Gonad examination reveals diandry and baseline DNA methylation in the three sexual phenotypes of the New Zealand spotty wrasse, *Notolabrus celidotus*

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“If things are hard, don't get mad, just live your life”

- Jessica Blas (aged 7)

Abstract

Understanding reproductive plasticity and how sexual phenotypes arise is of great interest to biology, particularly the form and function of sex changing fish. However, there are gaps regarding detailed descriptions of gonad morphology and structure of the different sexual phenotypes. Furthermore, the role of epigenetic regulation has yet to be applied to these studies.

Sexually mature terminal phase (TP) males were significantly larger in length, and weight (214 ± 7.74 mm standard length (SL), 146 ± 16.6 g), than the similar sized sexually mature initial phase (IP) males (169 ± 6.24 mm SL, 67 ± 9.52 g) and sexually mature females (167 ± 3.54 mm SL, 61 ± 4.07 g). There was a large overlap in size distributions with a vast size range in sexually mature males (147 – 240 mm SL, 42 – 221 g) and small sexually mature females (146 – 183 mm, 41 – 82 g). These results suggest small males use female mimicry and overlapping size ranges suggest diandry.

Three gonad types were identified, one ovary and two types of testis. Solid testes were more common in IP males than in TP males. It is proposed that the unique and complex shape of the solid testis arises through an evagination process in juvenile males. Hollow testes arise through sex change from female and are more common in TP males. Colour phase not always corresponding to testis type suggests the ability to transition between body colour phenotype according to environmental influences, and two testis types suggest two different developmental pathways to become a male (diandry).

Females and one male were identified as juveniles. All sexually mature males had similar testis lobular structure and somatic and germ cell arrangement. The structural arrangement of the solid testis was consistent with primary male testes, and the hollow testis was consistent with secondary male testes. Juvenile males and the existence of primary and secondary testes are associated with diandry in protogynous species.

Whole-genome percentage methylation analysis revealed no significant difference in global brain DNA methylation between females (71.4 ± 1.06 %), TP males (72.2 ± 0.5 %) or IP males (75.1 %). Global ovarian methylation (53.7 ± 1.16 %) was significantly lower

than both the similarly high global methylation in the IP male testis (82.2 ± 1.39 %) and TP male testis (86.8 ± 0.21 %). Ovaries during spawning season (67.3 ± 1.27 %) had significantly higher global methylation than ovaries outside of spawning season (51.4 ± 0.96 %). Global gonad DNA methylation variations suggest sex-specific differences and seasonal effects on reproduction are under epigenetic control.

The results of this study clearly support diandry in *N. celidotus* and provide insight into the fundamental differences in gonad morphology. Also, the results warrant further investigation into male developmental pathways and the role of epigenetic regulation. These findings provide critical knowledge towards developing the spotty as a model species for sex change research in temperate species.

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Chapter 1

General Introduction

1.1 Phenotypic plasticity

The ability to change the way genes are expressed is essential for organism survival to respond to a fluctuating world. For example, seasonal fluctuations, food availability, response to pathogens, competition with conspecifics, prey/predator densities, and life cycle are all changes that need alterations to organism phenotype for survival (Devlin & Nagahama, 2002). In response to internal and external environmental variation, regulation of gene expression can give rise to phenotypic plasticity (Duncan *et al.*, 2014). Changes in gene expression alter cell and tissue function and can subsequently lead to a change of phenotype (Best *et al.*, 2018). Phenotypic variation occurs at behavioural, biochemical, physiological or at developmental levels and these alterations arise from the same genotype. Biochemical and physiological changes can fluctuate over short periods, but some changes, such as development, may be a long-term modification or irreversible (Pigliucci *et al.*, 2006). Therefore, phenotypic plasticity allows an organism to alter its development, physiological state, or behaviour in response to environmental cues.

1.2 Phenotypic sex – genes and the environment

The fusion of gametes through sexual reproduction to produce young is nearly universal amongst vertebrate species, but the development of offspring into male or female can have varied mechanisms and triggers (Bachtrog *et al.*, 2014; Pennell *et al.*, 2018). Depending on the species, genetics and environmental conditions can both influence the phenotypic sex of vertebrate offspring. In genetic sex determination (GSD), sexual phenotype is not affected by the environment, but rather the genes which are inherited from the mother and the father and result in fixed sex at fertilisation. Whereas, in environmental sex determination (ESD), sexual phenotype is typically influenced by the conditions during embryonic development after fertilisation (Rigaud *et al.*, 1997; Kraak & Pen, 2002; Ezaz *et al.*, 2005). Types of environmental influences on the determination of sex include temperature, conspecific density,

photoperiod (Rigaud *et al.*, 1997). In fish, pH, social conditions, body size (Kraak & Pen, 2002) and salinity (Ezaz *et al.*, 2009) may also affect sex determination. Historically, animals have been placed in either GSD or ESD categories. However, recent studies have found phenotypic sex is more like a continuum which ranges from entirely genetic determination to wholly reliant on environmental conditions, and intermediate strategies can be influenced by both genetics and the environment (Stelkens & Wedekind, 2010; Dupoué *et al.*, 2019).

Genetic determination relies on sex chromosomes which are fixed when gametes combine at fertilisation. Mammalian sexual phenotypes are examples of genetic determination via chromosomes which differ between males (XY) and females (XX). In the mammalian reproductive system, females are homogametic and produce only X chromosomal gametes, and males, which are heterogametic, produce even numbers of X and Y gametes. Because males produce both X and Y gametes, their sperm is the deciding genetic factor in the offspring's phenotypic sex (Kraak & Pen, 2002; Graves, 2008). In contrast, bird GSD is associated with heterogametic female (ZW) and homogametic male (ZZ) chromosomes, which means female gametes are the genetic factor regulating sex (Kraak & Pen, 2002; Graves, 2008). The platypus has an interesting version of a GSD system of XX female and XY male, but it is much more complicated than mammalian systems. Platypus sex chromosomes are made up of a long chain of 10 chromosomes; females have 10 X chromosomes and males have five X and five Y chromosomes. During sperm production, the five X and five Y chromosomes polarise and divide into X and Y sperm. (Waters & Graves, 2009). GSD is also found in some species of reptile, amphibian and fish (Kraak & Pen, 2002).

At fertilisation, species exhibiting ESD will not have fixed sex. Alternatively, the environmental conditions during early development will determine sex (Stelkens & Wedekind, 2010). ESD is characteristic of fish, reptiles and amphibians (Kraak & Pen, 2002). Temperature is the most common environmental variable to influence the phenotypic sex in these groups, therefore is the most studied type of ESD (Dupoué *et al.*, 2019). Temperature related ESD is well studied in reptiles and falls into three distinct categories: type IA (MF) males are produced at low temperatures and females at high temperatures; type IB (FM) females are produced at low temperatures and males at high temperatures; type II (FMF) females are produced at low and high

temperatures, and males are produced at mid-range temperatures (Mitchell *et al.*, 2006). Many turtle species exhibit the MF pattern of sex determination. Some turtle, alligator and lizard species display FM patterns. FMF patterns are common in turtle, alligator, crocodile and lizard species (Kraak & Pen, 2002; Mitchell *et al.*, 2006).

An interesting example of genetic and environmental influences interacting to alter sexual phenotype can be observed in some species of reptiles. Although the central bearded dragon, *Pogona vitticeps,* have a ZZ male and ZW female chromosomal sex determination system, critical environmental factors can override GSD during early ontogeny (Ezaz *et al.*, 2005; Quinn *et al.*, 2007). For example, if incubation temperatures are elevated, hatchlings, which are genetically males (ZZ), are sex-reversed to phenotypically female. Sex-reversal, in this case, is thought to occur through high temperatures downregulating key male sex determining genes which eliminate the formation of a testis, so alternatively the gonad forms as an ovary (Quinn *et al.*, 2007). In contrast, the skink, *Bassiana duperreyi,* has an XX female and XY male chromosomal sex determination and under low temperatures, genetic females sex reverse, resulting in phenotypic males (Radder *et al.*, 2008).

1.3 Sexual phenotypes in fish

In comparison to other vertebrates, sexual phenotypes in fish are particularly plastic and, depending on the species, phenotypic expression may be environmentally or genetically determined or occur at various times during their lifecycle (Piferrer *et al.*, 2019). This relative plasticity maximises reproductive success through diversity of phenotype (Garcia *et al.*, 2016) and demonstrates how fish reproduction has evolved to fit the complex habitats, niches, life histories and interactions different species encounter (Devlin & Nagahama, 2002). The flexibility of fish gender not only allows for maximised reproductive success in the wild but when applied to aquaculture practices increases economic gain. Monosex cultures are sought after when aquaculture species have differential growth or desired traits between sexes (Helfman *et al.*, 2009). For example, females in sablefish, *Anoplopoma fimbria*, reach harvestable size one year faster than males (Luckenbach *et al.*, 2017) and sex-reversed male red tilapia, *Oreochromis niloticus*, in monosex cultures gain greater significant growth than mixed-sex cultures (Singh *et al.*, 2017). Other benefits of having a monosex culture are

removing the possibility of unwanted reproduction, reduced competitive or aggressive behaviours and higher quality final product at harvest (Luckenbach *et al.*, 2017).

1.3.1 Gonochorism and hermaphroditism

The flexibility of phenotypic sex ranges from low to high depending on the sex determining mechanisms of the species (Figure 1.1) (Navara, 2018). Although most fish species display gonochorism, fixed separate sexes throughout their life (Kobayashi *et al.*, 2018), some species display simultaneous or sequential hermaphroditism. Simultaneous hermaphrodites can produce both male and female gametes at the same time within the gonadal compartment. Whereas, sequential hermaphrodites can either transition from a male to a female (protandry) or female to male (protogyny) or transition bi-directionally (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Fennessy & Sadovy, 2002; Helfman *et al.*, 2009; Kobayashi *et al.*, 2018).

Low Plasticity ⟵ ⟶ High Plasticity

Gonochorism		Hermaphroditism			
Primary	Secondary	Sequential			Simultaneous
Undifferentiated gonad	Immature ovary	Protogynous (Female first)	Protandrous (Male first)	Bi-directional (Can change both ways)	Can produce male and female gametes
Maturity = Fixed as a testes or ovary for life					

Figure 1.1: Plasticity spectrum of gonad differentiation in fish. Modified from Navara (2018).

Although there is a high proportion of gonochoristic fish, sex chromosomes are relatively rare and account for only 10 % of fish species (Helfman *et al.*, 2009). Additionally, sex variability is not only observed in hermaphroditic fish species but under the right environmental circumstances, gonochoristic species of fish can also develop sexual phenotypes that differ from their genotype (Piferrer *et al.*, 2019). For example, at low temperatures (14 °C) during early development barfin flounder, *Verasper moseri*, produce a sex ratio of 1:1. However, if the temperature is increased to 18 °C, all undifferentiated juveniles develop into males (Goto *et al.*, 1999). Changes to sex occurring after embryonic development is termed environmental sex reversal (ESR). ESR can be initiated by changes in temperature, pH, social conditions, endocrine hormones, photoperiod and population structure (Stelkens & Wedekind, 2010).

1.3.2 Protogynous sex change

Sequential sex change in fish occurs in more than 350 species and 23 families (Frisch, 2004; Kobayashi *et al.*, 2018). However, a small group of families including; Labridae, Scaridae, Pomacentridae, Serranidae, and Sparidae are typically associated with sex change (Francis, 1992; Frisch, 2004; Godwin, 2009). Most protogynous species will sexually mature as females, but if the conditions are right, they can change sex to male. It is typical within a group of protogynous labrids to have a large terminal phase (TP) male. A TP male generally holds the most dominant position in the group and maintains a harem of females. TP males will use aggressive behaviours to retain their position in the group and a stable social structure. After a loss of a TP male, the largest and most dominant female usually initiates sex change (Devlin & Nagahama, 2002; Frisch, 2004; Munday *et al.*, 2009; Kobayashi *et al.*, 2018). Therefore, social structure is essential for maintaining sex within the group and has a direct influence on sex determination. However, in other protogynous species, some or all females will change sex when they reach a specific size or age (Dipper & Pullin, 1979; Devlin & Nagahama, 2002).

The process of protogynous sex change is a striking phenotypic change. Behavioural sex change appears to occur prior to gonadal restructure. Godwin *et al.* (1996) demonstrated that gonadectomised female bluehead wrasse, *Thalassoma bifasciatum,* undergo behavioural sex change shortly after the removal of male conspecifics. Subsequently, physiological changes occur to endocrine production, gene expression and gonad structure. Lastly, external morphology and colour changes often occur between female and male morphs (Shapiro, 1979; Frisch, 2004; Nozu *et al.*, 2012; Lamm *et al.*, 2015; Kobayashi *et al.*, 2018). So, teleost sex change, therefore, presents an interesting model of phenotypic plasticity affecting whole organism functionality.

1.3.3 Neuroendocrine control of sex change

Gonadal development and maturation result from the integration of environmental cues into a physiological signal via the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis consists of endocrine feedback between the brain and gonad, resulting in gametogenesis and reproduction (Nagahama, 1994; Devlin & Nagahama, 2002). When an environmental change occurs, the stimuli are transduced into chemical signals

through the neuroendocrine system. The stimuli are received at the hypothalamus region of the brain, and gonadotropin-releasing hormone (GnRH) is produced. GnRH signals the pituitary to release gonadotropins (GtHs) (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) into the circulatory system (Suzuki *et al.*, 1988; Swanson *et al.*, 1991). GtHs then stimulate the production of sex steroids (oestrogens and androgens) in steroidogenic cells in the gonads via their receptors, which regulate reproduction by stimulating oogenesis and spermatogenesis in female and male fish respectively (Kagawa *et al.*, 1982; Nagahama, 1994). Therefore, the gonadal endocrine environment directly influences sexual phenotype.

1.3.4 Aromatase and steroid hormones

During the process of protogynous sex change, genes that maintain the female pathway to ovarian development are silenced, and male pathway genes are upregulated to develop a testis. Sex change often begins with the downregulation of the aromatase (*cyp19a1a*) enzyme, which typically maintains oestrogen concentrations through the bioconversion of testosterone into 17 β estradiol (E2) (Nakamura *et al.*, 1998; Devlin & Nagahama, 2002; Navarro-Martin *et al.*, 2011; Todd *et al.*, 2016) and is therefore important in the expression of phenotypic sex. Because gonads in sex changing fish can produce male and female tissues, the influence of aromatase on sex hormone regulation could initiate or control female to male gonad development. For example, when European sea bass, *Dicentrarchus labrax,* (a species of fish that exhibits GSD in combination with ESD) are exposed to high temperatures before gonad differentiation, epigenetic silencing of the aromatase promoter occurs, and due to a lack of aromatase enzyme, the fish subsequently masculinises (Navarro-Martin *et al.*, 2011). Similarly, masculinisation of protogynous fish has been demonstrated using chemical aromatase inhibitors (AI) (Higa *et al.*, 2003; Nozu *et al.*, 2012; Todd *et al.*, 2016). However, if AI treatment is withdrawn during sex change (Wu *et al.*, 2015) or if exogenous oestradiol is administered (Higa *et al.*, 2003) the process of sex change may be inhibited. This evidence suggests that aromatase production is a vital factor in regulating sex.

Steroid hormone studies in sex changing fish began in the 1950s with Reinboth's studies of steroid synthesis (cited by Godwin, 2009). Since then it has been observed in several species of protogynous fish that when sex change commences there is often a

drop in plasma levels of E2 followed by an associated increase in 11-ketotestosterone (11-KT) (Nakamura *et al.*, 1989; Cardwell & Liley, 1991b; Bhandari *et al.*, 2006; Kobayashi *et al.*, 2018). In experimental conditions, male characteristics can be manipulated via alterations to sex steroid concentration. For example, when female common carp, *Cyprinus carpio*, are treated with methyltestosterone a high proportion sex reverse to male (Gomelsky *et al.*, 1994). Additionally, 11-KT treatment in bluehead wrasse, *T. bifasciatum* (Grober *et al.*, 1991), threespot wrasse, *Halichoeres trimaculatus* (Higa *et al.*, 2003) honeycomb grouper, *Epinephelus merra* (Bhandari *et al.*, 2006) and stoplight parrotfish, *Sparisoma viride* (Cardwell & Liley, 1991a) induces male colouration or complete sex change. Clearly, the relative balance of oestrogen and androgen concentration is critical in the process of protogynous sex change.

Additionally, changes in sex steroids can alter behaviour within a hierarchy and affect maturation of conspecifics. For example, aggressive behaviour from higher ranked members alters sex steroid regulation in subordinates, and consequently, gonad maturation and reproductive success are negatively impacted (Buston, 2003; Dzieweczynski *et al.*, 2006). Aggressive interactions can also prevent subordinates from feeding opportunities and increase stress and energy expenditure (Munday *et al.*, 2009). Therefore, aggressive behaviours can have a fundamental impact on the reproductive success of conspecifics.

1.4 Why become a male?

As demonstrated, there are high costs and a considerable transformation involved in changing sex from a female to a male, but being a large male in a protogynous group is a reproductively privileged position to be in. There is an increased opportunity to dominate territory, mate with many females, and produce many more offspring (Ghiselin, 1969; Warner, 1975; Godwin *et al.*, 2003). The leading theory behind the evolutionary driver of hermaphroditism is the size advantage model: where one sex benefits from being larger or smaller than the other (Ghiselin, 1969; Warner, 1975). This theory is true with protogynous sex change as there is an increase in fitness with size, meaning it is much more advantageous to reproduce as a large male than a small male (Kazancıoğlu & Alonzo, 2010). Additionally, the reproductive potential as a female is greater when they are smaller, but as the fish gets larger there is greater reproductive success as a male. Therefore, it is more advantageous for a protogynous

fish to be a small female or be a large male and mate with several small females than to be a small male and be excluded from mating opportunities (Jones, 1980; Devlin & Nagahama, 2002).

1.5 Types of males in protogyny – monandry and diandry

There are two forms of male systems in protogyny (Table 1.1). The first is monandry, where there is only one phenotype of male which always develops from a functional female. The second type of male system is diandry. Diandry exists when the population of a protogynous species is made up of two male phenotypes that have two distinct pathways of development; small primary males and large secondary males. Primary males become males at gonad sex differentiation and do not develop through a functional female phase. Secondary males either undergo sex change from a functional female or develop from a primary male (Warner & Robertson, 1978; Chan & Yeung, 1983; Sadovy & Shapiro, 1987; Devlin & Nagahama, 2002; Munday *et al.*, 2006; Sadovy & Liu, 2008; Helfman *et al.*, 2009; Kobayashi *et al.*, 2018). The term functional refers to the reproductive functional ability of mature male or female gonadal tissues (Sadovy & Liu, 2008). Thus, depending on the protogynous species, there may be one or two developmental pathways to becoming a male.

Table 1.1: Defining criteria of male systems in protogynous fish

Male system	Criteria
Monandry: one pathway to male	• All males go through a functional female phase before transitioning into male • Primary gonad: ovary • Secondary gonad: testis with membrane lined remnant ovarian lumen and peripheral sperm ducts • Small females and large males
Diandry: two pathways to male	• Males develop either directly following sexual immaturity to form a primary testis or through a functional female (ovarian) phase before developing a secondary testis • Primary testis: no ovarian cavity or remnant ovarian tissue. Central sperm duct and comparatively large testes • Secondary testis: membrane lined remnant ovarian lumen. Peripheral sperm ducts and comparatively small testes • Small females and males at overlapping size ranges

Diandry is common in wrasse (Labridae) and parrotfish (Scaridae) (Warner & Robertson, 1978; Nakamura *et al.*, 1989; Cardwell & Liley, 1991b; van Rooij *et al.*, 1996; Ohta *et al.*, 2008; Helfman *et al.*, 2009; Nozu *et al.*, 2012). However, to determine monandry or diandry, multiple aspects need to be evaluated. Studies need to include internal testicular morphology and reproductive function, body colour/morphology and gross testicular morphology to gain a complete understanding of male sexual phenotypes (Sadovy & Liu, 2008). There still can be confusion with the classification of monandric and diandric species, simply because the identification of the female sexual pathway may not always be apparent in the gonad structure. Monandric species usually have evidence of a membrane lined remnant ovarian lumen in males because they have all matured through a female pathway. If the juvenile develops directly into a male and lacks a female phase, they have not passed through the same developmental pathway and typically do not have the former ovarian structure and are therefore diandric (Sadovy & Shapiro, 1987). An additional confusing factor is that undifferentiated gonads can form immature oocytes that later regress and the gonad forms into a testis before maturation (Takahashi & Shimizu, 1983; Shapiro & Rasotto, 1993; Liu & Sadovy, 2004; Orban *et al.*, 2009). Immature ovaries before male maturity would not meet the criteria for monandry because the first function of the mature gonad would be male, not female, so are therefore diandric. Consequently, several aspects need to be analysed before determining monandry or diandry in a species.

The IP of a protogynous species is typically made up of a high number of females, a small proportion of males, and the TP is always male. For example, in stoplight parrotfish, *Sparisoma viride*, 90 % of the IP are female, and only 10 % are male (van Rooij *et al.*, 1996) and in Mediterranean rainbow wrasse, *Coris julis*, 10.4 % of the population are IP males (Alonso-Fernández *et al.*, 2011). However, the density of the population can influence the proportion of IP males. For instance, the proportion of IP male in bluehead wrasse, *T. bifasciatum*, increases with larger reef communities. This is thought to be because in small groups TP males can easily monopolise mating opportunities, and in larger groups it is harder to maintain territories, giving the smaller males a greater chance of reproductive success (Munday *et al.*, 2006). This suggests that social conditions after settlement influence the amount of IP males and

therefore social conditions affect the proportion of sexual phenotype at different stages of ontogeny.

Behaviour and colouration can differ between the two male phenotypes. IP males are generally small and mimic the dull female colouring and non-territorial behaviour. Whereas, TP males are larger than both IP males and females, generally have bright colouration and aggressively maintain territories (Warner *et al.*, 1975; Cardwell & Liley, 1991b; van Rooij *et al.*, 1996; Helfman *et al.*, 2009). However, in some species, there is no colour difference between males and females (Warner & Robertson, 1978; Dipper & Pullin, 1979). Reproductive behaviour can also differ between IP and TP males. TP males typically maintain a territory and pair spawn with females (Warner & Robertson, 1978; Munday *et al.*, 2009). In stoplight parrotfish, *Sparisoma viride*, TP males maintain harems of 2 - 16 females, but territorial behaviour also exists where no females are present. This behaviour suggests that in this species aggression is also used to defend areas they perceive females will like (Cardwell, 1989). Whereas, IP males normally have a sneaker or streaker spawning strategy where the IP male rushes in and releases his sperm with the TP males sperm during or after the TP has pair spawned with a female (Warner & Robertson, 1978; Cardwell & Liley, 1991b; van Rooij *et al.*, 1996; Helfman *et al.*, 2009) and in some species smaller males group spawn with single females (Hourigan *et al.*, 1991; Shapiro & Rasotto, 1993; van Rooij *et al.*, 1996). Overall, colour phase and behaviour can be different between male phenotypes and serve as an essential part of their reproductive strategy.

The act of reproduction can be costly for an animal, and the rate of energy expenditure is different depending on what sex the animal is (Garcia *et al.*, 2016). TP males expend their energy through aggressive interactions and maintaining a territory, which carries a high risk of damage or injury and usually requires them to leave the safety of a shelter. Whereas, females divert energy away from growth and energy storage to develop ovarian tissue which has a higher energetic cost in comparison to maintenance and development of testicular tissue (Villegas-Ríos *et al.*, 2014; Garcia *et al.*, 2016). Therefore, an IP male saves energy on behavioural aspects and the risk of harm as a TP male, and does not have to maintain the high costs of ovarian tissue development as a female. However, the benefits the IP male gains on energy allocation towards reproductive effort is adjusted by the low frequency and low input of

spawning opportunities (Helfman *et al.*, 2009). For example, in bluehead wrasse, *T. bifasciatum*, TP males maintain preferred spawning sites and pair spawn 40 - 100 times daily. Whereas, IP males, who group spawn, only spawn one to two times daily and because the spawning takes place in a group his sperm input is diluted by other IP male sperm output (Helfman *et al.*, 2009). TP males also have the advantage of preferred sexual preference by females (Warner & Robertson, 1978). Therefore, it is still a reproductive advantage to be a TP male in comparison to an IP male.

To combat low reproductive opportunities IP males typically have larger testes and greater sperm production in comparison to TP males (Warner & Robertson, 1978; Hourigan *et al.*, 1991; Shapiro & Rasotto, 1993). The larger testes are associated with the IP male's group or streaking spawning strategies, where a higher amount of sperm needs to be released to fertilise eggs in these competitive situations. Whereas, when the TP male pair spawns, he does not have the same competitive disadvantage (Warner & Robertson, 1978). Interestingly, larger gonads and greater sperm production do not equate to higher levels of plasma or gonadal 11-KT levels in IP males in saddleback wrasse, *T. duperrey*. (Hourigan *et al.*, 1991). Similar patterns have been observed in stoplight parrotfish, *S. viride*, where IP males have low levels of 11-KT and TP males have significantly higher levels of 11-KT (Cardwell & Liley, 1991b, 1991a). Elevated 11-KT concentrations have been associated with male secondary sexual characteristics, including behaviour and aggression in teleost (Borg, 1994).

1.6 Protogynous Labridae in New Zealand – *Notolabrus celidotus*

The spotty, *Notolabrus celidotus*, is a common, endemic wrasse to New Zealand. The wrasse family (Labridae) are a coastal marine fish found in the Atlantic, Indian and Pacific Oceans. Over 500 species of wrasse have been identified worldwide and are common in tropical and temperate waters (Helfman *et al.*, 2009; Parenti & Randall, 2011; Skiftesvik *et al.*, 2014). Although wrasse length can range from 5 to 230 cm (Helfman *et al.*, 2009), they are most commonly found under 45 cm (Morton *et al.*, 2008). All wrasse species are carnivorous and typically feed on molluscs and crustaceans (Parenti & Randall, 2000). It is common for labrids to change sex as part of their lifecycle. They typically begin life as a dull coloured female or an IP male and transition to a more vibrant and colourful male phase (Warner & Robertson, 1978; Dipper & Pullin, 1979; Parenti & Randall, 2000; Helfman *et al.*, 2009).

The genus *Notolabrus* is comprised of seven species (Parenti & Randall, 2011), which are mostly distributed in New Zealand and Australia (Table 1.2) (Parenti & Randall, 2000).

Table 1.2: Names and distribution of genus *Notolabrus*. Details obtained from Parenti and Randall (2000).

Scientific Name	Common Name	Distribution
N. celidotus	Spotty	New Zealand
N. cinctus	Girdled wrasse	New Zealand
N. fucicola	Banded wrasse	Southeast Australia & New Zealand
N.gymnogenis	Crimson banded wrasse	Eastern Australia & New Zealand
N. inscriptus	Inscribed wrasse	NSW Australia, Northern New Zealand, Lord Howe Id, Norfolk Id, & Kermadecs
N. parilus	Brown-spotted wrasse	Western & Southern Australia
N. tetricus	Blue-throated wrasse	Southeast Australia

N. celidotus grow to a total length (TL) of 260 mm, which is relatively small in comparison to other New Zealand *Notolabrus* species (*N. cinctus,* TL 350 mm; *N. inscriptus,* TL 600 mm (Doak, 2013) and *N. fucicola,* TL 600 mm (Denny & Schiel, 2002)). Like other labrids, the spotties diet mainly consists of molluscs and crustaceans, but they are also known the eat a range of other invertebrates (Doak, 2013). *N. celidotus* is a protogynous hermaphrodite with two body colour phases (Choat, 1965; Jones, 1980; Doak, 2013; Moraes, 2019). The two colour phases were once thought to be two different species, but under closer examination, it was discovered *N. celidotus* exhibits dichromatism (Choat, 1965). The IP colour phase consists of a yellow-brown body colour, a large black spot in the middle of their body and yellow pelvic and anal fins. The TP colour phase has a grey-brown body colour, a row of black spots that resembles a thick, black, stripe under the dorsal fin and grey-blue pelvic and anal fins with a gold stripe (Choat, 1965; Doak, 2013; Moraes, 2019). TP colour phase is always male, but IP colour phase can be either male or female, however females make up a more substantial proportion of the IP (Jones, 1980). Spotty sexually mature at around 100 – 110 mm (TL) (Jones, 1980) and sex change typically occurs from 130 – 190 mm (Francis, 2012). Therefore, there are three sexual phenotypes in *N. celidotus;* IP females, IP males, and TP males. In previous work, *N. celidotus* has been reported to be a monandric species (Jones, 1980). However, this study will look further into the differences between the two types of males to determine a developmental pathway and confirm monandry or diandry.

Pioneering studies into the ecology and biology of *N. celidotus* were conducted by Jones between 1976 and 1981 (Jones, 2013). He found that social behaviour has a central influence during the life of a haremic species such as the spotty. Juveniles (< 100 mm) aggregate in groups for shelter and protection, but as they get larger they are more likely to be distributed further apart due to increased aggression between conspecifics and less need for shelter (Jones, 1984). Mature females typically spend most of their time in shallow waters and travel to deeper waters for spawning. TP males actively defend territory against other TP males and territories sometimes overlap. Although there are aggressive interactions between TP males and females, females are not forced to stay within a territory by a TP male (Jones, 1981). *N. celidotus* spawning season takes place from August to December. TP males are territorial and pair spawn with females. Whereas, IP males mimic females in order to gain access to male territories to interfere by streaking during pair spawning. IP males also have large testes for sperm competition (Jones, 1980).

1.7 Thesis aims and objectives

The literature contains a wealth of knowledge on sex changing fish, and there are plenty of reviews on the role of the hypothalamic-pituitary-gonadal axis, sex steroids, potential genetic regulators, ecology, and socially induced sex change (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Baroiller *et al.*, 1999; Devlin & Nagahama, 2002; Godwin, 2009; Lamm *et al.*, 2015; Todd *et al.*, 2016; Todd *et al.*, 2019). However, few studies provide a detailed description of the two male testicular morphs, and epigenetic mechanisms, such as DNA methylation, are an emerging area of research for potential regulators of sexual fate.

Understanding sexual plasticity is of great interest to biology, particularly the form and function of reproduction in sex changing fish. The results of this research will give insight into the fundamental baseline of sex changing studies by increasing knowledge of differences between sexual phenotype, gonad morphology, gonad structure and a reference point for further research into the molecular mechanisms involved in regulating sex. Additionally, this research will further develop the spotty as a model species for sex change research in temperate species.

1.7.1 Aim

To describe the differences between the gonadal gross morphology, ultrastructure and baseline DNA methylation of the three sexual phenotypes and determine monandry or diandry in the temperate protogynous wrasse, *Notolabrus celidotus*.

1.7.2 Thesis structure

- Chapter 1: General introduction
- Chapter 2: Examination of the differences in external body morphology and gross gonad morphology and structure
- Chapter 3: Histological examination of the differences in gonad morphology and structure
- Chapter 4: Examination of the differences in global DNA methylation in the gonad and brain and ovarian seasonality
- Chapter 5: General discussion and determination of monandry or diandry

It is hypothesised there will be structural and morphological differences between the gonad that sets apart initial phase females, initial phase males and terminal phase males in *N. celidotus*. Additionally, it is also expected that there will be differences in global DNA methylation between seasons, tissue types and sexual phenotypes.

1.8 Animal ethics

This study was approved by the Toi Ohomai Institute of Technology (TIOT) Animal Ethics Committee. Committee approval was not necessary through University of Waikato because the study had already been approved via TOIT's Animal Ethics Committee (Animal Ethics Committee, University of Waikato, application number 1072). Standard operating procedures were followed for capture, handling and husbandry of fish and euthanasia and anaesthesia of fish. Wild capture of fish was allowed under a Ministry for Primary Industries special permit.

1.9 General methods (Chapters 2 and 3)

1.9.1 Capture and husbandry

Using a hook and line, 119 *N. celidotus* were wild captured from the southern basin of Tauranga Harbour, New Zealand (37°40'S 176°10'E), from 2018 to 2019. Once caught, the fish were transported to Toi Ohomai Institute of Technology's indoor aquaculture

facility in a large oxygenated bucket and then divided over two 1600 L recirculating aquaculture tanks.

During their housing period fish were fed three times a week with commercial fish food (Ridley Aquafeed, Ridley Corporation) until satiation, any uneaten food was removed after 20 minutes to maintain water quality. Photoperiod was kept to a 12:12 light:dark cycle and filters and tanks were cleaned daily. Water quality parameters were tested three times per week and water exchanges carried out as necessary (Table 1.3).

Table 1.3: Average values (±SE) of water quality parameters during *Notolabrus celidotus* housing period.

Tank	Temp (°C)	Salinity (‰)	Dissolved O^2 (%)	O^2 (ppm)	NH_4^-	NO_2	NO_3	pH
1	18 (±0.3)	35 (±0.1)	86 (±0.6)	7 (±0.1)	0.19 (±0.02)	0.04 (±0.02)	30 (±3.1)	7.7 (±0.02)
2	18 (±0.2)	35 (±0.1)	90 (±0.5)	7 (±0.1)	0.08 (±0.01)	0 (±0)	32 (±2.8)	7.7 (±0.01)

1.9.2 Sex identification

Fish use and dissection were reduced to a minimum to uphold the values of ethical consent. Sex was determined using external markings and gamete identification. IP males were identified amongst the IP fish by expressing milt using gentle abdominal pressure following anaesthetisation (0.6 ml L^{-1} 2-phenoxyethanol). Following sex identification IP fish recovered in clean seawater and were then assigned to different 500 L holding tanks according to sex until dissection.

Forty-five of the total 119 *N. celidotus* were used for DNA methylation research (Zebularine pilot study, Chapter 4). All 45 fish had initial phase colouration, and after dissection, histology confirmed three were IP males, and 42 were IP females. The remaining 74 fish went through the sex identification process, and 37 were identified as IP female, 11 IP male and 26 TP male (Table 1.4).

Table 1.4: Sex identification of 119 *Notolabrus celidotus* caught from Tauranga Harbour, New Zealand, 2018 – 2019. IP- initial phase; TP- terminal phase.

Research	IP female	IP male	TP male	Total
Zebularine pilot study	42	3	0	45
Sexual phenotype study	37	11	26	74
Total	**79**	**14**	**26**	**119**

1.9.3 Dissection

After sex was identified, 10 IP male, 11 IP females, and 12 TP males were randomly selected for dissection. Fish were placed in an aerated 10 L seawater bath of 0.6 ml L^{-1} 2-phenoxyethanol until heavily sedated. All fish were weighed (g), measured (standard length) and externally photographed. Fish were euthanised via decapitation. An abdominal incision was made to reveal the gonad, and additional photographs were taken of the gonad inside the abdominal cavity. The gonad was weighed (g) and photographed after excision. Gonads were preserved in Bouin's solution for no longer than 24 hours and then transferred to 70 % ethanol before being sent for histological processing (University of Otago, Histology Lab).

Statistical analyses were performed on Statistica, v13, software. Statistical significance was set to $p < 0.05$. Distribution normality was assessed with the Shapiro-Wilk test, and homogeneity of variance was assessed with a Levene's test. All data are presented as mean ± SE.

Chapter 2

Gross gonad and body morphology of *Notolabrus celidotus*

2.1 Introduction

Animal bodies are made up of several organ systems which can be investigated via morphological assessments to gain an understanding of the particulars of organ shape, structure and specific functions (Wakuri, 1991). Of particular interest is the form and function of reproductive systems of sex changing fish. Most vertebrate species sexually mature as male or female, which is fixed for life. However, in fish, sexual fate can be remarkably diverse and phenotypically plastic at different stages of ontogeny. Reproductive patterns range from gonochorism to several variations of hermaphroditism; simultaneous, protogyny, protandry and bidirectional changes (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Asoh & Kasuya, 2002; Devlin & Nagahama, 2002; Helfman *et al.*, 2009; Kobayashi *et al.*, 2018). With a vast diversity of reproductive patterns, sexual plasticity and with many species fertilising externally, it is expected that there will also be variation in body and gonad morphology relating to sexual phenotypes.

2.1.1 Development of secondary sexual characteristics and alternative male reproductive strategies

Development of secondary sexual characteristics and alternative mating behaviours between males is common in animals and is thought to have developed through intraspecific competition (Luttbeg, 2004). When reproductive competition arises between males, winners may gain reproductive access to females and losing rivals may be excluded from mating opportunities (Taborsky, 1994). However, the chances of becoming the winning male competitor can be increased by the development of secondary sexual characteristics. Large size and aggressive behaviour can be used to outcompete a rival male, and development of bright, vivid body colouration is typically associated with preferred female sexual selection (Majerus, 1986; Warner & Schultz, 1992; Kuwamura *et al.*, 2000). Therefore, male traits such as behaviour, morphology

and physiology are adaptations that can increase individual competitive advantage to allow their sperm to succeed in egg fertilisation over a rival's sperm (Parker, 1984; Stockley *et al.*, 1997; Taborsky, 1998).

Where there are large males and small males in a protogynous species, alternative reproductive strategies to gain access to females and mating opportunities may be required, especially if the male is in the smaller, weaker position and cannot compete successfully (Taborsky, 1994). In many protogynous fish, particularly wrasse, large males will dominate territories and successfully defend harems of breeding females (Robertson, 1972; Warner & Robertson, 1978; Nemtzov, 1985; Munday *et al.*, 2006). To counteract this, in some species, small males have evolved non-territorial and sneaking strategies to increase their chances of reproductive success (Warner & Robertson, 1978; Gross, 1991; Kuwamura *et al.*, 2000; Plaistow *et al.*, 2004). Because small males can be easily excluded from mating opportunities, they alternatively use female mimicry to sneak into pair spawning events to add their sperm to the mix of the large male's sperm in the water column (Jones, 1980; Taborsky, 1994; Devlin & Nagahama, 2002; Oliveira *et al.*, 2005). In some protogynous species, female mimicry involves having the size and external IP colouration of a female and non-territorial behaviour, but internally possessing a functional testis (Dipper & Pullin, 1979; Bentivegna & Rasotto, 1983; Nakamura *et al.*, 1989; Devlin & Nagahama, 2002). Therefore, colour phase and size does not always elucidate the sex of a protogynous species. However, a sneaking strategy has low reproductive success in comparison to large males due to reduced spawning opportunities, sperm dilution during spawning events and not exhibiting traits associated with female's preferred choice (Helfman *et al.*, 2009). Therefore, different sexual phenotypes potentially evolve from male-male competition, sperm competition and female sexual selection (Sadovy & Shapiro, 1987; Gross, 1991; Hourigan *et al.*, 1991; Plaistow *et al.*, 2004).

Another point of morphological distinction between male sexual phenotypes in protogynous species typically occurs in testicular morphology and structure. At a macroscopic level, comparisons can be made between sexual phenotypes by measuring a gonadosomatic index (GSI), which compares the size of gonad relative to body mass, and observations of shape and colour. Large males typically have a small testis to body size, and, in contrast, small males typically have a large testis in

comparison to body size (Choat & Robertson, 1975; Jones, 1980; Taborsky, 1998; Oliveira *et al.*, 2005). Differences in testis to body proportions are thought to relate to small males requiring a greater amount of sperm delivery to compete with larger males in pair spawning situations (Choat & Robertson, 1975; Hourigan *et al.*, 1991; Taborsky, 1998).

To summarise, in many species of fish where males monopolise females, there are two main male reproductive phenotypes; 1) the competitor: a large, often vibrantly coloured male who is invested in size, competition, and is preferred by females, and 2) the sneaker: a small, often dull coloured female mimic who is invested in sperm production and efficiency (Taborsky, 1998; Oliveira *et al.*, 2005). Each of these strategies has a different way to use morphology and behaviour to allocate their reproductive effort. However, in some species of wrasse, males have multiple sexual phenotypes. For example, the gonochoristic ocellated wrasse, *Symphodus ocellatus,* has four male sexual phenotypes; territorial males, sneaker males, satellite males and non-spawning males (Taborsky *et al.*, 1987) and the diandric protogynous bluehead wrasse, *Thalassoma bifasciatum,* has two male morphologies (IP and TP), but three male sexual phenotypes; IP males, territorial TP males and non-territorial TP males (Semsar & Godwin, 2004).

2.1.2 Protogynous sex change

For many protogynous species, transition between phenotypic sex occurs as a response to a social or environmental cue or at a specific age or size (Luttbeg, 2004). Morphologically, protogynous sex change is typically associated with a dramatic change to secondary body colouration and gonad function and morphology. However, there can be two pathways to become a male depending on the species reproductive systems. In a monandric system, all males pass through a functional female phase. In a diandric system, males develop directly from gonad differentiation (primary males) or through a functional female phase before transitioning to male (secondary male). In diandric species, large secondary males can develop from functional females or primary males (Warner & Robertson, 1978; Bentivegna & Rasotto, 1983; Chan & Yeung, 1983; Devlin & Nagahama, 2002; Sadovy & Liu, 2008).

2.1.3 Background and aim

To understand sex changing fish, such as *Notolabrus celidotus*, it is essential to study the morphological differences between sexual phenotypes to have a fundamental starting point for further research on sex changing mechanisms and reproductive biology. Despite the numerous studies on behaviour and histology in other species of fish, there appears to be limited knowledge on the differences in gross gonad morphology and structure between sexual phenotypes in protogynous species. In previous *N. celidotus* research, observations include two distinct colour variations, an IP and TP, with males existing in both colour phases (Choat, 1965; Jones, 1980; Moraes, 2019). Pair spawning and sneaking male reproductive behaviours have also been observed (Jones, 1980). However, there has not yet been an in-depth study that looks into the gross gonad morphology between the three sexual phenotypes; female, IP male and TP male. Therefore, this study will build upon the knowledge of morphology from earlier *N. celidotus* research.

2.1.3.1 Aim and objectives

To determine the differences between gross gonad and external body morphology of the three sexual phenotypes of the temperate protogynous wrasse, *Notolabrus celidotus*. Results will also be used to provide evidence towards the determination of monandry or diandry in Chapter 5 (General Discussion).

- Is there a difference in body size and/or colour between phenotypes?
- Is there a relationship between body size and gonad morphology within phenotypes?
- Is there a difference in gonad morphology between phenotypes?

2.2 Methods

To describe the differences in gonad and body morphology between the three sexual phenotypes of *N. celidotus* photographs and measurements were taken. After sex was identified (General methods, Chapter 1), 10 IP male, 11 females and 12 TP males were randomly selected for dissection. Fish were placed in an aerated 10 L seawater bath of 0.6 ml L^{-1} 2-phenoxyethanol until heavily sedated (reduced opercula ventilation rate,

loss of equilibrium and unresponsive to gentle prodding). Once sedated, fish were measured (mm, standard length), weighed (g), and external body photographs were taken. Fish were then euthanised via swift decapitation with a sharp knife. An abdominal incision was made along the ventral surface, and photographs were taken of the gonad *in-situ*. Gonads were excised and wet weighed (g). Additional photographs were taken of the dorsal and ventral surfaces of the gonads *ex-situ* alongside a ruler as a calibrating scale. Length of gonad lobes was digitally measured (mm) from photographs. All body and gonad photographs were taken on a Samsung Galaxy S10 smartphone.

2.2.1 Data analysis

Two IP male and one TP male testis samples were excluded from calculations due to severe deformities which were considered to be atypical pathologies of gonad size and morphology. After removal of these deformed samples, there was 8 IP males, 11 females, and 11 TP males for analysis. Gonadosomatic index (GSI) was calculated using the formula; gonad weight/body weight x 100. ANOVA was used to detect differences in GSI and body weight between the three phenotypes; however, square root transformation was required for body weight to fix data normality. A Newmans-Kuel test was used for posthoc testing. Body length did not meet the assumptions for parametric testing, so alternatively a Kruskal-Wallis test was used and a multiple comparisons test for posthoc testing. To compare the differences between the left and right lobe lengths for each gonad type a dependent t-test was performed. Data presented as mean ± standard error (SE) and body length as standard length. Statistical significance was set at $p < 0.05$.

2.3 Results

To describe the differences between the sexual phenotypes of *N. celidotus* photographs and measurements of the body and gonad were taken. At the time of dissection, three gonadal morphologies were apparent, an ovary and two types of testis. The testes types did not necessarily correspond with body colour phase. To adequately compare the types of testes, they were assigned as either a type 1: solid testis (see section 2.3.4.1) or a type 2: hollow testis (see section 2.3.4.2).

2.3.1 External morphology

Body morphology was compared between the three *N. celidotus* sexual phenotypes using photographs, weight and length measurements. TP fish were easily identifiable by external markings and were always male. TP males had typical *N. celidotus* secondary male colouring; with grey-brown body colour and white ventral surface, an elongated black spot spanning along the top of the body below the dorsal fin and pale blue-silver anal fin with a horizontal gold stripe. A bright blue line runs from the mouth, under the eye and across the gill plate. Body scales display bright blue ocelli (Figure 2.1a). In contrast, IP fish were made up of males and females. Females exhibited typical *N. celidotus* IP colouring; gold-brown body colour with a white ventral surface, a prominent black circular spot on the centre of their mid-upper body below the dorsal fin, between the pectoral and caudal fins four dark brown vertical bands extend from the dorsal to ventral surfaces and a bright yellow anal fin with two pronounced brown spots (Figure 2.1b). IP males appear almost identical to females (Figure 2.1c).

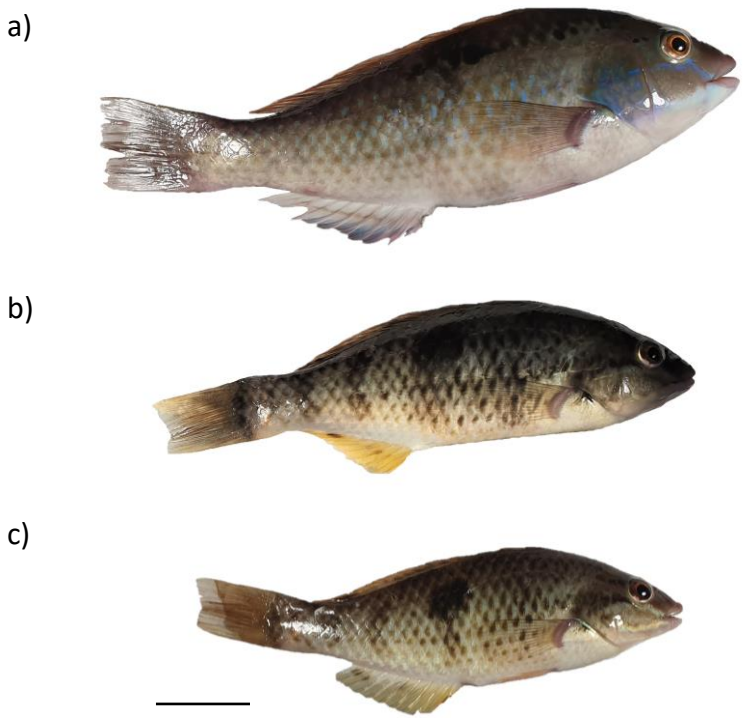

Figure 2.1: External colour differences between the three sexual phenotypes of *Notolabrus celidotus* (dorsal fin not extended). a) terminal phase male. b) initial phase female. c) initial phase male. Scale bar: 30 mm.

However, occasionally under close observation or during stress, IP males showed signs of TP male colouration with loss or dimming of yellow colouration, some displayed loss of anal fin spots and appearance of a gold stripe in the anal fin (pers obs – not shown).

Males were spread over a large size range (147 – 240 mm, 42 – 221 g), whereas, females were only observed at a smaller size range (146 – 183 mm, 41 – 82 g). However, when categorised by sexual phenotype, expected differences were detected in weight ($F = 14.71$, $p = < 0.001$) and length ($H = 12.04$, $p = 0.002$) (Table 2.1). TP males were significantly longer than either sex in the IP ($p = < 0.01$), whereas, females and IP males had a similar body length ($p = 1.0$). The weight differences between the sexual phenotypes followed the same pattern. TP males weighed significantly more than either sex in the IP ($p = < 0.001$). IP males and females had a similar weight ($p = 0.64$).

Table 2.1: Size ranges (±SE) of sexual phenotypes of *Notolabrus celidotus* during spawning season. IP – initial phase; TP – terminal phase.

Sex	Length (mm)		Weight (g)	
	Range	Mean	Range	Mean
Female	146 – 183	167 (± 3.54)	41 - 82	61 (± 4.07)
IP male	147 – 212	169 (± 6.24)	42 - 142	67 (± 9.52)
TP male	160 – 255	214 (± 7.74)	54 - 253	146 (± 16.6)

2.3.2 Gonad lobe morphology

The gonad of *N. celidotus* is made up of two elongated lobes which are separated anteriorly, and fused posteriorly, with a common genital opening. The gonadal lobes run laterally along the edges of the ventral surface of the swim bladder and upper lateral surfaces of the intestine (Figure 2.2). A horizontal mesentery (mesovarium and mesorchium for ovary and testis, respectively) supports the gonad and is also bound to the spleen and the swim bladder. Paired gonadal arteries travel into the gonad from the anterior end of each lobe. In this study, gonads were dissected during the spawning season, and all samples were in a ripe state. Seasonal changes to gonad size were not examined. Testicular and ovarian tissues were easily distinguishable by visual inspection at the time of dissection; these assumptions were followed up by histological analysis to confirm (Chapter 3).

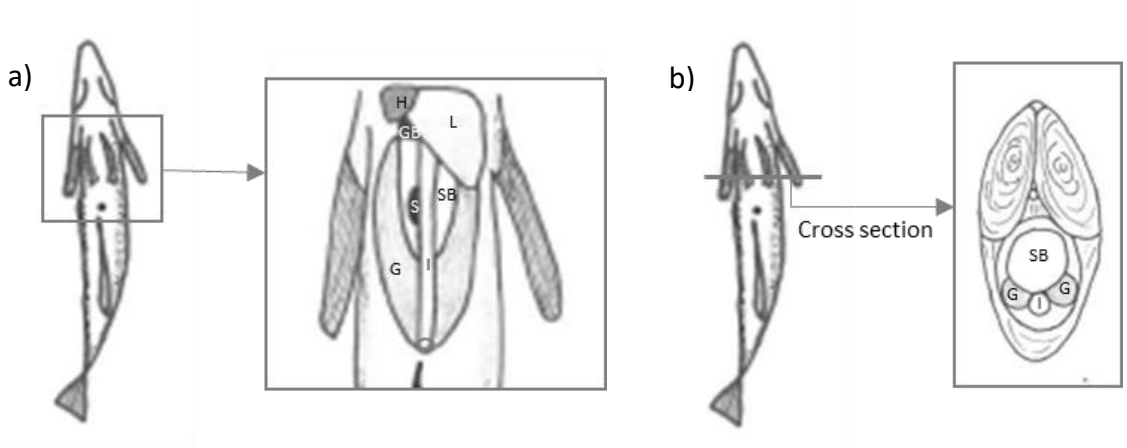

Figure 2.2: Diagrammatic representation of organ layout of *Notolabrus celidotus* relative to ripe gonad. a) ventral view. b) cross section. H – heart; L – liver; GB – gall bladder; S – spleen; SB – swim bladder; G – gonad; I – intestine

To calculate the proportion of gonad mass to body weight a gonadosomatic index (GSI) was calculated (Table 2.2). There was no significant difference in GSI between any of the three sexual phenotypes ($F = 2.96$, $p = 0.07$).

Table 2.2: Gonadosomatic index (±SE) between the three sexual phenotypes of *Notolabrus celidotus* during spawning season. IP - initial phase; TP - terminal phase.

Sex	Range	Average
Female	1.57 – 4.09	2.4 (± 0.2)
IP male	1.78 – 4.92	3.4 (± 0.4)
TP male	1.14 – 4.52	2.7 (± 0.3)

2.3.3 Ovaries

All *N. celidotus* that had ovarian tissue also had an external initial colour phase. Upon visual inspection, the ovary appeared distinct to testicular tissue. Ovarian tissue was much softer, was slightly more cream in colour than testicular tissue and did not seem to be as affected by the tissue shapes surrounding the gonad. Both ovarian lobes were a cylindrical shape and had the same convex shape on the dorsal and ventral surfaces (Figure 2.5a). The external membrane (tunica albuginea) appeared translucent, and ova could be seen inside it. In a small number of samples, the ovary was so ripe and full that the dorsal surface of the gonad appeared stretched and a translucent longitudinal line could be observed. All ovaries had external vascularity, particularly on the ventral surface, extending from the gonadal arteries at the anterior end of each lobe. The ovaries were of the cytovarian type with an oviduct connecting the ovary to

the genital pore. The average length of the right lobe (24 ± 0.77 mm) was similar to the left lobe (27 ± 1.52 mm) (df = 9; p = 0.95). However, the left lobe was slightly longer than the right lobe in 70 % of the ovaries.

2.3.4 Testes

Testicular tissue was found in both colour phases of *N. celidotus* and was visually distinct to ovarian tissue. At the time of sampling, the gonad was ripe, and before dissection, a gentle abdominal squeeze would release milt. It was apparent that there were two morphologies of testis. Both testis types were firmer and a paler colour than ovarian tissue. The following sections describe the testes according to their morphology, with reference to colour phase.

2.3.4.1 Type 1: Solid testis (typical IP male)

Most of the IP males (87.5 %) and a small proportion of the TP males (36.4 %) had a solid testis. Close examination of the solid testis suggests that the convoluted morphology may arise from a rupture along a presumptive seam and then an evagination subsequently occurs, causing the testis to fold back around itself (Figure 2.3). Evagination appears to relocate the outer integument wall into the centre of the testis and correspondingly the innermost core of the testis to the outer periphery. When viewed as a cross-section, the presumptive seam appears to have two arms at the dorsal apex which wrap around exposing a medial region characterised by an internal ridge.

The resulting shape of the solid testis after evagination appears to lead to a range of morphological variations (Figure 2.4). Differences in the morphology at the apparent testis ridge had an interesting impact on the overall shape of the solid testis. When the medial ridge became flattened, it appeared to induce a smooth concave lobe (Figure 2.4b). When the ridge presented as a convex shape, a square-shaped lobe was formed that tended to curl (Figure 2.4c). When the ridge was pronounced, there was a long distinct edge which ran longitudinally along the lobe (Figure 2.4d). Where testis arms 1 and 2 met after evagination the gonad curved inward towards the centre in a deep v-shaped groove (Figure 2.4e). An additional longitudinal split that was also observed in these testes appears to further increase the external surface area of the gonad, although this is not evident in all samples.

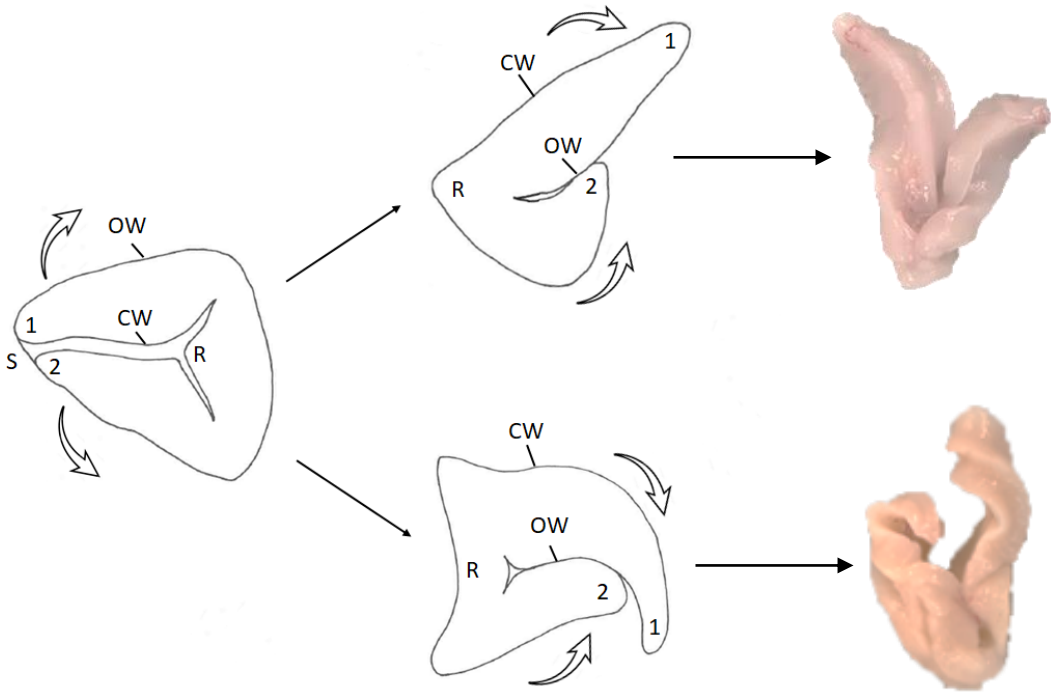

Figure 2.3: Diagrammatic representation of proposed testis evagination *in Notolabrus celidotus* to form a solid testis, viewed as a transverse cross-section. A rupture occurs along a weak presumptive seam on the dorsal apex and testis arms 1 and 2 fold back around themselves until they come together so the outer testis wall becomes centrally located and the central testis wall becomes the peripherally located. White arrows indicating direction of testis arm evagination. The shape of the solid testis after evagination may depend on the shape of the testis ridge – two of the many possible shapes showing. S – presumptive seam on the dorsal apex of the testis; R – testis ridge; 1 and 2 – testis arms; CW – central testis wall; OW outer testis wall.

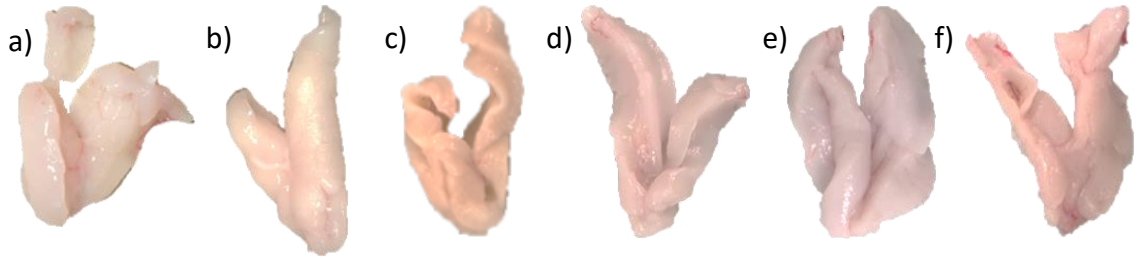

Figure 2.4: Variations in morphology of solid testis in *Notolabrus celidotus*

In general, solid testes, instead of being tube-shaped, like hollow testes (described below) or ovaries, are convoluted and more of a flattened shape and lack superficial vascularity on the external surface (Figure 2.5b). The right lobe (31 ± 1.36 mm) was shorter than the left lobe (36 ± 1.86 mm) (df = 9; p = < 0.001). The solid testis was

found in smaller sized males (< 225 mm, < 122 g), regardless of colour phase (Figure 2.6a&b). GSI of the solid testis was similar to the hollow testis (df = 17; p = 0.35) (Figure 2.6c).

2.3.4.2 Type 2: Hollow testis (typical TP male)

Most TP males (63.6 %) and a small proportion of IP males (12.5 %) had a hollow testis. Hollow testes had triangular-shaped lobes with the apex projecting dorsally into the visceral cavity. From the apex of the lobe toward the inner of the abdomen, the gonad curve was slightly concave following the shape of the expanded swim bladder. From the apex of the lobe toward the outer of the abdomen, the gonad curved convexly (Figure 2.5c). When dissected, the gonad did not curl, was hollow, and the central cavity was visible from the posterior end of the gonad.

Figure 2.5: Gross gonad morphology of *Notolabrus celidotus* during spawning season. a) typical ovary – cylinder shaped with external vascularity. Left – dorsal surface, right ventral surface; b) Type 1: solid testis – typical convoluted flattened shape of an initial phase (IP) male testis with lack of external vascularity. Left – dorsal surface, right ventral surface; c) Type 2: hollow testis – typical in-tact cylinder shape of a terminal phase (TP) male testis with external vascularity, showing hollow cavity at the posterior end. Left – dorsal surface, right ventral surface. Scale bar – 10 mm.

A seam ran longitudinally along the dorsal apex in a similar position to that observed in the ovary and where the solid testis possibly ruptures and evaginates from during

development. All testes with triangular-shaped lobes also had superficial, external vascularity. The average length of the right lobe (45 ± 1.04 mm) was similar to the left lobe (47 ± 1.51 mm) (df = 7; p = 0.06). However, the left lobe was slightly longer than the right lobe in 88 % of the samples. The hollow testis (type 2) was found in larger males (> 212 mm, 138 g), regardless of the colour phase (Figure 2.6a&b).

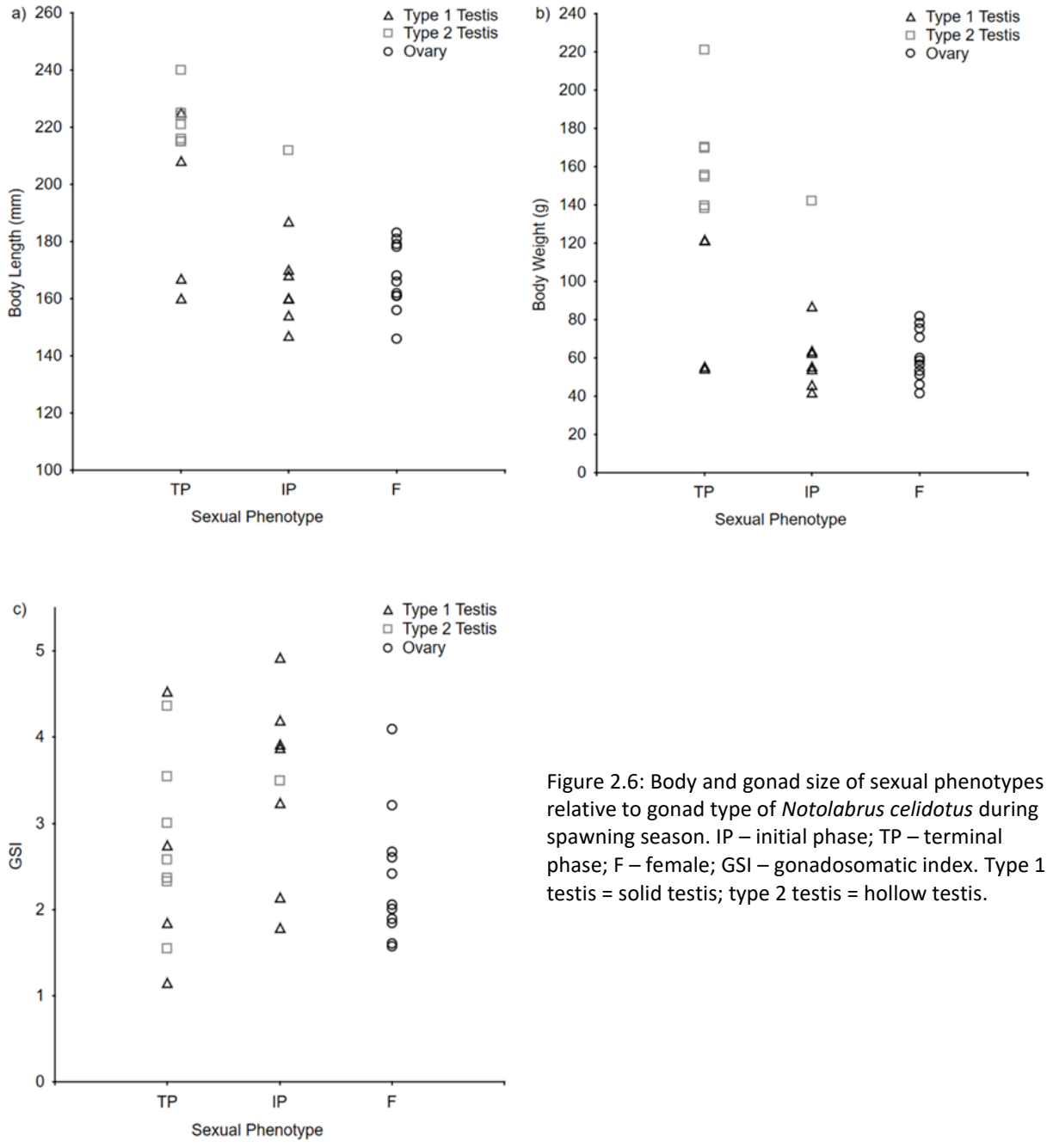

Figure 2.6: Body and gonad size of sexual phenotypes relative to gonad type of *Notolabrus celidotus* during spawning season. IP – initial phase; TP – terminal phase; F – female; GSI – gonadosomatic index. Type 1 testis = solid testis; type 2 testis = hollow testis.

2.4 Discussion

Morphometric analysis of *N. celidotus* has revealed the existence of three distinct morphologies of adult gonad (one type of ovary and two types of testis). Interestingly, testis types do not necessarily correspond with external body colouration. Males were present at all size ranges, whereas females were only present in small size ranges. The existence of two testis types and the wide range of male size provides evidence towards the possibility of *N. celidotus* having two male developmental pathways and meeting the criteria for diandry. However, gross morphology on its own is not enough evidence to determine diandry and will be further investigated via histological morphology in Chapter 3.

2.4.1 External body size and colour phase relative to sexual strategy

Photographic comparison and measurements of body morphology revealed expected results. *N. celidotus* is a dichromatic species with two distinct colour phases. The IP is made up of small females with dull coloured bodies and IP males which mimic female colour and size. TP males are large and exhibit bright and vivid body colour in comparison to the IP. Observations of colour pattern of *N. celidotus* from Tauranga matched the colour descriptions from previous research (Choat, 1965; Jones, 1980; Moraes, 2019). While some protogynous wrasse are monochromatic (Warner & Robertson, 1978; Shapiro, 1979; Kobayashi & Suzuki, 1990), such as Ballan wrasse, *Labrus bergylta* (Muncaster *et al.*, 2013), circle-cheeked wrasse, *Halichoeres miniatus*, (Munday *et al.*, 2009), and foxfish, *Bodianus frenchii* (Cossington *et al.*, 2010), the female mimicry of IP males and secondary colouring of TP males of *N. celidotus* are typical of diandric protogynous wrasse, such as rainbow wrasse, *Coris julis*, (Bentivegna & Rasotto, 1983), bluehead wrasse, *Thalassoma bifasciatum*, (Warner & Swearer, 1991), slippery dick, *Halichoeres bivittatus* (Laming & Ebbesson, 1984) and threespot wrasse, *H. trimaculatus* (Suzuki *et al.*, 2010).

By having the same colour pattern and external appearance as females, IP males can prevent being recognised and excluded from mating opportunities (Choat & Robertson, 1975). This mimicry strategy allows small males higher reproductive success by blending into the harem and allowing for opportunities to streak or spawn during or after a TP male and female pair spawning has taken place (Jones, 1980). For

example, in bluehead wrasse, *T. bifasciatum*, having IP colour allows small males to enter a TP males territory and to sneak into his spawning events (Warner & Robertson, 1978). In contrast, TP males that have a larger body size and display vibrant, distinctive colouring gain an advantage in territorial male-male competition as well as a preference for female sexual selection (Choat & Robertson, 1975; Kuwamura *et al.*, 2000). Therefore, once an IP male reaches a large enough size, it is likely to change to the TP colouring to attain the associated reproductive benefits (Warner & Robertson, 1978; Hourigan *et al.*, 1991).

Male *N. celidotus* in this study were observed at all sizes ranges, and females are only present in IP colour and smaller size ranges. However, males are present in different colour phases, with IP males generally smaller than TP males. Similar results for *N. celidotus* male size ranges and colour patterns were found by Jones (1980); however, TP males (182.0 ± 2.0 mm Jones; 214 ± 7.74 present study) and IP males (118.2 ± 5.6 mm Jones; 169 ± 6.24 present study) were observed in a larger size range in the present study. Jones also found that in Wellington and Leigh, IP males were present in the population before female maturity and were rarely found over 130 mm (Jones, 1980). In contrast, in Tauranga, all IP males were greater than 147 mm and up to 212 mm. Larger size of IP males may have various explanations including; targeted capture size (> 140 mm targeted in this study), regional differences in IP male length or an earlier shift in colour phase in males from Wellington and Leigh than males in Tauranga. However, Jones (1980) targeted all available size ranges of *N. celidotus* and compared 561 specimens from Leigh and 440 specimens from Wellington. Therefore, the smaller sizes detected by Jones may be a reflection of sampling from a more extensive size range and the greater number of specimens examined.

Because male size in *N. celidotus* has a large overlap with females, this suggests that sex change is not a developmental process where age or size initiates sex change. Overlapping size patterns between males and females in protogynous species tend to be consistent with diandric species (Warner & Robertson, 1978; Shapiro, 1979; Sadovy & Liu, 2008). In contrast, monandric species tend to have bimodal size frequencies (Shapiro, 1979; Sadovy & Shapiro, 1987). For example, monandric bimodal distribution of size between small females and large males is observed in labrid butterfish, *Odax pullus*, (Trip *et al.*, 2011); Ballan wrasse, *L. bergylta* (Muncaster *et al.*, 2013);

Mediterranean razorfish, *Xyrichthys novacula* (Candi *et al.*, 2004); pinklined wrasse, *C. dorsomaculata* (Tribble, 1982); and circle-cheeked wrasse, *H. miniatus* (Munday *et al.*, 2009) and diandric overlapping size distribution between male and female is observed in bluehead wrasse, *T. bifasciatum* (Warner & Schultz, 1992); cuckoo wrasse, *L. ossifagus* (Dipper & Pullin, 1979); rainbow wrasse, *C. julis* (Bentivegna & Rasotto, 1983); slippery dick, *H. bivittatus* (Laming & Ebbesson, 1984); saddleback wrasse, *T. duperrey* (Nakamura *et al.*, 1989); and threespot wrasse, *H. trimaculatus* (Suzuki *et al.*, 2010).

2.4.2 Gonad lobe morphology

Photographic comparison and measurements of gonads of *N. celidotus* revealed three distinct morphologies of gonad type, one type of ovary and two types of testis. All three gonad types had the basic teleost gonad shape of paired lobes, separated anteriorly and fused posteriorly with a supporting mesentery (Hastings, 1981; Shapiro & Rasotto, 1993; Fennessy & Sadovy, 2002; Koulish *et al.*, 2002; Lone & Hussain, 2009). As noted in other species, determination of ovarian and testicular tissue of *N. celidotus* can be examined macroscopically via shape and texture (Hastings, 1981; Kobayashi & Suzuki, 1990; Denny & Schiel, 2002; Fennessy & Sadovy, 2002). Suggesting these basic gonad features are relatively consistent among teleost on an evolutionary scale.

In wrasse, IP males typically have a larger testis relative to body size than TP conspecifics. Comparatively large testes are observed in IP males of several species of wrasse including; bluehead wrasse, *T. bifasciatum* (Shapiro & Rasotto, 1993), saddleback wrasse, *T. duperrey* (Nakamura *et al.*, 1989; Hourigan *et al.*, 1991), and rainbow wrasse, *C. julis* (Alonso-Fernández *et al.*, 2011). A larger testis in IP males is related to spawning strategy and sexual selection. It is advantageous to have a large testis in streaking and spawning events because large quantities of sperm are required for mating success due to sperm dilution and reduced mating opportunities. A TP male does not require a proportionally larger testis because they do not have the same limitations in access to females and during pair spawning their sperm does not suffer the same dilution effect relative to IP males (Choat & Robertson, 1975; Hourigan *et al.*, 1991; Alonso-Fernández *et al.*, 2011). While Uglem *et al.* (2001) found that IP male corkwing wrasse, *Symphodus melops,* had a greater GSI than TP conspecifics, they also investigated differences in sperm traits between the two phenotypes. Their work

showed no difference in fertilisation, deformity and hatching success between eggs fertilised by either phenotype, but IP males proved to have significantly greater sperm motility after 5 minutes compared to TP fish. Therefore, variations between testis types reflect the reproductive and spawning strategies of the different sexual phenotypes.

In the present study, there was no statistical difference in mean GSI among the three sexual phenotypes and testis types (solid and hollow testes) in *N. celidotus*. In contrast, previous research involving *N. celidotus* showed that IP males do have comparatively larger gonads than TP males and females (Jones, 1980). The fish sampled by Jones (1980) were generally smaller, captured in different biogeographical regions and across a wider seasonal range than the fish in this study and these factors cannot be discounted as influencing the variation in the results. However, differences in testis size between research could also indicate that smaller IP males may have a larger testis in comparison to TP males, but as they get larger the relative difference in gonad to body mass decreases or could be due to the present studies smaller sample size. Further studies with increased sample size would be necessary to verify this.

2.4.3 Ovaries

The macroscopic ovarian structure in *N. celidotus* appears to be typical of most teleost (Candi *et al.*, 2004; Muncaster *et al.*, 2010; Alonso-Fernández *et al.*, 2011). The paired lobes fuse at the posterior end with a cytovarian structure allowing eggs to be transferred from the ovarian lumen to the genital opening via an oviduct (Muncaster *et al.*, 2010). One of the more interesting features was the vascularity around the ovary, which was notably similar to the hollow testis, observed primarily in TP males. This shared structural feature in conjunction with the existence of a lumen in the hollow testis is strongly indicative of a shared ontogeny.

2.4.4 Testes

Two distinct morphologies of testis were observed in *N. celidotus.* A solid testis was more common in IP males and smaller TP males. In contrast, a hollow testis was more common in TP males but was also found in one large IP male. Therefore, testis morphology is independent of colour phase, but certain types are more likely to occur in specific colour phases or sizes. Similar patterns of skewed testis morphology are

found in other diandric species (Sadovy & Liu, 2008). For example, a secondary testis is skewed towards TP males, with a small proportion possessing a primary testis in saddleback wrasse, *T. duperrey*, (Hourigan *et al.*, 1991) and rainbow wrasse, *C. julis* (Bentivegna & Rasotto, 1983). Additionally, a primary testis is skewed towards IP males, with a small proportion possessing a secondary testis in some scarid species (Choat & Robertson, 1975). If the ontogenetic pathways to form the two gonad types are fixed, then this implies that the species described above may on occasion transition between external phenotypes.

Differences in testis morphology between IP and TP males in *N. celidotus* have previously been described as an evagination process that results in the testis opening along a split seam and folding around itself to form a superficial primary testis (Jones, 1980). Evagination seems to be a plausible explanation as to how the solid testis has obtained its characteristic shape and may be a normal process in the development of a primary testis in *N. celidotus*. The external structure of this testis was unlike testicular descriptions of other protogynous species in the literature, which most likely reflects a lack of detailed morphological description rather than a unique structure. However, histological evidence is required to clarify evagination and testis developmental pathways and will be further examined in Chapter 3.

The small occurrence of a hollow testis in IP males may be due to a TP male reverting the external appearance to a female mimic and assuming a sneaker strategy. IP males of *N. celidotus* can change their external colour to the TP colour phase rapidly in captivity (colour pattern changes within 30 minutes under stress or less than three weeks due to social conditions, pers obs). Therefore, it could be possible that a TP male with a secondary testis could also revert to the IP colour phase if the social environment favoured a sneaker strategy. Jones (1980) also states that IP males of *N. celidotus* appear to change their colour patterns over the spawning season. He explains that when an IP male is interacting a with TP male, IP colouration, such as the central spot, is exaggerated and when an IP male is not in the presence of a TP male, the TP colour pattern can alternatively be displayed. Furthermore, it has been demonstrated in the protogynous bluestreak cleaner wrasse, *Labroides dimidiatus*, that in the presence of a larger male, a smaller male can become subordinate and sex reverse back to female (Kuwamura *et al.*, 2002). Although sex reversal from male to

female has not been reported in *N. celidotus,* the observations in *L. dimidiatus* suggests that males can alter their reproductive strategy based on conspecifics. Personal observations and evidence from Jones suggest that colour pattern in *N. celidotus* can change rapidly, and this strongly suggests that males may take advantage of either colour phase or reproductive tactic depending on their surrounding environment.

2.4.5 Summary

Accurate biological criteria are necessary to identify the reproductive strategy used by a protogynous species. Historically, determination of male type (monandry or diandry) in protogynous species was concluded from the external body size and colour as well as spawning strategy. However, these elements, plus testicular morphology, became a more accurate way to determine male type and further criteria has been developed to determine male types (Sadovy & Liu, 2008). In monandric systems, all males go through a functional female phase before transition into a male and in diandric systems males develop either directly following sexual immaturity to form a primary testis or through a functional female phase before developing a secondary testis (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Devlin & Nagahama, 2002; Sadovy & Liu, 2008). It is clear from the results of this study that two macroscopically distinct morphologies of testis exist in *N. celidotus* and males and females have overlapping size distributions. However, further investigation of the internal structure will clarify if there are two pathways to male development. Because colour phase, body size, and gross gonad morphology cannot determine functional sex or gonad maturity, Chapter 3 will examine the histological structure of the gonads in *N. celidotus.*

Chapter 3

Gonad ultrastructure of *Notolabrus celidotus*

3.1 Introduction

In order to understand the form and function of reproductive systems, it is vital to study the internal ultrastructure. However, because these structures cannot be viewed externally, histological techniques are required to view the gonad internally to assess the differences between sexual phenotypes accurately. Histology is a valuable technique in studies involving reproduction, particularly identifying sexual patterns in hermaphroditic fish (Parenti & Grier, 2004; Alonso-Fernández *et al.*, 2011). Additionally, histomorphometric analysis can also be used for a variety of purposes in reproductive studies; such as internal morphology, verification of sex, stages of sex change, assessing fecundity, and identification of reproductive maturity (Blazer, 2002; Parenti & Grier, 2004; Alonso-Fernández *et al.*, 2011).

Labrids are the second largest marine fish family; therefore, it is not surprising that there is such a diverse range in sexual patterns observed within labrid species (Dipper & Pullin, 1979; Sadovy & Liu, 2008). Labrid sexual patterns range from gonochorism to several variations of hermaphroditism; simultaneous, protogyny, protandry and bidirectional changes (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Asoh & Kasuya, 2002; Devlin & Nagahama, 2002; Helfman *et al.*, 2009; Kobayashi *et al.*, 2018). Additionally, within protogynous species, there can be complex relationships between sex, colour phase, and social and reproductive behaviours (Dipper & Pullin, 1979). This diversity of sexual patterns and reproductive strategies also means there is a range of internal gonad morphological structure (Asoh & Kasuya, 2002).

3.1.1 Gametogenesis

During sexual reproduction, the maturation of gametes takes place through gametogenesis. Females go through oogenesis and produce a comparatively small number of large eggs and males go through spermatogenesis and produce a greater amount of small, motile, sperm (Schulz *et al.*, 2010). Each stage of gametogenesis has distinguishable characteristics that can be viewed histologically (Nagahama, 1983;

Blazer, 2002). Gametogenesis is initiated through the reproductive axis or the hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamic region of the brain, where it is secreted into the pituitary to stimulate the production and release of gonadotropins into the circulatory system (Suzuki *et al.*, 1988). The two main pituitary gonadotropins associated with the regulation of gonad development are luteinising hormone (LH) and follicle stimulating hormone (FSH). In the testis, LH acts on the interstitial Leydig cells to stimulate sex steroid production (Grier, 1981; Engel & Callard, 2007; Schulz *et al.*, 2010), while FSH acts on the Sertoli cells to stimulate growth factors that are responsible for maintenance and growth of germ cells (Schulz *et al.*, 2010). In the ovary, gonadotropins activate their corresponding cell surface receptors on the steroidogenic follicle bilayer of cells. In general, LH regulates final oocyte maturation, and FSH stimulates the rapid growth phase of vitellogenesis via the production of the sex steroid 17β- estradiol (E2). E2 activates the production of egg shell proteins (zona radiata) and vitellogenin in the liver (Nagahama, 1983; Patiño & Sullivan, 2002; Lubzens *et al.*, 2010). Sex steroid concentrations act in a feedback manner on the brain to modulate gonadotropin production (Lubzens *et al.*, 2010). Therefore, gonad development involves a coordinated process of physiological signalling and endocrine feedback between the brain and gonad to regulate germ cell development and maturation.

3.1.2 Ovarian development

Ovarian development in fish occurs in three ways: 1) synchronous – all oocytes develop at the same time; 2) group synchronous – two or more groups of oocytes develop simultaneously; 3) asynchronous – oocytes at all stages of development are present in the ovary (Nagahama, 1983; Blazer, 2002; Lubzens *et al.*, 2010). Each method of oocyte development relates to the spawning habits of the fish species. For example, a species with synchronous oocyte development will participate in a single spawning event. Whereas, a species with asynchronous oocyte development will have the capacity to spawn several times over an extended spawning season (Lubzens *et al.*, 2010).

Oogenesis involves maturation of oogonia through several stages of development to produce mature ova for fertilisation. Within the ovary, follicles are composed of a

steroidogenic bilayer of granulosa and thecal cells, essential to egg development and supporting oocyte growth (Patiño & Sullivan, 2002; Lubzens *et al.*, 2010). Follicle and oocyte growth progress through two main stages, primary growth and vitellogenic. Vitellogenic stages, when yolk granules are incorporated, are followed by oocyte maturation and ovulation (Blazer, 2002; Patiño & Sullivan, 2002). All stages of oocyte development are mediated through communication between the follicle layers and oocyte to allow for a coordinated process to produce an ovulated ova (Lubzens *et al.*, 2010). For a detailed review of oogenesis, see Lubzens *et al.* (2010).

3.1.3 Testicular development

Testis structure differs from species to species. However, the spatial organisation of testis germ cells can be categorised in two basic types: 1) restricted - spermatogonia are limited to developing near the periphery of the gonad wall; 2) unrestricted - spermatogonia occur throughout the testes (Grier, 1981; Parenti & Grier, 2004). There are also intermediate stages between restricted and unrestricted, where spermatogonia have a preferred location close to the gonadal wall (Schulz *et al.*, 2010).

Spermatogenesis involves a series of mitotic and meiotic divisions to proliferate and then differentiate from diploid spermatogonia to haploid spermatids. Spermatid then differentiates to spermatozoa via spermiogenesis to form motile, highly compact, mature sperm (Nagahama, 1983; Schulz *et al.*, 2010). Spermatogenesis occurs in the lobules of the testis (Engel & Callard, 2007; Hess & De Franca, 2009). Within the lobules, the germinal epithelium contains two types of cells; Sertoli cells and germ cells (Grier, 1981; Hess & De Franca, 2009; Schulz *et al.*, 2010). Sertoli cells are somatic cells which perform an essential role in the testis, nurturing and providing for the survival and development of germ cells. Sertoli cells also regulate spermatogenesis and phagocytosis (Hess & De Franca, 2009; Schulz *et al.*, 2010). In fish, Sertoli cells form spermatocysts which contain groups of germ cells at a synchronous developmental stage and neighbouring Sertoli cells house germ cells at different stages of development (Nagahama, 1983; Vilela *et al.*, 2003; Schulz *et al.*, 2010). For a detailed review of spermatogenesis and spermiogenesis, see Nagahama (1983) or Schulz *et al.* (2010).

3.1.4 Protogynous sex change

In hermaphroditic species, germ cells can be bipotential meaning male and female tissue can coexist in the gonad compartment simultaneously throughout their adult life or simultaneously for a short period during sequential sex change (Sadovy & Shapiro, 1987; Sadovy & Liu, 2008). During protogynous sex change, ovarian tissues breakdown and atresia of oocytes occur, spermatogenic tissues begin to proliferate, becoming more prevalent than female tissue over time. After complete gonad restructure, the ovary transitions to a functional testis, devoid of female tissue (Sadovy & Shapiro, 1987; Alonso-Fernández *et al.*, 2011; Muncaster *et al.*, 2013). Depending on the species, gonad organisation of male and female tissue types during sex change can be arranged in three ways: 1) delimited - a membrane separates female and male tissues; 2) undelimited - female and male tissues are separated but do not have a membrane dividing them; 3) also an undelimited type – however, female and males tissues are mixed throughout the gonad. The mixed undelimited type is typical of protogynous labrids (Sadovy & Shapiro, 1987) and occurs in *Notolabrus celidotus* (Moraes, 2019).

Testicular structure may also vary in protogynous fish because not all species have one male developmental pathway. In monandric species, all males transition from a functional female and form a secondary testis. In diandric species, males either develop directly from immaturity and form a primary testis or develop through a functional female phase before transitioning to a male and forming a secondary testis (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Devlin & Nagahama, 2002). Following sex change, the newly formed secondary testis will have an absence of ovarian tissues. However, there may still be signs of former female function, such as a remnant ovarian lumen. Furthermore, secondary testes characteristically have peripheral sperm ducts, which form longitudinally down the testis wall, and a vas deferens made up of several ducts (Choat & Robertson, 1975; Dipper & Pullin, 1979; Sadovy & Shapiro, 1987; Hourigan *et al.*, 1991). In contrast, because a primary male does not previously function as a female, the testis does not typically contain any ovarian features. The primary testis characteristically exhibits gonochoristic features, such as a solid testis, central sperm ducts and a singular central vas deferens (Choat & Robertson, 1975; Dipper & Pullin, 1979; Sadovy & Shapiro, 1987; Hourigan *et al.*,

1991). Therefore, histological analysis of the testis ultrastructure can provide evidence of the male developmental pathway.

3.1.5 Background and aim

To understand sex changing fish, such as *Notolabrus celidotus*, it is essential to study the histological differences between sexual phenotypes to have a fundamental starting point for further research on sex changing mechanisms and reproductive biology. Clearly, there will be a difference between male and female tissues; however, it will be an advantage to understand the internal structures between females and IP and TP males. Only limited information conducted approximately 40 years ago is available on the internal structure of the gonad of *N. celidotus*. These early studies do not include histological figures or diagrammatic representations of the gonad tissues. Therefore, this study will build upon the knowledge from earlier *N. celidotus* research and will inform future sex change studies.

3.1.5.1 Aim and objectives

To describe the differences between gonad internal morphological structure of the three sexual phenotypes of the temperate protogynous wrasse, *Notolabrus celidotus*. Results will also be used to provide evidence towards the determination of monandry or diandry in Chapter 5 (General Discussion).

- Is there a difference in internal gonad morphology between phenotypes, particularly testes in males?
- Is there a difference in morphology from different locations or orientations within the gonad?
- Does internal gonad morphology match with gross gonad morphology from Chapter 2?

3.2 Methods

To describe the differences in gonad morphology of *N. celidotus* histological samples were taken of the three sexual phenotypes. In addition to the gonads described in Chapter 2, five smaller initial phase (IP) fish were also sampled to gain a further representation of gonad type. All fish were measured to standard length.

After sex was identified (General Methods, Chapter 1), 10 IP male, 11 females, 12 terminal phase (TP) males, and five sexually immature IP fish were randomly selected for dissection. Although the number of individuals analysed for each phenotype is less than those used in some other studies, they were deemed sufficient to provide a comprehensive analysis of gonadal structure while respecting ethical requirements to reduce sacrificial fish. Individual fish were placed in an aerated 10 L seawater bath of 0.6 ml L^{-1} 2-phenoxyethanol until heavily sedated (reduced opercula ventilation rate, loss of equilibrium and unresponsive to gentle prodding). Once sedated, fish were euthanised via swift decapitation with a sharp knife. An abdominal incision was made along the ventral surface, and the gonad was excised from the visceral cavity. In the larger fish (> 140 mm), both gonad lobes were dissected from the fused posterior region of the gonad. One whole gonad lobe and the fused posterior region were preserved in Bouin's solution for 24 hours and then transferred to 70 % ethanol until sectioning. The gonads of the juvenile fish (< 116 mm) were too small to follow this process. Therefore, only one whole lobe was preserved for sectioning.

To gain a greater understanding of the gonad structure of each phenotype it was necessary to examine several locations and orientations within the gonads. A transverse cross-section was prepared at the posterior portion (where the lobes fuse), and two additional transverse cross-sections were taken from the gonad lobe, one slightly to the anterior of the fused posterior region and one from the anterior end of the lobe (Figure 3.1a). Cylindrical pieces of tissue were excised from the mid-portion of the gonad lobe and cut longitudinally. Histological sections were prepared from these hemispheres in either dorsal or longitudinal planes (Figure 3.1b). The juvenile fish had

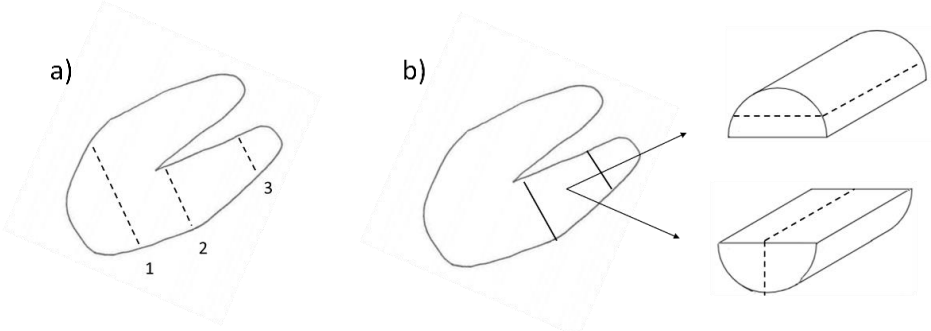

Figure 3.1: Diagrammatic representation of gonad histology locations and orientations for adult fish. a) transverse cross sections at: 1 – fused posterior region, 2 – posterior lobe, 3 – anterior lobe. b) mid-portion of gonad, top right, showing dorsal plane sectioning location; bottom right, showing longitudinal plane sectioning location.

small gonads, and consequently, one transverse cross-section was excised from the mid-lobe.

After the gonad samples were prepared, they were stored in 1.5 ml tubes, containing 70 % ethanol, before being sent for histological processing (University of Otago, Histology Lab). Gonad tissues were serially dehydrated, cleared, then infiltrated and embedded in paraffin for histological sectioning at 3-4 µm and stained with hematoxylin and eosin.

Histological slides were viewed under an Olympus BX53 compound microscope for close tissue examination and an Olympus SZ61 stereo microscope, with the objective removed, to view the whole tissue sample. The stereo microscope was fitted with an Olympus EP50 camera and connected to a laptop via EP View software. The compound microscope was fitted with an Olympus DP27 camera and connected to a laptop with CellSens Entry software.

3.3 Results

To describe the internal gonad morphology and structure of the three sexual phenotypes of *Notolabrus celidotus* histological sections were taken from several points of view. Results showed that none of the fish were intersex or transitioning individuals. Histology confirmed that of the five juvenile fish (88 – 115 mm), one was male and four were female. Of the adult fish (> 140 mm) there were 22 males and 11 females. Histological evaluation confirmed the existence of three broadly distinct gonad morphologies in the larger fish; one type of ovary and two types of testis.

3.3.1 Juvenile gonads

The body length of the juvenile female *N. celidotus* ranged from 88 – 115 mm. These fish had small, cylindrical shaped ovaries with oogonia and dominated by perinucleolar oocytes contained within a thin tunica albuginea (Figure 3.2a&b). There was no evidence of any further stages of oocyte development or atretic oocytes. The single male fish was 107 mm and had small, filiform, translucent testes that contained mainly spermatogonia with several spermatogenic cysts containing spermatocytes and no evidence of ovarian germ cells (Figure 3.3a&b). The presence of a central ovarian lumen could not be determined as the tissue did not remain intact during processing.

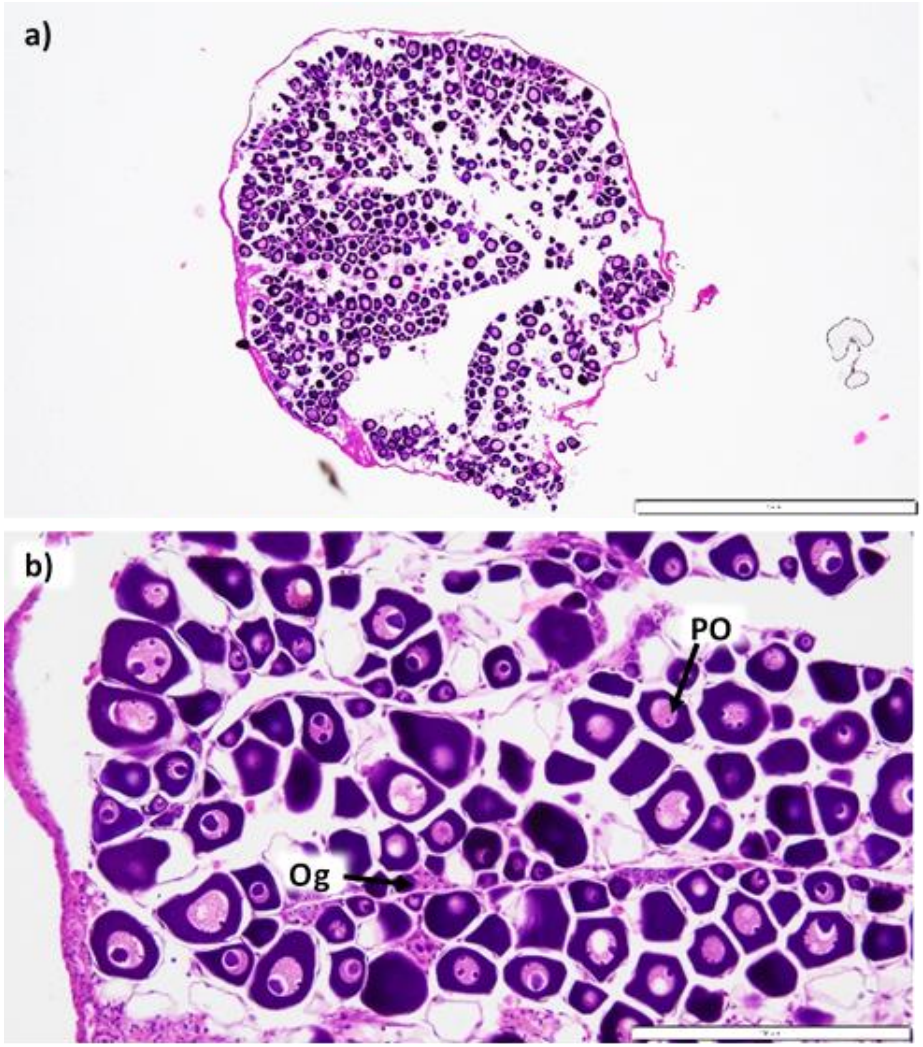

Figure 3.2: Transverse cross sections of a juvenile ovary of *Notolabrus celidotus*. a) whole section of sexually immature ovary (scale bar – 1mm); b) sexually immature ovary dominated by perinucleolar oocytes (scale bar – 200 µm). PO – perinucleolar oocyte, Og – oogonia

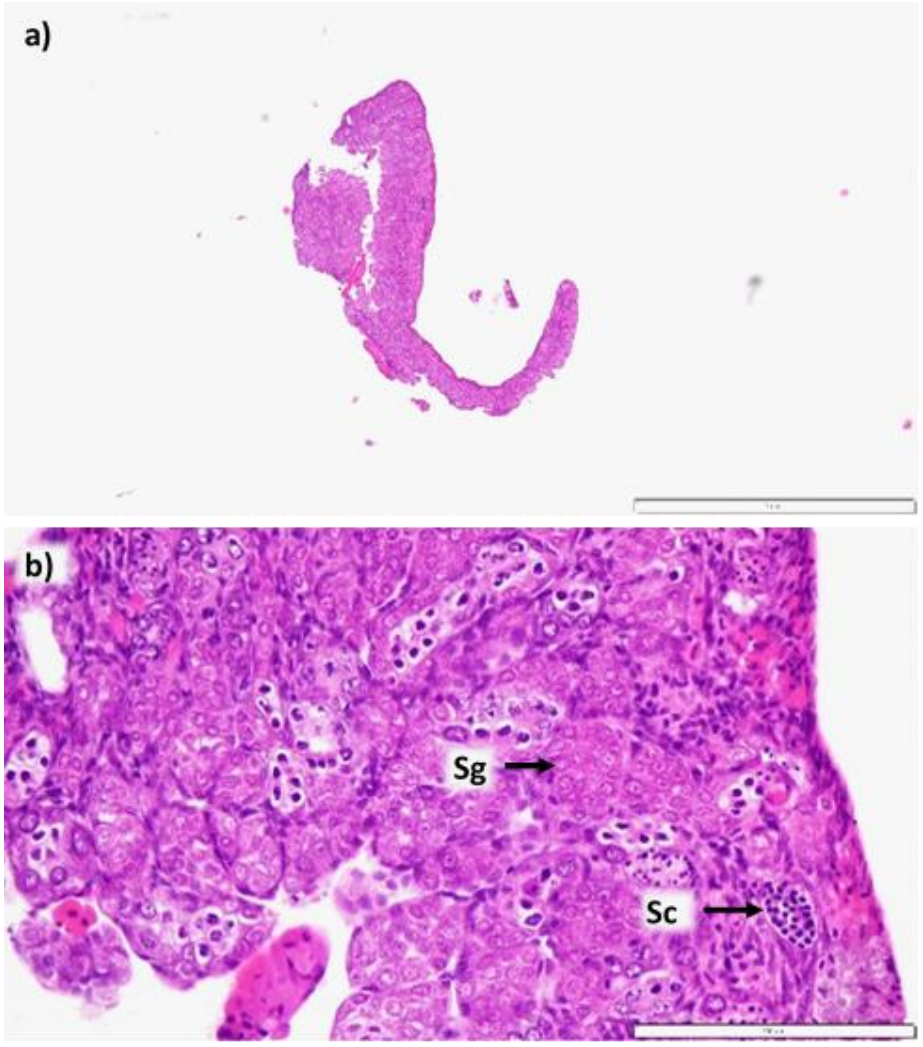

Figure 3.3: Transverse cross sections of a juvenile testis of *Notolabrus celidotus.*); a) whole section of sexually immature testis (scale bar – 1 mm); b) sexually immature testis containing spermatogonia and spermatocysts (scale bar – 100 µm). Sg – spermatogonia, Sc – spermatocyst.

3.3.2 Adult ovaries

Ovarian histology of the 11 sexually mature females over 140 mm revealed typical teleost ovarian structure with ovigerous lamellae projecting inward to a central lumen (Figure 3.4a&b). There was no evidence of coexisting spermatogenic tissues. A range of healthy oocytes ranging from perinucleolar to mature oocytes were observed (Figure 3.4b). Oocyte development was consistent along the length of the ovary (Figure 3.4c). The lumens of both lobes appear to converge to form a fused oviduct in the posterior region of the ovary (Figure 3.4d).

3.3.3 Adult testes

Both testis types contained spermatogenic cysts, enclosed in somatic Sertoli cells, that were arranged within lobules. Germ cells ranged from spermatogonia to mature sperm. Germ cells within individual cysts were of an identical developmental stage, whereas, adjacent cysts within an individual lobule contained different stages of germ cells simultaneously. Free spermatozoa had ruptured from their spermatocysts and were evident in the lumen of many lobules. Steroidogenic Leydig cells were present in the interstitial spaces between the lobule walls (Figure 3.5).

Two distinct gross morphological testicular structures were evident in sexually mature fish (> 145 mm). The first type of testis matched the type 1, solid testis, described in Chapter 2 (referred to as solid testis herein) and was more common in IP males (87.5 %) than in TP males (36.4 %). The solid testis was primarily characterised by central seminiferous, collecting ducts and blood vessels (Figure 3.6a&b). However, the thick central wall did not appear to be fused in all samples. When viewed along the longitudinal plane, lobules were observed, radiating laterally towards central collecting ducts and inner membrane (Figure 3.6c). When viewed from the posterior portion of the testis, where the two lobes had fused, mature sperm drained centrally from each lobe into a central vas deferens (Figure 3.6d). The external membrane of the solid testis was thin and closely resembled the germinal epithelium of the hollow testis (Figure 3.7a&b). Similarly, the central walls contained thick connective tissue and closely resembled the outer wall of the hollow testis (Figure 3.7c&d).

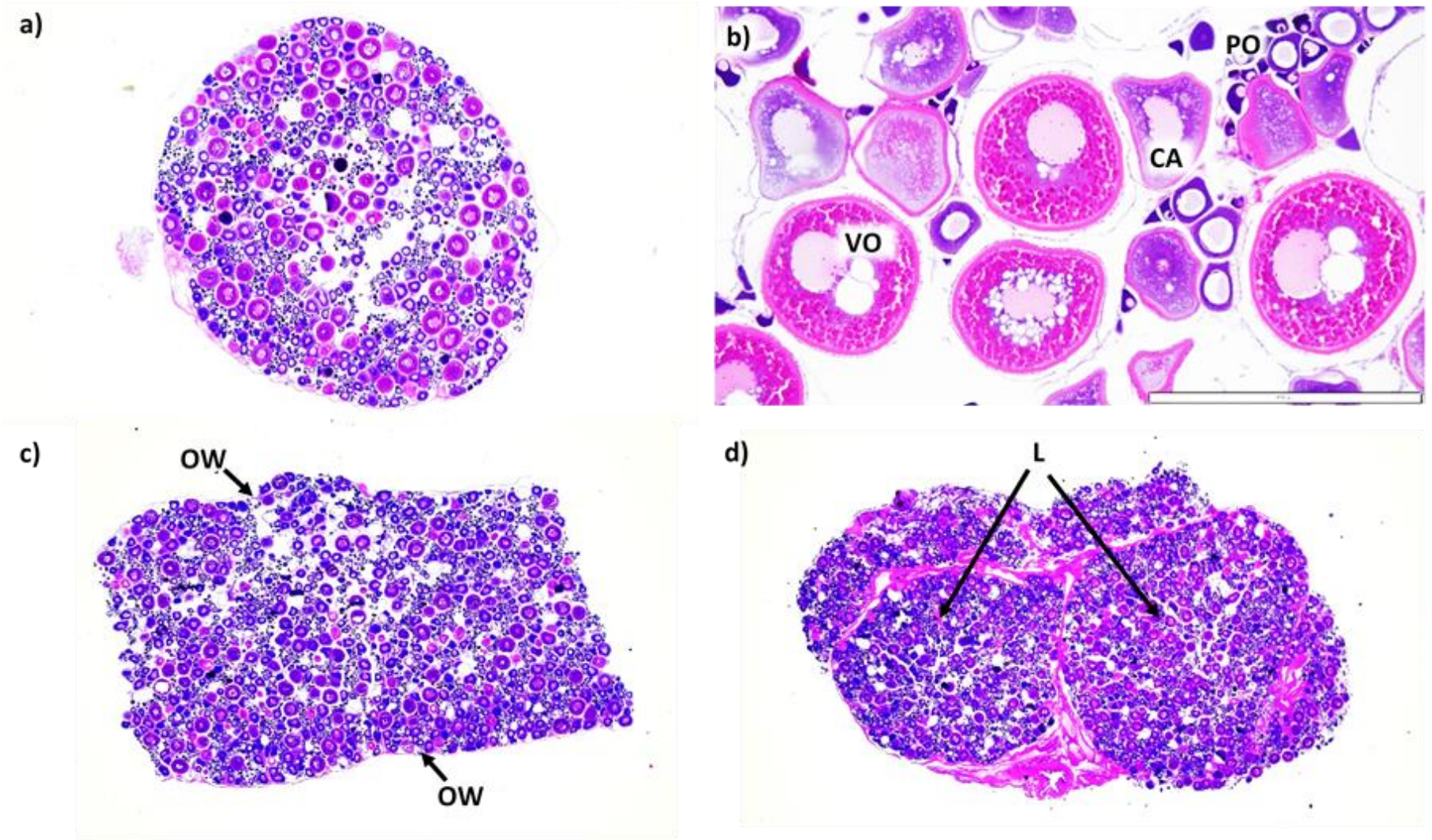

Figure 3.4: Adult ovary of *Notolabrus celidotus*. a) transverse cross section of lobe; b) multiple healthy oocytes at various stages of development (scale bar – 500 µm); c) mid-lobe dorsal plane; d) posterior transverse cross section, arrow pointing to where each lobe is beginning to converge PO – perinucleolar oocyte, CA – cortical alveoli, VO – vitellogenic oocyte, OC – ovarian cavity, OW – outer wall, L – lobe.

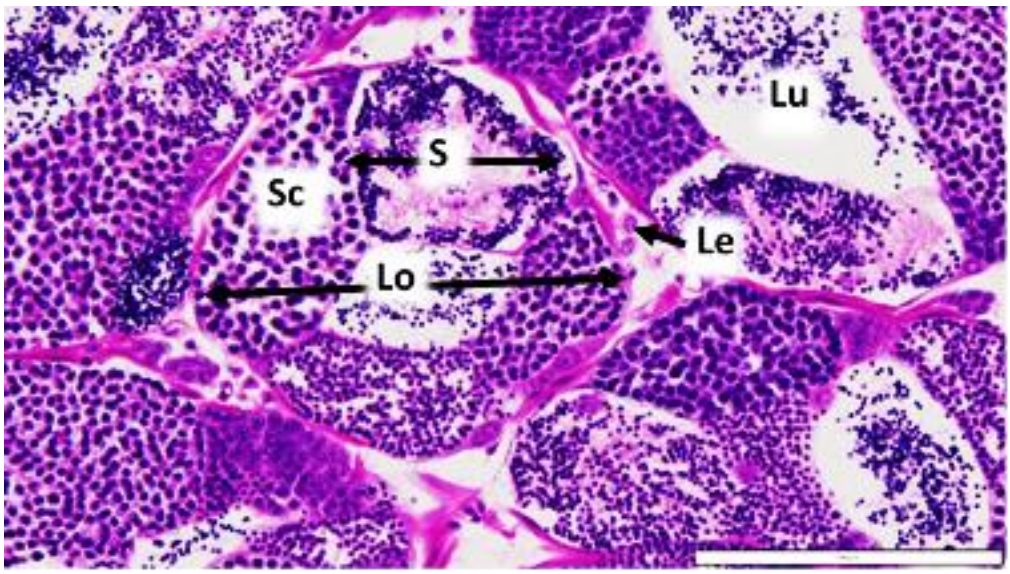

Figure 3.5: Spatial organisation of sexually mature testis of *Notolabrus celidotus* (scale bar – 100 µm). Sc - spermatocyst, S - Sertoli cell, Lo - lobule, Lu - lobule lumen, Le - Leydig cell.

The internal structure of the solid testis may be formed through the evagination of the testis (Figure 3.8). A rupture at a presumptive seam caused testis arms to fold back around themselves. As a result, the outer testis wall, blood vessels, and associated sperm ducts became centrally located, and the central testis walls became peripherally located to form a solid testis. The location of the presumptive seam was also located in the external membrane at the dorsal apex of the juvenile ovary, sexually mature ovary and the hollow testis (Figure 3.9). However, the ovary or hollow testis do not rupture or evaginate.

The second type of testis matched the type 2, hollow testis, described in Chapter 2 (referred to hollow testis in text) and was more common in TP males (63.6 %) than IP males (12.5 %). The hollow testis was characterised by a central remnant ovarian lumen (hollow cavity) and multiple collecting ducts around the periphery associated with the tunica albuginea (Figure 3.10a&b). Testicular tissue was arranged in large protuberances that loosely resembled lamellae projecting towards the lumen and were lined with a germinal epithelium. Similar to the solid testis, the longitudinal plane showed lobules radiated laterally towards the collecting ducts, however, instead of draining centrally, the lobules radiated towards the periphery where the sperm ducts had formed (Figure 3.10c). Collection ducts in the hollow testis appeared smaller than the solid testis; however, this was not measured. When viewed from the posterior portion of the testis, where the two lobes had fused, mature sperm drained from the periphery as a series of ducts into a central vas deferens (Figure 3.10d).

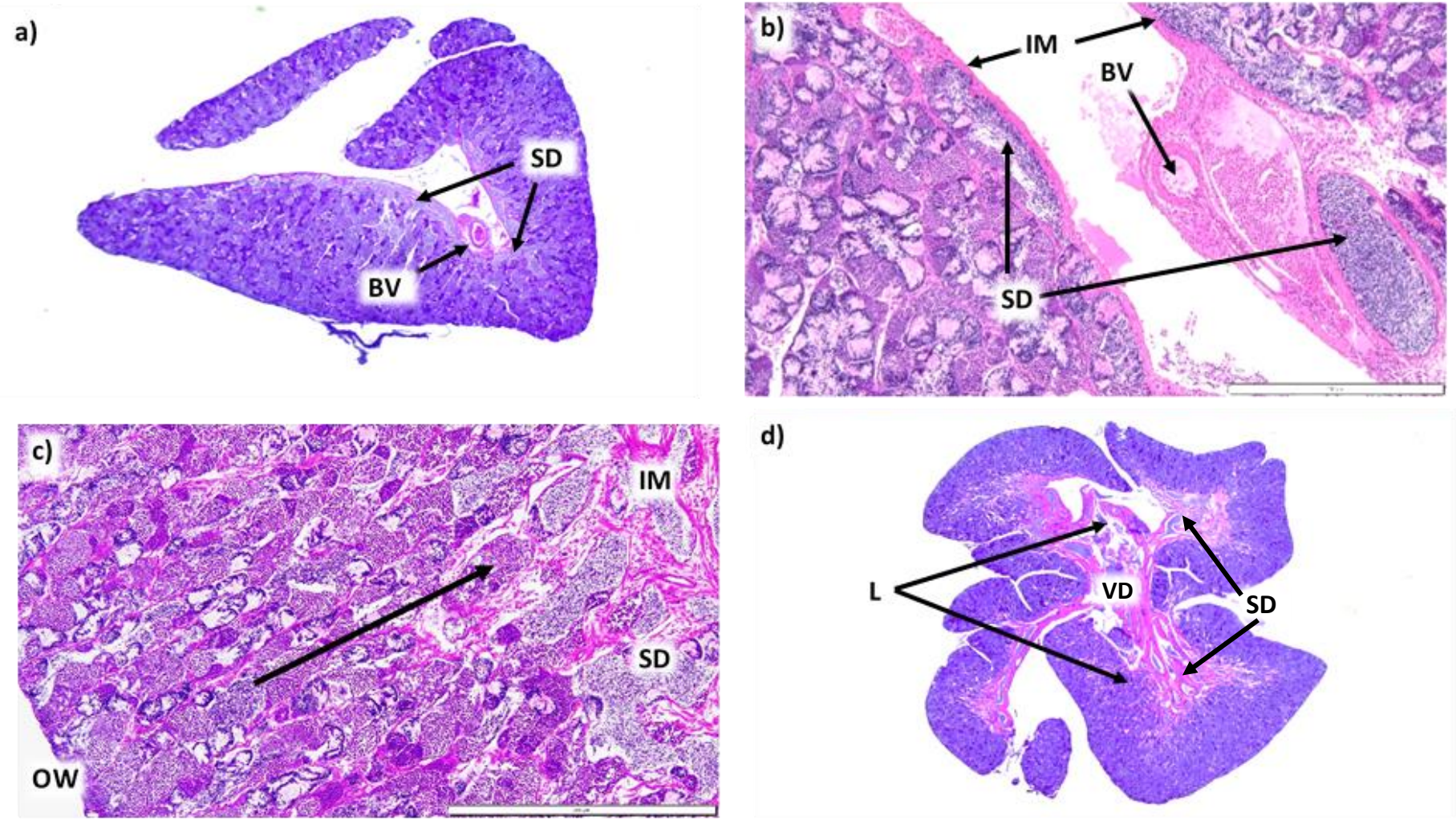

Figure 3.6: Type 1, solid testis – typical of initial phase (IP) males of *Notolabrus celidotus*. a) whole tissue piece of transverse cross section; b) closer examination of location of central blood vessel and sperm ducts (scale bar – 500 µm); c) sperm ducts radiating from the outer wall (left) towards central sperm ducts and inner membrane (right) viewed from the longitudinal plane (scale bar – 500 µm); d) posterior cross section showing centrally collecting sperm ducts towards central vas deferens. SD – sperm duct, BV – blood vessel, IM – inner membrane, OW – outer wall, L – lobe, VD – vas deferens.

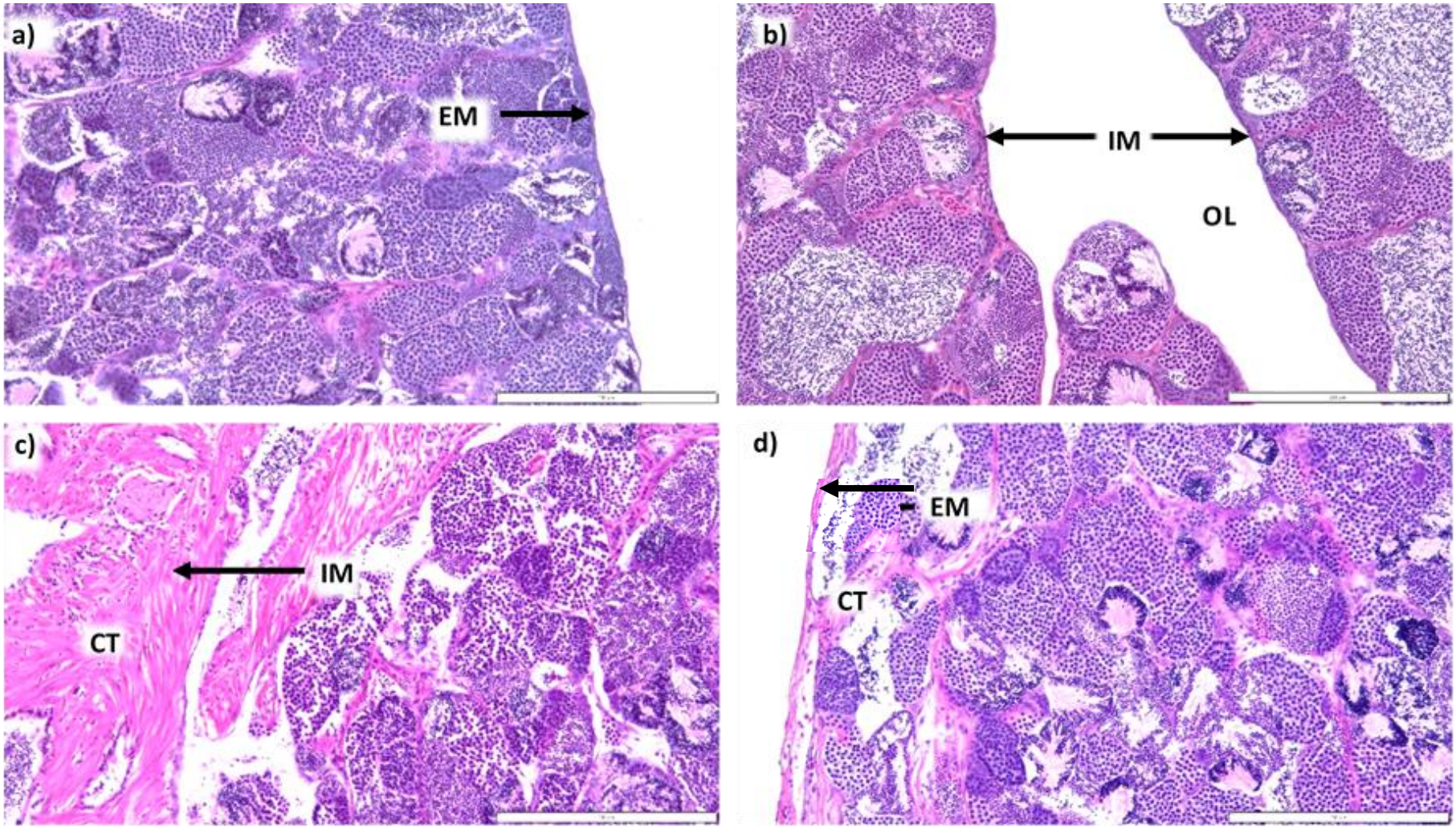

Figure 3.7: Comparison of the internal and external membranes between the two testis types of *Notolabrus celidotus*. a) thin external membrane of the type 1, solid testis, typical of initial phase (IP) males; b) thin internal membrane (germinal epithelium) of the type 2, hollow testis, typical of terminal phase (TP) males; c) thick connective tissue in the internal membrane of type 1, solid testis; d) thick connective tissue in the external membrane of type 2, hollow testis. EM – external membrane; IM – internal membrane, OL – ovarian lumen (hollow cavity), CT – connective tissue (scale bars all – 200 µm).

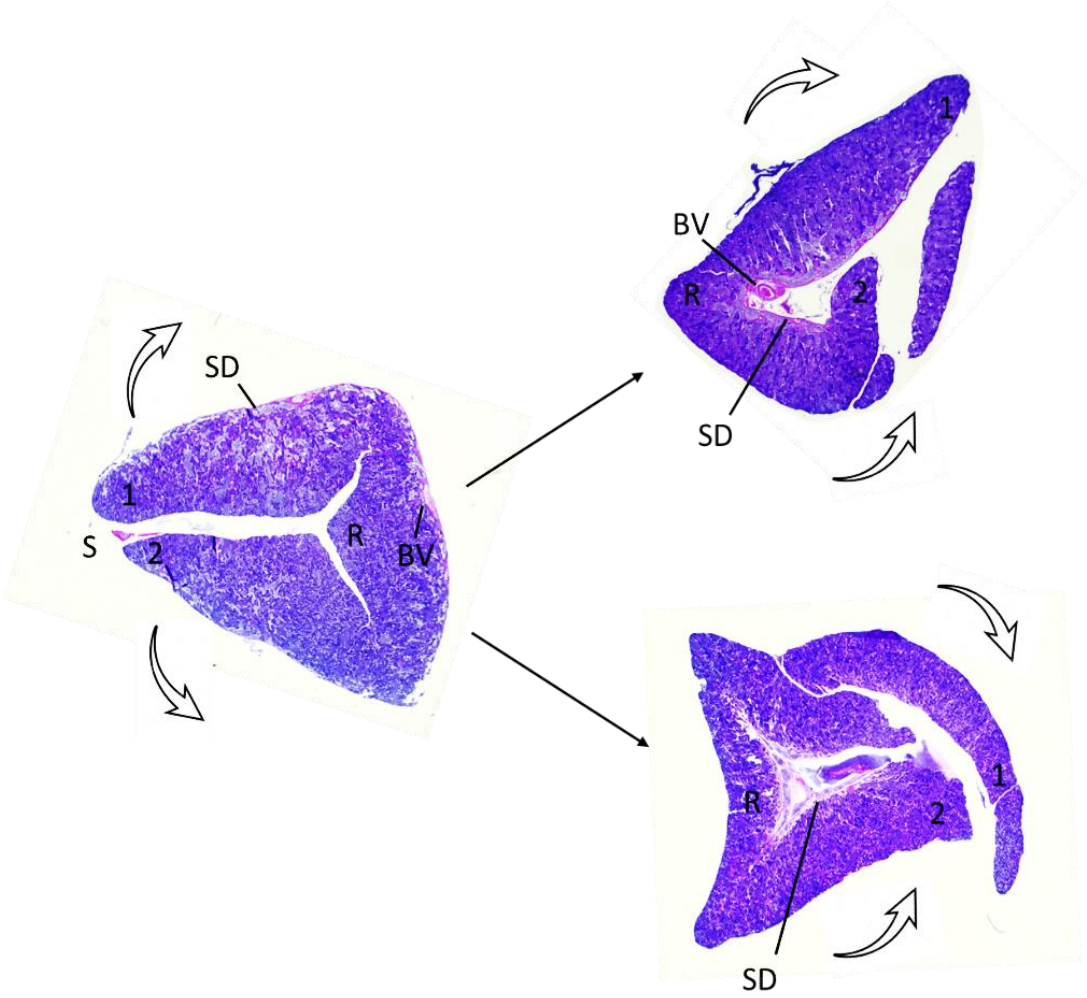

Figure 3.8: Representation of proposed testis evagination in *Notolabrus celidotus* to form a type 1, solid testis. Left histological transverse cross-section of an in-tact hollow testis (type 2) and right top and bottom transverse cross sections of two morphologies of solid testes with different shaped testis ridges. A rupture occurs along a weak presumptive seam on the dorsal apex and testis arms 1 and 2 fold back around themselves until they come together so the peripheral sperm ducts and blood vessels become centrally located and central testis wall becomes peripherally located. White arrows indicating direction of testis arm evagination. S – weak seam on the dorsal apex of the testis; R – testis ridge; 1 and 2 – testis arms; SD – sperm ducts; BV – blood vessel.

Figure 3.9: Weak point (presumptive seam) on the external membrane at the dorsal apex of *Notolabrus celidotus* gonads. a) juvenile ovary (scale bar – 1 mm); b) sexually mature ovary (scale bar – 500 µm); c) type 2, hollow testis, typical of terminal phase (TP) males (scale bar – 1 mm). EM – external membrane.

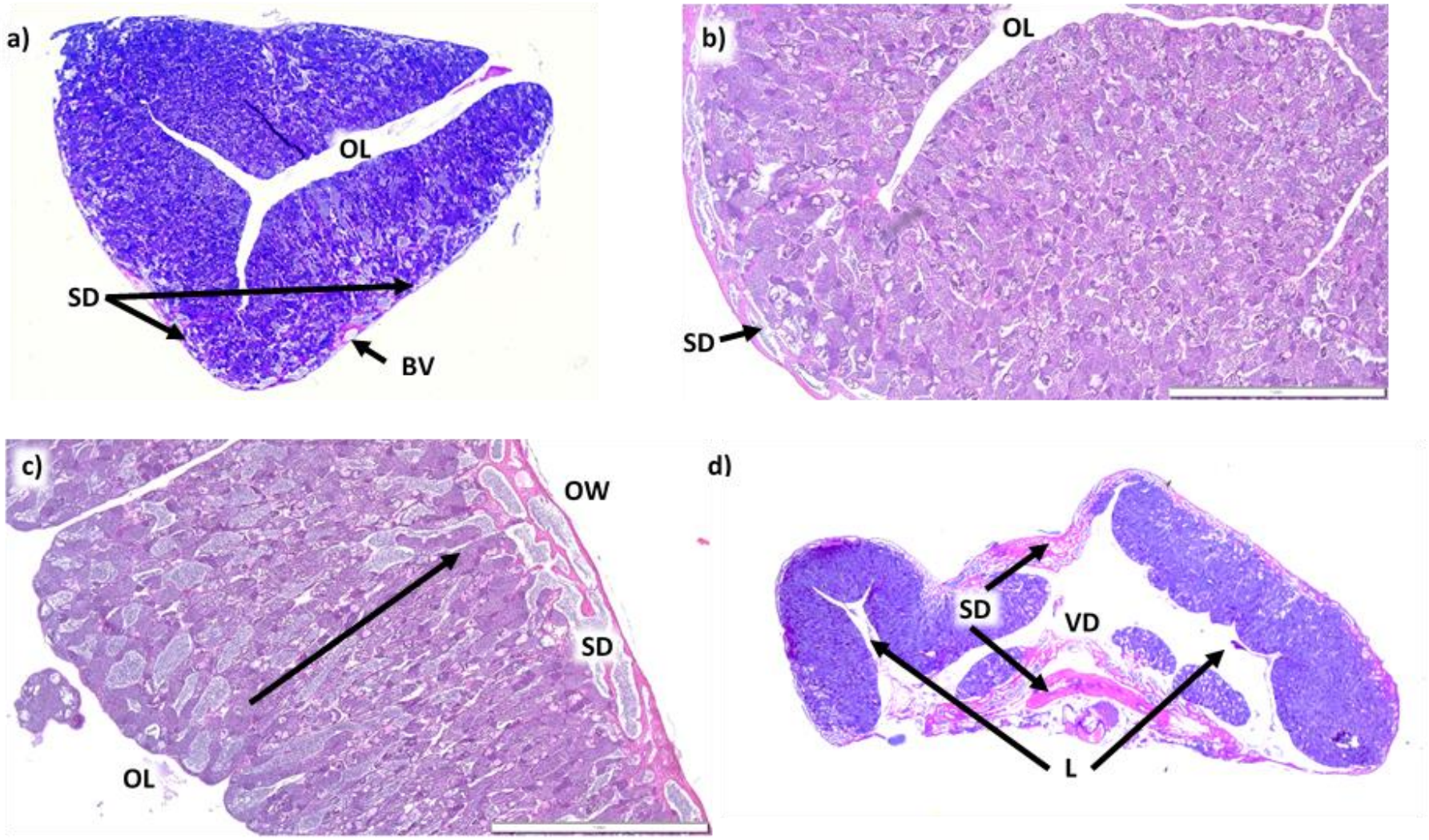

Figure 3.10: Type 2, hollow testis - typical of terminal phase (TP) males of *Notolabrus celidotus*. a) whole tissue piece of transverse cross section; b) closer examination of location of peripheral sperm ducts and remnant ovarian lumen (hollow cavity) (scale bar – 1 mm); c) sperm ducts radiating from the ovarian lumen (left) towards peripheral sperm ducts and outer wall (right) viewed from the longitudinal plane (scale bar – 1 mm); d) posterior cross section with peripherally draining sperm ducts towards central vas deferens. SD – sperm duct, BV – blood vessel, OL – ovarian lumen, L – lobe, OW – outer wall, VD – vas deferens.

3.4 Discussion

Histomorphometric analysis of gonads of the three sexual phenotypes of *N. celidotus* shows three gonadal ultrastructures, one ovary and two types of testes. The testis types matched the results found in Chapter 2 (type 1, solid testis, and type 2, hollow testis) and although more common in one colour phase than the other (solid testis common in IP males and hollow testis more common in TP males) the testis types did not always correspond with the external phenotype. The results provide further insight into the existence of an evagination process in the ontogeny of the testis of small males and evidence of diandry in *N. celidotus*.

3.4.1 Juveniles

To gain a better understanding of the ontogeny of the sexual phenotypes in *N. celidotus* it was important to look at the gonads of smaller, sexually immature fish. Sexual maturity in *N. celidotus* may occur between 100 – 110 mm (Jones, 1980); therefore, this size range was targeted. Although catching fish around this size range proved difficult, the five fish sampled between 88 to 115 mm had clearly sexually differentiated.

The ovaries of the four females are classified as sexually immature. The ovaries were dominated by differentiated perinucleolar oocytes which are in the primary growth stage of development (Patiño & Sullivan, 2002). No later developmental stage of oocyte was present, and there was no evidence of oocyte atresia. Oocytes at this stage of development are not sufficiently advanced enough for the current breeding season and are, therefore, classed as immature (Warner & Robertson, 1978; Lowerre-Barbieri *et al.*, 2011; Trip *et al.*, 2011; Farrell *et al.*, 2012). In female fish, early development is considered to have occurred when the first clutch of oocytes containing cortical alveoli appear, and puberty is deemed to have occurred when oocyte development reaches vitellogenic stages (Okuzawa, 2002; Patiño & Sullivan, 2002; Lowerre-Barbieri *et al.*, 2011). In *N. celidotus*, large females socially inhibit ovarian maturation of smaller females in order to spawn earlier in the season, resulting in small females ripening as late as December, which is towards the end of spawning season (Jones & Thompson, 1980). If the small females collected in December in the present study were participating in the current late spawning season, more advanced oocyte development

should be evident. Therefore, the histological evidence of oocyte development and time of the spawning season suggests that it is highly unlikely that these fish have entered puberty and remain sexually inactive.

Jones (1980) reported that all male *N. celidotus* have a secondary testis because of an evagination process where the ovary splits open along a weak seam to form a testis in small IP males. Based on evagination of the ovary, he determined all males are derived from females and consequently, labelled the species as monandric protogynous hermaphrodites. Furthermore, he also states that IP males mature at a similar time as females, if not, slightly before. However, using contemporary criteria to define monandry, all males must have reproductively functioned as female prior to sex change (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Devlin & Nagahama, 2002). Therefore, considering that females of a similar size are sexually immature, it is highly unlikely that IP males, such as the one in the present study, have previously functioned as females before sex change, and therefore, *N. celidotus* does not meet the criteria for monandry.

Additionally, passing through an initial female phase before gonad maturation is not uncommon in early ontogeny. During gonad development of the diandric bluehead wrasse, *Thalassoma bifasciatum*, oocytic gonads occur during immaturity that later develops into a testis before sexual maturity (Shapiro & Rasotto, 1993). The existence of early oocytic gonads before testis maturation is also observed in zebrafish, *Danio rerio* (Orban *et al.*, 2009), European eel, *Anguilla anguilla* (Colombo & Grandidr, 1996) and Sumatra barb, *Barbus tetrazona tetrazona* (Takahashi & Shimizu, 1983). Furthermore, in the diandric, protogynous reticulated damselfish, *Dascyllus reticulatus,* ovarian state in all immature gonads progress to primary growth follicles and development of an ovarian lumen, before oocyte atresia and development of a sexually mature testis (Asoh, 2005), this also occurs in the diandric, protogynous chocolate hind *Cephalopholis boenak* (Liu & Sadovy, 2004). Additionally, although being classed as diandric, these two species do not have a difference in testis structure between primary and secondary males. Therefore, the IP male testis of *N. celidotus* could still go through an early female phase as a normal part of ontogeny and the gonad could evaginate to form a primary testis without reproductively functioning as a female.

The single small male (107 mm) found in this study had technically reached puberty as cysts of spermatocytes were evident, indicating that the reproductive axis had been activated (Okuzawa, 2002). However, there was no clear sperm collection structure or mature sperm, and the basic structure of the gonad suggests that, although this fish was likely to be pubertal, it was unlikely to be breeding in the current spawning season. Furthermore, the testis did not contain any features of a secondary testis or indication of former female function (Shapiro, 1979; Cardwell, 1989; Sadovy & Liu, 2008). In particular, there was no evidence of oocytic tissues, oocyte atresia, or peripheral sperm ducts. The presence of a central ovarian lumen could not be determined as the tissue did not remain intact during processing. Collectively, this evidence strongly supports the development of primary males around the onset of puberty in *N. celidotus*.

3.4.2 Adult ovaries

Histology confirmed *N. celidotus* females over 145 mm were all sexually mature, and no transitional ovaries were observed. Nests of oogonia were visible within the germinal epithelium. Ovaries contained oocytes across a range of developmental stages from perinucleolar to maturing oocytes. Oocytes appeared to represent distinct clutches of oocytes that had been recruited into development. The range of oocyte stages developing in batches implies that *N. celidotus* have multiple group synchronous development and likely spawn batches of eggs during the spawning season. Multiple group synchronous ovarian development is common in teleost (Wallace & Selman, 1981) and is observed in other temperate species that spawn several times over a short annual breeding season, such as Ballan wrasse, *Labrus bergylta* (Muncaster *et al.*, 2010), snapper, *Pagrus auratus* (Scott *et al.*, 1993) and striped trumpeter, *Latris lineata* (Bransden *et al.*, 2007).

3.4.3 Adult testes

Histological evaluation of the testes collected of sexually mature *N. celidotus* showed that although there were two main types of testis, in general, the lobular structure and the associated somatic and germ cell arrangement were similar. Both types of testis contain spermatogenic cysts consisting of germ cells enclosed in somatic Sertoli cells. Sertoli cells provide a critical function for the maintenance of germ cells (Grier, 1981;

Schulz *et al.*, 2010; Uribe *et al.*, 2014). Also, Sertoli cells serve as a phagocytotic role in the removal of degenerating germ cells following the conclusion of breeding season (Grier, 1981; Schulz *et al.*, 2010; Uribe *et al.*, 2014). Steroidogenic Leydig cells were identified by their polygonal shape and location in the interstitial spaces between lobules (Grier, 1981; Nagahama, 1983). All germ cells within a cyst were at the same stage of development and neighbouring spermatocysts at different stages of development. Spermatocysts were arranged in a lobular structure. Therefore, the spatial arrangement of cysts in *N. celidotus* testis is an unrestricted lobular type testis described by Grier (1981) and is similar to other wrasse species (Nagahama, 1983; Candi *et al.*, 2004; Alonso-Fernández *et al.*, 2011).

Although there is a similarity in the spatial arrangement of germ cells and somatic elements in all testis of *N. celidotus*, the ultrastructure of the testis was noticeably different. In both phenotypes, the lobules are arranged radially and terminate in seminiferous collecting ducts. However, the direction of the lobule radiation is opposed in the two phenotypes. In the solid testis, the lobules radiate towards central ducts, and in the hollow testis, the lobules radiate towards peripheral ducts. While there is no difference in testis phenotype in some diandric species (Liu & Sadovy, 2004; Asoh, 2005; Alonso-Fernández *et al.*, 2011), more typically, a primary testis is characterised by a large, solid testis with centrally located sperm ducts and draining into a central vas deferens (Choat & Robertson, 1975; Dipper & Pullin, 1979; Sadovy & Shapiro, 1987; Hourigan *et al.*, 1991; Shapiro & Rasotto, 1993; Matsuyama *et al.*, 1997; Godwin *et al.*, 2003). The structural arrangement of the solid testis type of *N. celidotus* is consistent with primary male testes described in several other diandric protogynous hermaphrodites (Dipper & Pullin, 1979; Bentivegna & Rasotto, 1983; Hourigan *et al.*, 1991; Matsuyama *et al.*, 1997). In contrast, the structural arrangement of the hollow testis is consistent with a secondary testis commonly described in several monandric and diandric protogynous species (Dipper & Pullin, 1979; Bentivegna & Rasotto, 1983; Hourigan *et al.*, 1991; Matsuyama *et al.*, 1997; Cossington *et al.*, 2010). The secondary testicular structure arises through protogynous sex change from an ovary that has restructured into a functional testis and is typically characterised by a central remnant ovarian lumen, peripheral sperm ducts throughout the old ovarian wall and a vas deferens made up of several peripherally draining ducts (Choat & Robertson, 1975; Dipper & Pullin, 1979; Sadovy & Shapiro, 1987; Hourigan *et al.*, 1991; Shapiro &

Rasotto, 1993; Matsuyama *et al.*, 1997; Godwin *et al.*, 2003). Therefore, the differences in the ultrastructure of the two *N. celidotus* testis phenotypes are consistent with those described in other diandric fish with primary and secondary testes.

The evagination process in the solid testis seems unique or is not described in many other species. After close examination of the gross morphology and internal structure of the solid testis, it was hypothesised that an evagination process could result in the testis folding back around itself to centrally locate the sperm ducts and blood vessels and create the unique and complex morphologies observed in this phenotype. The thin germinal epithelial-like membrane around the exterior was similar to the germinal epithelium of the hollow testis. Furthermore, the thickened connective tissue with significant blood vessels and associated sperm ducts in the centre of the solid testis was similar to the thickened connective tissue, blood vessels, and associated sperm ducts in the tunica albuginea of the hollow testis. Collectively, these points indicate that the solid testis is an inverted version of the hollow, secondary testis. Similarly, Jones (1980) reported that the majority of testes he had examined had whole intact testes; however, a small proportion of the smaller males presented with evaginated testes. The evaginated testes Jones (1980) describes match the solid testis described in further detail in this study.

It is proposed that evagination is caused by rupture of a short region of thin integument on the dorsal apex of the testis. The inside of this region of membrane appears to be associated with a thin extension of the lumen that is found in juvenile ovaries, sexually mature ovaries and in-tact hollow testes. If the default immature gonad development is a basic ovarian type as in other teleost (Takahashi & Shimizu, 1983; Shapiro & Rasotto, 1993; Liu & Sadovy, 2004; Asoh, 2005), then the thin membrane on the dorsal apex is likely to be a feature in all juvenile gonads during early ontogeny. It is possible that with the proliferation of spermatogenic tissues when the testis is small, weakens the membrane causing a rupture at the thin point. The rupture of the membrane then causes the evagination process and solid testis formation associated with early IP male development. Whereas, in the TP male testis, the external membrane could thicken overtime during the mature ovarian stage and not experience the same pressure from spermatogenic tissue proliferation as in the

smaller testis of an IP male at maturity, therefore, not resulting in rupture of the membrane. While this explanation remains hypothetical, it fits with the internal and external morphology of these gonad types.

3.4.4 Summary

The testes in *N. celidotus* are reproductively comparable; both designed for producing viable sperm to reproduce. However, there are fundamental differences in morphology that appear to have arisen through testis evagination, and these differences are designed for different reproductive strategies and different fertilisation success. Due to greater sperm competition as a small male, the structure of central sperm ducts in a primary testis have a greater potential for increased sperm production and delivery which enhances fertilisation opportunities during sneaking or streaking spawning events (Choat & Robertson, 1975; Warner & Robertson, 1978; Hourigan *et al.*, 1991). Whereas, due to the reduced sperm dilution as a large male, peripheral sperm ducts in a secondary testis are designed for greater control in sperm delivery which would be an advantage during pair spawning with a single female (Choat & Robertson, 1975; Warner & Robertson, 1978; Hourigan *et al.*, 1991). Therefore, in duel male systems, being in the less favourable reproductive position and increased sperm dilution leads to the selection of a testis capable of greater sperm delivery to enhance reproductive success in smaller males.

Chapter 4

Global DNA methylation levels in the gonad and brain of *Notolabrus celidotus*

4.1 Introduction

One of the most striking things about teleost fish is the vast diversity of reproductive patterns and plasticity of sexual phenotypes. Unlike mammalian or bird sexual phenotypes, which are fixed for life at fertilisation (Kraak & Pen, 2002; Graves, 2008), fish reproductive patterns can range from fixed sexes to several kinds of hermaphroditism; simultaneous, protogyny, protandry or changes between male and female can occur bidirectionally (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Devlin & Nagahama, 2002; Helfman *et al.*, 2009).

Protogynous species exhibit a further range of gonadal development and sexual phenotypic expression. Typically, most of the population will sexually mature as females, and if the environmental or developmental conditions are right, they have the potential to change sex to male (Dipper & Pullin, 1979; Devlin & Nagahama, 2002; Frisch, 2004; Munday *et al.*, 2006). However, some protogynous species have more than one phenotype of male. Terminal phase (TP) males generally hold the most dominant position in a group, they have a large body size and are often brightly coloured, which is preferred by females and is associated with greater reproductive success. TP males typically use aggressive behaviours to retain their position in a group and regulate a stable social structure. Initial phase (IP) males use female mimicry (body size, often dull colour and non-territorial behaviour) to increase their reproductive success. IP males blend into groups of females and take advantage of pair spawning events by sneaking and adding their sperm to the mix of the larger TP male's sperm in the water column (Jones, 1980; Nakamura *et al.*, 1989; Taborsky, 1994; Devlin & Nagahama, 2002). Therefore, protogynous species can have more than one reproductive phenotype within a population.

Social status and seasonal cycles are essential to the regulation of sex in many protogynous hermaphrodites. Socially induced sex change has been demonstrated in

several protogynous species where the loss or removal of a dominant male can initiate rapid behavioural and physiological changes in the most dominant female and lead to a change in sex (Warner & Robertson, 1978; Devlin & Nagahama, 2002; Munday *et al.*, 2006; Godwin, 2009). Changing sex is an example of a dramatic shift in whole organism functionality; including changes to behavioural patterns, endocrine production, gene expression and gonad and body morphology (Godwin, 2009; Todd *et al.*, 2016; Gemmell *et al.*, 2019; Todd *et al.*, 2019). Periodic changes occur in the gonads of seasonal spawners where the ovary goes through seasonal cycles of regression and ripening, and sex change typically occurs during or just after the breeding season (Jones, 1980; Kobayashi & Suzuki, 1990; Candi *et al.*, 2004). The timing of the reproductive cycle in most teleost is regulated by changes in photoperiod (Björnsson *et al.*, 1998; Muncaster *et al.*, 2010). However, the precise mechanisms of how environmental cues, such as social conditions and photoperiod, are transduced into a change of sexual phenotype or reproductive phase are unknown. However, epigenetic regulation is likely involved in perception and translation, leading to change of sex.

4.1.1 Epigenetics

One of the emerging areas of research in sex changing fish, which has received growing interest over the last two decades, is understanding the role of epigenetic mechanisms (Burris & Baccarelli, 2014). Epigenetics refers to the molecular mechanisms which can physically modify DNA to shape the phenotype of an organism without altering the underlying genotype (Piferrer, 2013). These processes are mitotically heritable and are crucial to regulating gene expression relating to organism development, and physiological responses to internal and external environment (Piferrer, 2013; Duncan *et al.*, 2014; Best *et al.*, 2018). Changes in gene expression can occur through several different epigenetic mechanisms, including DNA methylation, histone modifications and the action of non-coding RNAs (Piferrer, 2013; Stoccoro *et al.*, 2013; Burris & Baccarelli, 2014; Labbé *et al.*, 2017; Best *et al.*, 2018). Therefore, internal and external stimuli experienced by a sex changing fish at varying stages of ontogeny may lead to development of sexual phenotype mediated by epigenetic mechanisms.

4.1.2 DNA methylation

DNA methylation is the best understood form of epigenetic modification (Piferrer, 2013; Best *et al.*, 2018; Fellous *et al.*, 2018), and has been linked in several studies regarding sex change or behaviour in fish (Navarro-Martin *et al.*, 2011; Shao *et al.*, 2014; Lenkov *et al.*, 2015; Best *et al.*, 2018; Todd *et al.*, 2019), so is, therefore, a good starting point for understanding the relationship between reproductive phenotypes and epigenetic modifications in sex changing fish. The action of DNA methylation transfers a methyl group to the fifth carbon of cytosine to guanine bases (CpG dinucleotides) to convert cytosine to 5-methylcytosine, resulting in epigenetic silencing of genes (Piferrer, 2013; Fellous *et al.*, 2018). Changing the state of DNA methylation regulates gene expression through altering the accessibility of transcription at gene promoter regions and modifying chromatin compaction (Stoccoro *et al.*, 2013). In summary, hypermethylation of DNA is typically related to inactive genes, and hypomethylation of DNA is related to active genes.

DNA methylation is a relatively new area of research in teleost fish. However, there is evidence to suggest that DNA methylation plays a role in behaviour and the regulation of sex. When European sea bass, *Dicentrarchus labrax,* are exposed to high temperatures before gonad differentiation, DNA methylation at the gonadal aromatase promoter (*cyp19a*) occurs (Navarro-Martin *et al.*, 2011). Aromatase is an essential enzyme in the bioconversion of androgens into oestrogens and is therefore vital to ovarian development (Nakamura *et al.*, 1998; Navarro-Martin *et al.*, 2011; Todd *et al.*, 2016). Therefore, when aromatase is downregulated via DNA methylation mediated gene silencing, the fish subsequently masculinises (Navarro-Martin *et al.*, 2011). In the ricefield eel, *Monopterus albus*, DNA methylation is lower at the gonadal aromatase promoter in the ovotestis and testis than in the ovary, and by using a DNA methylation inhibitor (5-aza-2'-deoxycytidine) natural sex change was reversed (Zhang *et al.*, 2013). Furthermore, DNA methylation regulated behaviour been demonstrated by chemically manipulating methylation levels in male African cichlid, *Astatotilapia burtoni.* When global DNA methylation was promoted with L-methionine, males were significantly more likely to become dominant, and when global DNA methylation was inhibited with zebularine, males were more likely to become subordinate (Lenkov *et*

al., 2015). Therefore, DNA methylation may influence the regulation of or facilitate sexual fate and dominant behaviours at a cellular level.

4.1.3 Background and aims

Notolabrus celidotus is a protogynous hermaphrodite, and sex change has a strong link to the social structure within the population. *N. celidotus* has three sexual phenotypes; females, IP males, and TP males (Choat, 1965; Jones, 1980; Moraes, 2019). *N. celidotus* has periods of gonad regression and ripening due to seasonal spawning between July until November (Jones, 1980). Despite many studies on sex changing fish, questions remain about the involvement of molecular mechanisms between reproductive phenotypes determining sexual fate. However, before targeted investigation into DNA methylation associated with sex change and specific gene expression can be performed in *N. celidotus*, it is essential to determine baseline levels and differences in global DNA methylation between the three sexual phenotypes.

4.1.3.1 Aim and objectives

To determine the differences in global (whole genome percentage methylation) DNA methylation in brain and gonad tissues between the three sexual phenotypes of the temperate protogynous wrasse, *Notolabrus celidotus*. Results have been gathered from a range of past investigations and will provide preliminary baseline DNA methylation levels between sexes and colour phases to inform future research and further develop *N. celidotus* as a model species for sex change in temperate fish.

- Is there a difference in brain global DNA methylation between sexual phenotypes?
- Is there a difference in gonad global DNA methylation between sexual phenotypes?
- Is there a seasonal effect on global DNA methylation in the ovary?

4.2 Methods

To determine the differences in DNA methylation between the three sexual phenotypes of *Notolabrus celidotus* low-coverage bisulfite sequencing of DNA was performed on data derived from eight brain tissue samples and 56 gonad tissue samples. Brain samples consisted of one IP male, four females, and three TP males

(Table 4.1). Gonad samples consisted of four IP male, 49 females, and three TP males (Table 4.2). Ovary samples were further divided into fish from spawning season (October – December) and non-spawning season (July and August) for analysis.

Table 4.1: Specimen source and collection dates of brain tissue samples of *Notolabrus celidotus* for DNA methylation analysis. IP - initial phase, TP terminal phase.

Specimen Source	Sample Date	Female	IP male	TP male
Social Induction Study	Dec, 2015	0	1	0
Incidental Capture	Nov, 2016	4	0	3

Table 4.2: Specimen source and collection dates of gonad samples of *Notolabrus celidotus* for DNA methylation analysis. IP – initial phase, TP – terminal phase.

Specimen Source	Sample Date	Female	IP male	TP male
Social Induction Study	Oct & Dec, 2015	3	1	0
Incidental Capture	Nov, 2016	4	0	3
Zebularine Pilot Study	Jul & Aug, 2019	42	3	0

The following details the experimental conditions of specimens that the tissue samples were derived from. The social induction study involved captive *N. celidotus* and manipulation of social groups by removal of a dominant male, leaving a group of females in permissive sex change conditions. Control tanks were also set up where the dominant male was not removed from female groups, leaving non-permissive sex change conditions. The gonad and brain samples obtained from this study were not in the process of sex change (as confirmed by gonad histology).

The zebularine pilot study was performed to understand the effects of chemically inhibiting global DNA methylation. Three treatment groups were administered; a 0, 200, or 400 ug/g dose of zebularine via abdominal subcutaneous injection to determine dosage level effects and fish were sampled on days 1, 2, 5, 10 and 20 post-injection to determine zebularine effects on methylation levels over time. The zebularine treated fish were determined fit for this study because gonad methylation levels were not significantly different between treatment dose (Kruskal-Wallis; $H = 0.98$, $p = 0.61$) or days post-injection (Kruskal-Wallis; $H = 2.0$, $p = 0.74$). Therefore, zebularine did not affect the baseline levels of gonad DNA methylation and allowed the samples to be utilised in this study. Upon dissection of the 45 *N. celidotus* used for the zebularine study, it was apparent that there were three IP males amongst the group, histological analysis was used to confirm this assumption. The IP males were

one from each zebularine treatment group, and their gonad DNA methylation levels were similar, so were included in this study.

DNA methylation data was generated by our collaborators at the University of Otago's Department of Anatomy and procedures were DNA extraction, and bisulfite sequencing followed methods described in Todd *et al.* (2019). Data produced was measured as CG (cytosine-guanine) methylation, which is a more specific term for DNA methylation because methylation occurs at CG bases. Herein, DNA methylation or simply methylation will be used to refer to CG methylation.

4.2.1 Data analysis

Dose and days post-injection of zebularine (methods section) did not meet the assumptions for parametric testing, so alternatively a Kruskal-Wallis test was used. An ANOVA was used to examine global differences in methylation in the brain amongst phenotypes. Homogeneity of variance could not be fixed by transformation for gonad methylation comparison between sexual phenotypes, so alternatively a Welch's F-Test was used and a Tukey test for posthoc testing. An independent T-test was used to compare methylation of ovary samples between spawning and non-spawning season. Data presented as mean ± standard error (SE) and statistical significance was set at $p < 0.05$.

4.3 Results

To identify the differences in DNA methylation levels between females, IP males and TP males of *Notolabrus celidotus* low-coverage bisulfite sequencing of gonad and brain tissues were performed to determine the genome-wide percentage of DNA methylation (measured as CG methylation).

Brain methylation did not differ significantly between females (71.4 ± 1.06 %), TP males (72.2 ± 0.5 %) or IP males (75.1 %) ($F = 1.82$, $p = 0.26$) (Figure 4.1).

Global methylation differed significantly between ovaries and testes (Welch $F = 366.8$, $p = < 0.001$) (Figure 4.2). Ovarian methylation (53.7 ± 1.16 %) was lower in comparison to IP male testis (82.2 ± 1.39 %) and TP male testis (86.8 ± 0.21 %) ($p = < 0.001$). However, testis methylation did not differ between males of either colour phase ($p = 0.75$). Females sampled during spawning season (67.3 ± 1.27 %) had significantly

higher methylation than females sampled outside of spawning season (51.4 ± 0.96 %) (df = 47, p = < 0.001) (Figure 4.3). The difference between spawning and non-spawning females did not affect the differences detected between ovaries and testes (Welch F = 340.67, p = < 0.001, spawning female's and non-spawning female's methylation both less than testis methylation).

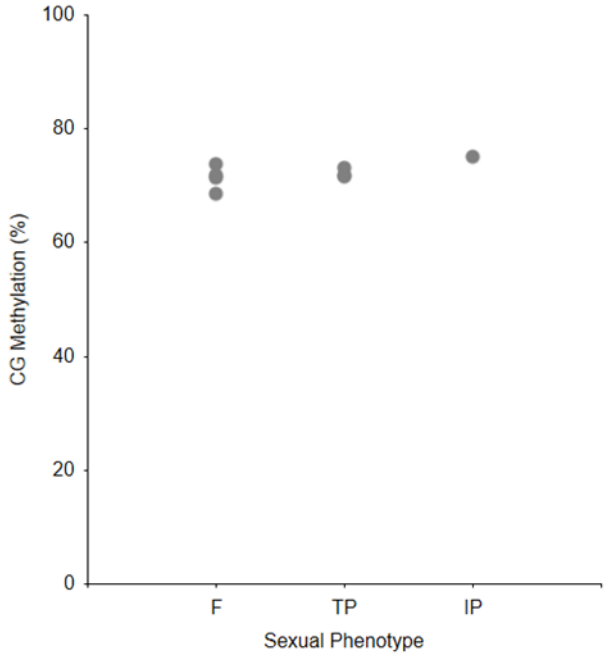

Figure 4.1: Global brain CG methylation of the three sexual phenotypes of *Notolabrus celidotus* during late spawning. F - female; TP - terminal phase; IP - initial phase; CG – cytosine- guanine

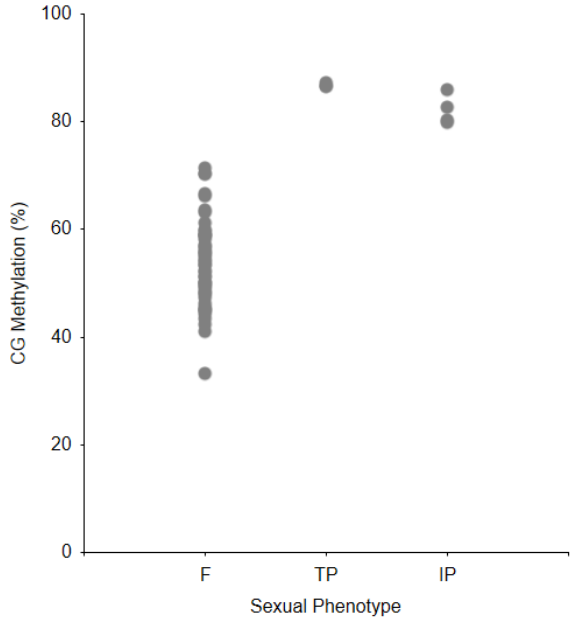

Figure 4.2: Global gonad CG methylation of the three sexual phenotypes of *Notolabrus celidotus*. F - female; TP - terminal phase; IP - initial phase; CG – cytosine-guanine

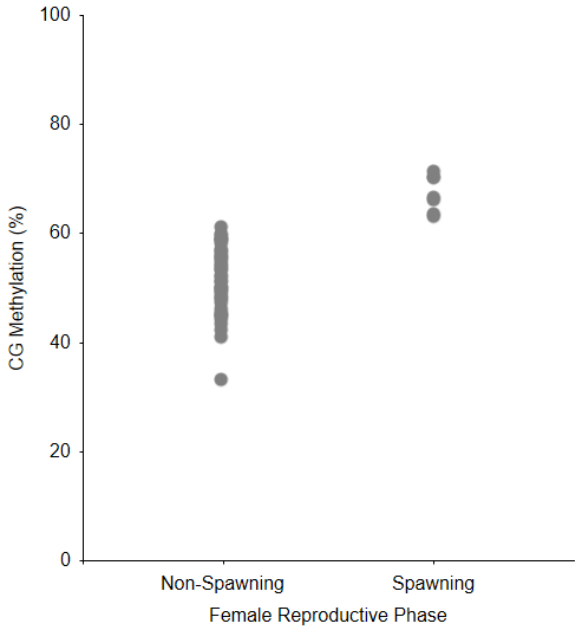

Figure 4.3: Global gonad CG methylation between females in non-spawning and spawning seasons in *Notolabrus celidotus*. CG – cytosine-guanine

4.4 Discussion

DNA methylation is a key epigenetic mechanism in maintaining and regulating gene expression leading to cellular fate and organism phenotype (Piferrer, 2013; Duncan *et al.*, 2014; Best *et al.*, 2018; Todd *et al.*, 2019). To determine differences in global DNA methylation levels among sexual phenotypes in *Notolabrus celidotus* genome-wide methylation in the brain and gonad were measured. The data for this study was drawn from three experiments, from three separate years, spanning over different reproductive seasons, and each study had different experimental conditions. Despite differences in sample origin, there were clear and consistent patterns in DNA methylation.

4.4.1 Global DNA methylation does not differ in the brain

Results confirmed that global brain DNA methylation between the three sexual phenotypes of *N. celidotus* did not differ. Brain DNA methylation levels, and the lack of sex-specific differences, are consistent with patterns observed in bluehead wrasse, *Thalassoma bifasciatum* (Todd *et al.*, 2019), zebrafish, *Danio rerio* (Chatterjee *et al.*, 2016) and mangrove rivulus, *Kryptolebias marmoratus* (Fellous *et al.*, 2018). Although no sex-specific differences were detected in overall DNA methylation in the brain, if subtle gene specific differences existed, they would not be picked up as a genome wide analysis. Therefore, DNA methylation does not have a significant impact on sex-specific differences in the brain among sexual phenotypes and to pick up on subtle differences between sexes, gene-level methylome sequencing would be required to detect specific gene differences.

4.4.2 Gonad DNA methylation is greater in the testis than the ovary

Sex-specific DNA methylation was detected in the gonads of *N. celidotus*. The ovary was characterised by low methylation states, and both IP and TP male testes had similarly high methylation states. If there were only subtle differences between sexes in methylation patterns, they would not be picked up at the global scale. Therefore, significant differences in global methylation between the ovary and testis suggest that methylation plays an important role in ovarian and testis fate. Gonadal DNA methylation levels in *N. celidotus* are consistent with patterns observed between sexes in the protogynous bluehead wrasse, *T. bifasciatum* (Todd *et al.*, 2019). Sex-specific

DNA methylation is also observed between pseudo-males, males and females in the half-smooth tongue sole, *Cynoglossus semilaevis*, where both types of males have higher gonadal methylation than females (Shao *et al.*, 2014). Similarly, gonadal methylation is greater in males compared to self-fertilising hermaphrodites in the mangrove rivulus, *K. marmoratus* (Fellous *et al.*, 2018).

4.4.2.1 Sex-specific DNA methylation in the gonad

The establishment and maintenance of ovarian or testicular fate are controlled by sex determining mechanisms which stimulate one pathway while actively suppressing the other pathway (Venegas *et al.*, 2016; Capel, 2017; Gemmell *et al.*, 2019; Todd *et al.*, 2019). Key genes in ovarian development include, *cyp19a1a, foxl2* and *wnt4*, and key genes in testis development include, *dmrt1, amh* and *sox9* (Todd *et al.*, 2016). In mammalian species, the female pathway is initially activated and maintained unless the male regulatory pathway intervenes (Sinclair *et al.*, 1990). Similarly, in zebrafish, *D. rerio*, and bluehead wrasse, *T. bifasciatum*, the gonad begins development as an ovary until the male regulatory pathway intervenes to develop a testis (Shapiro & Rasotto, 1993; Orban *et al.*, 2009). Therefore, in these cases, mechanisms are required to suppress the female pathway to allow for the male developmental pathway to activate.

DNA methylation is likely to be involved in maintaining genes related to major sex pathways. For example, in the half-smooth tongue sole, *C. semilaevis*, a species that exhibits genetic and temperature dependent sex determination, when sex reversal occurs, DNA methylation ensues at key genes to suppress ovarian development and subsequently activates vital genes related to testis development (Shao *et al.*, 2014). Other examples of temperature dependent sex determination exist in reptiles. After an initial similar methylation pattern in the gonad bipotential phase in the olive ridley sea turtle, *Lepidochelys olivacea*, sexual dimorphism exists in gonadal DNA methylation patterns, with methylation and demethylation events that lead to the development of either an ovary or a testis (Venegas *et al.*, 2016). Similarly, in the painted turtle, *Chrysemys picta*, DNA methylation targets regulators of gonad development resulting in sexually dimorphic gonad methylation (Radhakrishnan *et al.*, 2017). Therefore, DNA methylation patterns may be required to stabilise sex-specific gene expression in the gonad and differences detected in the global methylation patterns between the testis

and ovary of *N. celidotus* may reflect the antagonistic relationship between key sex-specific genetic pathways in maintaining gonadal fate.

4.4.2.2 Gamete-specific DNA methylation

One of the vital roles DNA methylation plays is not only in gene expression but also in the organisation of chromatin compaction (Miranda & Jones, 2007). Therefore, it is likely that part of the reason for sex-specific variations in gonad methylation may also come down to gamete-specific differences in chromatin compaction. The testis is designed to produce a large number of small, motile, mature sperm that have highly compacted chromatin acting as genome vectors delivering paternal DNA to the mature egg (Schulz *et al.*, 2010). In contrast, the ovary is designed to produce a comparatively smaller number of large mature eggs. Eggs are required to have all the molecules and nutritional requirements for the fertilised embryo's survival and the maternal contribution of DNA (Lubzens *et al.*, 2010), therefore, does not require the same amount of chromatin compaction and DNA methylation as the sperm. In several vertebrate examples, such as fish; zebrafish, *D. rerio* (Mhanni & McGowan, 2004; Laing *et al.*, 2018) and medaka, *Oryzias latipes* (Wang & Bhandari, 2019), and mammals; mice (Kobayashi *et al.*, 2012; Smith *et al.*, 2012), bovine (Jiang *et al.*, 2018) and human (Seisenberger *et al.*, 2013), sperm are hypermethylated. In contrast, oocytes are hypomethylated; this allows for gene expression related to the gamete-specific differences. Therefore, male and female gametes require specific methylation patterns and chromatin compaction and organisation for their uniquely specialised functions as a regular part of gamete development in the gonad.

4.4.3 DNA methylation may be involved in seasonal regulation of reproduction

Females had lower gonad methylation levels when they were out of spawning season compared to breeding season. Seasonal differences in gonad methylation suggest there could be seasonal effects on reproduction that are under epigenetic control. Timing of gamete maturation concerning reproductive season is essential to the successful production of offspring (Viitaniemi *et al.*, 2019). Some species, which are seasonal breeders, such as *N. celidotus*, may rely on cyclical epigenetic regulation to mediate reproductive behaviour or seasonal timing of gonad maturation in response

to environmental cues (Stevenson, 2018). Reproduction in many species is timed by seasonal fluctuations, such as changes in light or temperature, and epigenetics is the key regulatory link between environmental stimuli and changes in gene expression enabling response to the stimulus (Stevenson & Prendergast, 2013; Alvarado *et al.*, 2014). For example, temporal timing of DNA methylation throughout the breeding season in the blood of female great tits, *Parus major*, showed a small increase of DNA methylation over the breeding season (Viitaniemi *et al.*, 2019). In the Siberian hamster, *Phodopus sungorus*, photoperiod driven changes occur to DNA methylation at the hypothalamic *dio3* promoter, which influences reproduction (Stevenson & Prendergast, 2013). However, gene-level methylome sequencing would be required to detect specific gene differences, whereas, global analysis of DNA methylation would not. Furthermore, DNA methylation has also been suggested as a regulating factor in other seasonally related phenotypic changes, such as torpor, hibernation and coat colour (Alvarado *et al.*, 2014).

4.4.4 Summary

This study has identified that global DNA methylation in the brain did not differ between the three sexual phenotypes in *N. celidotus*. However, sex-specific gonad global DNA methylation existed, with ovarian methylation characteristically lower than testis methylation from either male phenotype. Seasonal differences were also detected in the ovary, with methylation lower during the non-breeding season compared to breeding season. Although these results are preliminary findings due to sample sizes and experimental conditions, they provide clear sex-specific differences in methylation that warrant further investigation.

Chapter 5

General Discussion

Understanding reproductive plasticity and how sexual phenotypes arise is of great interest to biology, particularly the form and function of sex changing fish. However, to conduct studies to determine the precise mechanisms of sex change and reproductive biology, it is essential to understand the fundamental differences in gonadal morphology and structure as well as factors that might influence this. The literature contains a wealth of knowledge on sex changing fish, and there are plenty of reviews on the role of the hypothalamic-pituitary-gonadal axis, sex steroids, potential genetic regulators, ecology, and socially induced sex change (Warner & Robertson, 1978; Sadovy & Shapiro, 1987; Baroiller *et al.*, 1999; Devlin & Nagahama, 2002; Godwin, 2009; Lamm *et al.*, 2015; Todd *et al.*, 2016; Todd *et al.*, 2019). However, there are gaps regarding detailed descriptions of gonad morphology and structure of the different sexual phenotypes in protogynous hermaphrodites. Furthermore, the role of relatively novel factors, such as epigenetic regulation, and the tools to assess this have yet to be applied to these studies. The goal of this thesis was to address these questions in *Notolabrus celidotus* to gain a better understanding of the different sexual phenotypes.

5.1 Diandric body size and colour

As previously reported, *N. celidotus* exhibits two colour phases; a dull coloured IP consisting of both males and females and a TP consisting of only bright coloured males (Choat, 1965; Jones, 1980; Moraes, 2019). Sexually mature IP males were statistically similar to females in weight and length. Whereas, TP males were larger than both IP males and females (Chapter 2). Large TP males compete for territories and have the advantage of female preference which relates to higher reproductive success. Small males can be easily excluded from mating opportunities and outcompeted by larger males relating to lower reproductive success (Choat & Robertson, 1975; Kuwamura *et al.*, 2000; Devlin & Nagahama, 2002). Therefore, a female mimicry strategy has evolved to allow small males the chance to blend into a group of females and thereby increase their reproductive success. Small males can use female mimicry to take advantage of

pair spawning events by sneaking and adding their sperm to the mix of the larger TP male's sperm in the water column (Jones, 1980; Nakamura *et al.*, 1989; Taborsky, 1994; Devlin & Nagahama, 2002). While dichromatic colour phases also exist in some monandric species, the IP generally consists of females, and the TP consists of males (Tribble, 1982; Candi *et al.*, 2004). Whereas in diandric species, dichromatism includes both males and females in the IP, and the TP is exclusively male (Dipper & Pullin, 1979; Bentivegna & Rasotto, 1983; Laming & Ebbesson, 1984; Warner & Swearer, 1991). Therefore, the colour patterns of *N. celidotus* are typical of diandric wrasse. However, because some monandric species are also dichromatic, further evidence is required to establish diandry in this species.

N. celidotus males have considerable overlap in size distribution with females (Chapter 2). In diandric species, juveniles can sexually mature as either male or female. Therefore, male size overlaps females and males are found in all size ranges (Dipper & Pullin, 1979; Bentivegna & Rasotto, 1983; Laming & Ebbesson, 1984; Warner & Swearer, 1991). Whereas, in monandric species, all juveniles sexually mature as females. Therefore, size distribution is typically bimodal due to the period as a female before transition to male through sex change (Tribble, 1982; Candi *et al.*, 2004; Munday *et al.*, 2009; Muncaster *et al.*, 2013). Additionally, in this study, IP males were found to already exist at the approximate time of puberty, with no evidence of recent sex change (Chapter 3). Jones (1980) also reported IP males at or around female maturity. Overlapping male and females size distributions and the presence of males before or around the time of sexual maturity in *N. celidotus* is consistent with diandry.

5.2 Two distinct testis types

By using gross morphology and histological techniques, this study was able to confirm two different types of testis (Chapter 2 and 3). The testes were described as a type 1, solid testis, which was typical of IP males, and a type 2, hollow testis, which was typical of TP males. Jones (1980) first proposed the concept of evagination to describe the solid testis and internal structure. The current study verifies this idea and provides further detail to explain the unique and sometimes complicated shape of the solid testis and the internal structure. Chapters 2 and 3 use evidence gathered from macroscopic and ultrastructural observations to build a hypothesis of a rupture and subsequent evagination of the juvenile gonad. The rupture of the gonad is proposed to

occur at a thin seam at the dorsal apex and is supported by evidence of the thin region of the external membrane with a fragile appearance at the same position on the juvenile ovary, mature ovary and the hollow testis. The rupture of the seam is thought to cause the testis to evaginate and fold back around on itself. This hypothesis not only explains the external morphology of the solid testis but also describes how the sperm collecting ducts and major vasculature could become centrally located. Therefore, the general features of this testis, solid germinal compartment and central sperm duct configuration, match those commonly associated with a primary testis (Dipper & Pullin, 1979; Shapiro, 1979; Bentivegna & Rasotto, 1983; Cardwell, 1989; Hourigan *et al.*, 1991; Matsuyama *et al.*, 1997; Godwin *et al.*, 2003; Sadovy & Liu, 2008).

In contrast, the hollow testis remained as a completely in-tact cylinder despite the presence of the seam described above. Sperm ducts were located around the periphery inside the tunica, and the hollow central cavity resembled a remnant ovarian lumen. In the posterior region where the lobes fuse, multiple sperm ducts drain from the periphery into a central vas deferens. These features are common in secondary testes (Dipper & Pullin, 1979; Shapiro, 1979; Bentivegna & Rasotto, 1983; Cardwell, 1989; Hourigan *et al.*, 1991; Matsuyama *et al.*, 1997; Godwin *et al.*, 2003; Sadovy & Liu, 2008). Primary and secondary testes are typically associated with IP and TP males, respectively, in diandric wrasse species. This further implies that *N. celidotus* are diandric.

Although testis type did not always correspond with colour phase, there were key trends with smaller males containing solid testes and larger males containing hollow testes. These findings are consistent with Jones (1980). The colour phase not always corresponding to testis type may reflect an ability of both IP and TP males to transition between body colour phenotypes and assume different sexual strategies according to environmental influences. In this instance, the gonadal structure would not alter and provides evidence for the developmental origin of individual fish.

5.3 Sex-specific DNA methylation in gonads and seasonal differences in ovary

Interestingly, there are striking differences between females, IP males and TP males in behaviour (General Introduction), body colour and size (Chapter 2) and gonad

morphology (Chapters 2 and 3). However, global brain DNA methylation did not differ between the sexual phenotypes (Chapter 4). Additionally, contrasting methylation patterns were detected between ovary and testis samples, with ovaries having significantly lower global methylation levels than both IP males and TP males (Chapter 4). Evidence suggests that the variances in global DNA methylation levels between the ovary and testis are due to sex-specific differences. Sex-specific differences reflect the mutual antagonism between male and female genetic pathways, where once one sexual pathway is activated, and it actively suppresses the opposite sexual pathway to determine gonadal fate (Venegas *et al.*, 2016; Capel, 2017; Gemmell *et al.*, 2019; Todd *et al.*, 2019). Gamete-specific methylation patterns could also have an impact on overall gonad methylation patterns with sperm typically hypermethylated and oocytes hypomethylated (Mhanni & McGowan, 2004; Kobayashi *et al.*, 2012; Wang & Bhandari, 2019). Therefore, while behaviour, body colour and gonad morphology are linked to sexual phenotypes in *N. celidotus*, methylation patterns differ significantly between the ovary and the testis. Differences in gonad methylation indicate that sex-specific DNA methylation regulates gene expression and gonadal fate. However, IP and TP males had similar global testis DNA methylation levels (Chapter 4). Similar methylation between testis types suggests that DNA methylation is not a major factor regulating testis type differences and warrants further investigation into gene-level methylome sequencing to detect specific gene differences between testis types.

Lower ovarian methylation levels were detected out of spawning season compared to breeding season (Chapter 4). Seasonal differences in ovarian methylation suggest there could be seasonal effects on reproduction that are under epigenetic control. *N. celidotus* is a seasonal spawner and spawning season takes place from late July until the end of November; however, small members of the group may remain ripe into December (Jones, 1980). Phenotypic changes due to seasonal fluctuations have been related to DNA methylation in other species (Stevenson & Prendergast, 2013; Alvarado *et al.*, 2014; Viitaniemi *et al.*, 2019). Therefore, epigenetics could be a key regulatory link between environmental stimuli and changes in gene expression, enabling a response to the stimulus.

5.4 Conclusions

To determine male development pathways in protogynous species three scenarios need to be considered; 1) all sexually mature males develop from juveniles (gonochorist), 2) all sexually mature males are derived through an initial functional female phase (monandry), 3) males can develop from both primary and secondary pathways (diandric) (Trip *et al.*, 2011). Previous studies have reported *N. celidotus* as being a monandric protogynous hermaphrodite. Therefore, not all males are gonochoristic because some are derived from sex change. The current study found evidence of a small IP male that was likely to be undergoing puberty and similarly sized females were deemed to be sexually immature, which indicates that males may occur without becoming sexually mature females. Therefore, not meeting the criteria for monandry.

Furthermore, evidence from macroscopic and ultrastructural analysis, demonstrates that two distinct testis phenotypes exist. One matches the general description of a primary testis while the other matches the criteria for secondary testes. The former is mostly associated with IP male phenotypes, which have a large overlap in body size with females, while the latter is with TP phenotypes. The two types of testes suggest two pathways of male development in *N. celidotus,* a primary testis that develops through evagination in juvenile males and a secondary testis that develops through sex change in large males. Therefore, not all the males in the population have passed through a functional female phase and cannot meet the criteria for monandry. It is proposed that *N. celidotus* are in fact diandric and the two male phenotypes can occasionally transition to adopt alternate colour and behavioural roles.

Additionally, DNA methylation analysis of the gonad suggests that epigenetic regulation appears to be a key regulator of sex-specific gonadal fate and response to environmental stimuli regulating seasonal changes to phenotypic expression.

5.5 Recommendations for future research

Understanding the basic diandric morphology and structure of the gonad, determining the baseline levels of brain and gonad DNA methylation and seasonal changes to global ovarian DNA methylation opens many opportunities for future research. A few questions remain unanswered that could be followed up by additional research. Rapid

changes in colouration of IP males were reported by Jones (1980) and were observed under periods of stress or when IP males were grouped during the present study, but these changes have not been measured or tested. Further investigation into IP male colour pattern changes would add to the hypothesis of male fish transitioning between IP and TP strategies. It would be interesting to quantify relevant sex steroids concentrations, such as 17β- estradiol and 11-ketotestosterone, in the different phenotypes to add to baseline understanding. It was noted that the sperm ducts of type 1, solid testis, appeared larger than the sperm ducts of the type 2, hollow testis; however, this was not measured. Future studies investigating the differences in sperm volume/numbers and duct size between the two testis types would help with understanding of the functional differences between testes. This study was conducted during the spawning season, so it would be valuable to understand gonad differences over other parts of the year. Further research is necessary to clarify if a link exists between testis evagination and development of the juvenile gonad. DNA methylation results could be further investigated by gene-level analysis to tie expression differences to methylation to assess the regulatory role of DNA methylation and further exploration into the seasonal differences related to gonad maturation.

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