Kruskal-Wallis H test, $x^2(4) = 99.168$, $p < 0.001$). The significance above each bar specifies the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) to the previous ovarian maturation stage.

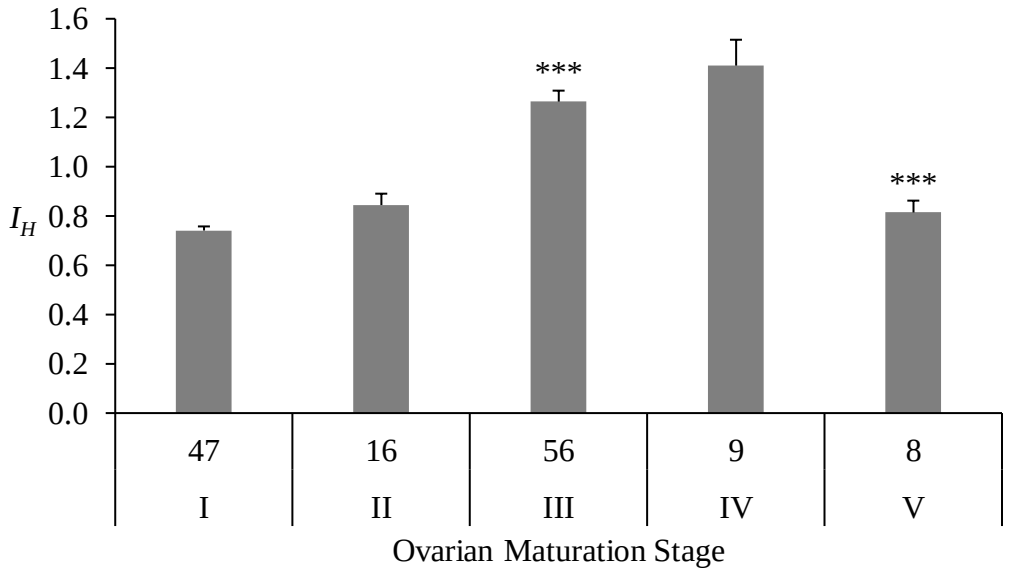

Figure 2.6: Mean ± S.E. I_H at different stages of ovarian development (see Table 2.2) in female *Rhombosolea leporina*. Numbers at the base of the bar indicate sample size. Significant difference is seen across ovarian stages (one-way ANOVA, $n = 136$, $P < 0.001$). The significance above each bar specifies the level of significance (*P < 0.05, **P < 0.01, ***P < 0.001) to the previous ovarian maturation stage.

2.3.4 Mean monthly values of organosomatic indices

Mean monthly I_G showed weak cyclic trends across the investigation, increasing from lows at the start of the calendar year. Mean monthly I_G was lowest in February 2016 (0.808 ± 0.028) and highest in June 2017 (6.348 ± 2.258) (**Figure 2.7**). Lows in mean monthly I_G are observed in the months of January and February and is repeated in the second year of this investigation. Mean monthly I_G appears to start increasing from March and April, and this elevated mean monthly I_G maintains through the end of the year, with some slight fluctuations, month to month.

Mean monthly I_H is comparatively lower to I_G, over the course of the investigation. However, mean monthly I_H shows a more cyclic trend than in I_G (**Figure 2.7**). The mean monthly I_H appears to peak near the middle of the calendar year. Mean monthly I_H appears to decline after August, in 2016. There was no apparent decline in mean monthly I_H in 2017 and when sampling ceased in October that year, the mean I_H is still elevated.

A Kruskal-Wallis H test found mean monthly I_G and I_H are significantly different across this investigation (I_G $x^2(19) = 62.850$, $p < 0.001$; I_H $x^2(19) = 79.706$, $p < 0.001$). Only one consecutive month pair is found to have significant mean I_G: February 2017 – March 2017 (Mann-Whitney, U = 4, $P = 0.025$). Nevertheless, in contrast to the lack of significant differences, there are visible changes in the mean monthly I_G across this investigation. This forms a very weak trend of increasing I_G from April (autumn) through to November (spring), repeating in the second year of this investigation.

More consecutive mean monthly values of I_H are observed to be significantly different (**Figure 2.7**). The majority of these differences are seen in late 2016 after the I_H peak in August that year.

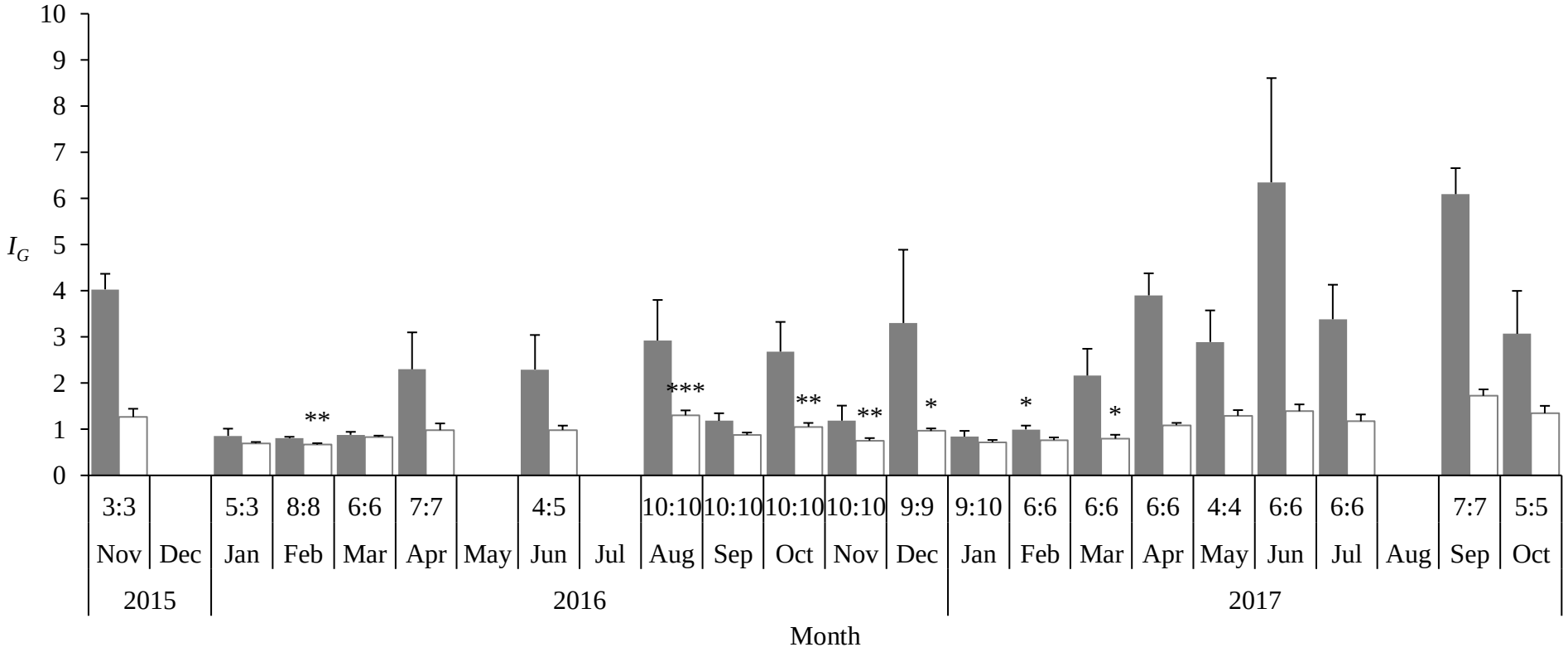

Figure 2.7: Mean ± S.E. monthly values of gonado-somatic index (I_G, ■) and I_H, □) with ovarian stage presence in female Rhombosolea leporina. Values below columns indicate sample size (I_G : I_H). A Kruskal-Wallis H test found significantly different between mean monthly values of I_G and I_H (I_G x2(19) = 62.850, p < 0.001; I_H x2(19) = 79.706, p < 0.001). Level of significance (*P < 0.05, **P < 0.01, ***P < 0.001) for the difference between consecutive months of mean monthly I_G and I_H, is marked above the column and indicates significance with previous month. Fish were collected by recreation drag nets and commercial set nets in southern Firth of Thames, New Zealand, from November 2015 – October 2017

2.3.5 Mean monthly values organosomatic indices and monthly frequency of ovarian maturity stages relation to environmental factors

Air temperature displays a moderate significant negative correlation to I_G and I_H (τb = -0.480, p = 0.003; τb = -0.596, p < .001 respectively) (**Table 2.3**). I_H also has a moderately significant, negative correlation to day length (τb = -0.463, p = 0.004). While I_G is only found to have a weak non-significant correlation. These correlations display seasonal trends across the study period (**Figure 2.8**).

Table 2.3: Kendall's tau-b nonparametric correlations of environmental variables, I_G, and I_H. Correlation is significant at the ** 0.01 level (2-tailed).

		Day Length (h)	Air Temperature (ºC)
Mean I_G (%)	Correlation Coefficient	-0.305	-0.480**
	Sig. (2-tailed)	0.060	0.003
	N	20	20
Mean I_H (%)	Correlation Coefficient	-0.463**	-0.596**
	Sig. (2-tailed)	0.004	0.000
	N	20	20

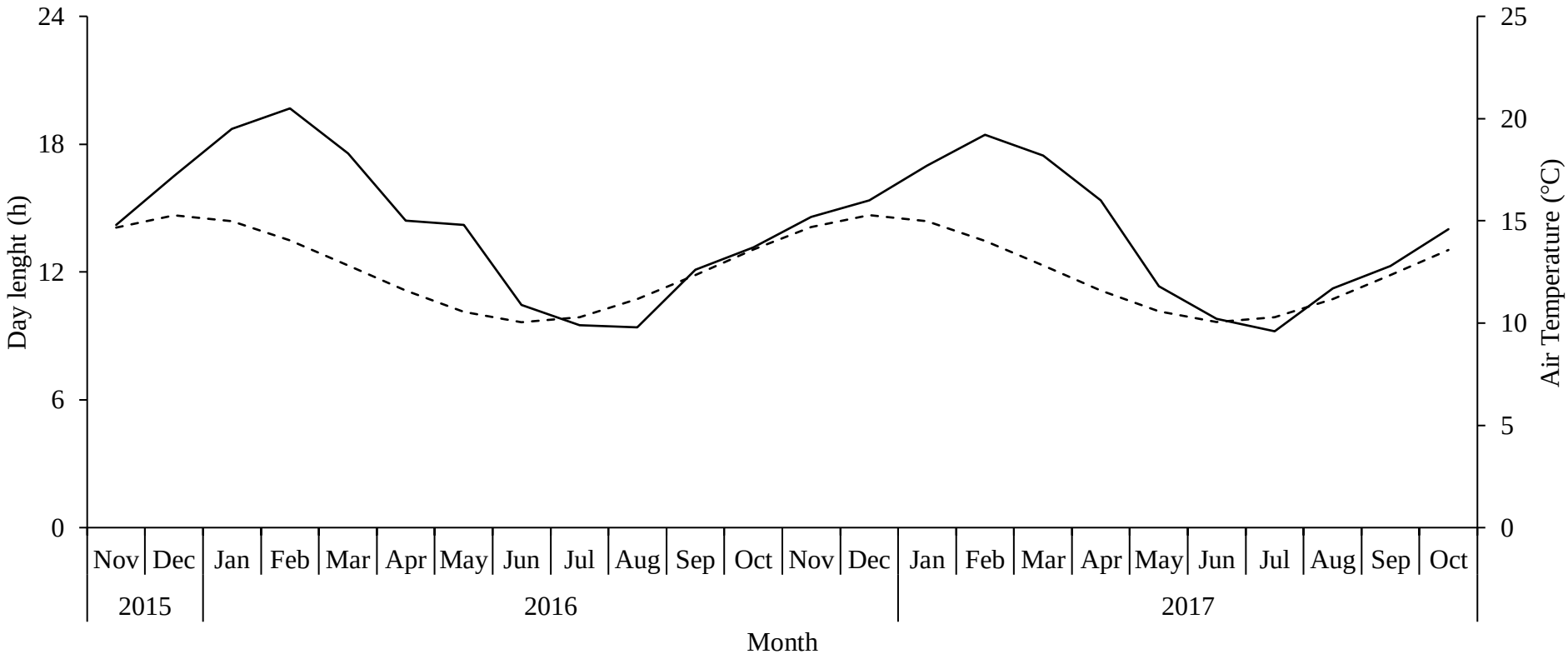

Figure 2.8: Mean monthly values of day length (….) and air temperature (___). Day length, at 37°10'00.0"S 175°29'00.0"E, was acquired through Astronomical Applications Dept. U. S. Naval Observatory, Washington, DC 20392-5420. Temperature was statistically calculated, from observations, for 37°12'54.0"S 175°27'00.0"E., acquired through the National Institute of Water and Atmospheric Research Ltd ("NIWA") CliFlo database.

2.3.6 Ovarian stage frequency

Most of the individuals are observed to contain stage I or III ovaries (**Figure 2.5**, **Figure 2.6**, and **Figure 2.9**). Stage IV is the most infrequent ovarian maturation stage observed. Generally, all stages of ovarian maturity showed evidence of atresia accompanying developing oocytes in at least one of their individuals.

The stage I ovaries consistently make up more of the monthly females around December through April (**Figure 2.9**). Stage II ovaries are far less common overall but had a marked presence in the females from August 2016 – November 2016. Stage III ovaries are only not seen during the calendar year in January and February. In 2016, they made up a significant amount of the females in June and August. The apparent reduction in their presence is seen in the months of September 2016 – November 2016. In 2017, Stage III ovaries pertained to a substantial amount of the females from April – October (**Figure 2.9**), more consistent than in 2016. Stage IV ovaries were only seen in months from June to December. This late maturation stage is only seen as a lone appearance in each month, except for October 2016, where four individuals with stage IV ovaries were present. Reabsorbing stage V ovaries were sporadically seen across the investigation; however, a marked presence is seen in September 2016.

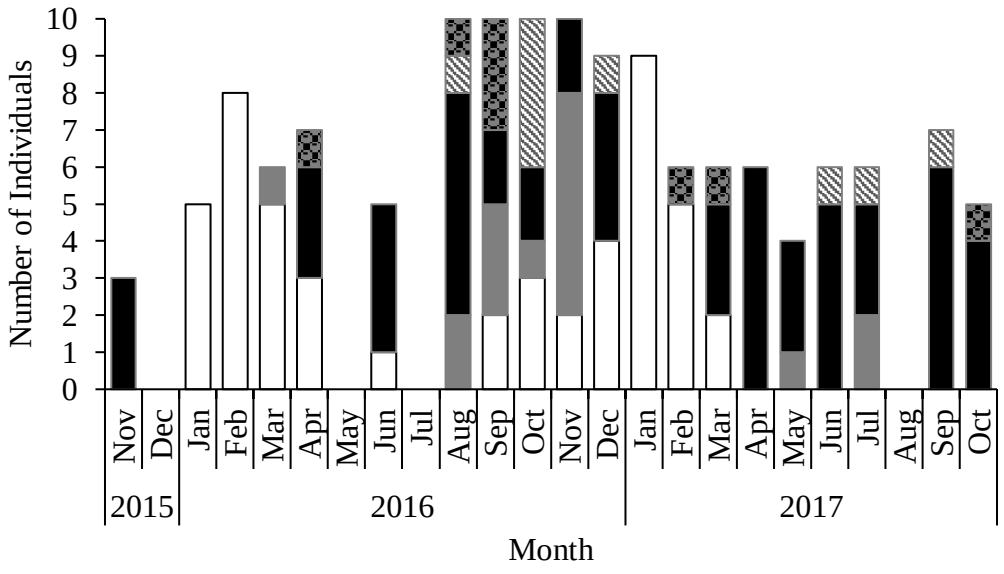

Figure 2.9: Monthly numbers of individual female *Rhombosolea leporina* in their respective ovarian maturity stages. Fish were collected from the southern Firth of Thames, New Zealand, from November 2015 – October 2017. Ovarian maturity stages from Table 2.2: Previtellogenic (I, □), Cortical Alveolar (II, ■), Vitellogenic (III, ■), Final Oocyte Maturation (IV, ▨), and Atretic (V, ▩).

2.3.7 Ovarian maturation stage size classes

The distribution of total length size classes varies between ovarian stages (Figure 2.10). The stages appear to contain unimodal distributions. However, separation of lesser peaks can be seen in ovarian stages I, III, and V. Noticeably, the distribution of ovarian stage III appears to be skewed to the right, containing more individuals of larger total length (mm) (**Figure 2.10**).

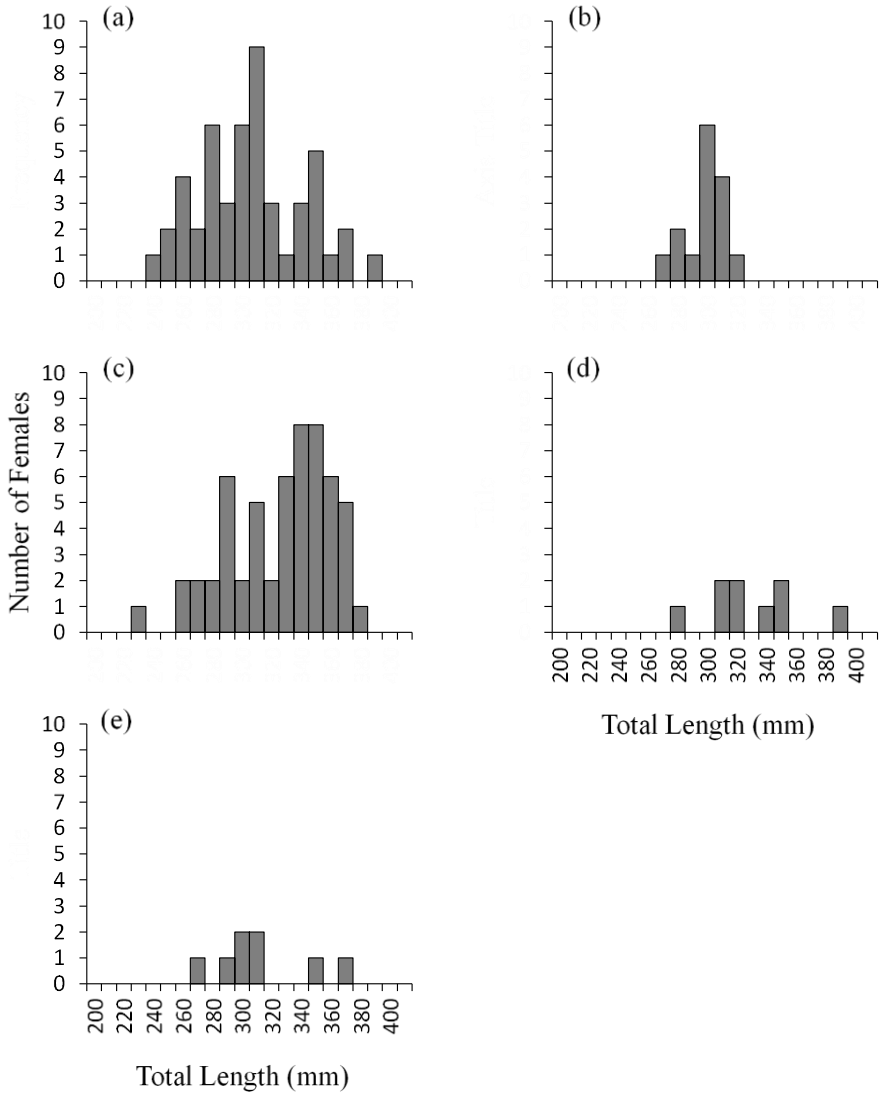

Figure 2.10: Number of female *R. leporina* grouped into 10 mm size classes (T_L) within their ovarian development stage group. (a) ovarian stage I; (b) ovarian stage II; (c) ovarian stage III; (d) ovarian stage IV; (e) ovarian stage V.

2.3.8 Uncertain maturity

One individual was observed to have low reserves of gametes. This individual was removed from the statistical analysis due to uncertainty around the abnormality seen in its histological sections being dominated by stromal tissues and abnormal gamete development (**Figure 2.11**). The individual displayed characteristics of a male, through visual examination in the field and with the aid of a bright light source (**Figure 2.11b**), yet histological observations revealed the individual to have oocytes (**Figure 2.12**).

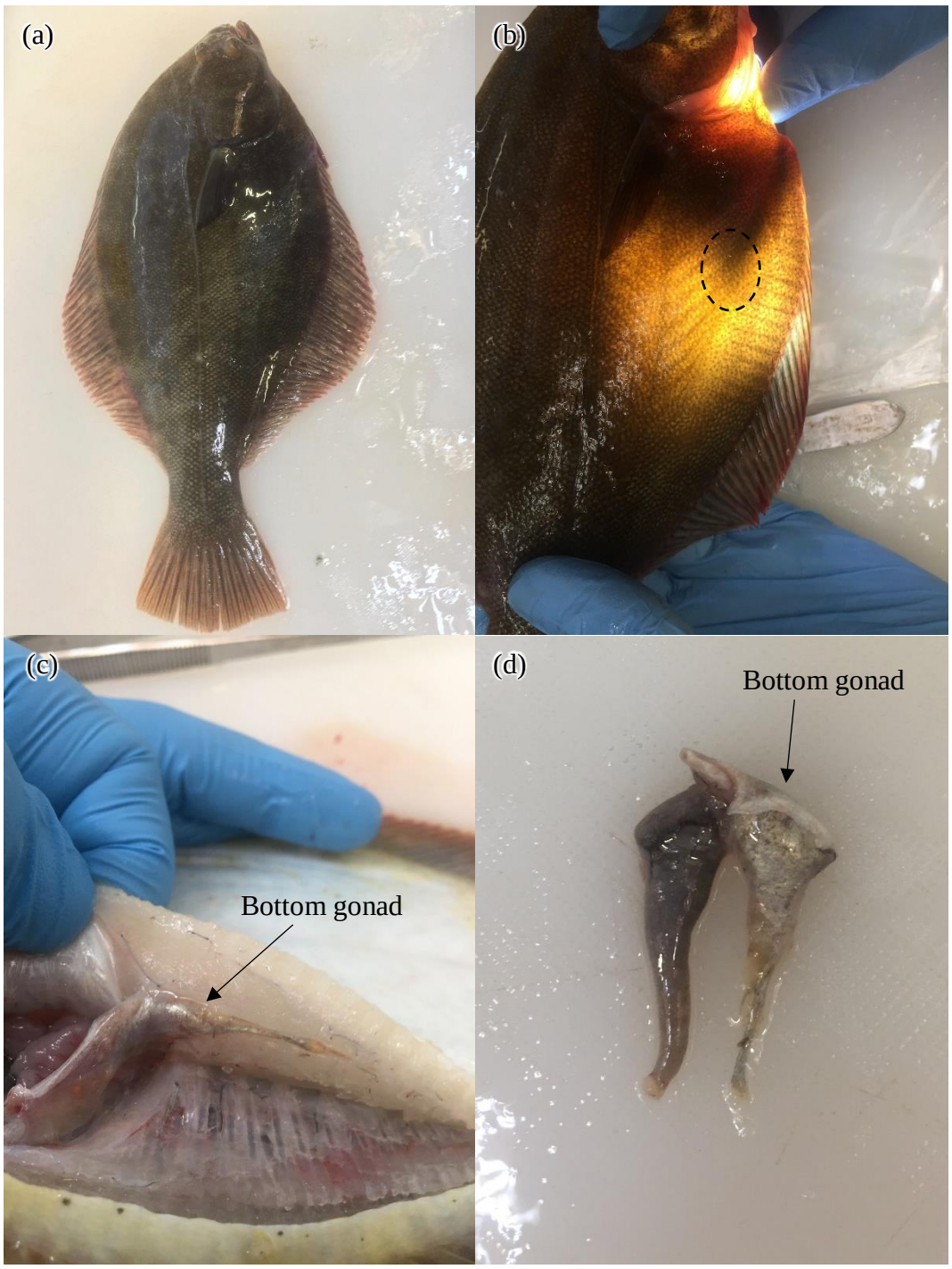

Figure 2.11: A female Yellow-Belly Flounder, *Rhombosolea leporina*, displaying evidence of abnormal gamete development. Uncertainties remain about maturity, fertility, and health of the individual. The female contained oogonia and chromatin nuclear oocytes. (a) overhead view; (b) overhead view of the body cavity and ventral side muscle, with backlighting. Dashed oval outlines the shadow from gonads; (c) gonad orientation on the underside of the individual; (d) the removed gonads.

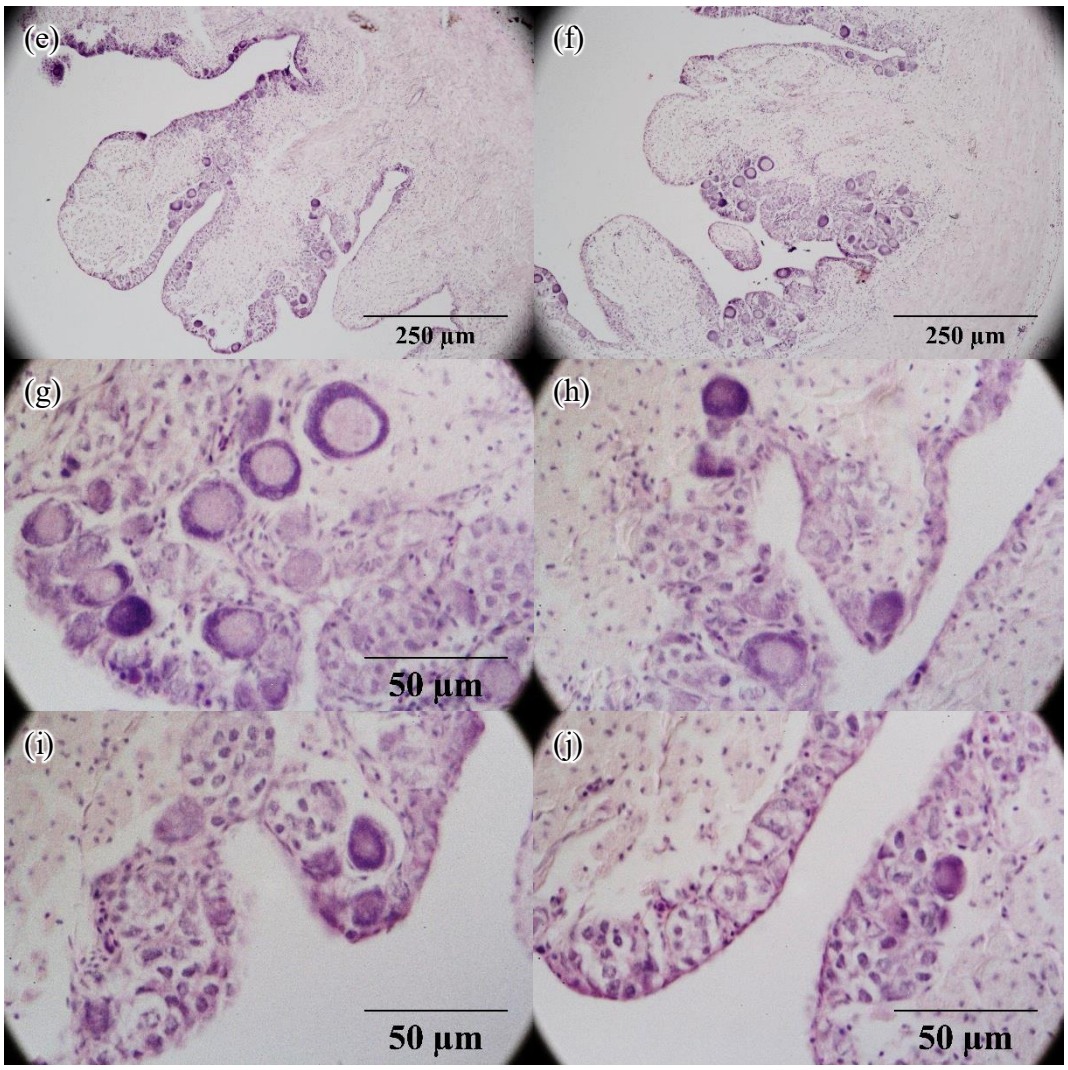

Figure 2.12: Individual female Yellow-Belly Flounder, *Rhombosolea leporina*, from previous Figure 2.11. Prepared histological sections, 7µm thick, of the above-stated female, displaying the absence of gametes within the gonad. (e – f) overviews of ovarian tissue; (g – j) magnified view of gamete organization from previous overview locations.

2.4 Discussion

This investigation characterizes the seasonal reproductive development of female *R. leporina*, based on I_G, I_H, oocyte diameter, and ovarian histology. Clear seasonal development of the ovaries is seen in *R. leporina* over the two-year investigation. Almost all individuals contained every less mature oocyte stage accompanying the LCO. In addition, there was also an increase in POFs during the protracted spawning season and apparent lack of a singular peak in I_G. This suggests that *R. leporina* display multiple group synchronous oocyte development.

Quiescence appears to occur in summer, between January and March, as stage I ovaries predominated, minimum values of I_G and I_H are observed, with air temperature rising to its peak, and as daylength is starting to decrease.

Ovarian recrudescence initiates in March and April when stage II and III females appear along with increases in I_G and I_H out of quiescent levels. This is when decreasing daylength and air temperatures are observed at the onset of autumn. These changing seasonal events may be transduced by the fish to cue reproduction through stimulation of the reproductive axis (DeVlaming, 1972; Crim, 1982; Bromage *et al*., 2001; Pankhurst & Porter, 2003).

The occurrence of key oocyte stages may provide some speculative insights into the timing of reproductive endocrine events in *R. leporina*. The formation of oocytes containing cortical alveoli (stage II) is known to be initiated by initial E_2 aromatization (Forsgren & Young, 2012) under FSH regulation in many teleosts (Nagahama 1994; Devlin & Nagahama, 2002; Lubzens *et al*. 2010; Brown-Peterson *et al*., 2011). It is, therefore, likely that in the Firth of Thames, around March and April indicates the seasonal activation of the pituitary leading to gonadotropin release in *R. leporina*. It appears that in this species, stage II females are closely associated with the appearance of females with stage III ovaries. Based on an extensive body of evidence in other species, this indicates the beginning of follicular steroidogenesis. It seems reasonable to expect that, although they were not measured in this study, plasma T and E_2 concentrations would be increasing in *R. leporina* at this time of year. E_2 induces the hepatic production of vitellogenin (Nagahama, 1994) and zona radiata proteins (Lubzens *et al*., 2010). These are released into the circulatory system and then subsequently sequestered by the oocytes (Tyler *et al*., 1991; Nagahama 1994; Devlin & Nagahama, 2002; Lubzens *et al*., 2010). This assumption is supported in *R. leporina* by the simultaneous

occurrence of oocytes containing vitellogenin granules and a thickened zona radiata in both March and April. This was observed in multiple fish at this time of year. This E_2 mediated increase in hepatic activity often shows as an increase in liver weight during vitellogenesis. This has been observed in European flounder *platzchtnys flesus* (Krosgaard-Emmersen & Emmersen, 1976), rainbow trout *Salmo gairdnerii* (Van Bohermen, Lambert, & Peute, 1981; Haux & Norberg, 1985) and American plaice *Hippoglossoides platessoides* (Maddock & Burton, 1999). Indeed, a significant increase in I_H was also observed in stage III *R. leporina*. This may further support the argument for E_2 activation of the liver. It is worth noting that in some species the liver is a major site of energy storage and which can also increase the hepatosomatic index (Lambert & Dutil, 1997). However, indicative evidence, histology and organosomatic indices indicate that spring seasonal cues appear to be important for activating the BPG in *R. leporina*. It is not possible though, to conclusively state the timing of key reproductive endocrine events in *R. leporina* without further assays of reproductive hormones.

Spawning appears to span from June, when the first ovulated female was observed close to the shortest day and approaching minimum air temperatures, through to December, when the last ovulated female was observed approaching peak air temperatures and maximum day length. Thus, *R. leporina* displays a protracted spawning season, spanning seven months. Although fish of various ovarian stages were observed during this time, fish with stage IV ovaries were only found between June to December, the same for ovaries containing POFs with LCOs of EVO – OV, evidence of the potential for batch spawning (Brown-Peterson *et al.*, 2011). This period also sees increases in the occurrence of stage V females with resorbing ovaries. The histological features of the leading cohort oocytes were characterized by some coalescence of the yolk granules and the off-center position of the nucleus. This is strongly indicative of final oocyte maturation and a switch from FSH to LH mediated signaling in the ovary (Kawauchi *et al.*, 1989; Nagahama, 1994; Devlin & Nagahama, 2002; Nagahama & Yamashita, 2008; Lubzens *et al.*, 2010). The production of the progestin, maturing inducing hormone (MIH) is stimulated in the granulosa cells by LH (Nagahama, 1994; Nagahama & Yamashita, 2008). The MIH is responsible for stimulating the completion of the first meiotic division and the expulsion of the first polar body out of the oocyte (Lubzens *et al.*, 2010). This is observed histologically as the nucleus migrates toward the oocyte periphery and

subsequently breaks down. The majority of fish with stage IV ovaries were found between August and October, indicating that peak spawning may be at this time. Further evidence is provided by the occurrence of maximum I_G values followed by a subsequent drop. This also corresponds to a significant decrease in I_H, which also occurs at the time of oocyte maturation in European flounder *Platzchtnys flesus* (Korsgaard-Emmersen & Emmersen, 1976). However, this may also indicate the cessation of feeding during spawning in flatfish (Maddock & Burton, 1999). This general pattern of late winter-spring spawning and summer quiescence supports the findings of previous reproductive studies in *R. leporina* (Colman 1973; Webb 1973; Mutoro 2001). The pattern of autumnal recrudescence, followed by spring spawning is similar to other Australasian flatfish such as turbot *Colistium nudipinnis*, brill *C. guntheri* (Poortenaar *et al.*, 2001), and greenback flounder *R. tapirina* (Barnett & Pankhurst, 1999). The closely related sand flounder *R. plebeia* has a similarly protracted spawning season, from June to November, when water temperatures are approaching near minimums (12 - 13ºC) (Colman, 1973). In his study, Colman (1973) thought that *R. plebeia* had an earlier spawning season than *R. leporina*, however, while this cannot be substantiated in this study, ovulated *R. leporina* were certainly found as early as June. Nonetheless, female *R. leporina* displays a clear seasonal pattern of reproduction with apparent multiple group synchronous ovarian development and the possibility of batch spawning.

While the peak of spawning appears to be consistent across *R. leporina* populations in New Zealand, there are potential latitudinal variations amongst reproductive duration in populations. *R. leporina* appear to display an inverse relationship between spawning duration and latitude, as evidence of spawning is found in this study, from June to December in the Firth of Thames (lat. 37ºS), while in the Avon-Heathcote estuary (lat. 43.5ºS) evidence of imminent spawning was only seen from July to October (Webb, 1973). The apparent decreased spawning duration may be a factor of the reduced water temperatures at higher latitudes, as *R. leporina* appear to cue the peak of spawning on warming temperatures (Colman, 1973), which is similar in the timing of peak spawning to air temperature in this investigation. In contrast, *R. plebeia* appear to display a positive relationship between spawning duration and latitude, as higher latitudes were found to maintain development and some spawning outside of the main season. *R. plebeia* are found to spawning from June to November in the Hauraki Gulf (Colman, 1973), imminent of spawning from

June to September and November to February in the Avon-Heathcote estuary (Webb, 1973), and peak spawning from winter to spring with lower amounts occurring the rest of the year in South Otago (lat. 45 – 46ºS) and is suggested to be temperature related (Setyono, 2005). The timing of reproduction might also have some relationship to the rate of change in day length across latitudes, which might play a role in accelerating or organizing the timing of spawning of *R. leporina*. Other flatfish have showed changes in timing of their spawning with adjusted seasonal daylengths: turbot *Scophthalmus maximus* (Imsland, Folkvord, Jónsdóttir, & Stefansson, 1997), summer flounder *Paralichthys dentatus* (Watanabe, Ellis, Elis, & Feeley, 1998), and Atlantic halibut *Hippoglossus hippoglossus*, which also delayed spawning when seasonal daylength was expanded beyond one year (Björnsson, Halldorsson, Haux, Norberg, & Brown, 1998). These introduce the possible importance that temperature or other latitudinally changing variable may have on reproductive development in *R. leporina*. Thus, there appears to be a latitudinally related variables at play, presumably temperature and potentially day length, which might be seasonal cues to reproductive timing in *R. leporina*.

Temperature may also be driving a secondary regulation on reproduction through growth and metabolism. The European flounder *Platichthys flesus* feeds during the summer, in the highly productive shallow waters, while in reproductive quiescence to restocking energy reserves for later ovarian recrudescence in autumn and winter (Korsgaard-Emmersen & Emmersen, 1976; Fonds, Cronie, Vethaak, & Van der Puyl, 1992). *R. leporina* is stated to increase their numbers in the shallows during summer months (Colman, 1973) and this current investigation found *R. leporina* to be in quiescence through summer. Thus, its most likely that *R. leporina* restocks energy reserves during quiescent summers for their oncoming recrudescence in autumn. This is further supported by the *R. leporina* congener *R. plebeia* in Canterbury waters, as maximum growth is seen in summer (Mundy, 1968). Interestingly, there might be some cogradient variation in relation to food availability, growth, and energy reserves affecting the reproductive development in female *R. leporina*. Differences in size and age at first sexual maturity across the North and South Island are apparent in *R. leporina*, being sexually mature at 230 – 270 mm (Colman, 1972) at about two years of age (Colman, 1974b) and at 260 mm in the end of their fourth year (Webb, 1973) respectively. The same trend also appears to be displayed in *R. plebeia* (Mundy, 1968; Colman, 1972; 1974b; Webb,

1973). It, therefore, appears that *R. leporina* restocks energy reserves during the productive months preceding the autumn gonadal recrudescence and may have some geographical variation in reaching sexual maturity.

Investigations into reproduction of *R. leporina* have also revealed that there is an apparent mature female migration to deeper waters to spawn in the Hauraki Gulf (Colman, 1973; 1974a) which also appears to be evident in the Avon Heathcote estuary (Webb, 1973). This seasonal migration of spawning has also been reported in the sand flounder *R. plebeia* (Mundy, 1968; Colman, 1973; Webb, 1973) and is presumed in greenback flounder *R. tapirina* (Crawford, 1984). Furthermore, mature female *R. tapirina* were seldom collected but were caught at their highest density in deep waters, which led Crawford (1984) to presume that duration of FOM to spawning is rapid. This might also be the case for *R. leporina*, as they are known to migrate to spawning grounds in the Hauraki Gulf (Colman, 1973; 1974a), which may be why there were the limited number of stage IV females collected from the shallows in this investigation. Thus, *R. leporina* may progress through FOM rapidly or only in deeper waters.

The backlighting of female *R. leporina* proved to be advantageous in the sexing and inferring the condition of the individual. The methodology to determine sex and gonadal condition were based on field observation as well as previously macroscopic descriptions for *R. leporina* (Colman, 1973; Webb, 1973). Similar methods to determine sex was used in a study of the congener greenback flounder *R. tapirina* (Sun & Pankhurst, 2004). Thus, backlighting fish is a comparatively reliable field technique to assess sex and gonadal condition in *R. leporina*.

The ambiguous cellular organization of the gonad in the individual considered to have uncertain maturity provides some evidence to speculate from. The individual appeared to lack the density of perinucleus oocytes as seen in other adult females of similar size in this investigation. The dab *Limanda limanda* also displayed similar lack of perinucleus oocytes in intersex (ovotestis) individuals (Stentiford & Feist, 2005). In contrast, the *R. leporina* individual is only observed to contain oocytes and could not confirm the presence of male reproductive cells. It was suggested that endocrine disrupting chemicals might have played a part in the observed intersex of *L. limanda* (Stentiford & Feist, 2005). While a pathological study of *R. leporina* observed myomas and high levels of atresia, there were no similarities to the observations from this investigation nor suggestion of the

influence of endocrine disrupting chemicals (Nenadic, 1998). This ambiguous observation of uncertain maturity in one individual *R. leporina* may suggest the potential influence of pollutants on the reproductive development. Thus, further investigation may be warranted if more observations are seen.

R. leporina displays clear seasonal patterns of ovarian development with recrudescence initiating in autumn and leading to a protracted spawning period covering seven months from winter until early summer. This investigation shows that *R. leporina* have multiple group synchronous ovarian development. It also provides strong evidence for the first time, that they are batch spawning within a single season. It is unknown as to whether this reflects repeat spawning activity of individuals over the entire length of the seven-month season. It may be that individuals start and finish spawning at different times during the season.

Chapter 3

Sexual differentiation in juvenile *Rhombosolea* spp.

3 Sexual differentiation in juvenile *Rhombosolea* spp.

3.1 Introduction

The growing production and interest in aquaculture appears to be focused on the increasing global population's need for a reliable food source (FAO, 2018). New Zealand flounder is a sought-after fish, being the 8th most harvested fish in a recreational panel survey during 2011 – 2012 (Wynne-Jones *et al*., 2014) and commercially landing 2464 tonnes in the 2014 – 2015 fishing season (MPI, 2016). Dimorphic growth has been identified in females of both yellowbelly flounder *Rhombosolea leporina* and sand flounder *Rhombosolea plebeia*, growing faster and larger than their male counterparts (Colman, 1972). As fast growth is important for production efficiency, the aquaculture industry has an interest in monosex stock creation with species that have sexually dimorphic growth. This can only be achieved with an understanding of the timing of sex differentiation. Sex in some flatfish is environmentally sensitive and may be influenced by factors such as temperature. These effects are expressed during sex differentiation. There is currently no information regarding environmentally sensitive sex determination in New Zealand flatfish. Complemented by the previous investigation in **Chapter 2**, understanding the gonadal development and size at sex differentiation in juvenile *R. leporina* presents a valuable asset of knowledge for both captive production and wild stock management.

Fish are a vastly diverse group of vertebrates, especially when it comes to reproduction. In general, the majority of teleosts are gonochoristic, where sexual differentiation yields male or female phenotype for the remainder of the lifecycle (Patzner, 2008). In gonochoristic fish, sexual determination is described by Penman and Piferrer (2008) as "the genetic or environmental process by which the sex (gender, male or female) of an individual is established in a simple binary fate decision." The sexual determination may be defined through genetics at fertilization, social interactions with others, or with environmental factors (Devlin and Nagahama, 2002). In genotypic sex determination (GSD) the sex is defined at fertilization from a set of sexually distinct genes provided from each parent, while

environmental sex determination (ESD) is defined after fertilization from the environment experienced and where genes of the sexes have little consistency (Penman and Piferrer, 2008). GSD is possibly polygenically controlled or by dominant highly evolved sex chromosomes, allowing for either heterogametic males or females in monofactorial systems or heterogameity of both sexes in multifactorial systems (Devlin and Nagahama, 2002; Penman and Piferrer, 2009), either of which initiates the sexual development cascade. ESD, on the other hand, is operated through changes to protein formation and biochemical processing, which regulate sexual development (Devlin and Nagahama, 2002), and may be controlled through temperature, social interactions, and pH (Luckenbach *et al*., 2009). While these determination methods are described as separate mechanisms, some species use a combination of the two classic determination modes, such as the Atlantic silverside *Menidia menidia* (Lagomarsino and Conover, 1993), European sea bass *Dicentrarchus labrax* (Vandeputte *et al*., 2007), and Pejerrey *Odontesthes bonariensis* (Yamamoto *et al*., 2014; Zhang *et al*., 2018). This use of both determining mechanisms also appears to include the flatfish, as environmental factors appear to influence the XX genotype (Luckenbach *et al*., 2009). Some flatfish that are known to express environmentally influenced GSD, such as Japanese flounder *Paralichthys olivaceus* (Yamamoto, 1999; Wang *et al*., 2017) and southern flounder *Paralichthys lethostigma* (Luckenbach *et al*., 2003), show male bias at high and low temperatures, while only suspected minor TSD influence on GSD is reported in turbot *Scophthalmus maximus* (Haffray *et al*., 2009). Identifying flatfish with the combination of GSD and ESD may be of high importance for management as there is a good chance that estuarine species experience significant variations in environmental variables and may lead to sex reversal in wild stocks (Luckenbach *et al*., 2009). This may be especially relevant in *R. leporina* as they have such a protracted spawning period at the community level. This provides just some of the variety in sexual determination that fishes have developed to suit their environment, habitat, and or population. However, these initial experiences which will initiate the cascade of development to the ultimate sex of an individual, are the most common for flatfish.

Reproduction is a highly endogenously regulated process in teleosts, including early determination and differentiation of the gonad. Sexual determination initiates the cascade of gene regulation in the sexual differentiation process to form initial male

testes or female ovaries (Devlin and Nagahama, 2002; Piferrer and Guiguen, 2008). This includes embryonic derived PGCs migration to the site of the primordial gonad anlage (Hamaguchi, 1982; Molyneaux and Wylie, 2004). This migration of PGCs is highly endocrine-regulated, by various and some species-specific, chemicals (Gurerrero-Estevez and Moreno-Mendoza, 2010). The path of the PGCs, shift along the peritoneal wall ventrally from the dorsal mesentery and upon arrival to the anlage, become surrounded by somatic cells, undergo proliferation, and develop into meiotic germ cells (Nakamura and Takahashi, 1973). Around this time of sexual differentiation of the gonad, the first signs of the endogenous endocrine regulation of gonadal development are observed (Nakamura *et al*., 1998). This precedes the cytological, histological, or morphological identifications of sexual differentiation and the appearance of steroid-producing cells and their physiologically present sex dominant steroids can help to identify the initial sexual differentiation (Devlin and Nagahama, 2002). However, these studies into the endocrinology of histologically undifferentiated teleost are challenging, and histological studies can supply adequate accuracy of observing initial gonadal differentiation for both sexes (Nakamura *et al*., 1998). While the endogenous regulation of sexual differentiation might appear to be a polished system, its complexity allows for possible disruptions to its process along the way (Devlin and Nagahama, 2002).

While species usually adhere to the criteria of these modes of sexual determination and differentiation, these processes are highly plastic, "flexible", in such that other environmental factors including temperature and pH, social conditions, and physiological factors, could affect the ultimate sex of the individual (Nakamura *et al*., 1998; Devlin and Nagahama, 2002; Guerrero-Estevez and Moreno-Mendoza, 2010). This plasticity has been used to artificially manipulate sex in fish, to create sex-reversed broodstock fish and ultimately monosex stocks of fish. Inducing sex reversal in fish requires the right timing of treatment (during physiological sex differentiation of the gonad), duration of treatment, and dose (Nakamura *et al*., 1998). While this pertains to hormone-induced sex reversal, which is just one method to adjust sex in fish, it illustrates the importance of understanding the time of sexual differentiation to ensure successful sex adjustment.

There is currently no literature available on sex differentiation in *Rhombosolea* spp. Previous work provides evidence for adult sexual maturity (Colman, 1972),

spawning biology and fecundity (Colman, 1973), adult movements (Colman, 1974a), and growth (Colman, 1974b) of both *R. plebeia* and *R. leporina*. These works provided fundamental insights into the biology of these two species and identifying potential sexual dimorphic size differences in both species (Colman, 1974b). *R. plebeia* and *R. leporina*, both displayed quicker growth rates than their male counterparts and appeared to reach different maximum lengths over their lifespan (Colman, 1974b). It would, therefore, be valuable to know the size of fish at sex differentiation. This would provide a better understanding of how and if environmental conditions affect the sex of wild and captive stocks.

3.2 Methods

3.2.1 Juvenile collection

Collection of *Rhombosolea* spp. juveniles occurred at the mud flats in Miranda, New Zealand (-37°08'47"S 175°18'18"N) in September 2017 (**Figure 3.1**). Two 30-meter dragnets were utilized to collect the juveniles, 2.5 mm and 114 mm sized mesh nets respectively.

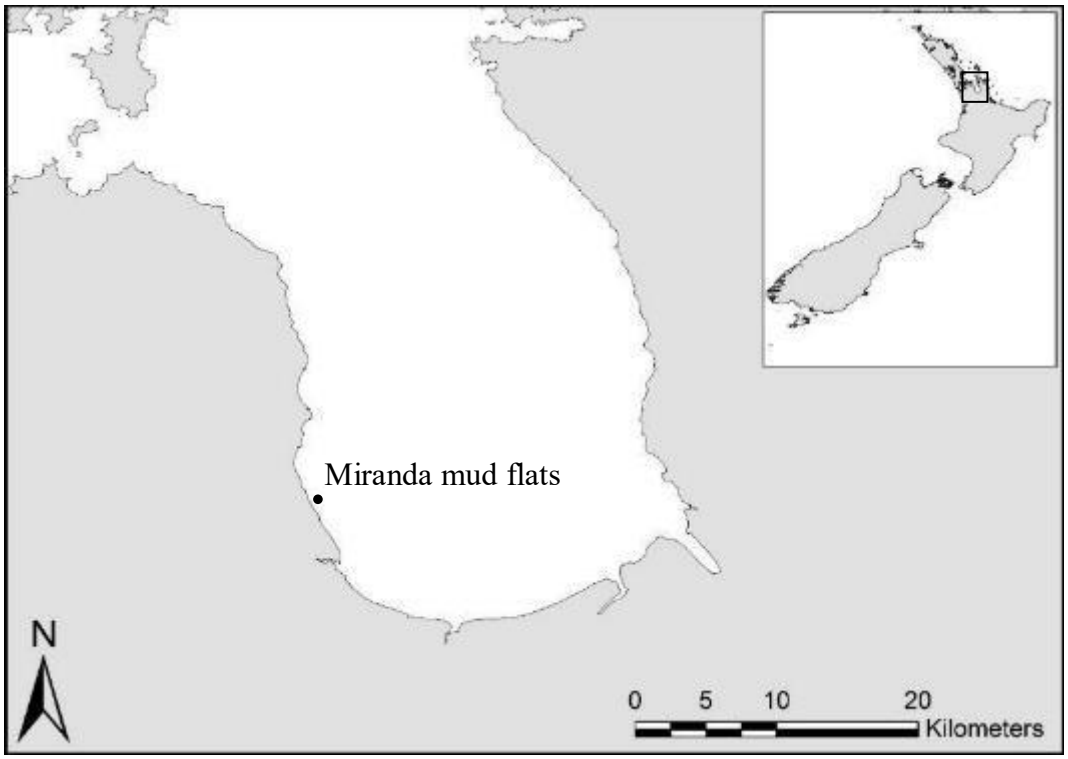

Figure 3.1: Collection site of juvenile *Rhombosolea* spp. Juvenile collection with recreational drag netting methods from the Miranda mudflats (-37°08'47"S 175°18'18"N) in September 2017.

The juvenile *Rhombosolea* spp. were transported live, in buckets filled with either filtered tanked saltwater or ambient water from the point of collection, back to the facilities at The University of Waikato, Coastal Marine Field Station, Sulphur Point, Tauranga, New Zealand 3110. At the facilities, the juveniles were held in 50-liter glass fish tanks filled with ambient seawater, collected at high tide from the Tauranga boat ramps used in **Chapter 2**. The tanks are maintained with a mechanical filter, > 50 % water changes every 12 hours, and were held in a

recirculating water bath held between 18º - 20ºC. The juveniles were sampled within 24 hours of arrival to the facilities.

An initial pilot investigation into juveniles > 80 mm total length (TL), showed all individuals containing gonads which sex was readily identifiable to the naked eye (not presented) by comparing gonadal silhouette to descriptions of gonadal shape from Webb (1973). Thus, the investigation in this chapter uses juvenile *Rhombosolea* spp. juveniles < 80 mm TL. Later on, lab measurements were then used to class fish into 10 mm TL size classes from 0 – 80 mm TL (**Table 3.1**).

Table 3.1: Number of juvenile *Rhombosolea* spp. sampled from each size range in this investigation.

Size range (TL; mm)	*n*
0.0 – 10.0	0
10.1 – 20.0	1
20.1 – 30.0	9
30.1 – 40.0	2
40.1 – 50.0	2
50.1 – 60.0	3
60.1 – 70.0	3
70.1 – 80.0	3
TOTAL	23

3.2.2 Species identification

Fish were sedated before identification, using a 5-liter tank of ambient seawater containing a dose of 2-phenoxyethanol (0.6 mlL^{-1}). Sufficient sedation is determined at the point where gill ventilation ceased for at least 1 minute and was assessed visually. At this point, the juveniles' species were presumptively identified only through analysis of the ray fins, following the guidelines of Eldon and Smith (1986) for *R. leporina* and *R. plebeia*.

The current investigation aimed to focus on the sexual differentiation of *R.* leporina, although, it became apparent that the different methods to identify between the two-congener species were not reliable to this investigation. The observations from the juveniles did not adhere sufficiently to the criteria provided in Eldon and Smith (1986) for ray fin morphology, ratio "snout" length to body length, body shape, or morphology of the premaxilla. Thus, there was no assurance of the accuracy of the distinction between the species. However, low numbers of juveniles did fit the ray

fin criteria of sand flounders and were therefore omitted. The remaining juveniles are used for the investigation, yet, are still presumed to be a combination of the *R. plebeia* and *R. leporina* and therefore referred to as juvenile *Rhombosolea* spp.

3.2.3 Dissection

After initial identification is completed, individuals' total weight (g) and total length (TL) to the nearest millimeter is recorded. The body weight is taken to the nearest 0.001 g (Mettler PJ360 DeltaRange scale, UOW: inventory: 32105). After the measurements are completed, the fish are swiftly dispatched. The juvenile is then portioned into blocks of fish which could allow for proper fixation, according to Mumford (2004), with Bouin's solution (Sigma-Aldrich®). These portions took into respect the zone of the body containing the developing gonad. For many of the larger individuals, the abdominal cavity was split into multiple sections. The tissue samples are fixed for 24 – 48 hours.

3.2.4 Histology

Juveniles were processed, sectioned, and analyzed, following the histological methodology in **Chapter 2**. However, a range of 3 – 10 µm thick sections were used for this investigation. The individuals are sectioned on one of the three different planes of fish: frontal plane, sagittal plane, or transverse plane (**Figure 3.2**).

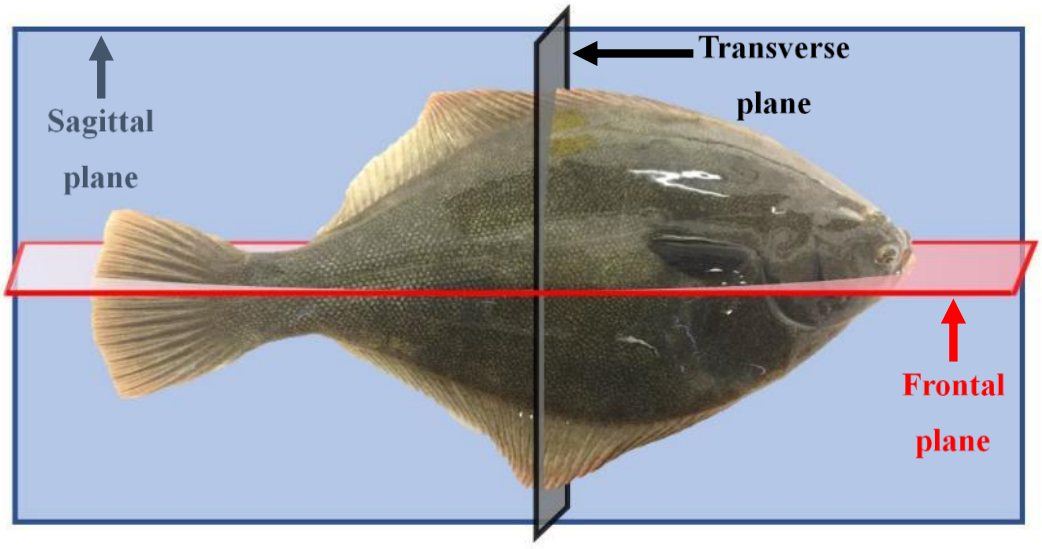

Figure 3.2: Anatomical planes of sections for yellowbelly flounder *R. leporina* histological sections.

Individuals were processed, sectioned, and analyzed from the largest to the smallest, working in one TL size class at a time. Sampling continued until both sexes are identified or juveniles of that size class were all sampled, before progression to the next smaller size class.

3.2.5 Gonadal cavities

Gonadal cavities were described as clear, partial, and irregular. Clear gonadal cavities undoubtedly contain a central cavity extending across the sectioned gonad. Partial gonadal cavities are not distinctly extending across the sectioned gonad yet remain centrally located. Irregular gonadal cavities are not distinctly extending across the sectioned gonad nor centrally located.

3.2.6 Digital measurements

Photos of prepared slides are taken through a microscope (Nikon Ni-U DIC light microscope) mounted digital camera (Canon 60D) and were processed in Image J (National Institute of Health; http://rsbweb.nih.gov/ij).

3.2.7 Male Reference/Differentiation Identification

Prepared histological sections of adult male *R. leporina*, provided from the collaboration with Toi Ohomai in **Chapter 2**, were used to aid identification of meiotic spermatocytes, spermatids, and spermatozoa in juvenile *Rhombosolea* spp. These two adults were 260 mm TL and 315 mm TL (**Figure 3.3a** and **b** respectively). Identification of male reproductive cells through sexual differentiation and spermatogenesis follows Schulz *et al*. (2010).

Female differentiated oogonia and oocytes were identified through descriptions obtained from previous investigations (Wallace & Selman, 1981; Le Menn *et al*., 2007).

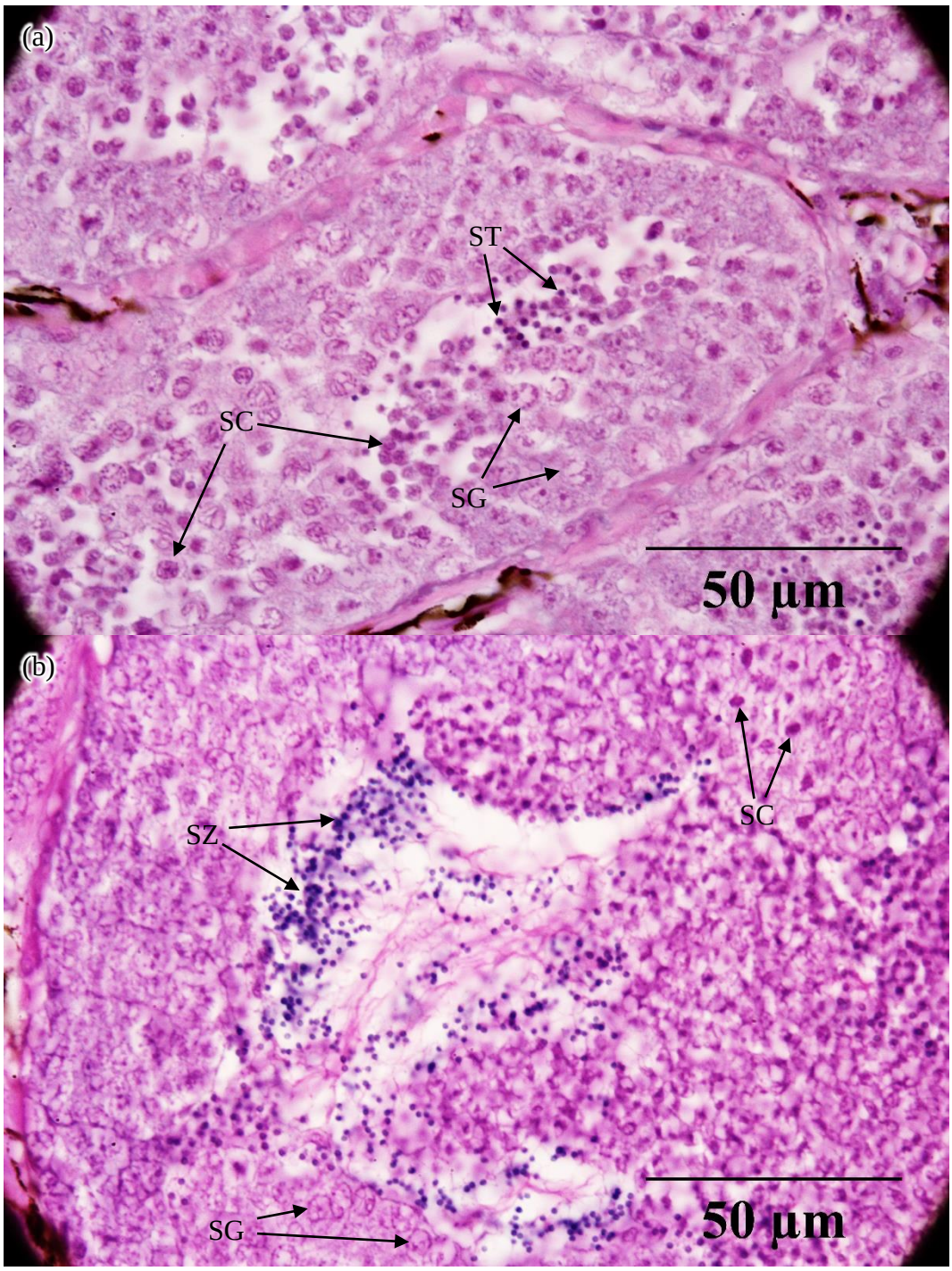

Figure 3.3: Photomicrographs of adult male *R. leporina* used for reference to identify meiotic male reproductive cells. (a) a 260 mm TL male containing spermatogonia (SG), spermatocytes (SC), and spermatids (ST); (b) a 315 mm TL male containing SG, SC, and spermatozoa (SZ).

3.3 Results

Gonads showed clear development and differentiation with increases in size (**Table 3.2**). The two gonads are separated by the ventral bones, which stem from the vertebrae and appear to protrude anteriorly into the caudal muscle from the abdominal cavity. It is not able to be determined if the protrusion is in part of the ventral bones, abdominal cavity, or concomitant of both. However, it is clear that final adult placement of the gonads is in the caudal muscle wall posterior to the abdominal cavity (CMPAC) and extending back into the caudal muscle, separated by the ventral bones, also seen in **Chapter 2** of this thesis. All individuals in the sample population were observed to have a developed gonad, located CMPAC.

The eye-side (ES) gonad appears to be larger in most individuals. However, for some individuals, both gonads were not observed. The ES gonad also appears to be further recessed anteriorly compared to the non-eye-side (NES) gonad, about the ventral bones. However, some individuals do not display with this characteristic, as the full extent of an elongated top gonad is visible (**Figure 3.6a**), or the orientation of the section along the sagittal plane is not allowing for this observation.

Most juveniles have pigmentation in the cortical gonadal tissue of the developed ES gonad (n = 16). Two juveniles showed pigmentation in the NES gonad, but to a far less extent, while the remaining could not have their gonad side determined due to histological orientation.

An observable clear gonadal cavity was seen in most of the juveniles (n = 14), while others contained partial gonadal cavities (n = 6) (**Figure 3.4**). Confirmed males did not have gonadal cavities but contain irregular gonadal cavities (n = 3).

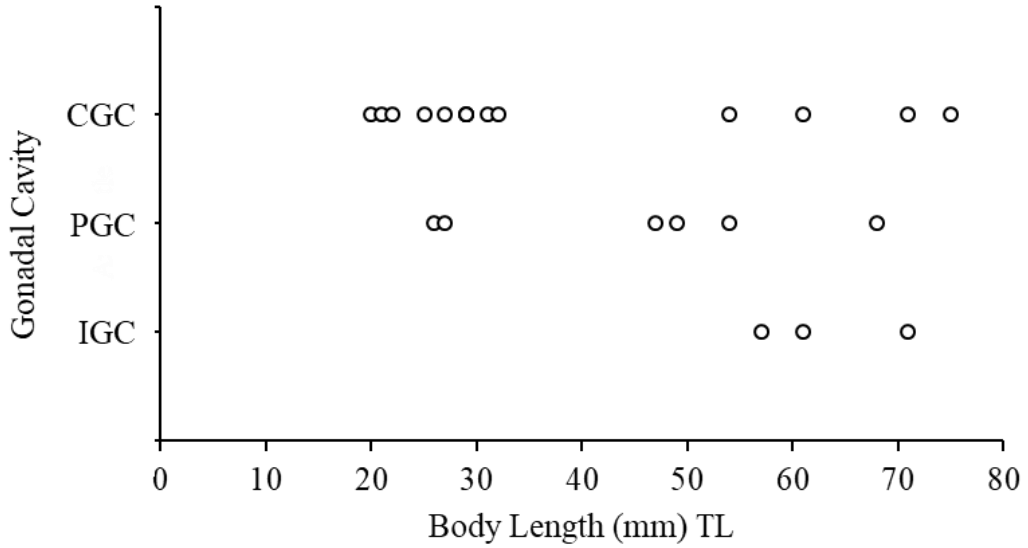

Figure 3.4: Schedule of gonadal cavity across body length (mm; TL). Gonadal cavities are IRL, irregular gonadal cavity; PGC, partial gonadal cavity; CGC, clear gonadal cavity.

The histological and sectioning protocols used, did not always provide positive identification of germ cells, oogonia, and spermatogonia. The thick (10 µm) sections did not provide sharp images for accurate identification of germ cells or initial differentiation into oogonia or spermatogonia. Therefore, sex was identified through the presence of meiotic oocytes or spermatocytes, for female and males respectively. Presumptively males were identified by lack of primary oocytes and difference in the cellular organization in comparison to similar sized differentiated females.

Table 3.2: Summary of histological events in the gonadal sex differentiation of R. leporina (adapted from Hendry *et al*., 2002).

L_{MT} (mm)	Stage in life cycle	Developmental stage	
		Female	Male
20.0	Post metamorphosis & settled	Gonads CMPAC and clear cavity in gonad	
26.0		Elongated gonad	
29.0 – 32.0		Gonad increasing in size (length and width) with clearly formed cavity and gonoduct.	
47.0	Female differentiation	Oogonial meiosis, identifiable primary oocytes	
49.0	Presumptive male		Clustering of presumptive spermatocytes in presumptive lobules.
54.0		Ovary increasing in size and number of lamellae.	
57.0	Male Differentiation		Meiotic proliferation of spermatocytes and possible spermatids. Presumptive efferent ducts also seen.
68.0		Primary oocytes increase in size and number.	
71.0			Spermatozoa present
75.0		Well defined lamellae and ovocoel.	

Caudal muscle wall posterior to the abdominal cavity (CMPAC)

At 20mm TL, a developed and migrated ES gonad are observed in the smallest post-metamorphic and settled juvenile (**Figure 3.5a**). Interstitial gonadal tissue consists of somatic cells and germ cells that could not be identified, thus are presumed undifferentiated. The gonad itself displays a large cavity in the interstitial gonadal tissue. The ES gonad is in the CMPAC with spots of pigmentation in the cortical tissue. The non-ES shows a cellular mass suspected of being the cortical tissue of the gonad in **Figure 3.5a** and with further sectioning confirmed the presence of the NES gonad (**Figure 3.5c**). The NES gonad is in line with the first ventral bone, however, appears to be in the CMPAC of the lower frontal plane.

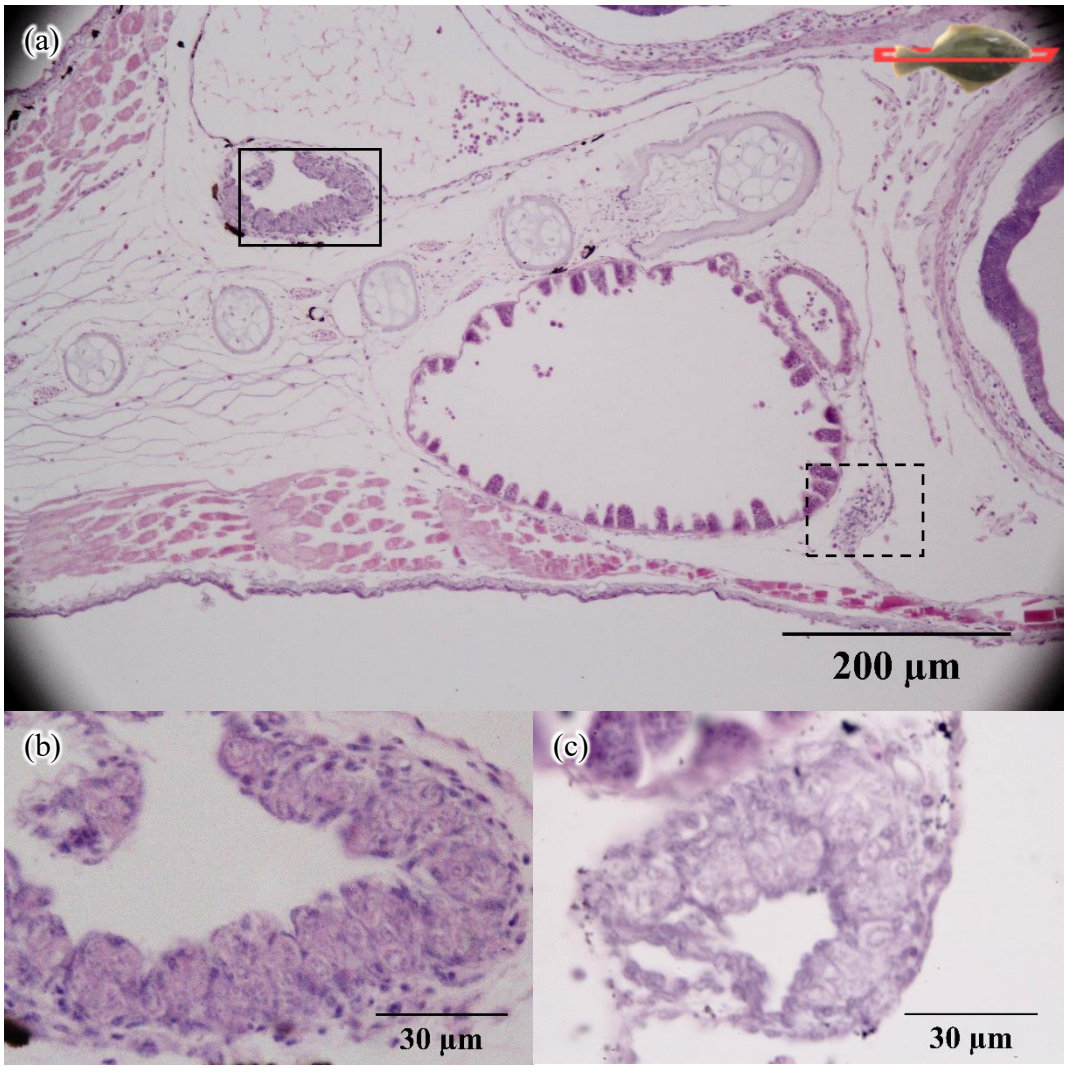

Figure 3.5: Photomicrographs of a juvenile *Rhombosolea* spp. at 20 mm TL with clear gonadal cavities. The sections are 5 µm thick and stained with standard H&E protocols. (a) posterior region on the body cavity and muscle tissue (10 µm). Boxes are denoting their respective magnified photos; (b) eye-side gonad (10 µm). Denoted in (a) by box (___); (c) non-eye-side gonad from further sectioning (5 µm). Location of gonad denoted in (a) by box (- - - -).

Ambiguously the ES gonad appears to be elongated anteriorly into the abdominal cavity or still developing posteriorly, in a 26 mm TL individual (**Figure 3.6**). The differentiation of germ cells still cannot be confidently identified. This individual has a partial gonadal cavity in the CMPAC located portions of the gonads.

The gonad is displaying clear connections to gonoducts in a 29 mm TL juvenile (**Figure 3.7**). Juveniles and gonads appear to concomitantly increase in size histologically, by length and width, from the initial 20 mm TL juvenile. The clear gonadal cavities are again apparent in juveniles from 29.0 – 32.0 mm TL, with some being exaggerated (**Figure 3.8**).

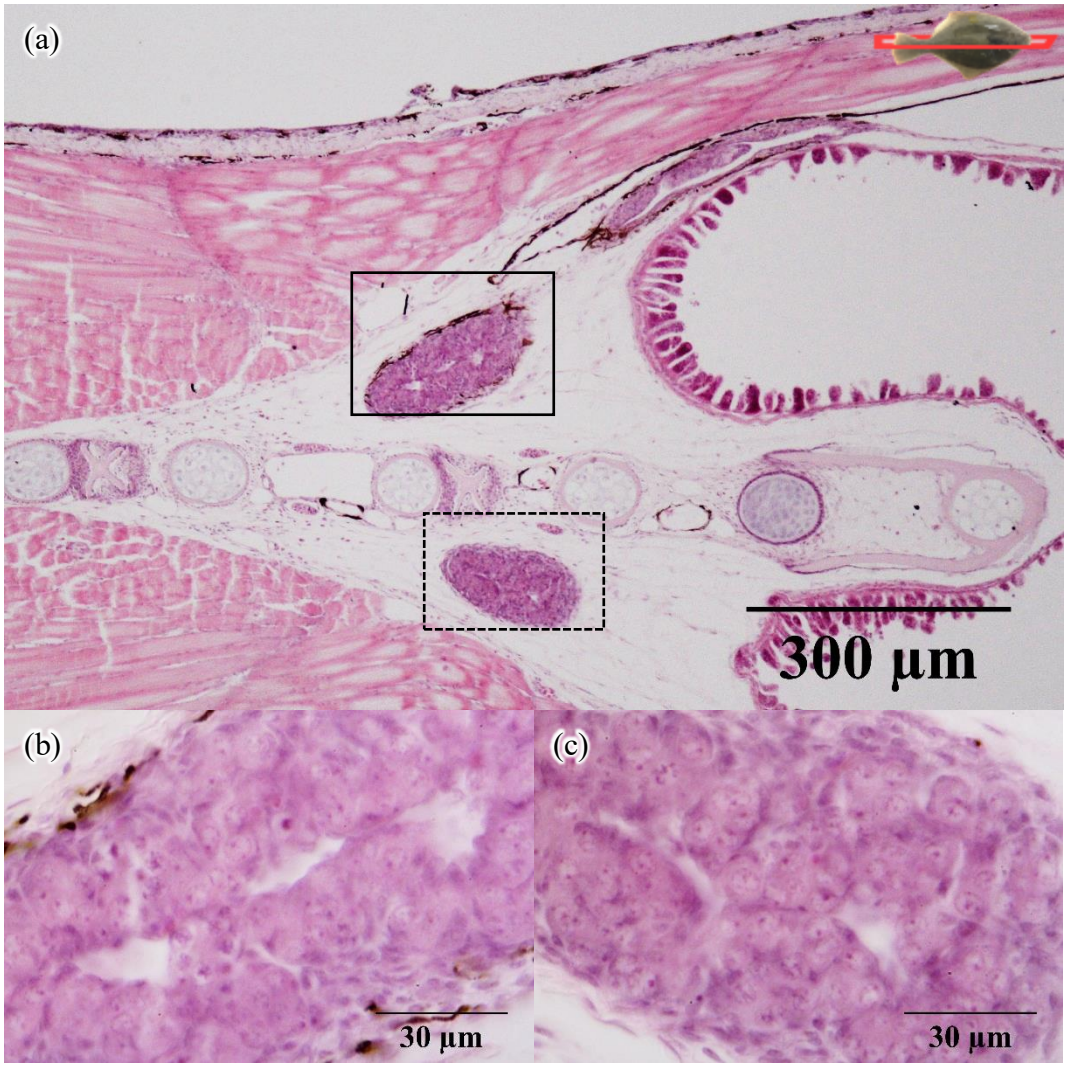

Figure 3.6: Photomicrographs of a juvenile *Rhombosolea* spp. at 26 mm TL with partial gonadal cavities. The sections are 10 µm thick and stained with standard H&E protocols. (a) posterior region on the body cavity and muscle tissue. Boxes are denoting their respective magnified photos; (b) eye-side gonad. Denoted in (a) by the box (___); (c) non-eye-side gonad. Denoted in (a) by the box (….).

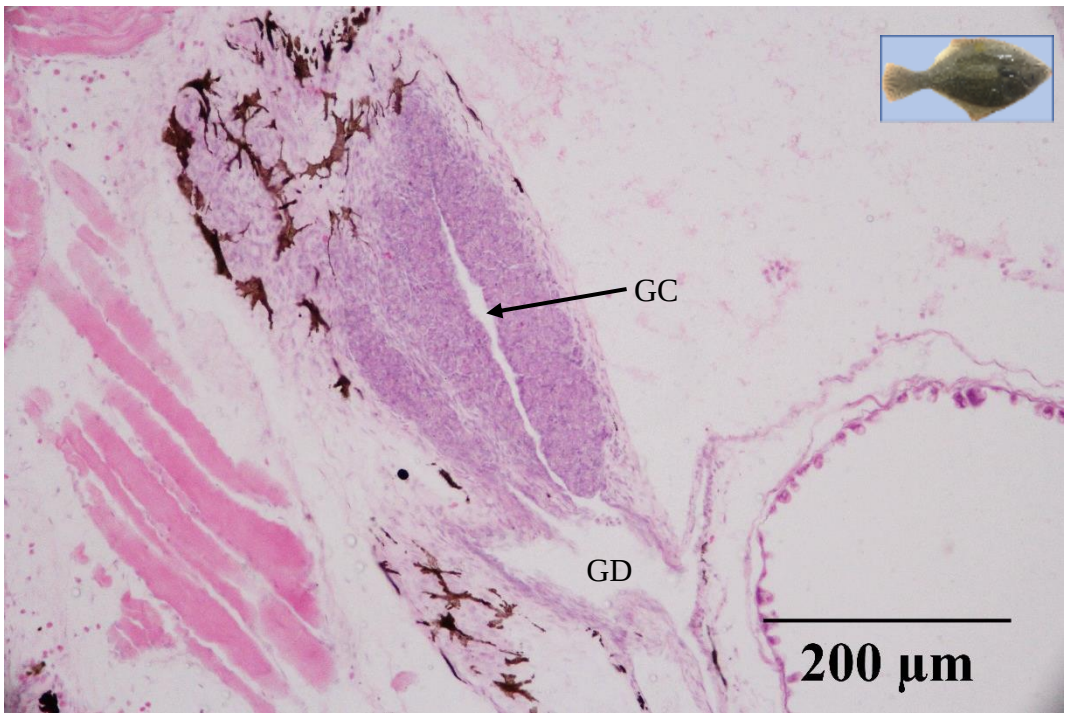

Figure 3.7: Photomicrograph of a 29 mm TL *Rhombosolea* spp. juvenile displaying apparent connection of the clear gonadal cavity (GC) to the gonoduct (GD) in the presumptive eye-side gonad (10 µm).

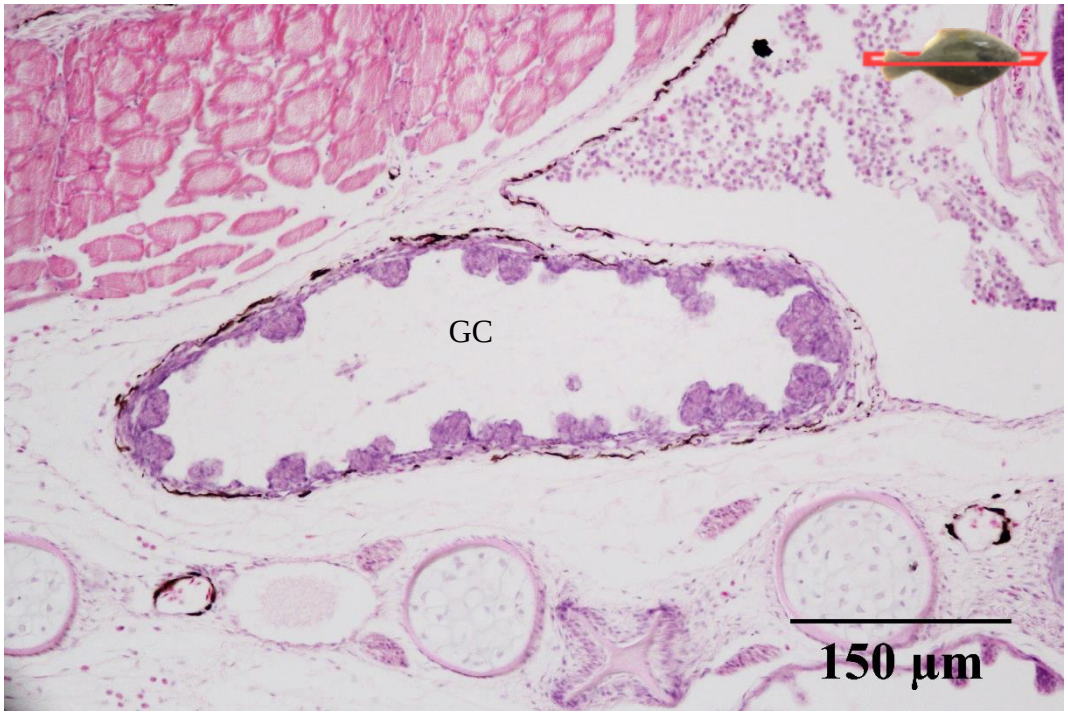

Figure 3.8: Photomicrograph of a 31 mm TL *Rhombosolea* spp. juvenile displaying ambiguously sized clear gonadal cavity (GC) in the eye-side gonad (10 µm).

The first confirmation of oogonial meiosis is seen in a 47 mm TL individual (**Figure 3.9a**). The NES ovary contains few early perinucleus oocytes, but no clear lamellae. The further increase in size (length and width) and amount of differentiation into primary oocytes, continues through females > 47 mm TL (**Figure 3.9b,c**). The largest individual, 75 mm TL, displayed increasing number and size of lamellae as the ovary continued to grow (**Figure 3.9d**).

The first presumptive differentiated male is a 49 mm TL individual, due to its clustering of presumptive spermatocytes (**Figure 3.10a,b**). The cells appear to be arranged into presumptive lobules, which did not resemble lamellae, but somewhat cube-shaped blocks of interstitial tissue. Adult male testicular structuring and various spermiogenic stages are presented in **Figure 3.3** and were used for comparison to identify lobule characteristics. Notably, this is the first individual not to display a continuous centralized clear gonadal cavity, which is more similar to the testis structure of larger confirmed males in this investigation.

An increase of presumptive lobules containing proliferated germ cells is seen in juveniles up to 61 mm TL (**Figure 3.10c**). The identification of the germ cells is still not possible. However, their stark contrast in internal gonadal structure and organization in relation to the identified meiotic females provides further evidence for their presumptive male status. Unusually large gonadal cavities are observed in some of the presumptive males (**Figure 3.9a, Figure 3.10a, Figure 3.11**). The peculiarly large cavities were not retained in the clearly meiotic males.

Clearly, differentiated meiotic males are seen in juveniles between 57 mm and 71 mm TL (**Figure 3.12**). These individuals are both undergoing spermiogenesis, with the latter undergoing development as present spermatozoa are observed. Space within the testes are not centered like in the other juveniles, but in contrast, contain space between the lobules instead.

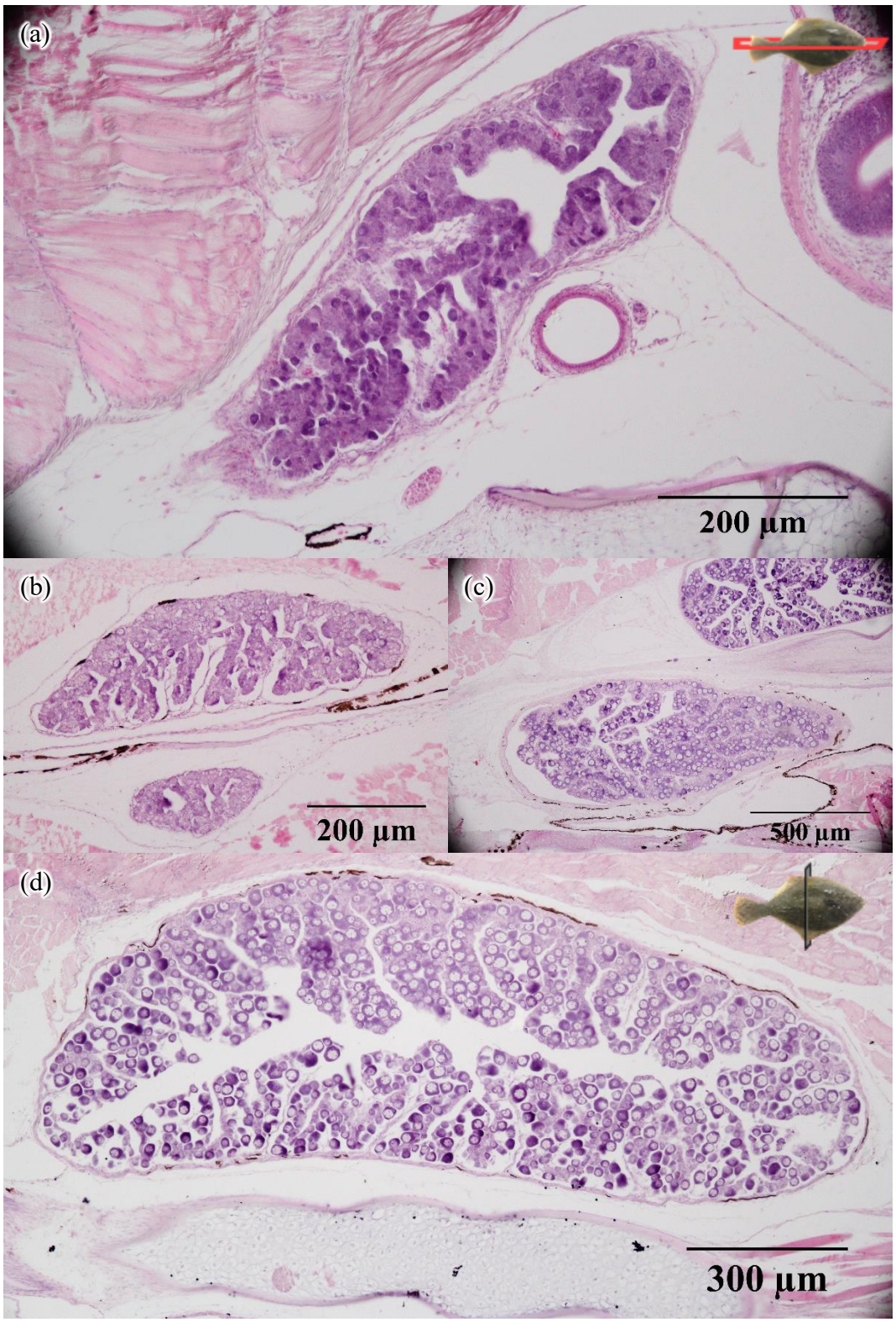

Figure 3.9: Photomicrographs of juvenile female *Rhombosolea* spp. containing meiotic primary oocytes. The sections are 10 µm thick and stained with standard H&E protocols. Partial gonadal cavities are seen in (a,b,c) while (d) displays a clear gonadal cavity. (a) a 47mm TL female's non-eye-side ovary containing few early perinucleus oocytes; (b) 54 mm TL female's ovaries with apparent lamellae. Sectioned through the transverse plane; (c) 68 mm TL female's ovaries displaying clear lamellae, increased number of primary oocytes, and growth of the overall ovary. Sectioned through the transverse plane.; (d) a 75 mm TL female's eye-side ovary containing well-defined lamellae, predominated by primary oocytes.

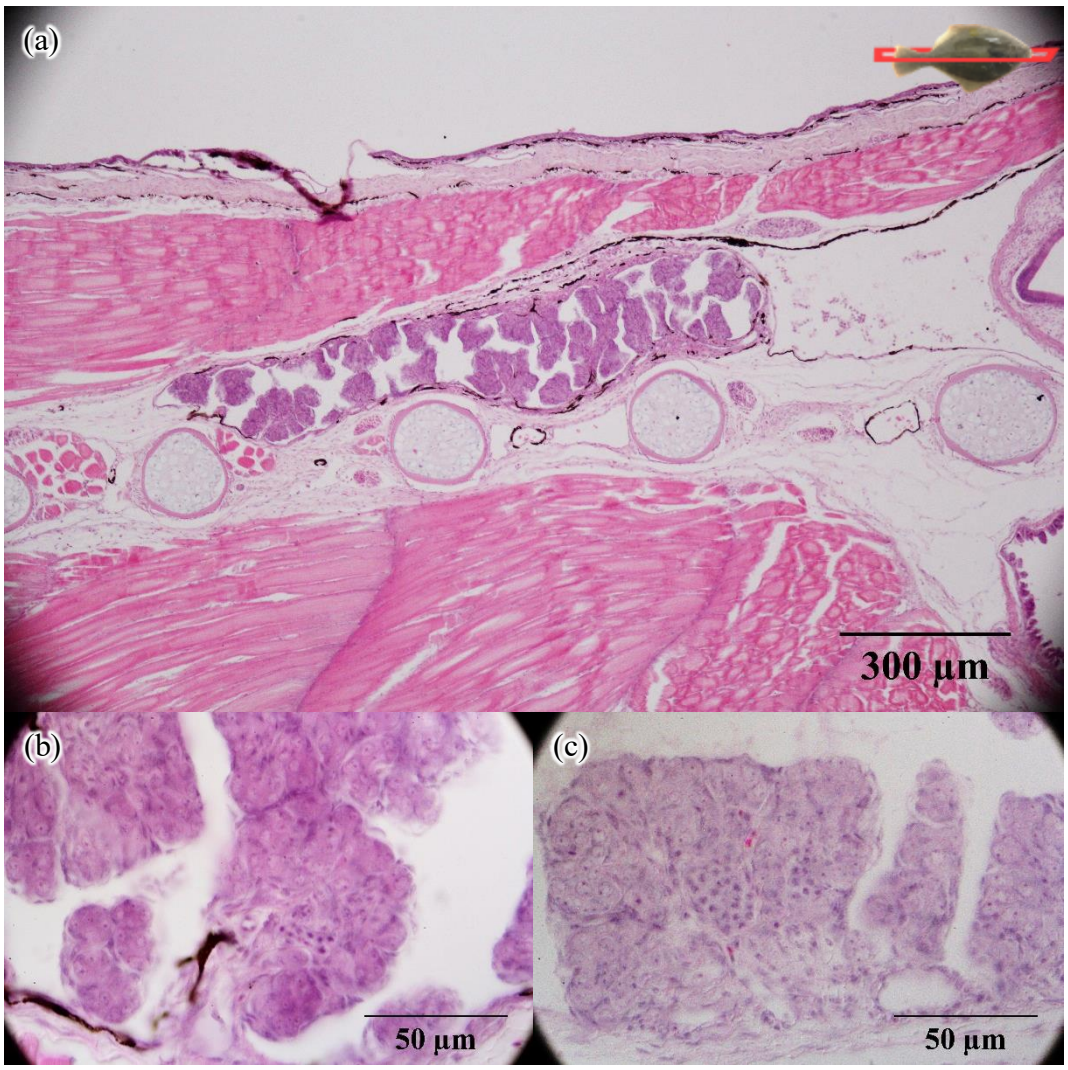

Figure 3.10: Photomicrographs of juvenile *Rhombosolea* spp. presumptive males containing clusters of gametes within cuboidal presumptive lobules. The sections are 10 µm thick and stained with standard H&E protocols. (a) a 49 mm TL individual showing the whole eye-side presumptive testis located CMPAC and an irregular gonadal cavity; (b) magnified view of the clustering of presumptive spermatocytes from the gonad in (a); (c) a 61 mm TL individual showing further clustering of gametes.

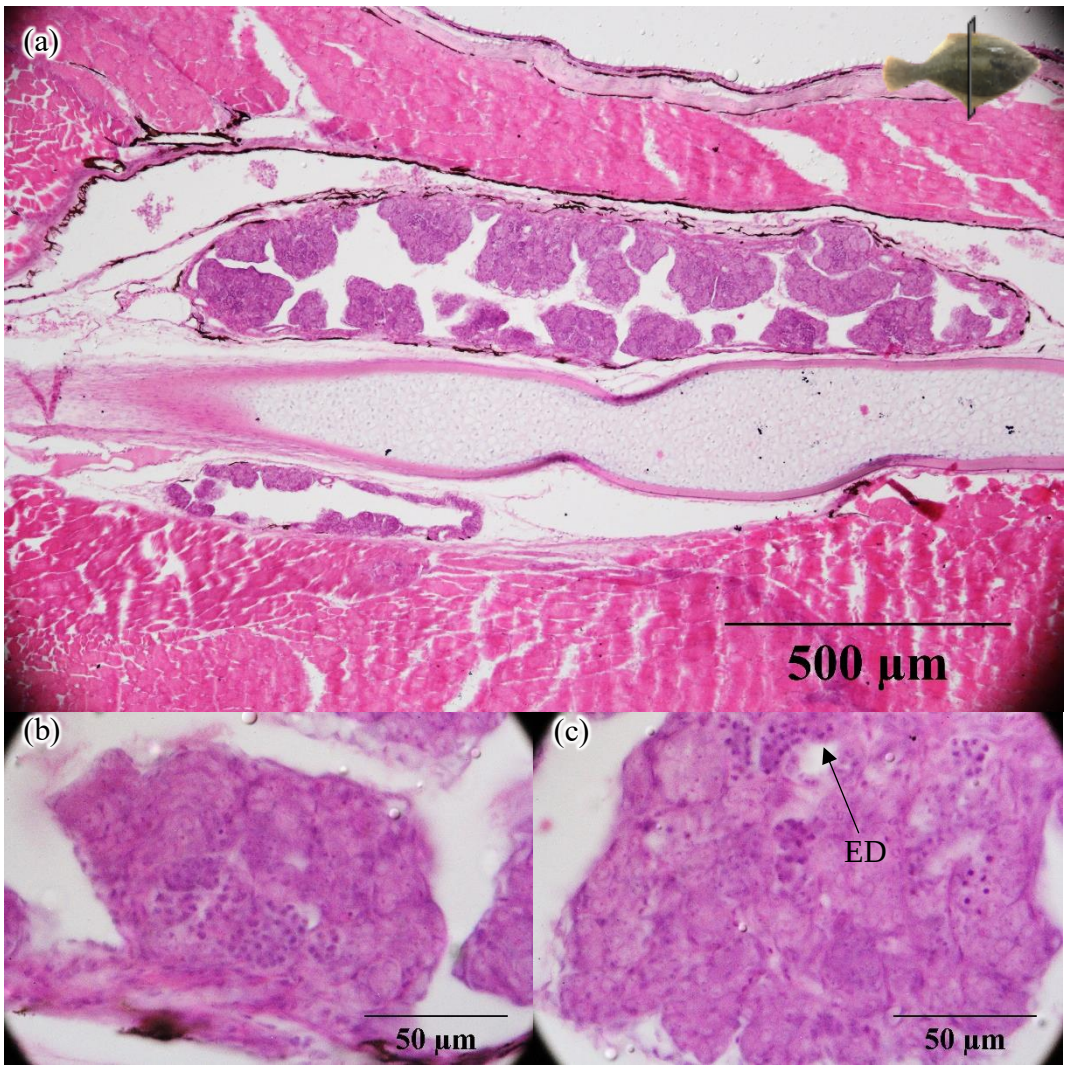

Figure 3.11: Photomicrographs of a *Rhombosolea* spp. juvenile presumptive male, 71 mm TL, containing clusters of gametes within cuboidal presumptive lobules and presumptive efferent ducts (ED). The sections are 10 µm thick and stained with standard H&E protocols. (a) placement of both presumptive testes CMPAC with irregular gonadal cavities; (b,c) magnified view of presumptive lobules of the eye-side presumptive testis in (a).

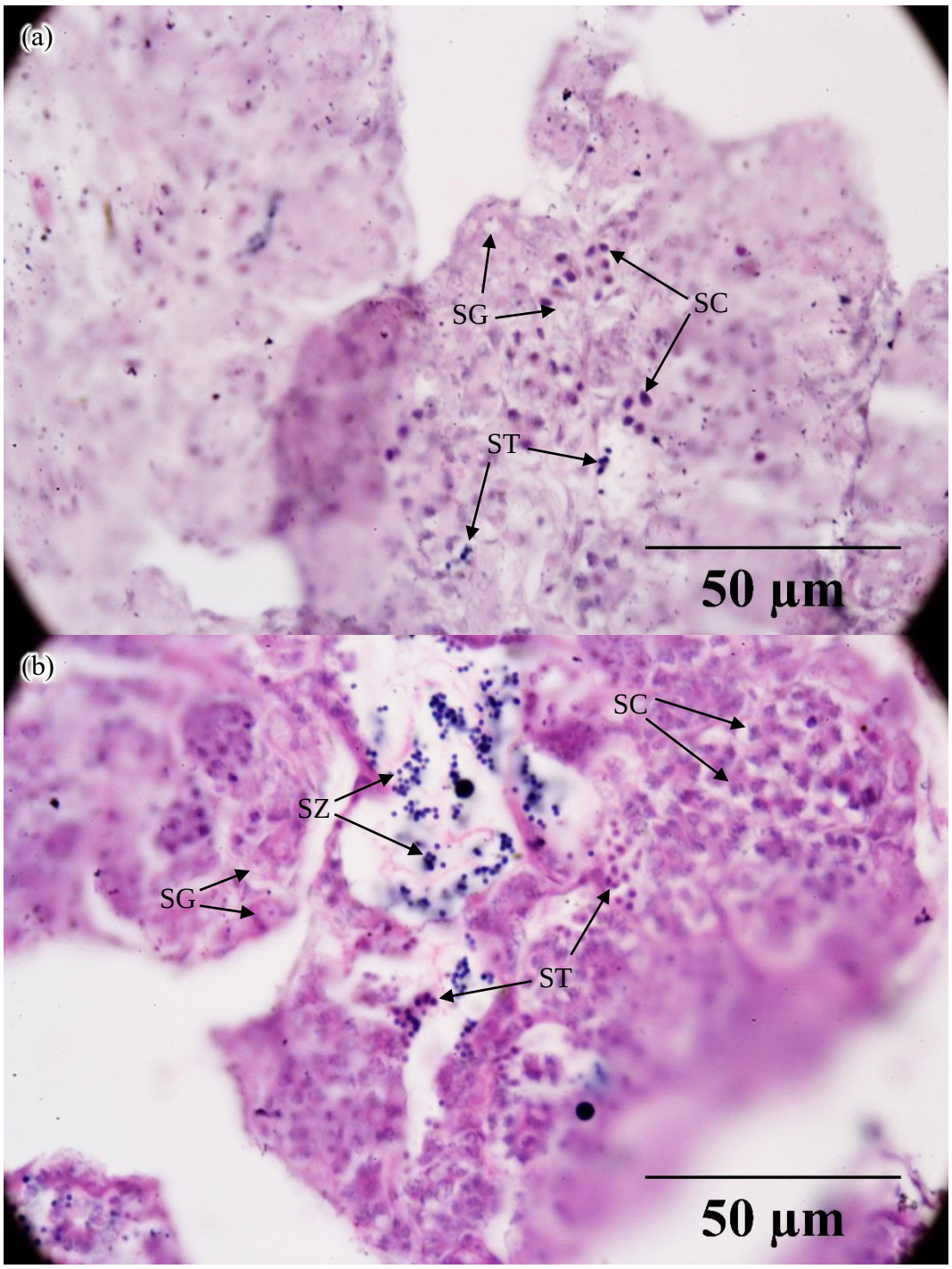

Figure 3.12: Photomicrographs of differentiated meiotic male *Rhombosolea* spp. undergoing spermiogenesis. (a) a 57 mm TL male with apparent spermatogonia (SG), spermatocytes (SC), and spermatids (ST); (b) a developing 71 mm TL male with SG, SC, ST, and spermatozoa (SZ).

3.4 Discussion

This investigation observed evidence of clear sexual differentiation in recently settled juvenile *R. leporina*. Sexual differentiation was determined by the presence of meiotic oocytes and spermatocytes, in females at 47 mm TL and males at 57 mm TL respectively. Undifferentiated gonads were seen in individuals below 32 mm TL.

Clear gonadal cavities were present in most juveniles becoming less apparent with increasing body length. The partial gonadal cavities may be evidence of the formation of a gonadal cavity or an artifact of the histological process and section orientation. Characteristically, testes do not contain gonadal cavities (Nakamura *et al.*, 1998). However, the current investigation observed irregular gonadal cavities in confirmed males, having a sizable amount of space between internal gonadal tissue, some even displaying similarly sized cavities as the females in this investigation.

The ovarian cavity is an accepted criterion for the histological identification of female sexual differentiation (Nakamura *et al*., 1998), being used for mud dab *Limanda yokohama* (Suzuki *et al*., 1992), barfin flounder *Verasper moseri*, (Goto *et al*., 1999), winter flounder *Pseudopleuronectes americanus* (Fairchild *et al*., 2007), and chub mackerel *Scomber japonicus* (Kobayashi *et al*., 2011). In contrast, Luckenbach *et al*. (2003) removed the use of the ovarian cavity criterion to identify southern flounder *Paralichthys lethostigma* females, due to the unpredictable timing of the ovarian cavity formation. Some *P. lethostigma* females had ovarian cavities forming from 75 – 100 mm TL and perinucleus oocytes appearing from 115 mm TL, while others had meiotic oocytes and no discernible cavity (Luckenbach *et al*., 2003). Thus, it was considered that the ovarian cavity was not a reliable criterion of sex. Similarly, gonadal cavity formation in *Rhombosolea* spp. juveniles do not appear adequate for identification of female sexual differentiation, due to their regularity among both sexes. Thus, the appearance of gonadal cavities could not be determined as only being a part of the female initial gonadal development in the congener species juveniles.

Also, the southern flounder *Paralichthys lethostigma* did not always have ovarian cavities forming before the onset of oocyte meiosis, some had cavity formation at the time of meiosis, and others displayed no apparent cavities in ovaries with primary oocytes (Luckenbach *et al*., 2003). In contrast, *Rhombosolea* species

juveniles do not display a delayed cavity formation post meiosis, most of the juveniles appearing to have clear gonadal cavities before meiotic oocytes are evident. However, the formation of the gonadal cavity in *Rhombosolea* species juveniles also appears to be substantially larger in some individuals of similar size and becomes less discernible in larger individuals. The orientation of histological sectioning may have influenced the definition or appearance of these gonadal cavities. However, it does not seem reasonable that they are responsible for the observed large size of some gonadal cavities seen in this investigation.

The fact that all the juvenile gonads had some formed cavity could suggest that *Rhombosolea* spp. are in fact undifferentiated gonochorists, as defined by Yamamoto (1969). Undifferentiated gonochorist pertains to juvenile hermaphrodism, where all individuals initially develop gonads with oocytes before sex reversal and differentiation into males as seen in zebrafish *Danio rerio* (Maack and Segner, 2003). This seems unlikely, however, as ovarian differentiation with meiotic oocytes did not appear to occur at a significantly smaller size to testicular differentiation. Therefore, *Rhombosolea* spp. juveniles do not appear to follow a similar pattern of sex differentiation to the proto-ovary observed in zebrafish. Residual ovarian cavities were, however, still observable in juvenile hermaphroditic male testes of Sumatra barb *Barbus tetrazona tetrazona* (Takahashi and Shimizu, 1983). While these bear little resemblance to the cube-like shape of the early developing presumptive testes in *Rhombosolea* spp., the possible retention of a proto-ovarian cavity provides an intriguing alternative explanation.

The apparently ubiquitous presence of a gonadal cavity in juvenile *Rhombosolea* spp. warrants more robust investigations. This would be best achieved through the measurement of sexually dimorphic expression profiles of key genes involved in sex differentiation. Ijiri *et al*. (2008) showed that *aromatase* (*cyp19a1a*) and *foxl2* were expressed as early as five days post hatch (dph) in female Nile tilapia, *Oreochromis niloticus*. While *cyp19a1a* is directly responsible for the production of 17β-estradiol, *foxl2* appears to be an important regulator of *cyp19a1a*. In contrast, *dmrt1, anti Müllerian hormone* (*amh*), and *Sox9* all showed male-specific expression patterns in Nile tilapia with the latter two genes being important during the later stages of testicular differentiation. *Sox9* is expressed in the Sertoli cells during sex differentiation and *amh* is consistently upregulated in the testis where it is important for inhibiting differentiation of dividing germ cells (Ijiri *et al*. 2008;).

Therefore, the development of such species-specific molecular tools coupled with the histological approach used in this study would yield a precise description of sex differentiation in *Rhombosolea* spp.

Obvious histological evidence of sexual differentiation of *Rhombosolea* spp. juveniles is seen in females at 47 mm TL, before males at 49 mm TL – 57 mm TL. The chub mackerel *Scomber japonicus* displayed a similar minimal difference in size at sexual differentiation between females and males, 76.41 ± 3.87 mm TL and 78.11 ± 4.38 mm TL respectively (Kobayashi *et al*., 2011). However, differentiation of Senegalese sole, *Solea senegalensis,* displays a more substantial variation in the timing of differentiation of females, 33.31 ± 0.89 mm TL, to males, 44.50 ± 0.83 mm TL (Vinas *et al*., 2013). It is expected that female differentiation precedes males (Devlin and Nagahama, 2002), which is seen in this investigation. However, *Rhombosolea* spp. juvenile sexual differentiation is limited as germ cell differentiation and gonadal cavities were not adequate evidence for differentiation in this investigation. Additionally, the low sample size may play a role in the presented difference in timing of sexual differentiation between the sexes of *Rhombosolea* spp. juveniles.

The apparent size at sex differentiation of the *Rhombosolea* spp. appears to range between that of some other flatfish species. For example, the 47 – 49 mm sexual differentiation size window found in this study is similar to winter flounder *Pseudopleuronectes americanus,* ≥ 41 mm TL (Fairchild *et al*., 2007) and Brazilian flounder *Paralichthys orbignyanus*, 41 – 75 mm TL (Radonic and Macci, 2009) and greater than that of the; mud dab *Limanda yokohama*, > 20.7 mm TL (Suzuki *et al*., 1992), barfin flounder *Verasper moseri, 35* mm TL (Goto *et al*., 1999), olive flounder *Paralichthys olivaceus,* 27 – 37 mm (Yamamoto, 1999), marbled sole *Limanda yokohamae,* 20.7 – 24.8 mm TL (Goto *et al*., 2000), and Atlantic Halibut *Hippoglossus hippoglossus*, 38 mm TL (Hendry *et al*., 2002). In contrast, sexual differentiation occurred at a smaller size in *Rhombosolea* spp. than southern flounder *Paralichthys lethostigma* 75-100 mm TL (Luckenbach *et al*., 2003) and summer flounder *Paralichthys dentatus* 190 mm TL (Colburn *et al*., 2009). Interestingly this investigation determined sexual differentiation in *Rhombosolea* spp. juveniles from the presence of clearly identified primary oocytes and spermatids. This size at sexual maturity was considerably earlier than female and male olive flounder *Paralichthys olivaceus* which saw meiotic divisions at 69 mm

TL and 129 mm TL respectively (Yamamoto, 1999). The difference of the morphological differentiation compared to the meiotic division in male olive flounder *Paralichthys olivaceus* provides insights to the initial differentiation in *Rhombosolea* spp., as this investigation only identified male differentiation at the first meiotic division in males. This suggests that the initial male differentiation of *Rhombosolea* spp. juveniles occurs before the currently reported length and as male differentiation generally occurs after female differentiation (Devlin and Nagahama, 2002), it can also be assumed female differentiation occurs earlier than currently reported. Thus, sexual differentiation in *Rhombosolea* spp. juveniles occurs in the range of reported timing of sexual differentiation of flatfish, however, may initially occur at a sizeably earlier time than currently reported. Therefore, the critical timing for sex manipulations and sexual differentiation occurs before the reported 47 mm TL for *Rhombosolea* spp.

Wild collection of smaller, < 50 mm TL, juvenile *R. plebeia* and *R. leporina* proved difficult. Smaller mesh sizes for dragnets, which would limit the smaller individual getting through, collects significantly more debris and is much more difficult to bring through the water. Thus, there was not an extensive collection of smaller sizes of juveniles as seen in Atlantic halibut *Hippoglossus hippoglossus*, where a commercial hatchery was able to provide 235 juveniles under 50 mm fork length (Hendry *et al*., 2002).

Adults are known to spawn from September to November (Colman, 1973) and the pelagic larval duration of the similarly related greenback flounder *R. tapirina* being about 69 days (Crawford, 1984), it is possible that the time of collection, being September, was not the correct time of the year to collect juveniles as they are settling on to the mud flats. Future studies would benefit from a higher number of smaller juveniles to increase sample size. However, smaller sizes may need to be reared unless planktonic larvae can be caught and identified.

Identification of the two *Rhombosolea* spp. at such a small size also proved to be a challenge. The work done by Eldon and Smith (1986) characterized juveniles of the two species to allow for quick identification. However, their results did not apply to this current investigation's *R. plebeia* or *R. leporina* juveniles collected in the southern Firth of Thames. The identification guide was based on fish collected from the South Island, suggesting that there may be more geographical variation in morphology than previously thought. Thus, in the course of this investigation an

attempt to gather genetic data to develop a species-specific polymerase chain reaction (PCR) assay was initiated. However, time constraints did not allow this work to be fully realized and this remains an ongoing project.

This study was limited in the ability to identify germ cell differentiation, due to thick histological sections. The *Rhombosolea* spp. juvenile females differentiate at 47 mm TL and males at 57 mm TL, from observation of present primary oocytes spermatids respectively. These may likely be overestimating the initial sexual differentiation as progression into meiosis succeeds germ cell differentiation (LeMenn *et al*., 2007, Schulz *et al*., 2010). Future studies would benefit from having the ability to produce adequately thin sections from wax, acrylic, or plastic embedding methods and the ability to increase the sample size of smaller juveniles. The results from this investigation demonstrated that *Rhombosolea* spp. have a period of undifferentiated gonads below 47 mm TL. However, it is possible that a greater sample size in the 30 – 40 mm size class may have yielded a smaller size at differentiation, as sexual differentiation was determined from meiotic oocytes and spermatocytes. Thus, earlier stages of oogonia and spermatogonia could appear at smaller sizes. These results are in agreement with other reported flatfish sizes at initial sexual differentiation. Future studies will need to be undertaken, containing more individuals and using genetic tools with greater sensitivity to identify the exact size of differentiation of *Rhombosolea* spp. Future studies would also benefit from the development of a species-specific genetic marker, to identify the two species at such a small size.

Chapter 4

General discussion

4 General discussion

This study investigated *R. leporina* with the aim to provide fundamental insights into its reproductive biology. *R. leporina* is a sought-after species for cultural, recreational, and commercial aspects. Together with its desired aquaculture characteristics provides the foundation of the rationale for this study. The results of these investigations provide fundamental knowledge on the reproductive biology for the advancement of its stock management and potential for aquaculture.

Robust stock management requires a detailed understanding of the species biology and how interactions with its natural environment or humans' effect it (Nakken *et al*., 1996; Morgan, 2008). This may include how removal from the stock occurs through fishing pressure, environmental changes, and reproductive success. Previously the biology of *R. leporina*, was limited to their seasonal movements (Webb, 1973; Colman, 1974a; Glova & Sagar, 2000; Jellyman, 2011), diet (Livingston, 1987; Mutoro, 2001), growth (Webb, 1973; Colman, 1974b; Mutoro, 2001), and a rough understanding of its reproduction (Colman, 1972; 1973; Webb, 1973; Mutoro, 2001). Previous insights into the reproductive biology were principally based on a macroscopic assessment of the gonadal condition to determine its spawning season. Macroscopic assessment of reproductive development has reduced accuracy of identifying the key timings of cellular events through the reproductive continuum (West, 1990; Kjesbu, 2009a). Histological insight provides superior detail into these events (Hunter & Macewicz, 1985b; West, 1990; Kjesbu, 2009b), which are endocrine-regulated (Nagahama, 1994; Nagahama & Yamashita, 2008; Forsgren & Young, 2012). Thus, can allow for the presumption of endocrinological regulation of the reproductive development. This study provided such histological detail into the reproductive development of adult female *R. leporina*, which revealed a protracted 7-month spawning season, one month longer than the previously reported spawning season (Colman, 1973). Furthermore, this investigation was able to determine that *R. leporina* utilizes a multiple group synchronous ovarian development and may contain batch spawning. This contrasts with previous investigations into the reproductive biology of *R. leporina* which did not identify the reproductive mode (Colman, 1973; Webb, 1973; Mutoro, 2001) or suggested that *R. leporina* only has single spawning potential (Colman, 1985). Furthermore, it was recently stated that the current state of flatfish

stocks in New Zealand is assessed based on tagging studies and morphology (Hannan *et al*., 2016), providing further evidence of the lack of usable biological data for management of *R. leporina* stocks. The detail provided from the histological assessment of reproductive development in this study provides these novel findings for *R. leporina*, which allows for robust management decisions and potential for propagation of this species for an aquaculture setting.

The careful orchestration of the cascade of the events, through reproductive development, are essential to ensure the progeny with the most advantageous environment to start life (Bromage *et al*., 2001; Lowerre-Barbieri *et al*., 2011). Reproduction in teleost is known to contain plasticity, especially in the undifferentiated stages of gonadal development during ontogeny (Baroiller & D'cotta, 2001; Kobayashi *et al*., 2013). Changes to the sex of the bipotential gonad may from temperature, pH, social conditions, and physiological factors could affect the ultimate sex of the individual (Nakamura *et al*., 1998; Baroiller, D'Cotta, & Saillant, 2009; Devlin & Nagahama, 2002; Guerrero-Estevez & Moreno-Mendoza, 2010). While many of these variables provide advantageous controls of sex ratios sought after in aquaculture, the wild stocks may experience such variables as well. Changes to sex ratios of wild stocks will subsequently affect the spawning potential of the stock upon reaching sexual maturity. Thus, there is added importance in understanding the initial reproductive development of larval and juvenile fish for wild stock management and its beneficial control for aquaculture. The results from this study's investigation into the sexual differentiation of juvenile *Rhombosolea* spp. provides the insight into the undifferentiated period of the congener spp. The results are new findings which have not yet been reported for *R. leporina*, identifying the undifferentiated period of the gonad to be < 47 mm TL. Additionally, this investigation found spermatozoa in a male *Rhombosolea* spp. juvenile at 71 mm TL. This finding is a notable size at which spermatozoa are present for the first time. This is supported by the previous speculation of sexual maturity in male *R. leporina* must be below 15 cm, as all males were sexually mature in that study (Colman, 1972). This provides essential knowledge for stock management to use for the assessment of potential stock sex ratios in the future. Furthermore the findings allows for the potential experimentation of the methods of artificial sex reversal during the critical timing of undifferentiation, such as hormone dosing (Nakamura *et al*., 1998) and gynogenesis (Mei & Gui, 2015) which has been done

in the flatfish *Solea solea* (Howell, Baynes, & Thompson, 1995), olive flounder *Paralichthys olivaceus* (Yamamoto, 1999), barfin flounder *Verasper moseri* (Mori, Saito, Kishioka, & Arai, 2004), turbot *Scophthalmus maximus* (Piferrer *et al*., 2004), southern flounder *Paralichthys* lethostigma (Luckenbach *et al*., 2004; Morgan *et al*., 2006), Atlantic halibut *Hippoglossus hippoglossus* (Tvedt, Benfey, Martin-Robichaud, McGowan, & Reith, 2006), half-smooth tongue sole *Cynoglossus semilaevis* (Chen *et al*., 2009), spotted halibut *Verasper variegatus* (Ji, Chen, Yang, Ma, & Jiang, 2010), and Senegalese sole *Solea senegalensis* (Molina-Luzon *et al*., 2015). Gynogenesis also opens the door for the ability to investigate the specific genetic mechanism for sexual determination in the future. Thus, the findings of the initial sexual differentiation in the *Rhombosolea* spp. provides valuable knowledge for the progression of scientific investigations and stock management.

In conclusion, this study provided investigations into *R. leporina* reproductive biology at different ends of the reproductive continuum. The insights from the first investigation into the adult reproductive cycle provided new findings in contrast to previous understandings, that *R. leporina* have a protracted spawning season over seven months and that they display the multiple group synchronous pattern of ovarian development with the potential of batch spawning. The second investigation provided a novel insight into the timing of sexual differentiation of the congener *Rhombosolea* spp. juveniles, displaying the critical period of sexual indifference < 47 mm TL and spermatozoa in a male at 71 mm TL. These findings provide fundamental background on the reproductive biology of *R. leporina* for more adequate management of the wild stocks, required for successful aquaculture, and for further studies into the reproductive biology of *R. leporina*

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