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Expression of gonadal immune genes during prepubertal sex change in spotty wrasse (*Notolabrus celidotus*)

A thesis
Submitted in partial fulfilment
of the requirements for
the degree of

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By

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Abstract

Teleost fish display exceptional diversity in their reproductive strategies, including sequential hermaphroditism, in which individuals undergo complete functional sex change during adulthood (monandry) and in some cases, a second male morph can arise prior to puberty (diandry). While the endocrine and genetic mechanisms underlying sex change have been extensively studied, the contribution of the immune system to gonadal remodelling remains poorly understood. This thesis investigates the role of immune processes during female-to-male prepubertal sex change in initial-phase members of the New Zealand spotty wrasse *(Notolabrus celidotus).* This study integrates histological analysis with gene expression profiling to examine immune involvement across transitional stages of gonadal sex change.

Histological examination revealed stage-dependent changes in gonadal leukocyte (eosinophilic granular cell) abundance and localisation, coinciding with ovarian degeneration and testicular development. Gene expression analyses demonstrated coordinated increases in immune-associated markers (*cd68*, *il-1β*, and *tnf-α*), particularly during late transitional and male stages. Notably, immune activation was not confined to early degenerative phases but persisted into the male phase, suggesting roles beyond debris clearance, likely including tissue organisation and maintenance. Although inter-individual variability limited statistical significance for some markers, consistent directional trends across genes and concordance with histological observations support a biologically meaningful pattern of immune modulation during sex change.

By building a body of evidence that links immune gene expression with histological evidence of leukocyte involvement, this study highlights a likely localised immune function response as a key mechanism associated with vertebrate sex change. Importantly, it identifies immune cells as active contributors to sex change rather than passive responders.

Acknowledgements

I want to express my sincere gratitude to my supervisor, Simon Muncaster, for his continued guidance, patience, and mentorship throughout the course of this research. I am also extremely grateful to my co-supervisor, Chloe Van Der Burg, for their guidance, constructive feedback, and support throughout this project. Her thoughtful advice and support were invaluable to the development of my gene expression analysis.

I am particularly thankful to Tessa Hamer for the considerable time, patience, and expertise she dedicated to teaching me the molecular techniques required for this project, as well as her support during troubleshooting and inevitable setbacks. My thanks also extend to Franz Ferguson for his assistance and time spent on histology and helping with data analysis.

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Upon PRR activation, myeloid cells such as macrophages and granulocytes (eosinophilic and basophilic) initiate downstream signalling pathways that induce the synthesis and secretion of pro-inflammatory cytokines (e.g., interleukin-1β (*il-1β*), interleukin-6 (*il-6*), and tumour necrosis factor-α (*tnf-α*), These cytokines orchestrate inflammatory responses, promote chemotaxis of additional leukocytes, and modulate gene expression related to immune activation. Innate effectors also include soluble antimicrobial peptides, lysozyme (Lyso G), and enzymes that degrade microbial components, which collectively limit pathogen proliferation at epithelial surfaces. Concurrently, recruited leukocytes engage in phagocytosis, degranulation, and reactive oxygen intermediate production, all hallmark innate defence activities described in teleosts. Signals generated during the innate responses provide essential cues for activation and shaping of the adaptive immune system, which in fish comprises T lymphocytes (T cells) and B lymphocytes (B cells). T cells mediate cellular immunity and assist B cells, while B cells differentiate into antibody-producing cells. Fish produce specific immunoglobulins, primarily IgM and the teleost-specific Ig class IgZ/IgT, which bind antigens to neutralise pathogens or facilitate their clearance.

The adaptive response confers enhanced and antigen-specific protection, with lymphocyte proliferation and antibody production increasing the efficiency of pathogen elimination. The figure also highlights regulatory mechanisms, including antioxidant enzymes (e.g., MnSOD, catalase) that mitigate oxidative stress and protect host tissues from self-damage resulting from intense immune activation.

Overall, this integrates key components of fish immunity, from PRR-mediated innate detection and inflammatory signalling to adaptive lymphocyte-driven antigen specificity, providing a comprehensive overview of how teleost fish recognise and respond to immunological challenges. Originally sourced from Chaves-Pozo et al. (2018) and adapted in Canva……………………………………………………………………………………………6

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Three major leukocyte types are shown: lymphocytes (Ly), antigen-presenting cells (AG), and macrophages (M∅). These immune cells express both nuclear receptors, which act inside the cell nucleus to regulate gene expression, and membrane-associated receptors, which trigger signalling events at the cell surface. The presence of question marks indicates that some receptor functions or signalling mechanisms remain incompletely understood in fish.

Nuclear androgen receptors (AR) and truncated nuclear androgen receptor (ARΔLBD) are depicted, reflecting fish-specific receptor variants reported in the literature. These receptors respond to androgens and can directly influence immune gene transcription. Nuclear estrogen receptors (ESR1) are also shown, highlighting the roles of estrogen in modulating immune cell activity. In Macrophages, estrogen receptor signalling can be upregulated upon activation, particularly involving ESR2a and ESR2b, indicating that immune stimulation (such as parasite exposure) enhances hormone sensitivity.

In addition to nuclear signalling, leukocytes express membrane estrogen receptors and membrane androgen receptors, which allow sex steroids to rapidly alter immune cell behaviour without directly changing gene transcription. These pathways are thought to influence processes such as cytokine release, antigen processing, and inflammatory responses. Overall, this figure visualises the multi-layer interaction between sex hormones and fish immune cells, helping explain why immune responses can differ between sexes, reproductive stages, or hormonal states.

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Chapter 1 – Introduction

Teleost fishes have a long and evolutionarily diverse history, with lineages diverging as early as the Jurassic period and accumulating 100-200 million years of independent evolution (Magnadottir, 2010; Nagahama et al., 2021). Over this time, teleosts have adapted to extraordinarily varied ecological niches, resulting in marked differences in feeding strategies, reproductive systems, and life histories (De Mitcheson & Liu, 2008; Magnadottir, 2010; Nagahama et al., 2021; Todd et al., 2016). These extensive ecological divergences have also shaped the architecture and function of the teleost immune system to support cellular function, combat disease and promote health. Consequently, producing a broad spectrum of immune capacities and response strategies has evolved across species (Magnadottir, 2010; Muiswinkel & Vervoorn-Van Der Wal, 2006; Mutoloki et al., 2014).

With this diverse group, sexual systems also exhibit remarkable plasticity (Todd et al., 2016). Sex determination in teleosts may be governed genetically, influenced by environmental cues such as pH, temperature, or population density, or shaped by interactions between these factors (Gemmell et al., 2019; Godwin, 2010; Shen & Wang, 2018; Nagahama et al., 2021; Todd et al., 2016). Sexual fate (becoming male or female), once thought to be permanently fixed following early developmental cascades, is now recognised as a dynamic and actively maintained state (Kamstra et al., 2024; Piferrer, 2021; Todd et al., 2016), especially among teleost fishes. These developmental cascades rely heavily on evolutionarily conserved transcription factors and steroid hormones; however, maintenance of sexual phenotype in adulthood requires continued suppression of the opposing sexual pathway (Gemmel et al., 2019). This biological "tug-of-war" between antagonistic male and female developmental networks provides the mechanistic flexibility that underpins sexual plasticity later in life.

1.1 Gonadal Plasticity and Tissue Remodelling During Sex Change

Sequential hermaphroditism represents one of the most striking examples of vertebrate phenotypic plasticity (Caballero-Huertas et al., 2025; Liu et al., 2017; Kamstra et al., 2024; Piferrer, 2021). More than 500 species undergo sex change as part of their natural life history (Muncaster et al., 2023), demonstrating that sexual plasticity is a widespread and adaptive

reproductive strategy, rather than a biological anomaly (De Mitcheson & Liu, 2008; Gemmell et al., 2019; Kamstra et al., 2024). Sex change in teleosts involves coordinated shifts in social behaviour, endocrine signalling, gonadal architecture, and external morphology, with social cues acting as the primary trigger for downstream endocrine and gonadal sex change (De Mitcheson & Liu, 2008; Gemmell et al., 2019; Kamstra et al., 2024; Piferrer, 2021).

Although the behavioural and endocrine correlates of sex change are well documented (De Mitcheson & Liu, 2008; Gemmell et al., 2019; Nagahama et al., 2021), the cellular processes supporting gonadal restructuring remain comparatively less resolved (Caballero-Huertas et al., 2025; Piferrer, 2021). The suppression of one sexual pathway and activation of the other necessitate precise coordination of apoptosis, cell clearance, proliferation, and tissue reorganisation (Garcia-Ayala & Chaves-Pozo; Liu & de Mitcheson, 2009; Nakamura et al., 1989; Valero et al., 2015). These features position sex change as a powerful biological model for investigating how adult tissues undergo regulated degeneration and regeneration in response to environmental and social cues (Gemmell et al., 2019; Piferrer, 2021).

1.2 Neuroendocrine and Molecular Regulation of Sex Change

The extraordinary diversity of sexual phenotypes observed in teleost fishes reflects a high degree of neuroendocrine flexibility that extends well beyond primary sex determination (Nagahama et al., 2021; Piferrer, 2021). In sequentially hermaphroditic species, this plasticity enables socially mediated sex change in adulthood, making teleosts a key model system for understanding environmentally induced reproductive transitions (De Mitcheson & Liu, 2008; Warner & Sweater, 1991).

In protogynous wrasses, sex change is typically triggered by the removal of a dominant male, which initiates a cascade of neuroendocrine events in the highest-ranking female (Campbell et al., 2021; Nagahama et al., 2021; Kamstra et al., 2024). The earliest gonadal response involves downregulation of aromatase (*cyp19a1a*), reducing estrogen synthesis, coupled with upregulation of male -promoting genes such as *amh* and *dmrt1*, which drive testicular differentiation and spermatogenesis (De Mitcheson & Liu, 2008; Gemmell et al., 2019). These processes are coordinated through the hypothalamic-pituitary-gonadal (HPG) axis, with luteinizing hormone (LH) and follicle-stimulating hormone (FSH) regulating steroidogenesis and gonadal restructuring (Campbell et al., 2021; Nagahama et al., 2021) (**Figure 1.1)**.

Steroid hormones are central to these transitions. Testosterone (T) can be converted into biologically potent androgens such as 11-ketotestosterone (11-KT), which are essential for testicular development and spermatogenesis (Chaves-Pozo et al., 2018; Nagahama et al., 2021), whereas estrogen synthesis via aromatase (*cyp10a1a*) is critical for maintaining ovarian structure and function (De Mitcheson & Liu, 2008; Pifferrer, 2021). The balance between these steroid pathways governs gonadal phenotype and progression of sex change (Gemmell et al., 2019) **(Figure 1.1)**.

Sex change also involves interactions between the HPG axis and the hypothalamic-pituitary-interrenal (HPI) axis, which regulates stress physiology (Goikoetxea et al., 2017; Szwejser et al., 2017; Wang & Belosevic, 1995). Social disruption elevates cortisol, which modulates reproductive hormones, gonadal gene expression, and immune activity in the gonad (Chaves-Pozo et al., 2018; Tort, 2011). These systems collectively modulate production of key steroids, including 17B-estradiol (E2) and 11-KT, that drive gonadal transformation. Typically, ovarian regression is accompanied by a decline in E2 and a rise in androgen synthesis. However, in temperate wrasses, where sex change often occurs outside the breeding season and circulating estrogen is already low, stress-related HPI-HPG crosstalk may play a proportionally greater role in initiating sex change and modulation of gonadal immune activity, including influencing cellular turnover and tissue restructuring (Gemmell et al., 2019; Nardocci et al., 2014; Chaves-Pozo et al., 2018; Tort, 2011).

At the molecular level, sex change involves extensive reprogramming of gonadal gene expression. Male-promoting genes such as *amh*, *dmrt1*, and *sox9a* are upregulated, while female-associated genes, including *cyp19a1a*, *foxl2a*, and *rspo1*, are downregulated (Muncaster et al., 2023; Thomas et al., 2019). Epigenetic regulation further refines these processes, with dynamic changes in DNA methyltransferase expression indicating chromatin remodelling and transcriptional plasticity during gonadal transformation (Muncaster et al., 2023; Thomas et al., 2019). Recent work in New Zealand spotty wrasse by Muncaster et al. (2023) demonstrated extensive epigenetic and transcriptional plasticity during sex change, reflecting the scale of molecular flexibility required for adult gonadal plasticity. These molecular networks conceptually mirror immune responses, which similarly rely on rapid transcriptional and chromatin-level regulation to adapt to physiological challenges (Secombes & Wang, 2012; Chaves-Pozo et al., 2018).

Together, these neuroendocrine, steroidal, and molecular mechanisms underpin the coordinated reorganisation of adult gonads, providing a framework to understand how social cues and endocrine signals integrate with tissue-level processes during sex change.

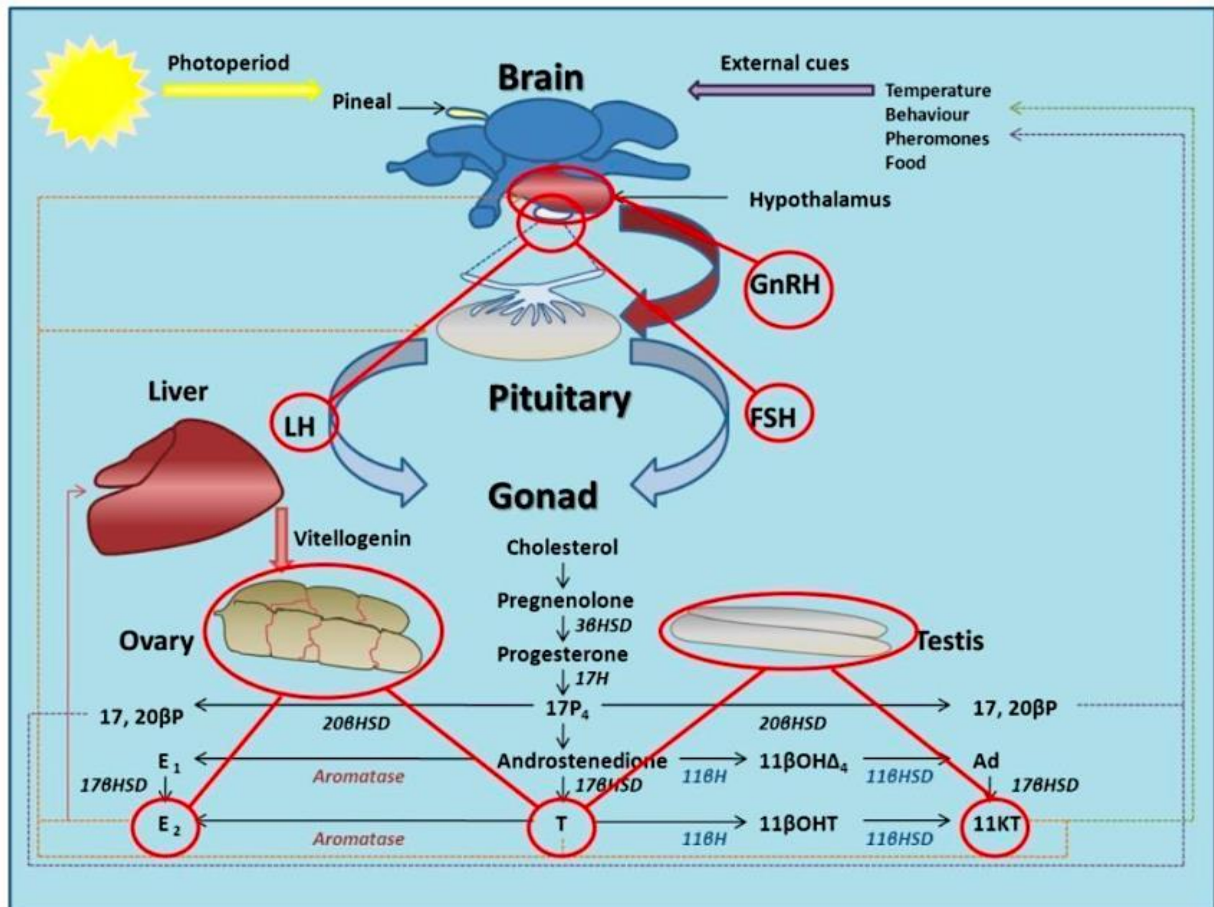
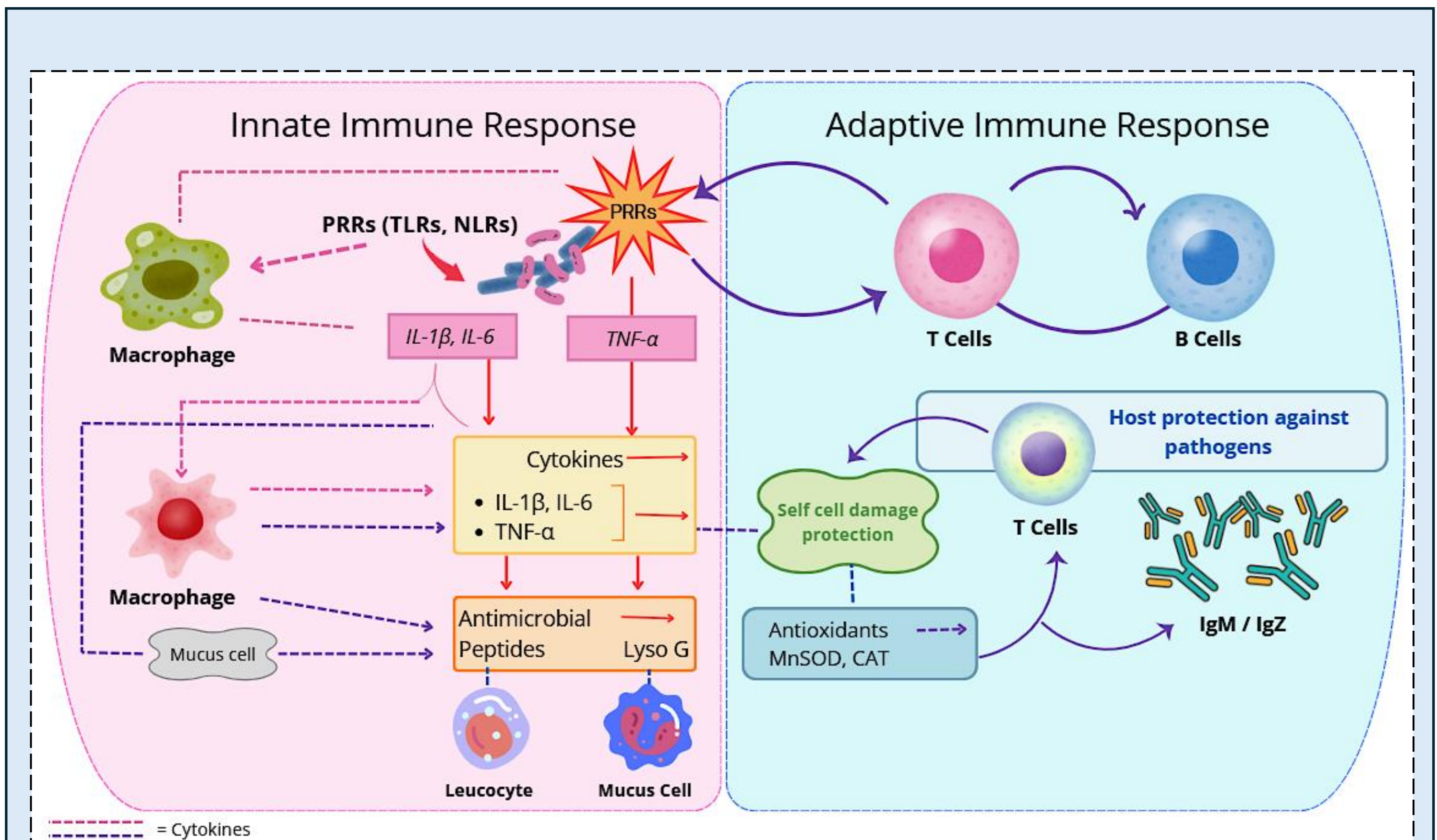


Figure 1.1: Illustration of the reproductive axis in teleosts, outlining the key processes involved in the maturation of the gonads. Sourced from Muncaster (2008).

1.3 Immune System, Tissue Remodelling, and Gonadal Plasticity

Teleost fishes possess a well-developed immune system comprising tightly interconnected innate and adaptive components (Aoki et al., 2008; Uribe et al., 2011). Innate immunity provides a continuously active frontline defence, including physical barriers (skin, gills, and mucus), cellular effectors such as macrophages, granulocytes, and natural killer-like cells (Magnadottir, 2010; Uribe et al., 2011), and humoral factors including cytokines, antimicrobial peptides, and lysozymes (Aoki et al., 2008; Secombes & Wang, 2012) **(Figure 1.2)**. Adaptive immunity in teleosts is mediated by B- and T-lymphocytes capable of antigen recognition and immunoglobulin production (Kordon et al., 2022; Mutoloki et al., 2014; Uribe et al., 2011), although responses are slower and less compartmentalised than in mammals (Tort, 2011; Uribe et al., 2011) **(Figure 1.2)**. The absence of lymph nodes and germinal centres, combined with the reliance on a limited immunoglobulin repertoire dominated by IgM, results in extensive crosstalk between the innate and adaptive pathways (Magnadottir, 2010) **(Figure 1.2)**. Teleosts typically rely on a single functional immunoglobulin class, IgM, which may act as both an adaptive antibody and a natural antibody with pattern-recognition properties (Magnadottir, 2010). The discovery that some fish B-cells exhibit phagocytic activity highlights the multifunctional nature of teleost lymphocytes (Kordon et al., 2022; Magnadottir, 2010) and raises the possibility that these cells may also contribute to tissue remodelling associated with sex change. This functional overlap highlights the versatility of teleost immune responses beyond classical pathogen defence and may be particularly relevant in tissues that undergo extensive structural reorganisation, such as the gonads during sexual plasticity and sex change (Liu et al., 2016).



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Signals generated during the innate responses provide essential cues for activation and shaping of the adaptive immune system, which in fish comprises T lymphocytes (T cells) and B lymphocytes (B cells). T cells mediate cellular immunity and assist B cells, while B cells differentiate into antibody-producing cells. Fish produce specific immunoglobulins, primarily IgM and the teleost-specific Ig class IgZ/IgT, which bind antigens to neutralise pathogens or facilitate their clearance.

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Overall, this integrates key components of fish immunity, from PRR-mediated innate detection and inflammatory signalling to adaptive lymphocyte-driven antigen specificity, providing a comprehensive overview of how teleost fish recognise and respond to immunological challenges. Originally sourced from Chaves-Pozo et al. (2018) and adapted in Canva.

Key immune tissues include the thymus (T-cell maturation), anterior kidney (hematopoiesis and antibody production), and spleen (immune surveillance) **(Figure 1.3)** (Kordon et al., 2022; Magnadottir, 2010). Immune cells are also present in non-immune tissues, including the gonads, where they are closely associated with regions of active turnover (Garcia-Ayala & Chaves-Pozo, 2009; Magnadottir, 2010). Macrophages and lymphocytes observed within gonadal tissue suggest roles in apoptosis, phagocytosis, and maintenance of local tissue homeostasis, supporting both degeneration of ovarian tissue and subsequent testicular development during sex change (Magnadottir, 2010; Muncaster et al., 2013).

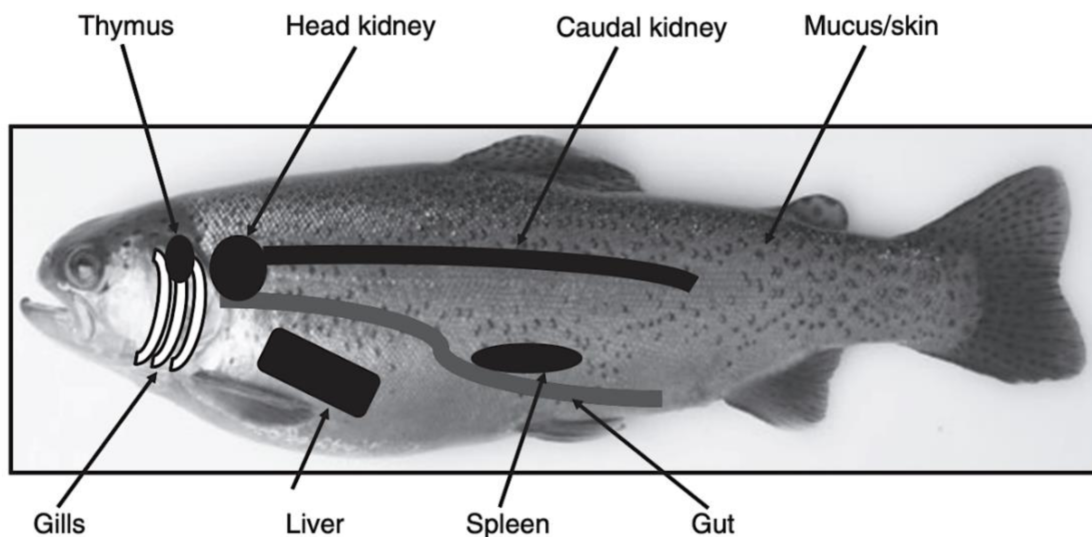
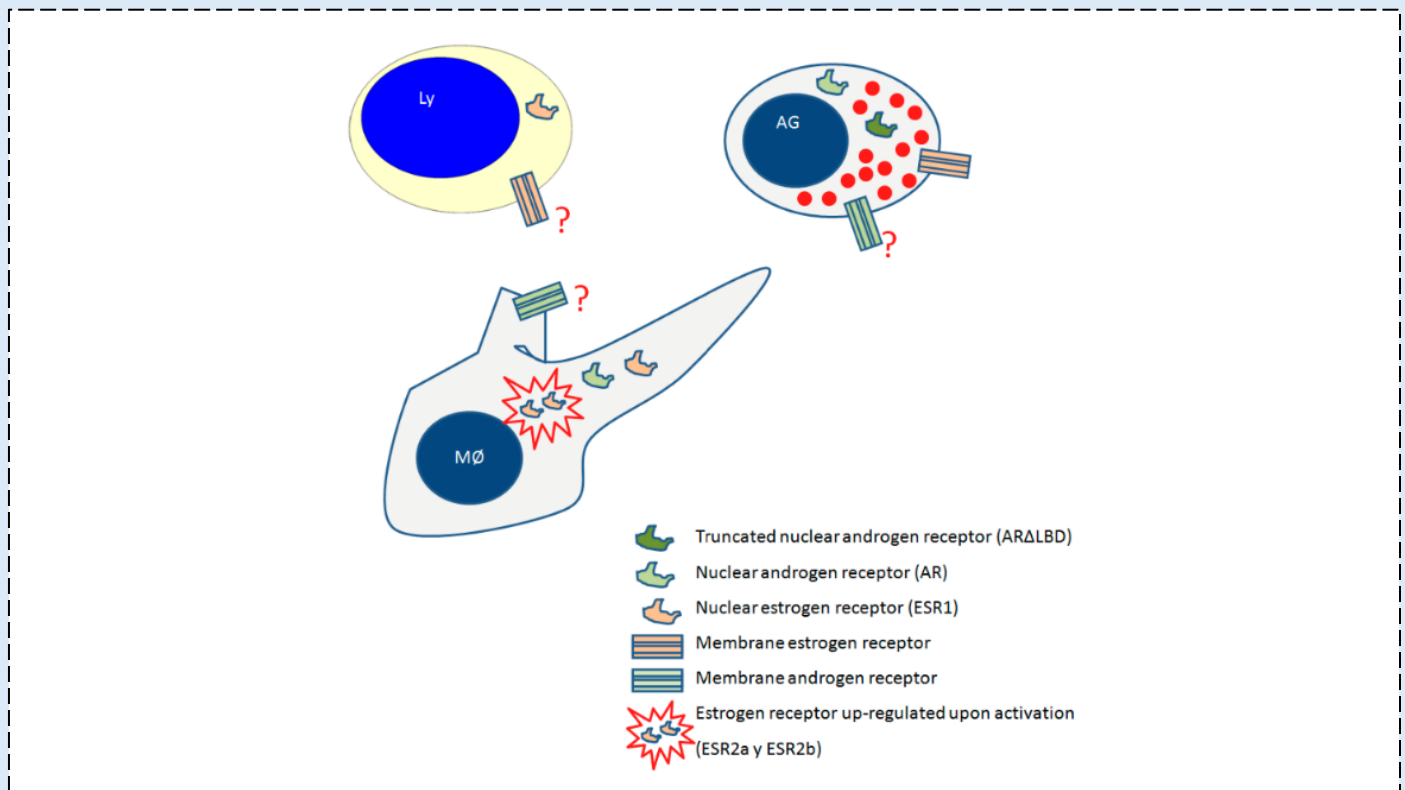


Figure 1.3: Representative of immune tissues relative to a rainbow trout (*Oncorhynchus mykiss*, highlighting the approximate immune tissue sites in teleost fish. Originally sourced from Secombes & Wang (2012).

Sex steroids and stress hormones interact directly with immune function, modulating cytokine expression and leukocyte distribution (Chaves-Pozo et al., 2018; Secombes & Wang, 2012; Valero et al., 2024). In hermaphroditic species such as gilthead seabream, the presence of both androgen and oestrogen receptors on leukocytes further supports the integration of immune and endocrine signalling across reproductive states **(Figure 1.4)**.

Seasonal and environmental factors such as temperature, light, and water quality further influence immune activity, producing predictable fluctuations that intersect with reproductive cycles (Magnadottir, 2010). Recent findings highlight that immune organs can respond to hormonal signals; for example, the teleost thymus shows sensitivity to sex steroids, linking reproductive status with adaptive immune development (Barraza et al., 2024).



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Three major leukocyte types are shown: lymphocytes (Ly), antigen-presenting cells (AG), and macrophages (M∅). These immune cells express both nuclear receptors, which act inside the cell nucleus to regulate gene expression, and membrane-associated receptors, which trigger signalling events at the cell surface. The presence of question marks indicates that some receptor functions or signalling mechanisms remain incompletely understood in fish.

Nuclear androgen receptors (AR) and truncated nuclear androgen receptor (ARΔLBD) are depicted, reflecting fish-specific receptor variants reported in the literature. These receptors respond to androgens and can directly influence immune gene transcription. Nuclear estrogen receptors (ESR1) are also shown, highlighting the roles of estrogen in modulating immune cell activity. In Macrophages, estrogen receptor signalling can be upregulated upon activation, particularly involving ESR2a and ESR2b, indicating that immune stimulation (such as parasite exposure) enhances hormone sensitivity.

In addition to nuclear signalling, leukocytes express membrane estrogen receptors and membrane androgen receptors, which allow sex steroids to rapidly alter immune cell behaviour without directly changing gene transcription. These pathways are thought to influence processes such as cytokine release, antigen processing, and inflammatory responses.

Overall, this figure visualises the multi-layer interaction between sex hormones and fish immune cells, helping explain why immune responses can differ between sexes, reproductive stages, or hormonal states.

In gonadal tissues, which are both hormonally active and immunologically sensitive, these receptor-mediated pathways are thought to play a key role in shaping local immune responses. Sex steroid signalling in gonadal leukocytes may regulate inflammation, immune tolerance, and pathogen defence. Originally sourced from Paul & Sahoo (2024).

These interactions suggest that immune processes are not merely defensive but actively participate in gonadal tissue remodelling in species capable of sequential hermaphroditism.

The conversion of ovarian tissue into testicular tissue during protogynous sex change is inherently dynamic, involving coordinated cycles of degeneration, apoptosis, proliferation, and structural reorganisation (Gemmell et al., 2019; Nakamura et al., 1989). These processes are frequently accompanied by immune-cell recruitment (leukocytes) and regulated inflammatory signalling (cytokines) within the gonad (Chaves-Pozo et al., 2018; Lutton & Callard, 2006; Magnadottir, 2010; Uribe et al., 2011). Histological studies in temperate wrasses report the redistribution of leukocytes within transitioning gonads, implicating immune cells in debris clearance and structural remodelling (Garcia-Ayala & Chaves-Pozo, 2009; Nakamura et al., 1989; Valero et al., 2015; Watts et al., 2001).

Crosstalk between the HPG and HPI axes provides a mechanistic link between social cues, stress responses, and immune activation (Goikoetxea et al., 2017; Szwejser et al., 2017) **(Figure 1.5)**. Cortisol elevation during social disruption may simultaneously promote gonadal sex change and modulate immune activity within the gonad, facilitating apoptotic clearance of ovarian cells and supporting the formation of functional testicular structures (Chaves-Pozo et al., 2018; Tort, 2011; Valero et al., 2015).

At the level of gene and protein regulation, key innate immune genes, including pro-inflammatory cytokines (*il-1β, tnf-α*) and macrophage markers (*cd68*), are expressed within both germinal and within the tissue space of the gonad (Chaves-Pozo et al., 2008; Valero et al., 2015). These genes regulate cellular turnover, degeneration, and regeneration, directly linking immune activation to the tissue remodelling processes that underlie sex change (Garcia-Ayala & Chaves-Pozo, 2009; Lieschke & Trede, 2009) **(Figure 1.5)**.

Together, these observations position the teleost immune system as an integral component of gonadal plasticity, actively supporting tissue breakdown, regeneration, and structural reorganisation during sex change. The combined action of endocrine signalling, stress pathways, and immune-mediated remodelling illustrates the tightly coordinated physiological orchestration required for adult sequential hermaphroditism.

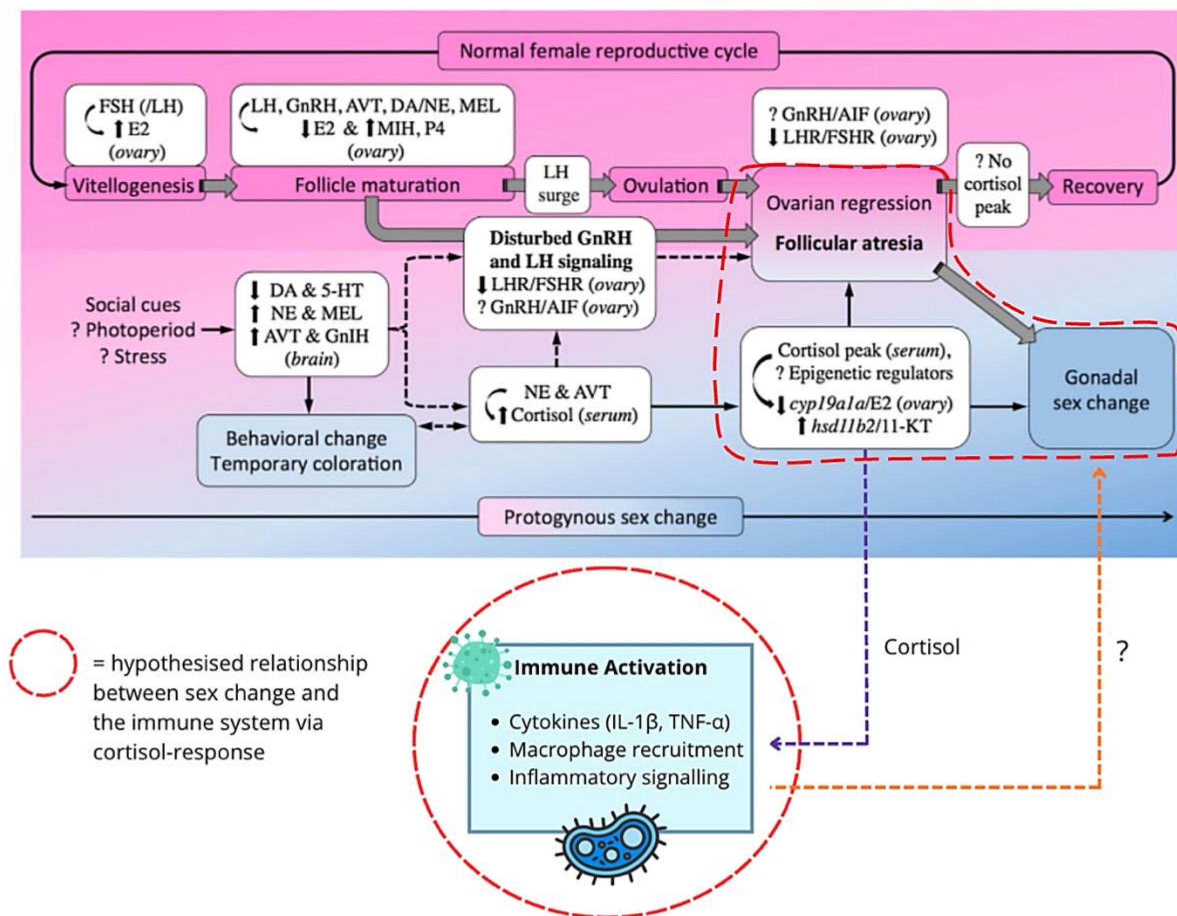

Figure 1.5: Conceptual model of the normal female reproductive cycle and the proposed initiation of socially induced protogynous sex change in teleost fish. Solid arrows indicate empirically supported interactions; dashed arrows represent hypothesised pathways, and question marks denote uncertain signalling pathways as explored in this study. Social cues and stress-related signals are proposed to disrupt hypothalamic-pituitary-gonadal (HPG) axis function, leading to ovarian regression and follicular atresia. Activation of the hypothalamic-pituitary-interrenal (HPI) axis and the associated cortisol response is hypothesised to link endocrine changes with immune activation, potentially contributing to gonadal tissue remodelling during sex change.

Liu et al. (2017) propose that socially induced sex change is initiated by rapid neuroendocrine reprogramming, with disruption of GnRH-gonadotropin signalling preceding ovarian regression and follicular atresia. Stress-associated endocrine pathways are suggested to modulate gonadal remodelling under socially unstable conditions, supporting the hypothesised role of HPI axis activation and downstream immune involvement depicted in this model. Originally sourced from Liu et al. (2017) and adapted in Canva.

Abbreviations: 5-HT, serotonin; AIF, apoptosis-inducing factor; AVT, arginine vasotocin; cyp19a1a, gonadal aromatase (gene); DA, dopamine; $E_{2,}$ 17β-estradiol; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; hsd11b2, 11β-hydroxysteroid dehydrogenase 2 (gene); LH, luteinizing hormone; LHR, luteinizing hormone receptor; MEL, melatonin; MIH, maturation-inducing hormone; NE, norepinephrine; P4, progesterone.

1.3.1 Candidate Immune Genes Examined in this Study

Teleost fish possess a well-developed and evolutionally conserved immune system in which innate immune responses dominate, mediating inflammation, cellular turnover, and tissue remodelling (Lieschke & Trede, 2009; Mokhtar et al., 2023; Rauta et al., 2012; Smith et al., 2019). In contrast to mammals, where adaptive immunity often drives specificity and memory, teleosts rely heavily on innate mechanisms that respond rapidly to tissue disturbance, physiological stress, and structural reorganisation (Dixon & Stet, 2001; Foey & Picchietti, 2014; Watts et al., 2001) **(Figure 1.2)**. Inflammatory responses involve increased vascular permeability and blood flow, facilitating the delivery of immune cells, antimicrobial molecules, and signalling factors to affected tissues, linking immune activation with tissue repair and remodelling (Bjørgen & Koppang, 2022; Lieschke & Trede, 2009).

Within this innate immune framework, immune signalling is mediated by cytokines, small, secreted proteins that coordinate immune cell recruitment, activation, survival, and apoptosis, and shape the local tissue environment (Lieschke & Trede, 2009) **(Figure 1.2)**. In teleosts, pro-inflammatory cytokines such as interleukin-1β (Il-1β) and tumour necrosis factor-α (Tnf-α) are central components of the innate immune system and are rapidly induced during periods of physiological change (Lieschke & Trede, 2009; Rauta et al., 2012; Secombes et al., 2009). These cytokines are expressed within both the germinal and tissue interstitial compartments of the teleost gonad, where they contribute to apoptosis, tissue restructuring, and immune-endocrine signalling rather than classical pathogen defence (Engelsma et al., 2002; Garcia-Ayala & Chaves-Pozo, 2009). The germinal compartment contains germ cells and their associated somatic support cells and is the site of gametogenesis, while the interstitial compartment comprises connective tissue, blood vessels, steroidogenic cells, and resident immune cells. Immune-related genes expressed in these compartments are strategically positioned to influence both germ cell fate and the broader tissue environment, including vascular dynamics, apoptosis, and tissue remodelling (Bjørgen & Koppang, 2022; Chaves-Pozo et al., 2008; Garcia-Ayala & Chaves-Pozo, 2009). The cytokines Il-1β and Tnf-α play key roles in regulating cellular turnover, degeneration, and regeneration during development and reproduction (Garcia-Ayala & Chaves-Pozo, 2009; Valero et al., 2024).

Macrophages are highly plastic effector cells that link inflammatory signalling with tissue remodelling, performing phagocytosis of apoptotic cells, secreting cytokines, and regulating

the local signalling environment (Belosevic et al., 2009; Leischke & Trede, 2009). The macrophage-associated protein marker Cd68 is widely used to indicate macrophage presence and activity within tissues, enabling direct comparison between immune gene expression and histological observations of macrophage-like cells during gonadal remodelling (Belosevic et al., 2009; Chaves-Pozo et al., 2008; Garcia-Ayala & Chaves-Pozo, 2009).

In species such as the protandrous gilthead seabream (*Sparus aurata*), pro-inflammatory cytokines and macrophage markers are constitutively expressed in the gonad and exhibit stage-specific regulation across the reproductive cycle (Chaves-Pozo et al., 2008; Valero et al., 2015). Steroid hormones that drive reproductive transitions, including androgens and oestrogens, exert immunomodulatory effects, influencing cytokine expression, leukocyte distribution, and immune sensitivity within reproductive tissues (Sang et al., 2020; Mokhtar et al., 2022) **(Figure 1.4)**. Experimental manipulation of steroidogenic pathways demonstrates that gonadal cytokine gene expression is tightly coupled to reproductive stage, indicating that immune signalling pathways respond directly to endocrine cues during sexual transformation (Cabas et al., 2012; Engelsma et al., 2002).

Together, the immune-related genes *il-1β*, *tnf-α*, and *cd68* constitute a focused panel capturing both inflammatory signalling and cellular immune involvement, providing a biologically meaningful framework for assessing immune activity during gonadal restructuring associated with protogynous sex change (Chaves-Pozo et al., 2008; Valero et al., 2015).

1.5 New Zealand spotty wrasse

The New Zealand spotty wrasse (*Notolabrus celidotus*) is a temperate, protogynous hermaphrodite in which individuals typically mature as females and may later transition to males (Jones, 1980). Sex change in this species is socially regulated: the removal of a dominant terminal-phase (TP) male triggers the largest initial-phase (IP) female to commence a behavioural, anatomical, and pigmentation transition characteristic of protogyny in Labrids (Gemmell et al., 2019; Hamer et al., 2025; Kamstra et al., 2024; De Farias e Moraes, 2019). Spotty wrasses are diandric, exhibiting distinct IP and TP colour morphs alongside alternative male reproductive strategies, features common within the family Labridae (Hamer et al., 2025; Kamstra et al., 2024) **(Figure 1.6)**.

As a seasonal breeder, unlike many tropical wrasses that reproduce year-round, *N. celidotus* follows a defined spawning period from late July to late November, with sex change occurring

primarily between November and May (Goikoetxea et al., 2022). Their high local abundance, manageable size, and the ease with which social hierarchies can be manipulated in captivity make them an effective model for studying socially induced sex change (Goikoetxea et al., 2021; Kamstra et al., 2024).

Experimental work demonstrates that spotty wrasse can complete female-to-male transition within roughly 60 days when induced through aromatase inhibition or controlled social restructuring (Goikoetxea et al., 2022). Interestingly, the early molecular events underlying transition differ from patterns observed in many subtropical species (Warner, 1984). In spotty wrasse, initial declines in plasma estradiol (E2) or gonadal aromatase (*cyp19a1a*) are not evident; instead, early upregulation of the masculinising factor Amh occurs, followed by later increases in glucocorticoid and mineralocorticoid receptor genes (*nr3c1* and *nr3c2*) (Goikoetxea et al., 2021). These dynamics suggest that endocrine and transcriptional reprogramming, rather than a universally conserved hormonal cascade, govern sex change in this temperate species.

Collectively, the biological and experimental features of *N. celidotus*, seasonal reproduction, strong social control, accessible field population, and distinct molecular signatures position it as a valuable system for investigating vertebrate sex change, including the associated histological and immune processes (Kamstra et al., 2024).

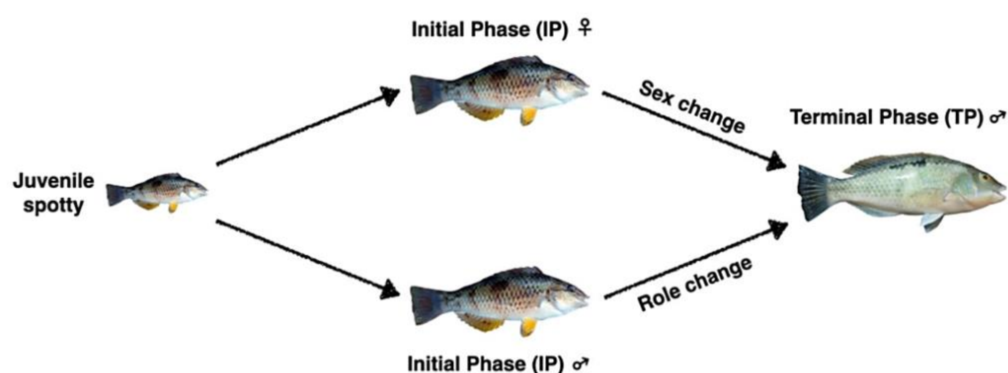

Figure 1.6: Schematic representation of the life cycle of the New Zealand spotty wrasse (*Notolabrus celidotus*). Juveniles develop into initial phase (IP) individuals, which may mature as either females or males. Initial phase females can undergo sex change to become terminal phase (TP) males, whereas initial phase males transition to the terminal phase via role change. This diagram illustrates the bidirectional developmental pathways characteristic of this species' protogynous reproductive strategy. IP New Zealand spotty wrasse image by Allan Burgess, TP New Zealand spotty wrasse image by Jodi Thomas. Sourced from Muncaster et al. (2013).

1.6 Aims and objectives

The overarching aim of this thesis is to investigate the role of immune-related processes during prepubertal sex change in juvenile IP spotty wrasse *(N. celidotus).* The specific objectives to achieve this aim are:

1.) Characterisation of the abundance and distribution of key immune cells (eosinophilic granular cells) across distinct histological stages of sex change in juvenile *N. celidotus* (Chapter 2).

2.) Characterisation of the expression profiles of three key immune genes, including two cytokine mediators of tissue restructure and maintenance (*il-1β*, *tnf-α)* and a macrophage marker (*cd68*) across the different stages of sex change in these juvenile individuals (Chapter 3).

Collectively, these objectives seek to explore a hypothetical role for immune gene expression as a complementary molecular indicator of gonadal transition alongside established reproductive and endocrine markers.

1.7 General Methods

The tissue samples used in the present study were obtained from legacy work, and the capture and processing are described in detail in Hamer (2023) and Hamer et al. (2025). In brief; fish were collected from shallow coastal waters (<6m depth) in the Tauranga Harbour (37.640411 °S, 176.181424 °E) during May and June 2022 by previous research students, using a Sea Harvester collapsible bait cage (25 x 25 x 45 cm). Individuals ranging from 50 to 150mm in total length (TL) were targeted, regardless of sex or phenotype. A total of 59 specimens were captured and immediately placed in aerated 20 L buckets containing seawater for transport to the Toi Ohomai Aquaculture Laboratory.

Upon arrival, fish were transferred to black polyurethane recirculating seawater tanks (1600 L capacity). Water quality parameters were monitored daily, and fish were fed fresh greenshell mussels (*Perna canaliculus*) three times per week until sampling.

For sampling, fish were deeply sedated in an aerated 10 L seawater bath containing 2-phenoxyethanol (0.6 mL L^{-1}) until loss of equilibrium and eye movement was observed. Each fish was then weighed (g), measured for total length (mm), and photographed externally. Euthanasia was performed by rapid decapitation using a sharp knife.

A ventral incision was made to expose the abdominal cavity, and the gonads were carefully excised and weighed where tissue size permitted. Samples that were too small or indistinct were recorded accordingly. Gonads were placed in 1.5 mL microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at -80 °C until further analysis.

Chapter 2 – Histology

2.1 Introduction

Protogynous sex change in teleost fish requires extensive remodelling of the gonad structure, involving the degeneration of ovarian tissue and the emergence of functional testicular structures within a single organ (Gemmell et al., 2019; Hamer, 2023). Histological studies consistently demonstrate that this transformation is characterised by progressive oocyte atresia, apoptosis of ovarian germ cells, expansion of interstitial tissue, and the proliferation of spermatogenic cells. These processes occur in a coordinated yet asynchronous manner, such that ovarian regression and testicular differentiation often overlap temporally rather than proceeding as discrete phases.

Histology provides a critical tool for investigating these changes, as it enables direct visualisation of gonadal architecture, cellular composition, and tissue-level organisation that cannot be inferred from molecular approaches alone. Accurate histological staging is particularly important in sex-changing species, where individuals may occupy transitional states for extended periods and exhibit substantial inter-individual variability (Gemmell et al., 2019). Establishing clear morphological criteria for female, transitional, and male gonads, therefore, underpins meaningful interpretation of both cellular and molecular data.

In addition to changes within the germinal compartment, gonadal remodelling during sex change is accompanied by marked restructuring of the interstitial compartment, which contains connective tissue, vasculature, steroidogenic cells, and resident immune cells (Garcia-Ayala & Chaves-Pozo, 2009). Teleost gonads are now recognised as immunologically active tissues rather than strictly immune–privileged organs, with leukocytes forming consistent components of the gonadal microenvironment (Garcia-Ayala & Chaves-Pozo, 2009; Magnadottir, 2010).

Histological observations in sex-changing wrasses indicate that leukocytes are frequently associated with regions of oocyte degeneration, stromal expansion, and emerging testicular tissue (Gemmell et al., 2019; Nakamura et al., 1989). These patterns suggest that immune cells may contribute actively to debris clearance, extracellular matrix remodelling, and the maintenance of tissue integrity during gonadal transformation. However, the timing and extent of immune cell involvement across distinct stages of sex change remain poorly resolved.

2.1.2 Immune Cell Involvement in Gonadal Remodelling During Sex Change

Gonadal remodelling in teleost fish is a tightly regulated biological process requiring coordinated interactions between endocrine, cellular, and immune mechanisms to maintain tissue integrity during periods of growth, regression, and transformation. In teleosts, immune cells are not confined to classical immune organs but are routinely present in peripheral tissues, including the gonads, where they contribute to tissue maintenance and remodelling (Garcia-Ayala & Chaves-Pozo, 2009; Magnadottir, 2010; Uribe et al., 2011).

Histological studies across multiple teleost species have demonstrated that leukocytes, particularly macrophages and granulocytes, are consistent residents of gonadal tissue and that their abundance and spatial distribution vary across reproductive stages (Garcia-Ayala & Chaves-Pozo, 2009). These cells are predominantly localised between the spaces of cells (interstitial compartments), which also contain Leydig cells, connective tissue, blood vessels, and structural cells such as fibroblasts, that contribute to extracellular matrix formation (Garcia-Ayala & Chaves-Pozo, 2009; Uribe et al., 2011; Valero et al., 2015). Notably, this compartment undergoes pronounced expansion during periods of gonadal regression and restructuring, suggesting a close association between immune cell activity and tissue remodelling (Garcia-Ayala & Chaves-Pozo, 2009; Valero et al., 2015).

The innate immune system dominates immune activity within the teleost gonad, with phagocytic leukocytes responding to internal danger signals generated by apoptosis, tissue degeneration, and cellular turnover (Magnadottir, 2010). These signals activate local immune responses that facilitate the clearance of apoptotic germ cells and extracellular debris while supporting tissue reorganisation. Importantly, such immune responses are tightly regulated and do not resemble classical inflammatory pathology, reinforcing the concept that immune involvement in the gonad is functional rather than detrimental (Garcia-Ayala & Chaves-Pozo, 2009).

Among the granulocytic immune cell populations described in teleost gonads, eosinophilic granular cells (EGCs) represent a particularly prominent and histologically recognisable type of immune cell. EGCs, also referred to as mast-like cells, are a distinct population of inflammatory cells commonly observed within fish connective tissues, including the gonad (Reite, 1998; Reite & Evensen, 2006). These cells are characterised histologically by their large size, round to oval morphology, and cytoplasm densely packed with coarse eosinophilic granules that are easily

visualised in routine haematoxylin and eosin (H&E) staining (Reite, 1998). Functionally, EGCs are associated with sites of tissue turnover and restructuring, where they are thought to contribute to local inflammatory signalling, modulation of vascular permeability, and interactions with other immune cell types (Reite, 1998; Reite & Evensen, 2006). Given their role in inflammation and tissue remodelling as well as the relative ease of their visualisation under H&E staining, EGCs were selected as a key histological indicator of immune system activity in the sex changing gonad.

During female-to-male sex change, ovarian regression produces large quantities of apoptotic oocytes and disrupted tissue architecture, creating conditions under which immune-mediated clearance and remodelling are essential. Histological observations in wrasse species undergoing sex change consistently report leukocytes in close association with degenerating ovarian tissue, expanding stromal regions, and emerging spermatogenic structures (Gemmell et al., 2019; Nakamura et al., 1989). These spatial patterns support the interpretation that immune cells actively contribute to gonadal transformation rather than representing incidental infiltration.

Beyond cellular clearance, immune cells participate in gonadal remodelling through cytokine-mediated signalling. Cytokines act primarily via autocrine and paracrine pathways to regulate apoptosis, cell proliferation, and extracellular matrix remodelling within the gonad (Garcia-Ayala & Chaves-Pozo, 2009; Lutton & Callard, 2006). In teleosts, cytokines are produced not only by infiltrating leukocytes but also by somatic support cells, including Sertoli and Leydig cells, highlighting the integrated nature of immune and reproductive signalling networks (Garcia-Ayala & Chaves Pozo, 2009).

From an evolutionary perspective, the involvement of immune pathways in reproductive tissue remodelling reflects a conserved strategy for balancing tissue protection, regeneration, and reproductive success (Lutton & Callard, 2006). In the context of sex change, controlled immune activation may facilitate efficient ovarian regression while supporting the differentiation and stabilisation of newly forming testicular biology; the timing, regulation, and functional consequences of immune cell activity during sex change remain incompletely understood (Gemmell et al., 2019; Muncaster et al., 2023).

2.2 Aims and Objectives

Building on this hypothesis that immune cells are not merely passive responders to tissue damage but active contributors to the coordinated tissue restructuring required for successful sex change, this chapter aims to characterise the dynamic changes in key immune cell presence, distribution, and abundance across female, transitional, and male gonads in the New Zealand spotty wrasse (*N. celidotus*). Light microscopy of haematoxylin and eosin-stained gonad sections is used to analyse the structural remodelling associated with sex change in conjunction with the localisation and abundance of leukocytes. These structural observations provide a morphological context for the molecular analysis of key immune cell markers examined in Chapter 3, which collectively build a multilayered insight of the role of the immune system in vertebrate sex change.

Such immune-endocrine interactions are consistent with histological observations from wrasse species undergoing sex change, which document extensive gonadal remodelling characterised by oocyte atresia, connective tissue proliferation, and the progressive development of testicular structures. These processes generate environments rich in apoptotic debris and regenerative signals, conditions under which immune cells would be expected to play active roles in clearing degenerating cells, modulating inflammatory responses, and facilitating tissue renewal within the expanding interstitial compartment. The coordinated involvement of innate and adaptive leukocytes therefore provides a plausible mechanistic link between endocrine restructuring and the cellular processes necessary for transforming ovarian tissue into functional testicular tissue.

2.3 Methods

Gonadal tissues from juvenile *N. celidotus* were originally collected and processed as part of a previous study, as described in the general methods (Chapter 1.7). Briefly, during dissection, the gonad lobes were removed from the fused posterior region of the reproductive organ (Hamer, 2023). One entire gonad lobe, along with the fused posterior tissue, was fixed in Bouin's solution for 24 hours and subsequently transferred to 70% ethanol for storage before histological preparation (Hamer, 2023). Fixed samples were processed at the University of Otago Histology Laboratory, where tissues underwent standard serial dehydration, clearing, and paraffin infiltration before being embedded for sectioning. Paraffin blocks were cut at 3-4 µm thickness and stained with hematoxylin and eosin (H&E) (Hamer, 2023).

Histological classification of the sexual stage

In addition to the 59 histological samples produced during the original study, a further 83 archived slides (collected 2019-2021) were incorporated to increase sample size and strengthen development stage comparisons (Hamer, 2023). These supplementary slides were derived from fish collected during the same seasonal period (May-June) and at the same location in Tauranga Harbour. There was no remaining tissue from these samples for further molecular analysis. All available slides were re-evaluated for histological evidence of immune cell activity that may be evident with H&E staining.

Individuals were assigned to one of six gonadal categories: juvenile female (JF; <110 mm TL), adult female (AF; >110 mm TL), early transitional (ET), mid-transitional (MT), late transitional (LT), and initial phase male (IP) **(Table 2.1)**. This classification followed previously established criteria (Bhandari et al., 2003; Goikoetxea et al., 2021; Muncaster et al., 2013; Muncaster et al., 2023; Hamer et al., 2025). Juvenile and adult females were distinguished primarily on size, as protogynous sex change in spotty wrasse is size-dependent rather than age-dependent, and not all females reach maturity in the same year (Gemmell et al., 2019; Jones, 1980). Individuals <100 mm TL exhibiting only previtellogenic oocytes were classified as juveniles due to sampling occurring outside the breeding season, which makes mature resting ovaries indistinguishable from immature tissue.

Sexually mature females were identified by the presence of cortical alveoli or more advanced oocyte stages. Male and transitional stages were defined by proliferating type A and B spermatogonia, more developed germ cells, or specific transitional features. Early transitional individuals, in particular, were distinguished by oocytes and the presence of nests of gonial cells, stromal cell accumulation, and cellular debris (Bhandari et al., 2003; Muncaster et al., 2013).

Table 2.1: Stages of gonadal sex change in New Zealand spotty wrasse as histology samples.

Stage	Description
Adult Female (AF) (>110mm)	Gonads contain either vitellogenic/mature oocytes or pre-vitellogenic oocytes, depending on the season. No evidence of testicular tissue or male structures.
Juvenile Female (JF) (<100mm)	Contains only early-stage (pre-vitellogenic) eggs. No developing or mature eggs present.
Early Transitional (ET)	Many eggs are breaking down (atresia). Gonial cells, granulocytes, and tissue debris (sometimes seen as yellow-brown bodies) are visible. No male tissue yet.
Mid-Transitional (MT)	Few eggs remain, and most are atretic. Early sperm cells (spermatogonia and spermatocytes) appear alongside stromal and granulocytes. Both egg and sperm cells may be present in similar amounts.
Late Transitional (LT)	Sperm cysts now dominate and begin forming lobule structures. Some stromal cells and a few degenerating eggs may still be seen.
Initial Phase Male (IPM)	Testes contain sperm and organised sperm cysts/lobules. Occasionally, a few remaining eggs are visible.

Histological analysis of gonadal immune cells

These established categories of gonadal sex change were retained in the current study; however, the primary analytical lens was shifted toward assessing the presence of immune cells across sexual stages, to compare immune features during key gonadal events such as ovarian maintenance, degeneration, and testicular development. As the available histological slides were H & E-stained, the range of immune cell types that could be resolved was limited. Consequently, focus was given solely to the presence of presumptive eosinophilic granular cells (EGCs), which appear as comparatively large (at least in comparison to erythrocytes) round to elliptical cells with a purple basophilic nucleus and bright pink eosinophilic cytoplasm. These cells contrast well under H&E staining of the germ cells and other somatic cells present in the ovary or testis **(Figure 2.1)**.

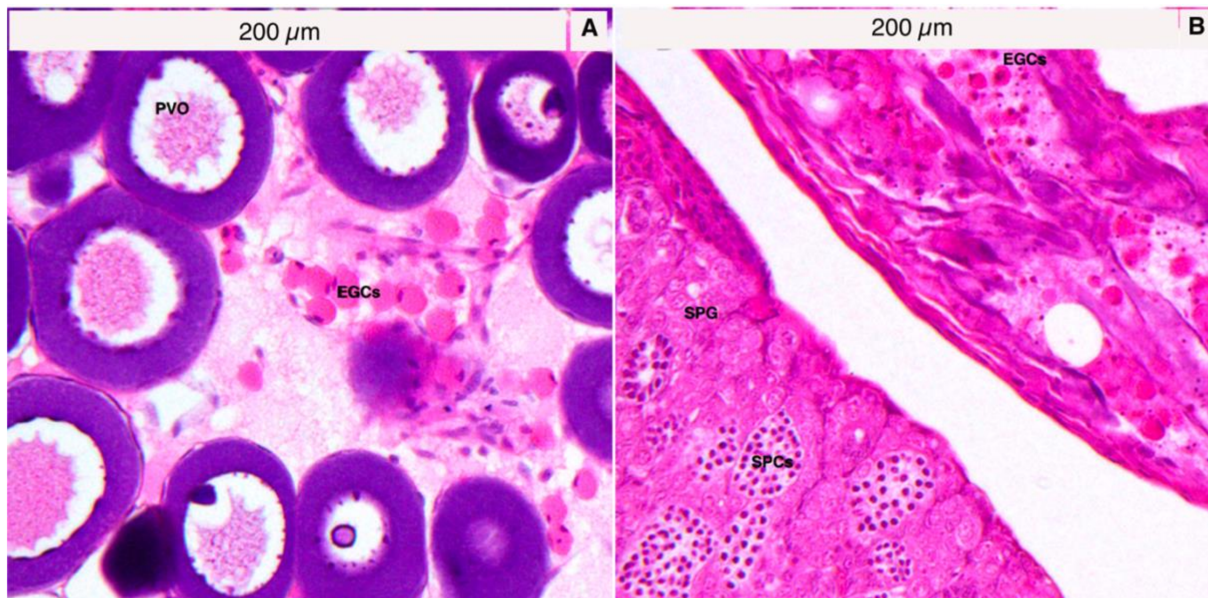

Figure 2.1: H&E-stained gonadal histology (40 x mag) demonstrating the appearance of gonadal germ cells and immune cells. **A:** ovary, **B:** testis. EGCs; eosinophilic granular cells, PVO; previtellogenic oocytes, SPC; spermatocytes, SPG; spermatogonia.

Image analysis software was used in an effort to identify, describe and quantify EGCs with the overarching aim of standardising histological interpretation and optimising efficiency. A total of five images (40 x magnification) were captured in separate, random areas of a single histological section for a total of 24 fish. These images were first adjusted for white balance (Corel Photopaint) and then uploaded into QuPath for training.

Twenty images were randomly selected across all five sex-change stages to serve as training images, ensuring representation across the full progression of gonadal transformation **(Figure 2.2 A)**. These images were imported into a single QuPath project and designated as the training dataset for EGC detection. Image type was set to *Brightfield H&E*, with the measurement scale calibrated to 0.1726 µm per pixel. These settings were applied uniformly to all training images using the script editor function (setPixelSizeMicrons (0.172600, 0.172600)).

Cell detection was performed using nucleus-based detection parameters, restricting identification to cells with a clearly defined nucleus. For each training image, regions of interest (ROIs) were manually annotated, and cells were classified as either EGCs or non-EGCs based on established histological criteria. Following manual annotation, the training images were used to train a classifier within QuPath **(Figure 2.2 B)**.

The trained classifier was subsequently applied to the remaining 100 images to identify and quantify EGCs automatically. Full-image annotations were generated using the 'Create Full

Image Annotation' function, after which the 'Cell Detection' tool was applied using the predefined parameters. Detection outputs and measurement data were recorded for all analysed images.

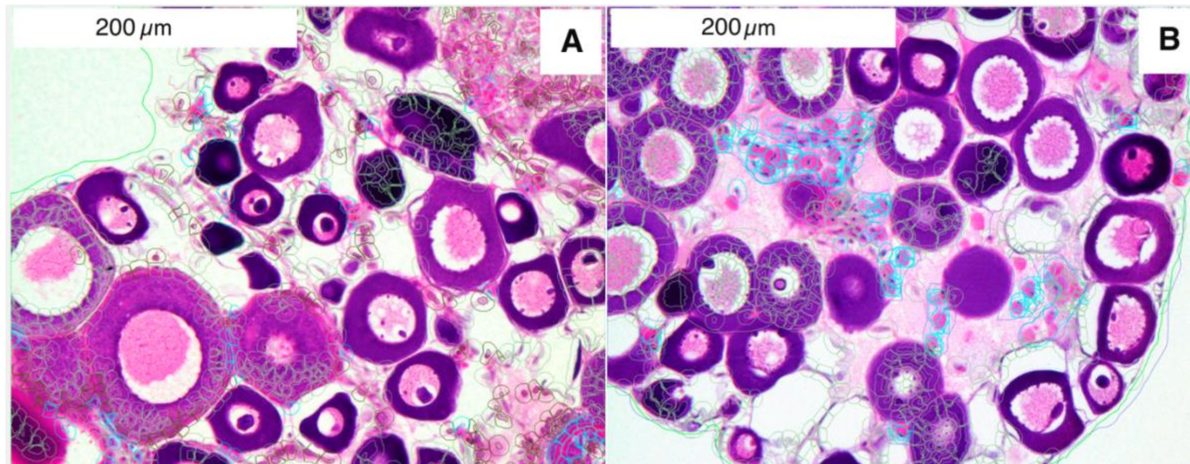

Figure 2.2: A: QuPath image output from the result of cell detection and classification, used to teach QuPath to classify different cellular components/types. B: Actual image used to train QuPath in identifying the characteristics of EGCs and differentiating from non-cellular types. This image shows the manual annotation to teach different cell types for the QuPath classifier.

Legend: cyan= EGCs; green = other egg cells.

2.4 Results

Histological ultrastructure changes across sexual stages

Structurally, gonadal sex change in juvenile *N. celidotus* follows a similar progression to that of other protogynous wrasses. The initiation in early transitional (ET) fish is marked by progressive ovarian follicle atresia and oocyte degeneration, followed by the proliferation of gonial cells, particularly within the peripheral ovarian lamellae (Goikoetxea et al., 2021; Hamer et al., 2025). Mid transitional stages were characterised by extensive tissue remodelling with the presence of stromal cells, cellular debris from degenerating oocytes and the first signs of spermatogenic germ cells that were already undergoing meiosis. The gonadal compartment was dominated by spermatogenic germ cells arranged into a typical testicular lobular structure in both LT and IPM stage fish. These samples frequently contained a few remnant atretic oocytes or cellular debris.

Immune cell presence across sexual stages

Presumptive EGCs were observed in the gonadal tissue of fish from all sexual stages, although not necessarily in all individuals. The model trained in QuPath detected a total of 2774 cells that it classified as EGCs. This indicated a reasonable sensitivity toward EGCs, successfully detecting nearly 80% of the EGCs present in the images (based on human-verified manual counts). However, the model was simultaneously plagued with poor precision as 76% of the detected cells were false positives, and a further 21% of actual EGCs were not detected at all. Due to time constraints further attempts to improve the resolution of the algorithm through additional training were abandoned, and the manual counts of the training images were used for final analysis. After eliminating the single LT sample and one of the AF fish that showed clear signs of ovarian atresia, a significant difference in the number of EGCs between sexual stages was evident using a nested linear mixed-effects model ($F_{4, 19}$ = 3.51, p = 0.029). Further post-hoc testing (Tukey's HSD test) indicated that ET and IPM had significantly more EGCs than JF fish (p=0.018; p = 0.042, respectively). One adult female vitellogenic fish exhibiting ovarian atresia was excluded from the EGC count analysis and treated as an 'outlier', as the atretic state substantially altered EGC counts.

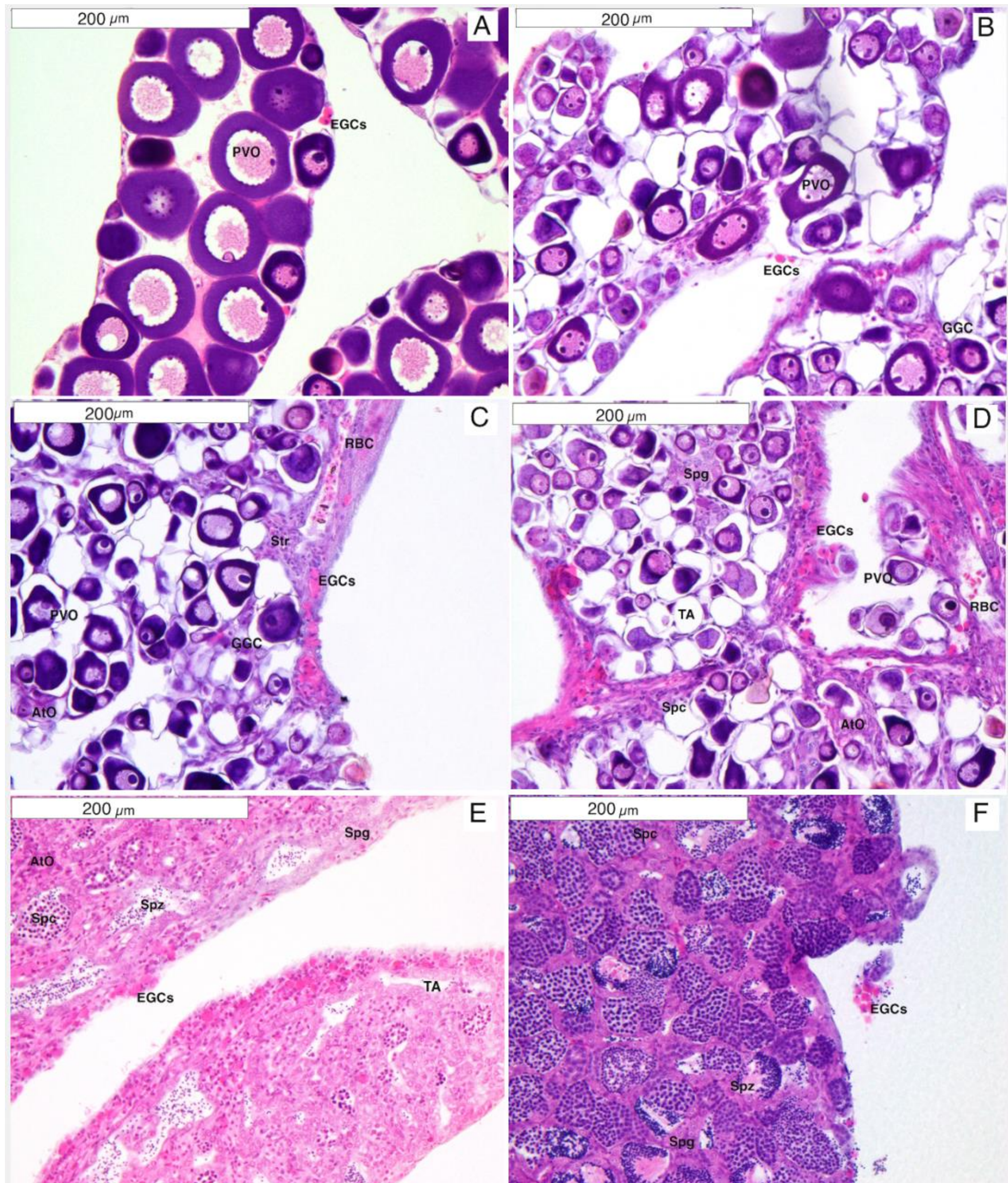

Figure 2.3: Histological progression of gonadal restructuring and immune distribution during protogynous sex change in the Zealand spotty wrasse. **A:** Adult female ovary containing pre-vitellogenic (PVO), with sparse leukocytes distributed among mature ovarian tissue. **B:** Juvenile female ovary showing developing pre-vitellogenic oocytes and gonial germ cells (GGC), with a limited and localised presence of leukocytes within the stromal compartment. **C:** Early transitional; characterised by the onset of oocyte atresia (AtO), increased leukocyte presence interspersed amongst degenerating ovarian material, and the appearance of stromal cells (StC) and red blood cells (RBC). **D:** Mid transitional; extensive oocyte degeneration accompanied by a marked increase in leukocyte abundance and the proliferation of spermatogonia (Spg) within reorganising tissue. **E:** Late transitional; developing spermatogenic cysts (Spc), localisation of leukocytes around emerging testicular structures and the tunica albuginea (TA), and minimal remaining atretic oocytes. **F:** Initial phase male; organised seminiferous tubules containing spermatozoa (Spz), with immune cells distributed within interstitial regions of the mature testicular tissue.

2.5 Discussion

Understanding how immune activity varies across the stages of protogynous sex change provides important insight into how gonadal restructuring is coordinated and whether leukocyte behaviour reflects underlying shifts in immune-gene expression. Sequential hermaphrodites such as the New Zealand spotty wrasse undergo a tightly regulated transition from a functional ovary to a functional testis, a process involving extensive ovarian degeneration, somatic remodelling, and regeneration of testicular tissue that is increasingly recognised as being shaped by crosstalk between hormonal systems (endocrine), immune, and stress-responsive pathways (Campbell et al., 2021; Chaves-Pozo et al., 2012; Chuphal et al., 2023; Gemmell et al., 2019; Klein & Flanagan, 2016; Muncaster et al., 2013; Nakamura et al., 1989; Presslauer et al., 2014). Immune cells such as macrophages, granulocytes, and cytokine-producing leukocytes (EGCs) play well-established roles in apoptosis, cellular debris clearance, and tissue remodelling in teleost fishes. Accordingly, histological analysis provides a powerful framework for assessing how leukocyte abundance, spatial distribution, and apparent activity change across stages of gonadal sex change (Aoki et al., 2008; Chaves-Pozo et al., 2012; Chuphal et al., 2023; Magnadottir, 2010; Zhu et al., 2013).

While efforts to use a trained image analysis model proved difficult, the software (QuPath) demonstrated potential for automated EGC detection. With additional time and an expanded training dataset, further optimisation may enable enhanced workflow and quantification of immune cells.

In juvenile females (JF) and adult females (AF), immune cells were generally sparse and primarily confined to the ovarian stroma or peripheral regions of the ovary **(Figure 2.3 A-B)**. This distribution is consistent with a gonad in a stable functional state, in which immune cells are present as part of normal tissue maintenance rather than being actively involved in structural reorganisation. Similar baseline immune surveillance has been described in teleost ovaries and is considered essential for maintaining tissue integrity while preserving reproductive function (Garcia-Ayala & Chaves-Pozo, 2009; Magnadottir, 2010; Nakamura et al., 1989; Uribe et al., 2011).

Importantly, even within otherwise healthy AF histology, some oocytes exhibited signs of atresia **(Figure 2.3 B; Figure 2.4)**. In these regions, leukocyte abundance increased locally, with mast-like cells appearing to infiltrate degenerating follicles and participate in the removal of apoptotic material. Ovarian atresia is a normal physiological process associated with

reproductive cycling, energetic limitation, stress, or ageing, and its immune component is well documented in teleosts (Aoki et al., 2008; Garcia-Ayala & Chaves-Pozo, 2009; Nakamura et al., 1989). The AF, illustrated in **Figure 2.4 (A)**, was therefore excluded from the EGC count analyses, as the presence of advanced atresia would confound interpretation by artificially inflating immune cell abundance.

Notably, the presence of presumptive EGCs within atretic ovarian tissue in AF demonstrates that immune involvement in gonadal tissue is not unique to sex change. Rather, it reflects a pre-existing and tightly regulated mechanism associated with normal tissue turnover and clearance processes that may be co-opted and amplified during sex-change associated ovarian regression (Aoki et al., 2008; Uribe et al., 2011). This distinction is critical, as it suggests that additional endocrine or social signals are required to shift immune function from routine atresia-related clearance to the large-scale remodelling characteristic of sex change (Gemmell et al., 2019; Muncaster et al., 2023; Warner & Sweater, 1991).

Early transitional (ET) gonads marked a pronounced shift away from baseline immune activity. Histological sections revealed widespread oocyte degeneration, structural disorganisation, and abundant cellular debris, producing a markedly mixed and disrupted tissue architecture **(Figure 2.3 C)**. Correspondingly, immune cell presence increased substantially, with presumptive EGCs frequently observed surrounding degenerating oocytes and within collapsing ovarian lamellae. This pattern suggests active engagement of the innate immune system in response to elevated apoptotic load, consistent with known roles of teleost phagocytes in recognising endogenous signalling generated by damaged or dying cells (Magnadottir, 2010; Uribe et al., 2011). The close spatial association between immune cells and degenerating oocytes supports a primary role for phagocytosis and debris clearance during the initiation of ovarian regression, consistent with observations in other teleosts where macrophages and granulocytes (eosinophilic and basophilic) are recruited to apoptotic germ cells and actively remodel gonadal tissue (Aoki et al., 2008; Garcia-Ayala & Chaves-Pozo, 2009; Nakamura et al., 1989; Valero et al., 2015). The peak in presumptive EGC presence during the ET stage may be consistent with the role of signals from the nervous and hormonal systems that regulate recruitment and activation of macrophages, granulocyte-like leukocytes, and cytokines (Gemmell et al., 2019; Nardocci et al., 2014; Tort, 2011).

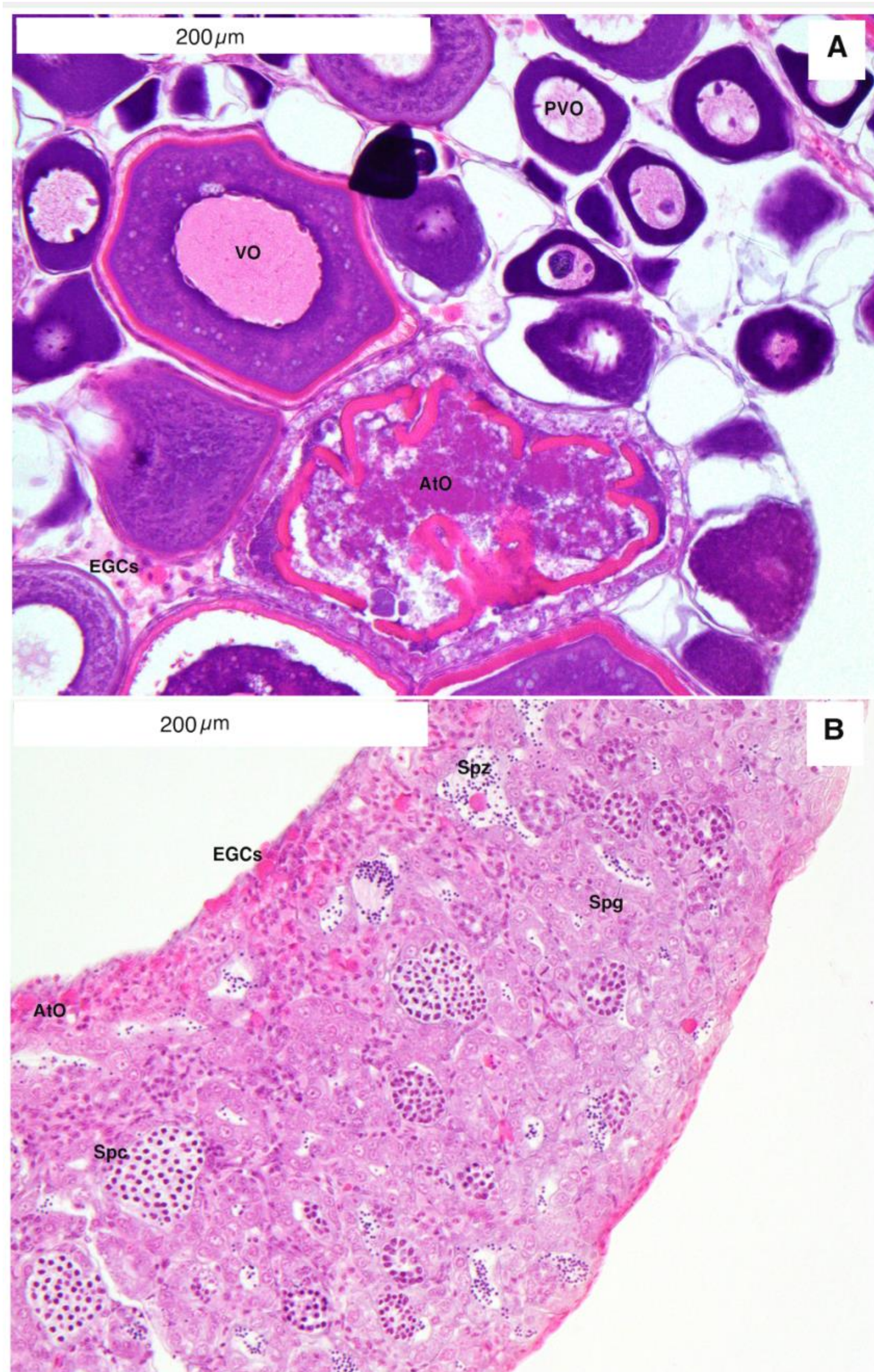

Figure 2.4: A: Adult female ovary exhibiting pronounced oocyte atresia (AtO), with increased presumptive eosinophilic granular cells (EGC) associated with regions of extensive degenerating material. B: Late Transitional; developing spermatogenic cysts (Spc), spermatogonia (Spg), and spermatozoa (Spz) with EGC localised around the periphery of the cysts and along outer cellular boundaries, as well as within small residual pockets of atretic ovarian tissue (AtO).

Goikoetxea et al. (2021) reported increased expression of androgen-associated enzymes in mid-transitional gonads in adult *N. celidotus*. These same enzymes are also central components of steroidogenic pathways involved in cortisol production, providing a potential mechanistic link between immune activity and stress-responsive endocrine signalling in teleost fish (Campbell et al., 2021; Chaves-Pozo et al., 2012; Harris & Bird, 2000). Although these enzymes were not directly measured in the current study, similar endocrine-immune interactions may operate during gonadal restructuring in the prepubertal *N. celidotus* examined here; however, this interpretation remains speculative and requires targeted investigation.

Consistent with this proposed integration of endocrine and immune signalling, mid-transitional (MT) gonads exhibited a strong qualitative level of immune-associated activity and represented the most structurally complex stage of sex change **(Figure 2.3 D)**. At this stage, extensive ovarian degeneration persisted alongside the emergence of early testicular structures, creating an environment characterised by simultaneous tissue breakdown and reorganisation. Presumptive EGCs were abundant and displayed a more structured distribution than in ET gonads, frequently clustering near degenerating ovarian remnants while also localising between and around emerging testicular cells. This spatial organisation suggests a transition from diffuse immune activation to more targeted immune involvement, potentially coordinating clearance of residual ovarian tissue with the establishment of new testicular architecture. Similar immune localisation within expanding tissue space has been described in other teleosts, including the protogynous wrasse *Thalassoma duperrey*, undergoing gonadal restructuring (Nakamura et al., 1989). In this species, immune cell accumulation during ovarian regression and testicular cord formation is indicative of a localised inflammatory response. Such inflammation promotes connective tissue remodelling and transiently increases tissue permeability, creating a microenvironment that permits the movement of ions, proteins and nutrients across cellular barriers during gonadal restructuring (Garcia-Ayala & Chaves-Pozo, 2009; Nakamura et al., 1989).

In the initial phase (IP) and terminal phase (TP) males, the spatial distribution of presumptive EGCs shifted again as testicular architecture stabilised **(Figure 2.3 E-F)**. Here, IP and TP gonads exhibited a more localised pattern of EGC distribution, with cells forming discrete clusters rather than a widespread presence. In IP gonads, small pockets of residual ovarian material persisted and were frequently associated with concentrated EGCs, suggesting ongoing localised clearance activity. In both developing and mature testes, EGCs were most commonly observed

around the periphery of testicular lobules, within interstitial spaces, and in close proximity to capillaries, consistent with previous reports of gonadal immune cell localisation in teleosts (Bjørgen & Koppang, 2022; Valero et al., 2015). This localised distribution is consistent with a shift from large-scale degenerative and remodelling processes towards maintenance and regulation, a pattern reported in several sequentially hermaphroditic teleosts, where immune cells are closely involved in ovarian atresia and early tissue remodelling, but later adopt more dispersed, interstitial positions as testicular tissue matures (Bhandari et al., 2003; Liu & De Mitcheson, 2009). Notably, the presumptive EGC abundance appeared higher in TP males than in mature AF ovaries **(Figure 2.3 F)**, suggesting that immune function within the testis differs fundamentally from that of the ovary **(Figure 2.4)**. This difference may relate to the immunological challenges associated with spermatogenesis, particularly the production of haploid germ cells during meiosis, which can be immunogenic if not carefully regulated. In vertebrates, including teleosts, the testis has traditionally been described as an immune-privileged organ, meaning that immune responses within the tissue are tightly restricted to prevent damage to developing germ cells, which may otherwise be recognised as foreign by the immune system (Garcia-Ayala & Chaves-Pozo, 2009; Klein & Flanagan, 2016). Early interpretations of immune privilege implied a relative exclusion of immune activity from the testis. However, immune privilege is now understood not as an absence of immune cells, but as a highly regulated and specialised immune environment (Dunn et al., 2024; Klein & Flanagan, 2016). Within the testis, immune cells such as macrophages and lymphocytes are present but operate under strict local control, contributing to functions including apoptotic cell clearance, tissue maintenance, and modulation of inflammatory signalling while avoiding excessive immune activation (Chaves-Pozo et al., 2018; Garcia-Ayala & Chaves-Pozo, 2009). This refined view of immune privilege highlights the importance of balanced immune activity in maintaining testicular structure and function, rather than complete immune suppression (Garcia-Ayala & Chaves-Pozo, 2009; Lutton & Callard, 2006). In teleosts, immune cells such as EGCs are strategically located within interstitial spaces and near capillaries, allowing rapid local responses while limiting tissue damage (Bjørgen & Koppang, 2022; Valero et al., 2015). Rather than being absent, immune activity is actively regulated to support spermatogenesis and tissue integrity.

In summary, the histological observations presented here highlight that both ovaries and testes maintain finely balanced immune environments, with immune cells strategically positioned to allow local surveillance while preserving tissue integrity. In the ovary, this may support controlled atresia and tissue remodelling throughout transitional stages, while in the testis, it maintains structural and functional homeostasis. Rather than reflecting complete immune suppression, immune activity appears actively regulated across gonadal types, highlighting the dynamic nature of immune privilege throughout the sex change processes

The peripheral localisation of EGCs observed in TP males may therefore reflect roles in maintaining barrier integrity, supporting germ cell turnover, or regulating local cell response (activation or cell death) during active spermatogenesis **(Figure 2.4 B).**

Collectively, these histological patterns demonstrate that leukocyte behaviour during sex change is dynamic, stage-specific, and closely aligned with the structural milestones of gonadal transformation. In stable female ovaries, immune cells seem to primarily perform sentinel surveillance activities and routine clearance associated with natural atresia (Aoki et al., 2008) **(Figure 2.3 A-B; Figure 2.4 A)**. During ET and MT stages, immune activity intensifies and becomes spatially linked to ovarian regression and tissue remodelling **(Figure 2.3 C-D)**. As testicular tissue consolidates, immune involvement becomes more localised, consistent with a transition toward regulatory and maintenance roles within the male gonad **(Figure 2.3 E-F)**. These observations suggest that immune cells function not only as responders to tissue damage but also as active participants in the coordinated tissue remodelling necessary for successful sex change.

Finally, the observed increases in EGCs presence during ET and IP sex change stages correspond with periods of heightened endocrine and stress-axis activity, suggesting that HPI-axis activation may modulate the recruitment of leukocytes within the gonad (Engelsma et al., 2022; Gemmell et al., 2019; Tort, 2011). Crosstalk between the HPI (cortisol) and HPG axes may therefore coordinate the recruitment, activation, and spatial redistribution of immune cells, providing a mechanistic link between environmental or social cues (Warner & Sweater, 1991), endocrine reorganisation, and immune-mediated tissue remodelling within the transforming gonad (Chaves-Pozo et al., 2018; Engelsma et al., 2002; Gemmell et al., 2019; Tort, 2011). Together, these findings provide strong qualitative evidence and establish a robust foundation for interpreting immune gene expression patterns examined in subsequent chapters.

Chapter 3 – Immune Gene Expression

3.1 Introduction

Gonadal transition in teleosts involves extensive ovarian degeneration followed by the establishment of functional testicular architecture. Histological analyses show that leukocytes are frequently associated with degenerating oocytes, stromal expansion, and emerging spermatogenic structures, suggesting roles in immune-mediated debris clearance and tissue reorganisation (Bravo et al., 2023). Importantly, experimental evidence demonstrates that macrophage activation can actively induce ovarian failure and masculinisation in zebrafish, indicating that immune cells can direct gonadal fate rather than simply responding to tissue degeneration (Bravo et al., 2023). Despite this, the molecular basis of immune involvement during sex change remains unresolved.

In spotty wrasse, histology revealed the consistent presence and stage-specific redistribution of EGCs throughout female-to-male sex change, supporting the hypothesis that immune cells contribute actively to the extensive tissue remodelling accompanying gonadal transformation. However, while histology provides essential insight into immune cell localisation and abundance, it cannot determine whether immune pathways are transcriptionally active or how immune-related signalling varies across sex-change stages. Quantifying immune-related gene expression, therefore, provides a necessary complementary approach for assessing whether transcriptional activation of immune pathways parallels observed cellular patterns.

Increasing evidence indicates that the teleost gonad is not strictly immune-privileged but instead functions as an immunologically active organ containing resident leukocytes that contribute to tissue homeostasis and reproductive function (Bjørgen & Koppang, 2022; Garcia-Ayala & Chaves-Pozo, 2009). Within the testis, macrophages and lymphocytes participate in steroidogenesis, germ cell development, and tightly regulated inflammatory responses, with cytokines acting as key mediators of apoptosis, cell proliferation, and tissue restructuring, processes fundamental to gonadal sex change (Garcia-Ayala & Chaves-Pozo, 2009).

Figure 3.1: Overview of the cytokine signalling network in teleost fish, illustrating interactions between innate and adaptive immune cells and their roles in regulating inflammation, cellular turnover, and tissue remodelling. Pro-inflammatory cytokines interleukin-1β (*il-1β*) and tumour necrosis factor-α (*tnf-α*) are depicted as central signalling molecules produced primarily by innate immune cells, including macrophages, monocytes, and neutrophils. Within this network, *il-1β* and *tnf-α* act as key initiators and amplifiers of local inflammatory responses, promoting immune cell recruitment, activation, and survival, as well as regulating apoptosis and tissue restructuring. These cytokines also influence crosstalk between innate and adaptive immune cells, shaping the local tissue environment during periods of active remodelling.

Macrophages, identified in this study using the marker *cd68*, occupy a central regulatory position within the cytokine network. Cd68-positive macrophages act as both sources and targets of cytokine signalling, integrating inflammatory cues with phagocytic clearance of apoptotic cells and debris. Through reciprocal signalling with other immune cell types and the production of cytokines such as Il-1β and Tnf-α, macrophages contribute to the coordination and resolution of inflammation. This localised inflammation response is essential for facilitating tissue breakdown, debris clearance, and subsequent remodelling by transiently increasing tissue permeability and activating pathways that support cellular turnover. Together, the components highlighted in this figure illustrate how cytokine-mediated immune signalling provides a mechanistic framework for linking immune cell activity with tissue degeneration, regeneration, and reorganisation, processes that are central to gonadal remodelling during sex change. Originally sourced from Zhu et al. (2013).

Immune activity within the gonad occurs in a dynamically regulated endocrine environment. Gonadal restructuring in marine teleosts is orchestrated by neuroendocrine signalling through the HPG axis, initiated by gonadotropin-releasing hormone (GnRH) and mediated by stage-specific changes in steroid hormone production (Sang et al., 2020). Sex steroids such as estradiol (E_2), testosterone (T) and 11-ketotestosterone (11-KT) regulate cell proliferation, differentiation, and apoptosis within the gonad and are also known to modulate immune responses (Garcia-Ayala & Chaves-Pozo, 2009; Sang et al., 2020; Valero et al., 2024). Shifts in steroid profiles during sex change may therefore indirectly shape immune gene expression by altering the gonadal microenvironment.

Importantly, immune cells are present within the gonad from the earliest stages of teleost development, before full sexual differentiation, indicating that immune involvement in gonadal tissue is developmentally programmed rather than solely reactive (Chaves-Pozo et al., 2009). This supports the interpretation that immune cells observed during sex change are not pathological infiltrates but normal residents whose activity may be re-engaged or amplified during gonadal restructuring **(Figure 2.2)**.

3.1.1 Genes Involved in Immune Responses During Sex Differentiation

Despite being described as immune-privileged, the teleost gonad, particularly the testis, due to the need to protect meiotic and haploid germ cells, maintains an active but tightly regulated immune environment that supports tissue maintenance and remodelling (Bjørgen & Koppang, 2022; Garcia-Ayala & Chaves-Pozo, 2009). Resident leukocytes produce cytokines and growth factors that regulate germ cell turnover, apoptosis, and stromal remodelling during reproductive cycling and differentiation (Garcia-Ayala & Chaves-Pozo, 2009; Valero et al., 2015). Accordingly, macrophage abundance and cytokine expression vary across reproductive stages and are closely associated with periods of gonadal degeneration and reorganisation, with *il-1β* and *tnf-α* showing coordinated regulation with steroidogenic activity (Valero et al., 2015). Early immune cell recruitment to the developing gonad further supports the view that immune pathways are intrinsically integrated into gonadal organisation (Chaves-Pozo et al., 2009).

Cytokines as mediators of gonadal remodelling

Cytokines regulate immune activity by activating intracellular signalling cascades that influence gene expression, cell fate, and tissue dynamics (Mokhtar et al., 2023) **(Figure 3.1)**. In teleosts,

Il-1β and Tnf-α function as proximal cytokines that initiate and amplify inflammatory signalling, including the induction of chemokines that direct leukocyte migration to sites of tissue remodelling (Secombes, Zou & Bird, 2009; Mokhtar et al., 2023). Importantly, cytokine expression within gonadal tissue is increasingly interpreted as evidence of local paracrine (short-distance) signalling associated with physiological remodelling rather than classical inflammation (Chaves-Pozo et al., 2009). During sex change, spatially and temporally restricted cytokine activity is therefore well positioned to facilitate apoptosis, debris clearance, and connective tissue reorganisation while preserving the integrity of developing testicular tissue.

Interleukin –1 beta (Il-1β)

In teleost gonads, Il-1β expression is associated with apoptosis, inflammatory signalling, and tissue remodelling during reproductive processes (Garcia-Ayala & Chaves-Pozo, 2009). It is a central mediator of innate immune responses and is synthesised as an inactive precursor, the bioactive form is induced via inflammasome activation following cellular stress or damage (Mokhtar et al., 2023; Secombes, Zou & Bird, 2009). Although predominantly produced by macrophages, Il-1β can also be expressed by other leukocytes and somatic cells. Gonadal Il-1β levels vary with reproductive stage and endocrine state, and experimental manipulation of estrogen signalling alters *il-1β* expression in a stage-specific manner in hermaphroditic species (Cabas et al., 2013; Chaves-Pozo et al., 2008; Engelsma et al., 2002). These findings indicate that Il-1β is closely integrated with steroid-dependent regulation of gonadal restructuring. Measuring *il-1β* expression provides insight into whether inflammatory signalling is temporally associated with specific stages of sex change and whether immune activity reflects active tissue remodelling rather than passive immune presence.

Tumour necrosis factor alpha (Tnf-α)

The pro-inflammatory protein Tnf-α is a multifunctional cytokine of the TNF superfamily that is expressed in teleost macrophages and T cells and plays a central role in macrophage activation, phagocytosis, and inflammatory amplification (Mokhtar et al., 2023; Secombes, Zou & Bird, 2009). In gonadal tissue, Tnf-α influences germ cell apoptosis and stromal remodelling and responds to both reproductive stage and hormonal environment (Cabas et al., 2013; Chaves-Pozo et al., 2008). Elevated *tnf-α* expression during periods of gonadal regression supports its role in immune-mediated tissue turnover (Valero et al., 2015).

Given that sex change involves large-scale ovarian breakdown and the establishment of testicular tissue, *tnf-α* is a strong candidate mediator of immune-driven degeneration and regeneration. Coordinated expressions of *tnf-α* and *il-1β* during transitional stages would support a role of cytokine signalling in helping to orchestrate controlled gonadal transformation rather than simply reactive inflammation (Cabas et al., 2012; Valero et al., 2015).

Cluster of Differentiation 68 (Cd68)

The functional protein Cd68 is a lysosomal glycoprotein widely used as a molecular marker of macrophages and monocyte-derived phagocytes. In teleost gonads, macrophages are abundant and become particularly prominent during periods of active remodelling, including seasonal regression and sex change (Chaves-Pozo et al., 2018; Valero et al., 2015). These cells localise to regions of germ cell degeneration, contributing to phagocytosis of apoptotic material and debris clearance (Bravo et al., 2023; Chaves-Pozo et al., 2008). Histological studies of protogynous wrasses consistently report increased macrophage-like cells during early and mid-transitional stages, coinciding with widespread oocyte atresia and stromal expansion (Gemmell et al., 2019; Nakamura et al., 1989). Given the plasticity of macrophages and their role in coordinating debris clearance and cytokine signalling, *cd68* expression provides a robust molecular indicator of macrophage recruitment and activity during gonadal restructuring (Belosevic et al., 2009). Quantifying *cd68* therefore, enables direct comparison between histological observations and transcriptional evidence of immune involvement during sex change.

3.2 Aims and Objectives

Immune-related gene expression reflects the cellular immune dynamics observed histologically during female-to-male gonadal sex change in *N. celidotus*. To achieve this, the expression of the pro-inflammatory cytokines (Il-1β and Tnf-α), together with the macrophage-associated marker *cd68*, was quantified across female, transitional, and male gonadal stages using quantitative PCR (qPCR). Patterns of immune gene expression were then compared with histological indicators of leukocyte presence and gonadal remodelling described in Chapter 2, to assess whether transcriptional changes align with ovarian regression and testicular differentiation. Through this integrated molecular and histological approach, this chapter

evaluates the potential involvement of macrophages and inflammatory signalling in coordinated gonadal restructuring during sex change.

3.3 Materials and Methods

Fish samples used in this study were obtained from individuals previously collected and dissected as described in the General Methods (Section 1.5). Gonadal histological slides were examined to confirm sex and reproductive stage (Results Section 2.4). Of the eight males previously identified, gonadal tissue with adequate RNA integrity for gene expression analysis was available for four initial phase (IP) males (77 – 105 mm total length, TL). To represent fully differentiated endpoints of the sex change pathway, gonadal tissue from five adult females (128-270mm TL) and thirteen juvenile females (73 – 106mm TL), none of which exhibited histological evidence of sex change, was included. Transitional stages were represented by seven early transitional individuals (52 – 95 mm TL), corresponding to the earliest detectable stages of gonadal restructuring, and five mid-transitional individuals (60 – 76 mm TL), allowing examination of gene expression changes following the initiation of sex change. Only one late transitional individual (95 mm TL) had sufficient tissue available for gene expression analysis and was therefore included. All gene expression assays and associated laboratory work were conducted by the investigator in the University of Waikato laboratories (G.02 and T.01), Durham Street, Tauranga, New Zealand.

3.3.1 Primer Design

Gene expression analyses were normalised using β-actin *(actβ2*) and glucose-6-phosphate dehydrogenase (*g6pd*), which are widely employed as reference genes in teleost qPCR studies due to their stable expression across tissues and physiological conditions (Goikoetxea et al., 2021; Zhu et al., 2013). The use of two reference genes improves the reliability and accuracy of normalisation across distinct stages of sex change.

Gene sequences for *N. celidotus* glucose-6-phosphate dehydrogenase (*g6pd*), β-actin 2 (*actbβ2*), interleukin 1-beta (*il-1β*), tumour necrosis factor alpha (*tnf-α*), and cluster of differentiation 68 *(cd68*) were identified from the available genome assembly (GCA_009762535.1, fNotCel1.pri). Based on these sequences, qPCR primers were designed following standard primer design guidelines (Bustin et al., 2009). Design criteria included: (i) a melting temperature (Tm) of 60 – 62 °C, (ii) a primer length of 20 – 25 nucleotides, (iii) a GC

content between 40 – 60%, and (iv) the inclusion of a GC clamp at the 3′ end. To ensure efficient amplification, the difference in Tm between forward and reverse primers was kept within 1 °C, and where possible, primers were designed to span exon – exon boundaries to minimise DNA amplification and acceptable amplification efficiencies. Annealing temperatures were optimised for each primer pair during this process. The final primer sets selected for gene expression analyses are listed in Table 3.1.

Table 3.1: Primer sequences used for qPCR amplification of target genes *(*cd68, tnf-α, il-1β*) and reference genes (*actb2, g6pd*)* in spotty wrasse are presented. For each gene, the mean amplification efficiency (± standard deviation) calculated across all qPCR plates is shown.

Abbreviations: *cd68*, cluster differentiation 68; *tnf-α*, tumour necrosis factor-α; *actb2*, β-actin; FW, forward; *g6pd*, glucose-6-phosphate dehydrogenase; RV, reverse; *il-1β*, interleukin-1 beta.

Gene	Primer sequence (5'-3')	Annealing temp. (°C)	Tm (°C)	Efficiency (Mean%±SD)
g6pd	FW: CGACGTCATGCAGAACCA	60	82.4	0.90 ±0.02
	RV: CAGCACCTTCACCTTTTCGT			
actb2	FW: CCCACTACCATGAAGATTAAGATCA	60	83.0	0.92± 0.01
	RV: AGTGTGTGTTTTGGGGGAGG			
cd68	FW: CCAAAGCAGAAGGAGGATGTAAG	60	81.4	0.89 ± 0.07
	RV: TCTGCAGCGCTCTTGTTG			
il-1β	FW: TCACCAAGATGCCCAAAGG	60	82.9	0.82 ± 0.33
	RV: CGCTCAAACACAACTTTCTCTTC			
tnf-α	FW: GGACACATCTGAGAATACAGATCAC	60	82.5	0.80 ± 0.19
	RV: GTTCTGTGGGATGACGATCTTG			

3.3.2 RNA extraction, quality assessment & cDNA synthesis

Total RNA used for gene expression analyses in this study was extracted from spotty wrasse gonadal tissue before downstream molecular analysis. RNA was isolated from 35 gonadal tissue samples using the Zymo Research Direct-zol™ RNA Miniprep Kit (Catalogue No. R2053),

following the manufacturer's instructions. Gonadal tissue (≤100 mg, following optimisation) was homogenised in TRIzol® reagent with a mixture of 0.1 mm and 0.5 mm silica beads using a Precellys® Evolution homogeniser. Total RNA was purified using Zymo-Spin™ columns, with an on-column DNase I treatment included to eliminate genomic DNA contamination. RNA was eluted in DNase/RNase-free water and was either used immediately for cDNA synthesis or stored at -20 °C to minimise degradation.

RNA concentration and purity were assessed using a DeNovix® DS-11 spectrophotometer (DeNovix, USA), with absorbance measured at 230, 260, and 280 nm. RNA purity was evaluated using A260/A280 and A260/A230 ratios, with an A260/A280 value of approximately 2.0 considered indicative of high-quality RNA. Only RNA samples that met acceptable purity thresholds were used for subsequent gene expression experiments.

Complementary DNA (cDNA) was synthesised from total RNA using the qScript™ XLT cDNA SuperMix kit (Quanta Biosciences, USA), which contains an engineered M-MLV reverse transcriptase, optimised concentrations of $MgCl_2$, dNTPs, recombinant RNase inhibitor, random primers, oligo(dT) primers, and stabilisers (Hamer, 2023). The volume of RNA used for cDNA synthesis was adjusted according to individual sample concentrations determined by spectrophotometric analysis, with 1 µg of total RNA used per reaction. Duplicate cDNA reactions were prepared for each RNA sample in 200 µL RNase/DNase-free PCR tubes, following the manufacturer's protocol, to a final reaction volume of 20 µL. Reaction mixtures were vortexed briefly and pulse-centrifuged before incubation in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA) under the following conditions: 25°C for 5 minutes, 42 °C for 60 minutes, 85 °C for 5 minutes, and a final hold at 4 °C. Synthesised cDNA was diluted 1:20 in molecular-grade water and stored at -20 °C until use in quantitative PCR analysis.

Gel electrophoresis

During the initial RNA extraction trials, the quality and integrity of *N. celidotus* gonadal RNA were assessed using agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving 0.25g of agarose powder (Fisher Biotec, Cat# AG100) in 25 mL of 1x Tris-acetate-EDTA (TAE) buffer. The solution was heated in a microwave until fully dissolved and visually clear. After cooling slightly, 25 µL of sodium hypochlorite (to a final concentration of 6%) and 2.5 µL of GelRed® nucleic acid stain (Sigma, USA) were added.

The agarose solution was poured into a gel mould fitted with a 12-well comb and allowed to set for approximately 20 minutes. Once polymerised, the gel was placed into a horizontal electrophoresis tank (Life Sciences, Mini Horizontal) and submerged in 1x TAE buffer. RNA samples were prepared by mixing 5 µL of RNA with 2 µL of loading buffer, and 6 µL of each mixture was loaded into the first lane as a size reference.
Electrophoresis was conducted at 90 V for 20 minutes using a PowerEase® 300 W power supply (Life Technologies). Following separation, the gel was visualised under ultraviolet illumination and imaged using the iBright™ CL750 Imaging System (Thermo Fisher Scientific, USA) to assess RNA integrity.

3.3.3 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was carried out using a 48-well Magnetic Induction Cycler (MIC) qPCR system (Bio Molecular Systems, Australia) with PerfeCTa® SYBR® Green FastMix ® (Quanta Biosciences, USA), in accordance with the manufacturer's instructions. This master mix contains a chemically modified DNA polymerase (AccuFast Taq) that is rapidly activated at 95 °C. Reaction components were combined in specified volumes **(Table 3.2)** in 200 µL RNase/DNase-free MIC PCR tubes (Dnature, New Zealand), to a final reaction volume of 20 µL.

Table 3.2: 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

All samples were analysed in duplicate, and no-template controls (NTCs) were included for each primer pair. NTC reactions contained all qPCR components except the cDNA template, which was replaced with molecular-grade RNase/DNase-free water, to detect potential

contamination. An inter-plate calibrator was also included in duplicate in each qPCR run to ensure consistency of Cq values and repeatability across assays.

Quantitative PCR amplification was performed using the following thermal cycling conditions: an initial denaturation step at 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 15 seconds and primer-specific annealing at the previously optimised temperature for 30 seconds **(Table 3.1)**. Fluorescence was measured at 60 °C at the end of each cycle. Melt curve analysis was conducted following amplification by recording fluorescence at 1 °C increments from 65 °C to 95 °C to confirm the amplification of a single, specific product.

To further verify amplification specificity, representative qPCR products were resolved on a 1% agarose gel stained with RedSafe and visualised under UV illumination using an iBright™ CL750 Imaging System (Thermo Fisher Scientific, USA) to confirm the expected amplicon size. Primer amplification efficiency for each run was calculated using the LinRegPCR algorithm (Ruijter et al., 2009). Gene expression levels of *il-1β*, *tnf-α*, and *cd68* were normalised against the reference genes *g6pd* and *actb2* (**Table 3.1**). Relative expression ratios were calculated using the geometric mean of the two housekeeping genes (Vandesompele et al., 2002) and the Pfaffl method (Pfaffl, 2001) for each tissue analysed.

Statistical Analysis

All statistical analyses followed a consistent analytical workflow. Sex stages represented by a single individual (LT; n = 1) were excluded before analysis to avoid unreliable group comparisons. Data were initially assessed using one-way analysis of variance (ANOVA), and model assumptions were evaluated using Shapiro-Wilk tests of residual normality. When normality assumptions were violated ($p < 0.05$), non-parametric Kruskal-Wallis rank-sum tests were used to assess differences among sex stages. Where appropriate, post-hoc pairwise comparisons were conducted using Dunn's tests with Bonferroni correction to control for multiple testing. In addition, targeted pairwise comparisons between female (AF, JF) and male (IPM) sex stages were evaluated using Welch's two-sample *t*-tests to accommodate unequal variances and sample sizes. This analytical approach allowed both overall group comparisons and focused evaluation of specific sex-change contrasts. As the data violated assumptions of normality and were analysed using non-parametric tests, box-and-whisker plots were generated to visualise median values and interquartile ranges **(Appendix I)**.

3.4 Results

Gel electrophoresis – RNA quality

Gel electrophoresis of qPCR products for the three immune-related genes of interest *(tnf-α*, *cd68* and *il-1β*) was used to assess amplification specificity and RNA quality. Visualisation of amplicons demonstrated that, following optimisation, each primer set consistently produced a single band of the expected size, with no amplification observed in no-template controls (NTCs), indicating no contamination of reagents or issues with primer dimerisation **(Figure 3.2)**. During initial optimisation trials, some primer sets demonstrated more than one product was visible, consistent with non-specific amplification or primer-dimer formation. To resolve this, annealing temperatures were incrementally increased, and cDNA template concentrations were systematically adjusted. Ultimately, diluting the original cDNA from 1:10 to 1:1000 proved most effective in eliminating non-specific products and reducing primer-dimer formation, resulting in robust and reproducible amplification of single target products across all genes **(Figure 3.3)**. These optimisation steps confirmed the specificity and suitability of the qPCR assays for subsequent gene expression analyses.

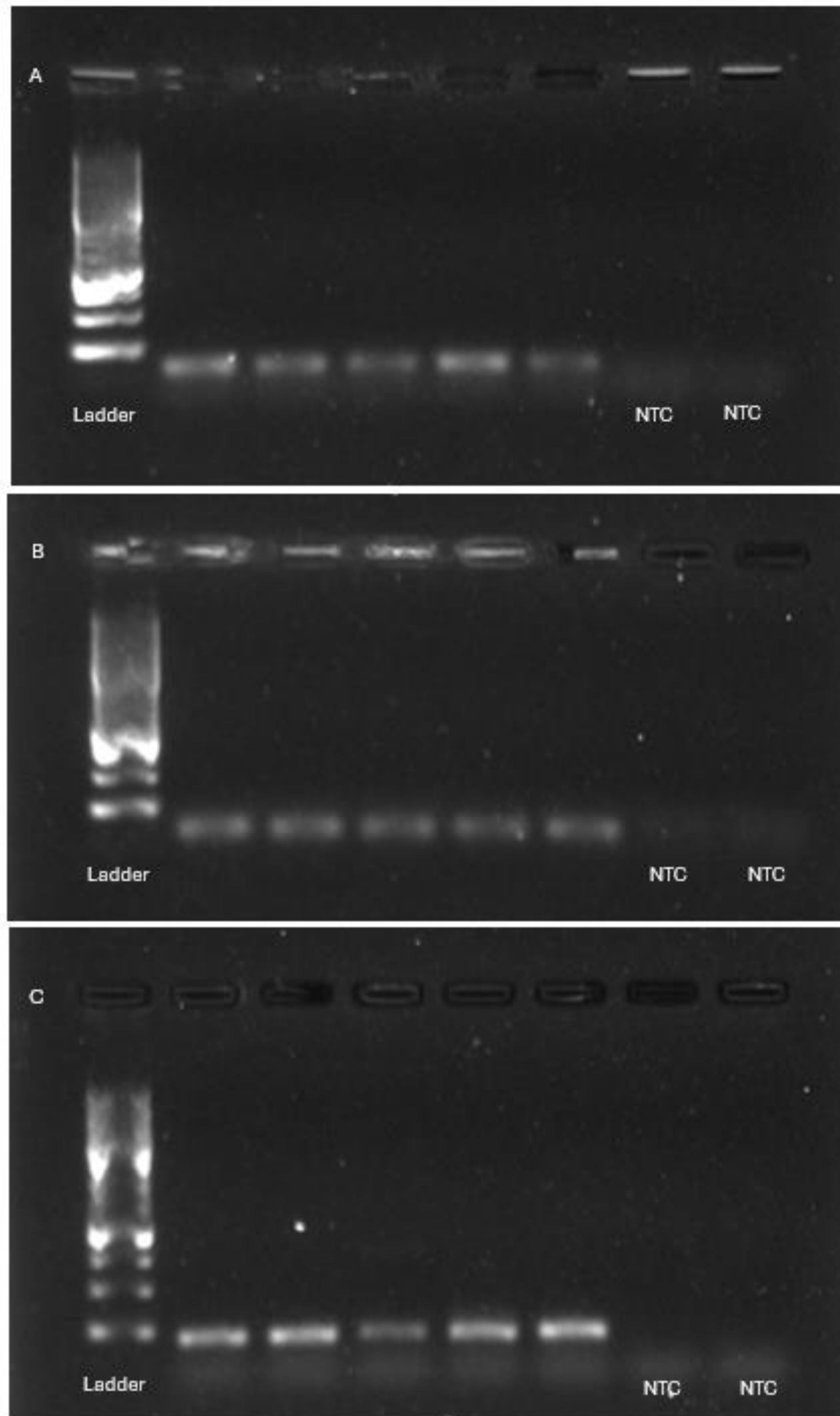

Figure 3.2: Gel electrophoresis analysis of real-time PCR products. **A:** *tnf-α* primer **B:** *cd68* primer **C:** *il-1β* primer. Each template generated a single band, with no band in the No Template Control (NTC). SolisDyne 1kb DNA ladder was added as a size reference.

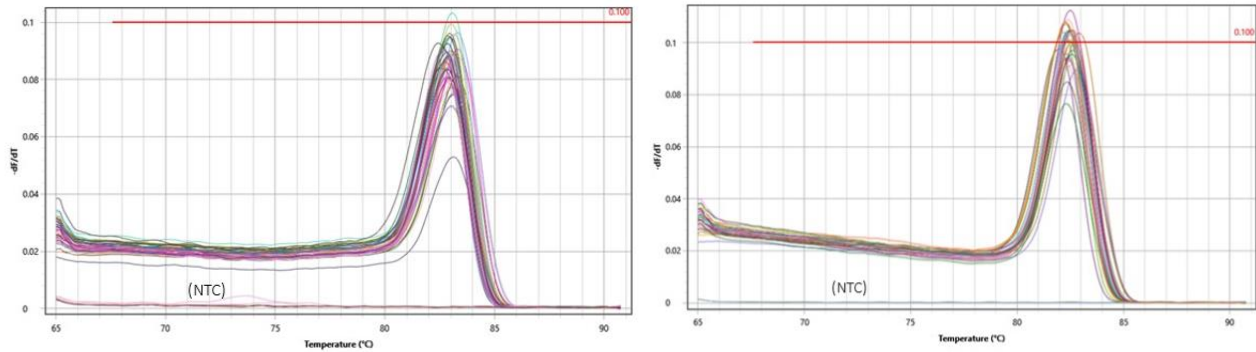

Figure 3.3: Melt curve analysis of real-time PCR products using micPCR v2.12.3. **A:** β-actin (*actb2*) primer, amplifying a single product across samples. **B:** *g6pd* primer, amplifying a single product across samples. Each template generated a single peak, with no amplification in the No Template Control (NTC).

Gene Expression

For all immune markers, the underrepresented sex change late transitional (LT) (n=1) was removed from the dataset before statistical analysis, as a single observation could not reliably contribute to group comparisons. For *cd68*, a one-way ANOVA indicated a significant effect of sex change (F = 16.48, df = 4.28, p = 4.74 x 10^{-7}). However, the Shapiro-Wilk test for residuals revealed a violation of normality (W = 0.660, p = 1.72 x 10^{-7}), indicating that parametric assumptions were not met. Consequently, a Kruskal-Wallis rank-sum test was used to compare *cd68* across sex stages, confirming a significant effect (χ^2 = 12.48, df = 4, p = 0.014). Post-hoc pairwise comparisons with a Dunn's test (Bonferroni adjustment) demonstrated significantly higher *cd68* expression in males (IPM) relative to adult females (AF) (p.adj = 0.015) and juvenile females (JF) (p.adj = 0.035), highlighting male-biased immune cell abundance.

For *il-1β*, one-way ANOVA suggested a marginal effect of sex stage (F = 3.016, df = 4.29, p = 0.0339), but residuals failed the Shapiro-Wilk normality test (W = 0.432, p = 2.41 x 10^{-10}), warranting the use of a Kruskal-Wallis test, which did not detect significant differences across stages (χ^2= 5.04, df = 4, p = 0.284).

Similarly, for *tnf-α*, ANOVA indicated a significant effect of sex change (F = 5.23, df = 4.29, p = 0.00269), but residuals again violated normality (W = 0.575, p = 9.44 x 10^{-9}). A non-parametric Kruskal-Wallis test showed no significant differences (χ^2 = 7.87, df = 4, p = 0.096).

Complementary Welch's two-sample *t*-tests were used to directly compare immune gene expression between female (AF, JF) and male (IPM) sex stages. For *cd68*, mean expression was higher in IPM than in AF (mean AF = 1.70, mean IPM = 50.96; t =-2.50, df = 2.01, p = 0.129) and JF (mean JF = 1.97, mean IPM = 50.96; t =-2.49, df = 2.00, p = 0.130). Although neither

comparison reached statistical significance, both reflected a consistent elevation of *cd68* expression in IPM relative to female stages.

Similarly, *il-1β* expression was higher in IPM compared with AF (mean AF = 0.84, mean IPM = 32.87; t =-1.20, df = 3.00, p = 0.318) and JF (mean JF = 0.89, mean IPM = 32.87; t =-1.19, df = 3.00, p = 0.318), with no statistically significant differences detected. Like *il-1β*, *tnf-α* followed the same pattern, with elevated mean expression in IPM relative to AF (mean AF = 1.42, mean IPM = 34.13; t =-1.68, df = 3.01, p = 0.191) and JF (mean JF = 2.84, mean IPM = 34.13; t =-1.61, df = 3.01, p = 0.206). Across all three immune markers, Welch's tests indicated a consistent directional pattern of higher expression in IPM compared with female stages, despite none of the pairwise comparisons meeting the conventional threshold of statistical significance ($p > 0.05$).

Graph Analysis

Relative expression of the immune-associated genes *cd68*, *il-1β*, and *tnf-α* varied across sex change stages (AF, JF, ET, MT, LT, IPM), with a consistent trend of increased expression during later transitional and male phases (**Figure 3.4 A – C**).

The expression of *cd68* showed the most pronounced variation across sex stages **(Figure 3.4 A)**. Mean expression levels were lowest in female stages (AF and JF), increased progressively through early (ET) and mid-transitional (MT) stages, and peaked sharply in males (IPM). Although one-way ANOVA initially indicated a significant effect of sex change, violation of normality assumptions necessitated non-parametric testing. The Kruskal-Wallis test confirmed a significant overall effect of sex stage on *cd68* expression. Post-hoc Dunn's tests revealed significantly higher *cd68* expression in IPM compared with both AF and JF, indicating a marked male-biased increase in this macrophage-associated marker.

In contrast, *il-1β* expression exhibited more moderate variation across sex stages **(Figure 3.4 B).** Mean expression remained relatively low and stable in AF, JF, and ET stages, followed by a gradual increase during MT and LT, and a substantial elevation in IPM. Despite this upward trend, non-parametric analysis did not detect statistically significant differences among sex stages, suggesting high within-stage variability, particularly in later transitional and male individuals.

Similarly, *tnf-α* expression followed a progressive increase across the sex change continuum. Female stages exhibited comparatively low expression, with incremental elevations during ET

and MT and markedly higher mean expression in LT and IPM **(Figure 3.4 C)**. While ANOVA suggested a significant effect of sex change, non-parametric analysis failed to detect statistically significant differences, again reflecting substantial inter-individual variation in later stages. Complementary Welch's t-tests comparing female (AF, JF) and male (IPM) stages revealed consistently higher mean expression of all three immune genes in IPM relative to females. Although none of these pairwise comparisons reached statistical significance, the directionality of these effects was consistent across *cd68*, *il-1β*, and *tnf-α*, supporting a pattern of increased immune gene expression associated with the male phase.

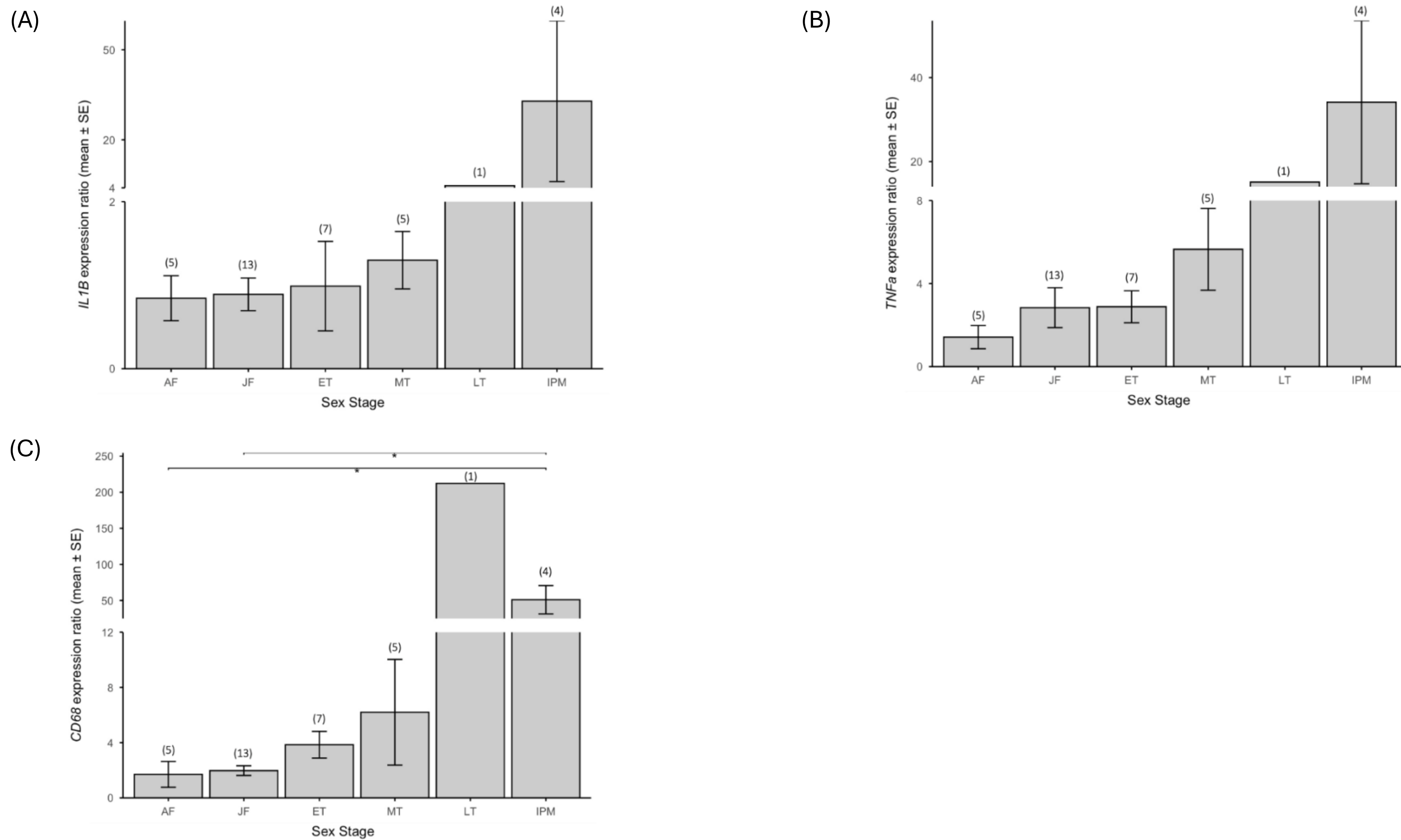

Figure 3.4: Relative expression of immune-associated genes across sex change stages. Bar plots show mean (± SE) relative expression of A: *tnf-α*, B: *il-1β*, and C: *cd68* across sex stages: adult female (AF), juvenile female (JF), early transitional (ET), mid transitional (MT), late transitional (LT), and male (IPM). Gene expression values were normalised to reference genes of interest and are presented as expression ratios. Due to underrepresentation (n=1), LT individuals were excluded from statistical analyses but are shown for descriptive purposes. Asterisks indicate significant differences identified by post-hoc Dunn's tests following Kruskal-Wallis analysis ($p < 0.05$). Note the broken y-axes used to accommodate large differences in expression among stages.

3.5 Discussion

Markers of key immune cells, including pro-inflammatory cytokines il-1β, tnf-α) and the macrophage-associated marker *cd68*, showed dynamic changes across female, transitional, and IP male gonadal stages, reflecting the cellular immune rearrangements observed histologically during sex change.

Overall, the coordinated elevation of immune gene expression observed during late transitional and male suggests that immune signalling is not merely a downstream consequence of gonadal remodelling but may actively contribute to the sex change process through regulated inflammatory and cellular pathways. In teleost fish, immune-related genes such as *cd68*, *il-1β*, and *tnf-α* are central components of innate immune function and are increasingly recognised as mediators of tissue remodelling, cell fate regulation, and endocrine-immune integration (Magnadottir, 2010; Zhu et al., 2013) **(Figure 3.1)**. Their upregulation during sex change, therefore, supports a functional role for immune processes in facilitating ovarian regression and testicular differentiation, rather than reflecting pathological inflammation alone.

The most robust transcriptional signal detected in this study was for *cd68,* a marker widely used to indicate macrophage abundance and activation **(Figure 3.4 C)**. The step-wise increase in *cd68* expression across transitional stages, together with a statistically significant elevation in males (IPM), strongly suggests increased macrophage presence or sustained macrophage activity within the gonad following sex change. In teleosts, resident macrophages are permanently present within the gonad and support tissue maintenance by clearing apoptotic cells, restructuring the extracellular matrix, and modulating the local hormone-producing environment (Garcia-Ayala & Chaves-Pozo, 2009; Chaves-Pozo et al., 2018). Given the extensive tissue degeneration that characterises ovarian regression, increased macrophage activity during late transition and IP male stages likely facilitates the clearance of apoptotic oocytes and cellular debris, while also contributing to the organisation and maintenance of newly formed testicular tissue. The persistence of elevated c*d68* expression in IP males further suggests that macrophages may play ongoing roles in testicular homeostasis rather than acting solely during acute tissue breakdown.

Macrophage activation is highly sensitive to sex steroid signalling, with estrogen and androgens known to influence macrophage polarisation, cytokine production, and metabolic state across vertebrate systems (Enright & Werstuck, 2024; Harris & Bird, 2000). In mammals, sex hormones shape macrophage phenotypes toward either pro-inflammatory or tissue-repair states,

influencing developmental and regenerative outcomes during reproductive transitions (Bird et al., 2008; Dunn et al., 2024). Comparable mechanisms are increasingly recognised in teleost fish, where leukocytes express sex steroid receptors and respond transcriptionally to endocrine cues (Chaves-Pozo et al., 2018; Liarte et al., 2011. During protogynous sex change, declining estrogen signalling coupled with increasing androgen dominance may therefore reprogramme macrophage behaviour within the gonad, shifting immune function from ovarian maintenance toward testicular reorganisation and stabilisation.

The concurrent trend of increasing *il-1β* and *tnf-α* expression observed in the gene expression analysis further supports a role for cytokine-mediated regulation during sex change, despite the absence of consistent statistical significance across stages **(Figure 3.4 A-B)**. Genes *il-1β* and *tnf-α* are key pro-inflammatory cytokines that regulate leukocyte recruitment, phagocytosis, extracellular matrix degradation, and apoptotic signalling (Magnadottir, 2010; Zhu et al., 2013). In mammalian systems, these cytokines directly influence gonadal function by suppressing steroidogenesis, modulating cell survival pathways, and shaping immune-endocrine feedback loops (Samir et al., 2017; Sinha et al., 2006). Although mechanistic data in fish remain limited, the conserved nature of cytokine signalling pathways suggests similar regulatory roles during teleost gonadal remodelling.

The lack of strong statistical support for cytokine expression differences is likely attributable to the inherently dynamic and transient nature of cytokine transcription. Cytokines are rapidly induced, tightly regulated, and often expressed in short-lived bursts in response to local stimuli (Lutton & Callard, 2006; Rauta et al., 2012). As such, their expression is highly sensitive to the timing of sampling relative to immune activation, tissue remodelling events, and individual physiological state. The underrepresentation of late transitional individuals in this study further limited the ability to detect short-lived immune activation associated with the final stages of gonadal transformation. Importantly, however, the consistent upward trends in mean *il-1β* and *tnf-α* expression across transitional and male stages **(Figure 3.4 A-B)** align with the timing of ovarian regression and testicular differentiation described histologically **(Figure 2.2)**, supporting their biological relevance despite statistical variability.

Cytokine signalling also plays a central role in coordinating the activity of other immune cell types likely involved in sex change. With respect to gene expression *il-1β* and *tnf-α* act as potent chemoattractants and activators of neutrophils and granulocytes (eosinophilic and basophilic), which are among the first responders during tissue degeneration and remodelling **(Figure 3.1)**.

In seasonal breeding teleosts, granulocyte infiltration into the testis is mediated by cytokine signalling and matrix metalloproteinase activity (MMP). MMP activity refers to the action of a group of zinc-dependent enzymes that break down and remodel the extracellular matrix, which is the structural scaffold surrounding cells in a tissue. In an immune context, immune cells such as macrophages and granulocytes (eosinophilic and basophilic) often produce MMPs, allowing them to penetrate dense tissue, clear cells that are degenerating and reshape the local environment during inflammation or regeneration, which can be linked to gonadal remodelling and leukocyte infiltration during reproductive transitions (Chaves-Pozo et al., 2008). Similar mechanisms may operate during ovarian breakdown in sex-changing wrasse, where neutrophil-mediated proteolysis, a critical immune mechanism in which activated neutrophils release specific enzymes that degrade extracellular matrix, activate cytokines and neutralise pathogens that could facilitate the dismantling of ovarian lamellae and the reorganisation of the fluid-filled, fibrous interstitial tissue that lies between cells, tissues, and organs (Mazzoni et al., 2018).

Beyond innate immune cells, cytokine-rich environments also influence monocytes, T lymphocytes, and B lymphocytes, shaping immune responses and immune tolerance within reproductive tissues (Zhu et al., 2013) **(Figure 3.1)**. Teleost gonads contain lymphocyte-like cells whose abundance varies with reproductive stage, although their specific roles remain poorly characterised (Garcia-Ayala & Chaves-Pozo, 2009; Zhu et al., 2013). In mammals, sex steroids and cytokines regulate lymphocyte trafficking and activation, contributing to sex-biased immune responses and tissue-specific immune privilege (Dunn et al., 2024; Torres-Chaves et al., 2011). The endocrine and inflammatory shifts accompanying sex change may therefore modulate adaptive immune dynamics within the gonad, potentially supporting immune tolerance during extensive tissue restructuring.

Evidence from other vertebrate systems further supports immune involvement as an active driver of sexual transformation rather than a passive response to tissue damage. In zebrafish, macrophage activation has been shown to directly induce ovarian failure and masculinisation, demonstrating that immune cells can function upstream of gonadal fate decisions (Bravo et al., 2023). More broadly, immune cells are increasingly recognised as key architects of sexually dimorphic tissue development across vertebrates, coordinating morphogenesis, cell turnover, angiogenesis, and endocrine responsiveness (Bravo, 2024).

In contrast, mammalian and invertebrate systems have revealed highly specialised and functionally diverse immune roles during developmental and reproductive transitions, demonstrating that immune activity frequently precedes and orchestrates tissue restructuring rather than merely responding to damage (Bird et al., 2008; Dunn et al., 2024). Macrophages, neutrophils, and lymphocytes are now recognised as integral regulators of apoptosis, extracellular matrix turnover, and endocrine signalling during follicular atresia, ovulation, pregnancy, and sexually dimorphic tissue development (Bird et al., 2008; Dunn et al., 2024). Invertebrate models similarly demonstrate conserved inflammatory and phagocytic mechanisms driving developmental plasticity. The relative lack of comparable mechanistic resolution in teleost fish, particularly during sex change, therefore, represents a substantial gap in current knowledge.

Collectively, the findings of this chapter partially support the hypothesis that immune gene expression reflects the cellular immune dynamics observed histologically during gonadal sex change. The significant elevation of the macrophage-associated marker *cd68* in males aligns with histological evidence of increased leukocyte presence and interstitial expansion during later stages of transition, indicating sustained macrophage involvement in gonadal remodelling. Although *il-1β* and *tnf-α* did not show statistically significant stage-specific differences, their consistent upward expression increased across sex-change to mirror the timing of ovarian regression and testicular differentiation. Together, the molecular and histological data converge on a model in which immune pathways form integral components of gonadal plasticity in sex-changing fish.

Chapter 4 – General Discussion

A defining feature of protogynous sex change is the degeneration of ovarian tissue through follicular atresia and germ cell apoptosis (Chapter 2). Extensive work in teleost fish has demonstrated that ovarian atresia is accompanied by transcriptional activation of genes associated with apoptosis, inflammation, and tissue remodelling (Gonzalez-Kother et al., 2020; Yamamoto et al., 2011; Yamamoto et al., 2016). These processes generate cellular debris and molecular signals that activate innate immune pathways, promoting macrophage recruitment and cytokine production within the gonad (Magnadottir, 2010). Reviews of follicular atresia consistently identify immune-related gene activity as a core feature of ovarian regression, operating alongside changes in local sex steroid signalling, particularly declining estrogen production, to facilitate controlled tissue breakdown and nutrient recycling (Corriero et al., 2021; Gonzalez-Kother et al., 2020). Importantly, because atresia occurs across multiple reproductive contexts, including seasonal reproduction and stress, immune involvement appears to represent a regulated component of gonadal remodelling rather than a passive consequence of sex steroid withdrawal alone.

In the present study, immune activation was prominent during early degenerative stages of sex change, likely facilitating ovarian apoptosis and tissue remodelling, and persisted into later transitional and male phases. This dynamic pattern highlights sustained immune involvement throughout gonadal transformation, supporting both tissue breakdown and reconstruction. The upward trends in *il-1β* and *tnf-α* expression align closely with molecular signatures reported during nutritionally induced follicular atresia in salmonids, where inflammatory and apoptotic pathways are co-activated during ovarian regression (Yamamoto et al., 2011; Yamamoto et al., 2016). In the context of sex change, ovarian atresia does not represent terminal reproductive failure but instead constitutes a programmed transitional phase preceding testicular differentiation (Corriero et al., 2021; Gonzalez-Kother et al., 2020; Weber & Capel, 2018). During this period, immune activation likely facilitates efficient clearance of degenerating ovarian tissue, contributing to a permissive microenvironment for subsequent gonadal reorganisation and testicular development (Chaves-Pozo et al., 2012; Garcia-Ayala & Chaves-Pozo, 2009; Hoffmann et al., 2023).

Peak expression of *il-1β, tnf-α* and *cd68* was observed in late transitional and IP male fish. Cytokines such as Il-1β and Tnf-α are known to act upstream of apoptotic cascades and extracellular matrix remodelling and have been implicated in coordinating tissue turnover and restructuring in teleost gonads (Garcia-Ayala & Chaves-Pozo, 2009; He et al., 2022). The elevated *cd68* expression observed in IP males supports the continued presence of macrophages following sex change, consistent with roles in tissue maintenance, immune surveillance, and regulation of spermatogenic organisation. Together, these findings support the hypothesis that immune activation contributes actively to gonadal remodelling during sex change, rather than simply reflecting a passive response to cellular degeneration.

The combined involvement of Cd68 macrophages, pro-inflammatory cytokines (Il-1β and Tnf-α) and eosinophilic granular cells (EGCs) further indicates that gonadal remodelling is supported by a coordinated immune response, rather than the action of a single cell type or marker. Macrophages, identified by *cd68* expression, are key regulators of tissue homeostasis and restructuring, mediating phagocytosis and producing cytokines such as *il-1β* and *tnf-α.* In mammalian reproductive tissues, these cytokines are integral to ovulatory and post-ovulatory processes, where they contribute to stromal activation, vascular permeability, and extracellular matrix remodelling (Carlberg et al., 2000; Machelon et al., 1995). Eosinophils, the functional homologues of teleost EGCs, are increasingly recognised as active participants in inflammatory and tissue-modelling environments, capable of responding to, and in some contexts producing, cytokines such as *tnf-α* (Costa et al., 1993; Moqbel et al., 1994). Moreover, *tnf-α* has been shown to enhance eosinophil activation and effector function, promoting oxidative activity and localised tissue impact (Slungaard et al., 1990). While direct evidence for cytokine production by fish EGCs remains limited, the conserved roles of eosinophil-like cells across vertebrates support their potential contribution as secondary effector cells within the gonad (Moqbel et al., 1994; Magnadottir, 2010). Taken together, these observations support an interpretation in which macrophage-derived *il-1β* and *tnf-α* initiate and regulate inflammatory signalling during gonadal restructuring, with EGCs contributing to downstream regulation of tissue structure, vascular function, and steroidogenesis. Such immune coordination likely facilitates controlled tissue remodelling while minimising pathological inflammation.

Collectively, these results indicate that immune pathways contribute not only to gonadal remodelling during transition but also to the stabilisation of male reproductive identity

following sex change. Rather than reflecting pathological inflammation, immune activation during sex change appears to reflect a tightly regulated, hormonally responsive system that coordinates tissue degradation, cellular clearance, and reproductive reorganisation. By linking immune gene expression with histological evidence of leukocyte presence, this study provides a focused molecular framework for understanding immune-endocrine interactions during female-to-male sex change. These findings reinforce the emerging view that immune cells are active participants in extreme reproductive plasticity in teleost fish and highlight promising directions for future research into cell-specific immune mechanisms and immune-endocrine interactions during sex change (Lutton & Callard, 2006; Magnadottir, 2010; Uribe et al., 2011; Zhu et al., 2013).

4.1 Connections between immune cells, sex steroids and gonadal remodelling

Teleost testes are known to contain resident leukocyte populations, including macrophages, granulocytes, and lymphocyte-like cells, which support germ cell turnover and are closely associated with steroidogenic cells. Collectively, these cells contribute to the maintenance of tissue homeostasis (Garcia-Ayala & Chaves-Pozo, 2009; Valero et al., 2015). The coordinated elevation of immune gene expression (*il-1β, tnf-α* and *cd68*) observed during late transitional and male stages likely reflects a close coupling between immune and endocrine signalling pathways during sex change. Sex steroids are well-established modulators of immune function in teleosts, influencing macrophage activity, cytokine production, and immune gene transcription, highlighting a shared relationship between endocrine and immune systems (Harris & Bird, 2000; Lutton & Callard, 2006). In support of this, transcriptomic analyses have shown that estrogen directly modulates immune, metabolic, and structural remodelling, gene expression in teleost macrophages, supporting a role for sex steroids in shaping macrophage function beyond classical immune defence (Liarte et al., 2011).

Experimental and comparative studies across vertebrates further demonstrate that immune cells express estrogen and androgen receptors and respond directly to hormonal signalling through altered cytokine production, metabolic activity, and inflammatory state (Chaves-Pozo et al., 2012; De Leon-Nava et al., 2009; Hoffmann et al., 2023). Estrogen signalling modulates innate immune activity and inflammatory gene expression, while androgens influence macrophage behaviour and innate immune responsiveness (Sheperd et al., 2021; Vancolen et al., 2023). Within the gonad, these hormonally responsive immune cells participate in follicular

atresia, apoptotic cell clearance, tissue turnover, and structural remodelling in a reproductive stage-dependent manner (Garcia-Ayala & Chaves-Pozo, 2009). Immune-related signalling pathways within teleost gonads have also shown to interact directly with steroidogenic and developmental processes, with immune mediators expressed dynamically across reproductive stages (Lutton& Callard, 2006). In this context, the elevated immune gene expression observed in IPM *N. celidotus* testes overlaps chronologically with active spermatogenesis as evidenced in the gonadal histology. Teleost spermatogenesis is androgen-driven (Nagahama, 1994; Schulz et al., 2010), and although steroids were not quantified in the current study, the timing of spermatogenesis would support the hypothesis of androgen-associated reprogramming of immune pathways in association with reduced estrogen signalling (Weber & Capel, 2018). Together, these findings provide a mechanistic basis for the hypothesis that, during female-to-male sex change, declining estrogen signalling and increasing androgen dominance may contribute to shifts in immune cell behaviour within the gonad, even though direct causative effects were not tested in the present study.

4.2 Study limitations

Despite clear directional trends across sex-change stages, statistically significant differences were not consistently detected for all immune markers. This likely reflects a combination of biological and methodological constraints. Cytokine expression is inherently dynamic and context-dependent, responding rapidly to endocrine signalling, cellular turnover, nutritional status, and stress exposure (Magnadottir, 2010; Secombes, Zou & Bird, 2009). In addition, the relatively small and uneven sample sizes available across all sex stages, particularly during late transitional stages, reduced statistical power and increased analytical sensitivity to inter-individual variation. Importantly, studies of ovarian atresia and gonadal regression in teleosts frequently report substantial variability in immune gene expression despite identifying consistent mechanistic links between immune activation, apoptosis, and tissue remodelling (Corriero et al., 2021; Yamamoto et al., 2011).

Many immune cells observed within the gonad are derived from primary haematopoietic tissues, including the head kidney and the thymus, which supply macrophages, granulocytes, and lymphocytes to peripheral organs (Garcia-Ayala & Chaves-Pozo, 2009; Valero et al., 2015). While this study focused on gonadal immune gene expression and local histological changes, immune activation during sex change is likely part of a broader systemic response. Leukocyte

recruitment to the gonad may be shaped by circulating signals and with other immune tissues, meaning that the patterns observed here represent only a subset of the immune dynamics operating during sex change. Future studies incorporating systemic immune profiling, particularly of the head kidney and thymus, would help clarify how central and peripheral immune responses are coordinated during sex change.

An additional limitation relates to the use of wild-caught fish, which were exposed to capture, handling, and transfer into a synthetic tank environment. These factors are known to elevate stress hormones and disrupt feeding behaviour, both of which can promote follicular atresia and immune activation independently of sex change (Harris & Bird, 2000; Yamamoto et al., 2011). Consequently, some atretic features observed in adult and juvenile females may reflect stress-associated endocrine-immune responses rather than sex-change-specific processes alone. The use of laboratory-bred cohorts acclimated to stable environmental conditions would help reduce these confounding influences in future research.

Nevertheless, these limitations do not detract from the broader significance of the findings. Instead, they highlight the complexity of immune-endocrine interactions during sex change and emphasise the importance of interpreting gonadal remodelling within the context of an integrated physiological system.

4.3 Future work

The present study contributes to a growing recognition that teleost gonads are immunologically active organs in which immune cells play essential developmental and regulatory roles. Despite extensive literature describing sex change and general features of the teleost immune system, the specific roles of immune cells during sex change remain poorly resolved, often noted only as generic ‘leukocyte clusters’ in histological descriptions. By integrating histological observations with immune gene expression data in a temperate protogynous wrasse, this study addresses a key gap in our understanding of how immune processes contribute to extreme sexual plasticity. Future work would benefit from expanded transcriptomic investigations of immune-related pathways across sex-change stages, particularly during transitional phases, to better resolve the timing and functional relevance of immune activation. In addition, much of the existing models indicate a likely influence of sex steroids on immune cell function and regulation. Considering the critical and dynamic role of estrogens and androgens in gonadal sex

change, it would be highly relevant to examine the influence of these sex steroids on gonadal immune cell expression.

While H&E staining provides essential structure information, it is not tailored for the precise identification of specific cell types or protein expression patterns, such as those associated with immune cells. Immunohistochemistry (IHC) offers a powerful alternative by using antibodies to detect specific antigens directly within the tissue sections, allowing more accurate localisation and quantification of immune markers in situ (Mebratie & Dagnaw, 2024). Unlike conventional H&E stains, IHC can reveal the spatial distribution and relative abundance of defined cell populations such as macrophages and lymphocytes, thereby greatly enhancing the interpretation of immune involvement during sex change. Incorporating IHC or related immunostaining techniques in future studies, particularly the use of *cd68* as a gold-standard marker for macrophages or Perl's Prussian Blue staining to visualise phagocytic macrophages, would therefore strengthen and extend the histological observations presented here and provide a more detailed molecular and cellular framework for understanding immune contributions to gonadal remodelling (Mebratie & Dagnaw, 2024).

4.3 Conclusions

Taken together, the evidence supports a model in which immune pathways are intrinsic components of gonadal plasticity in teleost fishes, although further targeted work is required to establish causality. Rather than reflecting pathological inflammation, immune activation during sex change appears to represent a regulated, hormonally responsive process that facilitates ovarian regression, cellular clearance, and tissue remodelling necessary for testicular differentiation. The present study contributes to a growing body of evidence indicating that immune-reproductive integration is an evolutionarily conserved feature of teleost reproduction and may be critical for effective gonadal restructuring in sex-changing fishes (Lutton & Callard, 2006; Uribe et al., 2011). Collectively, these findings provide a foundation for future research aimed at resolving the specific roles of immune cell populations and immune signalling pathways as functional drivers of gonadal sex change in teleosts.

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Appendices

Appendix I – Box and Whisker Plots for Non-Parametric Analysis