Protogynous sex change in sexually immature spotty wrasse (*Notolabrus celidotus*)

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Tessa E. Hamer

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Abstract

One of the most unique forms of sexual plasticity can be witnessed in sequentially hermaphroditic fish, which begin as one sex and change to another at some stage in their lifecycle. The endemic protogynous spotty wrasse (*Notolabrus Celidotus*), has historically been considered monandric although two male morphs appear to exist. To date the majority of research has focused on sexually mature females that are changing sex to become terminal phase males. The current study seeks to describe sex change in sexually immature females that are transitioning to become initial phase males. This was achieved through analysis of gonadal histology as well as expression of three key sex differentiation related genes (*amh*, *dmrt1* and *cyp19a1a*). Fish (n=141) were caught from the Tauranga Harbour ranging in size from 52-270 mm TL from May-June 2022. The smallest transitional fish identified was 52 mm TL and the smallest IP male detected in this study were 72 mm TL, indicating IP male spotty wrasse likely reach sexual maturity at a much earlier and smaller developmental stage than previously realised. Expression of *amh* and *dmrt1* showed a clear male-bias across sex change, with the former being upregulated in early sex change. The aromatase gene, *cyp19a1a* showed an overall female-biased expression pattern despite having some unexpected upregulation in the mid stages of sex change. The results supported previous reports that all juvenile spotty wrasse are female first before some individuals undergo pre-maturational sex change to become IP males. Having clarified the process of sex change leading to IP male formation and considering data from other studies of IP and TP testicular structure in this species, it seems no longer tenable to consider them monandric.

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Chapter 1 - Introduction

Approximately six percent of all fish species show the remarkable ability to reproduce as both sexes at some point in their life history (Sadovy & Liu, 2008). The female and male sexual state is extraordinarily plastic in teleosts, and this has led to divergent sexual strategies. These include gonochoristic species, which remain as one sex throughout their lives, as well as hermaphrodites, where individuals may breed as one or other species at either the same or different developmental time points (Devlin & Nagahama, 2002; Kobayashi, Nozu, Horiguchi, & Nakamura, 2018). Sex changing teleosts exist across more than 20 taxonomic families within 9 orders (Avise & Mank, 2009), with polygyny being the most abundant form of hermaphroditism, occurring in 66% of all hermaphrodites (Kuwamura, Sunobe, Sakai, Kadota, & Sawada, 2020). The precise regulatory mechanisms and exact timing of these changes are currently poorly understood. It is, however, relevant to investigate these mechanisms as many sex changing species have important social, economic, and environmental benefits.

With aquaculture being the fastest growing primary industry in the world (Subasinghe, Soto, & Jia, 2009), understanding the reproductive physiology and sex determination systems of fish is essential. Many commercially valuable aquaculture species are sex changing hermaphrodites such as the gilthead seabream (*Sparus aurata)* (Sola et al., 2007), red porgy (*Pagrus pagrus*) (Kentoyri, Papandroulakis, Pavlides, & Divanach, 1995), and barramundi (*Lates calcarifer*) (Domingos et al., 2018). Controlling sex ratios through the manipulation of sex change in farmed fish is crucial for managing the negative effects of sexual maturation (Budd, Banh, Domingos, & Jerry, 2015; Piferrer, Ribas, & Diaz, 2012; Siegfried, 2010; Todd, Muncaster, & Gemmel, 2016; Wang & Shen, 2018). Understanding the regulation of sex-determining mechanisms within a given species is also essential when applying modern technologies to farmed stock such as the production of mono-sex populations (Beardmore, Mair, & Lewis, 2001; Devlin & Nagahama, 2002; Penman & Piferrer, 2008; Singh, 2013; Stanley, 1976; Viñas, Asensio, Cañavate, Piferrer, 2013; Wang & Shen, 2018). These manipulations are desirable to maximise production efficiencies linked to species with sex-specific growth rates, and to minimise the onset of early puberty, sex related aggression, and genetic pollution (Frost, Evans, & Jerry, 2006; Qin, Mittiga, & Ottolenghi, 2004; Singh, 2013; Vøllestad & Quinn, 2003).

Recent studies have demonstrated that several species, including some fish, experience environmental sex determination (ESD) (Conover & Heinz, 1987; Godwin, Luckenbach, & Borski, 2003; Rigaud, Juchault, & Mocquard, 1997; Shen & Wang, 2018; Strüssmann, Yamamoto, Hattori, Fernandino, & Somoza, 2021). Thermal stress during sex differentiation, the period when a fish first develops either an ovary or testis, can lead to masculinisation of individuals regardless of their genetic sex (Baroiller & D'Cotta, 2001). This ESD has been described in the Japanese flounder (*Paralichthys olivaceus*), pejerrey (*Odontesthes bonariensis*), European sea bass (*Dicentrarchus labrax*), and Atlantic silverside (*Menidia menidia*) (Díaz, Ribas, & Piferrer, 2013; Fernandino, Hattori, Acosta, Strüssmann, & Somoza, 2013; Lagomarsino & Conover, 1993; Yoshinaga et al., 2004). This is believed to result from a crosstalk between the stress and reproductive axes stimulating increased 11-Ketotestosterone (11KT), the main fish androgen, as a result of elevated circulating cortisol concentrations (Fernandino, Hattori, Kishii, Strüssmann, & Somoza, 2012; Fernandino et al., 2013; Goikoetxea et al., 2021; Hattori et al., 2009). Interestingly, social stress and the associated increase in plasma cortisol have been implicated as potential drivers of female to male sex change in some sex changing fish (Goikoetxea et al., 2021, Goikoetxea et al., 2022; Liu et al., 2017; Muncaster et al., 2023; Nozu & Nakamura, 2015; Solomon-Lane et al., 2013; Strüssmann, Saito, Usui, Yamada, & Takashima, 1997; Todd et al., 2019). It seems that ESD and some sex changing species are susceptible to masculinisation by different stressors although this arises through a similar physiological pathway. The fact that these sex-changing fish can undergo masculinisation due to elevated cortisol levels raises intriguing questions regarding the potential consequences of rising global sea temperatures associated with climate change (Fausch, Lyons, Karr, & Angermeier, 1990; Hattori et al., 2009; Ospina-Alvarez & Piferrer, 2008; Strüssmann, Conover, Somoza, & Miranda, 2010; Strüssmann & Nakamura, 2002). Sex changing species such as Barramundi are susceptible to thermal stress (Budd, Robins, Whybird, & Jerry, 2022) which could create issues for both wild and captive populations of female to male sex changing fish. Understanding the mechanisms of sex change in these species is an essential step toward understanding their potential susceptibility to future sea temperature increases.

1.1 Sex determination and sex differentiation

Recent research has explored the diversity of sex determining mechanisms across vertebrate taxa and how these contrast to the relative stability of sex determination in mammals (Avise & Mank 2009; Devlin & Nagahama, 2002; Jalabert, 2005; Li et al., 2013). While a clear difference exists between ESD and genetic sex determination (GSD) species, the latter encompasses a range of variations including male (XX: XY) and female (ZZ: ZW) heterogamety as well as differences in sex chromosome number XX: XO or multi loci across different chromosomes (Chen, Zhu, & Hu, 2022; Moore, & Roberts, 2013; Nakamura, 2010; Singh, 2013; Viñas et al., 2013). Examples for each of these sex determining systems exist within the 30,000 extant teleost species. Much of the vertebrate research to date has demonstrated the role of master sex determining genes which favour either masculinisation (e.g., *dmrt1*; Doublesex and mab-3 related transcription factor 1) or feminisation (e.g., *foxl2*; Forkhead box protein L2). In either case, the expression of these genes leads to the upregulation of gene pathways that ultimately stimulate the production of specific sex steroids to induce gonadal sex differentiation. These estrogens and androgens direct ovarian and testicular development, respectively (Kagawa, Young, Adachi, & Nagahama, 1982; Liu et al., 2017; Nagahama, 1994; Ohta, Mine, Yamaguchi, & Matsuyama, 2008; Weltzien, Andersson, Andersen, Shalchian-Tabrizi, & Norberg, 2004). While the overarching systems regulating sex determination, and thereby sex differentiation, differ among fishes, equivalent genes within similar genetic pathways often orchestrate these events. The expression of these genes provides convenient targets from which the process of sex determination and/or sex differentiation may be characterised within a species.

Gonochorism and hermaphroditism

While the majority of fish have a fixed sex throughout their life, there are differences in the pattern of gonadal development between gonochoristic species. In primary gonochoristic species, early gonadal development proceeds from an undifferentiated gonad directly into either an ovary or testis (Yamamoto, 1969). Alternatively, in undifferentiated gonochorists, the gonads of all individuals initially develop ovarian tissue. This ovarian tissue then degenerates in approximately half of the population, and the gonad is subsequently invaded by additional somatic cells (Takahashi, 1977; Takahashi & Shimizu, 1983). The gonad then evolves into an intersexual state

before becoming a testis (Devlin & Nagahama, 2002). However, these fish will only functionally reproduce as one sex in their lifecycle.

In contrast, hermaphroditic fish can function as both female and male at some time point in their lives to produce mature eggs and sperm, respectively. While some teleost hermaphrodites such as black hamlet (*Hypoplectrus nigricuns*), chalk bass (*Serrunus tortugarum*), and harlequin bass (*Serranus tigrinus*), function simultaneously as male and female, others transit sequentially between sexes (Leonard, 1993). Sequential hermaphroditism exists in three forms: (1) protogynous (female-to-male) as described in species such as bluehead wrasse (*Thalassoma bifasciatum*) (Warner, Robertson, & Leigh, 1975), striped parrotfish (*Scarus iserti*) (van Rooij, Kroon, & Videler, 1996) and the honeycomb grouper (*Epinephelus merra*) (Bhandari, Komuro, Nakamura, Higa, & Nakamura, 2003), (2) protandrous (male-to-female), as described in *L. calcarifer* (Domingos et al., 2018; Roberts et al., 2021), and (3) serial bidirectional sex change, as seen in Maori coral gobies (*Gobiodon histrio*) (Todd et al., 2016). In all cases, functional sex reversal entails radical restructuring of the gonad plus changes in morphology and behaviour (Godwin, 2009; Nakamura, Kobayashi, Miura, Alam, & Bhandari, 2005; Ross, 1982; Todd et al., 2016; Warner, 1984).

The evolution of protogynous sex change is thought to be largely driven by a male size advantage. Here, large males use aggressive territorial defence to monopolise matings with females (Warner, 1984). This disadvantages small males and leads to strong selection for size-based protogyny and reproductive strategy (Warner & Swearer, 1991). By initially reproducing as a small female and then changing to become male at a larger size, in order to breed with multiple females, an individual may magnify its reproductive output and success (Charnov, 1982; Ghiselin 1969; Todd et al., 2016; van Rooij, et al., 1996; Warner, 1988; Warner et al., 1975). In contrast, a female biased reproductive size advantage favours protandry. This is associated with monogamy or systems without male territorial social structures (Munday, White, & Warner, 2006). This is seen in the anemone fish (*Amphiprion* and *Premnas spp*.) (Godwin, 1994) where a large dominant female lives with small male and smaller subordinate individuals. Loss of the female prompts protandrous sex change in her partner and maturation of the most dominant immature fish as the new breeding male. This leads to greater reproductive potential when the larger individual is female (Todd et al., 2016)

Protogynous sex change occurs within 15 teleost families and is the most common form of hermaphroditism, having been extensively described throughout the literature (Avise & Mank, 2009; Devlin & Nagahama, 2002; Frisch, 2004; Goikoetxea et al., 2021; Sadovy & Shapiro, 1987; Warner et al., 1975; Warner & Swearer, 1991). In some tropical protogynous fish, such as *T. bifasciatum*, sex change can occur year-round. Their small size and the fact that they can change sex during any season has meant that this species has become an ideal model for studying sex change (Godwin, Sawby, Warner, Crews, & Grober, 2000; Godwin et al., 2003; McCaffrey, Hawkins, & Godwin, 2011; Munday et al., 2006; Semsar, Kandel, & Godwin, 2001; Shapiro & Rasotto, 1993; Todd et al., 2019; Warner & Swearer, 1991). *T. bifasciatum* usually hatch as female and then sex change when they are large enough to compete successfully as territory holders (Warner et al., 1975). However, another less common male pathway also exists; initial phase (IP) males are small in size and use a sneaker spawning strategy to compete with dominant terminal phase fish rather than competing for territorial dominance (Warner et al., 1975). Interestingly, few studies have described sex differentiation in IP males from either tropical or temperate wrasse, and as such, the timing and regulation of sex change in individuals using this strategy remains uncharacterised.

Diandry and monandry

The existence of IP males in some protogynous species and not in others means that protogyny can be further separated into two divisions based on male reproductive strategy. In monandric protogyny, males are derived exclusively from mature functional females to form a characteristically structured secondary testis. Monandry has been observed in foxfish (*Bodianus frenchii)* (Cossington, Hesp, Hall, & Potter, 2010), hogfish (*Lachnolaimus maximus*) (McBride & Johnson, 2007), and Ballan wrasse (*Labrus bergylta*) (Dipper & Pullin, 1979; Muncaster et al., 2010). In diandric protogyny, males develop from one of two developmental pathways; either from sexually mature females, as in monandry, or directly from juvenile fish (Connington et al., 2010; Devlin & Nagahama, 2002; McBride & Johnson, 2007; Sadovy & Shapiro, 1987; Shapiro & Rasotto, 1993; Warner & Robertson, 1978). Typically, diandric males have two colour morphs; IP males tend to mimic female colour and patterning and are less abundant than TP males which usually have dimorphic markings in comparison. Interestingly, IP males can also transition to assume TP markings upon the death or removal of a dominant TP fish (Kazancıoğlu & Alonzo,

2010; Warner & Swearer, 1991). Diandric IP and TP male fish typically have different testicular structures. While TP males tend to have a remnant ovarian lumen in the centre of the secondary testis, IP males usually lack this lumen as has been described in *T. bifasciatum* (McCaffrey et al., 2011) and *Thalassoma duperrey* (Nakamura, Hourigan, Yamauchi, Nagahama, 1989). However, some species have been described as having no morphological testicular differences despite being labelled as diandric (Fennessy & Sadovy, 2002; Rasotto & Shapiro, 1992).

1.2 HPG axis and neuroendocrine regulation of sex change

Sex change and gonadal development in teleosts is cued through the transduction of environmental cues into physiological signals via the hypothalamic-pituitary-gonadal (HPG) axis **(Figure 1.1)** (Fernandino, Hattori, Kimura, Strüssmann, & Somoza, 2008; Godwin, 2010; Godwin et al., 2003; Nakamura et al., 1989). The HPG axis (also referred to as the reproductive axis) consists of endocrine feedback between the brain and gonad (Devlin & Nagahama, 2002; Nagahama, Miura, & Kobayashi, 2007). The stimuli from environmental changes such as temperature, or social structure (Soria, Strüssmann, & Miranda, 2008) are received at the hypothalamus region of the brain, and gonadotropin-releasing hormone (GnRH) is produced. GnRH signals the pituitary to release the gonadotropins (GtHs), luteinizing hormone (LH), and follicle stimulating hormone (FSH) into the circulatory system (Fernandino et al., 2008; Suzuki, Kawauchi, & Nagahama, 1988; Swanson, Suzuki, Kawauchi, & Dickhoff, 1991). The levels of FSH and LH expression may differ between the sexes (Ohta et al., 2008). These GtHs then stimulate the production of sex steroids (oestrogens and androgens) in the steroidogenic cells of the gonads via their respective receptors. This action directly regulates reproduction by stimulating oogenesis in females and spermatogenesis in male fish (Kagawa et al., 1982; Nagahama, 1994; Ohta et al., 2008; Weltzien et al., 2004). Therefore, the gonadal endocrine environment directly influences sex change and development. In socially regulated species, the cues for sex change are visual and induce rapid neurochemical changes in the brain to initiate behavioural responses that precede, and likely trigger, gonadal changes (Kobayashi, Alam, Horiguchi, Shimizu, & Nakamura, 2010; Thomas et al., 2019).

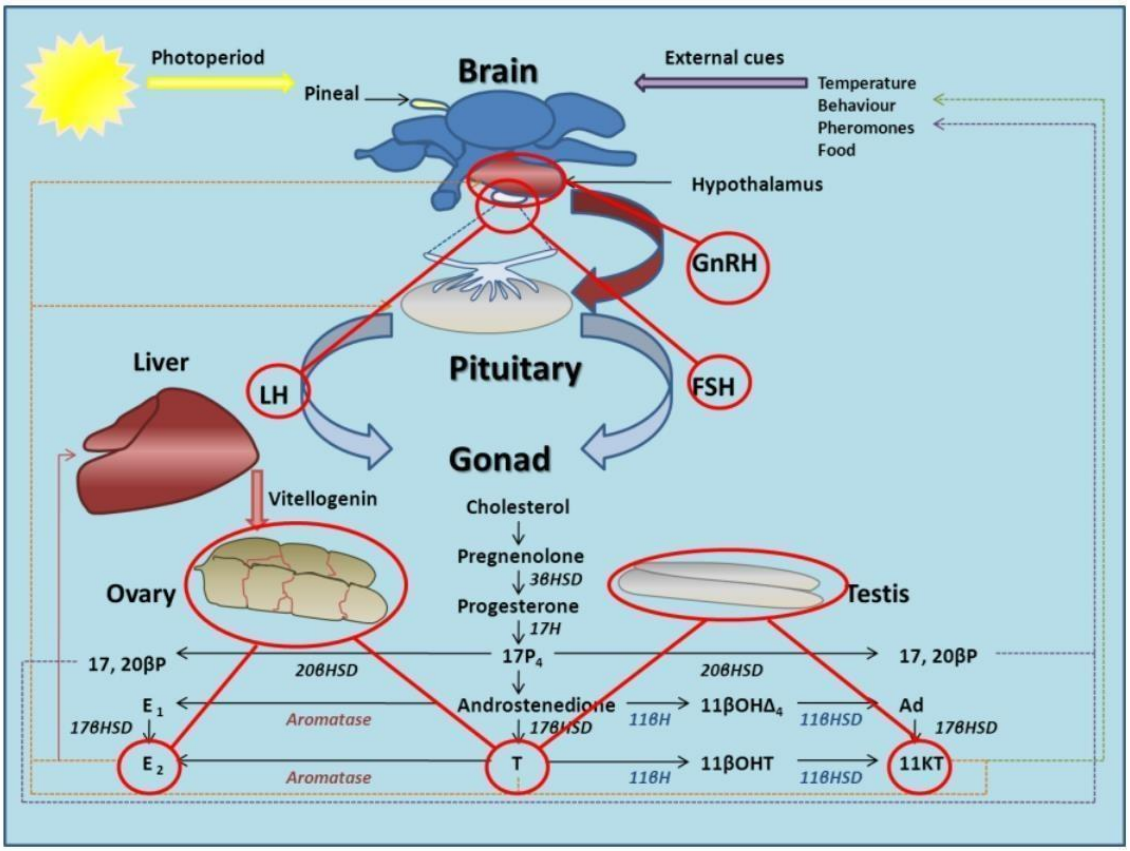

Figure 1.1: The reproductive axis in fish and the important hormones involved in the maturation of the gonads. Reproduced with permission from: (Muncaster, 2008).

1.3 New Zealand spotty wrasse

The New Zealand spotty wrasse (*Notolabrus celidotus*), or Paketi, is a small endemic protogynous wrasse (Jones, 1980; Goikoetxea et al., 2021; Muncaster et al., 2023). They are one of seven species within the genus *Notolabrus*, which are distributed throughout Australia and New Zealand (Parenti & Randall, 2000, 2011). Spawning occurs from late July until the end of November in this species and sex change typically occurs soon after this. Sex change is stimulated in response to social stimuli (changes in social structure), such as disappearance of the dominant male, the presence of smaller females within their territorial boundary or "harem" (Jones, 1980; Goikoetxea et al., 2021). As such, this species can be socially induced to change sex in the laboratory and has been proposed as a temperate model for studying sex change (Goikoetxea et al., 2021; Goikoetxea et al., 2022; Muncaster et al., 2023). Sex change in sexually mature spotty wrasse results in the formation of a secondary testis (Jones 1980; Thomas et al., 2019; Goikoetxea et al., 2021;

Muncaster et al., 2023). In spotty wrasse, TP colour change occurs slightly after sex change, and IP males appear to change colour during the spawning season (Jones, 1980). Initial phase males typically possess a primary testis, and mimic female IP patterning to suit their sneaker reproductive tactic. This type of phenotypic plasticity allows an organism to alter its development, physiological state, or behaviour in response to key environmental cues (Mank, Promislow, & Avise, 2006; Godwin et al., 2003; Robertson, 2020).

Despite the fact that spotty wrasse have two male morphs there is still confusion in the literature as to whether they qualify as a monandric or diandric species. Early research by Jones (1980) classified spotty wrasse as monandric, as all of the male individuals in this study were found to have secondary testes, with a residual ovarian lumen. Additionally, Jones suggests that all spotty wrasse begin as female which further supports monandry. A similar study by McBride & Johnson (2007) classified the hogfish, *L. maximus,* as monandric based on the same rationale. Terminal phase male spotty wrasse usually have a remnant ovarian lumen in the testes (Goikoetxea et al., 2021; Jones, 1980; Robertson, 2020). Whereas another testis type, mostly seen in IP males, also exists and hints at diandry. These testes appear solid without a central lumen and are believed to result from the testis rupturing along a seam in the tunicata albuginea and evaginating back on itself (Jones, 1980; Robertson, 2020). The fact that this species has two different male developmental strategies and morphologies, yet may always start life as female, makes classification of monandry or diandry equivocal. Little is known about the process of sex change in prepubertal IP spotty wrasse. This study seeks to clarify where in the life cycle spotty wrasse first begin to change sex and whether sex change is occurring prior to female puberty. This is achieved using histological and molecular analysis of juvenile and sexually mature *N. celidotus*. This study was executed in accordance with New Zealand animal ethics requirements.

1.4 Aims and objectives

This study aims to investigate the process of sex change in juvenile IP *N. celidotus.* Specific objectives include:

1.) A histological description of sex change leading to IP male formation (Chapter 2)
2.) Characterisation of size at sex change in prepubertal fish (Chapter 2)

3.) Characterisation of the expression of key genes involved in sex determination and sex differentiation during IP male sex change. (Chapter 3).

1.5 General Methods

Fish were captured in shallow water (<6m) from the wild using a Sea Harvester Collapsible Bait Cage (25 x 25 x 45 cm). Capture took place in the Tauranga Harbour (37°.640411, 176°.181424) from May-June 2022, targeting individuals 50-150 mm TL, not targeting any specific sex or phenotype. In total 58 specimens were collected over this period and placed in an aerated 20L bucket of seawater and transported to the Toi Ohomai Aquaculture Lab, where they were transferred into black polyurethane recirculating seawater tanks (1600L). Water quality parameters were monitored daily, and fish were fed fresh greenshell mussels (*Perna canaliculus*) three times a week until sampling. Fish were heavily sedated (loss of equilibrium and no eye movement) in an aerated 10 L seawater bath of 2-phenoxyethanol (0.6 ml L -1). They were then weighed (g), measured (mm, total length), and external body photographs were taken. Fish were then euthanized by rapid decapitation with a sharp knife. An abdominal incision was made along the ventral surface, gonads were excised, weighed where possible, as some tissues were unable to be found or too small for a scale reading. Gonads were then placed in 1.5ml tubes, flash frozen in liquid Nitrogen, and stored in -80^{o}C freezer ready for analysis.

Chapter 2 - Histology

2.1 Introduction

Histological analysis of gonad morphology using light microscopy provides direct insight into the reproductive status and physiology of a species (Parenti & Grier, 2004). Light microscope analysis enables verification of sex and stages of sex change as well as quantitative morphometric factors such as fecundity (Alonso-Fernández, Alós, Grau, Domínguez-Petit, & Saborido-Rey, 2011; Blazer, 2002; Robertson, 2020). Histological analysis is essential to understand the process of protogynous sex change as there is often a complex relationship between gonadal status/structure, external colour phase, and social behaviours (Asoh & Kasuya, 2002; Dipper & Pullin, 1979; Robertson, 2020).

2.1.2 Gametogenesis and HPG regulation of reproduction

Reproduction and gonadal development, or gametogenesis, is regulated by the HPG axis (Chapter 1.2). Early gonadal development in fish starts with the migration of primordial germ cells (PGCs) to the gonadal ridge where the gonadal compartment forms around them (Devlin & Nagahama, 2002; Fernández et al., 2015; Richardson & Lehmann, 2010; Yoshizaki, Takeuchi, Sakatani, & Takeuchi, 2000). These PGCs give rise to gonial germ cells in the form of either oogonia or spermatogonia in the ovary and testis, respectively (Braat, Speksnijder, & Zivkovic, 2004; Devlin & Nagahama, 2002; Lubzens, Young, Bobe, & Cerdà, 2010; Patiño & Sullivan, 2002; Yoshizaki et al., 2000). Under stimulation of the HPG axis oogonia will differentiate into previtellogenic oocytes in the ovary while spermatogonia will differentiate into spermatocytes in the testis (Schulz et al., 2010; Robertson, 2020). Increased 17ß-estrogen (E2) production typically drives further stages of oogenesis such as the formation of cortical alveoli and vitellogenesis. Alternatively, the main androgen 11KT drives spermatogenesis (Schulz et al., 2010; Nagahama, 1994). Both E2 and 11KT are produced by gonadal steroidogenic cells under stimulation of the pituitary hormone, follicle stimulating hormone (FSH). Gamete maturation is stimulated by a shift at the pituitary level from FSH production to luteinising hormone (LH) production (Nagahama & Yamashita

2008; Pankhurst & King, 2010; Patiño & Sullivan, 2002). LH stimulation of the gonadal steroidogenic cells leads to the production of the progesterone-based maturation inducing steroid (MIS) to complete gamete maturation (Lubzens et al., 2010; Nagahama, 1997; Nagahama & Yamashita, 2008; Pankhurst & King, 2010; Patiño & Sullivan, 2002). Puberty is a key developmental milestone when fish attain reproductive competence for the first time through initial activation of the HPG axis (Patiño & Sullivan, 2002; Taranger et al., 2010; Weltzien et al., 2004). Puberty occurs after sex differentiation and is associated with the full functional differentiation of the gonadal germ cell-supporting somatic cells which enable initial germ cell maturation. Puberty culminates in the first spermiation and sperm hydration in male fish or ovulation in females (Okuzawa, 2002; Patiño & Sullivan, 2002; Taranger et al., 2010; Weltzien et al., 2004).

2.1.3 Sex change

The relative balance of estrogen and androgen in the gonad can directly influence gonadal structure and function leading to either feminisation or masculinisation, respectively (Baroiller, Guiguen, & Fostier, 1999; Fenske & Segner, 2004; Guiguen, Fostier, Piferrer, Chang, 2010; Nakamura, 2010; Singh, 2013; Todd et al., 2016). Sex change begins with atresia of the ovarian follicles leading to complete oocyte degeneration (Devlin & Nagahama, 2002; Moe, 1969; Nakamura et al., 1989; Thomas et al., 2019). This is often accompanied by decreased plasma E2 levels and is typically followed by proliferation of spermatogonia and their supporting somatic Leydig cells within the ovarian lamellae. As the ovarian tissue breaks-down, increases in stromal and immune-like cells are also often evident (Elofsson, Winberg, & Francis, 1997; McBride & Johnson, 2007; Muncaster, Norberg, & Andersson, 2013; Nakamura et al., 1989). Over time, the degenerating oocytes are cleared from the gonad and the relative composition of cysts containing spermatogenic germ cells increases to yield a functional male testis which can produce sperm capable of fertilising eggs. This is typically associated with elevated plasma 11KT concentrations (Goikoetxea et al., 2021; Liu et al., 2017; Muncaster et al., 2013; Nakamura et al., 1989). In the tropical wrasse, *T. bifasciatum*, this process can be completed within 8 days (Warner & Swearer, 1991), with behavioural sex change occurring within hours (Godwin, 2010). Alternatively, this may take several weeks or months in temperate wrasses (Goikoetxea et al., 2021; Muncaster et al., 2013; Sadovy & Shapiro, 1987; Shapiro, 1981; Todd et al., 2016; Warner & Swearer, 1991). Previous

studies of sexually mature spotty wrasse show that they have undelimited gonads where both female and male germ cells may be interspersed during sex change rather than coexisting in separated compartments. At completion the resulting testis is of the unrestricted type (Grier, 1981; Parenti & Grier, 2004) with spermatogenic cysts of all developmental stages throughout the lobules (Goikoetxea et al., 2021; Muncaster et al., 2023). Analysis of gonadal histology using light microscopy is essential for characterising sex change and the process can be quantitatively described by classifying fish into discrete categories based on their gonadal development/status.

2.1.4 IP v TP testis structure

Testicular structure may vary in protogynous species depending on the male developmental pathway. In monandric species, all males arise through sex change from a functional female to form a secondary testis. In diandric species, males can develop either directly as sexually immature fish or as sexually mature females that change sex to form secondary testes (Devlin & Nagahama, 2002; Sadovy & Shapiro, 1987; Warner & Robertson, 1978). Differences in testis morphology between IP and TP males in *N. celidotus* have previously been explained by an evagination process (Jones, 1980, Robertson, 2020). This appears to result in the testis opening along a seam in the tunicata albuginea and folding back around itself to form a superficial primary testis with centralised sperm collecting ducts (Cossington et al., 2010; Jones, 1980). Secondary (TP) testes do not possess ovarian tissue but may retain an ovarian lumen as no evagination has occurred and sperm collecting ducts form in the peripheral regions (Jones, 1980). Depending on both the definition in the literature and the species being described, primary males can arise either through direct testis differentiation like gonochorists or through the formation of a secondary testis in sexually immature fish (Liu & Sadovy, 2004a; Liu et al., 2017; Munday et al., 2006). However, the vast majority of literature investigating protogynous sex change focuses on sexually mature fish (Alonso-Fernández et al., 2011; Bhandari et al., 2005; Candi, Castriota, Andaloro, Finoia, & Marino, 2004; Goikoetxea et al., 2021; Muncaster et al., 2023; Nakamura et al., 1989; Nozu & Nakamura, 2015; Shapiro & Rasotto, 1993; Thomas et al., 2019; Todd et al., 2019; Warner & Swearer, 1991). As a result, there are few studies that focus on the mechanisms of IP male development in sexually immature individuals. This section of the study seeks to describe sex change leading to IP male formation in prepubertal spotty wrasse based on gonadal histology. Key

aims are to identify the smallest size at sex change and re-evaluate the evidence to determine this species as either monandric or diandric.

2.2 Methods

During dissection, after sedating and euthanising fish (Chapter 1.5), 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. Fixed samples were 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 finally stained with hematoxylin and eosin. Histological slides were viewed under an Olympus BX53 compound microscope for close tissue examination, fitted with an Olympus DP27 camera and connected to a laptop with CellSens Entry software.

In addition to the 58 samples collected in this study, a further 83 slides of gonadal histology collected previously were used in the project. These additional slides were processed using the same embedding procedure and provided further data from spotty wrasse collected in different years (2019-2021) from the same season (May-June) and location in the Tauranga Harbour. From these tissues, we could not extract RNA for further gene expression analysis.

Spotty wrasse samples were categorised into 6 stages of gonadal development **(Table 2.1)** from Juvenile female (JF) (<110 mm TL), Adult female (AF) (>110 mm TL), Early transitional (ET), Mid-transitional (MT), Late transitional (LT) and IP male (IP). Adult and juvenile females were distinguished by size, as sex change is believed to be size-specific rather than age-specific and not all females mature in the same year (Jones, 1980). Maturity is defined as the size or age when the ovary or testis first enters the ripe condition and occurs at around 100-110 mm standard length (SL) in females (Jones, 1980). Individuals in this study were captured outside of the breeding season, therefore, it was impossible to distinguish between ovaries from immature fish and sexually mature fish with resting stage ovaries. Due to this ambiguity, and the fact that the focus of the study was on prepubertal fish, individuals less than 110 mm TL and with only previtellogenic oocytes were classified as a juvenile female. Sexually mature individuals were classified on the presence of cortical alveoli or later stage oocytes in females or proliferating type

A and B spermatogonia or later stage germ cells in males (Taranger et al., 2010). Early transitional fish were distinguished from juvenile females by having elevated oocyte atresia, including degenerating previtellogenic oocytes, and typically contained nests of gonial germ cells (presumed to later become spermatogonia), as well as some stromal cells and cellular debris (Figure below) (Bhandari et al., 2003; Muncaster et al., 2013).

Table 2.1: Histological stages of gonadal sex change in New Zealand spotty wrasse.

Stage	Description
AF (>110mm)	May contain vitellogenic/ mature oocytes or pre-vitellogenic oocytes depending on the season. No evidence of male structure.
JF (<110mm)	Predominantly pre-vitellogenic oocytes. Lacks vitellogenic or cortical alveolar oocytes.
ET	Atretic oocytes and nests of gonial cells are common. White blood cells and cellular debris, which may also be present as yellow-brown bodies, and stromal cells often evident. No evidence of male structure.
MT	Oocyte numbers diminished and mostly atretic. presence of stromal cells and proliferation of spermatogonia evident. Gonial cells and spermatocytes present in similar abundance to previtellogenic oocytes. May have increased white blood cell presence
LT	Number of spermatogenic cysts predominates over oocytes. Stromal cells often present, and a more structured arrangement of cysts into lobules. Some atretic oocytes (mostly previtellogenic) may be present.
Male (IPM)	Presence of spermatozoa and/or lobules, cysts of spermatogenic germ cells. May contain a few remnant oocytes.

Since the same individuals could not be sampled repeatedly, the differences in fish gonads were assumed to represent the normal progression of sex change at different stages (Nakamura et al., 1989).

2.3 Results

Sex change was observed in the histology of prepubertal spotty wrasse. Of the 141 samples analysed, 20.98% were adult females, 52.45% were juvenile females, 14.69% ET, 3.50% MT, 2.80% LT and 5.59% Male. Individuals ranged from 52-270 mm TL **(Table 2.2)**. JF ovaries were small, with oogonia dominated by peri nucleolar oocytes contained within a thin tunica albuginea.

Oocytes containing follicles were arranged in lamellae which extended into a central, membrane bound ovarian cavity or lumen.

Table 2.2: Size range and mean total length (±SE) of *Notolabrus celidotus* during May-June.

		Total Length (mm)	
Stage of development	**% Total**	**Average (±SD)**	**Range**
Adult Female (AF)	20.98%	153.64±31.43	112-270
Juvenile female (JF)	52.45%	89.30±11.81	64-110
Early transitional (ET)	14.69%	75.89±14.63	52-107
Mid-transitional (MT)	3.50%	70.2±6.02	60-72
Late transitional (LT)	2.80%	87.75±14.43	72-104
Male (IPM)	5.59%	95.5±19.34	77-137

Of the 105 females identified, one individual measuring 270 mm TL was sexually mature with vitellogenic oocytes. It is unclear whether this individual was in ripe condition from the time of capture or as a result of captive conditions before euthanasia. The 75 juvenile females identified had only previtellogenic oocytes and ranged between 64-110 mm TL (Mean = 89.30 ± 11.81 SD mm). It was sometimes difficult to distinguish juvenile females with small previtellogenic oocytes from early transitional individuals with degenerating oocytes. This was demarcated by the increase in presumptive white blood cell activity and presence of stromal cells and cellular debris associated with elevated oocyte atresia and sex change **(Figure 2.1)** (Elofsson et al., 1997; Muncaster et al., 2013; Muncaster et al., 2023; Nakamura et al., 1989; McBride & Johnson, 2007). Juvenile females were found as small as 64 mm TL with evidence of early sex change seen as small as 52 mm TL in ET fish. Both ET and JF samples contained pre-vitellogenic oocytes with no mature oocytes containing cortical alveoli.

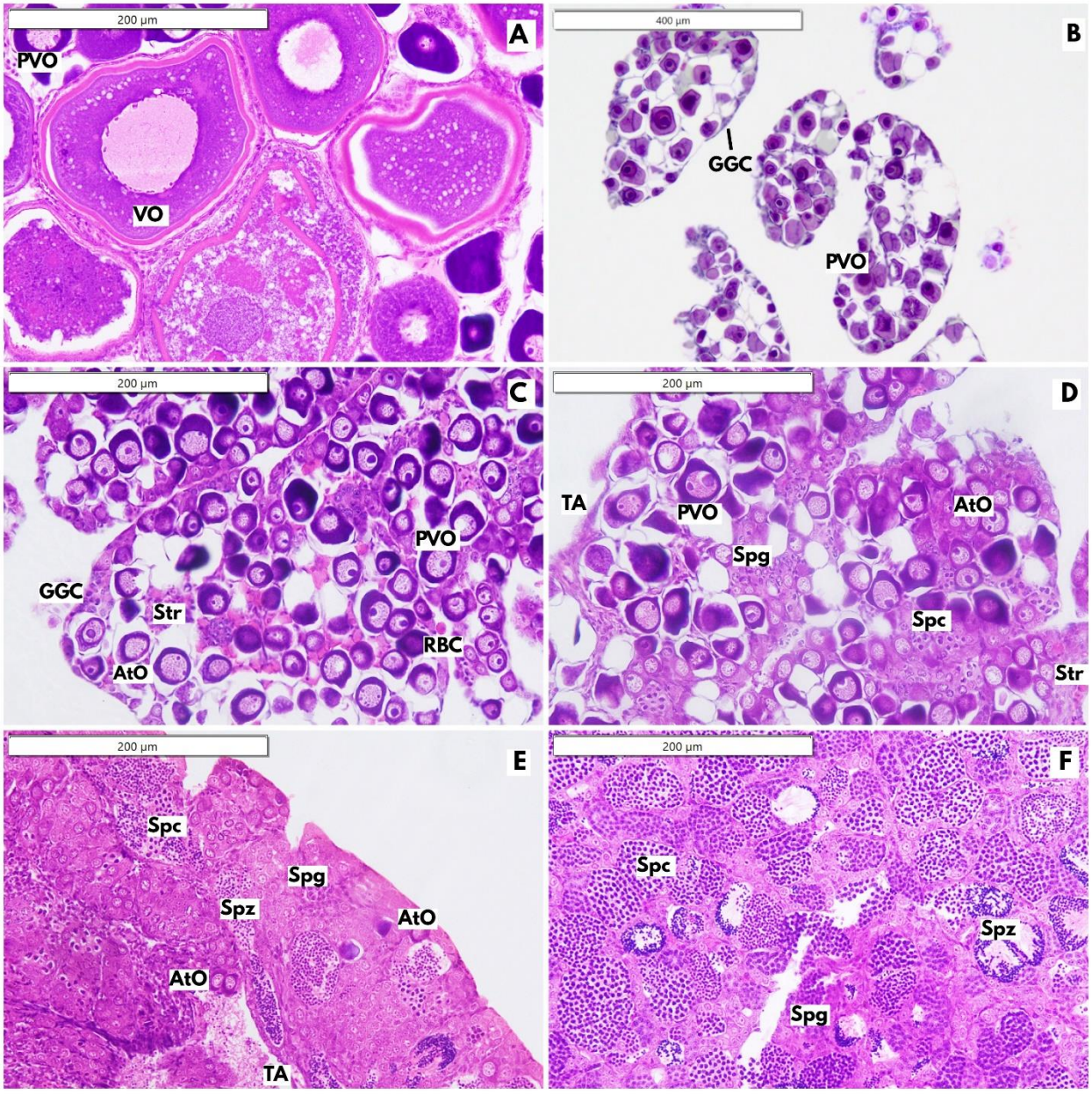

Figure 2.1: Histological stages of gonadal sex change in *Notolabrus celidotus* (stained with haematoxylin and eosin); A: Adult female with pre-vitellogenic (PVO) and vitellogenic oocytes (VO). **B:** Juvenile female predominated by pre-vitellogenic oocytes with gonial germ cells (GGC). **C:** Early transitional; atretic oocytes (AtO) and presence of stromal cells (StC) and red blood cells (RBC). **D:** Mid transitional; tunica albuginea (TA) evident, oocyte numbers diminished, and ovarian follicles were largely atretic, with proliferation of spermatogonia (Spg). **E:** Late transitional; spermatogenic cysts (Spc) predominate over atretic oocytes and TA becomes centrally located in solid testes. **F:** Initial phase male; mature testis with spermatozoa (Spz) in cysts arranged into seminiferous tubules. Scale bar, 200 µm (A, C, D, E & F) and 400µm (B).

Approximately 19 Early transitional individuals were identified, by the onset of oocyte atresia, (degenerating previtellogenic oocytes), nests of gonial germ cells (presumed to later become spermatogonia), masses of stromal cells and cellular debris. The Gonial germ cells and stromal cells were located near the periphery of the lamellae **(Figure 2.2A)**. In some cases, the nuclei of the oocytes appeared irregular in shape with irregular vesicles of various sizes in the cytoplasm. In addition, the ooplasm appeared to have shrunk, and irregularly shaped spaces appeared between the ooplasm and the surrounding immature granulosa cells **(Figure 2.2B)**.

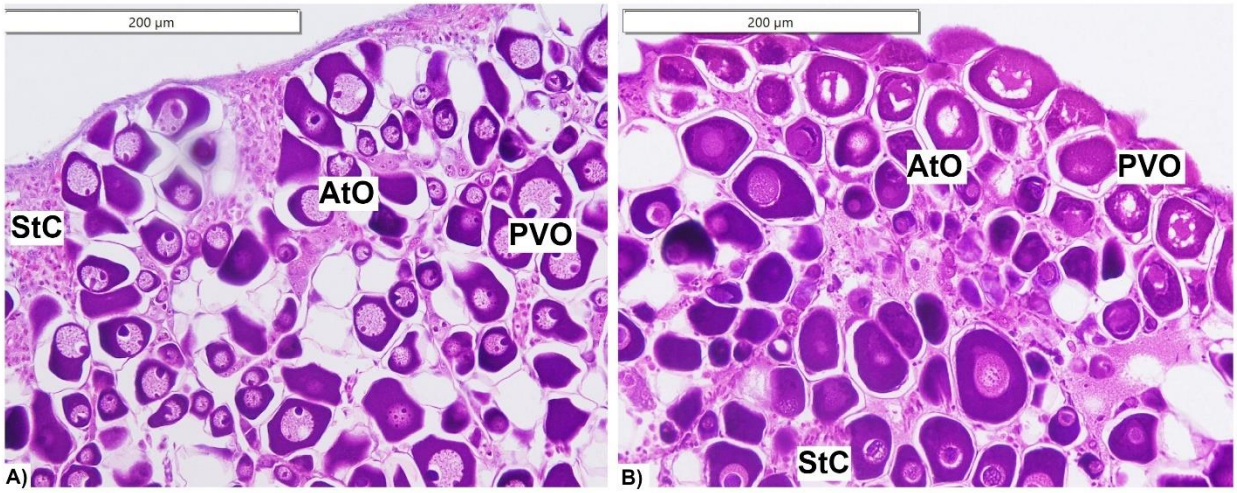

Figure 2.2: Early transitional *Notolabrus celidotus* gonads (stained with haematoxylin and eosin). With previtellogenic/atretic oocytes (PVO), stromal cells (StC) and cellular debris and atretic oocytes (AtO) **(A & B)** with irregularly shaped vesicles in the ooplasm **(B)** using a transverse cross sections of a sexually immature female ovaries of *Notolabrus celidotus*. Scale bar, 200 µm.

The five mid-transitional individuals were identified by reduced numbers of healthy oocytes and many atretic follicles; there was also an increased number of gonial cells which were presumed to be spermatogonia. Loose connective tissue and stromal cells were evident in the lamellae.

In the four late transitional individuals, spermatogenic cysts predominate over atretic oocytes. Atretic oocytes at the peri-nucleolus stage had disappeared completely by this stage, but a few cysts of oocytes at the premeiotic stage remain. spermatogonia were grouped into cysts where meiosis and spermatogenesis proceeded with lobules beginning to form.

The two smallest male specimens found both measured 77 mm TL and had small, filiform, translucent testes that contained spermatogonia with several spermatogenic cysts containing

spermatocytes and spermatozoa. All testes contained spermatogenic cysts, enclosed in somatic Sertoli cells, that were arranged within lobules. Germ cells ranged from spermatogonia to mature sperm. All testes identified appear to be solid testes, primarily characterised by central seminiferous, collecting ducts and blood vessels and the lack of a residual ovarian lumen. Some of these IP males still have residual oocytes and whilst still containing cysts of spermatozoa.

2.4 Discussion

This study provides an in-depth description of sex change in prepubertal spotty wrasse. Fish were able to be classified into three separate transitional states (ET, MT, LT) between female and IP male based on gonadal histology. This was characterised by atresia of previtellogenic oocytes and an apparent increase in gonial cells in ET fish. This leads to the proliferation of cysts of spermatogenic germ cells in MT individuals and these ultimately become arranged into a lobular testis formation in LT fish. This general pattern of gonadal sex change mirrors that seen in sexually mature spotty wrasse (Goikoetxea et al., 2021; Muncaster et al., 2023). Jones (1980) observed that sex change leading to IP male formation occurred between 80-90 mm SL and some individuals reached maturity at 100-110 mm SL. However, results obtained in the current study show early signs of sex change beginning in females as small as 52 mm. These results show that IP male formation in spotty wrasse occurs at a smaller size and therefore likely earlier in the life cycle than currently reported in the literature. Sex change in sexually mature spotty wrasse typically occurs between November and May (Jones, 1980). This also coincides with the timing of the IP fish capture in this research and considering that transitional fish from ET through to LT and IP male were found in this study, it seems indicative that prepubertal sex change also occurs at this time of year. The process of IP male sex change appeared to follow the undelimited model where both male and female germ cells were interspersed throughout the gonad, particularly in MT and LT fish. This is consistent with observations in adult spotty wrasse (Goikoetxea et al., 2021; Moraes, 2019; Muncaster et al., 2023; Robertson, 2020). Similarly, the proportion of IP males caught in this study (6.96%) is consistent with Jones (1980) data (7.1-10.6%). This is in line with observations in *T. bifasciatum* where males make up between 2-20% of the population depending on the ratio of TP to IP males (Warner et al., 1975).

The results of the histological analysis support the conclusion of Jones (1980) that spotty wrasse sexually differentiate as female first after hatch. All of the IP males identified were likely female prior to sex change based on the presences of residual oocytes in their testes. Moreover, no IP males were observed below 77 mm TL. The smallest transitional fish identified in this study was 52 mm TL. But it is important to note that this does not exclude the possibility of even smaller transitional or male fish existing in nature and therefore even smaller fish < 50 mm TL should be targeted in future work. The identification and dissection of gonads in such small fish can be problematic and would be best achieved using whole cross-sections prepared from the fish's trunk. Based on the fact that all fish appear to first differentiate as female, Jones (1980) designated spotty wrasse as monandric protogynous hermaphrodites with a single female to male pathway. Sexually immature transitional fish do seem to develop directly from females with a fully differentiated ovary containing previtellogenic oocytes and oogonial germ cells. None of these fish appeared to have entered puberty based on the absence of oocytes containing cortical alveoli or more advanced stages of development. These cellular inclusions are produced under the influence of FSH and are therefore key indicators of HPG axis activation in female fish and therefore puberty (Schulz et al., 2010; Taranger et al., 2010). Based on these observations IP male spotty wrasse appear to form exclusively from differentiated prepubertal females. These fish have presumably undergone sex differentiation to form an ovary from an undifferentiated protogonad during their early development.

Interestingly, other authors describe similar processes during the gonadal formation of IP males, but the terminology used to explain this can differ. For example, Liu & Sadovy (2009) refer to the development of a bisexual gonad leading to IP male formation in protogynous, orange-spotted grouper (*Epinephelus coioides*) and humpback grouper (*Cromileptes altivelis*). Here they describe the formation of an ovary from an *undifferentiated gonadal phase* which subsequently transitions through a *bisexual phase* containing both female and male germ cells. This bisexual gonadal phase concludes in a form of sex differentiation to yield *differentiated males* which are sexually immature. Similarly, zebrafish (*Danio rerio*) are labelled as undifferentiated gonochorists, where all fish first develop an immature ovary during prepubertal development before differentiation into a testis or ovary (Siegfried, 2010) also referred to as 'juvenile hermaphroditism' by Takahashi (1977). Examination of the histological descriptions of these different gonadal phases appears to correspond well to the key stages described in the present study. The fact that these authors

seemingly describe the same gonadal transformation in juvenile individuals suggests that different terminology is creating ambiguity in the literature.

IP male spotty wrasse likely reach sexual maturity at a much earlier and smaller developmental stage than previously realised. The smallest IP males detected in this study were 72 mm TL. Taranger et al. (2010) specify that puberty in male fish occurs the first time that type A spermatogonia differentiate into rapidly proliferating type B spermatogonia **(Figure 2.3)**.

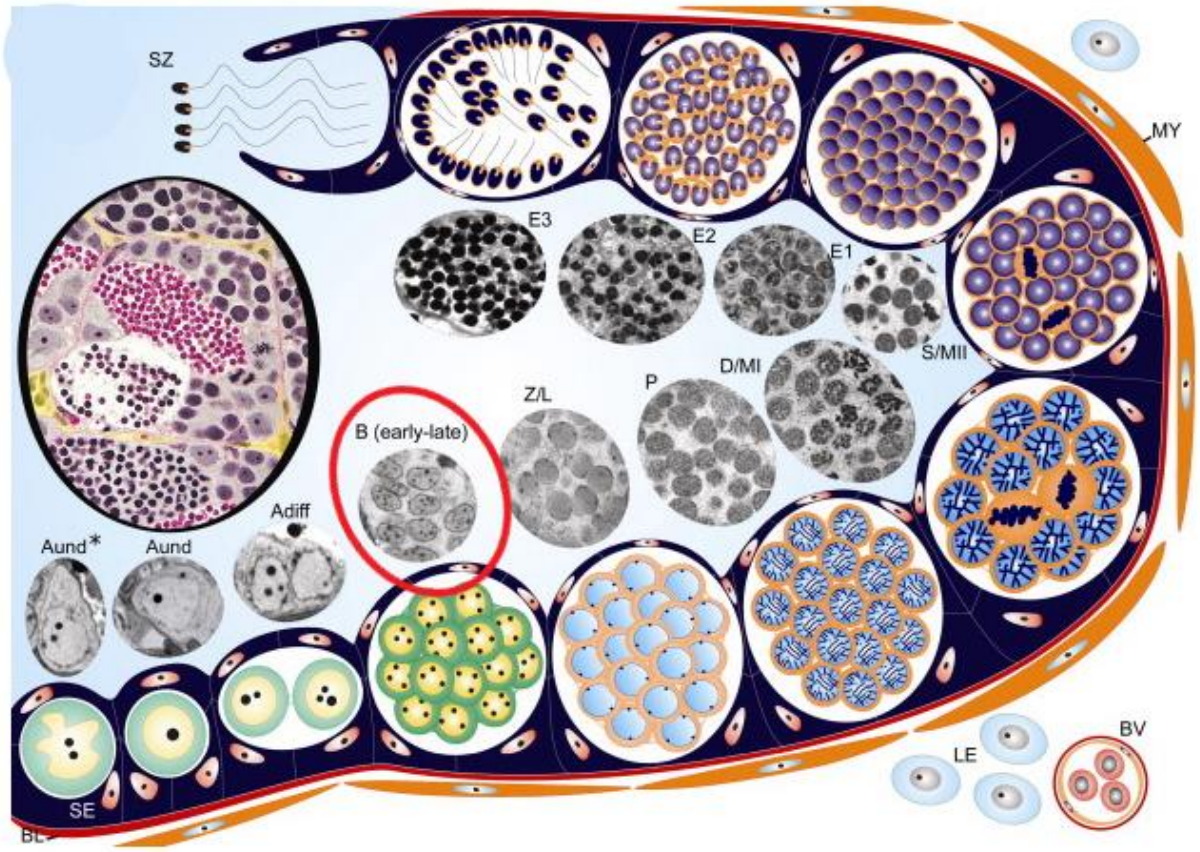

Figure 2.3: Zebrafish testis. Segments of spermatogenic tubules are shown to illustrate cystic spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). The interstitial Leydig cells (LE) and blood vessels (BV) are shown. Type A undifferentiated∗ spermatogonia (Aund∗); type A undifferentiated spermatogonia (Aund); type A differentiated spermatogonia (Adiff); spermatogonia type B [B (early–late)]; leptotenic/zygotenic primary spermatocytes (L/Z); pachytenic primary spermatocytes (P); diplotenic spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ). Red circle indicates the developmental point for puberty. Modified from Schulz et al. (2010).

The testes of IP male fish in the current study had cysts of spermatogenic germ cells at all developmental stages as well as evidence of spermatozoa in the collection ducts. Similar observations were also evident in the LT individuals, although the number of spermatogenic cysts appeared fewer in general in these fish. These observations infer that the testicular maturity of the fish was well past the point of the criteria outlined for puberty in male fish. Therefore, based on the population of fish collected in this study, where all female fish under 110 mm TL were sexually immature, it appears that the formation of IP males is closely associated with the onset of puberty. The ecological rationale for sexual maturity in IP male spotty wrasse to occur somewhat earlier in the life cycle than either IP females or most TP males is unknown. One possible explanation may be that posing as an apparently sexually immature female may allow these fish to more effectively execute their sneaker spawning strategy without attracting the attention of TP fish. Taborsky (1994) states that where two male forms exist, the smaller and weaker type will need to develop an alternative reproductive strategy to compete successfully to gain access to females. In spotty wrasse, dominant TP fish are presumably less likely to detect these individuals as possible competitors within their territory, allowing IP males to get closer to courting females to fertilise their eggs. In addition, with size being less of a barrier for male reproductive fitness due to the lower energetic cost of sperm production, these fish may increase their reproductive fitness by maturing earlier than their conspecifics. The Size Advantage Model is a widely accepted theory explaining the drivers of sex change in animals (Ghiselin 1969). The model clearly outlines that sex change is beneficial for a species when reproductive potential is greater as one sex when small and another sex when older and larger. Therefore, the timing of sex change should maximise lifetime reproductive success (Helfman, Collette, Facey, & Bowen, 2009; Warner, 1988; Warner et al., 1975; Charnov, 1982).

The fact that IP male sex change occurs prior to sexual maturity raises questions about the classification of spotty wrasse as monandric hermaphrodites. By definition, a monandric hermaphrodite should function reproductively as a female prior to sex change and only has one male morph (Devlin & Nagahama, 2002; Liu & Sadovy, 2004a; Sadovy & Shapiro, 1987; Warner & Robertson, 1978). However, the results presented in the current study indicates that this is not the situation in spotty wrasse. Initial phase males arise from females that have undergone sex change prior to having entered puberty and they have, therefore, never functionally reproduced as females. Sadovy & Liu (2008), and Connington et al. (2010) classify males that develop from

immature females as non-functional hermaphrodites. Other monandric species that have been described in the literature also tend to differ to spotty wrasse in that they have a different social structure (dominant territorial male and subordinate sneaker male), and two distinct male morphologies (Boddington et al., 2021; Candi et al., 2004; Cossington et al., 2010; McBride & Johnson, 2007). Robertson & Warner (1978) determined that members of the Scaridae family such as the yellowtail parrotfish (*Sparisoma rubripinne),* blue parrotfish *(Sparisoma chrysopterum),* stoplight parrotfish *(Sparisoma viride),* and redband parrotfish *(Sparisoma aurofrenatum),* were all monandric based on the fact that they only have one mating strategy and testicular structure. Contradictorily, Fennessy & Sadovy (2002) assert that some diandric species have no morphological testicular differences between IP and TP males despite being labelled as diandric. Munday et al. (2006) discuss differences between their work with *T. bifasciatum* and that of Liu & Sadovy (2004a; 2004b) in the rock cod (*Cephalopholis boenak*). *T. bifasciatum* have IP males that arise directly from undifferentiated gonads while rock cod follow a similar pattern to spotty wrasse, differentiating first as female and then undergoing pre-maturational sex change to form IP males. Munday et al. (2006) highlight that *T. bifasciatum* IP males have a primary testis while IP male rock cod possess secondary testes. These authors consider the structural difference between the two testis types to be of little functional relevance from an ecological point of view. Rather, they consider the existence of separate male spawning strategies of greater evolutionary importance. It may be that in spotty wrasse, becoming a small male with a sneaker spawning strategy allows individuals to reproduce earlier, albeit with limited success. However, the real advantage may be the ability to quickly transition to assume dominant spawning status when the existing TP male eventually disappears. Adult sex change in spotty wrasse can take approximately 60 days to complete (Goikoetxea et al., 2021). In contrast, IP males presumably need only increase existing androgen production to assume the secondary sexual characteristics of TP males (Muncaster pers. comm.). Considering that IP and TP male spotty wrasse appear to change sex at different life stages (pre- and post- maturation), and each is associated with a different reproductive strategy from the other, it no longer seems relevant to consider this species to be a monandric protogynous hermaphrodite.

Chapter 3 - Gene expression

3.1 Introduction

The development of molecular techniques has been greatly beneficial to global aquaculture research and fisheries management as it allows for a more in depth understanding of physiological processes and allows for fine-scale changes to be made to farming techniques (Thakur et al., 2008). For example, use of transcriptomic analysis of the Tibetan highland fish *(Gymnocypris przewalskii)* has revealed variations in sex hormone genes between reproductively active and dormant fish is influenced by photoperiod (Tian et al., 2019). In terms of sex differentiation, molecular research allows for characterisation of key genes that regulate the timing and progression of sex change. Genes that exhibit expression changes early on in sex change are of particular interest as proximal molecular regulators of the process (Thomas et al., 2019).

In teleost ovaries, steroids produced in the thecal cells such as testosterone are made available to the granulosa cells where the enzyme aromatase is expressed, resulting in the conversion of testosterone to estradiol-17β which is required for oocyte growth. The administration of sex steroids at the time of sex determination can strongly influence the course of sex differentiation in fish, suggesting that they play a critical role in assignment of gonad determination as well as subsequent differentiation (Devlin & Nagahama, 2002). If steroids are responsible for advancing sex differentiation or sex change rather than being produced as a result of the process, then steroid producing cells should be evident before morphological differentiation of the gonad (Nakamura, Kobayashi, Chang, & Nagahama, 1998). Aromatase is usually either not detectable or at low levels in testicular tissue; however, estrogen synthesis is required in both sexes and understanding differences in sex steroid profiles may be critical in understanding sex determination (Devlin & Nagahama, 2002). It is currently unknown where in the sex-change pathway sex-determination mechanisms first intervene to initiate alternate courses of steroid production and differentiation. It is assumed that in wrasse species, sex change is initiated by a signal from the brain to suppress estrogen synthesis (Nakamura, Bhandari, & Higa, 2003; Nakamura et al., 2005; Higa, Ogasawara,

Sakaguchi, Nagahama & Nakamura, 2003). This could be mediated by downregulation of the gene encoding the aromatase enzyme.

The developmental pathway leading to steroid production in gonadal somatic cells undoubtedly requires complex regulation of a multitude of genes. Therefore, a targeted approach to characterise their expression across sex change is warranted. Quantitative PCR (qPCR) is a technique that measures the expression of genes of interest, relative to a housekeeping gene, by targeting and amplifying specific genes using specifically designed primers (Kubista et al., 2006). Ultimately, this technique can allow the accurate measurement of gene expression in different life stages or when the organism is manipulated under different treatments.

3.1.1 Genes involved in sex differentiation

Molecular research on teleost fishes to date has identified numerous genes that are likely involved in sex differentiation. In particular: *sry* (Sex-determining region Y protein) (Capel, 1998; Siegfried, 2010), *dmy* (DM-domain gene, Y chromosome) (Siegfried, 2010), *dmrt1 (*Doublesex and mab-3 related transcription factor 1) (Banh, Domingos, Zenger, & Jerry, 2017; Herpin & Schartl, 2011a; Li et al., 2015), *cyp19a1a* (Aromatase enzyme) (Smith, Guzman, & Luckenbach, 2013; Tchoudakova, Kishida, Wood, & Callard, 2001; von Schalburg, Gowen, Messmer, Davidson, & Koop, 2014), *amh* (Anti-Mullerian hormone) (Li et al., 2013), *sox9* (SRY-box transcription factor 9) (Herpin & Schartl, 2011b), *dax1* (Dosage-sensitive sex reversal, adrenal hypoplasia critical region, chromosome X, gene 1) (Hu, Guo, Gao, Tang, & Li, 2015) and *vasa* (Ddx4, DEAD box polypeptide 4) (Yoshizaki et al., 2000). However, the master sex determining genes in fish are poorly understood due to lack of conservation among vertebrates (Sekido & Lovell-Badge, 2009). As the genes involved in sex differentiation are characterised in more species, the existence and role of master sex determination will become clear.

Aromatase (cyp19a1a)

The aromatase enzyme (*cyp19a1a*) is responsible for synthesising estrogens in both male and female fish and therefore it indirectly controls behavioural, physiological, and developmental responses that are crucial to reproduction (Blazquez & Piferrer, 2004; Sundaray, Ohta, Yamaguchi, Kitano, & Matsuyama, 2005). Aromatase facilitates the bioconversion of testosterone (T) to 17β-

estradiol (E2) and is produced in the granulosa cell layer of the oocyte follicle in order to maintain ovarian function (Devlin & Nagahama, 2002; Sundaray et al., 2005). In species such as the *T. duperrey* (Nakamura et al., 1989), *E. merra* (Bhandari et al., 2003), *T. bifasciatum* (Liu et al., 2017), and *L. bergylta* (Muncaster, 2013) an early decrease in estradiol concentration has been associated with the onset of sex change. This is further illustrated by manipulative experiments where chemical inhibition of *cyp19a1a* induces masculinisation (Afonso, Wassermann, & Terezinha de Oliveira, 2001; Bhandari, Alam, Higa, Soyano, & Nakamura, 2005; Chourasia & Joy, 2008; Diotel et al., 2010; Gao et al., 2010; Higa et al., 2003; Kroon, Munday, Westcott, Hobbs, & Liley, 2005; Kwon, Hashpanah, Hartudo, McAndrew, & Penman, 2000; Kwon, McAndrew, & Penman, 2002; Nozu, Kojima, & Nakamura, 2009; Singh, Singh, & Tripathi, 2012; Singh, 2013; Sun, Zha, Spear, & Wang, 2007; Vizziano et al., 2008).

The expression of aromatase in *T. duperrey* is rapidly arrested in transitioning females which coincides with a sharp decline in estradiol levels and the onset of ovarian atresia (Nakamura et al., 1989). Therefore, the arrest of aromatase expression may initiate gonadal sex change in protogynous species by interrupting the estrogen dependent processes that are responsible for ovarian function and maintenance (Todd et al., 2016; Liu et al., 2017). It is expected that the *cyp19a1a* gene will be upregulated during ovarian differentiation, and downregulated when male promoting genes are expressed. This would result in the prevention of estrogen production in the gonads of sex changing fish.

Anti-Mullerian hormone (amh)

In mammals, reptiles, and birds, *amh* is responsible for the regression of the Müllerian duct in the male foetus during early testis differentiation (Halm, Rocha, Miura, Prat, Zanuy, 2007). In teleost fish, *amh* is essential in early sex determination and gonadal development and its expression is tightly controlled (Luo et al., 2020). Production of *amh* occurs primarily in Sertoli cells surrounding undifferentiated spermatogonia, where it suppresses aromatase activity, germ cell proliferation and differentiation as well as steroidogenesis in the interstitial Leydig cells (Pfennig, Standke, & Gutzeit, 2015). This aromatase inhibition induces the undifferentiated gonad to develop into testes (Rey, Lukas-Croisier, Lasala, & Bedecarrás, 2003; Schulz et al., 2010). This expression profile, and the interaction between *amh* and other key sex determining genes such as *sox8* (SRY-Box Transcription Factor 8), *sf-1* (steroidogenic factor 1), and *gata-4* (GATA Binding

Protein 4) may differ between species (Luo et al., 2020). Particularly in mammals such as humans and mice (Anttonen, Unkila-Kallio, Leminen, Butzow, & Heikinheimo, 2005; Capel, 1998; Schepers, Wilson, Wilhelm, & Koopman, 2003), reptiles (Western, Harry, Graves, & Sinclair, 1999) and birds (Takada, Mano, & Koopman, 2005). Differences in *amh* expression patterns may also be evident in fish such as the Japanese flounder where *amh* is expressed most highly in males and is present in Sertoli cells surrounding spermatogonia but not in cells surrounding spermatocytes or spermatozoa, and not detectable in ovaries (Yoshinaga et al., 2004). Similarly, in the Japanese eel, *amh* expression is highest in the testis (Miura, Miura, Konda, & Yamauchi, 2002). In contrast, in medaka (*Oryzias latipes*) there is no expression pattern of *amh* during gonadal development and *amh* is expressed in both ovaries and testes (Klüver et al., 2007). Further differences in expression profiles can be seen in *D. rerio* (Rodríguez-Marí et al., 2005) where *amh* expression was seen most highly in undifferentiated gonads.

Doublesex and mab-3 related transcription factor 1 (*dmrt1)*

Doublesex and mab-3 related transcription factor 1 (*dmrt1*) encodes a transcription factor critical for male gonadal development (Herpin & Schartl, 2011a; Nagahama, 2005). *Dmrt1* is considered to be an important driver of sex determination for invertebrates (Baker & Ridge, 1980; Shen & Hodgkin, 1988), birds (Siegfreid, 2010) and mammals (Raymond, Murphy, O'Sullivan, Bardwell, & Zarkower, 2000). In fish, *dmrt1* has been recognized as playing a key role in the male sex-determination pathway in species such as the Atlantic cod (*Gadus morhua*) (Johnsen & Andersen, 2012), black porgy (*Acanthopagrus schlegeli)* (He et al., 2003) and rice-field eel (*Monopterus albus)* (Huang et al., 2002). Recent studies have indicated that *dmrt1* is also upregulated in adult bluehead and spotty wrasse during protogynous sex change (Muncaster et al., 2023; Todd et al., 2019). To date there are no studies examining the expression of these genes that directly influence sex differentiation in juvenile protogynous sex changing fish.

3.2 Aims and objectives

The aim of this study was to explore how key reproductive genes might influence sex change in prepubertal spotty wrasse. This was examined through the quantification of *cyp19a1a, amh,* and *dmrt1* expression across the different stages of prepubertal sex change.

3.3 Materials and Methods

Thirty-five samples were selected for gene expression analysis from the original 58 fish that had yielded gonadal tissue. These fish covered a range of stages of sex change based on histology (Results 2.3). Not all 58 samples were used as in some cases an excess of samples existed for a specific developmental stage or the quality of individual RNA samples was not high enough. Of the eight male samples previously identified, four IPM fish (77-105 mm TL) had tissue available for analysis, as the remainder were donated from previous research. In addition, five AF fish (128-270 mm TL) and 13 JF fish (73-106 mm TL), all of which showed no signs of sex change, were selected to illustrate the difference in gene expression for both a functional and fully formed male and female at each end of the sex change pathway. In addition, seven ET fish (52-95 mm TL) in the earliest detectable stages of transitioning and five MT fish (60-76 mm TL) were also selected and used to examine levels of gene expression once sex change began. Unfortunately, only one LT individual (95 mm TL) had tissue available for gene expression analysis, as the remainder were donated from previous research (**Table 3.1**). All assays and laboratory work for gene expression was conducted by the investigator in the University of Waikato Laboratories, G.02 and T.01 on Durham Street, Tauranga, New Zealand.

3.3.1 Primer design

N. celidotus Glucose-6-phosphate dehydrogenase (*g6pd*), β-Actin 2 (*actb2*), cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), Anti-Mullerian hormone (*amh*) and Doublesex and mab-3 related transcription factor 1 (*dmrt1*) gene sequences were first identified within the available genome (fNotCel1.alt; GenBank assembly accession: GCA_009762545.1). Using these gene sequences and the recommendations for typical primer design by X. Yang et al. (2006) as guidelines, qPCR primers were designed, using the following specifications: i) a melting temperature (Tm) between 60-62°C, ii) a length of 20-25 nucleotides, iii) a GC content of 40-60%, and iv) a 3’ end GC clamp. In addition, the Tm difference between the forward and reverse primer was within 1^{o}C to ensure efficient amplification and either the forward or reverse primer was designed to cross an exon/exon boundary within the gene to prevent genomic DNA amplification.

For each gene, two sets of forward and reverse primers were designed and tested (**Appendix I**). This was to ensure the final set of primers to be used in future expression work, gave no non-specific amplification (as shown by the melt curve and gel electrophoresis of the PCR products) and a good amplification efficiency (Primer efficiency for each run was obtained using the LinRegPCR algorithm) (Ruijter et al., 2009). During this time, the annealing temperature for each primer set was optimised. **Table 3.1** shows the final sets of primers that were used for the expression study.

Table 3.1: Specific primer sequences designed for qPCR amplification of target genes (*cyp19a1a, amh, dmrt1*) and reference genes (*actb2, g6pd*) in spotty wrasse. The average efficiency ± standard deviation of all the qPCR runs for one gene is shown. Abbreviations: *amh*, anti-müllerian hormone; bp, base pairs; *cyp19a1a*, gonadal aromatase; *Actb2*, β-Actin 2; FW, forward; *g6pd*, glucose-6-phosphate dehydrogenase; RV, reverse.

Gene	Primer sequence (5'-3')	Annealing temp. (^{O}C)	Tm (^{O}C)	Amplicon size (bp)	Efficiency (Mean%± SD)	GC (%)
cyp19a1a	FW: TGGACACTGTTGTTGGTGAC	60	62.5	161	0.91±0.03	50
	RV: AGGTACTCTATAGCCCTCTATGATG	60	62.2			44
amh	FW: GAAGAACTGAAACAAGATCTGCAC	60	62.2	134	0.90±0.02	42
	RV: GGATTCAAGCTGAAGGGAAGAG	60	62.6			50
dmrt1	FW: ACCTCTACAACTACCAACAATACC	61	62	205	0.91±0.02	42
	RV: AGACCTCCTGGAGAGAAAGAG	61	62.1			52
g6pd	FW: CGACGTCATGCAGAACCA	60	62.5	106	0.96±0.02	50
	RV: RV: CAGCACCTTCACCTTTTCGT	60	62.8			50
Actb2	FW: CCCACTACCATGAAGATTAAGATCA	60	62.2	200	0.95±0.03	40
	RV: AGTGTGTGTTTTGGGGGAGG	60	64.4			55

3.3.2 RNA extraction

Preparation of high purity RNA with no genomic DNA contamination is essential in achieving the best results from qPCR and helps to prevent the amplification of non-specific products. To achieve this, RNA was extracted from 35 (n=5 AF, n=4 males, n=13 JF, n=7 ET, n=5 MT, n=1 LT) spotty wrasse gonad tissue samples using the Direct-zol™ RNA Miniprep kit (Zymo Research, USA, Catalog Number: R2053). TRIzol® reagent allows the isolation of high-quality RNA from tissue lysate. Extraction of total RNA from ~100 mg of gonad tissue was carried out following the manufacturer's instructions. Initially, each of the 35 gonad samples were homogenised in 2 ml screw cap RNase/DNase free tubes containing 800 µL of TRIzol® reagent, and approximately 50 µl of a mixture of 0.1 mm and 0.5 mm diameter silica beads. A homogeniser (Precellys®

Evolution, France) was used at 6800rpm for three, 20 seconds bursts with 30 second intervals in between to allow the sample to cool. If the lysate remained cloudy, due to incomplete homogenisation, a second cycle in the homogeniser was performed. Once the lysate was clear, the supernatant was then pipetted out from the beads into a new 1.5ml RNase/DNase free tube and 800µL of 100% ethanol was added.

The tube was inverted by hand for 15 seconds, and the complete contents loaded into a Zymo-Spin™ column and centrifuged at 13500 rpm for 1 minute. The flow through was discarded and each sample then underwent a DNase I (Zymo Research, USA) treatment by first adding 400µL of RNA wash buffer to the column and centrifuging at 13500 rpm for 1 minute. After discarding the flow through 75µL of digestion buffer and 5µL of DNase I were directly pipetted onto the column matrix and each sample was incubated at room temperature (20-30°C) for 20 minutes. Following this, 400µL of Direct-zol Prewash (Zymo Research, USA) was added, centrifuged through the column at 13500 rpm for 1 minute and the flow through was discarded. This step was repeated, to ensure the removal of DNase from each sample. The final wash step involved adding 700µL of RNA Wash Buffer and centrifuging at 13500 rpm for 2 minutes to ensure removal of all ethanol. RNA was then eluted with 50µL DNase/RNase free water and centrifuged at 13500 rpm for 1 minute. The RNA concentration and purity was determined using the DeNovix® DS-11 spectrophotometer (DeNovix, USA). The mRNA was then used immediately for cDNA synthesis and then stored at -20°C to minimise RNA degradation.

3.3.3 RNA quality and quantity

Spectrophotometric Analysis

A DeNovix® DS-11 spectrophotometer (DeNovix, USA) was used to determine the concentration and purity of RNA (**Appendix II)**. This machine utilises surface tension to hold samples in place between two optical fibres and only requires 1 µl of sample to be used. The machine was blanked using 1 µl of DEPC-treated water, after which 1 µl of each sample was loaded and read. Kimtech Science™ KimWipes™ Delicate Task Wipes (Mediray, NZ) were used to clean the optical fibre ends between each sample. The software calculated sample concentrations by comparing absorbance readings at 260 nm between the blank and each sample. Purity was assessed by the A230/A260, and A260/A280 absorbance ratio, where a ratio of ~2.0

was considered pure for RNA. Nucleic acids absorb at 260 nm, whereas 280 nm is a good indicator of protein contamination (when ≥ 1.8, it indicates a pure DNA sample) and 230 nm is a good indicator of contamination probably caused by organic compounds or chaotropic agents (when ≥ 1.8, it indicates a pure DNA sample). Important to note, during the RNA extraction process, it was found that the amount of gonad tissue used in the RNA extraction made a considerable difference to final RNA quality when using the column-based method. During extraction, it was found that gonad tissue from this species often gave a high final RNA concentration (ng/µl) but very low purity values. It was found that such high quantities of RNA were blocking the Zymo-spin column and preventing the RNA from being efficiently washed. This was subsequently remedied by repeating extractions on remaining tissues using a much smaller section of gonad tissue and leading to suitably pure RNA.

Gel electrophoresis

During the early RNA extractions, RNA quality of *N. celidotus* gonad tissue was also tested by visualising it using gel electrophoresis. A 1% agarose gel was prepared by weighing 0.25g agarose powder (Fisher biotec, Cat#AG100) into a 50 ml conical flask and adding 25 ml of 1x Tris-acetate-EDTA (TAE). The solution was heated in a domestic microwave on full power until it began to boil, and the solution became clear, indicating the agarose had dissolved. As the solution cooled, sodium hypochlorite (25µl) was added to a final concentration of 6% and 2.5 µl of GelRed® Nucleic Acid Stain (Sigma, USA). GelRed® is a fluorescent nucleic acid stain which binds to RNA in the gel and fluoresces under ultraviolet light. Once cooled, the solution was carefully poured into a gel mould to avoid bubbles being introduced and a 12 well comb was placed into the mould. The gel was left to set for approximately 20 minutes, after which the gel combs were carefully removed. The gel was placed in an electrophoresis mini gel system tank (Life Sciences, Mini Horizontal) and enough 1x TAE buffer added to completely immerse the gel. Using Parafilm® (Sigma-Aldrich), 2µl of Loading buffer and 5µl of RNA template were mixed by pipetting up and down. Mixtures were then loaded onto the gel by pipetting 6 µl into each lane, excluding the first lane which was loaded with 4 µl of Solis BioDyne 1kb DNA ladder as a size reference. A power supply (Life Technologies. PowerErase®, 300W) was connected to the electrophoresis tank and activated for 20 min at 90 V, causing the RNA to travel towards the negative electrode and separate according to size. Following electrophoresis, the gel was placed

under a UV light and imaged using the iBright™ CL750 Imaging System (Thermofisher, USA) and photos were captured.

3.3.4 qScript™ complementary DNA (cDNA) synthesis

To synthesise cDNA, the qScript™ XLT cDNA SuperMix kit (Quanta Biosciences, USA) was used. This kit is an engineered M-MLV reverse transcriptase that contains optimized concentrations of $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer and stabilizers. RNA samples and kit reagents were thawed, mixed well, and held on ice. The volume of each RNA sample used for cDNA synthesis was dependent on its concentration (ng/µL) which had been determined using the previously obtained DeNovix results **(Appendix III)**. To obtain 1 µg of RNA, the following equation was used for cDNA synthesis:

$$RNA\ Volume\ (\mu l) = \frac{1000}{RNA\ Concentration\ (ng/\mu l)}$$

Using the qScript™ XLT cDNA SuperMix kit, duplicate cDNA from each RNA sample was synthesized according to the manufacturer's instructions, with various components added to 200 µl RNase/DNase free PCR tubes **(Table 3.2)**, giving a final volume of 20 µL:

Table 3.2: Volumes of reaction components required to make cDNA using qScript™ XLT cDNA SuperMix. Exact volumes used for each sample found in **Appendix II**.

Reaction component	Volume per 20µL reaction
qScript XLT cDNA SuperMix (5X)	4µL
RNA template	Variable
Rnase/Dnase-free water	Variable
Total volume (µL)	20µL

Each tube was thoroughly vortexed before being pulse spun by centrifugation, to collect the contents in the bottom of each tube. The contents were then placed into a SimpliAmp™ Thermal Cycler (Thermofisher, USA) and incubated at 25°C, for 5 minutes, 42°C for 60 minutes, 85°C for 5 minutes and held at 4°C. cDNA was diluted to 1:20 in molecular grade water and subsequently stored at -20°C until used for gene expression in in quantitative-PCR (qPCR).

3.3.5 Quantitative PCR (qPCR)

qPCR was performed using a 48-well Magnetic Induction Cycler (MIC) qPCR (Bio Molecular Systems, Australia), using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, USA) and following the manufacturer's instruction. This kit contains a chemically modified DNA polymerase (AccuFast Taq) which is activated instantaneously at 95°C. Each reaction component was added in a specific volume **(Table 3.3)**, to a 200 µl RNase/DNase free MIC PCR tube (Dnature, New Zealand), giving a maximum volume of 20 µL for each reaction.

Table 3.3: Volumes of reaction components for PerfeCTa® SYBR® Green FastMix®.

Reaction Component	Volume used per reaction (µl)
cDNA template	8
Forward primer (10nM)	1
Reverse primer (10nM)	1
2x PerfeCTa® SYBR® Green FastMix®	10

Each sample was carried out in duplicate, and No Template Controls (NTCs) were also performed for each primer pair. NTCs contained all of the reaction components except cDNA template which was replaced with molecular grade RNase/DNase free water. An inter-plate calibrator was also run in duplicate for each qPCR assay, to ensure consistency in cq values and repeatability across assays. Amplification was carried out using the following conditions: one cycle of 95 °C for 30 seconds, 40 cycles of 95 °C for 5 seconds and then an optimal annealing temperature that had been determined previously **(Table 3.1)**, for 30 seconds. The fluorescence signal output was measured at 60 °C for each tube after each cycle and a melting curve for each qPCR was determined at the end of the programme by reading fluorescence every degree from 65 °C to 95 °C to ensure a single product has been amplified. To also check that the correct product had been amplified, initial qPCR products were run on a 2% agarose gel, stained with RedSafe, and viewed under UV light and imaged using the iBright™ CL750 Imaging System (Thermofisher, USA), to confirm the correct band size. Primer efficiency for each run was obtained using the LinRegPCR algorithm (Ruijter et al., 2009) and expression levels of *cyp19a1a, amh* and *dmrt1* were normalised to two housekeeping genes, *g6pd* and *actb2*, **(Table 3.1)**. Expression ratio of *cyp19a1a, amh* and *dmrt1* was achieved using the geometric means of the two housekeeping genes (Vandesompele et al., 2002) and using the Pfaffl method (Pfaffl, 2001) in each tissue analysed. Data was tested for homogeneity of

variance using F test and transformed using natural log where necessary. Differences in gene expression among the gonadal stages was tested using Analysis of Variance (ANOVA) or Kruskal Wallis test if the data was not normally distributed (Shapiro Wilk's test). Tukey's HSD test was used for post hoc testing of differences between individual gonadal stages. Significance was determined using $p<0.05$.

3.4 Results

Gel electrophoresis - RNA quality

Gels showed a high volume of low molecular weight RNA **(Figure 3.1)**. This is considered to be a result of massive 5S rRNA amplification in ovaries, which masks the 18S and 28S rRNA fluorescence peaks making them unreliable estimates of RNA integrity (Liu, 2016; Goikoetxea et al., 2021). Similar patterns have been observed in studies involving ovaries/gonads of thick-lip grey mullet (*Chelon labrosus*) (Diaz de Cerio, Rojo-Bartolomé, Bizarro, Ortiz-Zarragoitia, & Cancio, 2012), Sharsnout Seabream (*Diplodus puntazzo*) (Manousaki et al., 2014) and *T. bifasciatum* (Liu, 2016). Various qualities of RNA were obtained for different samples and only the best were used in subsequent analysis.

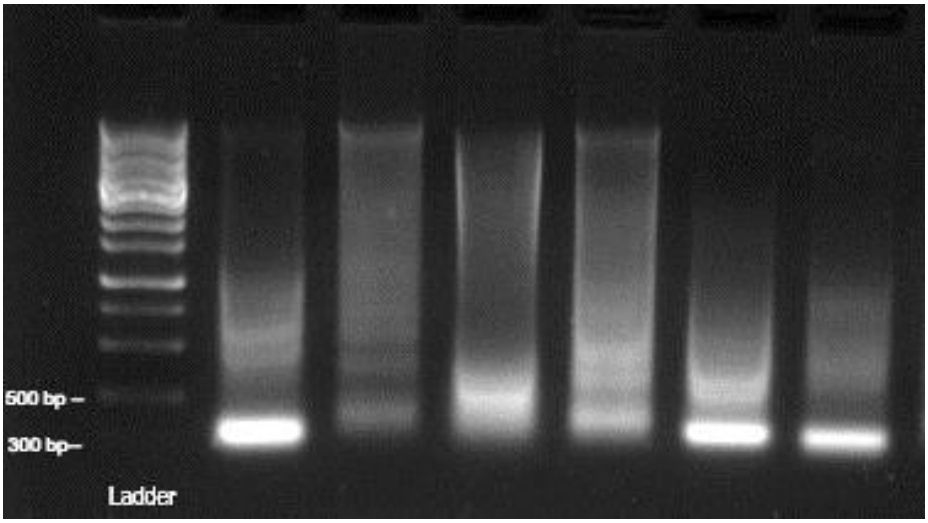

Figure 3.1: Example of gel electrophoresis showing the RNA quality of a sub-set of samples extracted with Direct-zol™ RNA Miniprep kit.

Non-specific amplification

Product visualisation determined that for most primer sets, a single qPCR product was being amplified and there was no amplification in NTCs. In cases where more than one product was visible due to primer-dimer formation or non-specific amplification, the annealing temperature was increased and annealing time decreased, leading to consistently producing single products. One of the two primer sets for the reference gene *Actb2* (β-Actin 2) resulted in consistent non-specific amplification **(Figure 3.2 & 3.3)**. Further investigation showed that the design of this primer sequence caused non-specific amplification using primers designed across an exon/exon boundary (**Appendix IV**).

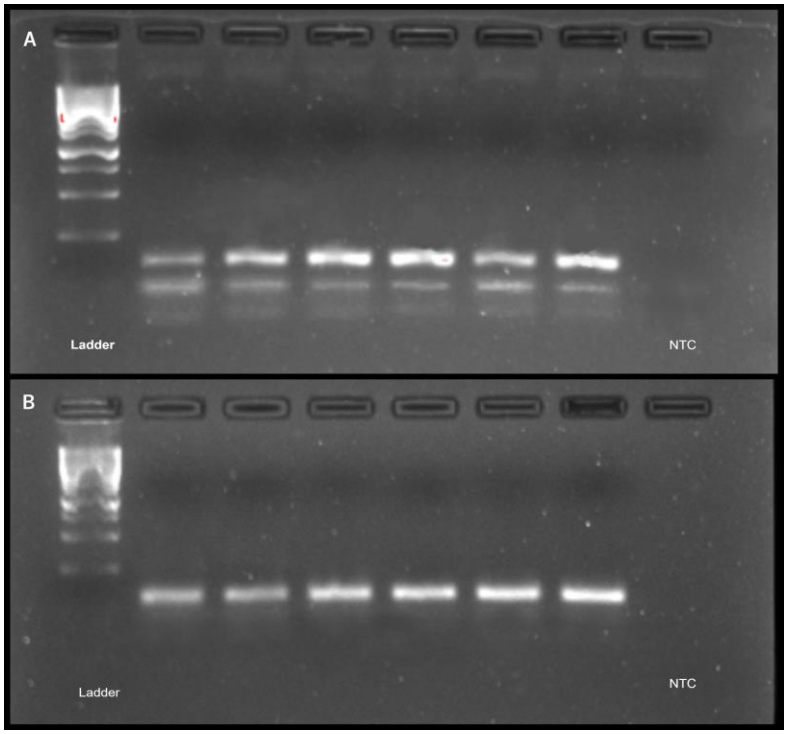

Figure 3.2: Gel electrophoresis analysis of real-time PCR products. **A)** β-actin primer set 1 amplifying two different products. **B)** Alternative β-actin primer set 2 amplifying a single product using the same samples. Each template generated a single band, with no band in the No Template Control (NTC).

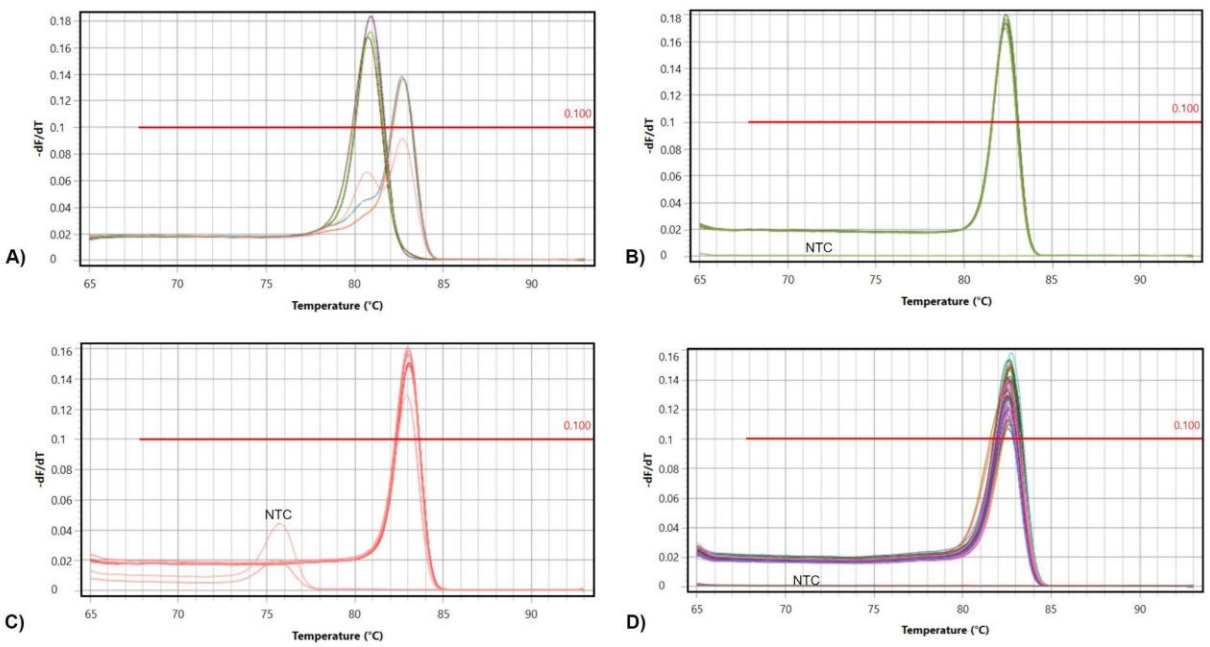

Figure 3.3: Melt curve analysis of real-time PCR products using micPCR v2.12.3. **A)** β-actin primer set 1, amplifying two different products. **B)** Alternative β-actin primer set 2 amplifying a single product using the same samples. Each template generated a single peak, with no amplification in the No Template Control (NTC). **C)** Amplification in β-actin set 2 NTC due to contamination. **D)** Final melt curve for all samples showing a single product and no amplification in NTC.

Gene expression profiles

The statistical analysis of expression was limited to include JF (13), ET (7) and MT (5) stages only. The number of LT (1) and IPM (4) fish were considered too few for statistical validity. The earlier stages of IP sex change were of greatest interest for the possible identification of molecular triggers of sex change. In addition, expression values of later stage fish (IPM) tended to greatly emphasise the general trend evident in the early-stage fish.

Anti-Mullerian hormone (amh)

Amh expression showed a strong trend of increasing expression across sex change ($F = 1.149$, $p = 0.001$, ANOVA), ranging from 1.65 ± SE 0.14 in JF to 163.7 ± 41.1 in IPM **(Figure 3.4)**. Of particular interest was the early changes directly proceeding JF. The relationship between JF (Mean = 1.65 ± 0.14) to ET (Mean = 3.14 ± 0.62) showed a significant increase in expression ($p < 0.05$), with a further increase in expression in MT fish (Mean = 5.37 ± 1.2, $p < 0.01$).

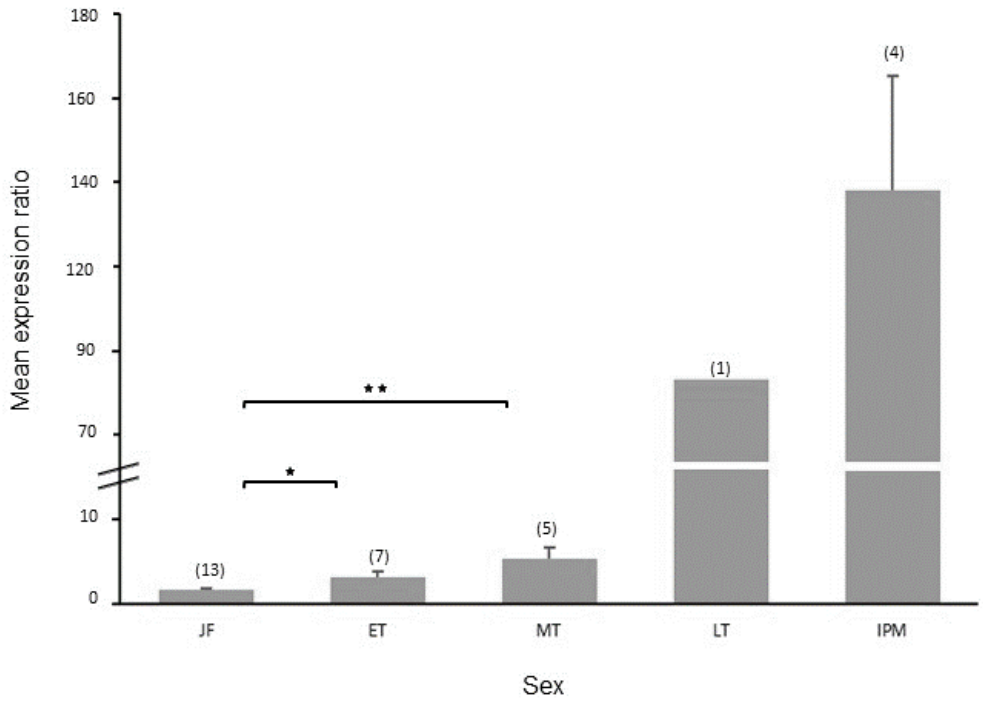

Figure 3.4: Mean expression ratio of *amh* (anti-mullerian hormone) mRNA for each sexual stage of *N. Celidotus* (JF; Juvenile female, ET; early-transitional, MT; mid-transitional, LT; late transitional, IPM; initial phase male). Error bars showing SEM. Expression levels are compared between JF, ET, and MT individuals using an ANOVA and Tukeys HSD post-hoc test. LT and IPM groups were not included in the analysis due to low sample numbers. Sample sizes (n) for each sexual stage is shown in parentheses above the bar. *Indicates the level of significance between groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

Aromatase (cyp19a1a)

Aromatase expression showed a significant change in expression across sex change ($F = 10.67$, $p < 0.001$, ANOVA). This change initially indicates a decreasing trend across sex change **(Figure 3.5)**, from JF (Mean = 3.67 ± 1.15) to near zero in IPM (Mean = 0.004 ± 0.0019). However, this was punctuated by an unexpected increase in gonadal *cyp19a1a* expression in MT fish (Mean = 19.65 ± 6.23). Although results suggest initial downregulation, there was no significant decrease in gene expression from JF to ET (Mean = 0.61 ± 0.11). However, there was significant upregulation from JF to MT ($p < 0.01$), and a further significant increase from ET to MT ($p < 0.001$).

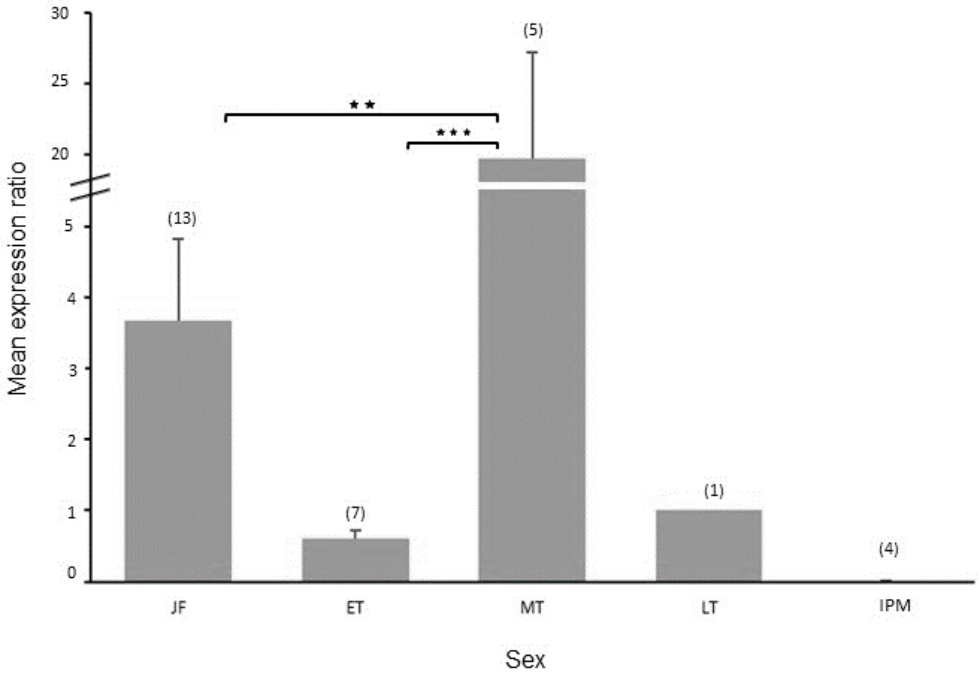

Figure 3.5: Mean expression ratio of *cyp19a1a* (Aromatase) mRNA for each sexual stage of *N. Celidotus* (JF; Juvenile female, ET; early-transitional, MT; mid-transitional, LT; late transitional, IPM; initial phase male). Error bars showing SEM. Expression levels are compared between JF, ET, and MT individuals using an ANOVA and Tukeys HSD post-hoc test. LT and IPM groups were not included in the analysis due to low sample numbers. Sample sizes (n) for each sexual stage is shown in parentheses above the bar. *Indicates the level of significance between groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

Doublesex and mab-3 related transcription factor 1 (*dmrt1)*

Expression of *dmrt1* showed a similar trend to *amh*, with increasing expression across sex change (H = 18.99, $p < 0.001$, Kruskal Wallis) **(Figure 3.6)**, from JF (Mean = 0.95 ± 0.2) to IPM (Mean = 15.62 ± 3.22). There was a significant increase in expression from JF to MT (Mean = 5.37 ± 1.32, $p < 0.001$) and ET to MT ($p < 0.01$).

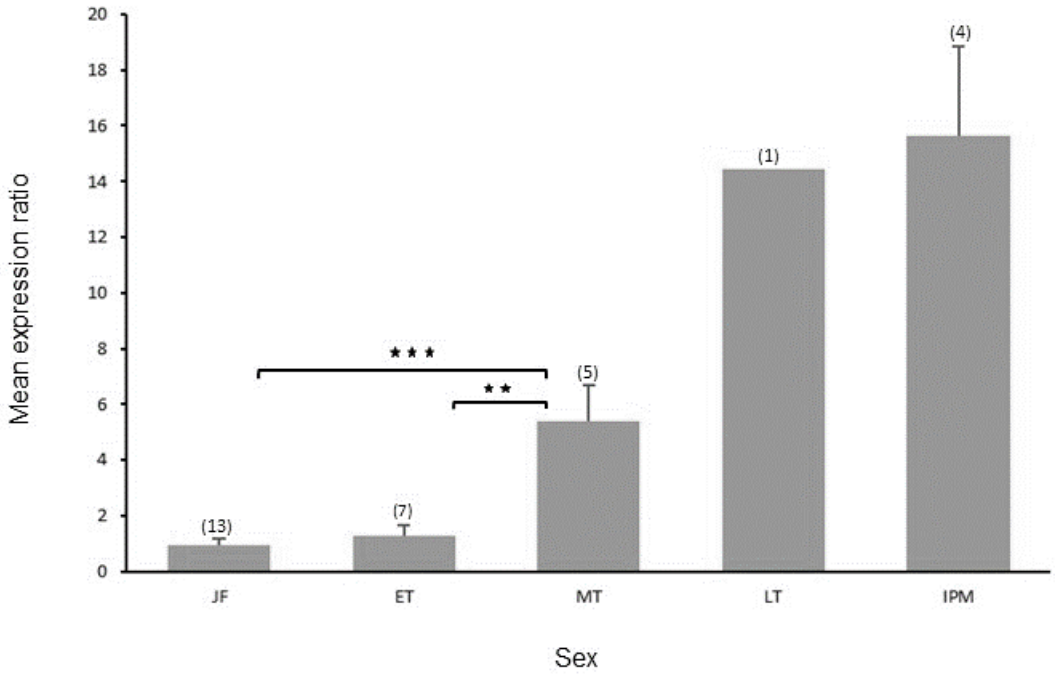

Figure 3.6: Mean expression ratio of *dmrt1* (Doublesex and mab-3 related transcription factor 1) mRNA for each sexual stage of *N. Celidotus* (JF; Juvenile female, ET; early-transitional, MT; mid-transitional, LT; late transitional, IPM; initial phase male). Error bars showing SEM. Expression levels are compared between JF, ET, and MT individuals using Kruskal-Wallis and Tukeys HSD post-hoc test. LT and IPM groups were not included in the analysis due to low sample numbers. Sample sizes (n) for each sexual stage is shown in parentheses above the bar. *Indicates the level of significance between groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

3.5 Discussion

Expression patterns of three genes related to either sex determination or sex differentiation (*amh, cyp19a1a, and dmrt1*) were assessed across different stages of sex change in prepubertal spotty wrasse. The expression of all three genes changed significantly during sex change in comparison to that of juvenile females. For the most part, these trends reflected the expected profiles based on their expression in other vertebrates (Hu et al., 2015; Pfennig et al., 2015; Todd et al., 2019; Wu, Li, Luo, Chen, & Chang, 2015). However, unexpected results were observed in the feminising aromatase gene, *cyp19a1a.* These results indicate that some of the ‘classical’ genetic regulators identified in vertebrate sex differentiation have a greater influence on the sex change of spotty wrasse than others.

Gonadal *amh* expression showed a clear trend of increasing across sex change and is expressed most highly in IP males. In sexually mature spotty wrasse, *amh* expression increased in a stepwise manner across transitional stages with peak levels observed in TP males (Goikoetxea et al., 2021). Expression of *amh* is believed to regulate spermatogonial proliferation in male fish and therefore, this hormone is potentially a key early mediator of female to male sex change (Goikoetxea et al., 2021; Liu et al., 2017; Skaar et al., 2011; Thomas et al., 2019; Todd et al., 2019). The pattern of increasing *amh* expression across sex change is consistent with observations in other teleost species. For example, *amh* upregulation is believed to be the male determining factor during female to male sex change in species with ESD, such as patagonian pejerrey (*Odontesthes hatcheri)* and some gonochoristic species such as nile tilapia (*Oreochromis niloticus),* (Hattori et al., 2012; Kwon et al., 2000; Li et al., 2015). In protogynous hermaphrodites such as *T. bifasciatum*, *amh* upregulation typically coincides with the arrest of *cyp19a1a* expression and therefore the beginning of sex change (Todd et al., 2019). Similarly, evidence in adult spotty wrasse suggests that upregulation of *amh* is influential in the onset of sex change (Goikoetxea et al., 2021). The results of the present study also implicate a critical role of *amh* upregulation in the earliest, histologically detectable, stages of IP male formation in this species. This adds further evidence that *amh* may be an early trigger of sex change.

Although not significant, *cyp19a1a* expression showed the opposite pattern to that of *amh*, with an overall down regulation across sex change. A notable exception was the greatly elevated expression in mid-transitional fish. While it is difficult to explain this apparent anomaly, it is possible that *cyp19a1a* expression does not typically fluctuate greatly across sex change and other external factors such as stress or captive conditions may influence individual expression. Blazquez & Piferrer (2004) report differences in *cyp19a1a* expression during development across species such as sea bass, goldfish (Tchoudakova & Callard, 1998), zebrafish (Kishida & Callard, 2001), and tilapia (Kwon et al., 2000). They suggest that differences in mRNA expression could be explained by individual differences between fish, the age of the fish or even their reproductive status. An alternative explanation may be that *cyp19a1a* expression differs in prepubertal sex change compared to that of functionally mature fish, although there is no obvious rationale for this. Previous studies in adult spotty wrasse have demonstrated that *cyp19a1a* expression does not differ significantly across sex change although it may be reduced in TP males (Goikoetxea et al., 2021; Thomas et al., 2019). In contrast, gonadal *cyp19a1a* is sharply downregulated during early

sex change in bluehead wrasse (Todd et al., 2019). While a clear explanation is lacking for the unexpectedly elevated expression in MT fish, the overall trend supports the conclusion that *cyp19a1a* is not an initiator of sex change in this species.

Expression of *dmrt1* followed the expected pattern increasing across sex change. This gene has been recognized as playing a key role in the male vertebrate sex-determination pathway (He et al., 2003; Huang et al., 2002; Johnsen & Andersen, 2012; Nagahama, 2005), While no significant change in expression was found between JF and ET females, significantly greater expression was observed in MT fish. This was not evident in adult sex changing spotty wrasse until LT and TP stages (Muncaster et al., 2023). In gonochoristic fish, *dmrt1* is closely linked with male differentiation and gonadogenesis in the testes (Herpin & Schartl, 2011a). In protogynous species, it has been reported that changes in *dmrt1* expression regularly appear downstream of other genes, suggesting that *dmrt1* may be more important in progressing rather than initiating sex change (Herpin & Schartl, 2015; Liu et al., 2017; Muncaster et al., 2022; Nozu, Horiguchi, Kobayashi, & Nakamura, 2015; Piferrer, 2013; Todd et al., 2019; Thomas et al., 2019).

These results show that some of the classical genes involved in vertebrate sex differentiation are also influential in prepubertal sex change. The timing of sex-specific changes in expression may differ slightly for individuals undergoing juvenile compared to adult sex change. The early upregulation of *amh* in ET and MT individuals supports the conclusion that this gene may be an early trigger of sex change and further validates the histological staging used in the study. Conversely the lack of significance of *cyp19a1a* across most stages indicates that this gene is not a key initiator of sex change in this species.

Chapter 4 - General discussion

Understanding teleost sex change has interesting implications for biomedical science, as well as the potential effects of climate change on marine communities and the management of many commercial aquaculture species. While many studies have described sex change in sexually mature hermaphrodites, very little is known about the mechanisms driving prepubertal IP male formation in diandric protogynous hermaphrodites. This study aimed to develop a histological description of sex change in prepubertal spotty wrasse, a species that has IP and TP male morphs. Other key aims were to identify the size at which IP male formation first occurs and to characterise the expression of key genes (*cyp19a1a, amh, dmrt1*) involved in sex differentiation across sex change. These results show that gonadal sex change in prepubertal IP female spotty wrasse follows a similar process to that of sexually mature conspecifics. This also broadly reflects sex change as described in other protogynous wrasses.

Using light microscopy, *N. celidotus* were classified into three different transitional states (ET, MT, LT) between female and IP male based on gonadal histology. Individuals identified as either initiating sex change or as sexually mature males were found to measure as small as 52 mm TL and 77 mm TL, respectively. This is considerably smaller than previously recorded in the literature (Jones, 1980). The study of sex change in diandric protogynous hermaphrodites has focused primarily on the transition from female to terminal phase male (Candi et al., 2004; Nakamura et al., 1989; Warner & Swearer, 1991; Shapiro & Rasotto, 1993; Thomas et al., 2019; Goikoetxea et al., 2021; Muncaster et al., 2023) and rarely, if ever, includes a detailed description of the prepubertal IP male formation. Considering that other species such as *T. bifasciatum* and *E. coioides* have two developmental pathways to becoming male it seems logical to determine any differences in development between IP and TP males. From the current study, it is evident that the developmental pathway from juvenile female to IP male closely resembles the TP pathway in spotty wrasse albeit with different timing. However, studies of further gonadal development shows that the vast majority of IP male spotty wrasse have solid testes lacking a remnant lumen while the opposite is true of TP males (Jones 1980; Robertson, 2020). Due to the size of dissected gonads from small IP males, we were unable to effectively determine the overall structural nature of the IP testis in terms of the persistence of an ovarian lumen or the evagination process leading to a

solid testis. However, it seems clear that in this species, both the solid evaginated testis and the 'hollow' testis with a remnant lumen develop first from an ovary, as either prepubertal or sexually mature fish **(Figure 4.1)**.

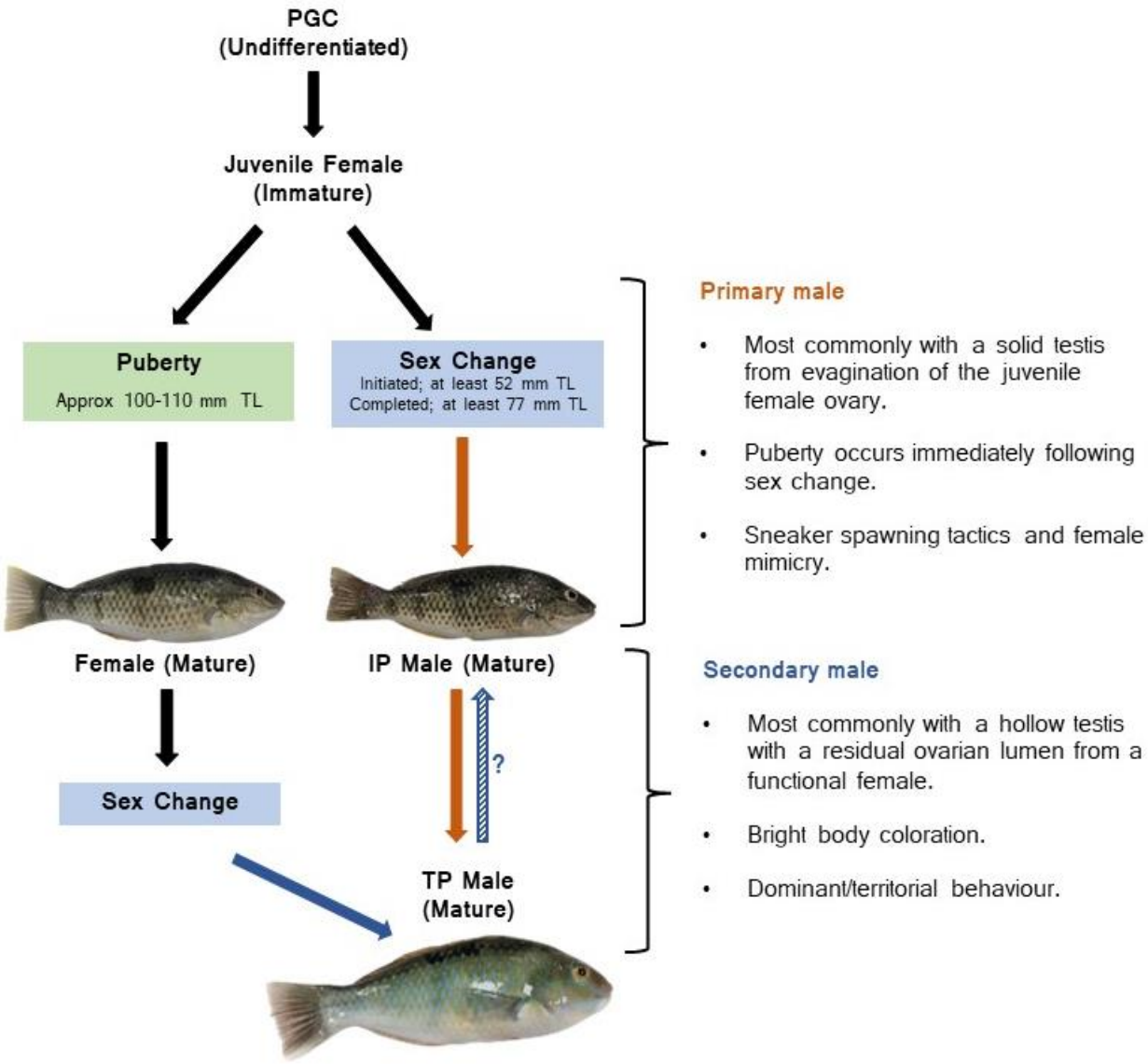

Figure 4.1: Proposed life history of the diandric protogynous spotty wrasse, *N celidotus*. The transition from TP to IP male has not been confirmed and requires further investigation.

The gene expression patterns of *amh*, *cyp19a1a* and *drmt1* provided further validation for the histological classifications of ET individuals, as it was often difficult to identify between oogonial and spermatogonial germ cells by morphology alone (Nakamura et al., 1989; Goikoetxea et al., 2021). All three genes of interest showed significant changes across sex change. Individuals classified through histology as early-transitional and mid-transitional showed significant upregulation in *amh* expression from juvenile female, and greater significance from juvenile female to mid transitional. Therefore, this data provides supporting evidence for conclusions made by Goikoetxea et al. (2021) and Muncaster et al. (2023) that *amh* may form part of the molecular trigger that initiates sex change, and its upregulation could be a useful early molecular marker for protogynous species. Expression of *dmrt1* increased significantly in mid transitionals, suggesting a key role in progressing rather than initiating sex change (Piferrer, 2013; Nozu et al., 2015; Herpin & Schartl, 2015; Liu et al., 2017; Todd et al., 2019; Thomas et al., 2019). While *cyp19a1a* showed an overall trend of decreasing expression with sex change, results indicated a drastic increase in *cyp19a1a* expression for mid transitionals, requiring further investigation in the absence of an obvious explanation. Collectively, these results indicate that all three sex differentiation genes play a similar role in prepubertal sex change leading to IP male formation as they do in TP formation in adult spotty wrasse (Muncaster et al., 2023).

Throughout the literature on early development in protogynous hermaphrodites, a common theme is a lack of cohesion in the terminology and different definitions used to describe early development. This is likely in part due to the vast diversity and plasticity across different teleost orders. For example, in some species considered to be gonochorists, all juveniles initially develop ovaries. Males form through the degeneration of the ovarian tissue and subsequent development of testes while still immature. These fish will remain male for life and have been termed *undifferentiated gonochorists* by some authors (Takahashi, 1977; Takahashi & Shimizu, 1983; Yamamoto, 1969). Yet this concept of gonochorism is contradicted by other definitions in the literature, where gonochorists are defined as having separate sexes which are fixed throughout their entire life cycle (Devlin & Nagahama, 2002; Kobayashi et al., 2018; Kraak & Penn, 2002; Kuwamura et al., 2020; Roberts et al., 2021). Certainly, definitions of sex differentiation based on the presence of specific stages of germ cell development would qualify these juvenile fish as being sexually differentiated females. Alternatively, other authors describe fish similar to undifferentiated gonochorists as *non-functional hermaphrodites* (Asoh & Kasuya, 2002; Hamilton

et al., 2007; Sadovy & Liu, 2008; Cossington et al., 2010). This terminology seems to align more closely with the gonadal transformations that occur in these all-female juvenile fish. In contrast to these fish, *functional hermaphrodites* have individuals who reproduce as one sex and then the other or both simultaneously. Although it is not necessarily the intent of these definitions, it could be argued that spotty wrasse technically have males that can arise as either non-functional or functional hermaphrodites. This highlights both the diversity of fish sexual strategies and associated terminology in the literature.

Assuming fish that have two male morphs with at least one arising after sexual maturity can be considered as functional hermaphrodites, then a lack of clarity still exists around the definitions of primary male formation. These definitions become important when distinguishing between monandry and diandry. Sadovy and Liu (2008) state that primary males arise prior to sexual maturity while secondary males arise from sex change after sexual maturation. In the literature, primary males often possess solid testes that lack an ovarian lumen, in contrast to the testes of secondary males. Robertson, Reinboth, & Bruce (1982) describe the existence of what they term 'functional analogues' of primary males among the parrotfish (*Leptoscarus vaigiensis, Calotomus spinidens* and *C. carolinus*). These fish have males arising from females prior to sexual maturity and retain a remnant ovarian lumen. Liu & Sadovy (2004a) first pose the question as to whether sex 'change' can be said to occur in pre-maturational individuals based on histological criteria. These authors further suggest that prepubertal development from female to male cannot be classified as *sex change* due to the lack of sexual function (Liu & Sadovy, 2008). Instead, these authors consider such pre-maturational transitions between ovary and testis to be an additional form of *sex differentiation* involving a transitory bisexual gonad. This further contrasts with Robertson & Warner (1979), who suggest that *pre-maturational sex change* appears common in parrotfish, which are also among the Labridae family. Here they report female individuals that change sex to become male prior to sexual maturity and then go on to spend their adult lives as males with functional testes. In the current study, IP male formation in spotty wrasse appears to follow a similar pattern to that of both the grouper studied by Liu & Sadovy (2009) and the parrotfish described by Robertson et al. (1982). The fact that all of these fish have a differentiated ovary prior to IP male formation yet use different descriptors for the same physiological transition highlights the ambiguity of the terminology in the literature. In this study preference is given to the term pre-maturational sex change to describe IP male formation.

Based on the results of the current study, the New Zealand spotty wrasse does not seem to meet the requirements for monandry. This is supported by the fact that all individuals appear to first differentiate as female before some undergo pre-maturational sex change to become IP males. This work indicates that these fish may also undergo puberty as they transition to become sexually mature males, showing that these fish reach sexual maturity at a smaller size than previously realised. The remaining females will delay puberty until they reach a larger size with some later undergoing post-maturational sex change. The fact that sex change occurs at different ontogenetic stages often leading to males with different gonadal structures and sexual strategies, adds further weight to the conclusion that spotty wrasse are, in fact, diandric protogynous hermaphrodites.

4.1 Future recommendations

Key sex differentiation genes such as *rspo1* (R-spondin-1) (Biason-Lauber, 2012; Chassot et al., 2014; Zhou et al., 2016), *wnt4* (wingless type MMTV integration site family) (Bernard & Harley, 2007; Biason-Lauber, 2012; Chassot et al., 2014; Todd et al., 2019) and *ctnnb1* (Catenin beta 1) (Chassot et al., 2014; Muncaster et al., 2023; Todd et al., 2019) have been seen in other studies to correlate with sex change. Further work characterising their expression would help clarify the regulation of pre maturational sex change and identify possible early-stage changes. In addition, investigation into the role of other emerging regulatory epigenetic markers such as *prc2* (Polycomb Repressive Complex 2), *kdm6b* (lysine (K)-specifc demethylase 6B, b), *jarid2* (jumonji, AT rich interactive domain 2b), *dnmt1* (DNA methyltransferase 1, *dnmt3aa* (DNA methyltransferase 3aa) as identified in *T. bifasciatum* (Todd et al., 2019) and adult spotty wrasse (Muncaster et al., 2023). It is also recommended that future research on prepubertal sex change in spotty wrasse, target even smaller fish. Although this study identified changes in development in a smaller size class than previously recorded, limitations on capture, equipment and processing did not allow for histological or gene expression analysis of fish <40mm. This would allow insight into the earliest developmental points during larval development where in this process the ovary first differentiates to form a lumen from an undifferentiated proto gonad (Banh et al., 2017). Future sampling of *N. celidotus* would include populations from different locations for comparison. In addition, repeated sampling from the same location would be avoided, as to not incorporate changes in growth and sexual pattern that may arise from decreasing densities (Jones, 1980). Individuals sampled would

also be dissected on the day of capture, to avoid the potential effects of changing social structure during captivity on sexual stage and development. In addition, with the availability of transcriptomics and single cell RNA seq approaches it would also be advantageous to use these to understand the role of the reproductive genes in finer detail within this investigation, as has been done in other studies (Liu et al., 2022)

4.2 Conclusions

This study has shown that IP male formation in spotty wrasse follows a similar process to that of adult sex changers, based on histology and expression of the key sex-related genes, *amh, dmrt1* and *cyp19a1a*. It has been identified that pre-maturational sex change leading to IP male formation occurs at a smaller size than previously thought and may also result in sexually mature fish. Further work characterising behavioural effects and molecular markers of sex change in temperate wrasses will be important for understanding how visual social cues are transduced to initiate the sex change pathway (Thomas et al., 2019). This will add to the growing pool of data on the regulation of sex change in this recently established, endemic model species.

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Appendices

Appendix I - Primer gene sequences

Supplementary figure 1: Genomic organisation of the Spotty *actb2* gene. Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. Primer sites are boxed.

ATGGAAGACGAAATCGCCGCCCTCGTTGTTGACAACGGATCTGGTATGTGCAAAGCTGGCTTTGCAGGAG
ATGATGCTCCTCGTGCTGTGTTCCCCTCCATTGTTGGACGTCCCAGGCATCAGgtacagtttcttattcc
aatccatttatttatatagcacattttaaaaacaacaaggttaccaaagtgttgcacaacaaatacaaa
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accatttagtgacttaaaaacaaatattaaaatcttaaaattaattctaaaatggacagggagccagtgg
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TGGTATGGGCCAGAAAGACAGCTATGTTGGTGACGAGGCCCAGAGCAAAAGAGGTATCCTGACCCTGAAG
TACCCCATTGAGCACGGTATTGTCACCAACTGGGACGACATGGAGAAGATCTGGCATCACACCTTCTACA
ATGAGCTGAGAGTTGCCCCTGAGGAGCACCCAGTCCTGCTCACTGAGGCCCCCCTGAACCCCAAGGCCAA
CAGGGAAAAGATGACCCAGgtaacacaggcttcaacactttatatttccttttctgtcatgaaacccactc
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CCTCATGAAGATCCTGACTGAGAGGGGTTACAGCTTCACCACCACAGgtaagcacagaagtgcaagttaa
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GTGATGTCGACATCCGTAAAGACCTGTATGCCAACACTGTGCTGTCTGGAGGTACCACCATGTACCCTGG
CATTGCTGACCGTATGCAGAAGGAAATCACAGCCCTGGCA**CCCACTACCATGAAGATTAAG**gtataactc

atcctcattactcaggctcacatcctccttgaggcagtgatgaaagtgttctagaaagagctttaactct
actgccacctggtggacaaacatggttgatgtagtttaaatctctagctagccttttacaacacaggat
tgaaatacttgcaggaattttaaaacatttttttttatatcttgctctctgcag**ATCA**TTGCTCCCCCTGA
GAGGAAGTACTCTGTATGGATTGGAGGCTCCATCCTGGCCTCTCTCTCCACCTTCCAGCAGATGTGGATC
AGCAAGCAGGAGTACGATGAGTCTGGCCCCAGCATTGTCCACAGGAAGTGCTTCTAAACAGACTGTCAGT
CC**CCTCCCCCAAAACACACACT**GCTACAAACCCAAACGACTGGCTCTGCATACATGCCTACACCAACACA
CTGGTGT

Supplementary figure 2: cDNA sequence showing the complete coding region for the Spotty *actb2* gene. Different exons are shown using a different colour. Primer sites are boxed, which give a product size of 200 bp.

ATGGAAGACGAAATCGCCGCCCTCGTTGTTGACAACGGATCTGGTATGTGCAAAGCTGGCTTTGCAGGAG
ATGATGCTCCTCGTGCTGTGTTCCCCTCCATTGTTGGACGTCCCAGGCATCAGGGTGTGATGGTTGGTAT
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ATTGAGCACGGTATTGTCACCAACTGGGACGACATGGAGAAGATCTGGCATCACACCTTCTACAATGAGC
TGAGAGTTGCCCCTGAGGAGCACCCAGTCCTGCTCACTGAGGCCCCCCTGAACCCCAAGGCCAACAGGGA
AAAGATGACCCAGATCATGTTCGAGACCTTCAACACTCCTGCCATGTATGTGGCCATCCAGGCTGTGCTG
TCCCTGTACGCTTCTGGTCGTACCACTGGTATTGTCATGGACTCTGGTGATGGTGTGACCCACACAGTGC
CCATCTATGAAGGCTACGCCCTGCCTCACGCCATCCTGAGGTTGGACCTGGCTGGCAGGGACCTCACAGA
CTACCTCATGAAGATCCTGACTGAGAGGGGTTACAGCTTCACCACCACAGCTGAGCGTGAAATCGTGCGT
GACATCAAAGAGAAGCTGTGTTACGTTGCACTGGACTTTGAGCAGGAGATGGGCACTGCTGCTTCCTCTT
CTTCCCTGGAGAAGAGCTACGAGCTGCCTGACGGACAGGTCATCACCATTGGAAATGAGAGGTTCCGTTG
CCCTGAGGCCCTCTTCCAGCCCTCATTCCTTGGTATGGAATCTTGCGGTATCCATGAAACCACCTTCAAC
AGCATCATGAAGTGTGATGTCGACATCCGTAAAGACCTGTATGCCAACACTGTGCTGTCTGGAGGTACCA
CCATGTACCCTGGCATTGCTGACCGTATGCAGAAGGAAATCACAGCCCTGGCACCCACTACCATGAAGAT
TAAGATCATTGCTCCCCCTGAGAGGAAGTACTCTGTATGGATTGGAGGCTCCATCCTGGCCTCTCTCTCC
ACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGATGAGTCTGGCCCCAGCATTGTCCACAGGAAGT
GCTTCTAAACAGACTGTCAGTCCCCTCCCCCAAAACACACACTGCTACAAACCCAAACGACTGGCTCTGC
ATACATGCCTACACCAACACACTGGTGT

Supplementary figure 3: Genomic organisation of the Spotty *g6pd* gene. Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. Primer sites are boxed.

ATGGCCAACATCCCCCTCTCACGCTCTGAGGTATTTGGGGAGCTGAGGAAGGAGCTGCATGAAGATGAGAAGTTCCA
CCAGTCAGATGCCCACATCTTCATCATCATGGGAGCATCGgtgagtgcaggggaaggggagcaataatctaaagaga
aagtgataacacaatacacttgtattaattcaaccagagtggatggttttactttgtgagttggaagcctaatgact
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AACTGAAGCTGACGACCTCGCAAAGAGGGTTGGATTCCGCTATGAAGGCACTTATAGATGGGTCAACCCTCACAGAC
TGTGA

Supplementary figure 4: cDNA sequence showing the complete coding region for the Spotty *g6pd* gene. Different exons are shown using a different colour. Primer sites are boxed, which give a product size of 106 bp.

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TGGTGCAAAACCTCATGGTGCTCAGGTTTGGGAACCGCATCTTTGGTCCGATCTGGAACAGGGACAGTGTGGCCTGC
GTCGTTCTCACCTTCAAAGAGCCGTTTGGCACTCAGGGGCGAGGAGGCTACTTTGATGATTTTGGCATCATTCGCGA
CGTCATGCAGAACCACTTGCTCCAGATGCTCTGCCTGGTTGCCATGGAGAAACCAGCATCAACCAGCTCGGATGATG
TCAGGGACGAAAAGGTGAAGGTGCTGAAGTGCATCGCCCCAGCGTCTATGTCAGACGTGGTGCTGGGTCAGTATGTG
GGGGATCCAGAGGGTGAAGGAGATGCCAAGCTGGGTTATCTAGATGATCCTACAGTTCCTAAAGGGTCCACTCAGGC
CACCTTCACCACGGCTGTGCTCTATGTGCACAATGAACGCTGGGATGGTGTTCCTTTCATCCTCCGCTGTGGAAAAG
CTCTGAATGAGAGGAAGGCTGAGGTGCGGCTGCAGTTCACAGATGTCCCAGGGGACATTTTTGGAAACACATGTCGC
AGGAATGAGCTTGTGGTGCGTGTGCAGCCCAATGAGGCTGTCTACGCCAAGATGATGAGCAAGAAGCCCGGCGTTTT
CTTTAGCCCTGAGGAAACTGAACTTGACCTCACCTACAAAAGTAGATACAAGGACGTGAAGCTCCCAGACGCCTACG
AACGCCTCATTCTTGACGTCTTCTGTGGGAGTCAGATGCACTTTGTTCGCAGTGATGAACTAAGGGAAGCATGGAGG
ATCTTCACTCCTCTTCTTCATCATATAGAGCGAGAGAAGCCCAAGCCCATTCCTTACAAATACGGAAGCCGTGGCCC
AACTGAAGCTGACGACCTCGCAAAGAGGGTTGGATTCCGCTATGAAGGCACTTATAGATGGGTCAACCCTCACAGAC
TGTGA

Supplementary figure 5: Genomic organisation of the Spotty *amh* gene. Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. Primer sites are boxed.

ATGTTGTTTGTGGATTTCTCCGTCTGTCGATCGTTGATGCTTTGCTGTACCGGGATGTGCGTGGCTCTGATCCCTCT
CTGTAAGCCCACAAAAACAGgtcagactgcacaatctacactaactaacccccctgcaaatatgtgcaccattagtca
tgctgttttatgcataatgtcatgttgtggagctactgttgcatagctcctcaataagtatgttttgagcacaggtt
gtactgtttgcatgctgtttctgcctccagcttttacctgttcctcaatttaaaatgtaatttgtttattttgcatt
tctctgaaagGAGATCACCCCATGACCAGCACAACAGAGACAGGAGACGGGTTAGATGTAACAAATGGAGCGCACAC
CTTATCAACAAAGACCTCATCAGGCTCCACAGTTTCACAACCCCTTCCTAACCTCTCACCATGCTTTGTGGACGATG
TGGTTGCAGAGTTATGTGACAGTGTTGGTGACAATGGTGAACTCACAAACGACACCATGACTCTGTTTGGAATCTGC
ACAGGATCTGAAAATTCCTCAGCCTCACTTTTAACAGAGCTTGCCAAAGAAACCAGCAGAAACCAGAGAAAGGGCTT
GGAGGTTTGGCATCTATCTGGAGgtaatagttccctctaactgtaactatttcccctgcttctttttcttaaagcat
gctttgttttctccatcagAGCTCTTGTCAGAGGGAGATGAGACAGGATCACTCAAGCTGACCTTTGACCTCCCACA
TTCTCATTTGCTGAAGTCAAACCCCGTGCTGCTCTTAGATTTTGAAAGTCCCCTTCCAGGAGCAAACCTGGAGGTTA
CTTTCACCAGCCGTGCACTGCATCCTAGCACACAGgtaaagaaaggtgtgatgagaatatttcatgattgaaaactg
ccccaagctcactaaatgatgttgtcacatttctttttgttgttttcctgctgtcgtttgcagACCGTGTGCATTTC
AGGAGAAACACAATACATAATGCTGGCAGGAAAAGAATCAGAGGGCAAAGTCCAGCGTAAATGGAGGATTTCTGCTG
AGACAAATCCCCCTGTTATGAgtaagatttaaaatgagacagacaaacatttttatttcatgtgcactctcctcac
acttcctctatttgtagAGCAAAGGTTAAGAAACATCTTTATTGGTGGAAAATCAGGAAGTAACTTCAGCATGACTC
CACTTCTGCTTTTCTCAAGGGAAAGAAGAACTGAAACAAGgttgacttagcttttttgttccctttgtttttgata
ttttaccacactcatttcagagcagtggtaagtcatttttaccctctttttttcagATCTGCACATGTTTCGGGTTCA
TCCATGACCTCCTCCTTCCTCTGTGAGCTGAGGCGGTTCCTTGGTGATGTCCTGCCTCAGAGCCACCCTCCCTCCTC
TTCCCTTCAGCTTGAATCCTTACAGTCCCTGCCTCCCCTGACCCTTGGCCTGTCCTCTAGTGAGACCCTGCTGGCAG
GACTGATCAACTCCTCGGCCCTCACCATCTTCTCCTTCTCCAGCTGGGGCTCCAGGTTTCAGGTGCATCATGGAGAG
TTGTCCCTGTCTCCTGCGCTGCTGGAGGAGCTCAGGCAGAGGTTGGAGCAGGCTATGGAGCAGATAACGACAGTGAT
AAGGGAGGAAGATGTGGGTCACAGAGCCATAGAGAAGCTGGGGAGGCTCAAAGAACACAGTGCATTCCCAAAGACAC
AACCTGCAGCAGgtgatgtgtggccaacatgaaaacaggcaactaaacagcattatcagattaaatctcatccagag
attttggataacttcaggttactttaactgtttaaaaactcctgtgaggaaatgtatgtttgtgttgaattttgcac
cccctgctcaaaaacaacttagctttttgctgatcttgttctgtgcttgcagtgtttgcttatgataaatatcatcc
tgctcatacatttaattcactgttacactttagagttgaaaatatacaaatacagccttttcggcaatcgcttctt
ttgataactaaacaaaaataatcaatcttgtctccagtgatctgagggttgatttctggttcatctagacaccacag
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taactgtttaaccacatcaagctaactttgtcatatctacactacaactgagctaacaacttcagctgttcacccat
catctctttgagctattttaacactttgttcatcagctactgttagttcatgatttcagttgtttatatagaaagtt
tattcaacttttcaactatatgaaattacttatggcctttccaactatttatccactaacaatgttttatgctaat
gtcgcagttgtctgttgcttttagctaagtatttgtttaagctgtgttcagctcatacctttaagctatctcaagtg
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ttatgtttaataattaaaaaactttagctatatttcacattattatttcagttctttaaatatattgcttttagcta
atattttccactatttatatactactttcagtatttcctaagtactaatacattctcaattgaaaacttttggtcta
cctggctcaatcatcctgctttttaaaaattagaaatgtctcaagtagagcacttgatgtaatttagatgtctttaa
taaacagtccccagttctatttttcatggatatctctgtttccccttctgcatgttacagGAGAAAGCCAATACTGT
GCGTTCCTGCTGCTGAAGGCTCTGCAGACAGTGGCCCGAGCGTATGAGGTGGAGAAGAGACTACGGGCCACCAGAGC
TGGCCGCAACAACTCAATGGGCAACGTCTGTAGGCTGAAAAGCCTCACCGTGTCCCTTGAAAGAACTCTAGTGGGTC
CAAACACTGCGAACATCAACAACTGCCATGGCTCCTGTGCCTTCCCCCTGGTCAACACCAACAACCACGCAGTTCTG
CTCAACTTCCACATTGAGAGCGGGAATGTGGACGAGCGGGCGCCATGCTGTGTGCCAGTGGCCTACGAACACCTGGA
GGTGGTCGACATGAACCAACATGGGACTTACCTCTCCATCAAACCAGATGTGGTGGCGAAGGAGTGTGGCTGCCGCT
AA

Supplementary figure 6: cDNA sequence showing the complete coding region for the Spotty *amh* gene. Different exons are shown using a different colour. Primer sites are boxed, which give a product size of 134 bp.

ATGTTGTTTGTGGATTTCTCCGTCTGTCGATCGTTGATGCTTTGCTGTACCGGGATGTGCGTGGCTCTGATCCCTCT
CTGTAAGCCCACAAAAACAGGAGATCACCCCATGACCAGCACAACAGAGACAGGAGACGGGTTAGATGTAACAAATG
GAGCGCACACCTTATCAACAAAGACCTCATCAGGCTCCACAGTTTCACAACCCCTTCCTAACCTCTCACCATGCTTT
GTGGACGATGTGGTTGCAGAGTTATGTGACAGTGTTGGTGACAATGGTGAACTCACAAACGACACCATGACTCTGTT
TGGAATCTGCACAGGATCTGAAAATTCCTCAGCCTCACTTTTAACAGAGCTTGCCAAAGAAACCAGCAGAAACCAGA
GAAAGGGCTTGGAGGTTTGGCATCTATCTGGAGAGCTCTTGTCAGAGGGAGATGAGACAGGATCACTCAAGCTGACC
TTTGACCTCCCACATTCTCATTTGCTGAAGTCAAACCCCGTGCTGCTCTTAGATTTTGAAAGTCCCCTTCCAGGAGC
AAACCTGGAGGTTACTTTCACCAGCCGTGCACTGCATCCTAGCACACAGACCGTGTGCATTTCAGGAGAAACACAAT
ACATAATGCTGGCAGGAAAAGAATCAGAGGGCAAAGTCCAGCGTAAATGGAGGATTTCTGCTGAGACAAATCCCCCT
GTTATGAAGCAAAGGTTAAGAAACATCTTTATTGGTGGAAAATCAGGAAGTAACTTCAGCATGACTCCACTTCTGCT
TTTCTCAAGGGAAA**GAAGAACTGAAACAAG**ATCTGCACATGTTTCGGGTTCATCCATGACCTCCTCCTTCCTCTGTG
AGCTGAGGCGGTTCCTTGGTGATGTCCTGCCTCAGAGCCACCCTCCCTC**CTCTTCCCTTCAGCTTGAATCC**TTACAG
TCCCTGCCTCCCCTGACCCTTGGCCTGTCCTCTAGTGAGACCCTGCTGGCAGGACTGATCAACTCCTCGGCCCTCAC
CATCTTCTCCTTCTCCAGCTGGGGCTCCAGGTTTCAGGTGCATCATGGAGAGTTGTCCCTGTCTCCTGCGCTGCTGG
AGGAGCTCAGGCAGAGGTTGGAGCAGGCTATGGAGCAGATAACGACAGTGATAAGGGAGGAAGATGTGGGTCACAGA
GCCATAGAGAAGCTGGGGAGGCTCAAAGAACACAGTGCATTCCCAAAGACACAACCTGCAGCAGGAGAAAGCCAATA
CTGTGCGTTCCTGCTGCTGAAGGCTCTGCAGACAGTGGCCCGAGCGTATGAGGTGGAGAAGAGACTACGGGCCACCA
GAGCTGGCCGCAACAACTCAATGGGCAACGTCTGTAGGCTGAAAAGCCTCACCGTGTCCCTTGAAAGAACTCTAGTG
GGTCCAAACACTGCGAACATCAACAACTGCCATGGCTCCTGTGCCTTCCCCCTGGTCAACACCAACAACCACGCAGT
TCTGCTCAACTTCCACATTGAGAGCGGGAATGTGGACGAGCGGGCGCCATGCTGTGTGCCAGTGGCCTACGAACACC
TGGAGGTGGTCGACATGAACCAACATGGGACTTACCTCTCCATCAAACCAGATGTGGTGGCGAAGGAGTGTGGCTGC
CGCTAA

Supplementary figure 7: Spotty *cyp19a1a* genomic DNA – Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. One set of primers were designed between the exons (fluorescent green and turquoise) and the other set of primers were designed between the two grey regions.

ATGGATCTGATCTCTGCTTGCGAACGGACCATGAGTCCTGTAGGTTTGGATGCTGAGGTGAAAGACCTGGTCTACAT
GTCCCAGAATGCAACTGTGGTGGGATTGCAGGGAGTGTCAATGGCAACCAGGACCCTGGTTCTGCTTTTTTTTGTGC
TGCTTGCTGCGTGGAACCACAAGGACAAGAGATCTGTACCAGgtaggttccctgggttctctcaatgttctttctct
atgttgcctatcttcctggttgtacctcgttactgaatttaatagaagcttgtgtgtatatttcctcaacagGCCCG
TCTTTCTATCTGGGTTTGGGTCCACTTCTATCATATTTAAGATTTATCTGGACTGGCATTGGTACAGCATCTAACTA
CTACAGCACCAAGTATGGAGACATTGTCAGAGTCTGGATCAATGGAGAGGAGAC**GCTCATACTCAGCAG**gtcagttt
tcttggctgaggcggtaaacttagttttaaaatagccgctcaacaccttagttgtaaatattattgttttcttcttg
tctttttttcaccacaag**GTCATC**AGCTGTGCACCACGTCCTGAAGAGTGGCCACTACACTTCACGTTTTGGAAGCA
AGCGAGGCCTCAGCTGCATTGGCATGGATGAAAGAGGGATCATATTTAACAACAATGTGCCTTTGTGGAAAAAGATA
CGCAC**CTATTTCACTAAAG**gtaacatttctatctgctttaagagacccttcaagtaacctttaacaatcttaaatca
gcctggcaaacctactaattgaagtatttttgcactgatttcagaaatgcaaaacatttaacctcataaatgttgat
gttctgtatcag**CGCTGACAG**GTCCAGGCCTGCAGCAGGCAGTAGAGGTTTGTGTCTCATCCACTCAGAGTCACCTA
GATGATCTGGACAGTTTGGATGATGTGGACGTGATCAATTTGCTGCGTTGCACCGTGGTGGATATCTCCAACAGACT
CTTCCTGGATGTTCCTGTGAATGgtgagcaggcagcagacatctcctaactttgtttaagttagcaagtcgtaactc
agggcattgcttcaaggatcaaacaaaccttagacgtgtgtgttatgtttgcgtgcagAAAAAGAGCTGATGCTGA
AGATTGAGAAGTATTTTGACACGTGGCAGACTGTGCTCATCAAACCAGACATTTACTTCAAGTTAGACTGGATTCAC
CAGAGGCACAAGACAGCAGCgtgagtcactccagcactcattgtaaaatgtgttttaatgcatgagccaattcaagt
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TGTGGAGCAGAAGAGGAGAGAAGTGGAGCAGGCAGAGAAACTAGACAACATCAACTTCACAGCTGAGCTCATCTTTG
CTCAGgtaagactcagagtgtgtatgttttgaaagaacagcacaaacttttagtcgtttcttcctaatcactggtc
agaaatacatttatggtaaacccagctcataatgtttgtatttgtgtgtgtattcagAGCCATGGTGAGCTGTCTGC
AGAGAATGTAAGGCAGTGTGTGTTGGAGATGGTGATCGCAGCACCTGACACTCTGTCCATCAGCCTCTTTTTCATGC
TGCTGCTCCTCAAAGAACATCCTGATGTGGAGCTGCAGTTGCTGCAGGAGA**TGGACACTGTTGTTG**gcaagtagaaa
ggagaatacttatttttgaaacagtcagattcacatattggctgatttcctgcagatgacagattggtttgaacaaa
cacagacggccaagttttgtggaaaatgtcaacagagcactttgcattcaatcaatatgaagattataaggtttagg
ttttcttgaagttgttcagaaacataacagctctattacctcatcctcttactgtag**GTGAC**GGACAGCTTCAGAAC
GGAAACCTTCAGAGGTTGCCATTGTTGGAGAGCTTCATCAACGAATGCTTACGCTTCCACCCTGTGGTGGACTTCAC
CATGCGTCGCGCCTTGTCGGATGA**CATCATAGAGGGCTATAGAGTACCT**AAAGGAACAAACATCATACTGAACACCG
GCCACATGCACAGGACAGAGTTTTTCCTCAAACCCAATGACTTCAGCCTGGAACATTTTGAAAAAAATgtaagtgaa
agaatttcattttacttttttggtctatctctgagatacagagccctacctccggtgacatctgaggaagaaatag
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tggactttggactacccctacttatgatatatgcagatgtagagcactggtacatgatagtgtttactactgaagtt
caaagaggttaataatacatcctgctgggatgtaattccagcattttcccaacgttacgcacgtccccacataaaca
caagctttcaaacgcaaaaaaggtagattaagcatgatcctcaaacattaccgcaattgccatcgatcactcataga
tggttccattacaaacactcacagtgtgtggctgtatgatccagcagattgaagagtgatgcgctgtgcgcccatgc
gccacatgcggatggccacctgcgcatccggccacacactgttagtgtatgtgtaatggaaccatctatatgtgatc
gatgaccctcgtggtaatgcctgagaactgtgacacatttcgcttttagttgagtgcttgggtttatgtgagcgcgt
gcgcaacggtggaaaaaaagctggagttgcatcactgcaggatgtattattaaattacagacttcaatagaaaaccg
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caaaaacactgttttcttacgacaaatgagtcccaaacagtctcaacagttctagatatttcacatgcaatatttct
gtgtttccttattcatccttcacctttcactgggttttcatagacagagcttcttagtgaagttatggtagataat
tatttgcagccattaagatatagcaacatttgatttggaatgaaccagtcacaccaccacagtccctataaataaag
cttgtttcactgaatataatatgtagagctatatgaagttttaagtgtttttttatttccttttatttgttaatattta
tgccctggacacagttttcagtctatatacctctcccctctgtcttccttcag**GCTCCTCGTCGTTACTTCCAGCCA**
TTTGGCTCAGGTCCTCGTGCGTGTGTTGGAAAGCACATCGCCATGGTGATGATGAAATCCATCCTGGTGACGCTGCT
GTCCCGGTACTCAGTCTGCCCTCACGAGGGTCTGACCCTGGACGGCCTCCCGCAGACCAACAACCTGTCCCAGCAGC
CTGTGGAGCACCATGAGGAGAACGAGCCGCTCGGCATGAGGTTCTTACCGAGGCAGAGAGGAAGCTGGGAAACTCTG
TGA

Supplementary figure 8: cDNA sequence showing the complete coding region for the Spotty *cyp19a1a* gene. Different exons are shown using a different colour. Primer sites are boxed and the second set of primers were used, which give a product size of 161 bp.

ATGGATCTGATCTCTGCTTGCGAACGGACCATGAGTCCTGTAGGTTTGGATGCTGAGGTGAAAGACCTGGTCTACAT
GTCCCAGAATGCAACTGTGGTGGGATTGCAGGGAGTGTCAATGGCAACCAGGACCCTGGTTCTGCTTTTTTTTGTGC
TGCTTGCTGCGTGGAACCACAAGGACAAGAGATCTGTACCAGGCCCGTCTTTCTATCTGGGTTTGGGTCCACTTCTA
TCATATTTAAGATTTATCTGGACTGGCATTGGTACAGCATCTAACTACTACAGCACCAAGTATGGAGACATTGTCAG
AGTCTGGATCAATGGAGAGGAGAC**GCTCATACTCAGCAGGTCATC**AGCTGTGCACCACGTCCTGAAGAGTGGCCACT
ACACTTCACGTTTTGGAAGCAAGCGAGGCCTCAGCTGCATTGGCATGGATGAAAGAGGGATCATATTTAACAACAAT
GTGCCTTTGTGGAAAAAGATACGCAC**CTATTTCACTAAAGCGCTGACAG**GTCCAGGCCTGCAGCAGGCAGTAGAGGT
TTGTGTCTCATCCACTCAGAGTCACCTAGATGATCTGGACAGTTTGGATGATGTGGACGTGATCAATTTGCTGCGTT
GCACCGTGGTGGATATCTCCAACAGACTCTTCCTGGATGTTCCTGTGAATGAAAAAGAGCTGATGCTGAAGATTGAG
AAGTATTTTGACACGTGGCAGACTGTGCTCATCAAACCAGACATTTACTTCAAGTTAGACTGGATTCACCAGAGGCA
CAAGACAGCAGCCCAGGATCTGCAAGATGCCATAGAGAGTCTTGTGGAGCAGAAGAGGAGAGAAGTGGAGCAGGCAG
AGAAACTAGACAACATCAACTTCACAGCTGAGCTCATCTTTGCTCAGAGCCATGGTGAGCTGTCTGCAGAGAATGTA
AGGCAGTGTGTGTTGGAGATGGTGATCGCAGCACCTGACACTCTGTCCATCAGCCTCTTTTTCATGCTGCTGCTCCT
CAAAGAACATCCTGATGTGGAGCTGCAGTTGCTGCAGGAGA**TGGACACTGTTGTTGGTGAC**GGACAGCTTCAGAACG
GAAACCTTCAGAGGTTGCCATTGTTGGAGAGCTTCATCAACGAATGCTTACGCTTCCACCCTGTGGTGGACTTCACC
ATGCGTCGCGCCTTGTCGGATGACAT**CATAGAGGGCTATAGAGTACCT**AAAGGAACAAACATCATACTGAACACCGG
CCACATGCACAGGACAGAGTTTTTCCTCAAACCCAATGACTTCAGCCTGGAACATTTTGAAAAAAATGCTCCTCGTC
GTTACTTCCAGCCATTTGGCTCAGGTCCTCGTGCGTGTGTTGGAAAGCACATCGCCATGGTGATGATGAAATCCATC
CTGGTGACGCTGCTGTCCCGGTACTCAGTCTGCCCTCACGAGGGTCTGACCCTGGACGGCCTCCCGCAGACCAACAA
CCTGTCCCAGCAGCCTGTGGAGCACCATGAGGAGAACGAGCCGCTCGGCATGAGGTTCTTACCGAGGCAGAGAGGAA
GCTGGGAAACTCTGTGA

Supplementary figure 9: Genomic organisation of the Spotty *dmrt1* gene. Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. Primer sites are boxed.

ATGAGCAAGGCCAAGCAGAGCAAGCAGGTACCGGAGCCCACCGAACCTCTGTGCCCATCAAAAGGCCCCAAAACACC
GAGGATGCCCAAGTGCTCCCGCTGTCGGAACCACGGCTTTGTGTCTCCTCTGAAGGGACACAAGCGCTTCTGCAACT
GGAGGGACTGCCAGTGTCCCAAATGCAAGCTGATAGCGGAGCGGCAGAGAGTGATGGCGGCCCAGgtaacatccttt
tatacatttgaatgagcgcgtgacgttagctgagaataacggttcaaacggtcagaggtcgtcgtgtaaataaaatg
ttaacggtcgagggaagccaaaaatacaacatttatttagaaaatactaaataatatcataaatattaacttttaaa
atacttttttatgatatgggcaacttttctttaatttctgtcgctttatttcatttattaaaccgtggcaattcag
aagtgcatctagctaacctagctaaatgtaaagcatcactcagctagcttacacacttttatactgttaataggtgt
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acctataaataatgaaaactccaaatgtttccctttggtttagcgcaaaaaagtacaagtacattctgaaaatgttc
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GAGGGACGAACCCCAACACCTACCGGATCGTCTTCTCTGGCTGTCACAGgttcagtattgccaactacatctctctc
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tgatttttccacagtcgaatattaattttcacaatcgaatctccatttttttttaatattcgaatatatattcgaa

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gtacaacgtgtcgagatctaaagtgtgcgttctttgtctctgcag**CTTTCTCTCCAGGAGGTCT**CTCCCCCGCTCAG
GACTCCGCCTTAACCTGCAGGTCCATCAGCTGCCTGGTCAACTCTGACATCACCACTGAGGCCAGCGGCGAGACCCA
AATCTTCACCTCCGTCATGGATGGTGACGCCGCCAAATAG

Supplementary figure 10: cDNA sequence showing the complete coding region for the Spotty *dmrt1* gene. Different exons are shown using a different colour. Primer sites are boxed, which give a product size of 205 bp.

ATGAGCAAGGCCAAGCAGAGCAAGCAGGTACCGGAGCCCACCGAACCTCTGTGCCCATCAAAAGGCCCCAAAACACC
GAGGATGCCCAAGTGCTCCCGCTGTCGGAACCACGGCTTTGTGTCTCCTCTGAAGGGACACAAGCGCTTCTGCAACT
GGAGGGACTGCCAGTGTCCCAAATGCAAGCTGATAGCGGAGCGGCAGAGAGTGATGGCGGCCCAGGTCGCCCTGAGA
AGGCAGCAGGCTCAGGAAGAGGAGCTTGGTATTTGTAGTCCTGTGACTCTGTCCAGCTCTGAGGTGATGGTGAAGGA
TGAGGCAGGACCAGACTGTTTGTACTCTGTGGAGGGACGAACCCCAACACCTACCGGATCGTCTTCTCTGGCTGTCA
CAGGGATTCGCTCGGCTTCGTCCCCCAGCCTGTCAGCTGGTTCCCGGGCTCACACCGAGGGACAGTCAGACCTGCTG
CTGGAAACCTCCTACTACAACTTCTACCAACCTGCTCGCTACCCGACCTATTACAGCA**ACCTCTACAACTACCAACA**
ATACCAGCCCCATGGAGAGAGCCCCCTCTCCAGCCACAGCATGTCCTCTCAGTACCTCATGCACTACCTGTCCCAGG
GCCTGGGCTCCACCATCTGTGTGCCGCCTCTCTTCAGCGTGGAGGACAACAACAATAACAACAACAACTACTCCGAG
ACCATGGCAGC**CTCTTTCTCTCCAGGAGGTCT**CTCCCCCGCTCAGGACTCCGCCTTAACCTGCAGGTCCATCAGCTG
CCTGGTCAACTCTGACATCACCACTGAGGCCAGCGGCGAGACCCAAATCTTCACCTCCGTCATGGATGGTGACGCCG
CCAAATAG

Appendix II

Supplementary table 1: DeNovix® DS-11 spectrophotometer results showing the concentration and purity of RNA for each sample.

Sample name	A260	A230/A280	A260/A280	Concentration (ng/μL)
H21	14.76	2.35	1.904	590.71
H24	4.17	2.11	2.006	166.987
SGON31	24.03	2.24	2.07	961.45
SGON39	15.72	2.18	2	628.98
SGON42	24.11	2.26	1.97	964.55
SGON44	31.94	2.5	2.05	1277.54
SGON47	61.54	2.31	2	2461.77
SGON48	52.04	2.2	1.98	2081.99
SGON52	46.96	2.25	1.96	1878.54
SGON53	14.13	2.22	2.02	565.28
SGON55b	8.79	2.304	1.908	351.643
SGON57	37.27	2.26	2.08	1491.09
SGON58	22.55	2.26	2.07	902.14
SGON60	24.73	2.26	2.08	989.107
SGON64a	9.06	2.17	1.99	362.35
SGON65a	2.35	1.92	1.99	94.31
SGON66a	27.25	2.259	2.084	1090
SGON69	44.58	2.265	2.04	1783.22
SGON70	31.7	2.25	2.06	1268.23
SGON71	42.7	2.01	2.033	1709.124
SGON72	7.31	2.15	1.97	292.42
SGON73	18.35	2.21	2.02	734.321
SGON74a	9.29	2.21	1.99	371.99
SGON75	25.41	2.24	2.06	1016.47
SGON77	36.66	2.09	2.04	1466.4
SGON79	27.06	2.5	1.97	1082.323
SGON80	39.87	2.23	2	1595.19
SGON82	17.84	2.23	1.96	713.81
SGON83	36.41	2.27	2.033	1456.41
SGON84	30.77	2.27	2.071	1230.84
SGON85	9.79	2.2	1.985	391.91
SGON87	15.28	2.31	2.03	611.204
SGON88	1.03	1.952	1.99	41.17
SGON89	11.53	2.2	2	461.4
SGON92a	14.57	2.228	2.02	583.04
SGON96	16.66	2.2	2.09	666.4

Appendix III

Supplementary table 2: Reagent volumes for qScript™ cDNA synthesis for each sample.

Sample name	RNA (µL)	Water	Supermix (µL)	Total (µL)
SGON31	4.2	11.8	4	20
SGON39	6.4	9.6	4	20
SGON42	4.2	11.8	4	20
SGON44	3.1	12.9	4	20
SGON47	1.6	14.4	4	20
SGON48	1.9	14.1	4	20
SGON52	2.1	13.9	4	20
SGON53	7	9	4	20
SGON57	2.7	13.3	4	20
SGON58	4.4	11.6	4	20
SGON60	4	12	4	20
SGON69	2.2	13.8	4	20
SGON70	3.1	12.8	4	20
SGON71	2.3	13.7	4	20
SGON72	13.7	2.3	4	20
SGON73	5.5	10.5	4	20
SGON75	3.9	12.1	4	20
SGON77	2.7	13.3	4	20
SGON79	3.7	12.3	4	20
SGON80	2.5	13.5	4	20
SGON82	5.6	10.4	4	20
SGON83	2.8	13.2	4	20
SGON84	3.3	12.7	4	20
SGON85	10.2	5.8	4	20
SGON87	6.5	9.5	4	20
SGON88	16	0	4	20
SGON89	8.7	7.3	4	20
SGON96	6	10	4	20
SGON55b	11.4	4.6	4	20
SGON64a	11	5	4	20
SGON65a	16	0	4	20
SGON66a	3.7	12.3	4	20
SGON74a	10.8	5.2	4	20
SGON92a	6.9	9.1	4	20
H21	6.8	9.2	4	20
H24	16	0	4	20

Appendix IV

Supplementary Figure 11: Genomic organisation of the Spotty actb2 gene. Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. Primer set 1 is boxed, which can potentially amplify two products, 138 bp from cDNA or 235 bp from genomic DNA.

ATGGAAGACGAAATCGCCGCCCTCGTTGTTGACAACGGATCTGGTATGTGCAAAGCTGGCTTTGCAGGAGATGATGC
TCCTCGTGCTGTGTTCCCCTCCATTGTTGGACGTCCCAGGCATCAGgtacagtttcttattccaatccattttattt
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cggatgctgcaggtttctatctttttgcgatgtgatgcatcaggaatgacttgttgcagcatggtcactgttggttt
ctatataaggcttgttttagactctctaatgcagacaggaagcgtctaactacacagatgcatgatgcaacagatga
cgaaagaaaagggtccttaactcatcttgtactttcaatatgacccaactaaagtgcagattactgtaatgactggt
ttcaggatggtgataatacttaaaacaaaactgccagtgtgctgtgcctctcttggtctgattgtactaaccatact
agctcacctaaggcatttgactcactgtttttcaatgactcacctttcatctctctctttccccagATCATGTTCGA
GACCTTCAACACTCCTGCCATGTATGTGGCCATCCAGGCTGTGCTGTCCCTGTACGCTTCTGGTCGTACCACTGGTA
TTGTCATGGACTCTGGTGATGGTGTGACCCACACAGTGCCCATCTATGAAGGCTACGCCCTGCCTCACGCCATCCTG
AGGTTGGACCTGGCTGGCAGGGACCTCACAGACTACCTCATGA**AGATCCTGACTGAGAGGGGT**TACAGCTTCACCAC
CACAGgtaagcacagaagtgcaagttaagactcatggatgagattgaaacttgaggaaacttgaaaaaagatgtaac
ttaaatgtttgttcttgtcttacagCTGAGCGTGAAATCGTGCGTGACATCAAAGAGAAGCTGTGTTACGTTGCACT
GGACTTTGAGCAGGAGATGGGCACTGC**TGCTTCCTCTTCTTCCCTGG**AGAAGAGCTACGAGCTGCCTGACGGACAGG
TCATCACCATTGGAAATGAGAGGTTCCGTTGCCCTGAGGCCCTCTTCCAGCCCTCATTCCTTGgtaagttaaatcag
ggaaactttcattttaaaggtggacacctagaggtcttgacagcaaagagaatctatcataaactaacttttacatt
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CGTAAAGACCTGTATGCCAACACTGTGCTGTCTGGAGGTACCACCATGTACCCTGGCATTGCTGACCGTATGCAGAA
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ACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGATGAGTCTGGCCCCAGCATTGTCCACAGGAAGTGCTTCTA
AACAGACTGTCAGTCCCCTCCCCCAAAACACACACTGCTACAAACCCAAACGACTGGCTCTGCATACATGCCTACAC
CAACACACTGGTGT

Supplementary Figure 12: Genomic organisation of the Spotty actb2 gene. Nucleotides in upper-case (coloured) indicate exons and nucleotides in lower-case indicate introns. Primer set 2 is boxed, which can potentially amplify only one product, 200 bp.

ATGGAAGACGAAATCGCCGCCCTCGTTGTTGACAACGGATCTGGTATGTGCAAAGCTGGCTTTGCAGGAGATGATGC
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GACCTTCAACACTCCTGCCATGTATGTGGCCATCCAGGCTGTGCTGTCCCTGTACGCTTCTGGTCGTACCACTGGTA
TTGTCATGGACTCTGGTGATGGTGTGACCCACACAGTGCCCATCTATGAAGGCTACGCCCTGCCTCACGCCATCCTG
AGGTTGGACCTGGCTGGCAGGGACCTCACAGACTACCTCATGAAGATCCTGACTGAGAGGGGTTACAGCTTCACCAC
CACAGgtaagcacagaagtgcaagttaagactcatggatgagattgaaacttgaggaaacttgaaaaaagatgtaac
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TCATCACCATTGGAAATGAGAGGTTCCGTTGCCCTGAGGCCCTCTTCCAGCCCTCATTCCTTGgtaagttaaatcag
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CGTAAAGACCTGTATGCCAACACTGTGCTGTCTGGAGGTACCACCATGTACCCTGGCATTGCTGACCGTATGCAGAA
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ttaaatctctagctagccttttacaacacaggattgaaatacttgcaggaatttaaaacatttttttatatctt
gctctctgcag**ATCA**TTGCTCCCCCTGAGAGGAAGTACTCTGTATGGATTGGAGGCTCCATCCTGGCCTCTCTCTCC
ACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGATGAGTCTGGCCCCAGCATTGTCCACAGGAAGTGCTTCTA
AACAGACTGTCAGTCC**CCTCCCCCAAAACACACACT**GCTACAAACCCAAACGACTGGCTCTGCATACATGCCTACAC
CAACACACTGGTGT