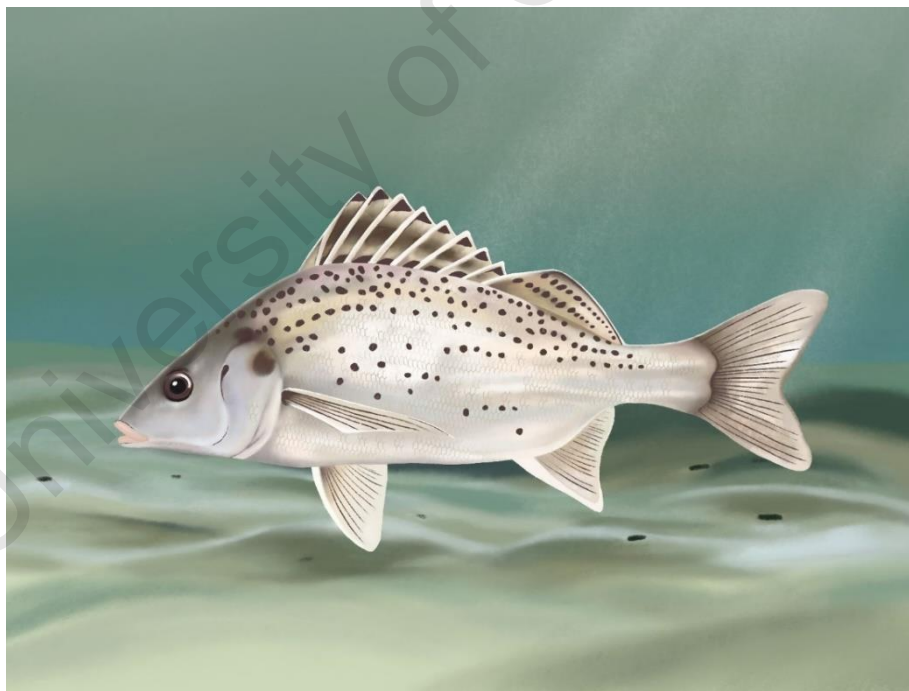


MORPHOLOGICAL, ETHOLOGICAL AND GENOMIC
DISCRIMINATION OF FUNCTIONAL STOCKS OF SPOTTED
GRUNTER, AN ESTUARINE-DEPENDENT FISH



Vivienne Nicole Coetzee

Thesis Presented for the Degree of
DOCTOR OF PHILOSOPHY
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Supervised by Associate Professor Sven Kerwath, Dr Denham Parker and Dr
Stephen Lamberth

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ABBREVIATIONS

ANOSIM	Analysis of Similarity	LFMM	Latent Factor Mixed Model
ANOVA	Analysis of Variation	LMM	Linear Mixed Model
CAP	Canonical Analysis of Principal Components	MAF	Minor Allele Frequency
CI	Confidence Interval	MS	Mean Squares
CTAB	Cetyltrimethyl ammonium bromide	mtDNA	Mitochondrial DNA
DAPC	Discriminant Analysis of Principal Components	nDNA	Nuclear DNA
ddRAD	Double Digest Restriction Site Associated DNA	Ne	Effective Population Size
DF	Degrees Of Freedom	NGS	Next Generation Sequencing
DFFE	Department of Forestry, Fisheries and the Environment	NTU	Nephelometric Turbidity Units
DNA	Deoxyribonucleic Acid	PCoA	Principal Co-Ordinate Analysis
F	Inbreeding Coefficient	PERMANOVA	Permutational Multivariate Analysis of Variance
FFT	Fast-Fourier Transform	PCA	Principal Component Analysis
FL	Fork Length	RAD-seq	Restriction Site Associated DNA Sequencing
F _{ST}	Fixation Index	SD	Standard Deviation
GBS	Genotyping By Sequencing	SE	Standard Error
GO	Gene Ontology	SIMPER	Similarity Percentages
H _E	Heterozygosity	SNFM	Sparse Nonnegative Matrix Factorization
Indels	Insertions And Deletions	SNPs	Single Nucleotide Polymorphisms
KDE	Kernel Density Estimates	SS	Sum Of Squares
LD	Linkage Disequilibrium	TL	Total Length

ABSTRACT

This thesis investigates the differences between spotted grunter *Pomadasys commersonnii* from two biogeographically distinct South African estuaries, 800 km apart, and relates these differences to the environmental properties of these estuaries, in particular turbidity. Understanding both the phenotypic and genotypic mechanisms that structure spotted grunter populations is beneficial for their effective conservation and management, especially in the advent of climate change and anthropogenic pressures.

The two study sites, the Breede Estuary and the Kei Estuary, represent low and high turbidity habitats, respectively. These properties affect the ability of fishes to use visual and acoustic modes of interaction. Moreover, recent literature has suggested the establishment of a recent divergent breeding population of spotted grunter in the Breede Estuary. Genetic and phenotypic differences, that drive population divergence, do not progress at the same rate and the divergence of these traits will occur at different timescales. This thesis provides a multifaceted approach that incorporates both phenotypic and genetic analyses of traits that can change over different timescales to identify any differences in fish originating from these two estuaries of contrasting turbidity. Consequently, four characteristics were chosen: (1) spot patterns, (2) otolith shape morphology, (3) acoustic repertoire and (4) genomic analysis.

Markings and colouration of fishes are traits associated with camouflage as well as inter and intra-specific communication. As the species name implies, spotted grunter have distinct spot patterns. It was hypothesised that the spot patterns of spotted grunter living in turbid environments would differ from those living in less-turbid environments. It was important to establish the degree of phenotypic plasticity in spot patterns prior to field studies. Thus, a controlled aquarium experiment was conducted in order to ascertain the degree to which spotted grunter individuals can alter their spot pattern in response to changes in turbidity. The experiment revealed that an individual cannot alter the number or position of their spots but can adapt their spot size and brightness in response to changes in turbidity levels. Changes to spot size and brightness were also found to be reversible. Subsequent findings from the field

revealed that spotted grunter from the less turbid Breede Estuary presented with more- and larger spots, for a given size range, compared to those living in the turbid Kei Estuary.

Acoustic communication involves the dual process of generating and receiving sounds, where the functionality of the vocal organs must be paired with an appropriate sensory counterpart. Therefore, one would expect changes in the acoustic repertoire to coincide with changes in otolith morphology. Otoliths are under dual regulation by both genetic and environmental factors. It was hypothesised that (1) spotted grunter from the two biogeographically distinct estuaries would exhibit differences in their otolith morphology and (2) there would be differences in the otolith outlines of spotted grunter captured in the Breede Estuary pre-2002 and post-2019. While each otolith is unique to some degree, the results revealed significant differences in the outlines of otoliths from spotted grunter samples, both spatially and temporally. Furthermore, these differences could be used to assign individuals to their specific populations.

As with most species of the Haemulidae family, spotted grunter produce rasping-or as the name suggests- 'grunting' sounds, by stridulation of their pharyngeal teeth, which are amplified by the swim bladder. These sounds are species specific in terms of a number of acoustic properties. With respect to turbidity, it was hypothesised that the acoustic properties of voicings or vocalisations of spotted grunter from turbid environments would differ from those in less turbid environments. The findings from the field experiment confirmed this hypothesis, albeit in a manner opposite to expectations. Individuals from the less -turbid Breede Estuary produced longer, louder sounds of a lower frequency; this is presumed to be due to differences in each estuary's ambient noise or due to spotted grunter communication behaviour whereby acoustic signals may only succeed visual signals.

The divergence of phenotypic stocks can eventually alter the genomic structure of a species. Despite differences in the phenotypic characters measured above, the genomic results suggested a single panmictic population of spotted grunter. Interestingly, a few highly differentiated loci were identified and some of these loci were associated with the measured

phenotypic characteristics. Therefore, despite evidence for high gene flow occurring between these two estuaries, the highly differentiated loci may suggest early stages of divergence.

This research marked the initial discovery that functional stocks of spotted grunter differ in aspects related to sound production, colouration and pattern. Currently, these differences are temporary, reversible phenotypic adaptations to local environments. However, if a new breeding population is established in a different biogeographic zone with unique environmental properties these differences could result in a genetically, morphologically and ethologically distinct population of spotted grunter, with distinct conservation and management requirements.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Rationale

Marine species range expansions and distribution shifts are gaining more and more attention, especially due to the rapidity at which these shifts are taking place (Pinsky, Selden and Kitchel, 2019). Over 30 estuarine-associated fish species have expanded their ranges along the South African coastline with more and more species moving into the warm/cool temperate transitions zones (Augustyn *et al.*, 2017). *Pomadasys commersonnii*, the spotted grunter, has received considerable attention. This species has entered the Western Cape region, in particular, the Breede Estuary, over the last few decades (Augustyn *et al.*, 2017). In the beginning this species was only documented to occur in the warmer summer months, but eventually reports of overwintering were confirmed. Acoustic telemetry studies demonstrated year-round residency (Kerwath *et al.*, 2005; Ziko *et al.*, 2023) and empirical evidence of spawning was published (Butler *et al.*, 2022).

How do these range shifts affect the ecology of local spotted grunter stocks? If local adaptation to environmental conditions occurs within the new range, it may be possible to delineate stocks of spotted grunter. Furthermore, environmental conditions are known to drive population divergence through local adaptation and in the absence of proper stock structure information, species will be managed as a single unit. This is problematic for species with multiple unique populations that require individual management strategies. Therefore, by identifying unique traits in spotted grunter, that may have developed due to local adaptation, these traits can be utilised and exploited as tools for stock discrimination.

1.2 Stock Concept

Stock identification is the process by which discrete units are identified within a species while stock discrimination classifies the individuals that make up each unit (Cadrin, 2000). There are many definitions for a stock, however, for this thesis, it is simply a self-producing population with its own demographics (Begg and Waldman, 1999; Lester and MacKenzie, 2009). Effective conservation for a species that straddles areas with different environmental and anthropogenic pressures necessitates the identification of stocks and the ability to confidently assign individuals to each stock. In cases where spatial and temporal overlap exists among stocks,

conservation strategies in one area may not have the same positive effect in another area (Lester and MacKenzie, 2009).

Characteristics, used for stock identification, can vary between populations and this variation can be influenced either genetically, environmentally or from a combination of the two (Swain and Foote, 1999; Cadrin, Karr and Mariani, 2014). Environmental influences can induce different phenotypes among populations; therefore, a stock can be identified as either phenotypic or genotypic. Stock identification using phenotypic characteristics can be advantageous as these reflect an individual's fitness; when a characteristic's efficiency in one environment is inversely related to its efficiency in an alternative environment (Martins, 2018). The biggest challenge with phenotypic characteristics is plasticity- the phenomenon whereby multiple phenotypes can arise from a given genotype (Dewitt and Scheiner, 2004). As selection acts on an individual's phenotype, and not its given genotype, it is the phenotype that is primarily responsible for an individual's fitness (Watkins, 2021).

Phenotypic plasticity has many definitions however, it is simply "environmentally induced variation" (Stearns, 1989). Phenotypic plasticity can be divided into two modes: developmental conversion and phenotypic modulation (Smith-Gill, 1983; Table 1.1). Developmental conversion refers to the use of environmental cues that identify the developmental 'path' that is selected from the genetic program and is considered irreversible once selected. In this case, the development is dictated by environmental cues which the organism is genetically adapted to detect. In contrast, phenotypic modulation depends on the organism's sensitivity to environmental cues and thus, responds passively to this variation by altering the degree or rate of expression of phenotypic characteristics (Smith-Gill, 1983; Cadrin, Karr and Mariani, 2014). Phenotypic modulation produces continuous phenotypic variation and is therefore reversible. Consequently, it is valuable to determine whether characteristics fall under developmental conversion or phenotypic modulation as only the former is suitable for stock identification.

In summary, a phenotypic stock is a discrete unit of individuals containing certain characteristics, which are not heritable but are expressed depending on the environment they inhabit (Swain and Foote, 1999). As such, phenotypic characters cannot be used as evidence of

genetic isolation between stocks but rather a difference in environmental regimes (Begg and Waldman, 1999).

Table 1.1: Two types of phenotypic plasticity taken from Smith-Gill, (1983)

Developmental conversion	Phenotypic modulation
Specific genetic basis	No specific genetic basis; reflects inherent general physiology of the taxon (perhaps of all cells/ species)
Specific response to a specific set of environmental stimuli	Nonspecific variation in developmental phenotype due to physiological constraints of the environment
Change in the developmental program	No change in the developmental program
Occurs at discrete developmental stages	At any time in life history; phenotypic results may be stage-specific
Produces discrete phenotypes or states	Continuous phenotypic variation
Adaptive	Not necessarily adaptive; may be nonadaptive

Contrastingly, a genetic stock is a reproductively isolated unit and the level of genetic divergence is dependent on the levels of selection, genetic drift and gene flow between populations (Cadrin, Karr and Mariani, 2014). The evolutionary clock hypothesis states that differences in proteins (genetic component) can reflect divergence time since proteins evolve at constant rates- this, however, is not true for phenotypic differences (Gorman and Kim, 1977). Stocks that lack genetic differentiation but show differences in phenotypic characteristics are interpreted to be from a recent origin. There are also cases where phenotypic similarity occurs despite high genetic differentiation which is expected to be due to environmental influences (Swain and Foote, 1999). Nevertheless, phenotypic and genetic stocks have been shown to evolve at different rates (Clayton, 1981) and different timeframes are associated with different stock definitions. Genetic stocks, in higher vertebrates, deal with processes acting over periods of millions of years to at least multiple generations (Clayton, 1981) whereas phenotypic stocks

could evolve within a cohort's lifespan (Scheiner and Holt, 2012). In conclusion, considering stock structure across different characteristics, as well as different timeframes may prove beneficial for the conservation management and resilience of a species.

1.3 *Pomadasys commersonnii*, The Spotted Grunter

Spotted grunter, *Pomadasys commersonnii*, belong to the Haemulidae family which is comprised of Plectorhinchinae (rubberlips) and Haemulinae (grunts) (Smith and Heemstra, 2012). Grunters, aptly named after the sounds they produce, are generally estuarine-dependent and feed on small invertebrates and fish (Smith and Heemstra, 2012). The largest grunter species, known as the spotted grunter (*Pomadasys commersonnii*), can attain a size of 870mm total length (TL) and weigh up to 9kg (Heemstra and Heemstra, 2004).

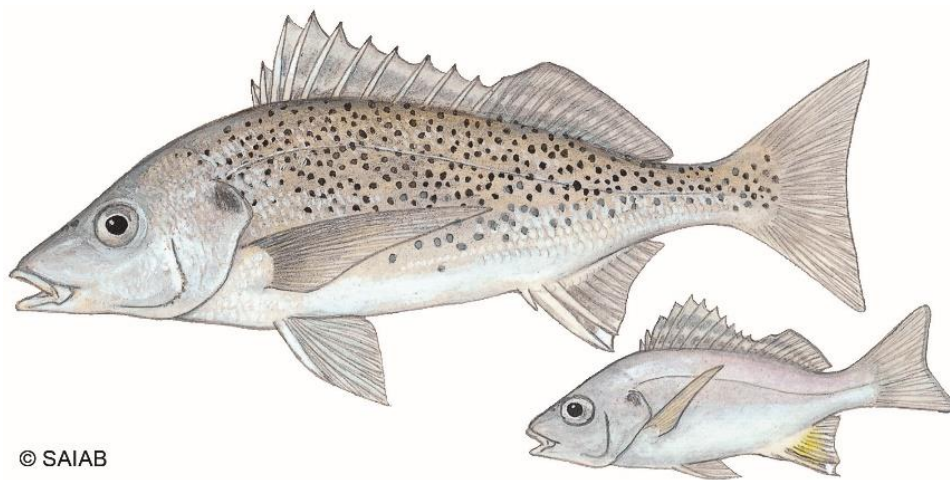


Figure 1.1. *Pomadasys Commersonnii*: Scientific drawing of *Pomadasys commersonnii*, the spotted grunter, showing both the adult and juvenile phases (Heemstra & Heemstra, 2004).

Spotted grunter are a demersal, west Indian Ocean coastal species ranging from India to False Bay in South Africa (Heemstra and Heemstra, 2004). Being eurytopic, they can be found in a wide range of environments (Whitfield, 2005). As its name suggests, adult spotted grunter can

be recognised by a mother-of-pearl sheen with distinct dark spots on the flank and dorsal surface; spots are mostly absent in 0+ juveniles (Webb, 2002; Smith and Heemstra, 2012). An unmistakable dark spot is located on the operculum in both adults and juveniles.



Figure 1.2. *Pomadasys commersonnii* distribution: World map showing the distribution of spotted grunter from India to South Africa. Image generated from AquaMaps (Kaschner *et al.*, 2019).

The spotted grunter exhibits opportunistic feeding with a wide range of prey species; this flexibility in the feeding regime is selected to promote the integrity of the population in a relatively unstable ecosystem (Webb, 2002). Despite being opportunistic feeders, they express a preferred diet at different life stages. Adult spotted grunter are crepuscular, macrobenthivores that use a powerful pump action, via the gill chamber, to force a strong jet of water into the benthic burrows of crustaceans and bivalves to force them out (Whitfield, 2019), while juveniles feed on pelagic zooplankton and microbenthic crustaceans (Webb, 2002).

The gonochoristic spotted grunter reaches sexual maturity at approximately three years of age with a total length of 300 mm in males and 360 mm in females (Childs *et al.*, 2008a). Adults often move into estuaries after spawning to regain their condition and feed on the high prey availability. The larvae develop in the nearshore coastal waters thereby avoiding the strong

Agulhas current (Connell, 2001). The juveniles recruit into estuaries where they generally remain until sexual maturity (Childs et al., 2008a).

Whitfield described five categories of fishes that utilise southern African estuaries; spotted grunter fall into category IIa: “euryhaline marine species which usually breed at sea but whose juveniles are dependent on estuaries as nursery areas” (Whitfield, 1994a). Juveniles remain mostly resident in their nursery estuary where they rapidly grow between 12- 15 mm per month during summer (Wallace, 1975; Childs et al., 2008b). The growth of spotted grunter is affected by both external factors, such as temperature and food availability, and internal factors such as energy partitioning (e.g., spawning) and stress (Webb, 2002). Due to their high residency to nursery habitats, juvenile spotted grunter can be assumed to show a level of environmental imprinting and will exhibit contrasting characteristics between estuaries that differ physico-chemically.

In addition to Whitfield’s definition above, the findings of Dames et al. (2017) indicated that spotted grunter are estuarine-associated at nearly all life stages. By monitoring adult spotted grunter movement, it was found that spotted grunter exhibit high levels of connectivity between the marine and estuarine environments (Kerwath *et al.*, 2005; Childs *et al.*, 2013). Despite being philopatric at all life stages, spotted grunter will take occasional short trips to sea or neighboring estuaries (Dames *et al.*, 2017). This connectivity increases the species' resilience when threats such as anthropogenic activities and environmental changes occur.

Following decommercialization in 1992, spotted grunter were listed as a no-sale “recreational” species (Childs *et al.*, 2007). They are an important line fish species caught by both subsistence and recreational fishers (Mann, James and Beckley, 2002; Kerwath *et al.*, 2005) and are one of the most caught bycatches by inshore prawn trawlers (Fennessy, Everett and Radebe, 2000). In all, spotted grunter comprise approximately 750 t (20%) of estuarine and 40 t (1%) of shore-based line and net fisher catches annually (Lamberth and Turpie, 2003; Van Niekerk *et al.*, 2019). They are considered vulnerable, overexploited and are currently on the SASSI no-sale red list (Van Niekerk *et al.*, 2019). An assessment conducted in 2015 indicates that the current fishing mortality rate is unsustainable with high probabilities of overexploitation (95.2%) and stock

collapse (25%; Winker, Attwood and Kerwath, 2015). Their overexploitation is directly linked to their previous commercial value, their estuarine dependence, their importance to small-scale subsistence fishers and their high value to recreational fishers for their appealing taste and texture (Mann, James and Beckley, 2002).

Currently, the spotted grunter are treated as a single stock (Childs *et al.*, 2007) and until recently, were only documented to spawn in the coastal waters of KwaZulu-Natal between August and December (Childs *et al.*, 2008a; Butler *et al.*, 2022). Klopper (2005) found a lack of genetic differentiation in spotted grunter samples along their South African distribution contributing to the idea of a single panmictic stock in South Africa. However, ripe-running (spawning) spotted grunter were first documented in the Breede Estuary in the late 1990s and subsequently also verified to be spawning in the Knysna Estuary in 2022, both estuaries being outside KwaZulu Natal on the Cape South Coast (Augustyn *et al.*, 2017; Butler *et al.*, 2022). Furthermore, recent telemetry studies found no evidence of Western Cape tagged spotted grunter migrating to KwaZulu-Natal waters (Ziko, 2021). It is thus hypothesised that the Breede Estuary in the Western Cape is home to a divergent population of spotted grunter, in an estuary where they were once nearly absent (Augustyn *et al.*, 2017). Resident contingents are important to identify as they may require unique management strategies. Due to their philopatry and the recently confirmed high residency of spotted grunter, it is hypothesised that there will be lower levels of mixing, compared to historical levels, between spotted grunter that have recruited into estuaries from these two different biogeographic regions. This disconnect between biogeographic regions could eventually result in population divergence and this phenomenon requires further investigation. It is therefore paramount to explore the hypothesis that the Breede Estuary is home to a recently established, resident stock of spotted grunter.

1.4 South African Coastline

South Africa has a long, predominantly linear and diverse coastline that is shaped by two dominant currents: The cold Benguela current on the west coast and the warm Agulhas current on the east coast. The cold-water Benguela current is a powerful, northward Atlantic upwelling system characterised by high primary productivity (Griffiths *et al.*, 2010). Moving southwards along the east coast is the powerful, nutrient-poor Agulhas current which retroflects along the Agulhas bank (Branch and Branch, 2018). At the southern tip of Africa, these two major currents form a convergence zone. As a result, the South African coastline forms a temperature gradient from the west to east coast creating four bioregions along its distribution (tropical, subtropical, warm-temperate and cool-temperate; Teske *et al.*, 2011). This temperature gradient coincides with levels of biodiversity, with species richness increasing as you move from the west coast to the east coast (Branch and Branch, 2018).

1.4.1 South African Estuaries

The South African coastline is a high-energy environment that lacks sheltered nursery habitats for species that occupy the nearshore marine environment; consequently, estuaries provide critical nursery grounds for many marine species including fish, crustaceans and mollusks (Baird, Marais and Daniel, 1996; Dames *et al.*, 2017; Van Niekerk *et al.*, 2019). These highly productive environments deliver multiple ecosystem services including valuable feeding grounds for both juvenile and adult marine species (Baird, Marais and Daniel, 1996).

Despite the importance of the estuarine ecosystem, they experience high fishing pressure from both subsistence and recreational fishers. Only 8% of the 290 South African estuaries are protected from fishing and the remaining estuaries receive high fishing pressure with an annual yield of 3 730 tonnes, with 58% of the catch coming from illegal fishing, mostly gillnetting (Whitfield *et al.*, 2020; van Niekerk *et al.*, 2022a). In all, 21% of the systems are severely impacted by overfishing (van Niekerk *et al.*, 2022a). There are approximately 80 different fish species captured in South African estuaries, with spotted grunter providing 20% of the catch: 400 – 760 tonnes per annum (Lamberth and Turpie, 2003; van Niekerk *et al.*, 2022a). Over the

past 20 years, there has been a decline in the CPUE of spotted grunter whilst at least 14 other estuarine-associated species are also considered over-exploited (Mann and Pradervand, 2007; Whitfield, 2019). Many of these fish species, such as spotted grunter, are caught in both estuarine and coastal environments, in two or more of the four bioregions, but are managed as a single stock. This has wide implications for the effective management and utilization of these species (Childs *et al.*, 2007; Whitfield, 2019).

The estuaries on either side of South Africa's coastline differ in several characteristics. On the west coast, the cool temperate estuaries receive mostly winter rainfall and due to the relative isolation of these estuaries, around 53% of their species are endemic to Southern Africa (Lamberth, Van Niekerk and Hutchings, 2008). Contrastingly, the warm-temperate estuaries of South Africa receive either summer or biannual rainfall and have a higher ichthyofaunal diversity, supplemented by tropical and subtropical species with around 35% being endemic to Southern Africa (Augustyn *et al.*, 2017).

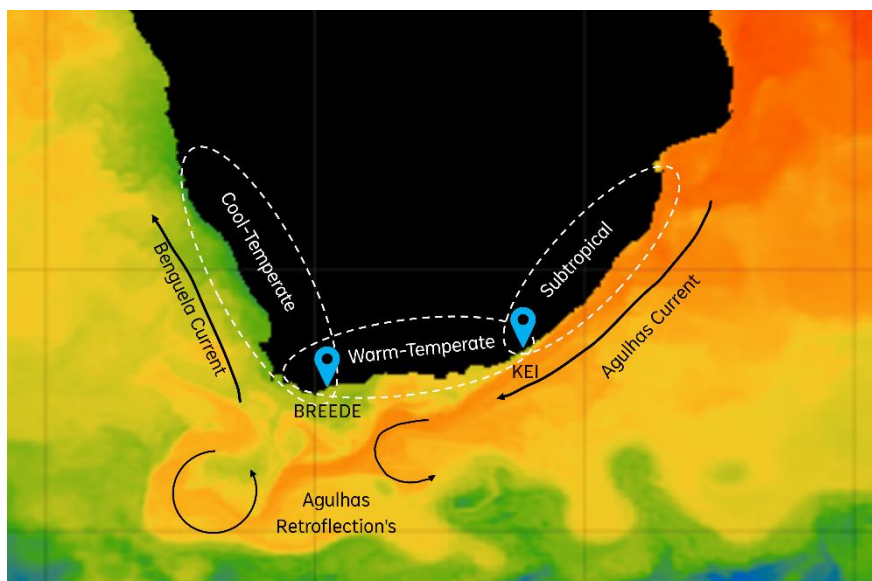


Figure 1.3. Map of Southern Africa: Major current systems, bioregions and the two study sites used for this thesis. The Breede Estuary on the left and the Kei Estuary on the right. Image generated using E.U. Copernicus Marine Service information.

1.4.2 Study Sites

Due to the biogeographical differences around the South African coastline, two contrasting estuaries were selected for this thesis. The Breede River, situated on the southwest coast of South Africa, 20°50'43.1951"E, 34°24'26.762" S, is 322 km long and enters the Indian Ocean in San Sebastian Bay. The Breede Estuary, 56km long, 1 789 ha in extent with 1171 ha open-water area, is one of the 20 largest estuaries in South Africa (James *et al.*, 2018). The estuary falls within the warm-temperate transition zone and is permanently open (van Niekerk *et al.*, 2022b). The catchment is approximately 12 600 km², falling predominantly within the winter rainfall region but also partly within the bimodal rainfall zone. Consequently, the estuary experiences high winter flows but also high variability due to the combined influences of the two rainfall zones (James *et al.*, 2018). Counterintuitively, this high variability contributed to the relative stability in species composition, abundance and community structure of the fish assemblage (James *et al.*, 2018). The Breede Estuary receives a Mean Annual Runoff (MAR) of $1034 \times 10^6 \text{ m}^3$ which is 57% of the reference or pristine (van Niekerk *et al.*, 2022b). The catchment drains the Cape Fynbos region over the quartzites and sandstones of the Cape Supergroup so water reaching the estuary is relatively clear but stained dark by the tannins leached out of the vegetation by low-pH (Flemming and Martin, 2021). Turbidity in the estuary is higher during the winter high-flow season with relatively clear conditions during the summer months. Tidal influence extends to about 50km from the mouth. The estuary has a high biodiversity with more than 110 species of fish from 60 families recorded and is a popular spot for recreational fishing and watersports with over 100 boats recorded over weekends and holiday periods (Lamberth, van Niekerk and Hutchings, 2008; van Niekerk *et al.*, 2022b).

The Great Kei Estuary, located in the Eastern Cape province of South Africa, 32°39'0" S, 28°22'0" E, is 15 km long, 226 ha in extent with 189 ha open-water area. The estuary falls within the warm-temperate, subtropical biogeographic region transition zone (van Niekerk *et al.*, 2022b). The catchment area is approximately 20 673 km² draining sediments of the Karoo Supergroup which results in a high silt deposition and turbidity (Plumstead, Prinsloo and Schoonbee, 1985; Harrison, 2004). Mean Annual Runoff is $649 \times 10^6 \text{ m}^3$ which is 67% of the reference or pristine (Van Niekerk *et al.*, 2019). The catchment falls within the summer rainfall region with peak flow

occurring in the late summer months. The estuary mouth is permanently open and is an important nursery ground for many estuarine-associated fish with more than 40 species recorded (Harrison, 2004; J. Nhleko DFFE In Litt.). While salinity and temperature are similar between these two study sites, the most distinct difference is in their mean turbidity values. Turbidity has been identified as an important factor for juveniles when recruiting into estuaries (Cyrus and Blaber, 1987; Whitfield, 1994b) which is particularly advantageous for juveniles avoiding predation.



Figure 1.4. Study Sites: Photographs highlighting the contrast in turbidity between two South African estuaries: Breede Estuary (left) and the Kei Estuary (right).

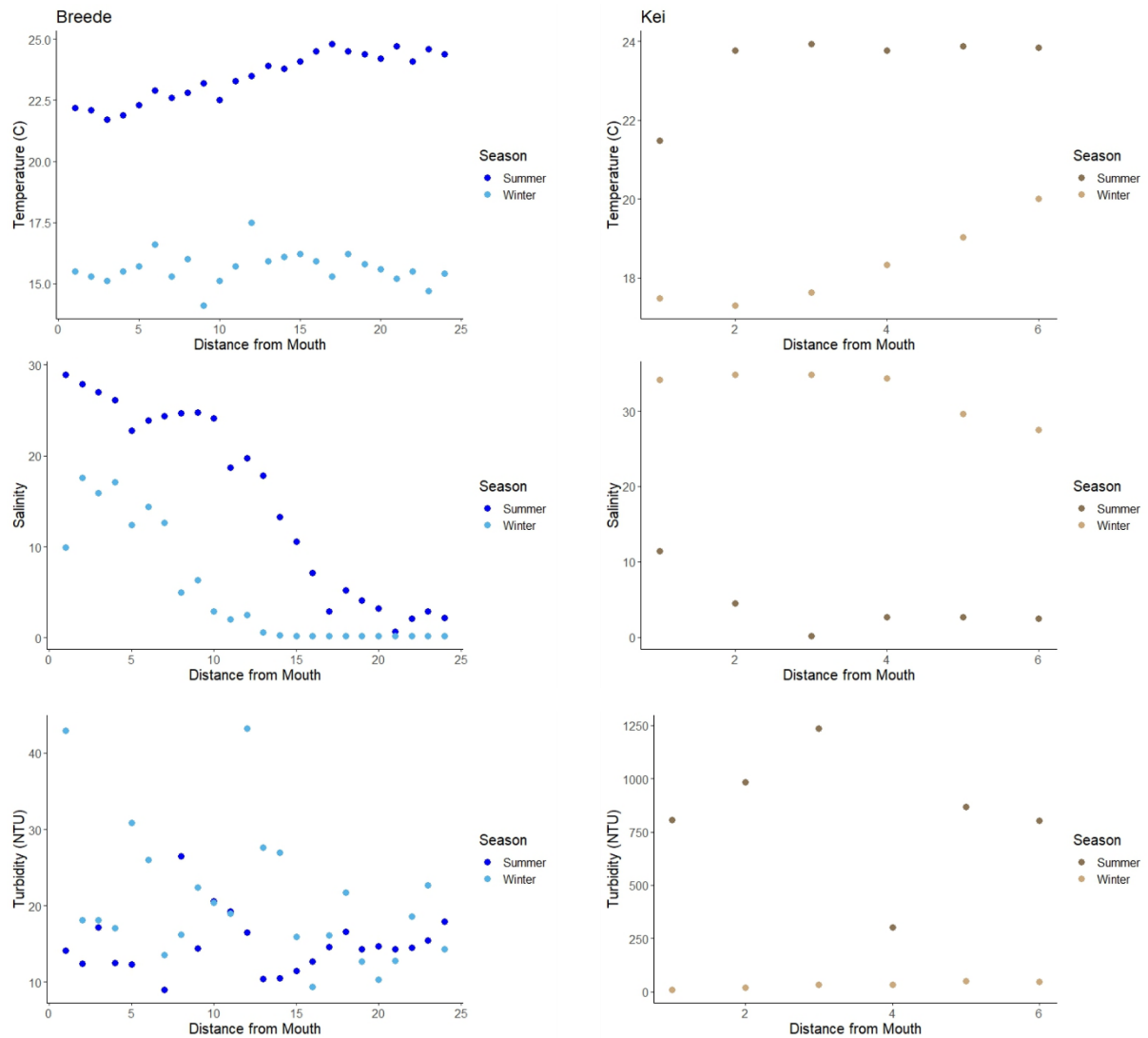


Figure 1.5. Average environmental conditions: Temperature (top), salinity (middle) and turbidity (bottom) throughout the Breede Estuary (left) and Kei Estuary (right) for both summer and winter seasons (Data supplied by Stephen Lamberth).

1.5 Aims and Methods

This thesis aimed to investigate whether a specific environmental variable (turbidity) would trigger local adaptation in *Pomadasys commersonnii* from two biogeographically distinct estuaries. To achieve this aim, several scientific methods were selected that focused on specific population features that were hypothesised to be affected by turbidity.

Since divergence due to genetic and phenotypic differences may not appear at the same time or progress at the same rate, a holistic, multifaceted approach that covers both phenotypic and genetic stocks, incorporating a wide range of timescales, would be beneficial for this thesis. Furthermore, using multiple methods will allow for any disparities between methods to be resolved. With the Breede estuary being a recent hotspot for spotted grunter research, a second study site in a different biogeographic region with contrasting turbidity values was selected, the focus then shifted to traits that could, based on the sensory drive hypothesis, differ between these two study sites. Therefore, it was decided to investigate differences in three traits related to their sensory systems: spot pattern analysis, otolith morphology and acoustic repertoire. Lastly, a genomic analysis was incorporated to determine whether any differences in phenotypic traits had a genetic basis that could ultimately lead to genetically isolated stocks/ subpopulations.

Divergence would act over different timescales for each of these methods (Figure 1.6). Variation in spot patterns is expected to occur within seconds (physiological change) to months (morphological change) and could represent the first phenotypic changes seen in a new stock undergoing speciation due to local adaptation (Stevens, 2016). Changes in otolith morphometry would be a result of ontogeny and occur over a period of months to years and the rate of change would also depend on local environmental variables. As otolith morphology changes, one could expect differences in the acoustic repertoire to occur as individuals adapt to new sensory conditions and these changes would take place over multiple years as a new “dialect” is developed. Lastly, divergence of a new stock would begin to alter the genomic

make-up of the species as sexual selection and inbreeding increases and these changes would occur over a much longer timeframe than phenotypic differences.

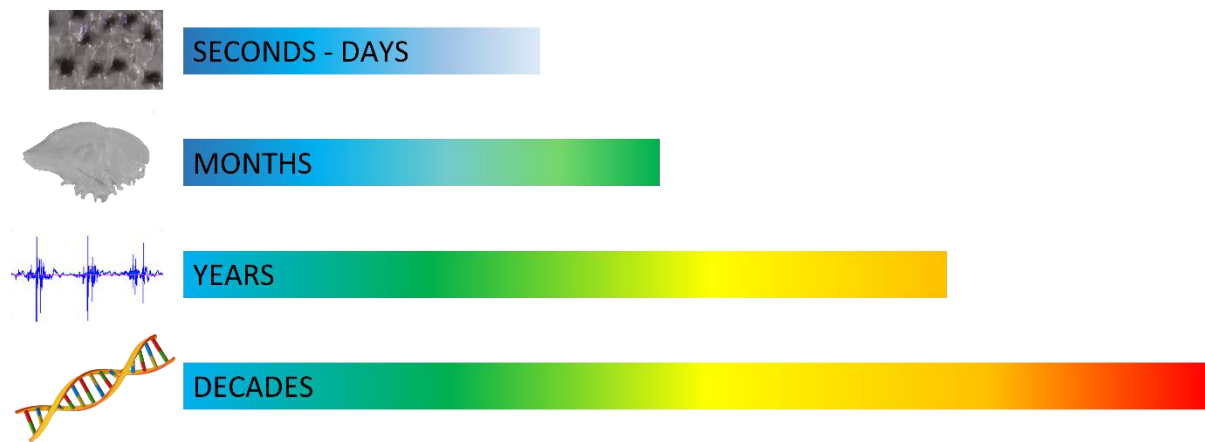


Figure 1.6. Divergence timeline: Infographic showing the expected timeframe for divergence to occur for each of the four methods used in this thesis. Spot patterns (seconds – days), otolith morphology (months), acoustic repertoire (years) and genomic differences (decades).

1.5.1 Spot Pattern Analysis

Animals possess a wide variety of colour patterns with an array of functions depending on different ecological niches (Cruz *et al.*, 2021). These natural body markings can be used for camouflage (both predator and prey), aposematic colouration, courtship behaviour and intra-specific communication. Changes in colour patterns can change instantaneously or gradually over a period of months; some are reversible while others are more permanent (Stevens, 2016). Therefore, there is a very important degree of plasticity related to animal colour patterns and this can complicate population studies. Regardless, spot patterns have been used successfully in the discrimination of different trout species, populations and wild vs. farmed individuals (Qadri, 1959; Aparicio *et al.*, 2005).

As their name suggests, spotted grunter are donned with elaborate spots. In terms of communication, turbidity would affect the efficacy of these spot patterns and it may not be energetically rewarding for spotted grunter living in turbid environments to invest in spot patterns. Therefore, it was hypothesised that the spot patterns of spotted grunter living in turbid environments would differ from those living in less-turbid environments. Expected differences include less spots, smaller spots or lighter spots for individuals living in turbid environments compared to those living in less turbid environments.

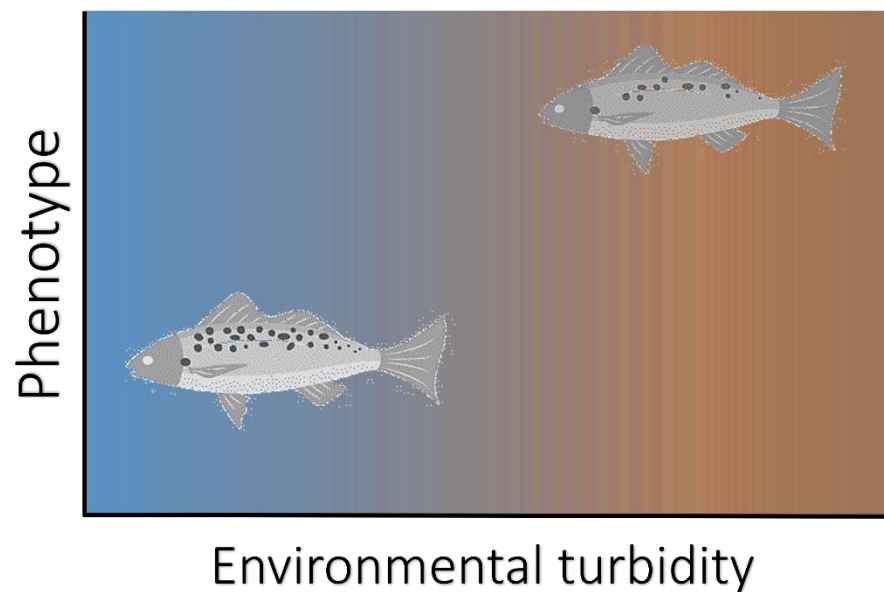


Figure 1.7. Spot pattern reaction norm: Visual representation of the hypothesis that *Pomadasys commersonnii* will have different spot patterns depending on the environment they inhabit; with the hypothesis that high turbidity will result in fewer spots compared to those living in less turbid environments.

1.5.2 Otolith Morphology

Morphometric analyses can be used to differentiate phenotypic stocks or groups of individuals with similar growth patterns. Variations in the morphology of metabolically inert hard parts are a result of ontogeny and therefore cannot be used to identify the genetic stock structure of a species (Cadriin, Karr and Mariani, 2014). However, phenotypic differences are important in understanding how each stock responds to exploitation as ontogenetic rates influence the

populations' fecundity and reproduction, which ultimately affects the dynamics of the population, such as its resilience to exploitation (Dewitt and Scheiner, 2004; Bonamour *et al.*, 2019). Hard structures are also ideal for morphometric analyses as they are not influenced by short-term variability in the environment (Cadrin, Karr and Mariani, 2014). Saggital otoliths are robust and reflect a permanent record of the environmental conditions experienced by the individual and are thus suitable for morphometric analyses (Vignon and Morat, 2010). For this method, the outlines of otoliths are analysed so that any shape variations can be evaluated within and among groups (Cadrin, Karr and Mariani, 2014). Due to environmental differences, including food availability, in each estuary it was hypothesised that the otolith outlines of spotted grunter captured will differ between the two estuaries. Furthermore, temporal changes in the otoliths shape of spotted grunter from the Breede Estuary are hypothesised to differ due to spotted grunter range expansion into this region over the last six decades (Lamberth, Van Niekerk and Hutchings, 2008).

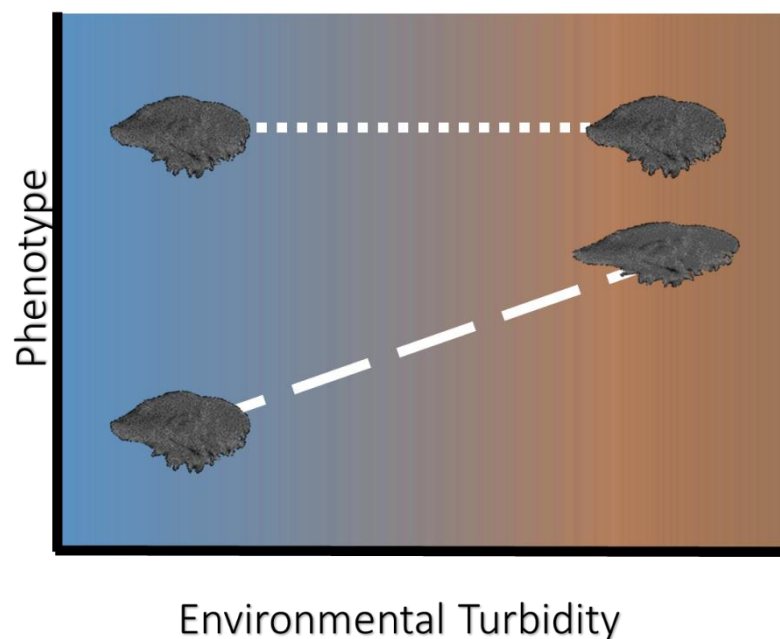


Figure 1.8. Otolith morphology reaction norm: Visual representation of the hypothesis that different environmental conditions, such as turbidity, may affect the otolith shape of *Pomadasys commersonnii*. The dotted line suggests that otolith shape is genetically controlled regardless of environmental conditions while the dashed line suggests that environmental conditions play an important role in the growth and shape of otoliths in *Pomadasys commersonnii*.

1.5.3 Bioacoustics

Fish species often rely on visual cues or stimuli during courtship and hunting however, in environments with poor visibility this is not an effective form of communication (Parsons *et al.*, 2009). Due to this limitation, these species are often forced to rely on other sensory systems for effective communication. Soniferous (sound-producing) fish inhabit a wide range of environments but are generally either nocturnal or found in areas with poor visibility. Soniferous fish species have been documented to produce sound for several life events (Parsons *et al.*, 2009) including courtship, foraging and spawning. There are four main sound producing mechanisms found in fish species including (1) percussion of a substrate, (2) vibration of muscles or appendages, (3) forced water flow through an opening and (4) stridulation. The spotted grunter uses a stridulation mechanism whereby they produce sound by rubbing their pharyngeal teeth together (Webb, 2002; Heemstra and Heemstra, 2004; Parsons *et al.*, 2009; Cadrin, Karr and Mariani, 2014). Fish sounds have been documented to differ in both spectral and temporal properties and these differences have been observed both between and within species and even populations (Clara and Amorim, 2015). The well studied Haemulidae species, *Haemulon aurolineatum*, exhibits a negative relationship between specimen size and dominant frequency suggesting size information is encoded in these signals (Millot *et al.*, 2021). Additionally, the grunts of this species were found to differ significantly in temporal properties among individuals.

Therefore, it was hypothesised that the acoustic repertoire of spotted grunter from geographically distant estuaries would differ in their acoustic properties. It is further hypothesised that spotted grunter living in turbid environments will rely more on acoustic communication than those living in less-turbid environments thereby producing louder, longer and lower frequency sounds that can travel further to reach the receiver.

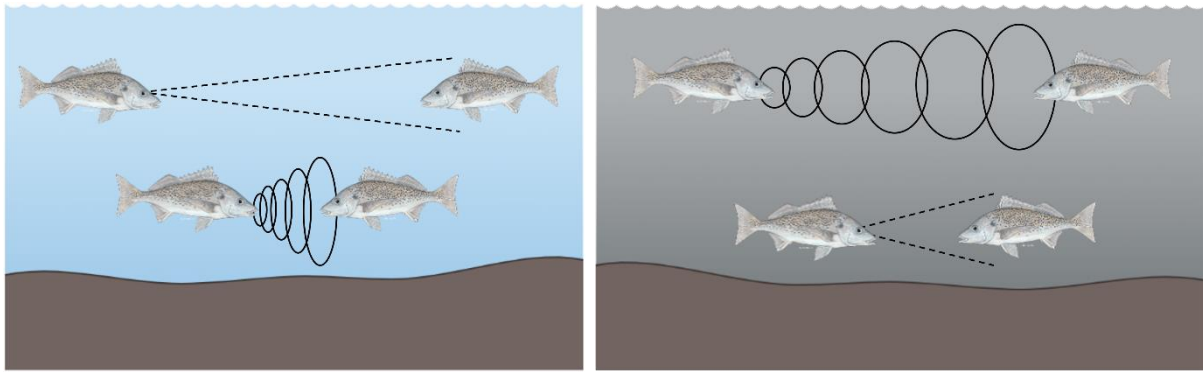


Figure 1.9. Sensory drive hypothesis visual: *Pomadasys commersonnii* living in clear water environments may favour visual communication over acoustic communication. *Pomadasys commersonnii* living in turbid environments may favour acoustic communication over visual communication to locate conspecifics in the poor visibility environment.

1.5.4 Genomic Analysis

Traditionally, marine conservation and management along the South African coastline has been based on ecological parameters (Reiss *et al.*, 2009) with very little knowledge on the historical population structuring and demography that has shaped the species distribution seen today (von der Heyden, 2009). Fortunately, genetics can be used to verify the degree of reproductive isolation between stocks or populations (Begg and Waldman, 1999). Genetic techniques that can be used for stock discrimination include protein variation, mitochondrial DNA and nuclear DNA. Mitochondrial DNA (mtDNA) has maternal inheritance and has been successfully used to separate fish stocks (Begg and Waldman, 1999). Klopper (2005) explored the genetic differentiation of spotted grunter, using mtDNA, and suggested that there is a single South African intermixing population that has high levels of genetic drift, diversity and connectivity. Klopper (2005) also found that genetic diversity was greater within a locality compared to among localities. However, mtDNA evolves much slower than other markers, such as microsatellites, and is often used to determine historical differences between stocks (Avise, 2000). Nuclear DNA (nDNA), including non-coding markers such as microsatellites, evolves much faster and can give a more recent description of any changes that have taken place

between populations (Begg and Waldman, 1999). However, high-throughput sequencing (HTS) has revolutionized molecular research by generating thousands of markers across the genome with a much greater level of detail (Davey *et al.*, 2011) which is particularly useful for population studies. A common HTS method, RADseq (Restriction Site Associated DNA sequencing) uses genomic DNA with the aim of discovering and genotyping thousands of single nucleotide polymorphisms (SNPs). Being genomic, this method is not only useful for assessing/investigating population genetic structure, as other traditional methods do, but it can also identify gene regions that are under divergent selection which may be linked to environmental variables (Baird *et al.*, 2008). Local adaptation to environmental conditions can initiate barriers to gene flow and these barriers can, over time, result in isolation and ecological speciation. The rate at which divergence occurs is variable but it is expected to occur rapidly when selection acts on environmental preferences (Momigliano *et al.*, 2017).

Therefore, SNP data generated through RADseq was used to determine if spotted grunter from two estuaries are genetically different. Additionally, these estuaries fall within different biogeographic regions and major genetic breaks have been found to coincide with these temperature-defined bioregions (von der Heyden, 2009; Teske *et al.*, 2019). Thus, it was hypothesised that spotted grunter from these two estuaries will have distinct traits, due to local environmental pressures, and if these traits have a genetic basis there may be some degree of genetic differentiation between them.

1.6 Outline of Thesis

This overall aim of this thesis was to apply scientific methods that could investigate whether turbidity produces local changes in *Pomadasys commersonnii* from two biogeographically distinct estuaries and potentially use these methods to answer an ecological question regarding a newly established population of spotted grunter. To achieve this, the thesis is divided into six chapters: An introduction chapter, four data chapters and a conclusion chapter. The introduction (Chapter 1) provided important background information and explained the thought process used to develop this thesis.

Chapter 2 (*Spot the Difference*) examines the spot patterns of spotted grunter. It details the preliminary experiment that was conducted to investigate the level of phenotypic plasticity in individual spotted grunter spot patterns. Thereafter, and based on the outcome of the preliminary experiment, the difference between spot patterns from the two study sites was investigated.

Chapter 3 (*Shaping the Future*) explores shape differences in spotted grunter otoliths. A proof-of-concept is outlined and thereafter the chapter examines differences in the otolith outlines of spotted grunter from two biogeographically distinct locations. Finally, the temporal stability in otolith shape morphology of spotted grunter captured in the Breede Estuary is investigated.

Chapter 4 (*Something Sounds Fishy*) investigates the acoustic sounds produced by spotted grunter. The rationale for this chapter was based on the differences observed in the hearing apparatus (otoliths – chapter 3). The chapter briefly details a proof-of-concept study that assessed whether it was possible to differentiate the sounds produced by fish from different families, genera and species. Thereafter, the acoustic grunts from spotted grunter were compared and discussed.

To determine whether any of the phenotypic differences identified in the previous three chapters have a genetic basis, chapter 5 (*Fishing for Answers*) uses double digest restriction-

site associated DNA sequencing (ddRADseq) to assess genomic variability in spotted grunter from the two estuaries. A brief phenotype/genotype association study was also conducted.

The final chapter, Chapter 6, concludes the thesis by summarizing the key findings of the research questions and reflects on their value and contribution. Lastly, it reviews limitations and proposes opportunities for future research.

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CHAPTER Two

SPOT THE DIFFERENCE: QUANTITATIVE
ANALYSIS OF SPOT PATTERNS IN
POMADASYS COMMERSIONII FROM TWO
CONTRASTING ESTUARIES

2.1 Abstract

Animal body markings such as spots, stripes and patterns play important roles in camouflage and visual communication. Camouflage is achieved through background matching and is advantageous for both predators and prey as they blend into their environment. Visual communication through body markings has a wide array of applications ranging from aposematic to nuptial colouration. In all cases, the ability for body colouration to be adaptive is advantageous. This chapter investigated the level of phenotypic plasticity in *Pomadasys commersonnii* spot patterns. First, an aquarium experiment was designed to examine the level of plasticity in spot patterns when exposed to various degrees of turbidity which indicated that *P. commersonnii* are able to adapt their spot size and brightness. However, the number and position of spots remained constant throughout the experiment, suggesting that spots could be used for individual identification. Next, spots of *P. commersonnii* (n = 158) from two estuaries, that vary in water clarity, were compared. *P. commersonnii* living in the less turbid Breede Estuary were found to have a greater number of spots, for a given size range, compared to the turbid Kei Estuary individuals. Furthermore, the spots from the Breede Estuary were, on average, larger than those from the Kei Estuary. These results can be explained by the sensory drive hypothesis that suggests individuals in turbid environments favour, and expend energy, on forms of communication other than visual. The exact purpose of these spot patterns is unknown, however, it is apparent that environmental variables, such as turbidity, play an important role in the phenotypic variation of *P. commersonnii*.

2.2 Introduction

Animals possess a wide diversity of colourful-patterned skins, integuments, pelages and plumages. These patterns can be so pronounced that species are named after them, such as the spotted grunter, *Pomadasys commersonnii*. Natural body markings can include stripes, blotches, patches, spots and even outlines and these visual signals are assumed to have one of two functions: camouflage and/or communication. Within a species, there are often notable differences in the natural markings of individuals.

For many species, the ability to blend into the background environment reduces the chance of detection and recognition by observers which is advantageous to survival (Duarte, Flores and Stevens, 2017). There are various forms of camouflage that animals employ; the two main forms of camouflage are background matching and disruptive colouration. Background matching is when an animal's body colouration is optimised to reduce the signal-to-noise ratio of visual predators (Michalis *et al.*, 2017). In contrast, disruptive colouration is used to confuse the observer by creating false boundaries and disrupting an animal's silhouette (Duarte, Flores and Stevens, 2017). Colour patterns are produced by chromatophores which are located in the dermis and are composed of layers of light-absorbing pigment cells (Seehausen *et al.*, 2008). These pigment cells absorb specific wavelengths of light which contribute to the wide variety of patterns and colours seen in vertebrates (Price *et al.*, 2008).

Visual signals are vital in many animal communication systems and colour patterns are often used to relay information to an observer (Morrongiello *et al.*, 2010). Aposematic colouration is used as a warning to predators that an individual is potentially toxic. This form of colouration has also produced many non-lethal mimics which have taken advantage of these widely understood visual signals (Briolat *et al.*, 2019). Colour patterns can also signal traits that express the quality or fitness of an individual and can be used for both sexual selection and intra-specific competition. For example, females often favour males with specific nuptial patterns; male *Gasterosteus aculeatus*, three-spine sticklebacks, present with blue eyes, bright red throats and anterior bellies during breeding season to attract females (Smith *et al.*, 2004).

Fish species also display ontogenetic colour changes, polyphenism, whereby they change colour from their juvenile to adult state (Figon and Casas, 2018). This is commonly found in species that change environments across life history stages and is assumed to be a function of camouflage in different environments. Ontogenetic colour change is also found in species that remain resident throughout their life. In this instance it is assumed that colour change represents sexual maturity and reduces adult male aggression towards juveniles during breeding season. This phenomenon has been observed in the emperor angel fish, *Pomacanthus imperator*, whereby juveniles were not identified as competitors by the adults (Fricke, 1980).

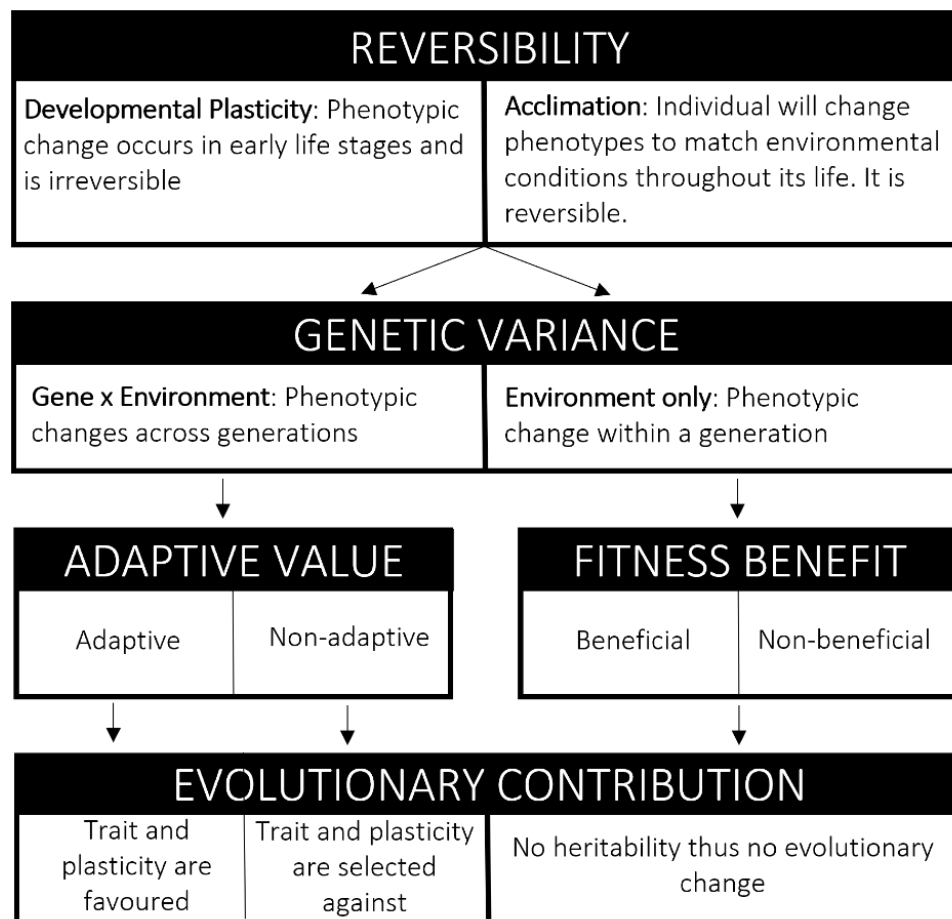


Figure 2.1: Breakdown of phenotypic plasticity: The response of plasticity to environmental conditions and how genetic variance and fitness influence the evolution of the phenotypic trait. Taken from Wada and Sewall, (2014).

Body colouration is associated with adaptation and the capacity for this trait to be plastic is essential for many species (Sköld *et al.*, 2016). Adaptation towards different environments is made possible by both genetic and phenotypic traits but plasticity provides a faster, more diverse response to environmental changes (Sköld *et al.*, 2016). An organism that can rapidly adapt to a changing environment is resilient and according to the sensory drive hypothesis, individuals may alter their sensory or signalling systems based on their local environmental conditions (Duarte, Flores and Stevens, 2017). Therefore, phenotypic plasticity is vital for cosmopolitan species that occupy a wide range of habitats (Price, 2006) and the capacity for an individual to perform colour change is a fundamental example of phenotypic plasticity (Sköld *et al.*, 2016). Plasticity in colour patterns is beneficial when balancing the many selective pressures that colour patterns undergo and these changes can occur either morphologically or physiologically.

Morphological colour change happens over time intervals in the order of weeks to months and involves changing the number and proportion of chromatophores (Sköld *et al.*, 2016); or chemical modification and degradation of the pigments within each cell (Figon and Casas, 2018). This method of colour change is most common in response to environmental conditions (UV, background matching, food availability) and during life-stage transitions. A well-studied example of this type of colour change is observed in crabs after a moult (Duarte, Flores and Stevens, 2017). Morphological colour change, to achieve optimal camouflage in a changing environment, has been studied in the *Oryzias latipes*. These fish increase their leucophore (white pigment) density and lower their melanophore (black/brown pigment) and xanthophores (yellow/orange pigment) when exposed to white backgrounds (Sugimoto, 2002).

Contrastingly, physiological colour change is rapid and involves the aggregation of pigments cells within chromatophores; an example of physiological colour change occurs in cuttlefish which can alter their colouration in under a second (Pérez-Rodríguez, Jovani and Stevens, 2017). Physiological colour change can be controlled in two ways: directly through pigment-containing cells and indirectly through the interaction of light and iridophore regulation (Price *et al.*, 2008). The paradise whiptail, *Pentapodus paradiseus*, can turn from blue to red in a fraction of a second by altering the structure of its iridophores; this adjusts which wavelength

of light is reflected through the xanthophore (Mäthger *et al.*, 2003). Silvery colouration in vertebrates is almost always due to short wavelengths being refracted by iridophore cells. Melanophores are more commonly altered in fish by changing the intensity/area of these brown/black and grey cells. This mechanism is induced by a hormone, melanocyte-stimulating hormone, which disperses the melanin granules within the melanocytes (Price *et al.*, 2008) and the size and shape of these melanin-based patterns can be altered dramatically in a matter of seconds.

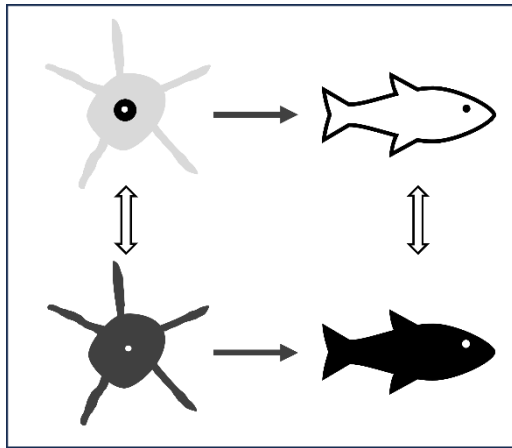


Figure 2.2: Schematic illustration of a melanocyte undergoing physiological colour change: The top melanocyte has a small area of tightly clustered black cells resulting in a lighter-coloured fish. Below, the melanocyte has dispersed the black cells creating a darker-coloured fish.

A trait needs to provide a certain level of fitness to be evolutionarily stable, but these benefits may be counteracted by the energetic costs needed to sustain these traits (Sugimoto, 2002). Recently, there has been an increased interest in the costs and benefits associated with colour change in fishes: sculpins have exhibited a reduced survival rate as a cost to colour change (Bergstrom, Whiteley and Tallmon, 2012) while guppies drastically increase their food consumption in response to repeated colour changes (Rodgers *et al.*, 2013). While much research still needs to be conducted on the costs involved with colour change, the consensus is that the cost incurred in each scenario is dependent on the speed and type of colour change.

Spotted grunter, characterised by pronounced black spots along their body, have a wide distribution and can withstand a vast number of environmental conditions. Rapid and successful adaptation of body colouration to local environmental conditions is vital for their success and they may favour certain modes of communication for each environment. It is assumed their spots are used for either camouflage or visual communication. The two study

sites selected for this thesis have vastly different turbidities which could play an important role in their spot pattern development.

Turbidity negatively affects the visual environment (Gray, Sabbah and Hawryshyn, 2011). For species that code visual information in their colour patterns, the efficacy of their signal depends on the receiver's ability to detect it. Detection is not only dependent on the observer's visual system but can also be compromised by changes in the visual environment (van der Sluijs *et al.*, 2011). Turbidity alters the wavelengths of light that penetrate certain water depths, generally decreasing the level of ambient light thereby shifting to a darker environment. Several fish species exhibit plasticity in their nuptial colouration when exposed to different environmental conditions: male pygmy perch (*Nannoperca australis*) maximise their nuptial signal efficacy in light environments by increasing the intensity of their red colouration (Morrongiello *et al.*, 2010) and male guppies living in turbid environments have a greater degree of structural colour compared to those living in less turbid environments (Adro'l *et al.*, 2018). Male cichlid fish in clear waters within Lake Victoria have distinct, bright colourations compared to those living in areas with poor visibility (Seehausen, van Alphen and Witte, 1997). Water clarity and visibility also affect the relationship between species diversity and colour diversity (Seehausen, van Alphen and Witte, 1997). *Pundamilia nyererei*, a species of cichlid in Lake Victoria, were found to have duller red colouration and reduced standards for mate choice when inhabiting turbid waters compared to those in clear water environments. Thus, turbidity degrades image quality and plays an important role in signalling and communication (Utne-Palm, 2002).

From the above literature, it is evident that environmental conditions can affect signal transmission and any environmental condition that affects visibility can have an impact on colour patterns. To date, no research has been done on the function of spotted grunter spots, however it is predicted that they play a role in either camouflage or visual communication. For spots to aid in camouflage, one would expect to see a higher spot density in complex, heterogenous environments as a means of background matching (Kelley, Fitzpatrick and Merilaita, 2013). Conversely, spotted grunter in turbid environments are hypothesised to

develop less spots thereby saving energetic costs and concealing the individual in the homogenously turbid environment.

If spots are used to communicate information, one would expect to see spotted grunter with fewer spots in turbid environments compared to those living in clear environments. The efficacy of visual signals in communication is contingent on the production of the signal itself, the observers' visual abilities and the environment through which the signal is sent (Morrongiello *et al.*, 2010). Therefore, the benefit of producing spots in an environment where signal transmission is low, such as turbid environments, is not worth the energetic cost and these individuals may opt towards a different mode of communication.

In this chapter, the aim is not to identify the function of spotted grunter spot patterns but rather identify whether there is variability in spot patterns between individuals and between study sites. Natural body markings have been successfully used to identify individuals in a wide number of species and can only be reliable for these studies if two assumptions are met: (1) the body markings must be distinguishable between individuals and (2) the markings need to remain constant over time (Merz *et al.*, 2012; Kristensen *et al.*, 2020). If individuals are distinguishable and their spots remain constant over time, then differences in these spot patterns can hopefully be used to distinguish phenotypic stocks of spotted grunter which may require unique management strategies. Given that technological advances in pattern recognition algorithms have reduced processing effort and enabled scientists to analyse large quantities of data, this method is now technically viable for stock delineation.

Before comparing spotted grunter from the two study sites, the level of phenotypic plasticity was evaluated in a proof-of-concept experiment which determined how/to what extent spotted grunter can alter their spot patterns. The outcome of the experiment was used to inform the direction for the main study which aimed to investigate whether spotted grunter from two different biogeographic regions, with contrasting turbidities, the turbid Kei Estuary in the Eastern Cape and the less turbid Breede Estuary in the Western Cape, will possess consistently different spot patterns that are detectable. It was hypothesised that spotted grunter living in more turbid environments (Kei Estuary) will have fewer, smaller spot patterns compared to

those living in less turbid environments (Breede Estuary). The objectives were to (1) determine whether spot patterns could be accurately identified and analysed using ImageJ and (2) compare the mean spot features between individuals from the two study sites.

2.3 Proof-of-Concept – Phenotypic Plasticity in Spot Patterns

Phenotypic plasticity can complicate stock delineation. For spot patterns to be used as an accurate method of stock identification in spotted grunter, it is important to assess the level of phenotypic plasticity in their spot patterns. It is unclear to what degree spot patterns are attributed to visual signalling although, it is generally accepted that patterns form an important part of camouflage and intraspecific communication in many species. If the former is true, and if maintaining colouration patterns is physiologically demanding then displaying spot patterns in areas with poor visibility may not be worthwhile. Turbid environments decrease the chance of the observer receiving visual signals compared to those observed in clearer, less turbid environments. According to the sensory drive hypothesis, it would be more beneficial for spotted grunter to expend energy on other modes of communication in environments with poor visibility.

With the above in mind, it was hypothesised that, if spot patterns are plastic, one would see changes in the number of spots, the colour of spots and/or the size of individual spots when spotted grunter are exposed to increased levels of turbidity (Figure 2.3). More specifically, high turbidity levels should result in less spots that are smaller in size and lighter in colour. Before determining whether the spot patterns of spotted grunter differ between the study sites, it was important to understand the biological mechanisms that underpin spot patterns. To do this, an experiment was conducted at the Sea Point research aquarium (Ethics approval: 2018/v10 /SK) and the following four questions were evaluated:

- I. Do spotted grunter change the number of spots displayed in response to increased levels of turbidity?
- II. Do spotted grunter change the shape/area of their spots in response to increased levels of turbidity?
- III. Do spotted grunter change the lightness of their spots in response to increased levels of turbidity?
- IV. If spotted grunter can change their spots in response to increased levels of turbidity, can this change be reversed if returned to low turbidity environments?

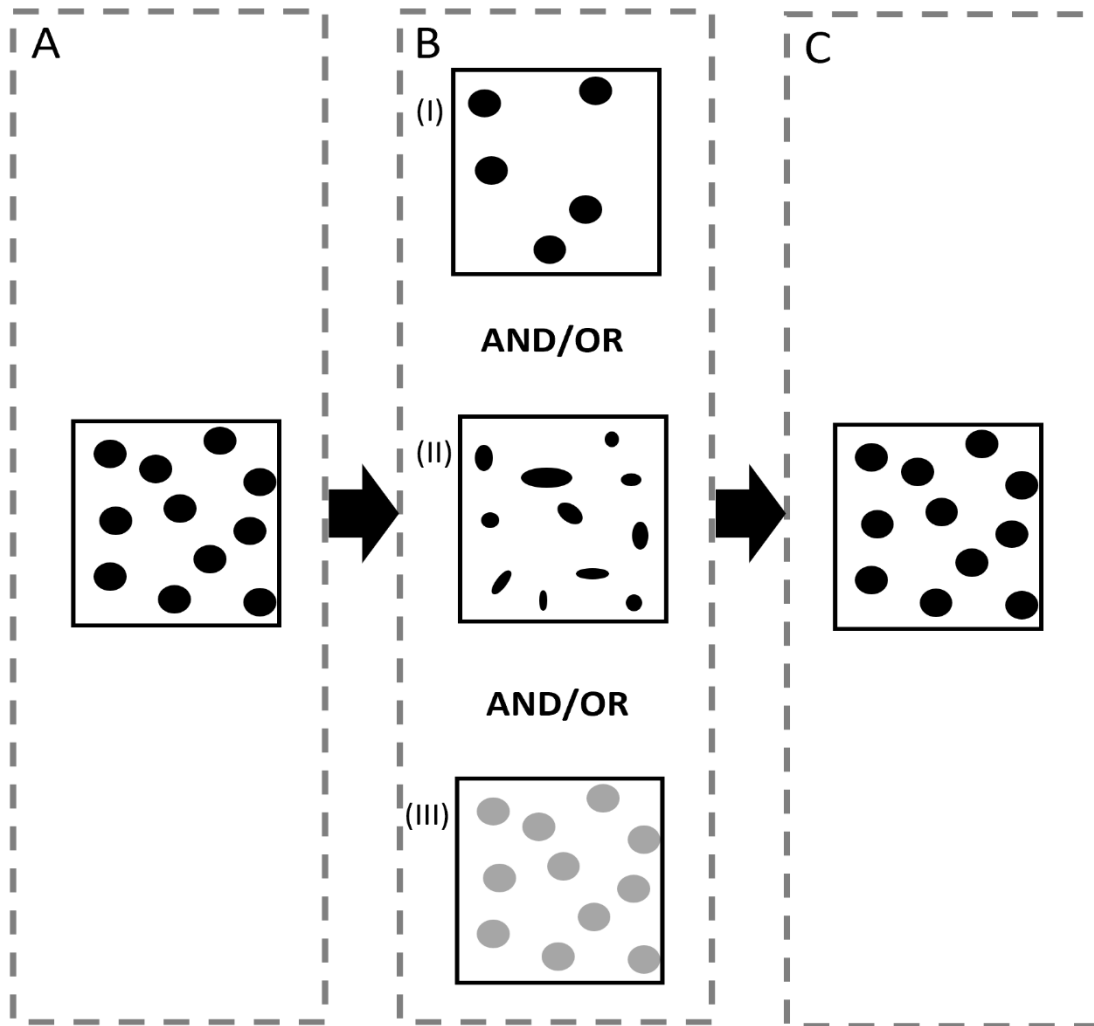


Figure 2.3: Spot Pattern Plasticity Infographic: Diagram indicating the predictions of spot pattern changes in *Pomadasys commersonnii* when exposed to varying levels of turbidity. (A) The hypothetical spot pattern of a *Pomadasys commersonnii* at the start of the experiment. (B) Indicates the predicted response in spot patterns of *Pomadasys commersonnii* when exposed to increased levels of turbidity: (I) fewer spots, (II) a change in spot size/shape and (III) a reduction in spot lightness. (C) The expected outcome of the experiment when returned to low turbidity environments i.e., reversal of change in patterns.

2.3.1 Experimental Set-up

The experimental units were 4kl tanks that were placed in a 2 x 2 grid-set up. The two treatment levels (Turbid vs Clear) were each applied to two of the 4kl tanks to avoid pseudoreplication. The tanks were all connected to the same system prior to the experiment resulting in uniform water variables. Once the experiment had begun, they were treated as closed systems. Six spotted grunter were added to each of the four tanks. An additional two 2kl tanks were used to store tank water while draining the larger tanks when capturing fish.

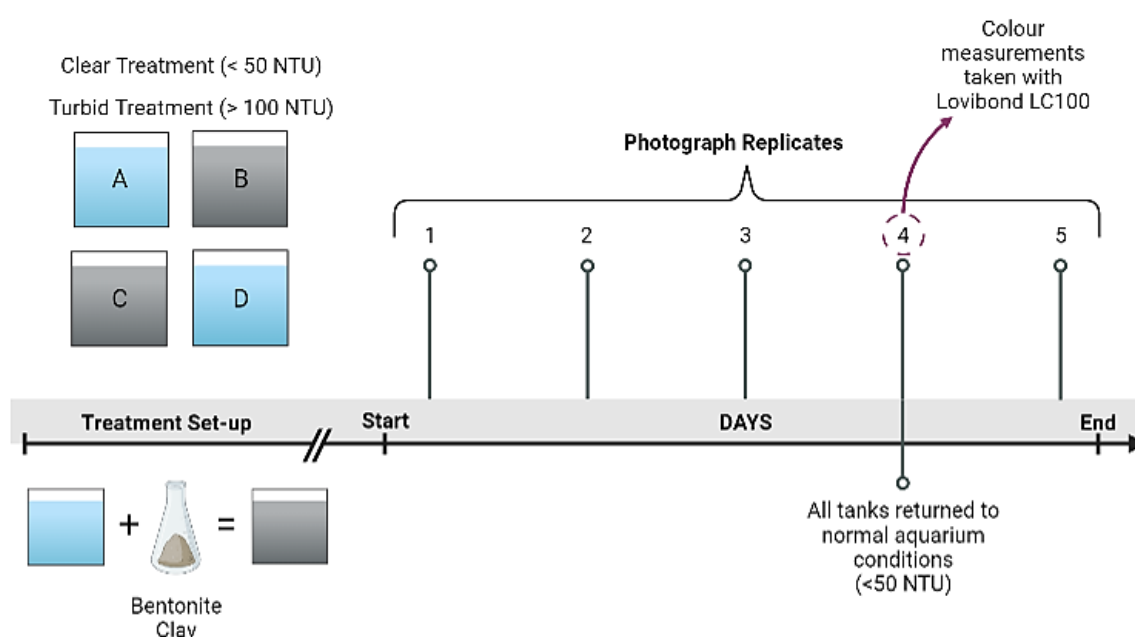


Figure 2.4. Experimental set-up and timeline: Schematic outlining the procedure and timeline that was followed for the experiment. Created in BioRender.com

PREPARATION OF TANKS

In preparation, the tanks were first disconnected from the main aquarium water system to prevent water mixing and ensure the maintenance of the chosen turbidities. Each tank had an independent oxygen source, however, other parameters such as water quality, temperature and salinity were kept constant across the tanks.

The modal NTU for the Kei estuary is > 120 , while the Breede estuary is < 20 (per comms Stephen Lamberth). It was therefore decided that the turbid treatment tanks would be maintained at a value > 100 NTU. The turbid-treatment tanks were increased in turbidity by adding bentonite clay to the system (Pusch, 2015). Bentonite clay has been successfully used in aquarium studies to alter water transparency/light reflectance by increasing inorganic turbidity (Benfield' and Minello, 1996; Madsen et al., 1999). A 10L bucket was filled with water from each of the two turbid-treatment tanks. Approximately 1.225kg of bentonite clay was slowly added to each bucket of water until the solution was thoroughly mixed. These buckets were then introduced back into each of the respective treatment tanks. This was repeated twice for each treatment tank. Individual pumps were added to the tanks to continuously stir the water, creating water movement to prevent the bentonite clay from settling. The tanks were monitored and measured with a turbidimeter to ensure that turbidity levels were maintained throughout the experiment.

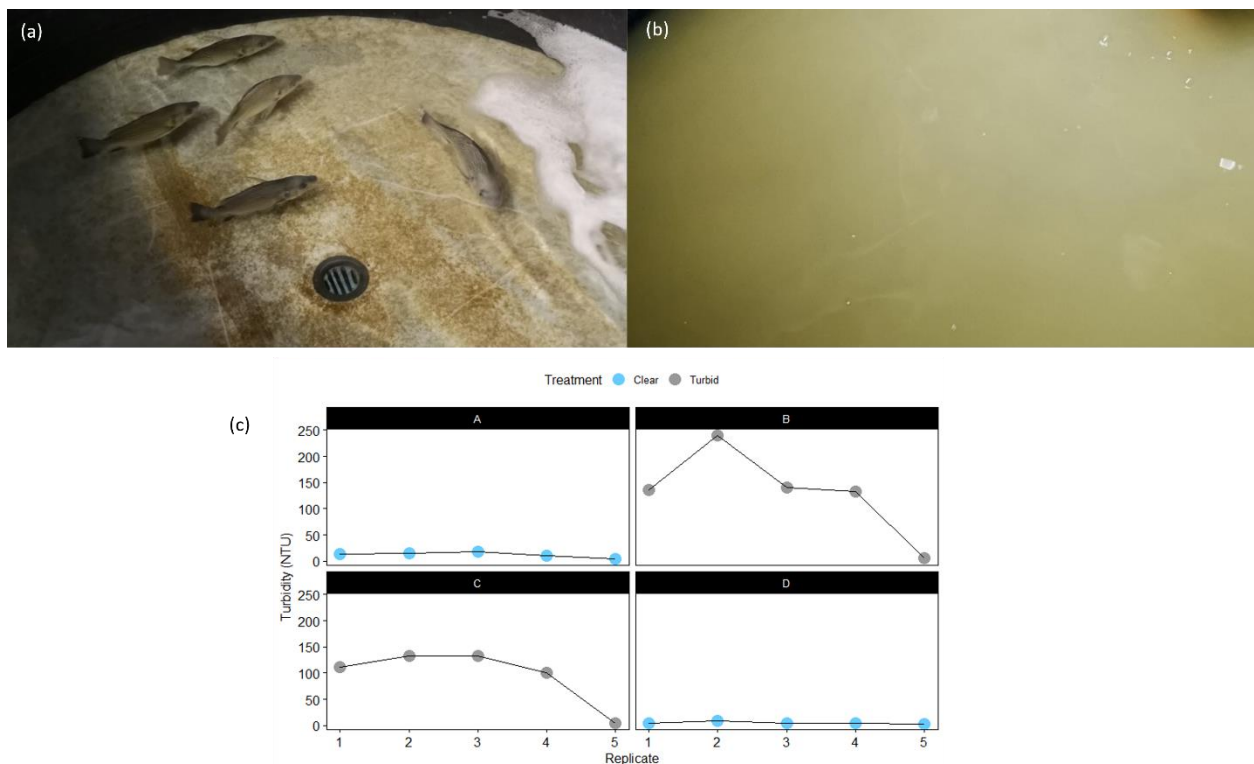


Figure 2.5. Photographs of treatment tanks: (a) Clear control tank and (b) turbid treatment tank. (c) The turbidity level of each tank was measured throughout the experiment. The black bars on top represent the tank number.

FISH SELECTION

The fish used for this experiment had been reared at the aquarium and were part of the long-term aquarium stock. The aquarium's ethical standards were always adhered to. There were approximately 40 spotted grunter individuals, ranging in length, to select from and a total of 24 spotted grunter, six per tank, were chosen. The six fish from each tank were comprised of two "small" (< 30mm), two "large" (>50mm) and two "medium" (30 – 50mm) individuals to maintain similar size ranges among tanks. After selection, each fish was photographed, measured (total length- mm) and given an identification number. The photographs were taken on a black background of the left-hand side of each fish. A scale card was included in each photograph. The fish remained in their tanks for a twelve-day period during which time they did not receive food so as to prevent nitrate build-up in the tanks.

REPLICATES – PHOTOGRAPHS

The fish were photographed every four days over the twelve-day period. Before capturing the fish, the water levels in each tank were lowered to a height of 20cm. The removed water was stored in the small holding tank to avoid wastage and maintain water quality parameters. To capture the fish, as per our ethics approval, 8 ml of 2-phenoxyethanol was added to the remaining water in each tank to sedate the fish (Deacon and Hecht, 1996). Once sedated, each fish was caught, measured, and photographed. They were then placed in a holding tank and monitored while the 2-phenoxyethanol water was drained from their treatment tanks and their original water was replaced. Once the fish had recovered from sedation, they were placed back into their treatment tanks.

SPOT BRIGHTNESS

On the 12th day of the experiment the intensity of spot colouration was measured using a Lovibond LC 100 spectrophotometer, this handheld device allows one to accurately pinpoint the colour area that one would like to measure (Lovibond Ltd, UK). Two colour measurements were taken of each fish: an area on the head where spots are absent and a spot above the

lateral line. Ideally, colour measurements would be taken within each tank. Since this was not possible, it was ensured that the colour measurement was taken as soon as each fish was removed from the treatment water.

REVERSAL OF SPOT PATTERNS

Once the twelve-day experiment was completed, the four tanks were drained, cleaned and reconnected to the aquarium water system. The fish were left for 3 weeks under normal aquarium conditions before being photographed again. Therefore, a total of five images (replicates) were taken of each individual fish.

2.3.2 Spot Image Analysis

Images of each spotted grunter were enhanced and analysed using the '*Particle Analysis*' function from the open-source imaging software **ImageJ** (Schneider, Rasband and Eliceiri, 2012). A section of spots from each individual was selected for analysis (Figure 2.6). The section remained constant over replicates and each section included approximately five–ten spots. Spot area was selected as the measure of spot size.

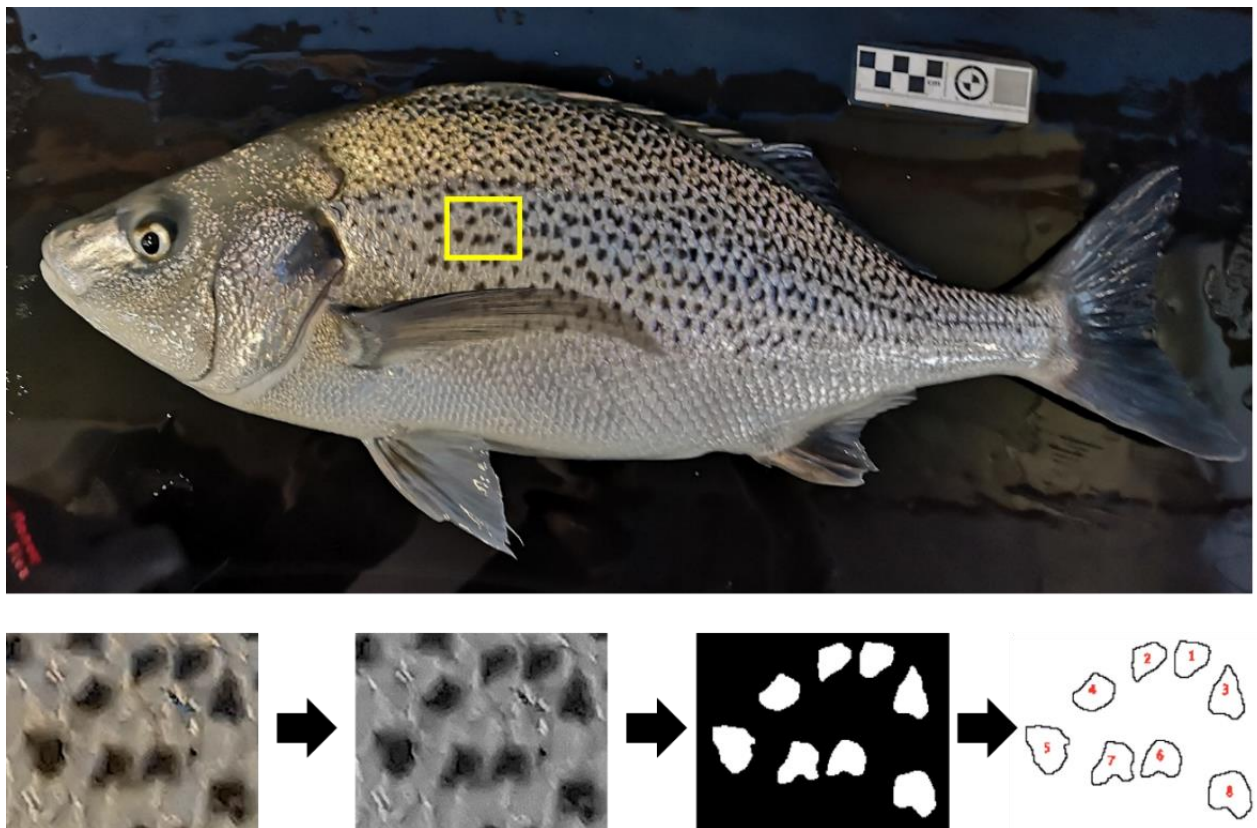


Figure 2.6: ImageJ Spot Analysis: (Top) Sedated fish B6, from the turbid treatment, is shown with its section of spots used for the duration of the experiment and the scale card is visible in the background. (Bottom) The section of spots as they move through the spot analysis process from **ImageJ**. From left to right: (1) Cropped image, (2) greyscale, (3) threshold adjusted (4) spots analysed and numbered.

2.3.3 Statistical Analysis

ASSESSING STABILITY IN SPOT PATTERNS

To assess whether spotted grunter could alter the number of spots displayed, the images were assessed by eye. The five photographs of each individual were compared visually to determine whether any spots had appeared/disappeared.

ASSESSING STABILITY IN SPOT SIZE

The area of each spot pattern was converted to view the proportion of change in spot size over the five replicates. The first photograph was used as the baseline for spot size. The spot area of each additional replicate was divided by the spot area of the first replicate as given by the equation:

$$\text{Proportion of Spot Size} = \frac{\text{SpotArea}_{Rx}}{\text{SpotArea}_{R1}} \quad \text{Equation 2.1}$$

Where R is the replicate for x values 1 – 5. Spot size was used for the remaining statistical analyses. T-tests were conducted on the effect of treatment on spot size per replicate to determine if there were any significant differences in the spot size between the two treatments over time.

Two-way repeated measure analysis of variations (ANOVAs) were conducted to rule out any effect that tank may have had on spot size during the experiment (Table 2.1). ANOVAs are used to test the variation about the means of two or more populations. Thus, it is a measure of the variation present in the experiment by examining the differences in the mean values of the dependent variable associated with the effect of the independent variable/s (Keselman, Algina and Kowalchuk, 2001). The output gives an F-statistic which is based on the F-ratio. One would expect to find no influence of tank on the outcome of spot size, therefore, accepting the null

hypothesis which states that the category means are equal in the population. This was performed in R Studio using the R function ‘*aov*’ (R Core Team, 2020; RStudio, 2020).

Table 2.1: Repeated measure ANOVA models used to determine the effect of tank over time for spot patterns of *Pomadasys commersonnii* exposed to two levels of turbidity.

	Response variable	Formula	Random Effect
Replicate 2-6	Spot Area	$Y \sim \text{Treatment} + \text{Tank}$	Error (Individual)
All replicates	Spot Area	$Y \sim \text{Treatment} + \text{Tank}$	Error (Individual + Replicate)

Linear mixed models (LMM) allow both fixed and random effects and allow for modelling non-independent hierarchical/multi-level data (Gomes, 2022). They can also be used for repeated measures that are taken from the same statistical units. Our experiment contains both hierarchical data (spots, nested in individuals, nested in treatment. The tank effect was already evaluated above), and repeated measures (replicates) on the same spots (statistical units). Thus, an LLM was conducted to test how spot size was affected by treatment and repeated measures (replicates; Table 2.2). The LMM was conducted using the ‘*lmer*’ function from the R package “*lme4*” (Bates *et al.*, 2015).

Table 2.2: Linear Mixed-effects model Formula to determine the effect of turbidity on spot size (area) in *Pomadasys commersonnii* over time (replicate).

Response variable	Formula	Random Effect
Spot Area	$Y \sim \text{Treatment} + \text{factor (Replicate)}$	1 Individual

ASSESSING STABILITY IN SPOT BRIGHTNESS

The Lovibond LC 100 measures colour using the CIELAB colour scale where: L^* defines lightness, a^* defines the red/green value, b^* gives the yellow/blue value and C denotes chroma or saturation (Figure 2.7). The two measurements of interest: are L^* which correlates to the level of pigmentation in the skin and B^* which relates to pigmentation and tanning (Ly *et al.*, 2020).

Two-sample t-tests were conducted to determine if there were any tank effects on fish colouration within a treatment. To accept the null hypothesis, there is to be no observed difference in fish colouration between tanks within the same treatment. Once the tank effect was ruled out, ANOVA tests were conducted on the five colour measurements to pick up any variation about the means of these measurements from the two treatments. This was performed in R Studio using the R function 'aov' (R Core Team, 2020; RStudio, 2020).

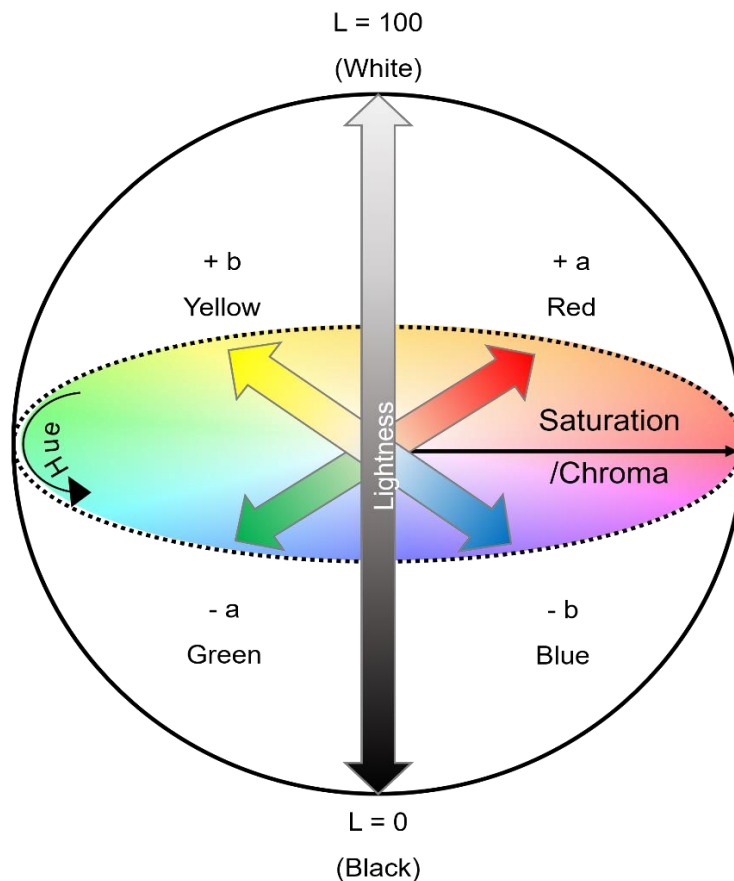


Figure 2.7. CIELAB colour space diagram: This system represents the relationship of colour using three axes: a^* and b^* which represent chromaticity coordinates and L^* denoting lightness. Lightness, the vertical axis, ranges from a value of 0 (black) to 100 (white). The a^* value gives the red (+a) and green (-a) colour values while b^* gives the yellow (+b) and blue (-b) colour values. The distance from the centroid, which represents neutral/achromatic, represents the saturation, or Chroma of the colour. Hue is given by the angle around the chromaticity axes.

2.3.4 Results

ASSESSING STABILITY IN SPOT PATTERNS

By visual inspection of the five replicate photographs, it became apparent that the number and position of spots remained constant over time for each individual. None of the spots disappeared over time and no new spots developed during the duration of the experiment (Figure 2.8).

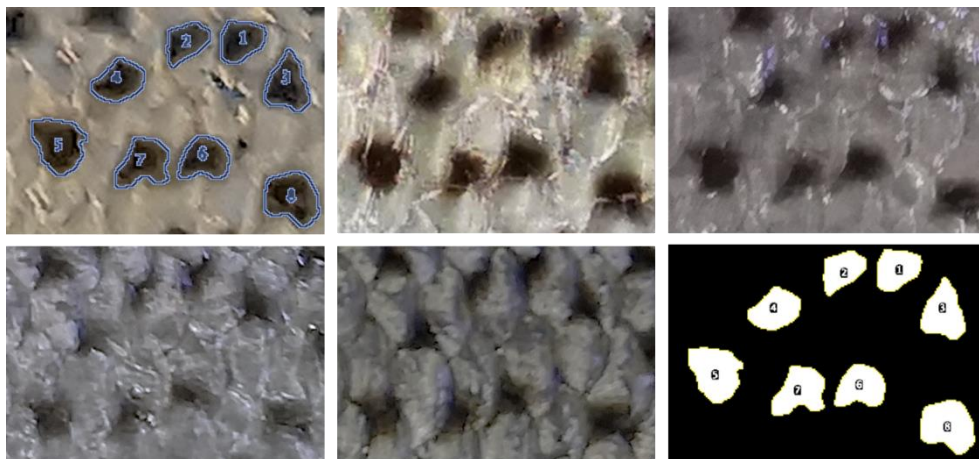


Figure 2.8. Spots over time: Spots (1-8) from a *Pomadasys commersonnii* (B6) exposed to high turbidity levels over time. (Top: left-right) Replicate one (with the over-lying spot numbers), Replicate two and replicate three. (Bottom: left-right) Replicate four, replicate five and the particle analysis outline produced by ImageJ shows spot numbers.

ASSESSING STABILITY IN SPOT SIZE

The results from the experiment suggest that turbidity levels affect spot size in spotted grunter, with higher turbidity resulting in smaller spot size (Figure 2.9). When comparing treatments, at a 95% confidence interval, spot size was significantly different for all replicates (Table 2.3). There was a noticeable decline in the spot size of individuals in the turbid treatment when first exposed to high levels of turbidity (Replicate 2: Figure 2.9). Spot size continued to decrease during the next two replicates in the turbid treatment (Figure 2.9). Contrastingly, the individuals in the clear treatment tank had negligible variation in their spot size throughout the treatment. This contrast in results is shown by the increasingly significant differences in the mean spot size

between the two treatments: replicate 2 ($t = 3.6$, $df = 138.96$, $p = 4.0e-04$) to replicate 4 ($t = 4.0$, $df = 141.2$, $p = 2.7e-06$).

Spot size was not statistically significant for different tanks during the experiment ($F = 0.725$, $p = 0.497$) nor during each of the replicates: replicate 2 ($F = 0.889$, $p = 0.427$), replicate 3 ($F = 0.822$, $p = 0.454$), replicate 4 ($F = 0.057$, $p = 0.945$) and replicate 5 ($F = 1.155$, $p = 0.335$). Therefore, one can assume that individual tanks had no notable effect on the outcome of spot size, regardless of treatment (Table 2.3). There was a main effect of treatment, with a smaller spot size in the turbid treatment compared to the clear treatment ($\beta = -0.18$, $SE = 0.06$, $t = -2.85$, $p < 0.009$; Table 2.4). The main effects for replicates show a decrease in spot area for replicate two ($\beta = -0.11$, $SE = 0.03$, $t = -4.11$, $p < 0.0001$), replicate 3 ($\beta = -0.13$, $SE = 0.03$, $t = -4.98$, $p < 0.0001$) and replicate four ($\beta = -0.18$, $SE = 0.08$, $t = -6.75$, $p < 0.0001$; Table 2.4). Replicate five suggests that the spot area increased in size once all individuals returned to normal aquarium conditions ($\beta = 0.14$, $SE = 0.03$, $t = 5.36$, $p < 0.0001$).

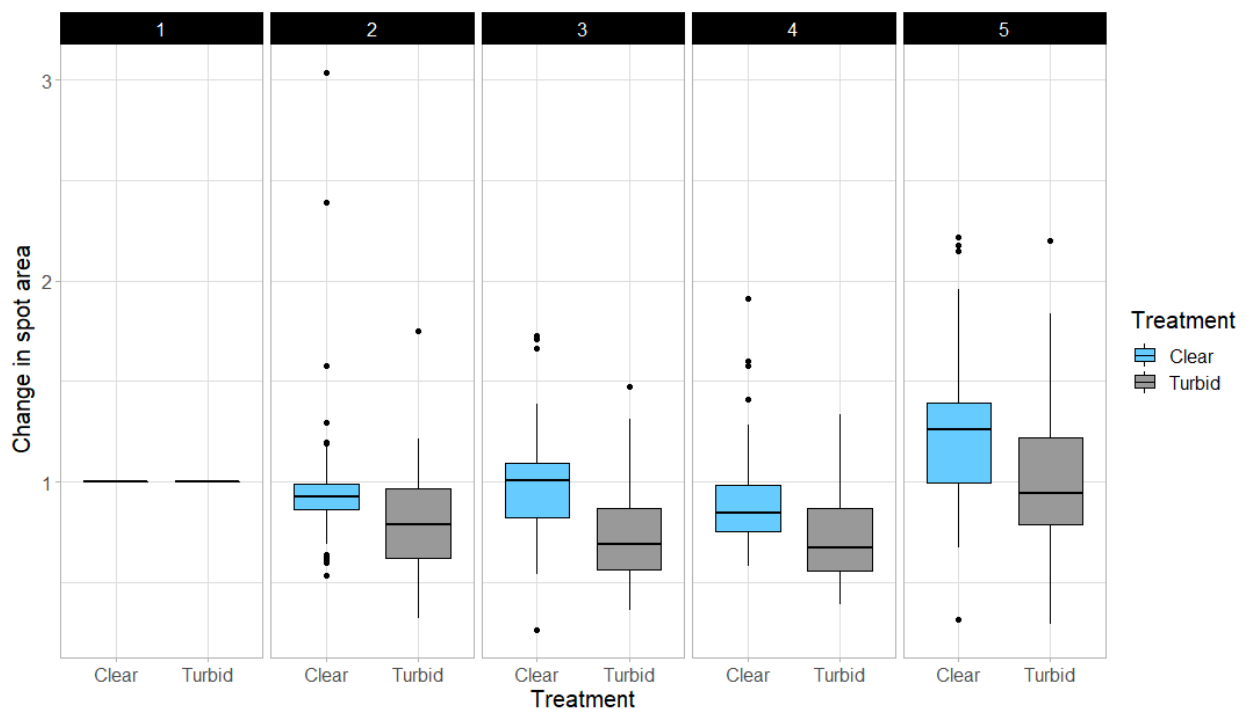


Figure 2.9: Change in spot size: *Pomadasys commersonnii* spot size when exposed to two levels of turbidity over five replicates (indicated by the black bars on top). The 24 *Pomadasys commersonnii* were exposed to either low turbidity levels (blue) or high turbidity levels (grey).

Observed changes in spot sizes within each individual were consistent, regardless of treatment type, suggesting that spot regulation is uniform across the body (Figure 2.10). The changes in spot size for individual A5, exposed to clear treatment, and individual B5, exposed to turbid treatment, show some correspondence in spot size with turbidity values (Figure 2.10). There is an obvious decrease in spot size with an increase in turbidity that is most noticeable from replicate 1 to replicate 2 in the turbid treatments. Individuals from both the clear and the turbid tanks had increased spot size in replicate 5 (Figure 2.11). This increase in spot size for the turbid treatment suggests that their phenotypic plasticity is reversible and in constant flux. The increase in spot size for the clear treatment individuals was unexpected but could be due to all individuals being returned to “normal” or optimal aquarium conditions with cycling water and normal feeding routines.

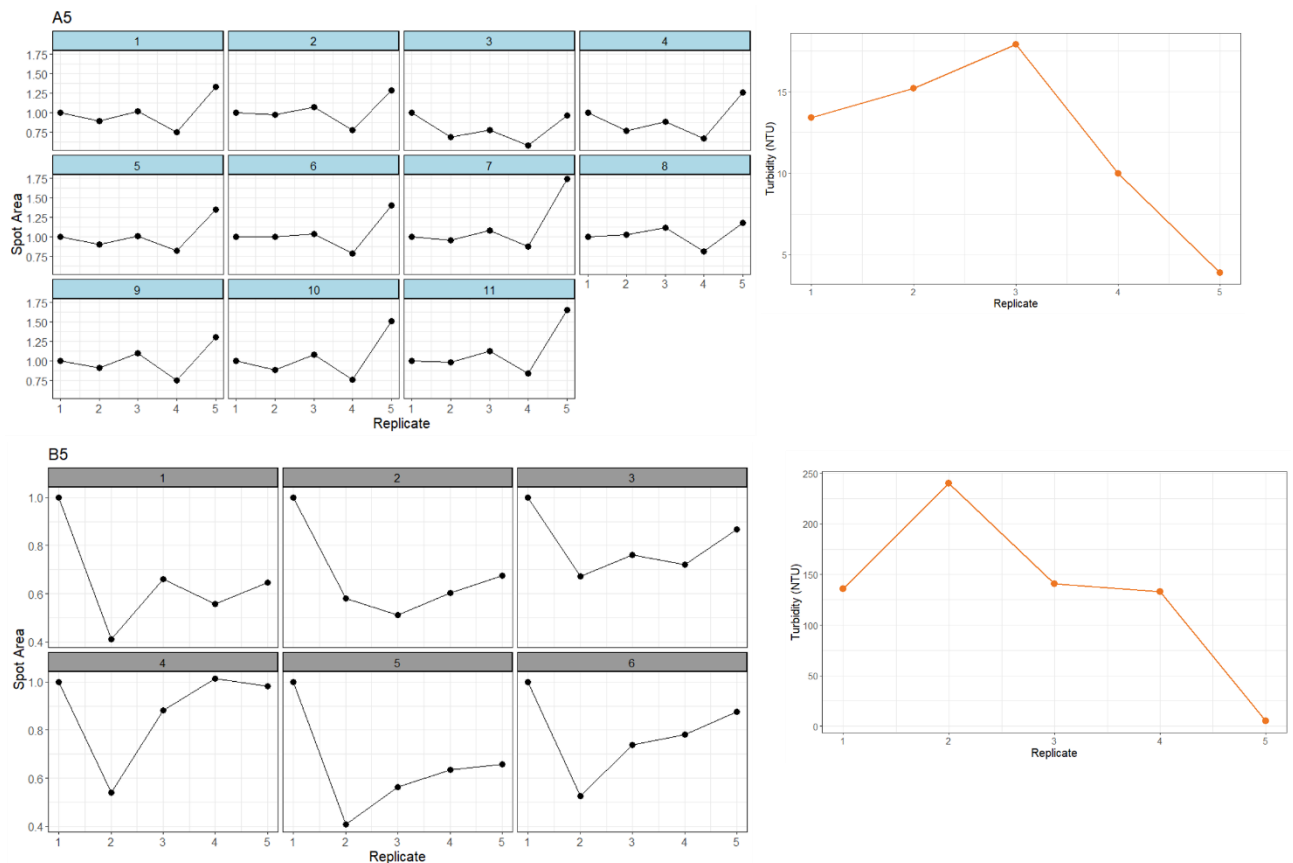


Figure 2.10: Change in size of individual spots: Spot size of two individuals over five replicates exposed to either low (blue) or high (grey) turbidity levels. (Top) Change in the size of 11 spots from individual A5 from the clear treatment Tank A. (Bottom) Change in the size of 6 spots from individual B5 from the turbid treatment tank B. The corresponding turbidity levels for each replicate in each tank are also shown (Right).

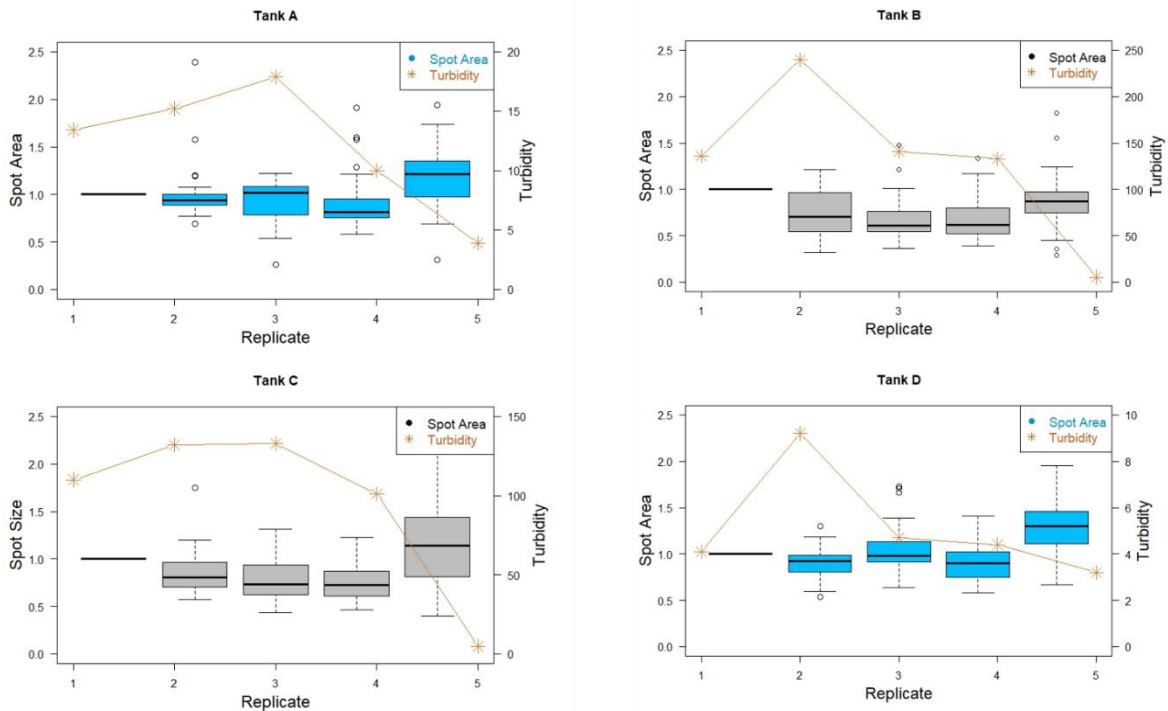


Figure 2.11. Effects of turbidity on the spot size: *Pomadasys commersonnii* spot size from four different treatment tanks over five replicates. Tank A and D (blue) experienced low turbidity levels while tank B and C were exposed to elevated levels of turbidity. The orange stars represent the corresponding turbidity levels for each replicate.

Table 2.3: Repeated measure ANOVA results for changes in spot size for *Pomadasys commersonnii* exposed to one of two treatments in one of four tanks for replicates 2 – 5, as well as an overall analysis including the repeated measures. The sum of squares (SS), mean squares (MS) and degrees of freedom (DF) are given. The non-significant p-value for the tank effect is in bold.

Replicate	Source of variation	DF	SS	MS	F-Value	p-value
2	Treatment	1	1.062	1.062	3.205	0.089
	Tank	2	0.589	0.295	0.889	0.427
	Residuals	20	6.626	0.331		
	Within	124	5.143	0.041		
3	Treatment	1	2.349	2.349	9.223	0.007
	Tank	2	0.419	0.209	0.822	0.454
	Residuals	20	5.094	0.255		
	Within	123	2.796	0.023		
4	Treatment	1	1.195	1.195	4.813	0.040
	Tank	2	0.028	0.014	0.057	0.945
	Residuals	20	4.967	0.248		
	Within	121	2.319	0.019		
5	Treatment	1	1.881	1.881	3.025	0.098
	Tank	2	1.436	0.718	1.155	0.335
	Residuals	20	12.439	0.622		
	Within	123	6.811	0.055		
All	Treatment	1	5.046	5.046	7.588	0.012
	Tank	2	0.964	0.482	0.725	0.497
	Residuals	20	13.301	0.665		
	Error (Replicate)	1	0.662	0.662		
	Within	710	44.7	0.063		

Table 2.4: Effect of turbidity and time on spot size. Results of the LMM model to test the effect of turbidity and time (replicate) on the spot size of *Pomadasys commersonnii*. The standard error (SE), degrees of freedom (DF) and the level of significance are given in each case: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Independent	Predictor	Estimate	SE	DF	t- value	p
Spot Size	Intercept	1.09	0.05	27.99	23.27	***
	Turbid	-0.18	0.06	21.66	-2.85	**
	Replicate 2	-0.11	0.03	706.67	-4.11	***
	Replicate 3	-0.13	0.03	706.68	-4.98	***
	Replicate 4	-0.18	0.03	706.71	-6.75	***
	Replicate 5	0.14	0.03	706.68	5.36	***

ASSESSING THE STABILITY IN SPOT BRIGHTNESS

The spots of fish from the clear treatment tanks were, on average, darker, more saturated and had more positive b^* values compared to those in turbid environments (Table 2.5; Figure 2.12). ANOVA results suggest that turbidity plays an important role in fish colouration. For spot colouration, all measurements except for a^* (red/green) were significantly different between the two treatments while background colouration was only significantly different between the two treatments for lightness (L^* ; Table 2.6). There were no significant differences found in the colouration of fish from different tanks within a treatment ($P > 0.05$; Table 2.5) suggesting no tank effects on fish colouration.

The findings suggest that fish colouration can be actively altered in response to environmental turbidity. The fish from the clear treatment tanks have a greater contrast in their colouration with darker spots containing more red and yellow pigments that would stand out against the clear, blue water environments. The spots from the turbid tank were lighter with lower pigment values, this might suggest these individuals do not expend energy on spot colouration in poor visibility environments.

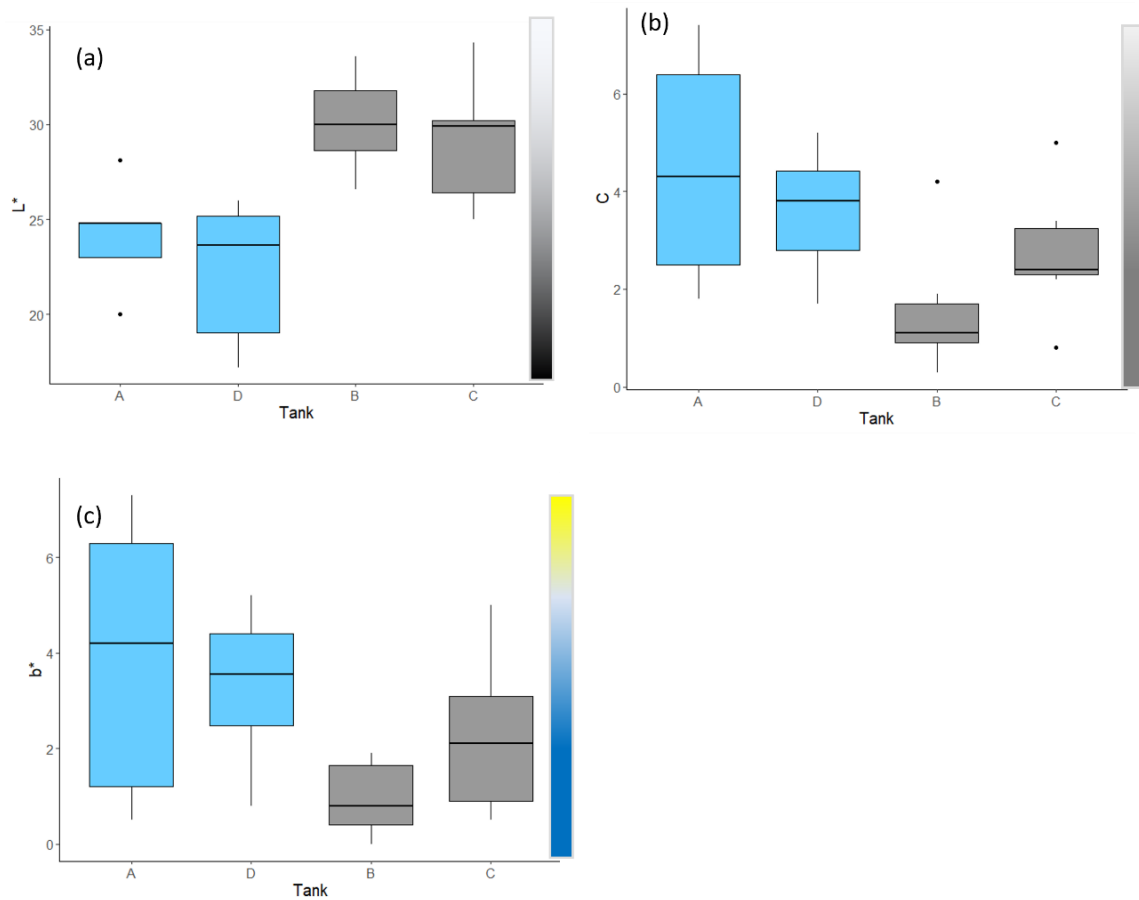


Figure 2.12. Spot colouration measurements: *Pomadasys commersonnii* spot colouration when exposed to two different treatments: turbid (grey) and clear (blue). Three colour measurements are shown: (a) Lightness, (b) Chroma/ Saturation and (c) Blue/yellow pigment. The coloured bars on the right of each graph offer only a visual example of each colour measurement and are not accurate for the given values.

Table 2.5: Spot colouration measurements. Measurements of spot and background colouration of *Pomadasys commersonnii* from four tanks (A-D) exposed to one of two treatments: clear and turbid. L* denotes lightness, a* gives the red/green value, b* gives the blue/yellow value and C denotes saturation. The mean $\pm$ standard deviation (minimum value – maximum value) is given in each case. The p-values for the t-tests for the colour measurements between tanks within a treatment are given.

Measurement	Location	Treatment and Tank			
		Clear		Turbid	
		A	D	B	C
L*	Background	54.58 $\pm$ 12.89 (32.3 – 75.9)	51.02 $\pm$ 13.50 (32.9 – 79.5)	55.52 $\pm$ 8.90 (45.8 – 63.9)	57.08 $\pm$ 8.02 (40.7 – 71.6)
	Spot	24.14 $\pm$ 2.96 (20 – 28.1)	22.28 $\pm$ 3.86 (17.2 – 26)	30.16 $\pm$ 2.60 (26.6 – 33.6)	28.92 $\pm$ 3.28 (25 – 34.3)
	P-value	P > 0.05		P > 0.05	
a*	Background	7.08 $\pm$ 5.0 (0.3 – 16.1)	10.7 $\pm$ 6.5 (4.3 – 21.5)	6.84 $\pm$ 5.9 (0.3-20)	7.43 $\pm$ 5.0 (0.1-16.3)
	Spot	1.36 $\pm$ 0.7 (0.8 – 2.5)	0.92 $\pm$ 0.7 (0 – 1.7)	0.94 $\pm$ 1.3 (0.1 – 3.8)	1.1 $\pm$ 0.8 (0 – 2.1)
	P-value	P > 0.05		P > 0.05	
b*	Background	14.69 $\pm$ 4.9 (7.7- 23.7)	8.36 $\pm$ 7.0 (1.6 – 24.5)	7.13 $\pm$ 6.0 (1.1 – 21.1)	9.23 $\pm$ 7.5 (0.7 – 21.9)
	Spot	3.9 $\pm$ 3.0 (0.5 – 7.3)	3.32 $\pm$ 1.6 (0.8 – 5.2)	0.97 $\pm$ 0.8 (0 – 1.9)	2.23 $\pm$ 1.6 (0.5 – 5)
	p-value	P > 0.05		P > 0.05	
C	Background	16.83 $\pm$ 5.5 (7.9 – 24.4)	14.78 $\pm$ 7.3 (5.8 – 25.2)	10.5 $\pm$ 7.6 (2.6 – 24.7)	12.73 $\pm$ 7.6 (0.7 – 23.2)
	Spot	4.48 $\pm$ 2.4 (1.8 – 7.4)	3.6 $\pm$ 1.3 (1.7 – 5.2)	1.54 $\pm$ 1.3 (0.3 – 4.2)	2.76 $\pm$ 1.3 (0.8 – 5)
	p-value	P > 0.05		P > 0.05	

Table 2.6: ANOVA results for fish colouration measurements. Results from eight ANOVA tests comparing four measurements of fish spot and background colouration of *Pomadasys commersonnii* exposed to two different treatments. L* denotes lightness, a* gives the red/green value, b* gives the blue/yellow value and C denotes saturation. Degrees of freedom (DF), the sum of squares (SS) and mean sum-of-squares (MS) and the level of significance are given in each case: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Measurement	Source of Variation	Spot					Background				
		DF	SS	MS	F-value	p	DF	SS	MS	F-value	p
L*	Treatment	1	253.00	252.98	25.38	***	1	2644	2643.90	4.72	*
	Residuals	23	229.30	9.97			21	11770	560.5		
C	Treatment	1	21.08	21.08	8.24	**	1	11.6	11.60	0.25	0.62
	Residuals	23	58.84	2.56			21	981.1	46.73		
b*	Treatment	1	24.19	24.20	7.42	*	1	6	5.99	0.15	0.71
	Residuals	23	74.96	3.26			21	855.2	40.72		

2.3.5 Discussion

The following four questions were examined in the above experiment:

- I. *Do spotted grunter change the number of spots displayed in response to increased levels of turbidity?*
- II. *Do spotted grunter change the shape/ area of their spots in response to increased levels of turbidity?*
- III. *Do spotted grunter change the lightness of their spots in response to increased levels of turbidity?*
- IV. *If spotted grunter change their spots in response to increased levels of turbidity, can this change be reversed in low turbidity environments?*

The results from the proof-of-concept experiment suggest that the number of spots displayed by spotted grunter are consistent regardless of turbidity levels. However, there is a degree of phenotypic plasticity with regard to spot size and colouration. The experiment demonstrated that the spot patterns of spotted grunter may be used to identify individuals due to the persistence of spots across treatments. Furthermore, the two assumptions required for natural body markings to be used for long-term monitoring were met: (1) the body markings must be distinguishable between individuals and (2) the markings need to remain constant over time (Kristensen *et al.*, 2020). While the spot patterns persist over time, the high degree of plasticity in response to turbidity levels is a key finding for this study. The experiment shows that when exposed to higher levels of turbidity spotted grunter respond by decreasing the size of their spots and adjusting the lightness of their colouration. The reduced spot size and lighter, less pigmented colouration of spots in the high turbid treatment is most likely both an energy-saving tactic as well as a background-matching response (Rodgers *et al.*, 2013; Polo-Cavia and Gomez-Mestre, 2017). Lastly, the experiment demonstrated that the change in spot patterns can be reversed when the environment is altered. This suggests that this level of phenotypic plasticity is not developmental in nature but rather an acclimation/modulation process with continuous phenotypic variation. An implication of this continuous plasticity is that these spot patterns may not be suitable for stock identification, however, the local adaptation to environmental conditions is still an important aspect to explore. The above results support our hypothesis that the spotted grunter from the turbid Kei Estuary will display smaller, duller spots compared to those in the less turbid Breede Estuary.

2.4 Methods and Materials

2.4.1 Data Collection

Data collection took place at both the Breede Estuary in the Western Cape and the Kei Estuary in the Eastern Cape. A total of 158 (76 in Breede; 63 in Kei) spotted grunter were photographed from these two estuaries. Breede sampling was conducted in September 2018, April 2019 and October 2019. Kei sampling took place in October 2018, June 2019 and January 2020. Spotted grunter were captured via a beach seine net, the total length (TL) and fork length (FL) of each fish was recorded and a fin clip was taken. A full-body photograph of the left, lateral side of each fish was taken with a scale card and against a black background. Ethics permit numbers: 2018/v10/SK, RES2020/86, RES2021/56, RES2022/72.

2.4.2 Image Analysis – Quantification of Spot Patterns

Spot patterns of individuals were analysed using **ImageJ**, an open-source, Java-based program specifically designed for image processing and analysis in the scientific field (Abràmoff, Magalhães and Ram, 2004). Each image is analysed independently from start to finish.

IMAGE ENHANCEMENT AND SCALING

Each image is first length-calibrated (in cm) using the '*Set scale*' command which converts the pixel values to the specified unit using your given scale factor (Figure 2.13) using the scale card beside each fish in the photographs. The images were then cropped to focus on the spot patterns and converted to 8-bit greyscale and a bandpass filter (Fast Fourier Transform) was applied to enhance the contrast of the images (Figure 2.13(3)). To further enhance the contrast of the spots, the images were thresholded to create a binary image. This was done using the **ImageJ** '*Adjust*' → '*Threshold*' function. Low quality images were discarded at this point.

SPOT DETECTION AND PARTICLE ANALYSIS

A polygon was drawn around the spot patterns in each fish to create a region of interest that will be used to analyse spot measurements (Figure 2.13(5)). Using the 'set measurements' function, one can select the measurements to measure for each spot in the region of interest. The following six measurements were selected: Area, Perimeter, Bounding rectangle, Fit ellipse, circularity and Feret's diameter (Table 2.7). Once the measurements have been selected, the 'Analyze particle' function is selected. An outline image is produced to relate the spot measurements to the specific spot on each fish. The images are saved and the measurements are exported as CSV files.

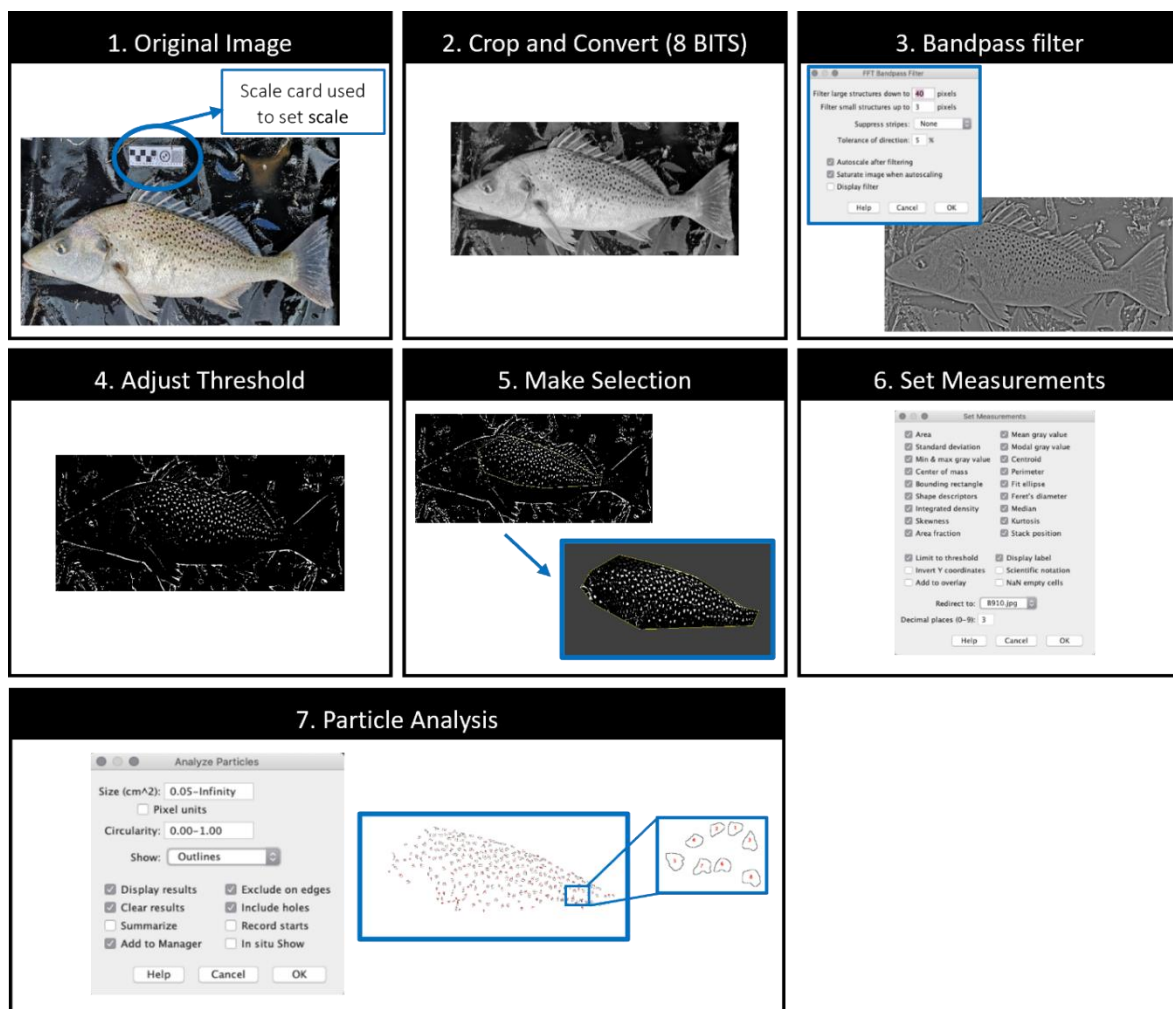


Figure 2.13: Particle analysis: Flow chart to show the steps taken to conduct the spot analysis of *Pomadasys commersonnii* in ImageJ.

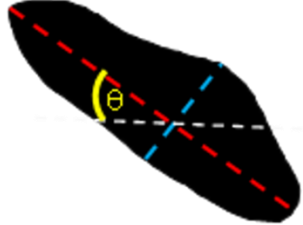
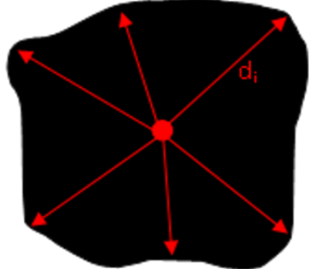
Table 2.7: ImageJ ‘Particle Analysis’ Measurements used to analyse the spot patterns of a fish species: *Pomadasys commersonnii*. A description and depiction are given for each measurement.

Measurement	Description	Diagram
Area	The area of each spot is calculated in cm ²	
Perimeter	The length of the outside boundary of each spot is calculated in centimetres.	
Bounding rectangle	There are two measurements taken for this measurement: Width and Height. This is the measurement of the smallest rectangle enclosing the selection.	
Fit Ellipse	The best fitting ellipse to each spot. Measurements given include the primary (Major) and secondary (Minor) axis of the best-fitting ellipse.	
Circularity	A value to indicate the circularity of each spot where a perfect circle will have a value of 1 and elongated shapes will have values closer to 0. Equation 2.2: $Circularity = 4\pi \frac{Area}{Perimeter^2}$	
Feret's Diameter	The longest distance between any two points on the spot boundary.	

PAT-GEOM PLUGIN

PAT- GEOM is an open-source **ImageJ** plugin that measures seven properties of animal patterns: marking shape, marking size, marking distribution, marking randomness, marking shape directionality, marking distribution directionality and pattern contrast (Chan, Stevens and Todd, 2019). Not every measurement is suitable, depending on the particular animal patterns, and only three of the seven measurements were considered suitable for spotted grunter spot patterns. The three measurements used for this analysis include marking shape (Elliptical Fourier analysis) which approximates the shape of each spot using harmonically related trigonometric functions, marking size which produces an independent measure of size by calculating the average centroid size and marking shape directionality which measures how elongated a marking is as well as patterns overall direction of orientation (Table 2.8; Chan, Stevens and Todd, 2019).

Table 2.8: PAT-GEOM measurements selected for spot pattern analysis of *Pomadasys commersonnii*. PAT-GEOM is a free open-source ImageJ plugin.

Measure	Description	Depiction
Marking shape – Elliptical Fourier analysis	Approximates the outline of each spot using a specified number (20) of harmonics. Each harmonic describes the shape of the marking using a numerical descriptor. The greater the number of harmonics (n), the more detailed the outline.	
Marking shape Directionality	Aspect ratio: calculated by dividing the length of the minor axis (blue) by the length of the major axis (red). Orientation (θ): a measure of the angle between the major axis and a horizontal line (white) across the spot.	
Centroid size	Average centroid size of each spot. This is calculated using the distance (d) from the centroid to the boundary of each spot by a given number of points (N). Equation 2.3: $= \frac{1}{N} \sqrt{\sum_{i=1}^N d_i^2}$	

2.4.3 Statistical Analysis

All statistical analyses were conducted in **R (v.4.2.0)** and **R studio** (R Core Team, 2020). Despite best efforts, there was a lack of comparable size ranges (fork length) of spotted grunter captured in the two estuaries. Therefore, the mean fork lengths were tested for significance between the two estuaries with a non-parametric Mann-Whitney U test.

A Kernel density estimate (KDE) was performed on the length data to further compare size structure between the two estuaries. KDEs are a non-parametric method used to estimate the probability density function of fork lengths between the two estuaries (Samiuddin and El-Sayyad, 1990; Stanislaw Weglarczyk, 2018). It overcomes the problem of using discrete bins to analyse continuous variables by estimating the shape of the frequency distribution $f(x)$ by using kernel (K) and smoothing parameter (h):

$$\hat{f}(x) = \frac{1}{nh} \sum_{i=1}^n K\left(\frac{\hat{x} - x_i}{h}\right) \quad \text{Equation 2.4}$$

Where K is the kernel, h is the bandwidth (smoothing parameter), and n is the number of observations. The kernel density was fitted using the '*ggdensity*' function in the R package '*ggplot2*'.

ALLOMETRIC EFFECTS ON SPOT MEASUREMENTS

A correlation matrix was conducted to summarize the correlation coefficients between measured variables. Fish size was included in this matrix to identify allometric patterns in the data. The Pearson correlation coefficients were calculated using a significance level of $p < 0.05$ and a correlation matrix was constructed between the following measurements: fork length, area, perimeter, circularity, centroid size, aspect ratio, Feret angle, Feret diameter and number of spots.

Morphometric analyses are sometimes complicated by allometry i.e., how morphometric characteristics change as an individual grows (Leonart, Salat and Torres, 2000). Fish size has been shown to affect morphological features such as otolith size and body weight. Therefore, it is essential to evaluate whether fish size has an influence on spot patterns. Thus, a simple linear regression was created to visualize whether fork length can be used to predict the number and size of spots in spotted grunter. To ensure an equal overlap in size ranges between the two populations, the regression analyses were restricted to individuals in the following size range: 300mm < fork length < 480mm. The assumptions of normality, linearity and homoscedasticity were met for the above analyses.

DO THE SPOT PATTERNS OF SPOTTED GRUNTER DIFFER BETWEEN THE TWO ESTUARIES?

Summary statistics were calculated for the spot measurements of spotted grunter from each estuary and the variance about the means was tested for significance. As the assumption of normality was not met, non-parametric Mann-Whitney U tests were used. Due to the correlation between fish size and spot measurements, all measurements were standardized by the individual's fork length to remove the effect of body size for the remainder of the analysis.

A principal component analysis (PCA) allows one to visualize the data by reducing the dimensionality of multivariate data (Daffertshofer *et al.*, 2004). By condensing the data, the PCA aims to display explained variation in the data through principal components. The PCA assumes the directions in the data with the greatest variances are the most influential thereby identifying which variables contribute most to the variability between areas. The PCA was calculated with the R function '*prcomp*' and graphically displayed showing the first two principal components, PC1 and PC2 respectively. All variables were standardized and normalized prior to analysis. The following variables were included in the PCA: perimeter, circularity, centroid size, aspect ratio and number of spots.

Analysis of similarity (ANOSIM) was performed to test for significance between the spot patterns of spotted grunter from the two estuaries. This non-parametric test is based on distance measures that are used to compare two or more samples (Clarke, 1993). The ANOSIM

was performed on the length-standardized spot measurements and the R statistic produced by the ANOSIM measures the degree of separation between the two estuaries. An R-value of zero would indicate no difference between the spot measurements of an individual from the two estuaries whereas an R-value of one would indicate that the spot measurements are statistically different.

The ANOSIM R statistic is calculated as:

$$R = \frac{(r_B - r_W)}{\frac{(N(N-1))}{2}} \quad \text{Equation 2.5}$$

Where R is the difference of mean ranks between groups (r_B) and within groups (r_W) and N is the number of samples (Clarke, 1993).

Permutational multivariate analysis of variance (PERMANOVA) was conducted to calculate the Bray-Curtis distances between samples. This is a non-parametric, dissimilarity measure which operates on a distance matrix. The R function '*vegdist*' from the R package '*vegan*' calculates the ratio of the variation between centroids to the variation within clusters. Similarity percentages (SIMPER) were estimated to determine which spot measurement contributed the most to the dissimilarities between the two estuaries i.e., it estimates the proportion that each spot measurement contributes to the Bray-Curtis distance between the two estuaries. The simpler analysis was performed using the function '*simper*' from the R package '*vegan*.'

2.5 Results

The lateral side of 139 spotted grunter from two disparate estuaries was analysed for spot patterns: 76 from the Breede Estuary and 63 from the Kei Estuary. Only individuals whose photographs displayed clear, visible spots with minimal glare were analysed. Some smaller individuals lacked spots and a few individuals were discarded due to injuries that morphed a large section of spots (Figure 2.14).

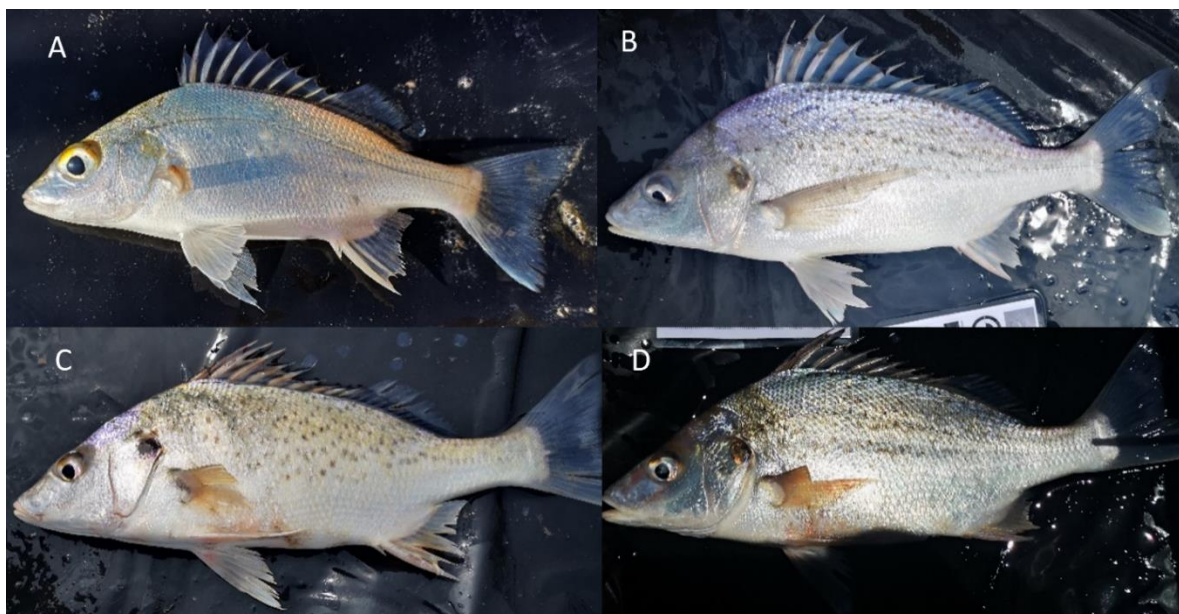


Figure 2.14. Examples of discarded photos: Unsuitable photos for analysis of spot patterns. (A) Small individual with no spots, (B) Glare makes it difficult to enhance spots for analysis, (C) individual has damage to the skin which alters the spot patterns and (D) Glare on skin makes it impossible to analyse spot patterns.

Table 2.9. *Pomadasys commersonnii* size range. Summary of *Pomadasys commersonnii* fork lengths (mm) from two estuaries. The Mann Whitney U-test level of significance is given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Estuary	Size range (Fork length – mm)	Mean $\pm$ Standard deviation	p-value
Breede	298 – 615	414.9 $\pm$ 89.35	***
Kei	121 – 585	332.4 $\pm$ 92.02	

There was a significant difference in the size ranges of spotted grunter from the two estuaries ($p < 0.001$, Table 2.9) with the Breede Estuary being composed of larger individuals (Figure 2.15). The 79 spotted grunter from the Breede Estuary had fork lengths ranging from 298mm to 615mm (414.9 ± 89.35) and the 63 spotted grunter from the Kei Estuary ranged from 121mm to 585mm (332.4 ± 92.02).

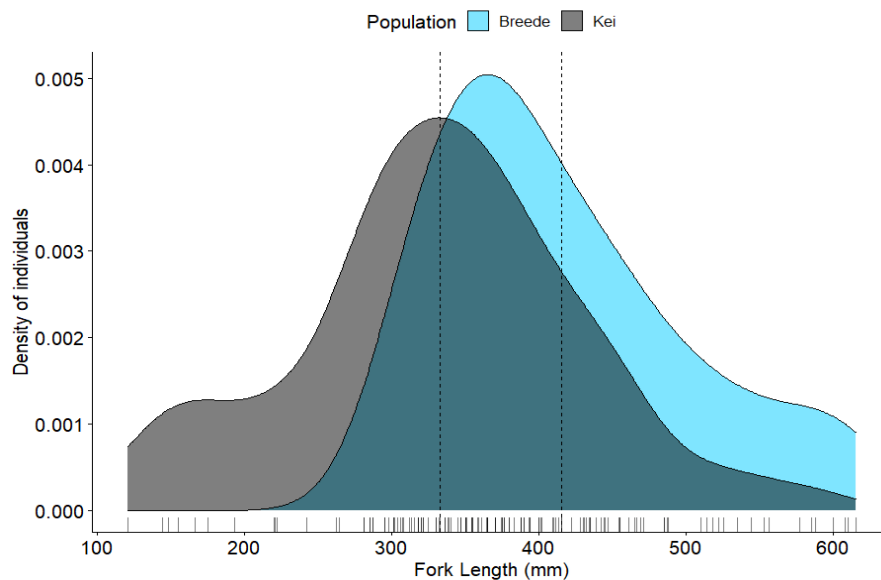


Figure 2.15. Kernel density estimate (KDE) of fork lengths: *Pomadasys commersonnii* fork lengths from two different areas- Kei Estuary (grey) and Breede Estuary (blue). The dotted lines represent the mean fork length for each sample and the rug lines above the x-axis represent each fish measured in the study.

2.5.1 Allometric Effects on Spot Measurements

There were strong correlations between fish size and the following spot measurements: number of spots ($r = 0.82$), area ($r = 0.81$), perimeter ($r = 0.71$), Feret diameter ($r = 0.8$) and centroid size ($r = 0.63$; Appendix 2.1). Simple linear regression was used to assess if fish size significantly affected the number and area of spots. The overall regression was significant for both number of spots ($R^2 = 0.32$, $F_{1,95} = 45.69$, $p < 0.001$; Appendix 2.2) and area of spots ($R^2 = 0.24$, $F_{1,95} = 31.38$, $p < 0.001$; Appendix 2.3) with fish size significantly affecting these two characteristics ($p < 0.001$). The linear relationship for fish size and number of spots between

the two estuaries suggests that spotted grunter from the Breede Estuary have more visible spots than those from the Kei Estuary (Figure 2.16).

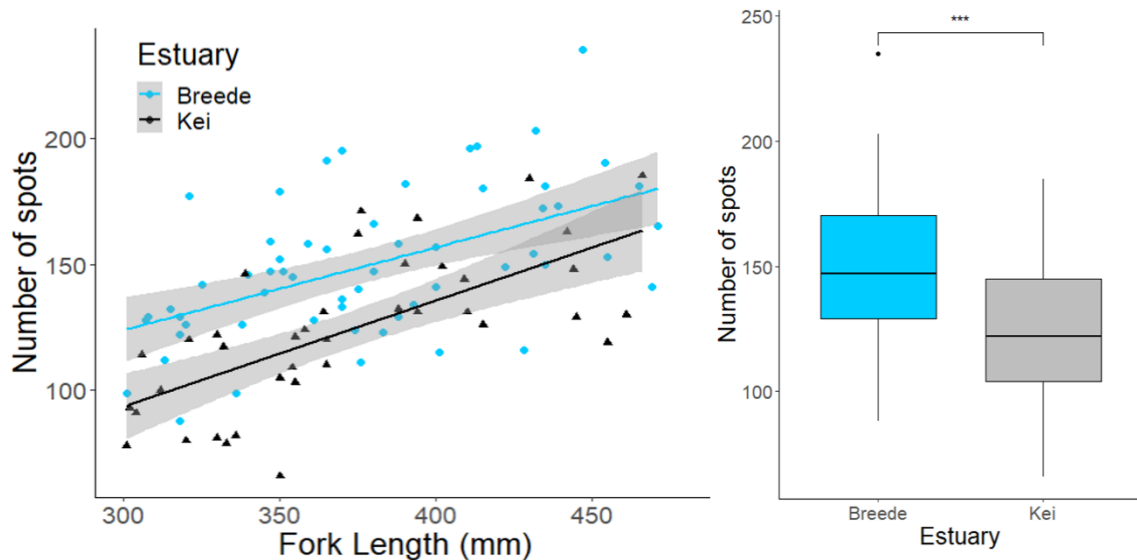


Figure 2.16. Number of spots in *Pomadasys commersonnii*: (Left) Linear relationship between fork length and the number of spots of spotted grunter from two estuaries- Breede (blue) and Kei (black). A 95% confidence interval for each regression is shown. (Right) Box plot showing a significant difference in the number of spots between these two estuaries.

2.5.2 Comparison of Spot Measurements

When standardized to account for size differences there was a significant difference in the number of spots displayed by spotted grunter between the two estuaries ($p < 0.001$). On average, the spotted grunter from the Breede Estuary displayed 163.16 ± 41.08 spots compared to 107.79 ± 37.41 spots displayed by individuals from the Kei Estuary (Appendix 2.5). The spots of spotted grunter from the Breede Estuary were significantly wider ($p < 0.001$), with larger centroid sizes ($p = 0.04$) and longer perimeters ($p = 0.03$) compared to the spots of spotted grunter from the Kei Estuary. In comparison, the spots of spotted grunter from the Kei Estuary were more circular ($p < 0.001$), with a greater aspect ratio ($p < 0.001$) and angle of orientation ($p < 0.001$; Appendix 2.5).

The principal component analysis represents the variation in spot patterns between the length-standardized spot measurements. Figure 2.17 represents the difference in the spot patterns from the two estuaries by plotting the first two principal components. The first two principal components explained a large amount of variation in the spot patterns of the two estuaries with a total of 80.7% (57.26% and 23.44% respectively). The Breede Estuary individuals are tightly clustered together compared to the Kei Estuary which has a much broader distribution of points (Figure 2.17). The biggest contributing variables for PC1 were aspect ratio and number of spots, while the biggest contributing variable for PC2 was perimeter and circularity (Appendix 2.4).

According to the Similarity Percentages (SIMPER) results the top contributing measurements to the dissimilarity in spot patterns between the two estuaries are centroid size (92.8%) and angle of orientation (6.8%). The analysis of similarity (ANOSIM) output was statistically significant with an R statistic of 0.07 which suggests that while the diversity of spotted grunter spot measurements are significantly different between the two estuaries, there is an even distribution of ranks both within and between areas (R stat = 0.07, significance = 0.001). The p-value for the PERMANOVA was also statistically significant ($p = 0.01$), suggesting differences in the group means of spot measurements between spotted grunter from these two estuaries, however, the R^2 values were very low which indicates that the estuary only accounts for 4% of the variation seen in the data (Appendix 2.6). The multivariate dispersion test was statistically significant and the two estuaries' dispersions were not homogenous ($F = 12.014$, $p = 0.002$; Appendix 2.7).

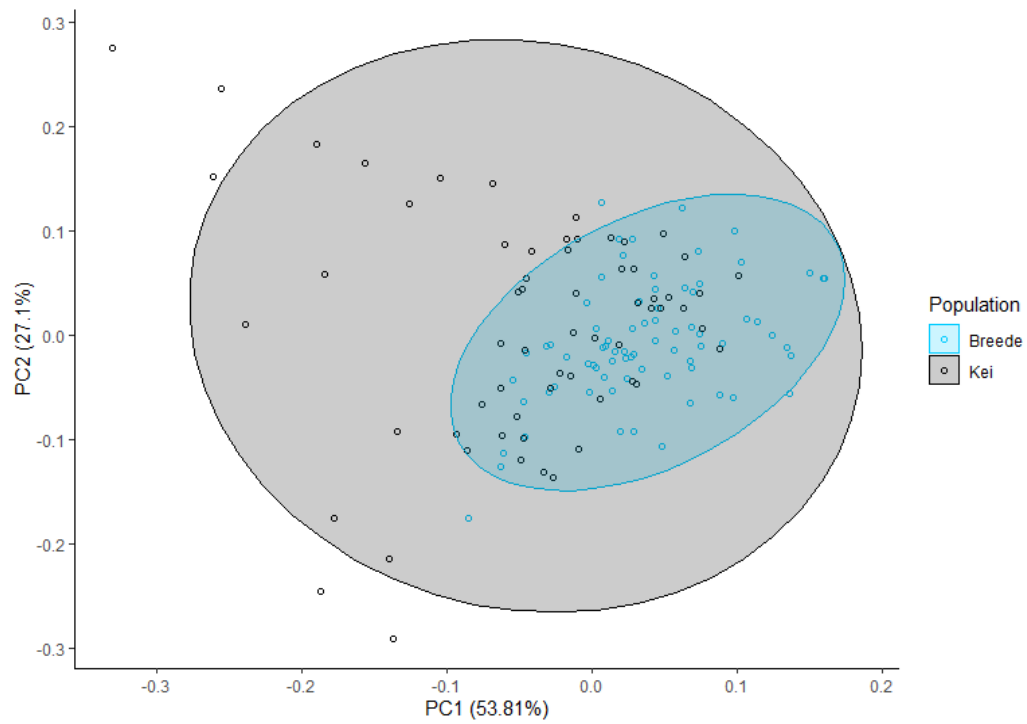


Figure 2.17. Spot pattern PCA: The first two axes of spot measurements from *Pomadasys commersonnii* spot patterns from two estuaries: Breede (blue) and Kei (grey) with 95% probability ellipses shown. Each point represents an individual fish.

2.6 Discussion

This study provides the first evaluation of spot patterns in spotted grunter, *Pomadasys commersonnii*. The aim was to investigate whether spotted grunter from two estuaries in different biogeographic regions, with contrasting turbidities, will differ in their spot patterns. Results demonstrated that there are notable differences in the spot patterns of fish from the two different estuaries.

The proof-of-concept study is the first study of its kind to investigate the short-term changes of spot patterns in spotted grunter. In the experiment, the number of spots displayed by spotted grunter did not change in response to increased turbidity. This persistence in spot patterns makes them suitable for long-term monitoring (Merz *et al.*, 2012) as being able to successfully identify individuals within a population is useful for demographic studies. Traditionally, invasive tagging methods have been used to identify individuals for capture-mark-recapture research (Mettouris, Megremis and Giokas, 2016). However, technological advances have made photographic identification a possible non-invasive, low-cost alternative for individual identification (Carpentier *et al.*, 2016). Studies using natural markings to identify individuals have been widely successful across animals including fish (McInnes *et al.*, 2020), amphibians (Mettouris, Megremis and Giokas, 2016; Glimm *et al.*, 2021), crustaceans and mammals (Nipko, Holcombe and Kelly, 2020; Topličanec *et al.*, 2022).

The stability of spot presence over time, regardless of turbidity, has been well documented in other species but it is important to note that there was a positive relationship between fish size and the number of spots observed in fish from both estuaries. As a fish grows, the surface area of the skin increases which not only 'stretches' their existing patterns but also provides an opportunity for new spots to develop. This phenomenon has been found in other species of fish such as Salmon (Stien *et al.*, 2017; Jørgensen *et al.*, 2018) and trout (Kocabaş *et al.*, 2011) where, although the number of spots increased throughout an individual's life, the initial spots from early life stages remained constant. Atlantic salmon, *Salmo salar*, develop new spots as they mature and grow, however, their initial spots (visible at the start of the experiment) remain consistent over time and are the most prominent of spots (Stien *et al.*, 2017).

Additionally, there was a significant difference in the number of spots observed in spotted grunter from the two estuaries. Differences in the number of spots produced by individuals within a species are not uncommon and environmental conditions are known to affect spot patterns. In *Oncorhynchus mykiss* and *Salmo salar*, the number of spots observed has been linked to an individual's physiological response to environmental stress (Kittilsen *et al.*, 2009). The results from this thesis suggest that the emergence of spots may be influenced by turbidity levels during the developmental program of spotted grunter. There is not yet evidence of when spot ontogenesis begins in spotted grunter, however, other species have been shown to develop spots around 197 – 230 days from conception (Merz *et al.*, 2012). Juveniles of estuarine-dependent species show a preference for turbid environments, such as the Kei Estuary, probably due to the reduced visibility which provides a natural camouflage while they mature (Cyrus and Blaber, 1987). The Kei Estuary sample consisted of predominantly small individuals with minimal to no spots and due to the reduced visibility in the Kei Estuary, it may not be necessary to expend energy on the development of spot patterns (Rodgers *et al.*, 2013; Polo-Cavia and Gomez-Mestre, 2017). On the other hand, individuals in the Breede Estuary may need to develop spots sooner as a method of communication or camouflage. The above hypothesis aligns with the following result: in the turbid Kei Estuary, the spotted grunter produced fewer spots, for a given size range, compared to those in the clearer water of the Breede Estuary.

The proof-of-concept also highlighted the high degree of plasticity in spotted grunter spot patterns. Spotted grunter can alter the size of their spot patterns in response to changes in turbidity with larger spot sizes appearing in low-turbidity environments. Likewise, spot size (linked to centroid size) contributed most to the dissimilarity of spot patterns between individuals sampled from the two estuaries. The Breede Estuary spotted grunter have, on average, larger, more elongated spots compared to the spotted grunter from the Kei Estuary. In high-visibility environments, the detection distance would be much greater than in turbid environments. Therefore, the larger spots produced by spotted grunter in the Breede Estuary can be seen over a greater distance. In addition to size, another factor that would affect spot detection is the intensity of spot colouration.

Pigment changes are considered to be a natural response to light conditions (turbidity) or are a means of adapting to habitat changes through different life stages (Carr, 1969). The development of fish patterns is gradual and can be influenced by environmental conditions (Polo-Cavia and Gomez-Mestre, 2017; Salis *et al.*, 2019). The proof-of-concept demonstrated how spotted grunter alter the lightness and/or pigmentation of their spots in response to changes in turbidity. This finding is consistent with (Urho, 1996) where the pigmentation patterns of perch were documented to be much duller in turbid waters compared to those in non-turbid waters. Whether this alteration is under morphological or physiological control is unknown, however, the proof-of-concept results show that these colour changes are reversible and do not fall under polyphenism (Polo-Cavia and Gomez-Mestre, 2017; Yang and Pospisilik, 2019). Unfortunately, spot colouration was not measured in the field but changes in the degree of pigmentation may provide more insight into spotted grunter spot patterns and this would be a beneficial next step in understanding the role that turbidity plays in spot patterns.

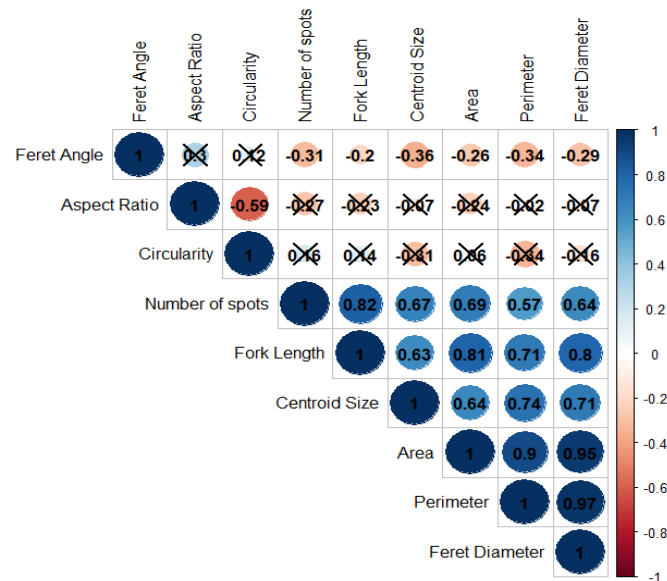
Phenotypic plasticity is common in fish species and variation thereof is often a result of genetic factors, environmental factors or a combination of the two. In some fishes, such as *Salmo trutta macrostigma* (the brown trout) spot patterns and colouration have had a genetic basis (Kocabaş *et al.*, 2011). Aparicio *et al.*, (2005) found that three genotypes of brown trout had significant differences in body spot patterns. Whether the observed differences in spotted grunter spot patterns have a genetic basis is unknown, however, it is still important to consider for the identification of adaptive stocks. Whether these spot patterns evolved as a method of camouflage or communication remains unclear. In terrestrial carnivores, spotted coats evolved primarily for camouflage by background matching (uniformly dark pattern) and spots have been suggested to provide good camouflage in aquatic environments (Ortolani, 1999). Similarly, it has been suggested that spot patterns provide a disruptive effect which breaks the boundary of an individual thereby confusing the observer (Duarte, Flores and Stevens, 2017). Price *et al.*, (2008) proposed that horizontal stripes in fishes may assist in identifying school mates both within and among species; viewed from a distance the spots of spotted grunter may appear to form horizontal stripes or, as an animal moves the spots would appear as stripes. These suggestions would further motivate the hypothesis that spotted grunter living in turbid

environments rely less on their spot patterns compared to those living in less turbid environments.

Image analysis of spotted grunter spot patterns has proved to be successful for identifying individuals and for quantitatively analyzing spot patterns. This is a positive step for citizen science as spotted grunter are a popular angling species and it would be easy for avid fishermen to upload photos of their spotted grunter catches. Further improvements to the study include following the development of spot ontogenesis in spotted grunter with long-term studies that monitor spot changes over time. More in-depth studies that investigate the physiological change in spot colour or the degree of pigment expression between stocks would be beneficial for stock studies but also as a possible marker for environmental change as we deal with increased pollution in our estuaries. A drawback to this study was the high number of spotted grunter with skin abrasions from the seine net, a less-invasive capture method which preserves the integrity of the fish is encouraged. Low-quality photos remain an issue for all image analysis studies as angles, lighting and camera quality can all affect the outcome of the results (Merz *et al.*, 2012).

In conclusion, this study provided the first attempt to analyse spot patterns in spotted grunter and highlights the effectiveness of spot pattern analysis in both individual identification and as a method to compare phenotypic variation between estuaries. The results show, as hypothesised, that there are significant differences in the spot patterns of spotted grunter from two estuaries with differing environmental regimes. This provides a platform to expand our knowledge on how spotted grunter respond to environmental changes. This is valuable for stock delineation and subsequent fisheries management. The exact reason for the differences in spot patterns has yet to be determined and whether these differences have a genetic basis or are simply a result of phenotypic plasticity remains unknown. Regardless, this study provides the first research on the spot patterns of spotted grunter in South Africa and suggests that environmental conditions, such as turbidity, may play an important role in the phenotypic variation of this species.

2.7 Appendix



Appendix 2.1: Correlation matrix of eight spot measurements and their correlation to fish size (fork length). The colour and sizes of the circles depict the correlation value with darker, larger circles showing high correlation while smaller, lighter circles show low correlation values. A cross is given to those correlations with non-significant p-values.

Appendix 2.2: Linear regression output to show the effects of fish size on the number of spots displayed by *Pomadasys commersonnii*. The level of significance is given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

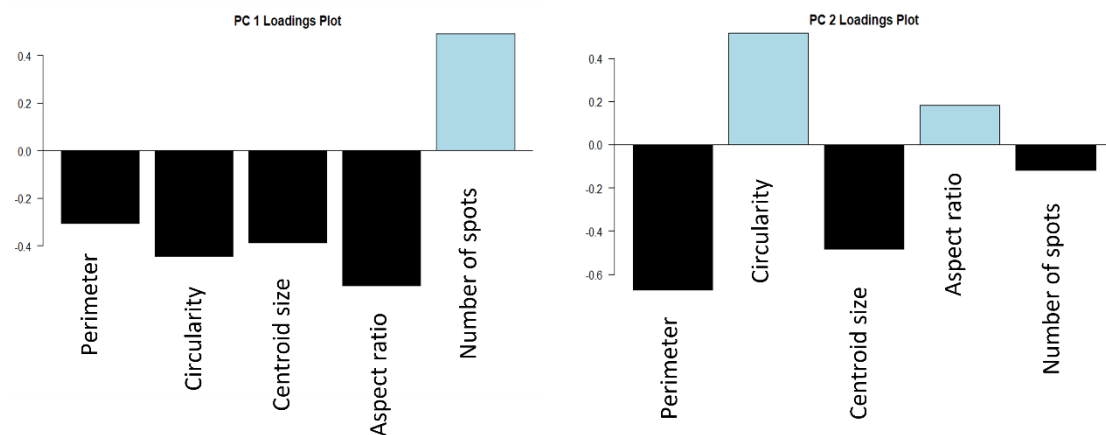
Regression statistics	
Adjusted R ²	0.32
F- statistic	45.69
Degrees of freedom	1 and 95
p-value	***

	Coefficients	Standard Error	t value	p-value
Intercept	-5.81	21.61	-0.27	0.788
Fork Length	0.39	0.06	6.76	***

Appendix 2.3: Linear regression output to show the effects of fish size on spots area displayed by *Pomadasys commersonnii*. The level of significance is given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Regression statistics	
Adjusted R ²	0.24
F- statistic	31.38
Degrees of freedom	1 and 95
p-value	***

	Coefficients	Standard Error	t value	p- value
Intercept	-1.07e-02	1.65e-02	-0.64	0.525
Fork Length	2.454-04	4.37e-05	5.60	***



Appendix 2.4. PCA loadings for the first two principal components of *Pomadasys commersonnii* spot pattern measurements. This shows the contribution that each measurement gives to the principal component in each case.

Appendix 2.5: Spot measurements of *Pomadasys commersonnii* captured from two estuaries: The Breede and the Kei Estuary. The level of significance for each measurement is given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Measurement	Mean $\pm$ Standard Error (Min-Max)		p-value
	Breede	Kei	
Number of spots	163.20 $\pm$ 41.08 (88 – 255)	107.8 $\pm$ 37.41 (29 – 186)	***
Area	0.10 $\pm$ 0.04 (0.02 – 0.71)	0.079 $\pm$ 0.03 (0.01 – 0.16)	0.080
Perimeter	1.58 $\pm$ 0.31 (0.91 – 2.56)	1.36 $\pm$ 0.40 (0.43 – 2.37)	*
Width	0.40 $\pm$ 0.08 (0.25 – 0.65)	0.35 $\pm$ 0.10 (0.12 – 0.54)	***
Height	0.44 $\pm$ 0.08 (0.27 – 0.67)	0.37 $\pm$ 0.10 (0.12 – 0.61)	0.133
Major	0.44 $\pm$ 0.08 (0.29 – 0.70)	0.38 $\pm$ 0.10 (0.13 – 0.59)	**
Minor	0.26 $\pm$ 0.05 (0.17 – 0.39)	0.22 $\pm$ 0.06 (0.08 – 0.36)	0.193
Circularity	0.50 $\pm$ 0.08 (0.29 – 0.70)	0.49 $\pm$ 0.09 (0.30 – 0.73)	***
Feret's diameter	0.52 $\pm$ 0.10 (0.32 – 0.80)	0.47 $\pm$ 0.12 (0.15 – 0.70)	*
Feret's Angle	102.14 $\pm$ 7.05 (81.09 – 121.34)	110.51 $\pm$ 8.02 (92.23 – 125.70)	***
Centroid size	2706 $\pm$ 433.43 (1754 – 4288)	2327.2 $\pm$ 680.90 (998.5 – 3787)	*
Aspect ratio	1.75 $\pm$ 0.13 (1.52 – 2.2)	1.84 $\pm$ 0.16 (1.59 – 2.34)	***
Angle of orientation	99.17 $\pm$ 6.80 (78.34 – 113.56)	107.12 $\pm$ 8.00 (91.56 – 122.57)	***

Appendix 2.6: PERMANOVA Results. Permutational multivariate analysis of variance for the spot measurements of *Pomadasys commersonnii* from two estuaries. Tests are based on Bray-Curtis dissimilarity distances with 999 permutations. Degrees of freedom (DF), sum of squares (SS), mean square (MS) are given. The level of significance is given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

	DF	SS	R ²	F-value	p-value
Estuary	1	0.06	0.04	5.38	0.012
Residual	137	1.57	0.96		
Total	138	1.63	1.0		

Appendix 2.7. Multivariate homogeneity of group dispersions analysis for the spot measures of *Pomadasys commersonnii* from two estuaries. Tests are based on Bray-Curtis dissimilarity distances with 999 permutations. Degrees of freedom (DF), sum of squares (SS), mean square (MS) are given. The level of significance is given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

	DF	SS	MS	F- value	p-value
Estuary	1	0.05	0.05	12.01	**
Residuals	137	0.52	0.003		

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CHAPTER THREE

SHAPING THE FUTURE: OTOLITH SHAPE
ANALYSIS AS A METHOD OF STOCK
DISCRIMINATION FOR *POMADASYS*
COMMERSIONII

3.1 Abstract

Morphology- an organism's structure, shape and form – is a fundamental aspect of many scientific discoveries and has been successfully used to differentiate stocks in fisheries research. Despite having a genetic basis, many phenotypic characteristics are influenced by environmental conditions. The fish otolith is an example of a morphological structure that is under dual regulation by both genetic and environmental factors and has been successfully used to aid in fish stock delineation. This chapter examined whether differences in otolith shape outlines could be used to successfully delineate potential stocks of *Pomadasys commersonnii*. It was hypothesised that (1) spotted grunter from two biogeographically distinct estuaries would exhibit differences in their otolith shape outlines and (2) there would be differences in the otolith shape outlines of spotted grunter captured in the Breede Estuary pre-2002 and post-2019 based on the latest spotted grunter movement research. Otolith shapes become increasingly complex as an individual grows and the size ranges of the samples can influence the effectiveness of this method. Therefore, only overlapping size ranges were used for this analysis. The findings revealed significant differences in the outlines of spotted grunter otoliths both spatially and temporally. It is suggested that while the overall otolith shape is genetically constrained, finer details may vary under environmental influences. These differences could indicate local adaptation of their sensory systems to environmental conditions which in time could lead to stock separation and species divergence.

3.2 Introduction

In fisheries, the identification of groups of individuals with unique, stable life histories is important for stock assessment and management (Begg and Waldman, 1999; Cadrin, 2000). There are many definitions for a stock, however, it is essentially a local population adapted to living in a particular environment (Swain and Foote, 1999). These populations will have slightly different morphological adaptations to survive in their given environment and these adaptations may or may not have a genetic basis. Morphological variations such as body shape or meristic count have been used to delineate phenotypic stocks (Cadrin, Karr and Mariani, 2014). Differences in morphological traits were assumed to have a genetic basis but research has now shown that they are under dual regulation by both environmental and genetic factors, a function of phenotypic plasticity (Swain and Foote, 1999; Torres, Lombarte and Morales-Nin, 2000; Cadrin, 2005). Thus, many studies now aim to delineate “phenotypic” stocks using morphological variation.

Morphology deals with organism structure, shape and form and is an important field in biology for understanding function by comparing the usage of homologous structures among different groups of animals with similar lifestyles (Wray, 1999). Morphometric differences between populations living in different environments can be used to understand environmentally induced variations and population dynamics (Hanif *et al.*, 2019).

Morphological variation can be caused by developmental disparities from the combined influences of the environment, genetics and selection (Cadrin, 2000, 2005; Vignon and Morat, 2010). Ontogeny, the development of an organism, can provide morphological variation in two ways: isometric size (due to growth) and allometric shape from developmental form changes i.e. when a variation in size is connected to a variation in shape (Lleonart, Salat and Torres, 2000; Monteiro *et al.*, 2005). Ontogeny can sometimes correct errors in development, however, some features are permanent and can provide information on the individual’s life history (Cadrin, 2000). From this standpoint, morphometric variation within a species can reflect differences in the environment during critical developmental stages (Cadrin, 2000; Stransky, 2014).

Consequently, morphometric analysis is often used to differentiate phenotypic stocks or groups of individuals with similar growth patterns. However, not all morphometric structures are suitable for phenotypic stock delineation. Desirable structures are those that are modified by development and correlated to life history events, such as maturation; these are beneficial as they represent differences in growth rates and environmental conditions (Cadrin, 2000). It is also important to exclude structures that could confound stock delineation such as those that are affected by temporal variables. Sampling should therefore not vary in time as any differences exhibited could be due to distinct temporal events rather than stock differences (Cadrin, 2000).

Structures that can be used in morphometrics include soft structures, such as organs, or hard structures such as bones and otoliths. A common hard part used in fish stock delineation is the ear bone which is generally inert and robust but can also reflect changes in environmental conditions (Cardinale, Kastowsky and Mosegaard, 2004). They also represent a permanent record of environmental conditions as no reabsorption occurs (Vignon and Morat, 2010).

Fish have three pairs of ear bones, known as otoliths, which are calcareous structures that are attached to end organs and are associated with two functions: balance and sound detection (Cruz and Lombarte, 2004). Otoliths are submerged in endolymph within the labyrinth located behind the brain and thus isolated from the external environment (Pannella, 1971). They can detect and give direction to sounds in the environment (Lombarte *et al.*, 2010). Otolith shape has been documented to exhibit species-specific, population-specific (Schulz-Mirbach *et al.*, 2011) as well as stock-specific (Tuset, Parisi-Baradad and Lombarte, 2013) differences. Multiple hypotheses have been proposed on how otolith morphometry affects acoustic communication however the role of otolith shape is still unclear.

Cruz and Lombarte, (2004) found that sound-producing fish, such as haemulids, have a greater otolith size compared to species that do not communicate via sound. Others have suggested that otolith size has an adaptive capacity related to sound sensitivity (Montgomery and Pankhurst, 1997) and acuity (Paxton, 2000; Cruz and Lombarte, 2004). Sound acuity, the ability to discriminate between frequencies, is important to identify sounds from various sources and

species within the soundscape (Cruz and Lombarte, 2004). While sensitivity is related to the range of frequencies that a species can hear.

Otolith size has also been related to different communication strategies (Cruz and Lombarte, 2004) as well as different environments (Paxton, 2000; Parmentier, Vandewalle and Lagardère, 2001). The relationship between otolith shape and fish ecotypes is well documented with pelagic species showing distinct rostrums while benthic species have rounder otoliths (Volpedo and Diana Echeverría, 2003; Schulz-Mirbach *et al.*, 2019). As with most biological findings, this observation is not ubiquitous (Lombarte *et al.*, 2010) but elongated otoliths have also been shown to correlate with fast-swimming species (Schulz-Mirbach *et al.*, 2019).

The research to date has recognized dual regulation on otolith shape: overall shape is genetically regulated (Cardinale, Kastowsky and Mosegaard, 2004; Vignon and Morat, 2010) with strong variability related to age, cohort and sex (Begg and Brown, 2000; Gagliano and McCormick, 2004) and by environmental factors such as water temperature, depth and substrate (Hüssy, 2008; Vignon and Morat, 2010; Vignon, 2012) affecting the shape (Vignon and Morat, 2010). Otolith growth continues throughout an individual's life and while the shape may be genetically constrained, environmental factors will influence the rate of deposition of material deposited during otolith formation (Vignon, 2012). Therefore, environmental conditions express metabolic and physical constraints (Vignon and Morat, 2010).

A study by Vignon and Morat, (2010) compared otoliths from native and introduced non-indigenous coral reef snapper (*Lutjanus kasmira*), they found that cohabiting individuals from different lineages are influenced by both genetic and environmental conditions while the otolith shape morphology of introduced individuals was mostly influenced by environmental conditions. Therefore, it is assumed that overall otolith shape (elliptic, oval etc.) is regulated by genetics while any variability in the outlines, density, size etc. is regulated by environmental factors. Thus, when comparing otoliths among groups, populations or stocks the focus should be on the smaller, finer characteristics instead of the overall shape for phenotypic stock delineation.

Bardarson *et al.*, (2017) conducted a study in Ireland to determine if otolith shape can be related to mating behaviour in Atlantic Cod. Based on migratory strategies, two ecotypes were

found within the cod stocks: frontal and coastal. Frontal ecotypes feed in deeper waters while coastal ecotypes are found in shallow water year-round. Using morphometric analyses of otoliths from Atlantic cods belonging to each ecotype they found a 90% and 70% success rate in identifying cod from coastal and frontal ecotypes respectively (Bardarson *et al.*, 2017).

Generally, the largest pair of otoliths, the sagittae- composed of calcifying tissue, is used in otolith studies. Many studies have analysed otolith growth rings as a means of ageing fish (Pannella, 1971; Williams *et al.*, 2015). Microchemistry is another commonly used method in otolith studies (Nowling *et al.*, 2011). The chemical composition of otoliths can be used to detect life history boundaries, especially in species that experience steep environmental gradients (Cadrin, 2000). Recent studies have shown that the use of otolith morphometrics could potentially replace these older, costlier methods as there is a strong relationship between age and otolith morphometrics. Williams *et al.*, (2015) investigated the relationship between otolith morphometrics and age compositions of deep-water snapper and concluded that otolith weight and thickness were the most crucial measurements that could provide accurate estimates of mortality rates, age compositions and other biological indicators. However, this is only true for stable, non-fluctuating environments as environmental conditions can influence calcium deposition (Loeppky *et al.*, 2021).

Modern morphometric analyses, used for phenotypic stock delineation, include the analysis of morphometric landmarks and morphometric outlines. Morphometric landmarks examine homologous structures to determine whether there are any variations among populations (Ibañez, Cowx and O'Higgins, 2007) while morphometric outline analysis deals with quantifying the outlines of hard parts and then using these to evaluate variations in outline morphology among groups (Cadrin, Kerr and Mariani, 2014; Song *et al.*, 2018; Wiff *et al.*, 2019). Both have been successfully used in stock delineation however, the landmarks method is time-consuming and requires the scientist to be familiar with the morphology of the selected structure (Tuset *et al.*, 2003; Ibañez, Cowx and O'Higgins, 2007). The scientist needs to know common key features, viz landmarks, on the hard parts that need to be identified and selected before analysis can begin, these landmarks should be common across all species or individuals that are used in the analysis (Ibañez, Cowx and O'Higgins, 2007). Therefore, the morphometric outlines method is more suitable as a method of stock delineation and the analysis is a relatively

quick process due to advances in the automated detection of shape outlines (Libungan and Pálsson, 2015). Otolith shape analysis, using **ShapeR**, has been used to successfully separate stocks of *Merluccius capensis*- the shallow-water hake (Shoopala, Wilhelm and Paulus, 2021), *Larimichthys polyactis*- the yellow croaker (Song *et al.*, 2018) and *Genypterus blacodes*- ling (Wiff *et al.*, 2019).

In respect to the above studies, one can assume that otolith shape outline is a function of the environmental conditions with which the spotted grunter inhabits and is thus, suitable for the use of phenotypic stock delineation. This study aimed to determine if individuals from different localities could be reassigned to their specific estuary based on the characteristics of their sagittal otolith shape outlines. It was hypothesised that there would be notable differences in the otolith shape outlines of spotted grunter from the Breede Estuary and the Kei Estuary- based on their environmental differences. Based on previous studies, it was presumed that fish size would have confounding effects on otolith shape analysis, therefore, the relationship between fish size and otolith shape morphology was evaluated and accounted for when comparing these areas for stock delineation. Lastly, this study aimed to determine whether there were any differences between the current and historical otolith samples from the Breede Estuary. It has been hypothesised that there could be differences between these two temporal populations based on the latest research of spotted grunter migration (Ziko, 2021). To address these aims, spotted grunter otoliths were photographed and analysed before the otolith outlines from each of these samples were compared.

3.3 Methods and Materials

3.3.1 Proof of Concept: Historical populations

A preliminary analysis was conducted to determine if spotted grunter otoliths would be viable for shape analysis. The objective was to determine if it was feasible to accurately photograph otoliths and then use the images to detect variations in the otolith morphology of spotted grunter from two different areas. To conduct this analysis, spotted grunter sagitta otoliths previously captured in the Breede Estuary and adjacent Heuningnes Estuary between 1998 and 2002 were used. Both these estuaries are situated on the south coast of South Africa and receive winter rainfall (Brown and Rajkaran, 2020; Flemming and Martin, 2021b). The Breede Estuary mouth is permanently open while the Heuningnes Estuary mouth is artificially kept open through the introduction of marram grass, *Ammophila arenaria*, and dune stabilisation with brushwood and afforestation with indigenous vegetation (McCord and Lamberth, 2009; Brown and Rajkaran, 2020). Both estuaries are popular fishing spots for spotted grunter and the Heuningnes Estuary is where one of the first spotted grunter populations in the southern cape established (de Villiers, 2018). There was a total of 61 and 40 samples available from the Breede and Heuningnes Estuary, respectively. For this study, only left otoliths were used in conjunction with fish length (mm).

3.3.2 Data collection

After the preliminary study, sampling was conducted in the Breede Estuary in the Western Cape and in the Kei Estuary in the Eastern Cape during standard DFFE estuarine fish surveys (Ethics permit numbers: 2018/v10/SK, RES2020/86, RES2021/56, RES2022/72). Fish were sampled using a 120m long, 4m deep, 48 mm stretched-mesh beach-seine net set from a rowboat and hauled onto the bank. The spotted grunter were removed from the net and euthanized by immersing them in water mixed with an anaesthetic clove oil. Bycatch species captured in the net were released as quickly as possible to avoid further loss of life. Before each fish dissection the total length (TL) and fork length (FL; mm) was measured. A fin clip was taken and stored in ethanol for genomic analysis. During dissection, the otoliths were removed.

3.3.3 Image acquisition

To capture images of the otoliths, each otolith was raised, with the use of a small pipe, on a black background and captured using a Leica camera. The camera, with consistent preset settings (Appendix 3.1) was placed on a stand above the otolith. The otoliths were positioned with the rostrum to the left and the sulcus facing upward. Two cool-light 5W LED light bulbs were placed on either side of the otoliths. The images were captured in full colour and saved as jpeg files. The images were enhanced using **Photos**, a program running on macOSX. An image of a scale bar was taken under the same conditions that were used to scale otolith measurements in millimeters. The images were calibrated in **ImageJ**.

3.3.4 Image analysis

The R-package, **ShapeR** (R Core Team, 2014; Libungan and Pálsson, 2015), was used to detect the outlines and shape coefficients of the otoliths. **ShapeR** uses an outline analysis approach that can extract and generate shape data. **ShapeR** has built-in functions that are able to transform the outlines using either normalized elliptic Fourier analysis or a discrete wavelet analysis. These analyses generate coefficients for the otolith outlines.

OTOLITH OUTLINES

The **ShapeR** package transforms the otolith pictures into grayscale and then the images are binarized, using the specified threshold pixel value (0.1 or lower). The software is then able to detect the outlines from the binarized images and allows you to view the outlines on each otolith to ensure that they are correct (Figure 3.1a). Contour smoothing is suggested to remove high-frequency pixel noise to increase the accuracy of the analysis. The pixel noise is eliminated by a weighted average of three successive points; these averages will smooth multiple outlines into one distinct outline. Lastly, the shape coefficients are calculated by positioning a horizontal axis across the longest axis of the otolith. A centroid for each otolith is calculated as the mean of the x and y coordinates of the outline. A polar axis is drawn horizontally from the right of

the horizontal axis, which corresponds with the 0° of the otolith outlines (Figure 3.1b). Polar coordinates are calculated from these radials and are collected in an anti-clockwise motion towards the 360° point.

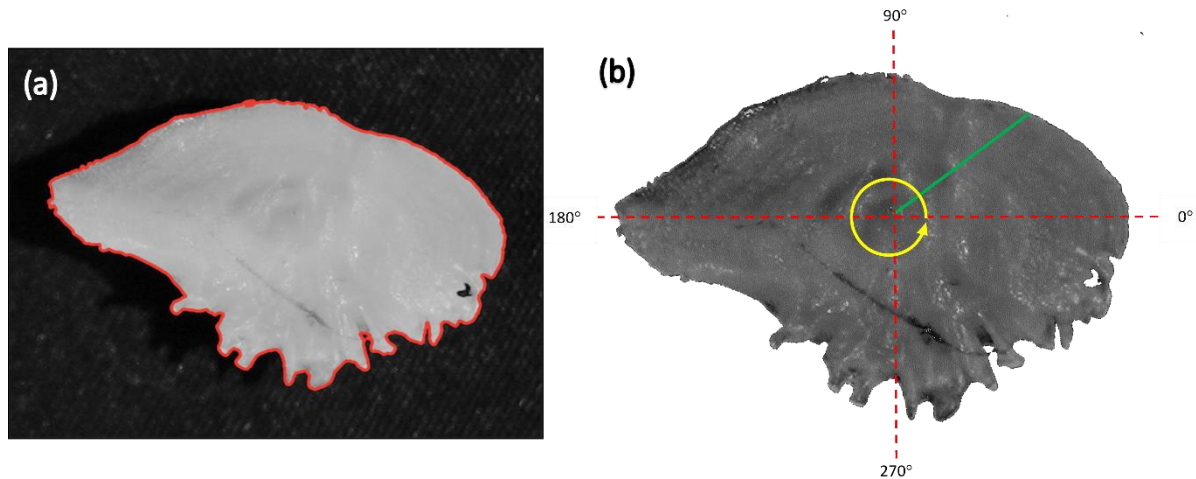


Figure 3.1. Otolith outline analysis. The original otolith picture with the given outline for review (a) and the radial length extraction which will be used to calculate shape coefficients (b).

SHAPE COEFFICIENTS

There are two transformations that can be used to calculate shape coefficients: the elliptical Fourier transformation and discrete Wavelet transformation; they differ in method and power. The Fourier transformation makes use of sine and cosine functions to represent the otolith contour and thus can be used to characterize the otolith shape (Equation 3.1). The Fourier transform is unable to locate and discriminate abrupt changes in contour (Bird and Eppler, 1986; Parisi-Baradad *et al.*, 2005) and is the less robust method for detecting differences in shape.

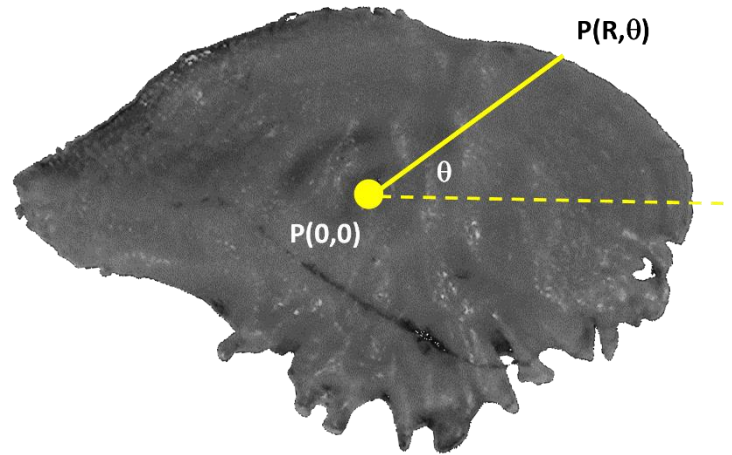


Figure 3.2: Otolith elliptical Fourier analysis: Depiction of an otolith demonstrating how the elliptical Fourier transformation is derived.

$$R(\theta) = A_0 + \sum_{n=1}^{\infty} A_n \cos(n\theta - \phi_n) \quad (\text{Equation 3.1})$$

Where θ is the polar angle (from a random reference point), A_0 is the mean radius of the shape and ϕ is the phase angle of the n^{th} harmonic (Yuan *et al.*, 2014).

The wavelet transformation uses a rapidly decaying oscillation from a function known as the mother wavelet. The deviation between the reconstructed wavelet otolith outline and the original outlines is used to assess the appropriate number of wavelet coefficients needed for analysis (Libungan and Pálsson, 2015). This transformation can locate and measure abrupt changes in shape (Parisi-Baradad *et al.*, 2005) and is thus the more powerful transformation. The wavelet transformation computes the otolith radius to a wavelet function (ψ_s), which has zero mean and a finite length:

$$W_s \rho(\varphi) = \rho * \psi_s(\varphi) = \int_{-\infty}^{+\infty} \rho(\beta) \psi_s(\varphi - \beta) d\beta \quad (\text{Equation 3.2})$$

The radius is given as ρ and the scale is given as s .

The function is dilated by a given scaling factor, where s is seconds:

$$\psi\left(\frac{t}{s}\right) \quad s > 0 \quad (\text{Equation 3.3})$$

3.3.5 Statistical analysis

All statistical analyses were conducted and achieved in R (v.4.2.0) and R Studio (RStudio, 2020). An initial evaluation was conducted on the size ranges of fish from each estuary. The assumptions of normality (Shapiro-Wilk normality test) and equal variance were evaluated and a two-sample t-test was used to determine whether there was a significant difference in the TL of fish (mm) between the two estuaries.

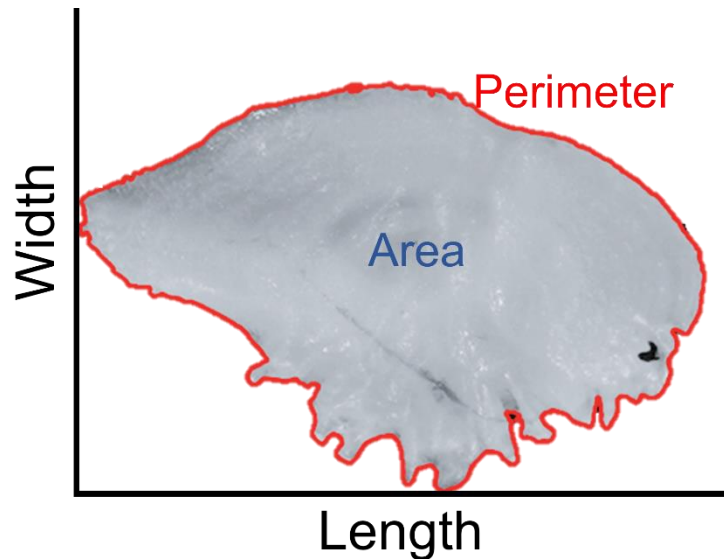


Figure 3.3. Otolith morphometric measurements: Schematic to show the four basic morphometric measurements taken from a sagittal otolith of a *Pomadasys commersonnii*.

To adjust the otolith shape with respect to allometric growth, coefficients were evaluated with the Bonferroni adjustment. Four coefficients were removed due to their interaction ($p < 0.05$) with fish length, these coefficients are omitted automatically. The mean of the otolith's width,

length, area and perimeter were determined for each sample. These four measurements were used to calculate further size descriptors:

$$\text{Roundness} = \frac{4\text{Area}}{\pi\text{Otolith Length}^2} \quad (\text{Equation 3.4})$$

$$\text{Form Factor} = \frac{4\pi\text{Area}}{\text{Perimeter}^2} \quad (\text{Equation. 3.5})$$

$$\text{Circularity} = \frac{\text{Perimeter}^2}{\text{Area}} \quad (\text{Equation 3.6})$$

$$\text{Aspect ratio} = \frac{\text{Otolith length}}{\text{Otolith width}} \quad (\text{Equation 3.7})$$

$$\text{Ellipticity} = \frac{(\text{Otolith length} - \text{Otolith width})}{(\text{Otolith width} + \text{Otolith length})} \quad (\text{Equation 3.8})$$

$$\frac{P}{A} = \frac{\text{Perimeter}}{\text{Area}} \quad (\text{Equation 3.9})$$

Analysis of variation (ANOVA) tests were run to determine if there were any differences in the otolith shape and size descriptors between populations. First wavelet and Fourier reconstructions were conducted to determine how the shape coefficients deviate from the otolith outline. Next, to determine how the variation in the coefficients is dependent on the position along the outline, the mean and standard deviation of the coefficients were plotted against the angle of the otolith. A canonical analysis of principal coordinates (CAP) was conducted to visually analyse the variation in shape indices between the two populations. For graphs with only two populations, an unconstrained CAPS analysis was used. An ANOVA-like permutation test was also performed to determine the partition of variation among groups

using 1000 iterations. Lastly, a test was conducted to determine the misclassification error, which indicates the effectiveness of the analysis in correctly identifying which population an individual would belong to.

3.4 Results

The spotted grunter sagittal otoliths have a rounded, oval shape that becomes elongated as the fish grows. They are thick and become increasingly laterally concave as the fish grows. They can be characterised by a broad, prominent rostrum, a rounded, smooth dorsal margin and a complex, jagged ventral margin. A total of 256 *Pomadasys commersonnii* otoliths were photographed for this study. After analysis, 27 of these otoliths were discarded due to incorrect outlines, resulting in a final total of 228 samples (Appendix 3.2). Incorrect outline detection generally occurred due to thin, jagged edges on the otoliths that were not in focus (Figure 3.4).

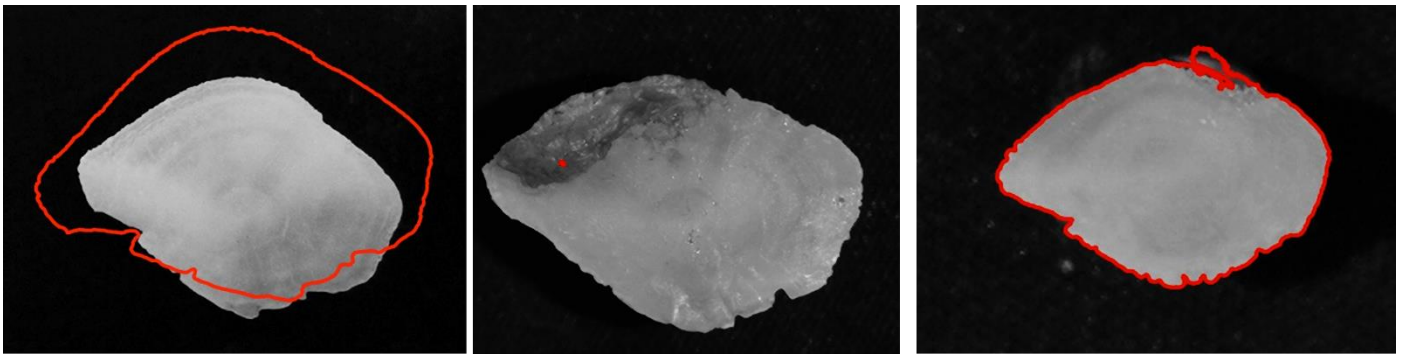


Figure 3.4. Incorrect outline analysis: Examples of otoliths that were discarded from further analysis due to incorrect outlines.

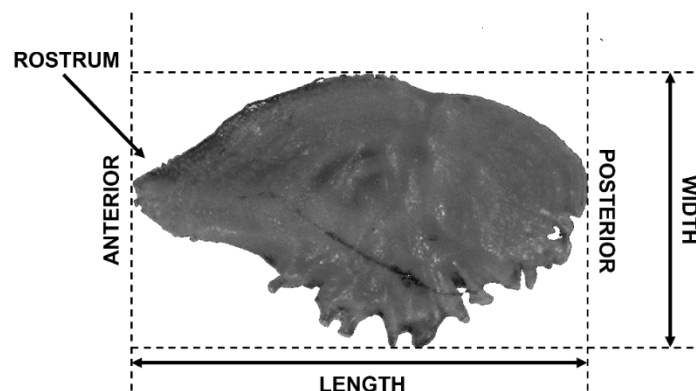


Figure 3.5: Otolith characteristics: Morphology used to describe differences in the outlines of *Pomadasys commersonnii* otoliths.

3.4.1 Proof of Concept: Historical Populations

A total of 117 individuals were sampled from the Breede and Heuningnes Estuary pre-2002 (Appendix 3.2). There was no significant difference between the length of fish from these two estuaries (F-value= 0.913, $p > 0.05$) however, both samples were composed of large fish (TL > 405mm) which consequently led to elongated otoliths with complex boundaries.

Only three of the ten otolith shape descriptors and indices showed significant differences between these two samples: otolith area, otolith roundness and otolith width (Appendix 3.4; $p < 0.05$). However, all three measurements had high F-values which suggests that the variation within the samples was high (Appendix 3.4), particularly in the Heuningnes Estuary. Therefore, these measurements may not be sufficient in accurately identifying differences between spotted grunter populations.

When using a Wavelet analysis, differences in otolith outlines generally occurred on the dorsal-ventral axis (Figure 3.6a). The otoliths from the Heuningnes samples were slightly thinner and more irregular on the ventral margin compared to the Breede samples. The Breede samples are also slightly broader on the dorsal margin. The canonical analysis of principal components represents the variation in the otolith shape of the length-standardized wavelet coefficients. The first two discriminating axes explained a total of 49.9% of the variation in otolith shape (Figure 3.6b). The linear discriminant analysis gave a classifier error rate of 40.17%, therefore, there is only a 59.83% chance that an individual from either of these historical samples will be correctly assigned to their estuary.

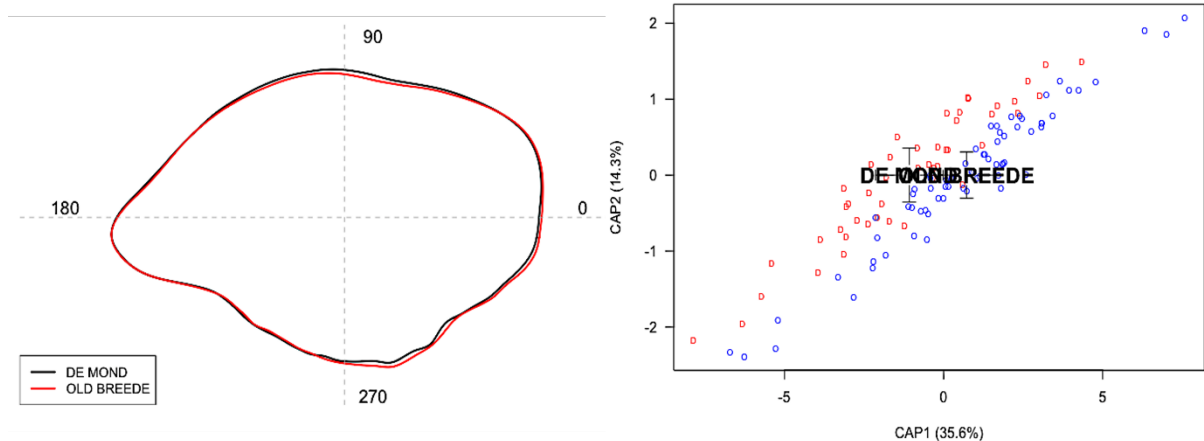


Figure 3.6: Average otolith outlines: *Pomadasys commersonnii* otolith outlines from wavelet coefficients for the historical Breede and Heuningnes (De Mond) Estuary samples (left). An unconstrained canonical analysis of principal coordinates, using wavelet coefficients, for the outline of *Pomadasys commersonnii* otoliths for these two estuaries ($P < 0.05$; right).

3.4.2 Spatial differences: Breede vs Kei Estuary

A total of 111 samples were used for this analysis (Appendix 3.2). There was no significant difference in the total lengths of fish used for this experiment from the two estuaries sampled (Breede and Kei; Appendix 3.3). The ANOVA tests run on the different otolith shape and size descriptors between the Breede and Kei samples identified only two of the ten otolith shape descriptors to have significant differences: otolith area and roundness (Figure 3.7; Appendix 3.4). Both otolith area (F -value = 4.9, $p < 0.05$) and otolith roundness (F -value = 6.12, $p < 0.05$) had a high degree of variation within the Breede Estuary. Therefore, these single measurements may not be accurate in determining differences between spotted grunter from different environments.

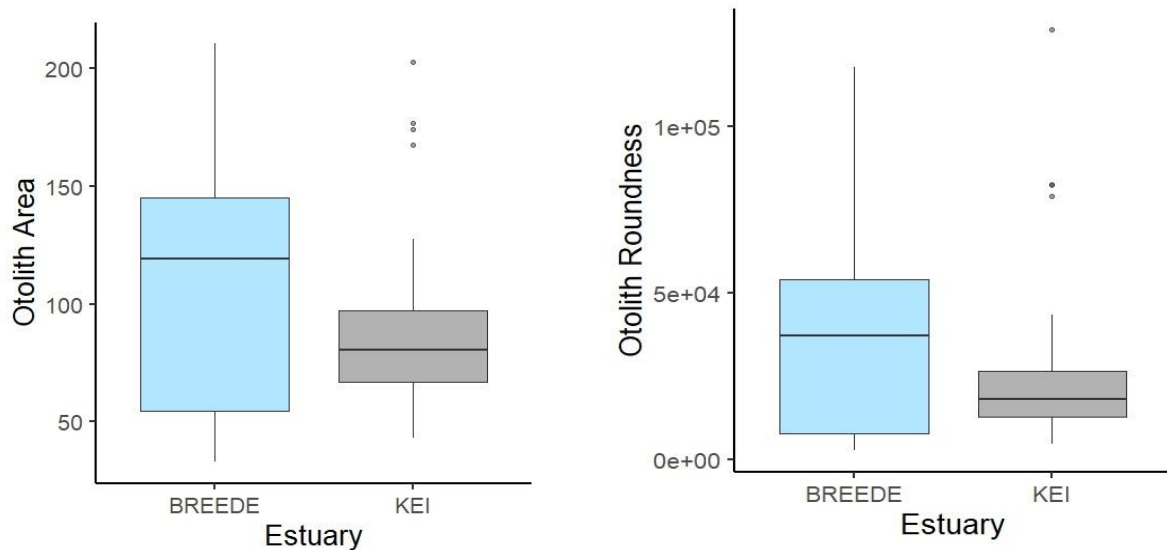


Figure 3.7: Otolith shape descriptors: Boxplots showing the otolith area (left) and otolith roundness (right) of *Pomadasys commersonnii* from two estuaries: Breede and Kei. Both shape descriptors were significantly different between these two estuaries ($P < 0.05$).

The otolith outline shape was modelled using both a Wavelet transform and Fourier analysis. The quality of the outline reconstructions can be assessed by the number of Wavelet levels (coefficients) and Fourier harmonics needed for 98.5% accuracy. The deviations decrease quickly for both analyses, however, five wavelet levels reflect 98.5% accuracy, while 12 Fourier harmonics are needed for the same accuracy in otolith outline reconstruction (Appendix 3.5). Therefore, the wavelet transform has a higher modelling performance compared to the Fourier transform.

The wavelet analysis provided an efficient method to analyse the mean outline of otoliths from each estuary (Figure 3.8). The mean outlines allow us to determine general characteristics of outline shape that differ between sample areas. There were a few observed differences between the mean otolith outlines of spotted grunter from the two estuaries (Figure 3.8). The mean otolith outline of spotted grunter from the Kei Estuary is slightly broader in the dorsal-ventral axis and shorter in the anterior-posterior axis compared to those from the Breede

Estuary, resulting in a more circular shape. The Breede Estuary samples are slightly more elongated in the anterior-posterior axis. This difference is substantiated by the ANOVA test that found roundness to be significantly different between these two areas (Appendix 3.4). The observed differences in figure 3.8a can be quantitatively assessed in figure 3.8b which indicates where the main differences in the average otolith outline occurred between the two estuaries. There are three main areas along the outlines that differ between these two areas. The first, between 300° and 30° , occurs at the anterior region of the otolith where the Kei Estuary is slightly narrower. The posterior region, where the rostrum occurs, between 170° – 200° also varies as the Breede Estuary has a slightly longer, broader rostrum. Intraclass correlation peaks at 50° – 70° and 100° – 140° , which is the smoother dorsal margin; this suggests that this is the most stable part of otolith shape in spotted grunter (Figure 3.8b). The main differences in otolith shape morphology occur between 280° – 340° and so this may be the most important region to look at for stock characteristics.

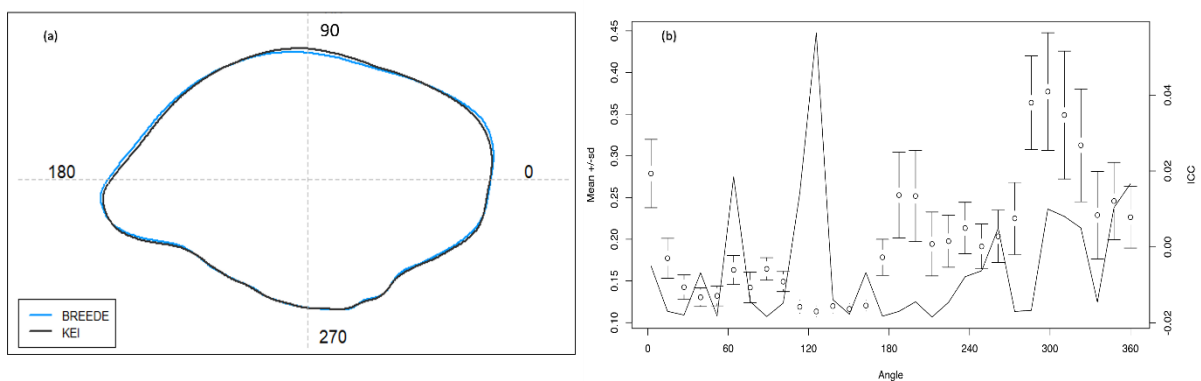


Figure 3.8: Otolith outlines: (a) Average otolith shapes of *Pomadasys commersonnii* produced from wavelet coefficients for two estuaries: Breede and Kei. The numbers represent the polar coordinates, and the dotted lines represent the otolith centroid. (b) The variance in outline between the two samples of *Pomadasys commersonnii* (via mean and standard deviations) and the intraclass correlation (solid line) of the wavelet coefficients is given.

Statistically, the fish from these two areas have significantly different outline shapes according to the ANOVA-like permutation test for both the wavelet and Fourier transforms, however, the wavelet analysis had a greater level of significance (Appendix 3.6, $p < 0.05$). The canonical analysis of principal components gives the variation in the otolith shape of the length-standardized wavelet coefficients. These graphs represent the differences in otolith outlines from the two estuaries by plotting the first and second discriminant axes, CAP1 and CAP2. The first two discriminating axes explained a total of 42.7% of the variation in otolith shape using wavelet coefficients and 52.2% of the variation when using Fourier harmonics (Figure 3.9). The Breede Estuary has a widespread distribution of data points compared to the Kei Estuary suggesting that the Breede samples have a greater variability in otolith shapes compared to the Kei samples. The linear discriminant analysis gave a classifier error rate of 37.6%, therefore, there is a 62% chance that an individual from either the Breede or Kei Estuary will be correctly assigned to their population.

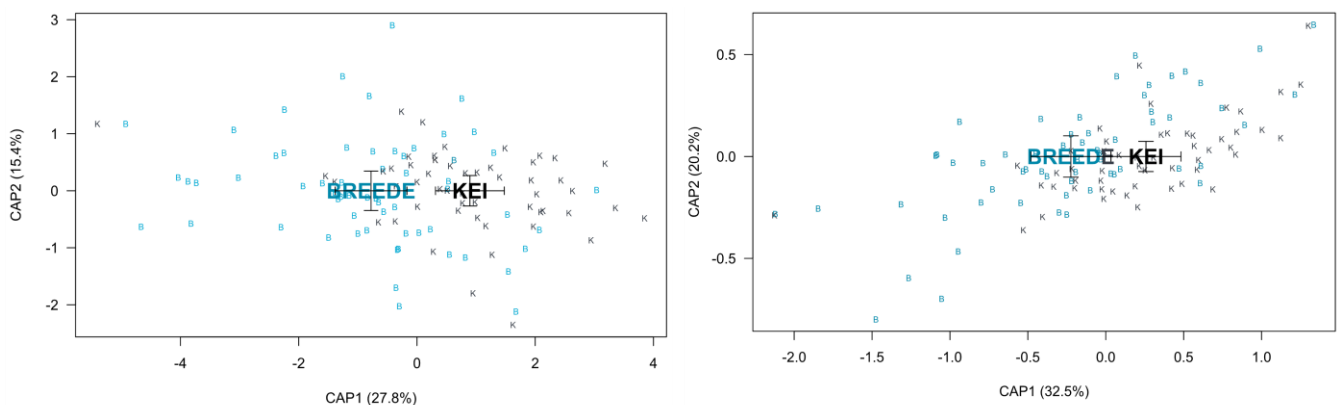


Figure 3.9: Unconstrained canonical analysis of principal coordinates: Wavelet coefficients (left) and Fourier harmonics (right) for the shapes of *Pomadasys commersonnii* otoliths from the Breede and Kei Estuary. The name of each population represents the mean canonical value and the surrounding interval represents one standard error.

3.4.3 Temporal Differences: Historical Breede vs Current Breede

The fish from the historical Breede samples were much larger than the samples collected from Breede Estuary for this study (Appendix 3.2). To test the effect of size on the **ShapeR** results two analyses were run to compare the current and historical Breede Estuary populations. The first analysis used all fish from both samples regardless of total length (mm) while the second analysis only used fish with overlapping size ranges between the two samples (Figure 3.10, Appendix 3.7).

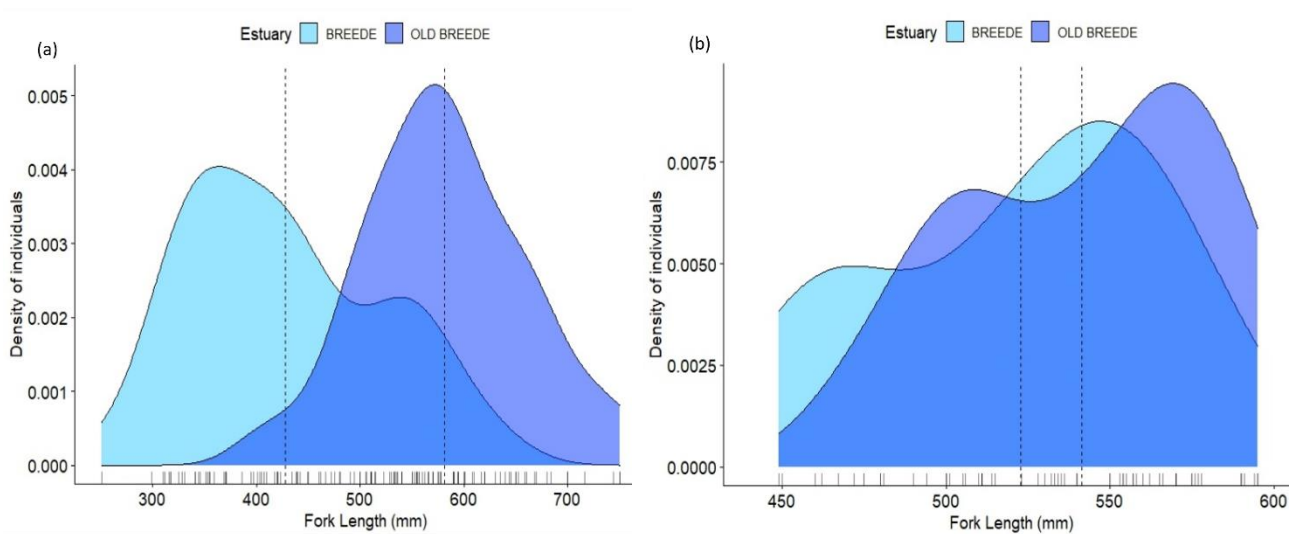


Figure 3.10: The total lengths of *Pomadasys commersonnii*: Size range of spotted grunter from two different populations, the historical Breede (Blue) and current Breede (pink). (a) Shows all samples from the two populations ($P < 0.05$) while (b) only shows samples with overlapping size ranges ($P > 0.05$)

Graphical representation of the average otolith outline for each population enables us to see where exactly differences in these outlines may lie. There were obvious differences between the otolith outlines of these populations when using different size ranges (Figure 3.11a). The historical Breede population has a more elongated otolith shape, with a more prominent rostrum while the current population is more compact in the anterior-posterior axis. The current population is slightly broader in the dorsal-ventral axis compared to the historical

population. The main variation between these populations occurs at the posterior region between $170^{\circ} - 250^{\circ}$ which corresponds to the rostrum. These differences were statistically significant when running an ANOVA-like permutation test on the shape coefficients for both the Wavelet and Fourier transformations (Table 3.1).

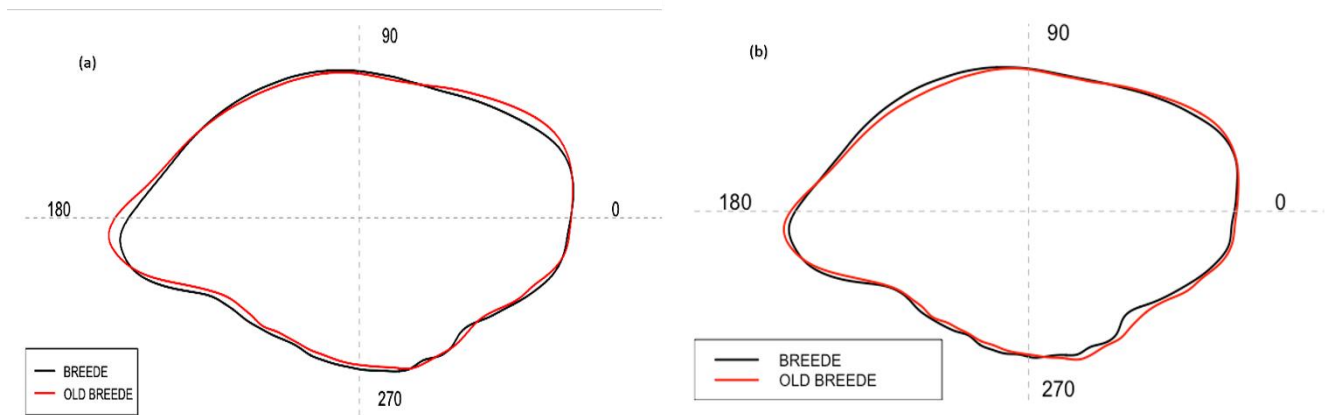


Figure 3.11: Average otolith shapes: *Pomadasys commersonnii* otolith outlines produced from wavelet coefficients for two *Pomadasys commersonnii* populations: current Breede and historical Breede. (a) represents the outlines of these two populations when using all size samples while (b) shows the average outlines when using overlapping size ranges for the two populations. The numbers represent the polar coordinates and the dotted lines represent the otolith centroid.

However, when comparing the outlines of the two populations using overlapping size ranges, there were different observable features (Figure 3.11b). The current population is still broader in the dorsal-ventral axis while the historical population is still longer in the anterior-posterior axis. The main differences now lie between $280^{\circ} - 310^{\circ}$ where the current Breede population has a more irregular anterior-ventral margin compared to the historical population which shows higher irregularity in the posterior-ventral margin. These differences were also statistically more significant than the differences using all size samples, according to the ANOVA-like permutation test for both Wavelet and Fourier transformations ($p < 0.05$; Table 3.1).

Table 3.1: Results from ANOVA-like permutation tests of *Pomadasys commersonnii* otolith-shapes between the current Breede population and the historical Breede population. ‘All samples’ refers to using all sampled fish regardless of total length and the ‘size-adjusted’ refers to only using fish samples that had overlapping size ranges between the populations ($P > 0.05$). This wavelet test for shape reconstruction was performed with 1000 iterations. The misclassification error for correctly assigning an individual to one of these populations is also given for each case.

Populations	df		Wavelet			Fourier			Misclassification error
			Var.	F-ratio	p-value	Var.	F- ratio	p-value	
All samples	Pop	1	7.7	15.0	0.001	0.03	6.1	0.002	16.2%
	Res	128	66.0			0.6			
Size adjusted	Pop	1	2.7	3.9	0.001	0.02	3.9	0.006	25%
	Res	58	39.6			0.3			

The canonical analysis of principal components gives the variation in the otolith shape of the length-standardized wavelet coefficients (Figure 3.12). These graphs represent the differences in otolith outlines from the two populations by plotting the first and second discriminant axes, CAP1 and CAP2. The first two discriminant axes explained a total of 51.1% of the variation in otolith shape using wavelet coefficients for all size samples while the restricted size range samples had a total of 59.8% of their variation explained by the first two discriminating axes.

In both cases, the populations each have unique linear clusters. The linear discriminant analysis gave a classifier error rate of 16.2% for all size samples and 25% for the restricted size range samples. Therefore, there is an 83.8% chance of being correctly assigned to the originating population when using all-size samples while only a 75% chance of being correctly assigned when using the overlapping size classes. Despite the lower misclassification rate for all-size samples, it is important to note the higher degree of variation in otolith shapes when using all-size samples compared to restricted-size samples. This suggests that the classifiers that have

larger sample sizes will give more accurate results when assigning individuals to their populations.

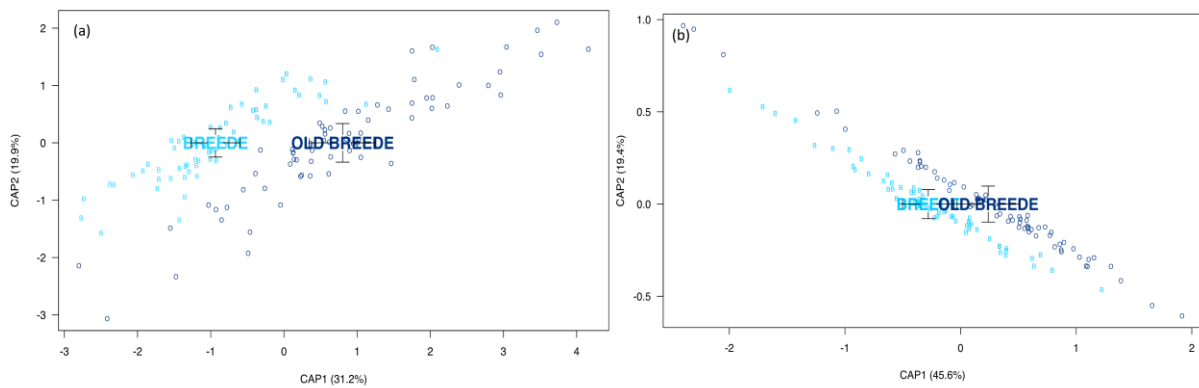


Figure 3.12: Unconstrained canonical analysis of principal coordinates: Wavelet coefficients for the shapes of *Pomadasys commersonnii* otoliths from the current Breede and historical Breede population river. (a) Gives the CAP analysis for all size samples while (b) gives the CAP analysis for the overlapping size range samples. The name of each population represents the mean canonical value and the surrounding interval represents one standard error.

3.5 Discussion

This study provides the first evaluation of spotted grunter otolith shape morphology. It was hypothesised that, since spotted grunter inhabit a wide range of environments, differences in physicochemical properties will affect the shape of their otoliths. The results demonstrated that there are significant differences between the otolith shapes of spotted grunter in both space (Breede vs. Kei Estuary) and time (historical Breede vs. current Breede). Therefore, the findings support the use of otolith shape morphology as a method of phenotypic stock delineation in spotted grunter. Additionally, this research supports claims that fish size can impact the results of comparing otolith shapes between stocks with different-sized individuals. Thus, it is important to have similar size ranges between samples being evaluated for phenotypic stock delineation when using otolith shape morphology.

Otolith shape measurements such as width, length and area have previously been used to successfully discriminate between fish species (Bostanci *et al.*, 2015) and populations (Begg and Brown, 2000; Ponton, 2006; Shoopala, Wilhelm and Paulus, 2021). However, these measurements are highly correlated to fish size and require data adjustments which greatly reduce discrimination efficiency (Song *et al.*, 2018). Depending on the estuaries used, only two/three out of the ten measurements showed any significant differences between samples which suggests that these morphometric measurements are not sufficient for phenotypic stock delineation in spotted grunter. Similarly, morphometric measurements have also been found to lack efficiency for stock delineation in previously studied fish species such as *Larimichthys polyactis* (Song *et al.*, 2018) and *Serranus cabrilla* (Tuset *et al.*, 2003).

Recent technological advances in image processing and contour modelling have enabled us to readily quantify otolith outlines. This offers a more powerful method of assessing shape differences between samples and has been beneficial in this study on spotted grunter otolith shape. Furthermore, studies on *Genypterus blacodes* (Wiff *et al.*, 2019), *Pennahia argentata* (Song *et al.*, 2020) and *Argyrosomus japonicus* (Ferguson, Ward and Gillanders, 2011) also found otolith shape outline analysis more useful for stock delineation studies than otolith shape measurements.

The results have shown that there are differences in otolith outlines, using both Fourier and Wavelet analysis, between the Breede and Kei Estuary spotted grunter, suggesting that these estuaries may contain two different phenotypic stocks of spotted grunter. However, a previous study suggested a single panmictic population of spotted grunter in South Africa when using mitochondrial DNA (Klopper, 2005). It is not uncommon for genetic differences to be absent in the presence of phenotypic differences in otolith shape (Stransky *et al.*, 2008). Three populations of *Albatrossia pectoralis*, the giant grenadier, with distinctly different otolith shapes, were also found to have no genetic differences (Rodgveller *et al.*, 2017). Another South African species, *Lophius vomerinus*, showed significantly different numbers of vertebrae and pectoral fin rays among fishing areas but there was no genetic variation across these geographic areas (Leslie and Grant, 1990). The linear discriminant analysis gave a 62% chance of correctly classifying a spotted grunter individual from one of the two estuaries. While this percentage is not extremely high, it does fall within ranges seen for other species such as *Albatrossia pectoralis* (48 – 69%; Rodgveller *et al.*, 2017), *Lutjanus johnii* (61 -77%; Sadighzadeh *et al.*, 2014) and *Gadus morhua* (60 – 81%; Stransky *et al.*, 2008). A future improvement would be to use a greater sample size as this did seem to increase the classification accuracy in other studies.

Phenotypic differences in the otolith outlines of spotted grunter from these two estuaries were statistically significant, however, whether these differences are genetic or environmentally induced is still unclear. Cardinale, Kastowsky and Mosegaard, (2004) studied the relative importance of genetic and environmental conditions on otolith morphometrics and found that unique populations living in similar environmental conditions had similar growth rates but differed significantly in otolith shape. They also found that individuals from the same stock living in different environmental conditions had significantly different growth rates and otolith shapes. They concluded that genetic differences influence otolith shape while environmental conditions affect otolith growth rates which in turn can modify otolith shape. These two study sites differ in many environmental aspects and there were significant phenotypic differences in the otolith outlines of their spotted grunter. Consequently, it can be assumed that the growth rates between these two estuaries differ and this ultimately affects the otolith outlines.

However, another important study on *Lutjanus kasmira* suggested that local variations, mainly in the rostral and antirostral parts, in otolith shape are due to genetic variation (Vignon and Morat, 2010). The Breede and Kei Estuary spotted grunter showed differences in both the rostrum and antirostrum margins which could therefore be due to genetic differences and not environmental differences. However, without proper age estimates for each individual used in this study, it is impossible to conclude whether these differences are due to genetic or environmental factors. Future studies could quantify the differences in growth rates of this species across environments to get a better understanding of the impacts of genetic and environmental factors on otolith outlines.

Phenotypic variation that occurs to improve communication in different environments is also an important factor to consider under the sensory drive hypothesis. The sensory drive hypothesis states that speciation can occur when environmental factors induce adaptations to sensory systems (Jacobs *et al.*, 2017). Therefore, changes in otolith morphology may reflect an adaptation to optimise communication in different environments - rockfish have been reported to have an increase in otolith size with environmental depth. Those individuals living in deeper, darker environments cannot accurately rely on visual signals for communication and thus may optimise their hearing capabilities by modifying their acoustic apparatus, such as otolith shape (Tuset *et al.*, 2016). Therefore, the differences seen in otolith shape between these estuaries could influence the acuity or sensitivity of these individuals to sounds; however, the exact influence that otolith shape has on acoustic abilities has yet to be determined.

ShapeR has a built-in function that standardizes otolith shape and size descriptors with fish length. Simoneau, Casselman and Fortin, (2000) showed that comparing otolith shape between *Salvelinus namaycush* (lake trout) individuals from the same populations with varying size ranges resulted in observable differences despite this standardization. A similar result was found in this study and it is believed that this observable difference is due to allometric growth (Tuset, Parisi-Baradad and Lombarte, 2013). The smaller individuals from each population had more circular otoliths while the larger individuals had otoliths with distinct rostrums and complex margins. The above study found negative allometric growth in the length and height

of lake trout otoliths. They showed that otolith height grew at a slower rate than otolith length in lake trout; therefore, over time, the otoliths became more elongated (Simoneau, Casselman and Fortin, 2000). Consequently, when using both smaller and larger individuals in the analysis, there will be a high degree of variation in otolith outline shape which cannot be fully corrected with fish size. This variation makes it difficult to determine an accurate “mean” outline for the population which negatively affects classification accuracy. Mapp et al., (2017) showed that fish age, and thus size, contributed most to classification accuracy and that stock classification using otolith outlines is more beneficial with older/larger fish. Furthermore, Shoopala, Wilhelm and Paulus, (2021) compared otolith morphometrics in two populations of shallow water hake, *Merluccius capensis*, and found that the differences in measurements between these two populations increased with age. These results suggest that the effectiveness of this method for stock identification is dependent on the range of size classes used from each population with larger individuals being preferential. However, it is also important to note that the increasing variability in otolith outlines with increasing fish size may be driving the higher degree of “significance” seen between the populations. With this in mind, an ANOVA, which tests the variance about the mean, may not be the appropriate test to use moving forward.

Furthermore, in species such as spotted grunter, there are trade-offs with using larger individuals. Larger individuals will undoubtedly provide more complex otoliths which ensures more noticeable differences between populations but this is complicated by the fact that spotted grunter are also known to utilise the marine environment more frequently as they mature (Childs et al., 2008). Spotted grunter are known to migrate to the inshore coastal waters of KwaZulu-Natal to spawn (Næsje et al., 2007) and research has shown that although spotted grunter have a high degree of philopatry, they have a tendency to visit other estuaries and take sea excursions (Dames et al., 2017). This can complicate the interpretation of the results as it is known that otolith shape is under dual regulation by both environmental and genetic impacts. Therefore, the otoliths of larger individuals, with high connectivity between different environments, will represent a narrative of the movements they have made. Hence, juveniles that have not yet left the estuary and are thus growing within the same environment

would be more beneficial for these studies but the differences between their otoliths will be less pronounced.

The differences in otolith outlines between the current and historical Breede samples gave some evidence to suggest that the Breede Estuary may be home to a new spawning population. One of the largest observable differences between these two samples was located around the rostrum and as mentioned above, local differences in this region of otoliths have been connected to genetic differences (Vignon and Morat, 2010). Temporal differences in otolith morphology have not been studied as intensely as spatial differences, however, a study on Icelandic cod looked at the temporal stability of otolith shape and found that there was a greater difference in otolith shape in space than in time (Jónsdóttir, Campana and Marteinsdottir, 2006). The clear differences between these two populations, separated only by time, again suggest that there may be some level of stock separation occurring within this population. The notable differences between these two populations were statistically confirmed by the high percentage of correct classification (> 75%) which not only represents the variability between these two populations but also confirms the effectiveness of otolith shape morphology as a tool for stock delineation in spotted grunter.

In conclusion, the results show that, as hypothesised, there are distinct differences in the otolith shapes of spotted grunter from two estuaries with differing environmental conditions. This suggests that these two samples might represent unique functional stocks that could be undergoing some form of diversification due to adaptations in their sensory systems relating to their local environment. It was also found that there has been a shift in the otolith shape of Breede Estuary spotted grunter which supports studies that have suggested the Breede Estuary is home to a new spawning aggregation. Otolith shape morphology has proven to be successful as a method of discrimination between spotted grunter. These results can be used to improve management strategies and future efforts can incorporate genetic data to validate whether these differences have a genetic basis or are purely a result of phenotypic plasticity. Regardless, this study provides the first evidence of differences in the otolith shape morphology of spotted grunter in both space and time.

3.6 Appendix

Appendix 3.1: Leica camera settings used to capture images of *Pomadasys commersonnii* otoliths

Setting	Value
Exposure compensation	-1.7
Shutter speed	1/100
ISO	100
Zoom	6x

Appendix 3.2: Number of *Pomadasys commersonnii* samples used for this study and the corresponding size ranges from each sample.

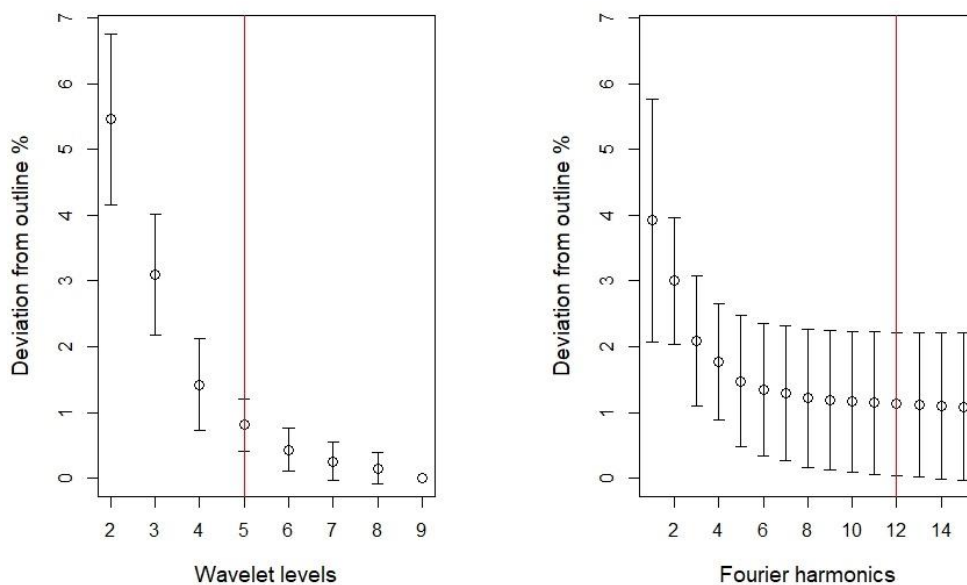
Sample	Total length (Minimum – Maximum; mm)	Number of individuals
Breede	251 – 635	60
Kei	232 – 635	51
Historical Breede	405 – 780	70
Heuningnes	405 – 783	47

Appendix 3.3: *Pomadasys commersonnii* size ranges. Table to show differences between the total lengths of *Pomadasys commersonnii* captured from two areas, the Breede Estuary and the Kei Estuary. Both samples had equal variances and non-normal distributions. A non-parametric test confirmed that there was no significant difference between the total lengths of individuals in these two estuaries ($p > 0.05$)

Total length (mm)	Breede	Kei
Mean	428,7	417,2
Standard deviation	94,1	76,2
Variance	8854,4	5810,9
n	60	51

Appendix 3.4: Differences in the otolith shape descriptors of *Pomadasys comersonnii* from different estuaries using ANOVAs. Results are given for the differences between the historical samples (Breede and Heuningnes) and the current samples (Breede and Kei). The level of significance is given in each case: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Otolith descriptor	Historical Breede and Heuningnes		Breede and Kei	
	F-Ratio	p-value	F-ratio	p-value
Length	3.56	0.06	2.51	0.12
Width	4.09	*	3.61	0.06
Area	5.80	*	4.91	*
Perimeter	2.15	0.15	2.69	0.10
Roundness	8.40	**	6.12	*
Ellipticity	0.23	0.63	1.85	0.18
Circularity	2.14	0.17	0.27	0.61
Form Factor	2.28	0.13	0.12	0.73
Aspect Ratio	0.45	0.50	1.90	0.17
Perimeter/ Area	2.69	0.10	0.83	0.36



Appendix 3.5: Quality of otolith outline reconstructions for (left) wavelet and (right) Fourier analysis. The red lines represent the level of wavelets/harmonics needed for 98.5% accuracy of the reconstruction.

Appendix 3.6: ANOVA-like permutation test results for the shapes of *Pomadasys commersonnii* otoliths from four different samples. The wavelet test for shape reconstruction was performed with 1000 iterations. The misclassification error for correctly assigning an individual to one of these samples is also given for each case. HB = Historical Breede, H = Heuningnes, B = Breede and K = Kei. The level of significance is given where: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Sample		Degrees of freedom	Wavelet			Fourier			Misclassification error
			Var.	F-ratio	p-value	Var.	F-ratio	p-value	
HB and H	Pop	1	1.8	3.0	*	0.01	2.1	0.078	40.2%
	Res	115	70.0			0.6			
B and K	Pop	1	1.6	3.3	**	0.01	2.7	$p < 0.05$	37.6%
	Res	109	52.5			0.4			

Appendix 3.7: *Pomadasys commersonnii* size ranges captured in the Breede estuary from two different time periods: Historical (1998 - 2008) and current (2018 - 2021). The range (minimum – maximum), mean and standard deviation (SD) are given for the total length of fish in each case. The p-value for the t-test assessing differences in these sizes ranges is also given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Size Range	Population	n	Total length (mm)		
			Range	Mean $\pm$ SD	p-value
All size samples	Current	60	251 - 635	428 $\pm$ 93	*
	Historical	70	405 - 750	581 $\pm$ 77	
Overlapping size samples	Current	22	449 - 591	522 $\pm$ 44	0.10
	Historical	44	460 - 595	541 $\pm$ 38	

3.7 References

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CHAPTER FOUR

SOMETHING SOUNDS FISHY: ASSESSING THE
EFFECT OF ENVIRONMENT ON THE
ACOUSTIC FEATURES OF SOUNDS
PRODUCED BY *POMADASYS*
COMMERSONNII FROM TWO ESTUARIES

4.1 Abstract

Animals have adapted a wide range of communication modalities and each is best suited to a particular environment. It is advantageous for species to have multiple communication systems and the ability to shift between them is critical for adaptability in an ever-changing environment. This is particularly important for species that inhabit a wide range of environments. The acoustic repertoires of spotted grunter, *Pomadasys commersonnii*, a soniferous haemulid species, were compared among two estuaries from different biogeographic areas in South Africa. It was hypothesised that (1) fishes from geographically distant (>1000 km) estuaries would exhibit different acoustic repertoires and (2) the differences in the acoustic parameters would correspond to differences in the turbidity levels of these two estuaries. Using an HTI-96 min hydrophone, the sounds of spotted grunter (n = 107) were recorded under standardized conditions. Significant differences between the acoustic parameters of spotted grunter from the two estuaries were observed, albeit in a manner opposite to what was expected. Grunter from the less turbid Breede Estuary emitted longer, louder sounds of lower frequencies. Two *a priori* hypotheses were proposed: (1) the Lombard Effect in response to anthropogenic boat noise on the Breede River, a recreational boating hub and (2) spotted grunter communication behaviour whereby acoustic signals may only succeed visual signals. In this instance, turbid environments would have a much closer visual, and thus acoustic, detection distance.

4.2 Introduction

The ability to communicate effectively with other animals in the local environment is essential to a species' survival (Maiditsch and Ladich, 2021). Through the evolution of sensory systems, animals can acquire information about their environment, an adaptation that increases their overall fitness (Oteiza and Baldwin, 2021). Information is anything that reduces the uncertainty about some aspect of an animal's environment (Rendall, Owren and Ryan, 2009; Carazo and Font, 2010). The definition of communication is widely disputed and ranges from 'the sharing of information between entities' (Smith, 1997) to 'an act or structure that alters the behaviour of other organisms' (Smith and Harper, 2003). However, for the purpose of this study communication will simply be defined as the sharing of information.

Animals communicate through one or more communication systems including visual, acoustic, chemical and electric signals (van der Sluijs *et al.*, 2011). The sensory drive hypothesis states that environmental characteristics drive selection on sensory systems that are more effective for the local environment (Seehausen *et al.*, 2008), indicating that different environments may favour certain modes of communication. Therefore, in species that inhabit a wide range of environments, one may see local signal divergence (Endler, 1992), that could eventually result in reproductive isolation.

Acoustic signals are one of the most common modes of communication and animals have evolved a wide range of mechanisms to generate sound (Elemans, Heeck and Muller, 2008). Research on sound communication has been studied in a range of phyla and across many different social contexts including courtship, distress, predator avoidance and agonistic signalling (Wysocki and Ladich, 2002; Bertucci *et al.*, 2014; Smith *et al.*, 2018). Acoustic communication is used in a range of habitats but sounds generated in water have a quick distribution (almost five times faster than air) and a slow attenuation (energy loss) rate (Kasumyan, 2008; Farrell, 2011). Therefore, aquatic and marine environments are well suited for acoustic communication as signals have a better chance of reaching the receiver. Despite this, there are fewer sound-producing species in water compared to those in terrestrial

environments- assumed to be a consequence of physiological constraints and costs involved with producing sound in water (Clara and Amorim, 2015).

Despite the small number of soniferous marine species, they have the largest variety of sound-producing mechanisms among vertebrates (Ladich, 2004). There are over 800 known sound-producing fishes (Smith *et al.*, 2018) and their mechanisms have evolved independently in various lineages (Parmentier and Fine, 2016). Teleosts possess four basic sound-producing mechanisms which include (1) percussion of a substrate, (2) vibration of appendages/organs or muscles, (3) forced water flow through an opening and (4) stridulation (Bertucci *et al.*, 2014; Parmentier and Fine, 2016). However, the two most common sound-producing categories are: Swim bladder vibration using sonic muscles and stridulation of bony products (Smith *et al.*, 2018). Acoustic studies, dating back to as early as 1954, describe differences in the sounds produced by these two mechanisms (Kaatz, 2002). They describe swim bladder sounds as rapid contractions of the sonic muscles, which deform the swim bladder walls, producing hollow, low frequency (50 – 1500 Hz) sounds (Kaatz, 2002; Bertucci *et al.*, 2014) while stridulation sounds are broadband (50 – 10 000 Hz), rapid, repeated pulses with limited acoustic properties and no harmonic structure (Kaatz, 2002; Ladich, 2004; Clara and Amorim, 2015). Stridulation sounds occur when bony components are rubbed together causing friction and are further amplified via the swim bladder (Bertucci *et al.*, 2014; Parmentier and Fine, 2016).

Despite the various sound-producing mechanisms, fish have a limited acoustic repertoire with only one or two distinct sounds per species (Clara and Amorim, 2015). Fish produce two types of sounds: tonal and pulsed sounds. Tonal sounds are longer vibrational sounds produced by repeated contraction of the sound-producing muscles; the frequency of these sounds does not depend on fish size. Pulsed sounds are produced by a single sonic muscle contraction which creates one pulsed sound. The frequency of these pulses depends on the sound-producing mechanism and the size of the individual (Farrell, 2011). Pulsed sounds typically consist of repetitive, low-frequency pulses that vary in number and duration (van der Sluijs *et al.*, 2011; Clara and Amorim, 2015) and generally have poor amplitude and frequency modulation (Clara and Amorim, 2015). Fish vocalizations vary in their spectral (frequency) and temporal (duration) properties. The biggest variability in fish sound has been found in the time domain with

differences in the rate and number of pulses (Clara and Amorim, 2015). Winn, (1964) demonstrated that *Opsanus tau* communication is greatly dependent on the temporal properties of sounds and Fay, (1985) concluded that temporal processing has been adapted for fish auditory systems while processing in the frequency domain is fairly poor. This has led researchers to believe that communication is coded in the time domain rather than in the frequency domain for marine species (Wysocki and Ladich, 2002). The Haemulidae family is the most well-researched soniferous family whose main frequencies occur between 100 – 500 Hz (Ladich, 2004).

Haemulidae, commonly known as grunts, are well known among the fishing communities for the loud sounds that they produce when captured. It is assumed that they grunt during distress situations (Bertucci *et al.*, 2014) and competitive interactions (Milot *et al.*, 2021). Many Haemulidae species have been observed producing sound during manual stimulations: *Haemulon album*, *Haemulon carbonarium*, *Pomadasys corvinaeformis* and *Orthopristis chrysopterus*. Two Haemulidae species, *Haemulon flavolineatum* (Bertucci *et al.*, 2014) and *Haemulon aurolineatum* (Milot *et al.*, 2021) have received more extensive research on their acoustic properties. *Haemulon aurolineatum*, the tomtate grunt, shows variability in both pulse period and sound duration. Smaller specimens produce isolated grunts while larger specimens produce a series of grunts when handled (Milot *et al.*, 2021). Tomtate grunt show a negative relationship between specimen size and dominant frequency, a common phenomenon described in other animals, including several other teleost species. This suggests that there may be size information about the sender encoded in the frequency of these grunts (Colley *et al.*, 2009). To date, no studies have explored the acoustic repertoire of the largest Haemulidae species, *Pomadasys commersonnii*, the spotted grunter.

Spotted grunter have a wide distribution and being eurytopic, they live in a wide variety of environmental conditions. This can be problematic as different communication modalities have specific costs and benefits associated with differing environments. Most animals can communicate in more than one sensory modality and this multimodal communication can be vital to the evolution and survival of the organism (Partan, 2017). Consequently, fish may show a preference to a specific modality depending on their local environment (van der Sluijs *et al.*,

2011). A multimodal shift in communication can be advantageous for organisms dealing with rapid environmental change, especially anthropogenic change. This study sought to determine whether spotted grunter alter their acoustic communication based on their local environmental conditions. A growing body of literature has investigated the effects of environmental factors on sensory systems with several studies focusing on water clarity and turbidity (van der Sluijs *et al.*, 2011). Turbidity not only affects visual signals (Utne-Palm, 2002) but also affects the attenuation of sound as the suspended particles reflect and diffract sound energy (Brown *et al.*, 1998). In high turbidity environments, which have reduced visibility, one could expect a higher degree of reliance on acoustic communication. Whereas those individuals living in clearer environments could rely predominantly on visual signals.

This chapter aimed to investigate whether spotted grunter from two different biogeographic estuaries, with contrasting turbidities, differ in their acoustic repertoires. It was hypothesised that spotted grunter from a turbid environment (Kei Estuary) would produce louder (higher energy/amplitude) sounds that travel farther (low frequencies) compared to the sounds produced by spotted grunter in a less turbid environment (Breede Estuary). Individuals living in less turbid environments may opt to rather take advantage of visual signals, such as behaviour and body patterns/colouration, for communication. Spotted grunter, as the name suggests, are covered in spots that may also be used to communicate information. Thus, it was hypothesised that spotted grunter from two different estuaries, the turbid Kei Estuary in the Eastern Cape and the less turbid Breede Estuary in the Western Cape, will have significantly different acoustic repertoires.

4.3 Proof-of-Concept

To date, there has been minimal focus on fish bioacoustics in South Africa and as such there is limited equipment/technology specifically targeted for this research. It was unknown whether unique differences in spotted grunter sounds could be detected successfully. Therefore, a proof-of-concept study was required. The objective for the proof-of-concept was to investigate if sounds could be recorded, analysed and differentiated from fish in the following three scenarios:

- i. Two soniferous fish families that differ in sound apparatus: Haemulidae (stridulation mechanism) and Sciaenidae (drumming mechanism).
- ii. Four genera within the Haemulidae family that use the same sound apparatus: *Conodon*, *Haemulopsis*, *Orthopristis* and *Haemulon*.
- iii. Seven species within the *Haemulon* genus: *H. album*, *H. aurolineatum*, *H. carbonarium*, *H. flavolineatum*, *H. parra*, *H. plumierii* and *H. sciurus*.

4.3.1 Methods and Materials

Recordings of two soniferous fish species: *Argyrosomus japonicus* (the dusky kob: Sciaenidae) and *Pomadasys commersonnii* (the spotted grunter: Haemulidae) were taken at the Two Oceans Aquarium and the Sea Point research aquarium in Cape Town, South Africa. An HTI 96-min hydrophone (sensitivity: -163.9 dB re 1 V μPa^{-1} ; flat frequency response range 2 Hz – 30 kHz; High Tech, Inc. Long Beach, ms, USA) attached to a DR-44WL Tascam recorder (sampling frequency: 9600 Hz, 24-bit resolution; TEAC, Wiesbaden, Germany) was used for recordings. The hydrophone was placed in the predator tank at the Two Oceans Aquarium over two consecutive nights. The predator tank has both dusky kob and spotted grunter, however, only dusky kob vocalizations were recorded. The hydrophone was also placed in both dusky kob and spotted grunter tanks at the Sea Point research aquarium over five consecutive nights. Dusky kob recordings were captured on all five nights however no spotted grunter recordings were made. Finally, Haemulidae recordings were downloaded from FishBase (Froese, Pauly and Editors, 2021) for analysis (Appendix 4.1).

All recordings were analysed in **Raven Pro v. 1.5**. ('Raven Pro: Interactive Sound Analysis Software', 2022) and the following measurements were made on each sound: dominant frequency (Hz), center frequency (Hz), delta frequency (Hz) and delta time (ms).

Principal component analyses (PCA) were used to analyse the multivariate acoustic data. A PCA aims to condense variability in the data into principal components. Observations are then depicted on a map which can be used to interpret the similarity between observations (Abdi and Williams, 2010). The PCAs were created using the R function '*prcomp*' and the first two principal components were displayed graphically in each scenario. For the family PCA, the acoustic variables were averaged, standardized and normalized for sounds produced by the same individuals.

4.3.2 Results

For this proof of concept, a total of 271 individual grunts from 12 species of fish were analysed. The first test, to determine if two families of fish with different sound apparatus could be distinguished by their acoustic parameters, was successful. The PCA groups the 10 Haemulidae species together while the Sciaenidae species is separated from the rest (Figure 4.1A). The first two axes account for 77.97% of the variation between these two families.

A similar result was seen when comparing four genera within the Haemulidae family with over 80% of the variation explained in the first two axes (Figure 4.1B). While the variation between these species is not as distinct as that between families, the species are well clustered together within their genus.

Lastly, when comparing the acoustic properties of seven species within the *Haemulon* genus there was over 74% variation explained by the first two axes (Figure 4.1C). Seven of the *Haemulon* species (*H. album*, *H. aurolineatum*, *H. carbonarium*, *H. parra*, *H. plumierii* and *H. sciurus*) have tight distributions while *H. flavolineatum* shows a broader distribution of acoustic parameters.

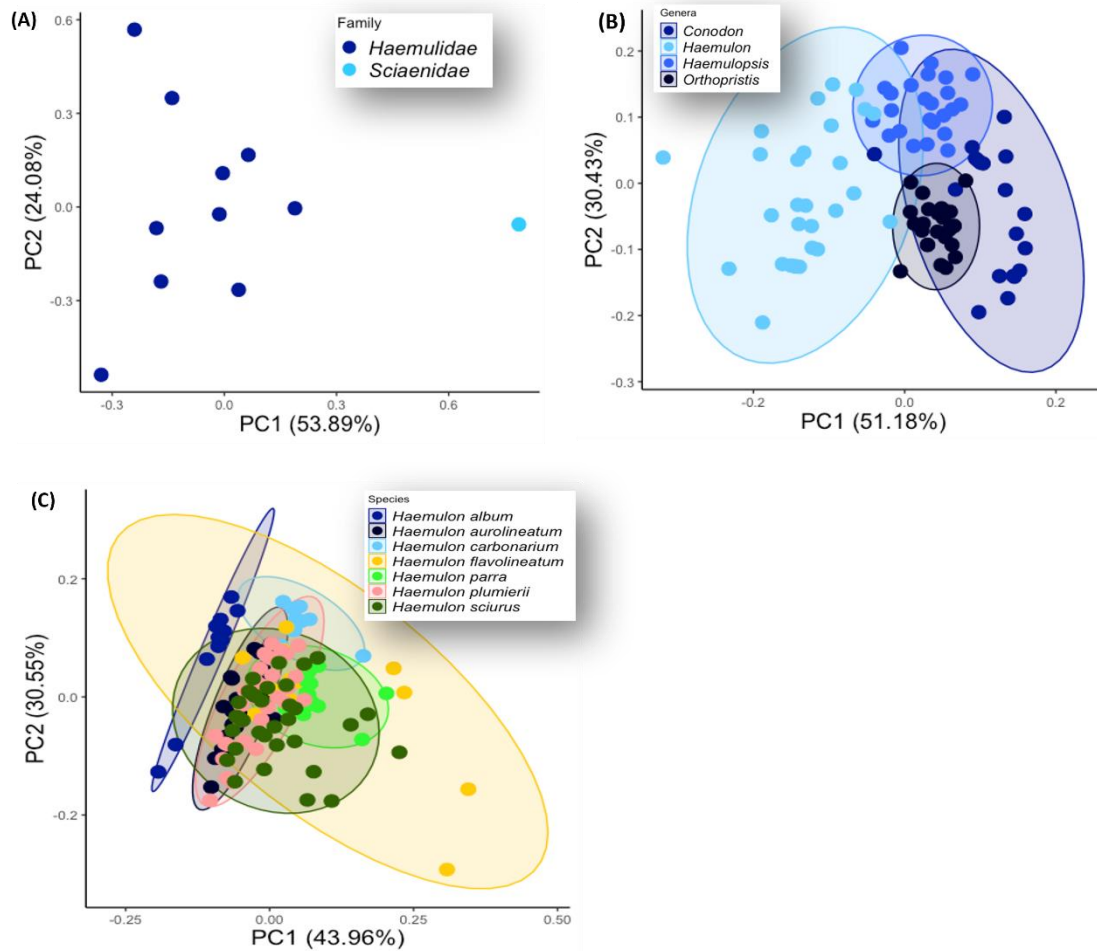


Figure 4.1. PCA of acoustic characteristics: Sounds produced by fish from (A) two different families of fish, (B) four different genera within the *Haemulidae* family and (C) seven species from the *Haemulon* genus. The first two principal components are displayed in each case and data ellipses are shown in (B) and (C).

4.3.3 Conclusion

The proof-of-concept suggested that it is possible to differentiate the sounds from different fish in the following three scenarios:

- ✓ Two soniferous fish families that differ in sound apparatus: Haemulidae (stridulation mechanism) and Sciaenidae (drumming mechanism).
- ✓ Four genera within the Haemulidae family that use the same sound apparatus: *Conodon*, *Haemulopsis*, *Orthopristis* and *Haemulon*.
- ✓ Seven species within the *Haemulon* genus: *H. album*, *H. aurolineatum*, *H. carbonarium*, *H. flavolineatum*, *H. parra*, *H. plumierii* and *H. sciurus*.

Since the above three scenarios were possible, it was believed that it would be possible to detect differences that might exist between spotted grunter captured from two estuaries that differ significantly in levels of turbidity.

4.4 Methods and Materials

4.4.1 Acoustic Recordings

Acoustic data were collected over three years (2018 – 2021) at two different sites during five week-long trips: three at the Breede Estuary in the Western Cape and two weeks at the Kei Estuary in the Eastern Cape (Ethics permit numbers: 2018/v10 /SK, RES2020/86, RES2021/56). An HTI- 96 min hydrophone (sensitivity: - 163.9 dB re 1 V μPa^{-1} ; flat frequency response range 2 Hz – 30 kHz; High Tech, Inc. Long Beach, ms, USA) attached to a DR-44WL Tascam recorder (sampling frequency: 9600 Hz, 24-bit resolution; TEAC, Wiesbaden, Germany) was used to record spotted grunter sounds for the duration of the study.

Three separate attempts were made to capture spotted grunter sounds under ‘natural’ conditions:

- i. Passive listening from an anchored boat over known spotted grunter territory, during both the day and night.
- ii. Passive listening of spotted grunter, captured via hook and line, placed in holding nets in a quiet harbor overnight.
- iii. Passive listening of spotted grunter as they were reeled in after being captured via rod and line.

Unfortunately, all three attempts were unsuccessful. However, several studies have had success in capturing sounds when fish are restrained/held in a tank (Raick *et al.*, 2020; Millot *et al.*, 2021). This method was successfully implemented for the remainder of the study.

Spotted grunter were captured via a beach seine net (120m long x 4m deep with a mesh size of 48mm; cod end: 4m wide x 8m long with 44mm mesh size) as part of the routine estuarine surveys conducted by DFFE. Individual grunter were then measured (fork length and total length) and placed in a large tub for acoustic recordings. To prevent the grunter from creating ambient noise through movement and to encourage sound production, they were all gently

handled for the duration of the recordings. The hydrophone was secured to a long pole and placed in the tub to record.



Figure 4.2. Fieldwork images: The setup used to capture and record the sounds of *Pomadasys commersonnii*. *Top:* An example of a sampling location used along the Breede Estuary. *Middle left:* A beach seine net is being pulled onto the bank to catch *Pomadasys commersonnii*. *Bottom left:* A *Pomadasys commersonnii* being lowered into the water to capture sound recordings. This shows how the *Pomadasys commersonnii* were handled throughout the recording. *Right:* The hydrophone is set up to capture recordings while listening in real-time to ensure good-quality sounds.

4.4.2 Acoustic Pre-Processing and Analysis

Acoustic files were viewed and analysed in **Raven Pro v. 1.5** ('Raven Pro: Interactive Sound Analysis Software', 2022) a program that detects grunts visually and aurally via spectrograms and oscillograms. Each acoustic sound file was viewed in 60s windows with a frequency range of 0 to 500Hz and created using a Hann window (50% overlap) with a fast Fourier transform (FFT) of 8192.

Each sound file was adjusted for brightness and contrast to best detect individual grunts. Individual grunts were selected and measured for dominant frequency (Hz) and energy (dB; Table 4.1). Additional temporal measurements were made including grunt duration (ms), inter-grunt interval (ms), inter-pulse interval (ms) and series duration (s; Figure 4.3). A series was defined as two or more grunts separated by less than one second. When a series occurred, the number of grunts per series was also recorded. Good-quality grunts with minimal background noise were preferred for analysis and as such some sound files had to be discarded. All measurements were then extracted from **Raven Pro** as text files and combined into csv files.

Table 4.1: Raven Pro Acoustic measurements used to characterize sounds produced by spotted *Pomadasys commersonnii*.

Measurement	Unit	Explanation
Grunt duration	ms	Time from beginning to end of individual grunt
Inter-grunt interval	ms	Time from the end of one grunt until the start of the following grunt within a series
Inter-pulse-interval	ms	The time from the peak of one pulse to the peak of the following pulse within a grunt
Series duration	s	The time from the start of the first grunt until the end of the last grunt within a series.

The total energy of the grunt is given by:

$$\left(\sum_{t=t_1}^{t_2} \sum_{f=f_1}^{f_2} (W_0 \cdot 10^{\left(\frac{S_{t,f}}{10}\right)}) \right) \Delta f \quad (\text{Equation 4.1})$$

Energy	dB	Where: F_1 and f_2 are the lower and upper-frequency limits T_1 and t_2 are the beginning and ending frame numbers. W_0 is the power dB reference value (equal to unity) $S_{t,f}$ is the spectrogram power spectral density in frame t at frequency f (in dB) Δf is the frequency bin size
Dominant frequency	Hz	The frequency at which maximum power occurs within the selection

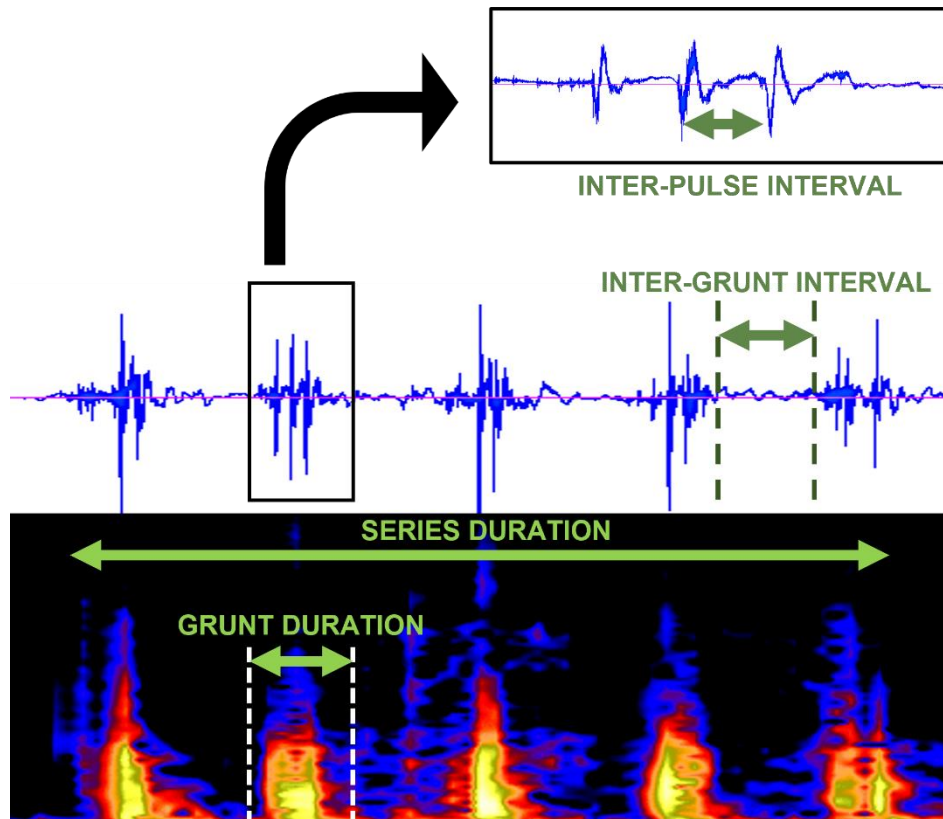


Figure 4.3: Temporal grunt measurements: Oscillogram (top) and spectrogram (bottom) of four grunts produced by *Pomadasys commersonnii*. Four temporal measurements are shown: inter-pulse interval, inter-grunt interval, series duration and grunt duration. Spectrogram colours indicate the relative sound intensity (Red = high, blue = low).

4.4.3 Statistical Analysis

SIZE COMPOSITION

To determine differences in the size structure of spotted grunter the mean fork lengths were tested for significance between the two sample areas. As the length of spotted grunter from the Breede Estuary did not demonstrate a normal distribution, a non-parametric Mann-Whitney U test (from the R package 'exactRankTests') was selected.

Kernel density estimates (KDEs) were performed on size frequency data to compare population size structure between the two sample areas. The KDE is a non-parametric method that

estimates the probability density function of lengths (x) from the two sampled areas. Unlike other density estimates, KDEs do not require discrete bins to represent continuous variables (Samiuddin and El-Sayyad, 1990; Silverman, 2018). It overcomes this problem by estimating the shape of the frequency distribution $f(x)$ by using the kernel (K) and smoothing parameter (h):

$$\hat{f}(x) = \frac{1}{nh} \sum_{i=1}^n K\left(\frac{\hat{x} - x_i}{h}\right) \quad (\text{Equation 4.2})$$

Where K is the kernel, h is the bandwidth (smoothing parameter) and n is the number of observations. The kernel density was fitted using the ‘*ggdensity*’ function in the R package ‘*ggplot2*’.

Fish size has been shown to affect certain acoustic parameters such as grunt duration and dominant frequency (Bertucci *et al.*, 2014; Millot *et al.*, 2021). Therefore, the relationship between fork length (mm) and each of the following variables was explored using linear models: dominant frequency (Hz), energy (dB) and grunt duration (ms).

SOUND PARAMETERS

The mean and standard deviation of the acoustic parameters were calculated for each sample area and depicted using boxplots. The data were assessed for normality using Shapiro-Wilks’s normality test. As the assumption for normality was not found, a non-parametric Mann-Whitney U test (from the R package ‘*exactRankTests*’) was used to evaluate the variance around the means between the two sampling areas.

MULTIVARIATE STATISTICS

All acoustic parameters were accounted for size effect to reduce any effect of body size (Malavasi, Collatuzzo and Torricelli, 2008). To investigate the correlation between measured variables a correlation matrix was conducted using the function ‘*rcorr*’ from the R package

'Hmisc'. A Spearman's correlation matrix was performed between fork length (mm), grunt duration (ms), series duration (s), inter-grunt interval (ms), inter-pulse interval (ms), dominant frequency (Hz) and energy (dB). The output from the above correlation matrix provides both the correlation coefficient and correlation p-value for each pair of variables.

A Principal component analysis (PCA) was used to condense the acoustic data into fewer dimensions to assist in analysis. The following standardized and normalised variables were included in the PCA: energy (dB), dominant frequency (Hz), Grunt duration (ms), series duration (s), inter-grunt interval (ms) and inter-pulse interval (ms). By reducing the dimensionality of multivariate data, one can visualize the data graphically with minimal data loss (Daffertshofer *et al.*, 2004). Thus, the PCA aims to show explained variation in the data through principal components. It assumes the directions in the data with the largest variances are the most important and therefore assists in identifying which acoustic parameters were most influential to the variability between the sample areas. The first two axes, PC1 and PC2, were estimated and displayed graphically using the R function '*prcomp*'.

To test for homogeneity of group dispersions a Principal coordinate analysis (PCoA) was conducted on the length-standardized acoustic parameters. To calculate the Bray-Curtis distances between samples the '*vegdist*' function was used from the R package '*vegan*.' The multivariate dispersion was calculated using the '*betadisper*' function from the R package '*vegan*.' This function is analogous to the univariate Levene's test for homogeneity (Levene, 1960) and is an implementation of Marti Andersons Permdisp (Anderson, 2004). An ANOVA test was performed on the output to determine if group dispersions were homogenous between the two sample areas. The PCoA produces orthogonal axes that summarize the variability in the data. Objects that are ordinated closer together have smaller dissimilarity values than those further apart. The PCoA is displayed with one standard deviation confidence ellipse about the group medians.

An analysis of similarities (ANOSIM) was conducted to test for significant differences between the acoustic repertoire of the two sample areas. This non-parametric test is based on distance measures that are converted to ranks which are used to compare two or more samples (Clarke,

1993). The non-parametric ANOSIM test was conducted on the length-standardized acoustic parameters by using the '*anosim*' function from the R package '*vegan*.' The R statistic produced by the ANOSIM measures the degree of separation between the two sampled areas and a value of zero would indicate no difference between the acoustic repertoire of the sampled areas whereas an R value of one would indicate that the acoustic repertoire between the two sample areas was completely different. Similarity percentages (SIMPER) were estimated to determine which acoustic parameter contributed most to the dissimilarities between the sample areas (Clarke, 1993). This estimates the proportion that each acoustic parameter contributes to the Bray-Curtis distance found between the two sample areas.

4.5 Results

4.5.1 Size Comparison

Sounds of spotted grunter were recorded from two areas: the Breede Estuary and the Kei Estuary. Only those specimens that produced sounds were used for this study and some recordings were discarded due to high background noise. A total of 1416 grunts were analysed from 107 individuals. There was a significant difference in the size range of spotted grunter from the two sample areas with the Kei Estuary being composed of smaller individuals compared to those found in the Breede Estuary (Mann Whitney U test, p -value $< 2.2e-16$; Figure 4.4). The 59 spotted grunter from the Breede Estuary had fork lengths ranging from 298mm to 615mm (405.3 ± 87.0) and the 47 individuals from the Kei Estuary ranged from 139mm – 585mm (349.8 ± 87.3).

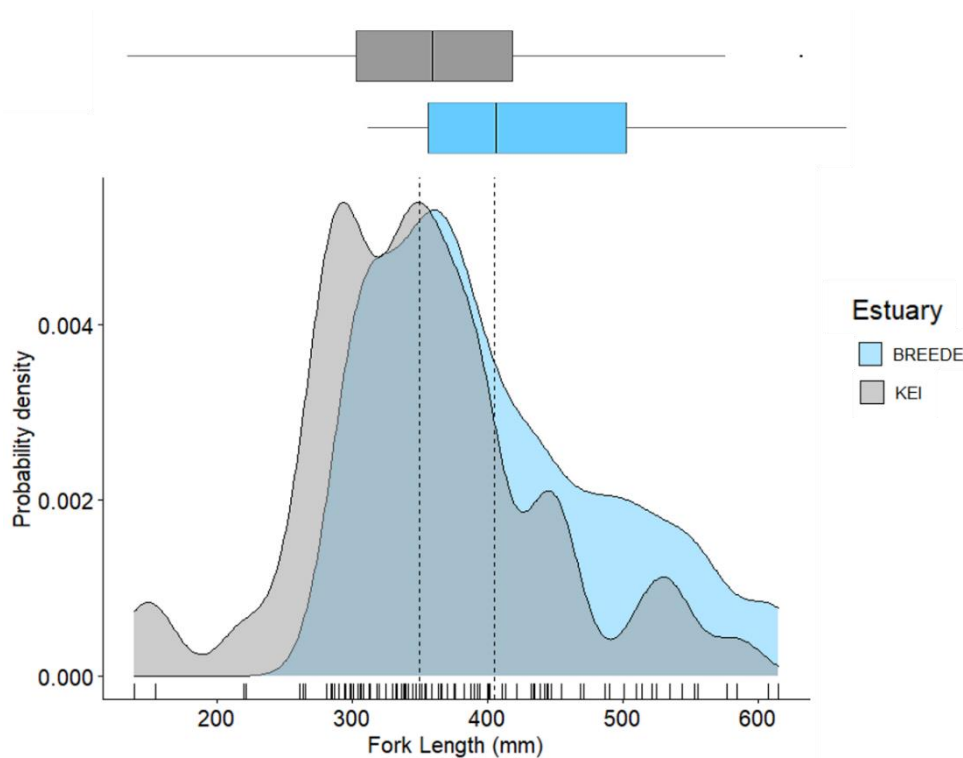


Figure 4.4: Length of *Pomadasys commersonnii*: length of individuals sampled from two different areas: Breede Estuary (blue) and Kei Estuary (grey). Top: Boxplots depicting the size range for each estuary. Bottom: Kernel density estimate (KDE) probability function of spotted grunter fork lengths from two sample estuaries. The dotted lines represent the mean fork length for each sample and the rug lines above the x-axis represent each spotted grunter measured in the study.

Fish size had low correlation ($-0.4 < r < 0.4$) coefficients with all acoustic parameters (Appendix 4.2). Fork length had a non-significant negative correlation with dominant frequency (Hz; $r = -0.18$; $p > 0.05$; $n = 107$) and a significant positive correlation with energy (dB; $r = 0.35$; $p < 0.05$; $n = 107$) and grunt duration (ms; $r = 0.23$; $p < 0.05$; $n = 107$). Both areas showed positive correlations between fish size and grunt duration (Figure 4.5). Grunt duration varied between the two areas with the Breede Estuary having, on average, longer grunts (ms) compared to the Kei Estuary. The Kei Estuary had a larger variability in the dominant frequency (Hz) of sounds compared to the Breede Estuary and both estuaries have weak negative correlations between dominant frequency and fish size. The energy (dB) of the grunts were significantly higher for the Breede Estuary compared to the Kei Estuary ($p > 0.001$; Figure 4.5).

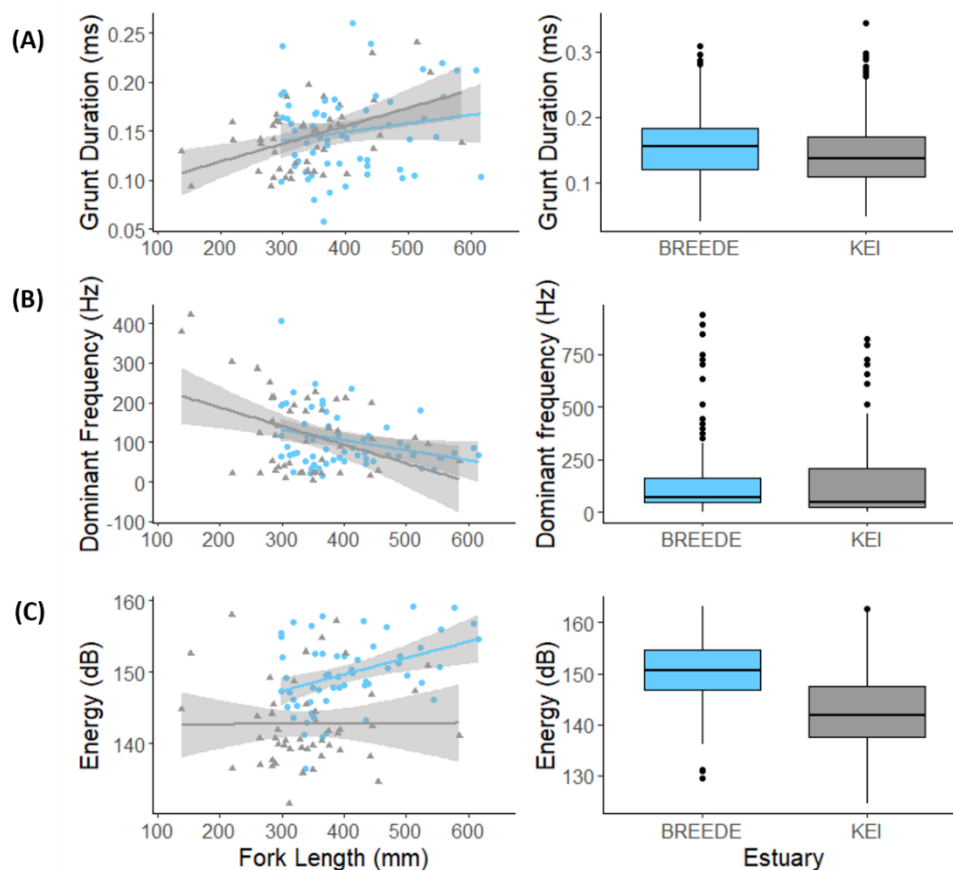


Figure 4.5: Acoustic features of *Pomadasys commersonnii*: Sound characteristics of individuals captured in the Breede Estuary (blue) and Kei Estuary (grey). Left: Relationship between acoustic features and fork length for sounds produced by *Pomadasys commersonnii*. Right: Boxplots showing the difference between acoustic features of *Pomadasys commersonnii* between two estuaries. (A) Grunt duration (ms), (B) Dominant frequency (Hz) and (C) energy (dB).

4.5.2 Acoustic Parameters

Six acoustic parameters were measured for this study: dominant frequency, energy, grunt duration, inter-grunt interval, inter-pulse interval and series duration (Table 4.2). The mean values of five acoustic parameters were significantly different between the two sample areas; the sixth variable, inter-grunt interval, did not differ significantly between the two areas (Table 4.2). The mean dominant frequency was significantly higher in the Kei Estuary (120.4 ± 138.3 Hz) compared to the Breede Estuary (113.7 ± 111.8 Hz), while energy was significantly higher in the Breede Estuary (150.6 ± 5.7) than in the Kei Estuary (142.9 ± 7.2). The mean values of three temporal measurements were significantly different between the two areas: grunt duration, inter-pulse interval and series duration. All three of these temporal measurements were longer in the Breede Estuary compared to the Kei Estuary (Table 4.2).

Table 4.2: *Pomadasys commersonnii* sound measurements of grunts produced by individuals from two sample areas: The Breede and Kei Estuary. Measurements are given as mean $\pm$ Standard deviation (minimum value – maximum value). The corresponding p-value for the mean difference of these characteristics between the two sample areas is also given: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Acoustic variable	Breede	Kei	p-value
Dominant Frequency (Hz)	113.7 ± 111.8 (23.5 – 937.5)	120.4 ± 138.3 (23.4 – 820.3)	***
Energy (dB)	150.6 ± 5.7 (129.5 – 163.2)	142.9 ± 7.2 (124.5 – 162.7)	***
Grunt duration (ms)	155.1 ± 45.8 (41.0 – 309.0)	142.7 ± 44.9 (47.3 – 343.1)	***
Inter-grunt interval (ms)	234.9 ± 82.0 (112.3 – 554.0)	245.4 ± 72.0 (110.4 – 455.6)	0.295
Inter-pulse interval (ms)	25.9 ± 9.4 (6.0 – 61.2)	20.0 ± 9.2 (0.6 – 42.2)	***
Series duration (s)	2.2 ± 1.8 (0.5 – 10.1)	1.2 ± 0.6 (0.4 – 3.6)	***

The multivariate dispersion test was significant for differences in dispersion distances of acoustic measurements for the two sampled areas ($F = 5.4$; $p = 0.022$). Figure 4.6. depicts the large variability within acoustic measurements recorded from fish sampled from the Kei Estuary compared to those sampled from the Breede Estuary (Figure 4.6). The ANOSIM output was significant, suggesting that the acoustic properties of spotted grunter differ between the two sample areas ($R \text{ stat} = 0.05477$, significance = 0.0097).

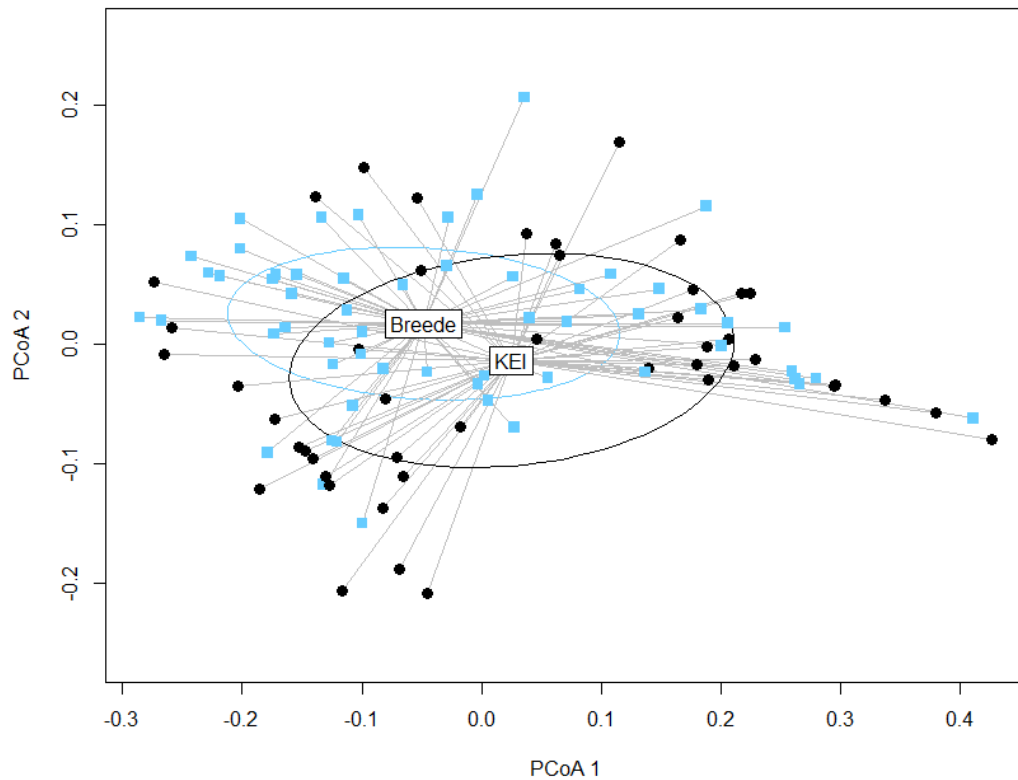


Figure 4.6: PCoA of acoustic parameters: *Pomadasys commersonnii* acoustic features from a Bray-Curtis dissimilarity matrix. *Pomadasys commersonnii* from two areas are shown: Breede Estuary (Blue) and Kei Estuary (Black). One standard deviation ellipse is given for each area's dispersion.

The principal component analysis describes variation in acoustic characteristics between the length-standardized acoustic features from fish sampled in the two different estuaries. This variation is then depicted by plotting the first and second discriminating axes, PC1 and PC2 (Figure 4.7). The first two principal components explained a large amount of variation in the acoustic features with a total of 65.55% (with 49.51% and 16.04% respectively; Figure 4.7).

Results of a SIMPER test suggest that the primary contributing acoustic feature to this distribution is dominant frequency (Hz; Appendix 4.3).

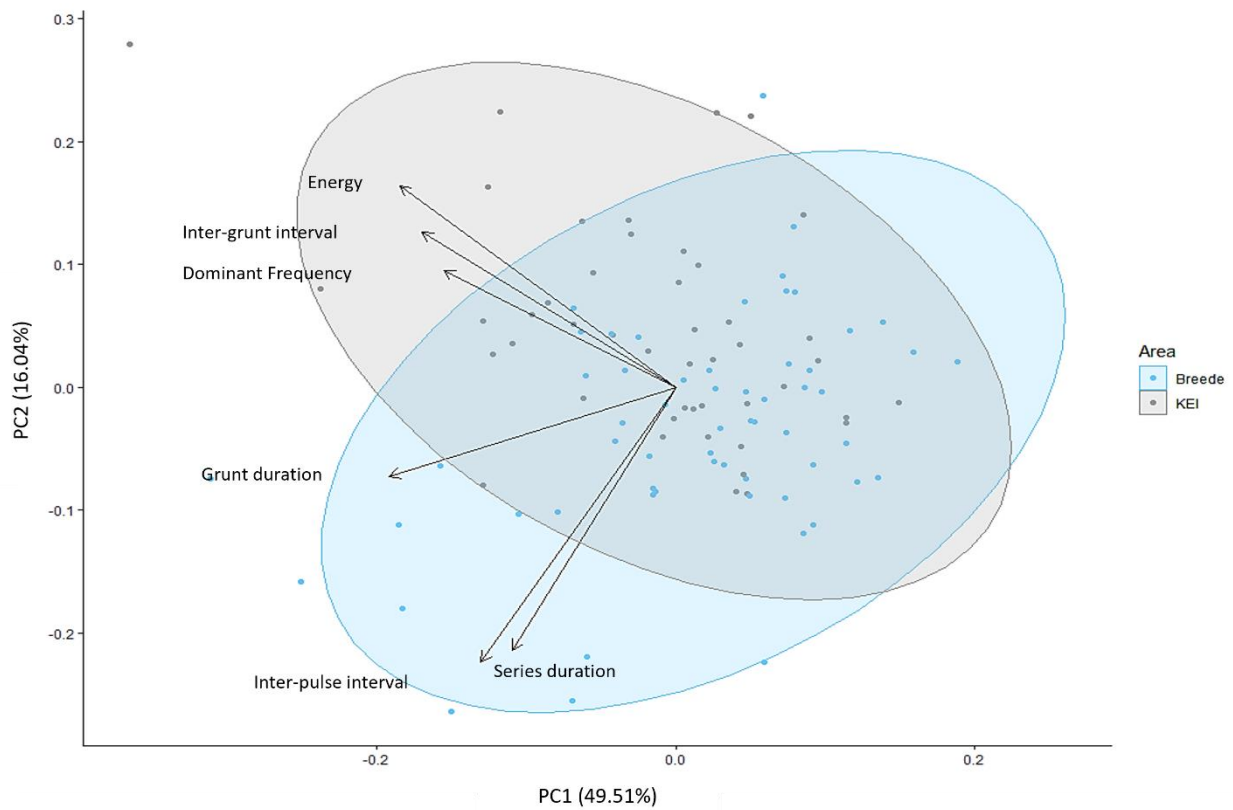


Figure 4.7: PCA of acoustic characteristics: Sounds produced by *Pomadasys commersonnii* from two sample areas: Breede Estuary (blue) and Kei Estuary (grey) with their corresponding 95% probability ellipse. The first two axes are depicted and the eigenvectors for this distribution are shown in black: Energy (dB), Inter-grunt interval (ms), Dominant frequency (Hz), Grunt duration (s), Inter-pulse interval (ms) and Series duration (s).

4.6 Discussion

Acoustic communication plays an invaluable role in the survival and success of fishes in all environments but is particularly beneficial in turbid and dark environments. How the acoustic signals of species are influenced by local environmental conditions is important for future conservation efforts. This study provided the first evaluation of the acoustic sounds produced by spotted grunter, *Pomadasys commersonnii*. It was hypothesised that in-situ differences in environmental physicochemical properties will affect communication modalities in spotted grunter, and the results confirm this. Significant differences were found in the acoustic parameters of sounds produced by spotted grunter from two different estuaries, particularly dominant frequency, energy and grunt duration. The analysis also revealed that many acoustic parameters were significantly correlated to fish size – an important aspect to consider when interpreting the results.

Body size and acoustic features have been shown to correlate across a wide range of animals (Henglmüller and Ladich, 1999; Amorim, Vasconcelos and Fonseca, 2015). There was a negative relationship between dominant frequency and increasing body size in spotted grunter. Furthermore, as the size of spotted grunter increased, so did their sound energy and grunt duration, also demonstrated in *Haemulon aurolineatum* (Millot *et al.*, 2021) and *Haemulon flavolineatum* (Bertucci *et al.*, 2014). Consequently, these sounds could be considered index signals that are constrained physiologically and thus provide honest information about the sender to the receiver. There are no explanations for how the frequency and amplitude of these sounds are affected by fish size, however, grunt duration could be explained by the surface area of the sound apparatus. The pharyngeal teeth of spotted grunter (and haemulids in general), which produce the grunt sound when rubbed together, would increase in surface area as the fish grows thereby increasing the duration of sound production. Regardless of the mechanisms involved in these differences, these results suggest that sender information is coded in acoustic characteristics which would be particularly beneficial in agonistic and courtship encounters.

While the contrast between the acoustic parameters of spotted grunter vocalizations from the two estuaries was expected, the outcome was inverse to expectations. With spotted grunter

living in both turbid and non-turbid environments, it was predicted that, due to the sensory drive hypothesis, spotted grunter living in turbid environments would rely more on acoustic communication compared to those in non-turbid environments. To this end, the acoustic sounds of spotted grunter in the turbid Kei Estuary were hypothesized to have louder, low frequency grunts that can be heard from a greater distance compared to those in the Breede Estuary. However, the results suggested that the Breede Estuary has, on average, louder grunts with lower frequencies. Two *a priori* hypotheses could offer possible explanations: (1) the effects of anthropogenic ambient noise and (2) differences in spotted grunter communication behaviour due to different turbidities.

Fish have evolved sound communication in the presence of natural background noise caused by rain, currents, tides and other species (Merchant *et al.*, 2015). With growing human populations and technological advancements, there has been a substantial increase in background noise levels from anthropogenic activities (Codarin *et al.*, 2009; Slabbekoorn *et al.*, 2010). A considerable amount of research has been conducted on the negative effects of noise on goldfish – *Carassius auratus* (Smith, Kane and Popper, 2004), catfish (Amoser and Ladich, 2003) and Oscars – *Astronotus ocellatus* (Hastings *et al.*, 1996). Additionally, during the early hydrophone experimentation for this study, it was noted that boats could be heard on the hydrophone well before they could be detected visually. These unnatural, loud sounds can be harmful to an animal's anatomy and physiology (Amoser and Ladich, 2003; Smith, Kane and Popper, 2004). Scholik and Yan, (2000) illustrated that the sound from an idling (single 55-horsepower outboard) motorboat significantly reduced the hearing sensitivity of some fish from only two hours of sound exposure.

The anthropogenic noise experienced by spotted grunter in each of the two estuaries is vastly different. The Kei Estuary is remote with only a small rural community near the mouth. There is very little activity on the estuary and apart from one small vehicle ferry that operates near the mouth, there is, on average, only one boat launched on a given day (Kei Mouth Skiboat Club launch register). Furthermore, the majority of boats launched travel out to sea and do not make use of the estuary. Contrastingly, the Breede Estuary is lined with farmlands and communities and is a popular tourist destination (Flemming and Martin, 2021a). Historically,

the Breede Estuary was used by both recreational and commercial fishers but is now predominantly used by recreational fishers, bait collectors, wakeboarders and kite surfers (Turpie and Goss, 2014). On average, there are about 21 boats on the Breede Estuary at any given time and it is particularly busy over holiday periods and weekends where more than 100 boats have been recorded (LBRCT vessel monitoring; Lamberth, Van Niekerk and Hutchings, 2008).

Boat noise in marine protected areas has been shown to markedly reduce the detection distance of fish sounds (Codarin et al., 2009). Vasconcelos, Amorim and Ladich, (2007) investigated the effects of ship noise on the detectability of acoustic signals in the Lusitanian toadfish, *Halobatrachus didactylus*, and found that the auditory threshold was significantly affected by ship noise. Furthermore, the ability of toadfish to receive courtship mating calls from conspecifics was also reduced in the presence of ship noise (Vasconcelos, Amorim and Ladich, 2007). Boat sounds significantly mask the sounds produced by many species and this effect is most pronounced within the frequency ranges used by fish to communicate (Codarin et al., 2009). Hearing-damage coupled with the masking effect could decrease the distances at which individuals can communicate. Therefore, to counteract the effects of noise, fish have been found to either produce sounds more frequently (Picciulin et al., 2012; Ladich, 2019), to increase the signal-to-noise ratio by increasing the sound level (energy) of their calls (Ladich, 2019) or modify their call frequencies (Amoser and Ladich, 2003). This response to sound has been termed the Lombard effect, whereby an individual modifies their sounds when exposed to noisy environments (Holt and Johnston, 2014).

Apart from spectral sound changes, the spotted grunter from the Breede Estuary also produced sounds with longer temporal features. Fish sounds have generally been found to vary in temporal features rather than spectral features (van der Sluijs et al., 2011). The survival of organisms is dependent on their ability to respond to environmental changes, whether natural or human-induced. The spotted grunter from the Breede Estuary may be adapting their sound communication in response to anthropogenic boat noise by producing longer grunts with a longer series duration. By increasing the duration of their calls, they have a greater chance of their signals being received when there is a break in boat noise (Ladich, 2019). The above

research could explain why the sounds produced by spotted grunter in the Breede Estuary are louder, longer, lower frequency sounds compared to those in the Kei Estuary as a response to increased anthropogenic boat noise.

To date, there is limited research on Haemulidae communication and no literature specifically related to spotted grunter. Therefore, this study was based on the logical assumption that fish in turbid, poor-visibility environments would be more reliant on acoustic signals for communication. Intriguingly, on testing the hypothesis, the influence of behavioural responses to anthropogenic (motorboat) noise also came to the fore. Up to now, several studies have reported that acoustic communication is succeeded by a visual encounter. Fish will generally only start vocalizing after visually detecting conspecifics, and rarely respond to acoustic cues alone (Chabrolles *et al.*, 2017; Hyacinthe, Attia and Rétaux, 2019). The blacktail shiner, *Cyprinella venusta*, was reported to produce sounds almost exclusively with visual cues (Holt and Johnston, 2014), male African Cichlids, *Astatotilapia burtoni*, were found to only produce sounds in close proximity to females (Maruska, Ung and Fernald, 2012) and several species did not follow advertisement calls without additional stimuli, such as visual cues (Amorim *et al.*, 2013). Collectively, these studies suggest that acoustic signals are not used for long-distance communication and did not evolve solely to compensate for a lack of visibility but rather as an added method for close-range communication (Hyacinthe, Attia and Rétaux, 2019).

Detection distance would be much further away in non-turbid environments compared to turbid environments and this visual detection distance would be similar for both conspecifics and predators. In this case, spotted grunter in turbid environments do not need to produce loud, far-reaching sounds when their detection distance is very small. Additionally, producing sounds that exceed a fish's visual range can be dangerous as some predators may eavesdrop on these signals through passive listening (Ladich, 2019). There is evidence that some predators take advantage of fish sounds to locate their prey, this is illustrated by the diet of bottlenose dolphins which is predominantly composed of soniferous fish (Gannon *et al.*, 2005). Therefore, spotted grunter living in areas of poor visibility may need to adjust their acoustic signals to become less conspicuous to predators. This hypothesis provides a possible explanation for why

the spotted grunter from the Kei Estuary produced quieter grunts of higher frequencies compared to those from the Breede Estuary.

The observed differences in the acoustic parameters of spotted grunter from these two areas could eventually result in isolation due to behavioural/ethological differences. Changes in sensory systems can lead to speciation through the sensory drive hypothesis (Bernardi, 2013) and as such, monitoring changes in communication modalities could be a potential means of quantifying the rate of divergence. The high degree of variation within acoustic parameters from the Kei Estuary compared to that from the Breede Estuary could be interpreted as, and potentially support the idea of, the Breede Estuary being a newly established spawning population. When a new population is established, one would expect much less variation in features compared to older, more diverse populations. From this interpretation, one would expect, over time, to see greater differences in the acoustic repertoire of sounds produced by spotted grunter from these two estuaries as they adapt to enhance communication modalities for their local environments. However, these interpretations require further studies.

To further improve this study, a wider range of environmental variables should be considered. For example, a study on *Opsanus tau* found that the incidence of grunt vocalizations was positively correlated to water temperature (Maruska and Mensinger, 2009). Fundamental frequency and pulse rate have also been found to correlate with water temperatures in batrachoidid fishes. In Syngnathidae fishes, acoustic sounds, linked to feeding activity, were affected by low oxygen levels (Ripley and Foran, 2007). Additionally, recordings from different times of the day should be incorporated as some species may be more vocal at night. An important drawback to this study is the artificial setup that was used to capture spotted grunter vocalizations. This setting should be considered carefully when interpreting the results for research outside of this study. Haemulids are known to grunt under distress both in and out of the water; however, a study on white grunts, *Haemulon flavolineatum*, found a significant difference in the temporal characteristics of grunts produced in handheld distress calls versus those produced while foraging (Bertucci *et al.*, 2014). Despite best efforts to capture distress calls in a natural environment, all attempts were unsuccessful. As such, it is not known whether natural distress calls and those produced in this setup are significantly different. Despite this,

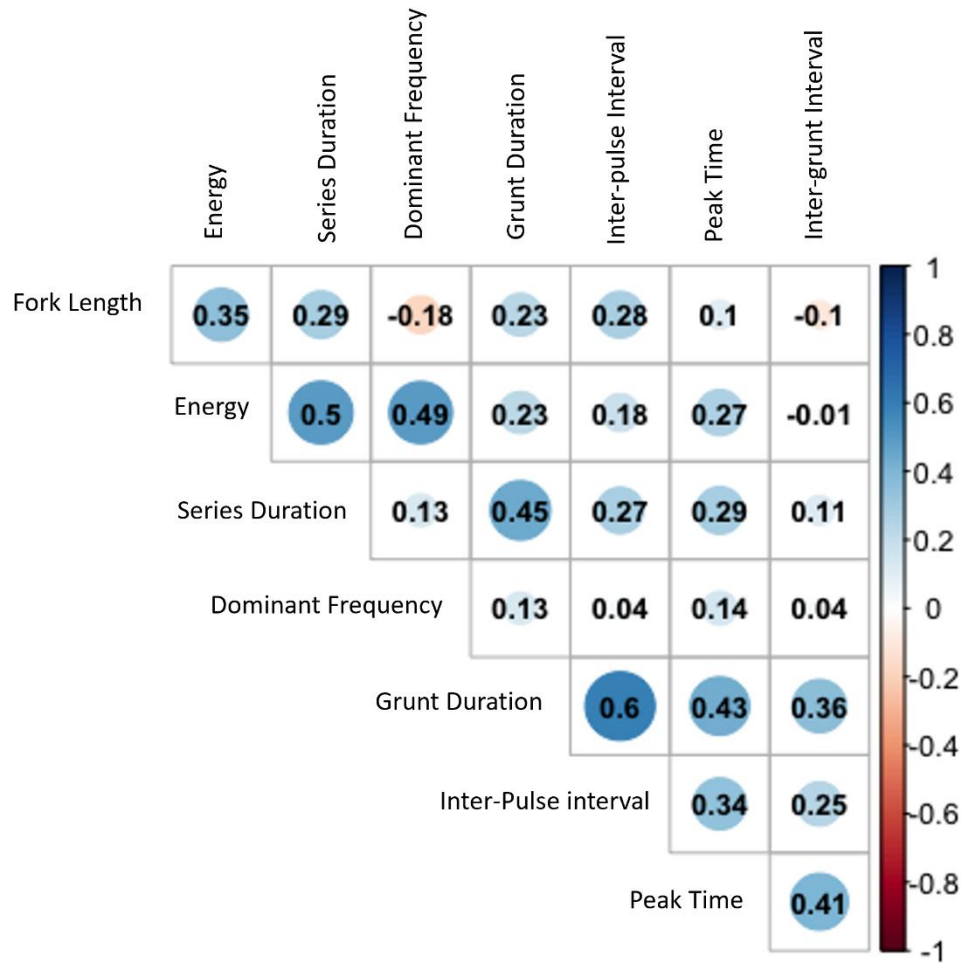
all recordings used in this analysis were taken within a constant artificial environment and are thus comparable.

In conclusion, this pioneering study demonstrated that it is possible to record and analyse the sounds produced by spotted grunter. It further demonstrated that it is possible to identify differences in the acoustic structure of vocalizations from spotted grunter living in two estuaries with vastly different environmental regimes. Environmental conditions are known to influence communication modalities and this has been demonstrated in the above study. These results can hopefully drive future research and interest into the biological function of spotted grunter vocalizations to monitor how this species adapts to local environmental conditions. Whether the difference in acoustic parameters between the two estuaries is a function of phenotypic plasticity or has a genetic basis has yet to be determined. Regardless, this is the first research of its kind in South Africa to not only analyse the acoustic repertoire of spotted grunter but to also report differences in the acoustic features of spotted grunter living under different anthropogenic noise levels and environmental regimes.

4.7 Appendix

Appendix 4.1. Species sounds downloaded from FishBase and used for the proof-of-concept study.

Haemulidae Species	Common name
<i>Haemulon album</i>	White margate
<i>Haemulon aurolineatum</i>	Tomtate grunt
<i>Haemulon flavolineatum</i>	French grunt
<i>Haemulon parra</i>	Sailors grunt
<i>Haemulon plumieri</i>	White grunt
<i>Haemulon sciurus</i>	Bluestriped grunt
<i>Conodon nobilis</i>	Barred grunt
<i>Haemulon carbonarium</i>	Caesar grunt
<i>Haemulopsis corvinaeformis</i>	Roughneck grunt
<i>Orthopristis chrysoptera</i>	Pig fish grunt



Appendix 4.2: Correlation matrix for *Pomadasys commersonnii* size (fork length) and acoustic parameters from vocalizations.

Appendix 4.3: SIMPER results for the acoustic parameters of *Pomadasys commersonnii* from two samples areas. The p-value is given where: * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Acoustic parameter	Cumulative contribution	P value
Dominant frequency	0.7147	*
Energy	0.9892	$p > 0.05$
Series duration	0.9986	$p > 0.05$
Inter grunt interval	0.9995	$p > 0.05$
Grunt duration	0.9999	$p > 0.05$
Inter-pulse interval	1.000	$p > 0.05$

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CHAPTER FIVE

FISHING FOR ANSWERS: GENOMIC DIFFERENTIATION OF *POMADASYS* *COMMERSONNII* FROM TWO ESTUARIES

5.1 Abstract

Application of molecular methods in fisheries management and marine conservation is increasingly crucial as the impacts of climate change, overfishing and habitat degradation drive many species to the brink of extinction. Current evidence suggests a recently established population of *Pomadasys commersonnii* (Haemulidae) exists in the Breede Estuary in South Africa, an area where they were not previously found. Consequently, the objective of this study was to survey genomic diversity in this new population and determine if *P. commersonnii* from the Breede Estuary could be genetically differentiated from an established population of the same species in the Kei Estuary located in the Eastern Cape of South Africa. Using Restriction site-associated DNA sequencing, 97 *P. commersonnii* were successfully sequenced and passed a rigorous quality check and a total of 7 175 single nucleotide polymorphisms were found. Subtle genomic differences were detected between the two populations suggesting some level of panmixia or recent co-ancestry between these two populations. Although genetic differentiation was low for the majority of loci, a small proportion of single nucleotide polymorphisms exhibited comparatively high levels of genetic differentiation, suggesting the potential presence of local adaptation. In addition, many of these outlier loci were associated with phenotypic traits that have been shown to differ significantly between *P. commersonnii* from these two estuaries. These could reflect different physiological responses as a result of environmental pressures. Thus, these heritable loci, or loci linked to them, may trigger early stages of population differentiation, possibly due to local adaptation to heterogeneous environments.

5.2 Introduction

Natural selection and the ability for populations to adapt to local environmental conditions is crucial for the long-term evolutionary success of a species (Allendorf, Ryman and Utter, 1987; Frichot and François, 2015). Global climatic change is fast becoming an increasing area of concern for the survival of many species and as a result, understanding the molecular bases that drive local adaptation in natural populations is fundamental for the establishment and successful implementation of conservation plans (Tigano and Friesen, 2016; Bernatchez *et al.*, 2017). It is commonly believed that the resilience of populations in the face of future climatic challenges is directly linked to their current level of genetic diversity (Cano *et al.*, 2008).

In conservation and fisheries management, 'stocks' or 'populations' are regarded as the relevant management unit. Furthermore, populations are also broadly accepted to represent the level at which evolutionary change typically occurs (Hauser and Carvalho, 2008). Accordingly, population genetics provides the framework needed to understand how genetic factors such as natural selection, gene flow, and genetic drift shape the genetic makeup of populations over time (Hohenlohe *et al.*, 2010; Sunde *et al.*, 2020). Without proper stock structure information, species are often managed as a single unit despite significant genetic, and consequent, differences. When there are multiple genetically distinct populations across the species range this management technique could result in a loss of genetic diversity (Reiss *et al.*, 2009) and this negatively affects the long-term evolutionary potential and the survival of these populations (Berntson and Moran, 2009).

While traditional approaches in managing fisheries are focused on short-term quantitative changes such as maximum sustainable yields (Hauser and Carvalho, 2008), molecular studies focus on qualitative data such as how the genetic composition of a population affects both short-term phenotypic variation and long-term adaptability to environmental pressures (Hart and Reynolds, 2002; Hauser and Carvalho, 2008). The inclusion of genetics has thus advanced research into population differentiation (both spatially and temporally), population connectivity and adaptive evolutionary change (Manuzzi *et al.*, 2022).

There are four main drivers responsible for genetic differentiation among populations: (1) mutation, (2) selection, (3) genetic drift and (4) gene flow with the latter being recognized as the most impactful evolutionary force behind population differentiation (Hart and Marko, 2010; Marko and Hart, 2011). Many terrestrial populations are separated by physical barriers such as mountains and rivers. These physical barriers decrease the level of gene flow and over time, due to localized selection or random drift, these populations can become genetically distinct from one another. However, the presence of genetically distinct populations with no apparent physical barrier is a popular area of research in population genetics.

Marine environments are connected via a continuous medium and its inhabitants are generally characterised by high dispersal capabilities, broad geographic ranges and large effective population sizes; these characteristics generally result in considerably higher rates of gene flow between marine organisms (Palumbi, 2003; Benestan, 2019). As a result, it is widely accepted that in marine environments, which lack physical barriers to gene flow, species are panmictic and show little genetic differentiation, despite their large dispersal opportunities (Palumbi, 2003; Neethling *et al.*, 2008; Werner *et al.*, 2020). However, several studies have found strong genetic structure in marine species, such as *Platichthys flesus* (Hemmer-Hansen *et al.*, 2007), *Clupea harengus* (Bekkevold *et al.*, 2005) and *Gadus morhua* (Nielsen *et al.*, 2003).

Genetic structure in marine populations generally fall within one of the three broad models: panmictic, stepping-stone and isolation-by-distance (Çiftci and Okumu, 2002; Benestan, 2019; Nikolic *et al.*, 2023). The panmictic model refers to a single intermixing population across the entire species distribution. The stepping-stone model refers to small, isolated populations that lack pronounced gene flow between one another, and the isolation-by-distance model is characterised by individuals within a continuous population that only exchange genes with those within their local geographic range (Wright, 1942; Teske *et al.*, 2018).

Genetic variation across a species distribution range is rarely uniform and is a facet of both historical and contemporary factors (Provan, 2013). Furthermore, panmictic populations can undergo divergence due to natural processes over time that can ultimately result in reproductive isolation (Hemmer-Hansen, Therkildsen and Pujolar, 2014).

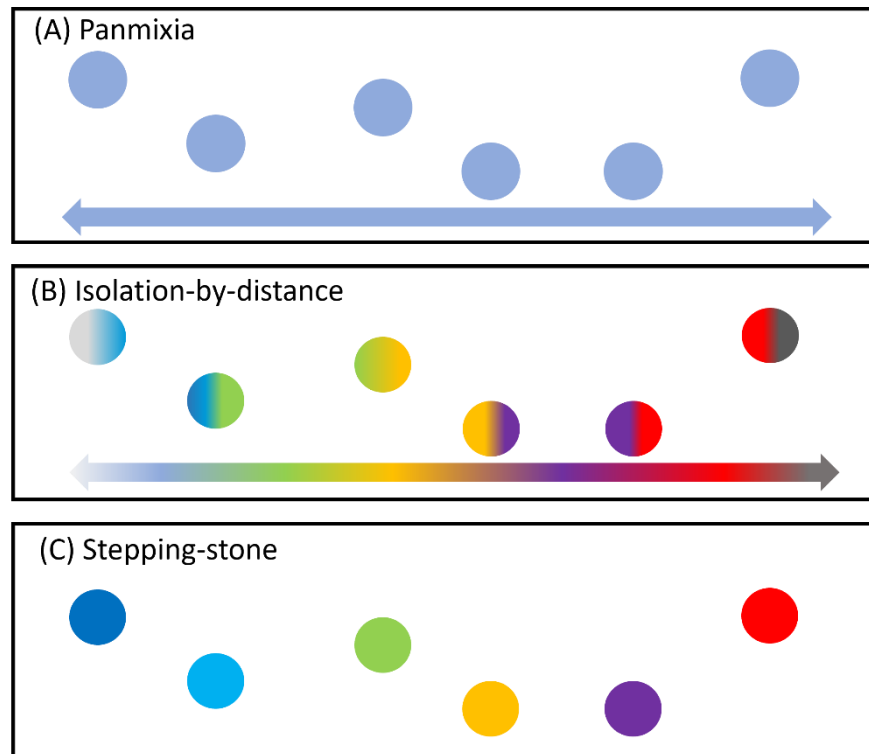


Figure 5.1. Gene Flow models: Panmictic model refers to an even distribution of alleles and genetic diversity across a species range. Isolation-by-distance is when gene flow only occurs with individuals within a close geographic range. The Stepping-stone model is characterised by isolated populations which lack gene flow between each other.

Population genetic studies based on genetic markers began in the early 1960s and have witnessed remarkable advances since then (Crawford and Oleksiak, 2016). Until recently, population genetic studies were based on DNA markers, such as numerically more abundant mitochondrial markers and microsatellites which only cover a small proportion of the overall genomic variation in populations (Bergey *et al.*, 2013). Furthermore, the traditional view that these markers are generally neutral has been challenged and studies point to the limited power of these markers to accurately reflect genetic structure within a species (Bergey *et al.*, 2013; Teske *et al.*, 2018). While there is undoubtedly value in these traditional methods, next-generation sequencing (NGS) has revolutionized molecular studies by enabling scientists to generate thousands of markers across the genome that provide an opportunity to explore the

evolutionary processes with an unprecedented level of detail (Davey and Blaxter, 2010; Davey *et al.*, 2011).

These studies focus on identifying single nucleotide polymorphism (SNP) markers across the genome by sequencing thousands of markers in a single step (Davey *et al.*, 2011; Andrews *et al.*, 2016). The design of such studies makes it possible to generate SNP panels that have high data quality, deep genomic coverage and low error rates (Milano *et al.*, 2011). By analysing thousands of SNPs there is a greater chance of detecting informative highly differentiated, polymorphic loci that are undergoing divergent selection (Hohenlohe *et al.*, 2010; Crawford and Oleksiak, 2016). Therefore, these genome-wide studies can provide information on the molecular bases of adaptation and speciation which is indispensable to population management practices (Hemmer-Hansen, Therkildsen & Pujolar, 2014). Sequencing the whole genome in a large number of individuals is still prohibitively expensive. As a result, several methods have been developed to reduce the size and complexity of the genome to make both sequencing and analysis more affordable.

Among these methods, RADseq provides a method of genotyping and SNP discovery that is both economical and efficient (Bergey *et al.*, 2013) and has thus become increasingly popular for phylogenomic, evolutionary and population structure studies (Díaz-Arce and Rodríguez-Ezpeleta, 2019). This method benefits from specific properties of restriction enzymes (Baird *et al.*, 2008; Davey and Blaxter, 2010) that cut the genome by recognizing a specific DNA sequence and can score thousands of genetic markers by sequencing multiple short fragments of DNA. By using restriction enzymes that target a specific subset of DNA sequence, RADseq ensures that the same genomic regions are sampled across individuals (Arnold *et al.*, 2013) thereby increasing the depth of coverage per locus (Hemmer-Hansen, Therkildsen and Pujolar, 2014; Andrews *et al.*, 2016). RADseq has been successfully used to answer a range of ecological and evolutionary questions: estimating effective population sizes in salmon (Larson *et al.*, 2014), identifying genomic regions related to colour pattern adaptations in butterfly species (Nadeau *et al.*, 2014), resolving phylogenomics in cichlids (Wagner *et al.*, 2013) as well as delineating salmon populations (Dann *et al.*, 2013). RADseq is particularly beneficial in non-model organisms since it can identify thousands of polymorphic genetic markers across an

entire genome without any prior genomic information (Andrews *et al.*, 2016). This makes them the marker of choice for genetic studies on species without a pre-existing genome (Baird *et al.*, 2008; Davey and Blaxter, 2010; Andrews *et al.*, 2016), such as spotted grunter.

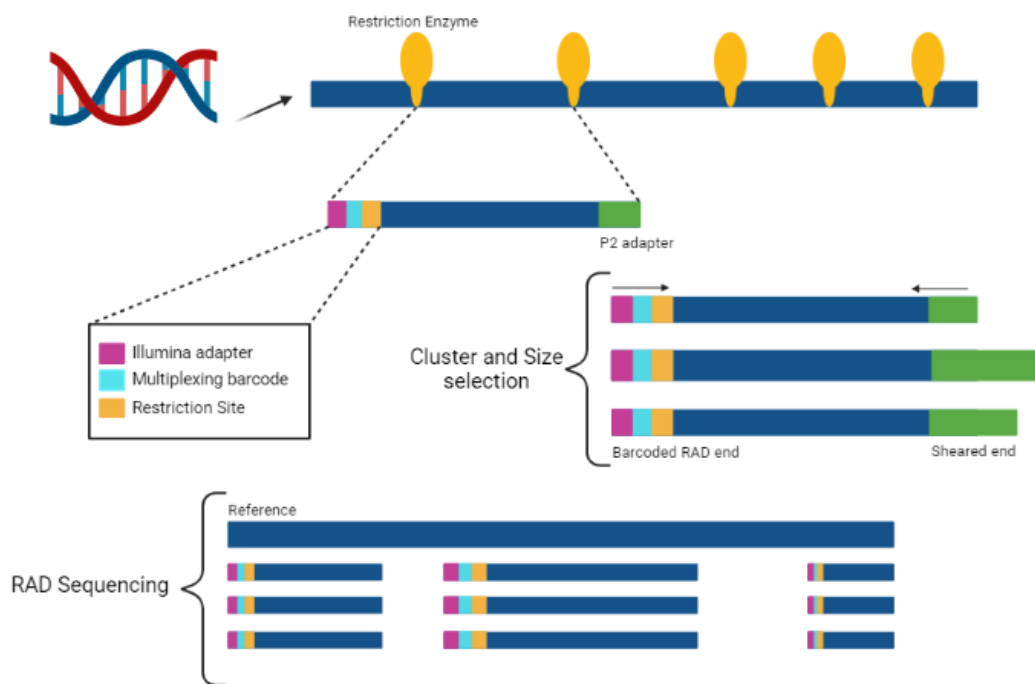


Figure 5.2. Schematic process of Restriction site-associated DNA sequencing (RADseq): Genomic DNA is digested via a specific restriction enzyme. Thereafter, adapters and barcodes are added to each fragment. Fragments are then size selected and amplified. Lastly, using NGS, the fragments are sequenced. Schematic created with BioRender.com.

The spotted grunter is found across a number of heterogeneous environments along South Africa's coastline. Due to the wide variety of environments inhabited by spotted grunter, it is assumed that local adaptation may be taking place as a response to the specific environmental pressures existing across different habitats (Bourret *et al.*, 2013; Phair *et al.*, 2015). Several biogeographical features have been found to create dispersal barriers in marine species such as ocean currents and eddies, upwelling cells and freshwater mixing (Teske *et al.*, 2011). These

features create gradients across the region which generally create biodiversity hotspots (Teske *et al.*, 2006). The two estuaries selected for this thesis fall in different biogeographic regions: the Breede Estuary in the cool temperate and the Kei Estuary in the warm temperate bioregion. Major genetic breaks have been found to coincide with these temperature-defined bioregions suggesting that thermal gradients may drive ecological divergence through local adaptation (von der Heyden, 2009; Teske *et al.*, 2019).

The spotted grunter is commonly caught in the Breede Estuary, an area where they were previously absent indicating an expansion in their distribution range (Augustyn *et al.*, 2018). Breede river spotted grunter display high residency and many reproductively ripe individuals have been captured (Augustyn *et al.*, 2018; Ziko, 2021). It was previously proposed that spotted grunter migrate to the KwaZulu-Natal area to spawn (Webb, 2002) however, the latest research found no evidence of tagged spotted grunter from the Western Cape migrating to the KwaZulu-Natal area during spawning season (Ziko, 2021; Ziko *et al.*, 2023). Furthermore, empirical evidence of spotted grunter spawning in the Knysna Estuary in the Western Cape has been published (Butler *et al.*, 2022). These observations could suggest that a new population of spotted grunter is forming in the Western Cape region. In population studies, the central hypothesis is that populations are panmictic and population studies generally aim to determine the validity of this hypothesis. When phenotypic studies are inconclusive, it is important to consider genomic approaches.

Investigations into spotted grunter genetics are restricted to a single MSc thesis. Klopper, (2005) investigated the variation in spotted grunter mitochondrial DNA (mtDNA) across South African estuaries. His results suggested a single panmictic population. The author also found that genetic diversity was greater within a locality compared to among localities. Mitochondrial DNA has a high mutation rate and has been widely used for taxonomic, phylogenetic and biogeographic relationships however, it is maternally inherited and thus limited in its use for recent demographic studies (Avice, 2000; Wang *et al.*, 2013). Since the level of genetic variation is positively associated with time since divergence in neutral markers, the recent evidence about the non-neutrality of the mitogenome can negatively affect estimates that are based on

this marker. Methods such as RADseq are suited to picking up outlier loci that could be involved with divergent selection due to local adaptation (Funk *et al.*, 2012; Bowen *et al.*, 2014).

The aim of this study was to assess the population connectivity, using RADseq, in spotted grunter from two estuaries in different biogeographic regions along South Africa's coastline. The previous chapters identified differences in specific morphological and ethological traits of spotted grunter from these two estuaries, therefore, an objective of this study was to determine whether any of these differences have a genetic basis. Furthermore, due to spotted grunter migration and spawning research, it has been hypothesised that the Breede Estuary is home to a newly formed population and these individuals may have evolved fine-scale local adaptations to environmental conditions.

5.3 Methods and Materials

5.3.1 Specimen collection

Spotted grunter were collected from the Breede and Kei Estuary via a beach seine net (120m long x 4m deep; mesh size 48mm in wings and 44mm cod end). A small tissue sample from the upper edge of the pectoral fin was removed using forceps and small scissors. Approximately 0.5 – 2cm in length of tissue was removed depending on the size of the individual. The tissue was immediately transferred into a vial containing absolute ethanol and preserved in a -20°C freezer for later DNA extraction. Ethics permit numbers: 2018/v10/SK, RES2020/86, RES2021/56, RES2022/72.

5.3.2 Phenotypic Data

Phenotypic data collected from Chapter 2 (Number of Spots and Spot Size) and Chapter 4 (Peak frequency of grunts) were used for those individuals which had successful DNA extraction.

5.3.3 DNA Extraction and RAD-Seq Library Preparation

Extraction of genomic DNA was personally conducted at the Centre for Ecological Genomics and Wildlife Conservation at the University of Johannesburg. The DNA was extracted from the fin clips of 254 spotted grunter using the cetyltrimethylammonium bromide (CTAB) method described by (Doyle, 1990). The quality of DNA extractions were checked on a 2% agarose gel electrophoresis with GelRed acid stain (Biotium) and 96 samples with highest quality and least amount of observed degradation were selected for genomic library preparation and were subsequently sent to LGC Genomics GmbH (Germany) for ddRAD (double digest restriction associated DNA) library construction:

I. RESTRICTION DIGEST

To prepare the restriction digest of the samples, the genomic DNA (100 – 200 ng) from each of the 96 samples were digested with restriction enzyme MspI (200 units) in Cutsmart buffer

(20 µl volume) at 37°C for an hour. Thereafter, the restriction enzymes were heat inactivated for 20 minutes at 80°C.

II. DDRAD LIBRARY CONSTRUCTION

Ligation Reaction

Each restriction digest (10 µg) was mixed on ice with an inline-barcoded forward MsiI adaptor (1.5µl; pre-hybridized, concentration 5 pM/µl) and Ligation master mix (20 µl; contains: 15 µl NEB Quick ligation buffer, 0.4 µl NEB Quick Ligase, 5 pM pre-hybridized common reverse MsiI Adaptor). This was then transferred to a 96-well PCR plate and incubated for one hour at room temperature followed by heat inactivation at 65°C for 10 minutes.

Library purification

Agencourt XP beads (50µl) were added to each reaction, first diluted with 30µl TE 10/50 (10nM Tris/HCl, 50mM EDTA, pH:8.0), and then incubated at room temperature for 10 minutes. This was followed by a further five minutes on a magnetic plate for bead collection. The beads were washed twice with 80% Ethanol (200µl) and air dried for 10 minutes; the supernatant was discarded. Libraries were eluted in Tris Buffer (20µl; 5mM Tris/HCl pH:9).

Library amplification

Each of the 96 libraries (10 µl) were amplified separately in PCR reactions (20 µl) using MyTaq (Bioline) and standard Illumina TrueSeq amplification primers with a maximum of 16 cycles following instructions provided by manufacturers.

III. POOLING AND CLEANUP OF DDRAD LIBRARIES

PCR primer and small amplicons were removed by Agencourt XP bead purification using one Volume of beads and then 10 µl from each of the 96 amplified libraries. An additional purification on Qiagen MinElute columns was done to remove the PCR enzyme and the pooled library was eluted in 20 µl Tris Buffer (5 mM Tris/HCl pH:9) before performing normalizations step.

IV. NORMALIZATION

The resulting libraries were normalized using Trimmer Kit (Evrogen). For this purpose, 1 µg of pooled ddRAD library was mixed with hybridization buffer and denatured at 98°C for three minutes, followed by incubation at 68° for five hours to allows DNA fragments to reassociate. Samples were then incubated for 10 minutes at 66°C after receiving DSN master buffer. Thereafter, a unit of DSN enzyme (1U/µl) was added and the reaction was further incubated for 30 minutes. DSN Stop Solution was added to terminate the reaction. It was then purified on a Qiagen MinElute Column and eluted in Tris Buffer.

V. REAMPLIFICATION

To reamplify using MyTaq (Bioline), the normalized pooled library was added to 100 µl PCR reaction. To index the library, an i5-Adaptor primer was used which allows parallel sequencing of multiple libraries on the Illumina NovaSeq 600 sequencer (limited to 14 cycles).

VI. SIZE SELECTION AND SEQUENCING

Size selection for the ddRAD library was done on an LMP-Agarose gel by removing fragments less than 250 bp and larger than 500 bp. Sequencing was done on the Illumina NextSeq 500/500 v2 (150bp paired end) following manufacturer's instructions.

5.3.4 Sequence Assembly and Quality Checking

The remaining analyses were personally conducted under the guidance of the Centre for Ecological Genomics and Wildlife Conservation at the University of Johannesburg.

QUALITY CHECKING

FastQC (Andrews, 2010) was used to visualize the quality of the raw sequences as well as to estimate major sequence quality statistics such as sequence lengths distribution, mean total

length, the presence of adapter and over-represented sequences and GC content. The estimated summary statistics for all samples were then summarized into a single report by using **MultiQC** version 1.12 (Ewels *et al.*, 2016).

IPYRAD PIPELINE

The quality-controlled sequences were assembled *de novo* using the *ipyrad* pipeline (version 0.9.89; (Eaton and Overcast, 2020)). The *ipyrad* pipeline is a widely used Python package that provides a robust workflow that enables users to directly assemble GBS (genotyping-by-sequencing) sequences from raw Illumina sequence data. Briefly explaining, the *ipyrad* workflow consists of seven steps: (i) demultiplexing/ loading raw reads, (ii) trimming, filtering and quality control, (iii) identifying and clustering reads from the same locus within samples, (iv) calculating sequence error rate and sample heterozygosity, (v) consensus base calls within samples, (vi) identifying orthologs across samples (vii) apply final filters, generate statistics and write output files (Eaton and Overcast, 2020) that can be further analysed by downstream population genetic pipelines. A summary of parameters that were used for the filtering steps and assembly is provided in Table 5.1.

VARIANT FILTERING

A combination of ‘**VCFtools v.0.1.16**’ and ‘**BCFtools v.1.18**’ were used to filter assigned variable sites (Danecek *et al.*, 2011, 2021). All indels (insertions and deletions), variant sites with a Minor Allele Frequency (MAF) of less than 0.05 in all populations, sites with more than 10% missing data across two populations, and those with a Linkage Disequilibrium (LD) exceeding 60% of were removed from downstream analysis. The only exception was the dataset for estimating effective population size (see below) based on the LD method for which no LD filtering was applied since pruning the sites based on an arbitrary LD value could have negatively impacted the estimation. Only a subset of unlinked biallelic sites (i.e., sites with a maximum of two alleles) that were covered with a minimum of 50 and a maximum of 500 quality filtered sequences were used for the population genetic analysis that follows.

Table 5.1: *ipyrad* filtering and assembly parameters used for *Pomadasys commersonnii* sequences. Default settings were specified except for those in bold.

Parameter Name	Parameter Setting	Definition
Restriction Overhang	RTG	To filter out any adapters/primers from the reads
Maximum low-quality bases	5	Sets the upper limit on the number of ambiguous sites (N's) allowed in the reads
Phred Qscore offset	33	Phred score quality threshold for trimming reads
Statistical Minimum Depth	6	Minimum depth at which heterozygous base calls can be identified
Majority Minimum Depth	6	Minimum depth for majority rule base calls to be made
Maximum Depth	10 000	Remove repetitive regions of genome with high depth clusters
Cluster Threshold	0.85	Level of sequence similarity at which sequences are identified as homologous
Maximum Mismatched Barcodes	0	Maximum number of allowed mismatches between barcodes file and sequences reads
Filter Adapters	Strictly filter	To remove Illumina adapters from data if present
Minimum trimmed length	35 base pairs	Minimum length of trimmed reads to be kept
Maximum alleles	2	Maximum alleles per site in consensus sequences
Maximum Ns	0.05	Maximum uncalled bases in consensus sequences
Maximum heterozygotes	0.05	Maximum heterozygotes in consensus sequences
Minimum samples/locus	65	Minimum number of samples per locus for output
Maximum SNPs/ locus	0.2	Maximum number of SNPs per locus
Maximum INDELS/locus	8	Maximum number of INDELS per locus
Shared heterozygous sites/loci	0.5	Maximum number of heterozygous sites per locus
Trim raw reads edges	0,140,0,140	Trim raw read edges (R1 >, <R1, R2>, <R2)
Trim locus edges	0,0,0,0	Trim locus edges (R1 >, <R1, R2>, <R2)

5.3.5 Population Genomic Analysis

All statistical analyses were conducted in **R** (v.4.2.0) and **R Studio** (R Core Team, 2020) unless explicitly said otherwise.

GENETIC DIVERSITY

Genome-wide summary statistics, such as the level of heterozygosity, inbreeding, and measures of population differentiation, were calculated using a combination of R packages: **SambaR** (de Jong et al., 2021a), **vcfR** v.1.13.0 (Knaus and Grünwald, 2017), **snpR** v.1.2.4 (Hemstrom and Jones, 2022) and **dartR** (Gruber *et al.*, 2018).

POPULATION DIFFERENTIATION

To investigate population differentiation two measures were used: Neis G_{ST} and F_{ST} . Neis G_{ST} measures genetic differentiation among subpopulations relative to the total genetic variation among subpopulations. It is traditionally used to estimate time since separation with large values, closer to one, equating to populations that have been separated for longer periods (Nei, 1972). G_{ST} values will reflect the effects of sampling and was estimated in the **vcfR** package (Knaus and Grünwald, 2017).

Conversely, F_{ST} (the fixation index) measures genetic differentiation among subpopulations relative to the genetic variation within subpopulations. Larger values closer to one will indicate complete differentiation between populations. This parameter is the most widely used descriptive statistic in population genetics (Holsinger and Weir, 2009) and was calculated using the Weir and Cockerham's (1984) formula in **snpR** v.1.2.4 (Hemstrom and Jones, 2022) and **SambaR** (de Jong et al., 2021a). The estimated F_{ST} values for each locus were visualised using a Manhattan plot that was generated using the **qqman** v.0.1.8 R package (Turner, 2017). A Manhattan plot is a specialised scatterplot used to visualise loci across the genome. By comparing single-locus F_{ST} estimates across a genome one can identify regions which deviate

from background rate of genetic differentiation, and these regions could represent regions of the genome that are under different selective forces.

POPULATION STRUCTURE

To investigate population structure of spotted grunter a Principal component analysis (PCA) was performed in **adeigenet** v. 2.1.8 (Jombart, 2008). A PCA is a powerful tool used to analyse multivariate data; by reducing the dimensionality of the data it provides an effective way to assess the presence of spatial patterns in the data (Daffertshofer *et al.*, 2004). Then, a Discriminant Analysis of Principal Components (DAPC) was performed using the same R package with predefined populations. **Adegenet** bridges the gap between multivariate analysis packages, such as **ade4** (Dray and Dufour, 2007), and packages specifically designed to analyse genetic data (Jombart, 2008). Therefore, **adeigenet** offers a powerful, robust and simple means to analyse population genetics.

Contrastingly to PCA, in a DAPC, the genetic variation is maximized between population clusters and simultaneously minimized within each population. DAPC is sensitive to the number of principal components that are selected for the discriminant analysis. To select the optimal number of PCA components an alpha analysis method was conducted (Jombart, 2008) and the DAPC function was then used to transform the data using the estimated optimal numbers of PCA axis to decipher the presence of population structure.

SELECTION ANALYSIS

Populations that are undergoing local adaptation are likely to have distinct loci and these loci typically show higher genetic differentiation among populations compared to neutral loci (Whitlock and Lotterhos, 2015). Therefore, by identifying loci with unusually large F_{ST} values one may find the genes responsible for local adaptation.

SambaR identifies SNP's which may be under diversifying or balancing selection using three different methods: **OutFLANK** (Whitlock and Lotterhos, 2015), **pcadapt** (Luu, Bazin and Blum,

2017) and **GWDS** (de Jong, Lovatt and Hoelzel, 2021b). **OutFLANK** has lower false positive rates compared to other approaches and predicts the distribution of F_{ST} for loci that are not influenced by balancing selection (Whitlock and Lotterhos, 2015). **Pcadapt** assumes candidate markers are outliers and detects genes under selection based on the powerful Mahalanobis distance (Luu, Bazin and Blum, 2017). **GWDS** infers loci under selection on an SNP-by-SNP basis (de Jong, Lovatt and Hoelzel, 2021b) and is similar to the **OutFLANK** method except it utilises Fisher exact tests for population differentiation instead of an F_{ST} metric.

GENE ONTOLOGY ANALYSIS

After identification of putative outlier loci the full sequences containing the outlier SNPs were extracted from **ipyrad** 'pseudo assembly' using the **ipyrad** analytical toolbox (Eaton and Overcast, 2020). Since the *Danio rerio* (Zebrafish) genome is the most complete annotated genome of all fish species, the resulting sequences were **BLAST**-searched (Altschul *et al.*, 1990) against the Zebrafish reference genome (GRCz11; Cunningham *et al.*, 2022).

All matches with more than 80% identity to a known gene in Zebrafish were selected. The biological significance of these matches in terms of three Gene Ontology (GO) categories (Cellular Component, Molecular Function, and Biological Process) were determined using **Ensembl BioMart** online tool (Cunningham *et al.*, 2022) and were summarised in **WEGO** webserver (Ye *et al.*, 2006, 2018).

GENOTYPE/PHENOTYPE ASSOCIATION STUDY

The association between genotypes and phenotypes for individuals were investigated using the R package **LEA** (Frichot and François, 2015). **LEA** is specifically designed for analysing multiple loci which is typical of population genomic studies. This method makes it possible to unravel the genotype-environment association by assuming an unknown underlying population structure that are considered as latent variables into the model (Frichot and François, 2015). To consider the unknown population structure, this method uses two classical approaches to analyse population structure: principal component analysis and admixture

analysis using sparse nonnegative matrix factorisation (snfm; Gain and François, 2021). Three phenotypic datasets were used to perform the association tests: Peak frequency of grunts, number of spots and the average spot size per individual.

Before conducting association studies for each of the three datasets, the missing genotypes for individuals were imputed using a kinship analysis based on cluster similarity using the function *impute()* in the same packages. Then a Latent Factor Mixed Model (LFMM) was used to measure the correlation between each genotype and its corresponding phenotypic values. The model was run independently for each phenotype using the (K=1 to K=3), and the estimated p-values for the association tests were visualised with Manhattan plots. To correct the multiple testing issue that is typical of association studies, the built-in correction method implemented in the same package was used to adjust all p-values.

5.4 Results

A total of 96 spotted grunter samples were sent for ddRADSeq sequencing, resulting in 346 600 000 raw pair-end sequences: 197 400 000 sequences from 52 Breede Estuary individuals and 146 200 000 sequences from 41 Kei Estuary individuals. The Breede Estuary sequences had an average length of 143.40 base pairs with a GC content of 42.94%. Similarly, the Kei Estuary had an average length of 143.05 base pairs and a GC content of 43.13%. The quality of the generated sequences showed that the majority of bases had Phred Scores above 30 (Figure 5.3) and the mean quality of each sequence was also of high quality with the majority having a Phred score of 35 (Figure 5.3).

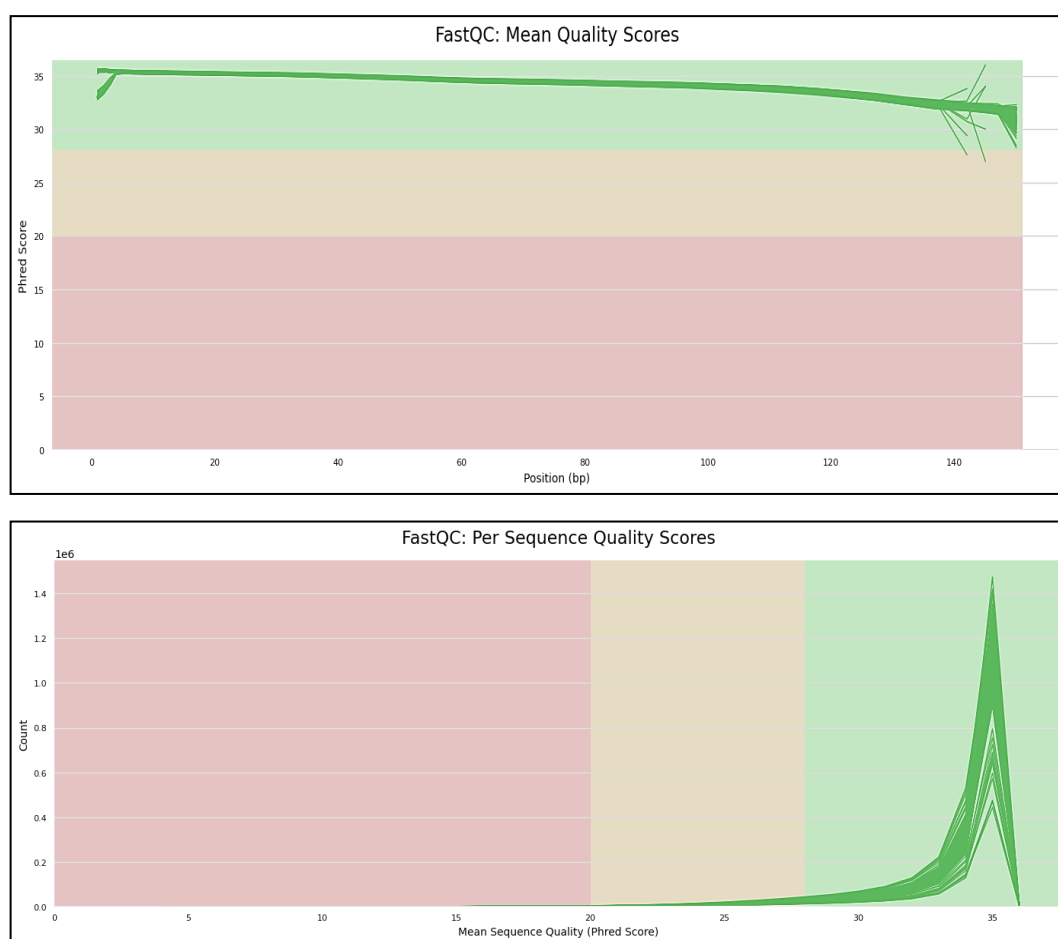


Figure 5.3. Sequence Quality Scores: (Top) The average quality value (Phred score) across each base pair in the sequence for *Pomadasys commersonnii* genomic samples. (Bottom) Distribution of average sequence quality scores for *Pomadasys commersonnii* genomic samples. The higher the Phred Score, the greater the quality with colours serving as indicators: green (good quality), orange (reasonable quality) and red (poor quality).

The ipyrad 7 step *de novo* assembly resulted in 40 226 filtered loci across all samples (Table 5.2) with only 7.7% missing sites. The samples from the Breede Estuary had an average of 38 442.69 loci while the Kei Estuary had fewer loci at 36 245.27. The post assembly filtering resulted in 9 711 sites and after linkage disequilibrium a total of 7 175 unlinked sites of good quality were used for downstream analysis. Of the 7 175 unlinked, the Breede Estuary samples had an average of 6 980.91 ($\pm$ 229.29) retained per individual while the Kei Estuary had 6 376.65 ($\pm$ 1 476.61) retained per individual.

Table 5.2: Number of loci removed by each filter during the seven step *ipyrad* workflow.

Filter	Total Filters	Applied Order	Retained Loci
Total Prefiltered Loci	0	0	194 127
Filtered by rm duplicates	7428	7428	186 699
Maximum INDELS	3098	3098	183 601
Maximum SNPs	0	0	183 601
Maximum shared heterozygosity	2253	2160	181 441
Minimum Sample	141 215	141 215	40 226
Total filtered loci	153 994	153 901	40 226

Four individuals, all from the Kei Estuary, had a lower number of retained polymorphic sites compared to the remaining individuals with 1005 sites from individual K307 having the lowest number of retained loci (Figure 5.4). However, these four individuals were not removed from analyses as they still had more than 1000 high-quality SNP sites that passed all strict quality checks. These individuals were tagged during the analysis to make sure no unexpected behaviour due to comparatively lower variable sites could be attributed to these individuals.

The minor allele frequencies were similar for both estuaries and followed a normal distribution with a mean of around 0.2 suggesting no obvious sequencing errors (Figure 5.5).

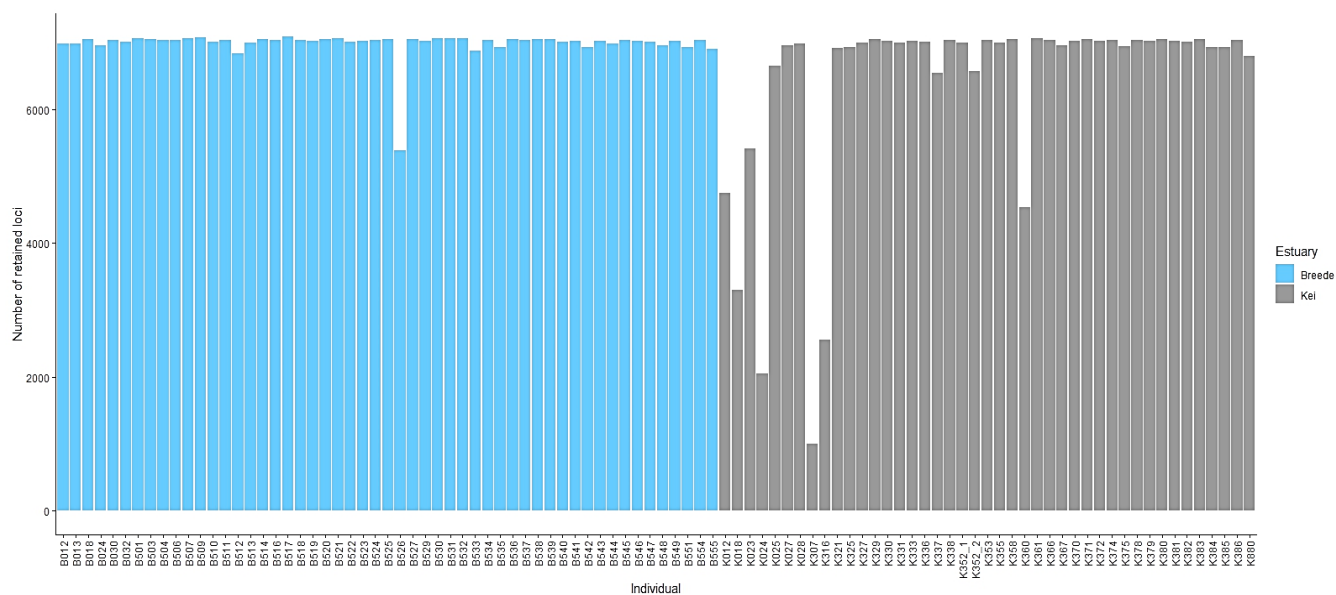


Figure 5.4. Number of retained loci per individual from one of two estuaries: Breede Estuary (Blue) and Kei Estuary (Grey)

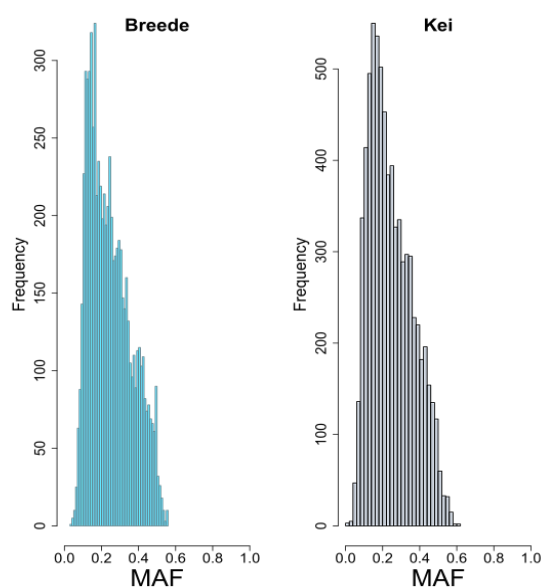


Figure 5.5. Minor allele frequencies: *Pomadasys commersonnii* MAF sampled from two estuaries: Breede Estuary (Blue) and Kei Estuary (grey).

5.4.1 Population Genomic Analysis

GENETIC DIVERSITY

The degree of heterozygosity was similar in both estuaries at around 0.35 for both sample and locus heterozygosity (Figure 5.6; Appendix 5.1). Two individuals from the Kei Estuary showed slightly higher levels of heterozygosity (0.40 - 0.56) which corresponded to a greater degree of missing data (Figure 5.6c) however these two outliers were still incorporated into the study as their variant sites were of good quality. Besides these two outliers, the general trend suggested no relationship between heterozygosity and proportion of missing data.

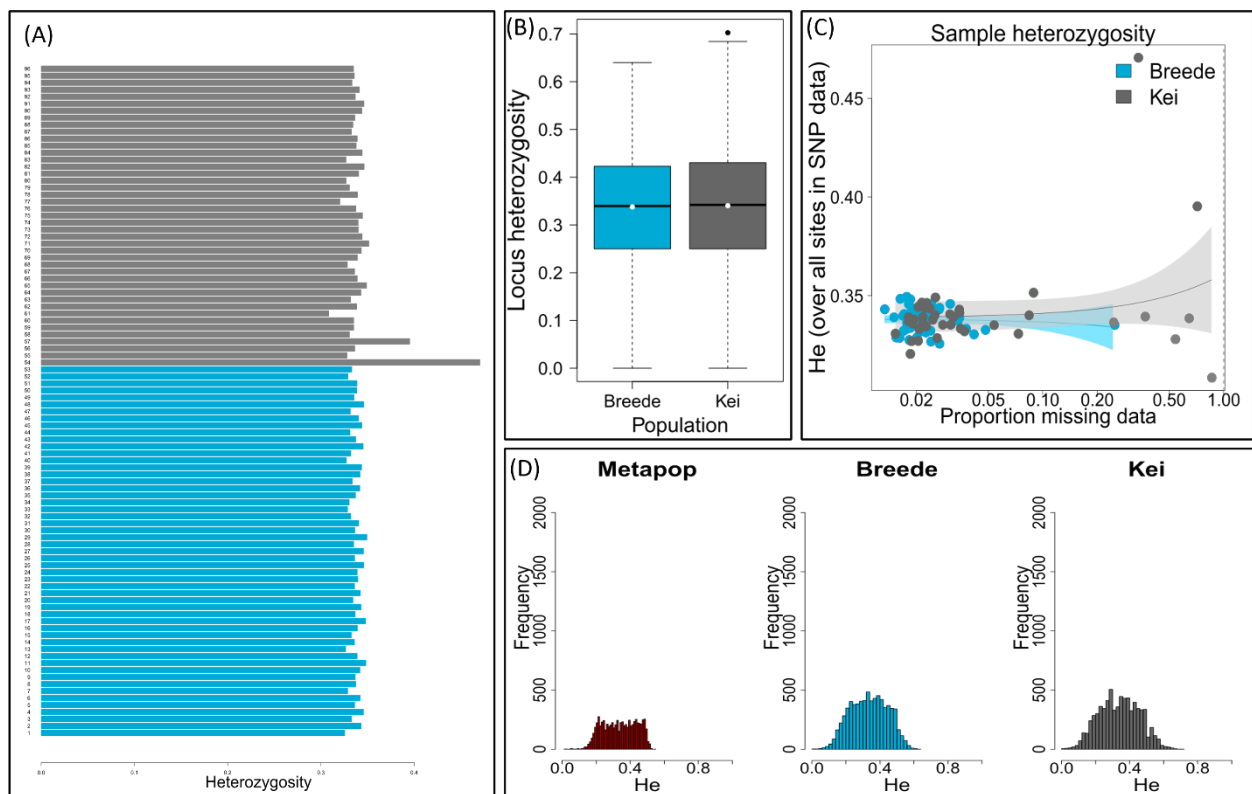


Figure 5.6. Heterozygosity of *Pomadasys commersonnii*: (A) Average heterozygosity of samples with each bar representing one of the 96 individuals. (B) Locus heterozygosity across all individuals from each of the two estuaries. (C) Relationship between heterozygosity and proportion of missing genomic data for each sample. (D) The frequency of heterozygosity for the two estuaries and the resulting metapopulation. The samples are from one of two estuaries: Kei Estuary (Grey) and Breede Estuary (Blue).

The inbreeding coefficient, which is the probability that an individual carries two identical alleles at a locus, was low for both estuaries (Figure 5.7; Weir, Anderson and Hepler, 2006). The mean inbreeding coefficient for individuals from the Kei Estuary was 0.03 (± 0.07) while the Breede Estuary was slightly higher at 0.04 (± 0.02 ; Figure 5.7). The Kei Estuary had two individuals with negative inbreeding coefficients (also known as out-bred), these are assumed to be hybrids which show higher levels of heterozygosity than can be expected for the given population.

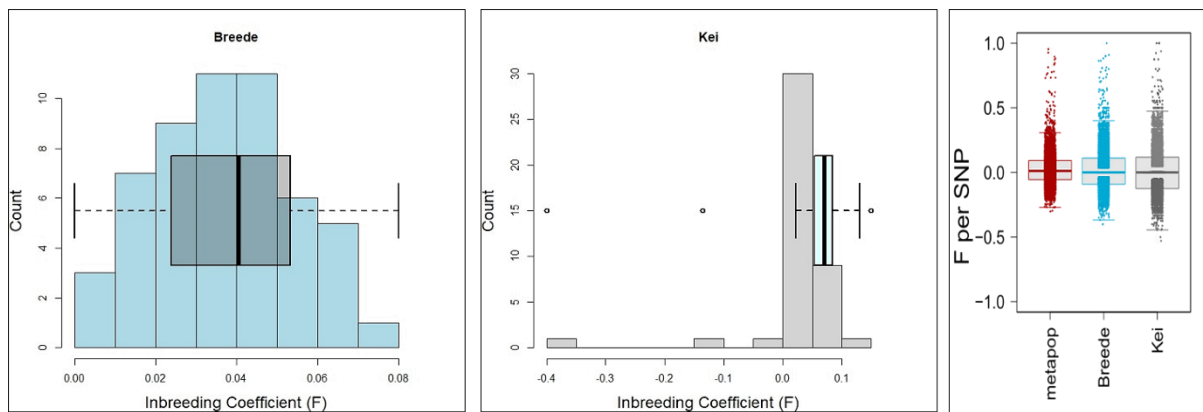


Figure 5.7. Inbreeding Coefficients: *Pomadasys commersonnii* in breeding coefficients from the Breede Estuary (left; blue) and the Kei Estuary (right; grey). The histogram represents the number of individuals for each inbreeding coefficient and the box and whisker show the distribution of points.

POPULATION DIFFERENTIATION

Neis G_{ST} gave a value of 0.007 suggesting that the genetic differentiation between these two estuaries is less than 1% (Appendix 1). F_{ST} values remained constant as the level of heterozygosity increased (Figure 5.8). The plot for pairwise F_{ST} values follows the typical ‘L shape’ with most loci having lower F_{ST} values which is typical of populations undergoing early stages of divergence (Figure 5.8; Bonin, 2008)). However, there were loci with higher F_{ST} values: 306 sites had F_{ST} values > 0.05 . The estimated pairwise F_{ST} values for each SNP from the two estuaries ranged from 0 to 0.256 with a mean of 0.0018. The generated Manhattan plot displayed three loci with significantly larger F_{ST} values highlighting a higher degree of differentiation between the two estuaries for these loci (Figure 5.9).

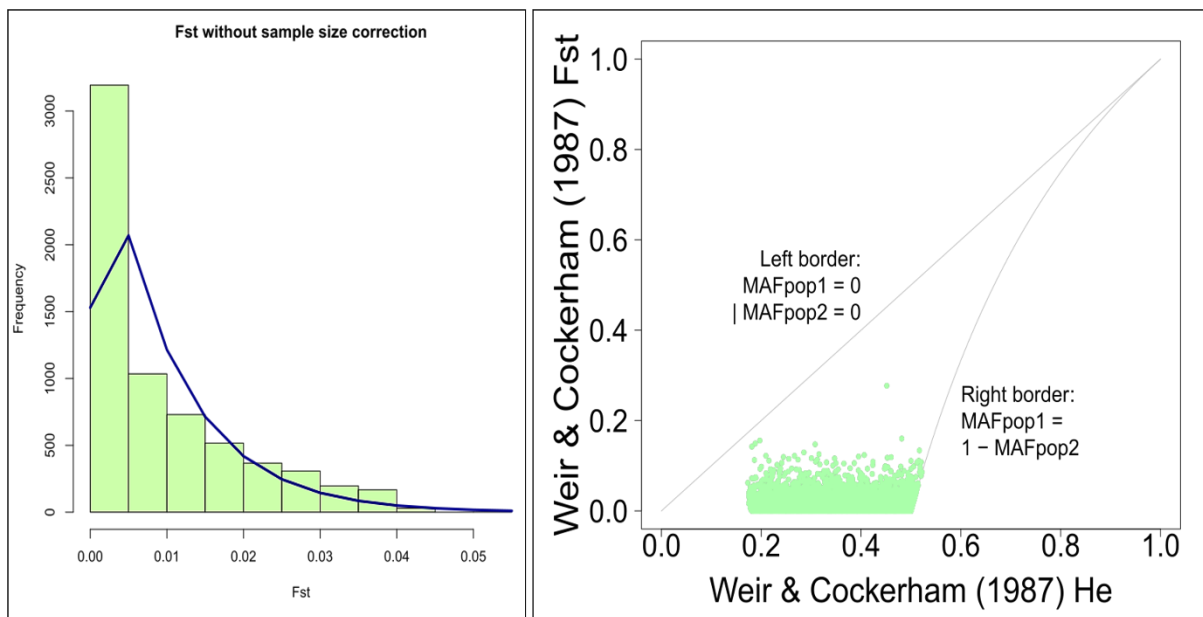


Figure 5.8. Average pairwise fixation index: F_{ST} values across the genome (left) and the relationship between pairwise F_{ST} values and the level of heterozygosity (right) in *Pomadasy commersonii* from two estuaries.

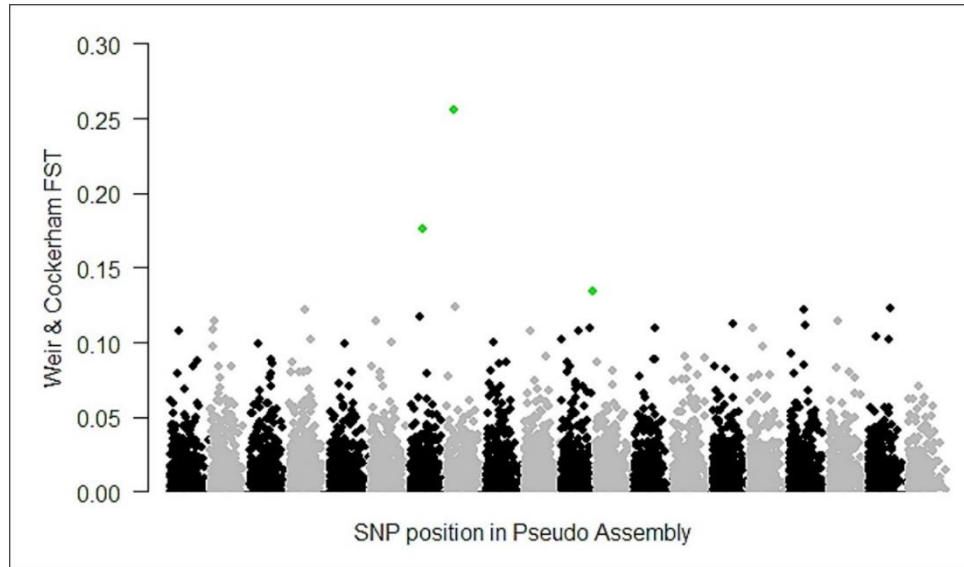


Figure 5.9. Manhattan plot of pairwise F_{ST} value: per SNP for two estuaries in a pseudo assembly. Three outlier loci with higher F_{ST} values are highlighted in green.

POPULATION STRUCTURE

The PCA represents the variation in SNPs of spotted grunter from the two estuaries and is visualized with the first two principal components. There are four major outliers from the Kei Estuary with the remaining individuals tightly clustered around 0 (Figure 5.10). The PCA tries to identify SNPs which may differentiate the two areas; there is little variation between the remaining individuals across both PC1 and PC2 suggesting some level of panmixia. The four outliers from the Kei Estuary did not have a high level of missing data and were thus not excluded from the analysis.

The alpha method suggested 13 principal components would be optimal for the DAPC. The DAPC compoplot, enforcing $K = 2$, suggested considerable admixture between the two estuaries even though individuals had, on average, higher membership probability for their given estuary (Figure 5.11). This lack of genetic structure further suggests a single, panmictic population.

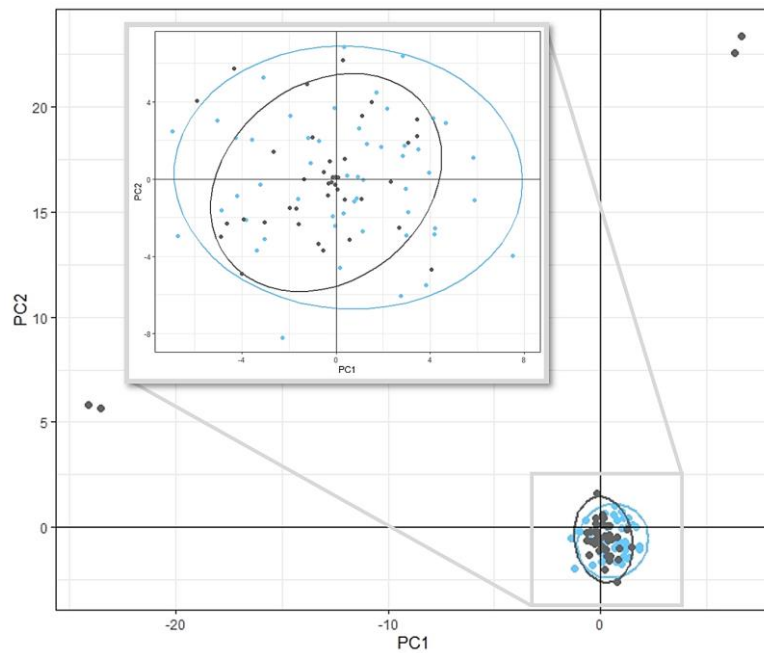


Figure 5.10. Principal component analysis (PCA) of variation in allele frequencies: *Pomadasys commersonnii* allele frequencies from two estuaries: Breede Estuary (blue) and Kei Estuary (grey). An enlargement of the area excluding outliers is also given. The first two principal components are shown.

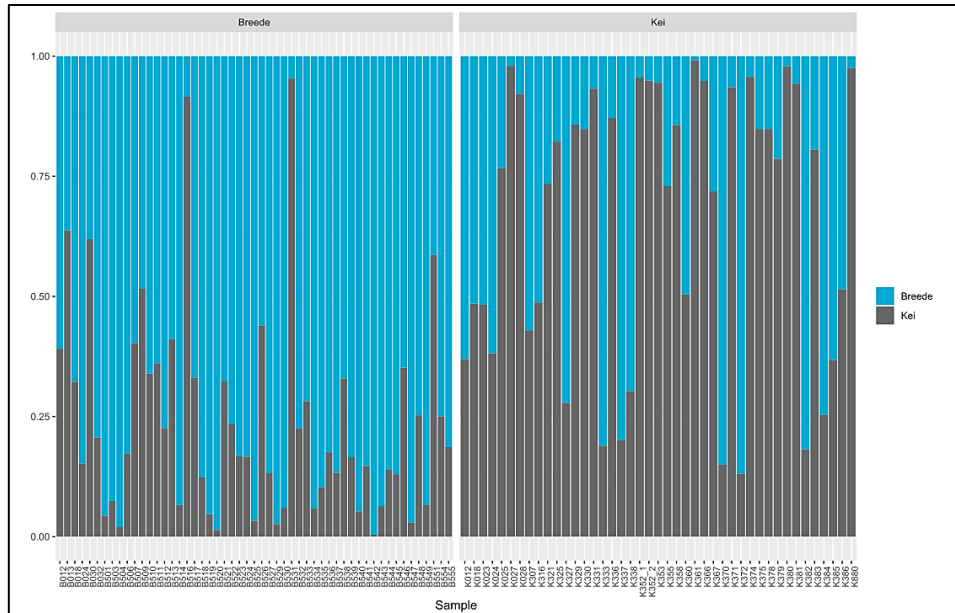


Figure 5.11. Discriminant analysis of Principal components: (DAPC) compoplot for spotted grunter, *Pomadasys commersonnii* based on a priori grouping ($K = 2$). Each individual is represented by a vertical column and colours indicate the probability of an individual's membership to one of the two estuaries: Breede Estuary (blue) and Kei Estuary (grey).

SELECTION ANALYSIS

Outlier loci were identified using three different established methods that undergo strict corrections. GWDS identified a single outlier locus (Figure 5.12). This single locus was also identified by OutFLANK and PCadapt which both further identified 14 other outlier loci (Figure 5.12). These candidate loci were used for the gene ontology analysis.

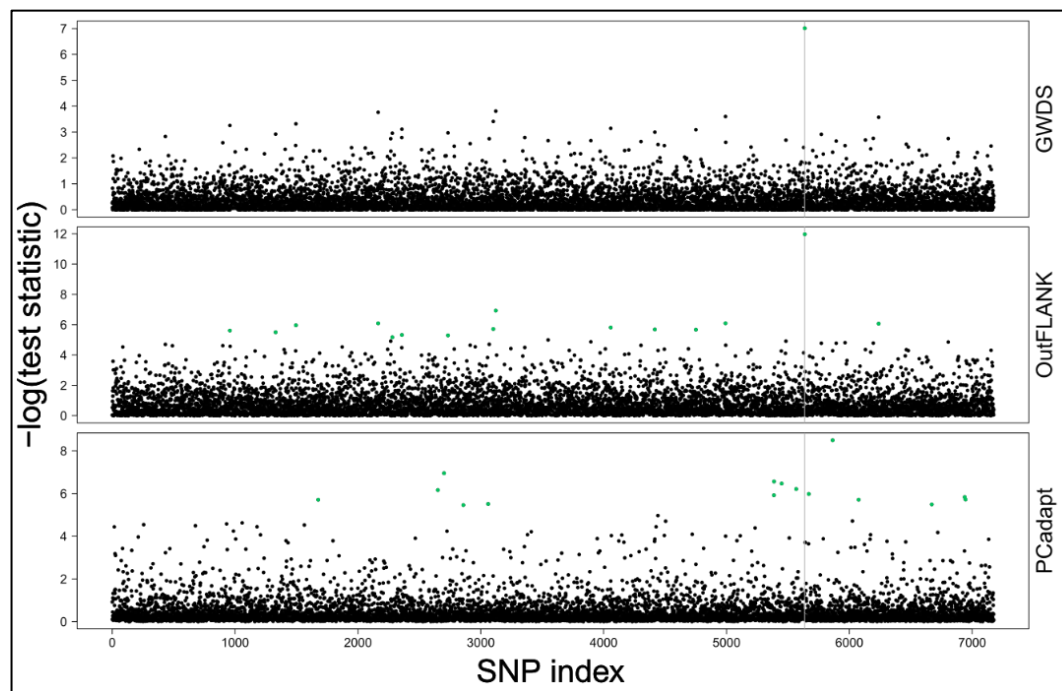


Figure 5.12. Selection analysis: Candidate loci (green) for local adaptation in *Pomadasys commersonnii* identified by one of three methods: GWDS, OutFLANK and PCadapt.

GENE ONTOLOGY ANALYSIS

The full sequences including the outlier SNPs identified in the genome scans above (Figure 7 and 10) were annotated based on *Danio rerio* reference genome (Zebrafish). The sequences with at least 80% identity to a zebrafish gene were mainly involved in protein binding, metabolic processes and cellular processes (Figure 5.13). All matches used for the GO analysis with at least 80% identity are given in appendix 5.2.

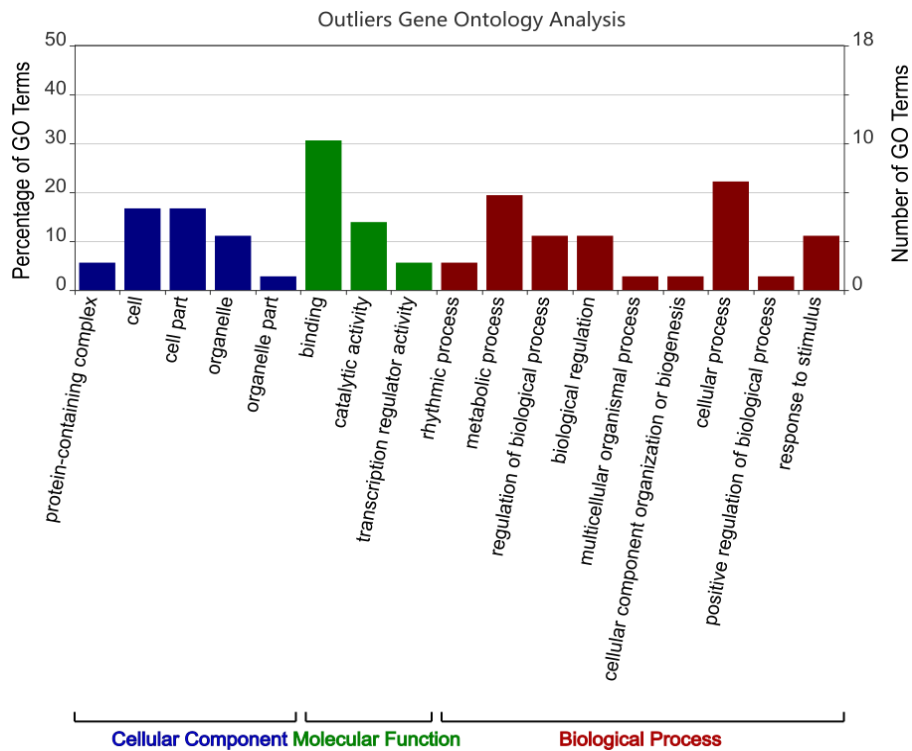


Figure 5.13. Gene Ontology (GO) assignment plot: The classification for candidate loci for adaptive differentiation in spotted grunter from two different estuaries.

PHENOTYPE ASSOCIATION STUDY

The phenotype association analysis identified several loci associated with variations in phenotypic traits. A total of 122 putative candidate sites were identified across the three phenotypic traits using the criterion: $p\text{-value} > 0.005$. Of the 122 candidate sites, 27 were associated with the peak frequency of grunts, 54 with the number of spots produced and 41 candidate loci were associated to spot size/area (Figure 5.14). However, after implementing the $p\text{-value}$ correction for multiple testing, none of the $p\text{-values}$ remained significant. While these putative candidate loci could still be associated with the measured phenotypic traits the number of phenotypic traits available to conduct this analysis was small (Peak frequency $n = 40$, Number of spots $n = 71$, Spot area $n = 72$), and the statistical power to conclusively link these loci to the phenotypes was limited. As such these results were inconclusive.

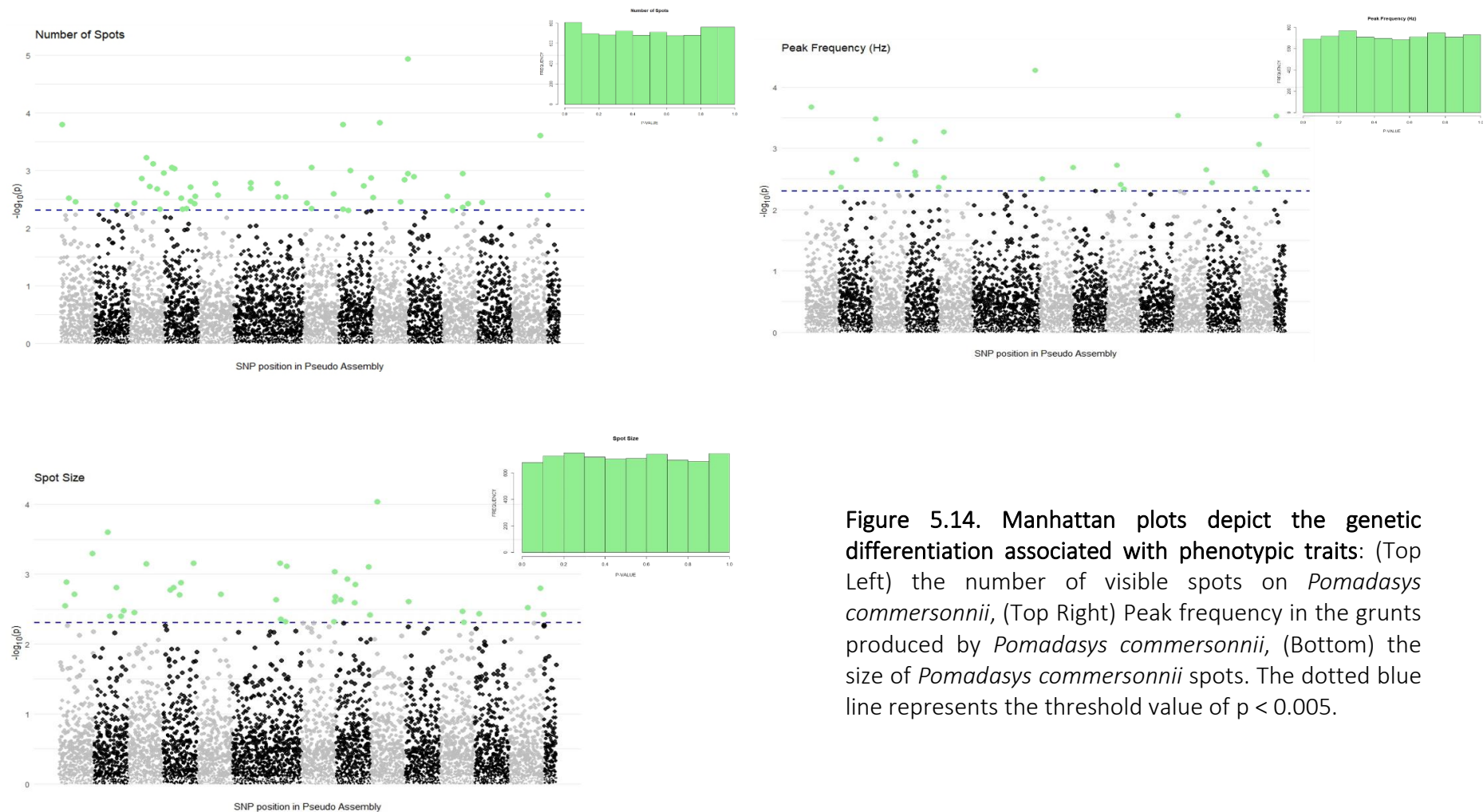


Figure 5.14. Manhattan plots depict the genetic differentiation associated with phenotypic traits: (Top Left) the number of visible spots on *Pomadasys commersonnii*, (Top Right) Peak frequency in the grunts produced by *Pomadasys commersonnii*, (Bottom) the size of *Pomadasys commersonnii* spots. The dotted blue line represents the threshold value of $p < 0.005$.

5.5 Discussion

The evolutionary potential of an organism for adaptation is vital for a species' long-term survival in an ever-changing environment, thus investigating the genetic basis of these adaptations is paramount for conservation efforts. This study provided the first genome-wide study on spotted grunter, *Pomadasys commersonnii* from two biogeographically diverse estuaries in Southern Africa. It was hypothesised that in-situ differences in environmental conditions will potentially promote some level of fine-scale local adaptation and could lead to genomic differences between spotted grunter from these two estuaries. Understanding the dynamics of local adaptations in these populations that may be undergoing early stages of ecological divergence will benefit the establishment of sustainable management and conservation efforts. The findings revealed a presence of subtle genomic differentiation between spotted grunter from these two estuaries in general while, at the same time, numerous loci displayed a high degree of population differentiation that could point to their involvement in the fine-scale local adaptations.

The implemented protocol to generate a reduced representation of spotted grunter genome through RADseq method proved to be successful for this non-model organism. This is not surprising since this method has been shown to be informative enough to find population structure in several species including *Gasterosteus aculeatus*, the three spine stickleback (Hohenlohe *et al.*, 2010). In this study, the authors showed that marine populations had little genetic differentiation from one another however, the freshwater populations of this species had become phenotypically differentiated. Furthermore, this methodology was able to identify specific gene regions, that were highly differentiated between populations, and their potential biological function (Hohenlohe *et al.*, 2010).

Marine species are distributed across marked environmental gradients (Hemmer-Hansen, Therkildsen and Pujolar, 2014) and differences in environmental conditions often lead to local adaptation and the development of unique ecological traits. However, the genomic dynamics and the associated biological processes that stimulate the observed level of genetic diversity and phenotypic variation within naturally occurring species was unknown for decades.

Although, with the current advancement in genomic methods, it is now possible to not only identify unique populations within a species that are morphologically indistinct (von der Heyden, 2009) but also identify the underlying genomic structures that underpin ecologically important traits that have evolved due to local adaptation (Arnold *et al.*, 2013). Similarly, investigating genomic variation makes it possible to identify outlier loci that are typically associated with environmental or phenotypic characteristics that could be involved in local adaptation (Stinchcombe and Hoekstra, 2008; Frichot and François, 2015).

It is now possible for researchers to conclusively determine whether there is a genetic basis to these adaptations (Miller *et al.*, 2007; Davey and Blaxter, 2010). This is important since if environmental differences result in phenotypic differences, due to local adaptation, one expects to see that specific loci (and their associated phenotypes) dominate the observed level of diversity in populations by removing all but the optimal phenotypes, thereby reducing the genetic variation within the local population while simultaneously increasing the differentiation between populations.

Investigating the extent of genetic relationships among individuals is a basic steppingstone for population studies (Rousset, 2002). Genetic relatedness measures can, potentially, be used to quantify the effects of migration, drift and selection in populations. However, understanding these mechanisms requires a clear definition of two major parameters that are used to represent the measured level of genetic variation in populations. Briefly, Heterozygosity (H_E) is when two different alleles are present at a single locus in an individual within a population. The understanding of heterozygosity in wild populations is fundamental since a low level of heterozygosity is an alarming indication of low evolutionary potential and this can hamper population adaptability to future ecological challenges (Gillespie, 1998; Weiss *et al.*, 2022). Low levels of heterozygosity are typically observed along comparatively higher levels of inbreeding. Inbreeding (f) can be defined as the deviation of observed heterozygotes compared to what is expected for an ideal population under Hardy-Weinberg equilibrium (Holsinger and Weir, 2009). The extent of inbreeding could reflect the degree of mating between relatives in a population. Highly inbred populations are known to be at a higher risk of local extinction.

In the current study, the overall degree of genetic differentiation between the two estuaries was comparatively low (average pairwise $F_{ST} = 0.0018$) with both estuaries exhibiting similar levels of heterozygosity and low inbreeding values. The observed level of genetic differentiation is on par with those reported from other marine fish populations ($F_{ST} = 0.062$; Ward, Woodward and Skibinski, 1994). Furthermore, the multivariate analyses highlighted a lack of clear population structuring and a considerable level of admixture occurring between individuals from the two study estuaries. These findings support an earlier study by Klopper, (2005) who suggested the presence of a single panmictic population of spotted grunter.

Panmictic population structuring is common across South African coastal species with several species showing low levels of population differentiation including the white steenbras, *Lithognathus lithognathus*, (Bennett *et al.*, 2017), the dusky kob, *Argyrosomus japonicus* (Mirimin *et al.*, 2016), Cape Stumpnose, *Rhabdosargus holubi* (Oosthuizen, 2006) and *Caffrogobius caffer* (Neethling *et al.*, 2008). Low levels of genetic differentiation in neutral markers, as was seen in spotted grunter, is a general rule in marine fish populations and is attributable to two main factors: (1) large effective population sizes and (2) significant levels of gene flow (Cano *et al.*, 2008).

Effective population size (N_e), defined as the number of interbreeding individuals that would be needed to explain the observed inbreeding rates and the variances in gene frequencies in the sample population, is important for the long-term survival of a wild population (Husemann *et al.*, 2016). It is a fundamental parameter for population genetics as it can be used to generate an idealized population with the same genetic diversity as the real populations being studied (Husemann *et al.*, 2016). As a result, it can then be used to predict the current populations' adaptive capability to predicted environmental change while incorporating stochastic forces such as genetic drift (Hare *et al.*, 2011). Large N_e would reduce the effects of genetic drift and mutation while low N_e values would decrease genetic variability and the chance for the accumulation of deleterious alleles that could negatively affect species' long-term survival (Cano *et al.*, 2008). This study had a small sample size with only two sample areas and as such it was not feasible to get an accurate measure of N_e , however future efforts could focus on this measure (Hauser and Carvalho, 2008; Hare *et al.*, 2011).

Low levels of genetic differentiation typically correspond with high levels of gene flow. Since the South African coastline is absent of physical barriers to gene flow, three hypotheses exist to explain the low level of genetic differentiation observed between spotted grunter from the two study estuaries. The first hypothesis, originally proposed by Klopper, (2005), is that all spotted grunter in Southern Africa belong to a single panmictic stock whereby spawning capable adults conduct migrations to the coastal waters of KwaZulu-Natal to spawn. Recent acoustic telemetry studies have revealed that some spotted grunter tagged in the Breede Estuary conducted migrations to Eastern Cape estuaries which suggests that there could be some levels of gene flow occurring between our two study sites however, there was no evidence for any spotted grunter tagged in Western Cape estuaries migrating to the KwaZulu-Natal areas to spawn (Ziko, 2021). This contradicts the hypothesis of a single panmictic stock of spotted grunter that spawn in KwaZulu-Natal. Furthermore, spawning aggregations of spotted grunter have recently been documented in the Knysna Estuary (Butler *et al.*, 2022) indicating more than one spawning population of spotted grunter in South African waters. Therefore, the hypothesis of a single panmictic stock of spotted grunter is unlikely as novel research has suggested otherwise. There is, however, some evidence towards an isolation-by-distance scenario since some Western Cape tagged spotted grunter migrated to the Eastern Cape and some Eastern Cape tagged spotted grunter migrated to the KwaZulu-Natal regions during spawning season. Further investigations would need to be conducted to confirm this.

The second hypothesis is that there is extensive mixing occurring during the pelagic larval phase of this species. Larvae are passively distributed across the South African coastline via coastal currents, thereafter it is thought that they actively recruit into estuaries (Whitfield, 2019). South Africa has two major currents that are thought to aid in the dispersal of pelagic larvae and eggs along the coastline. For spotted grunter in particular, the Agulhas current would distribute spotted grunter larvae southwards while retroflexion of the Agulhas current together with a northward flowing coastal current would aid in a northward distribution (Augustyn *et al.*, 2018). Even very small amounts of gene flow are often sufficient to reduce genetic differentiation between unique spawning populations (Begg and Waldman, 1999). Consequently, even in the case of unique spawning populations, mixing and distribution of

juveniles into estuaries along the South African coastline would increase gene flow and genetic variability within estuaries. High gene flow capabilities combined with limited adult migration have also been documented in the South African *caffrogobius caffer* (Neethling *et al.*, 2008), *Lithognathus lithognathus* (Bennett *et al.*, 2017) and *Chrysoblephus laticeps* (Teske *et al.*, 2010). Further research on larval distribution would contribute to understanding the dispersal potential of this species.

The last hypothesis, with some support from the previous chapters, is that the Western Cape may be home to a newly formed, resident population of spotted grunter. Several loci exhibited higher values of differentiation between the two estuaries which could be indicative of early stages of divergence. Furthermore, several of these highly differentiated loci were associated with phenotypic characteristics that were significantly different between spotted grunter from the two study estuaries. While these putative loci need to be interpreted with caution, they could represent the presence of some level of local adaptation due to varying selection pressures. Consequently, these loci could potentially indicate important adaptive traits for spotted grunter living under different environmental regimes. Genome-wide studies on multiple marine species have demonstrated the presence of highly differentiated loci that could represent early stages of divergence. For instance, a study of population structure in *Poecilia latipinna* reported 11 loci having significantly high F_{ST} values with an average of 0.118 (Nunez *et al.*, 2015) while only 2% of loci in *Clupea harengus* showed evidence of substantial divergence with F_{ST} values = 0.128 (Corander *et al.*, 2013). Similarly, 306 loci in spotted grunter had F_{ST} values ranging from 0.05 up to 0.256.

There is evidence in marine ecosystems that gradual effects of isolation-by-adaptation may occur due to local adaptation whereby outsiders will have reduced fitness in environments that they are not adapted for, and over time this could result in the formation of clear population differentiation. For example, research on *Haliotis laevis*, a broadcast spawner, was conducted using RADseq which revealed fine-scale adaptive variation that corresponded to minimum sea surface temperature and oxygen concentration (Sandoval-Castillo *et al.*, 2018).

It is noteworthy that while in this study some putative loci with high levels of genetic differentiation were identified and their functions were investigated, future studies will considerably benefit from a larger sample size and a fully annotated reference genome that can be used to better investigate the biological functions of these loci. In the same way, genotype-phenotype association studies typically require larger sample sizes to conclusively link a genotype to its observed phenotype. Therefore, given the comparatively small sample size used in this study, caution must be taken when interpreting these results and future efforts can focus on classifying phenotypic traits in a larger subset of the populations to increase the chance of conclusively identifying candidate loci.

The scope of this study was limited in the fact that only two estuaries, which make up a subset of estuaries inhabited by spotted grunter across their South African distribution, were investigated. Consequently, future efforts should include a greater number of estuaries, across all bioregions, which not only increases the number of samples used but also gives greater coverage across the species distribution area. This will increase the knowledge and depth of understanding of population connectivity and structuring across South Africa. It may also be worthwhile to investigate the four significant outlier individuals that were captured in the Kei Estuary. Perhaps these individuals are hybrids from further north (i.e. KwaZulu-Natal). Hybridization is not uncommon in marine fish species (Hart and Reynolds, 2002) but should be monitored closely since it can have important conservation implications.

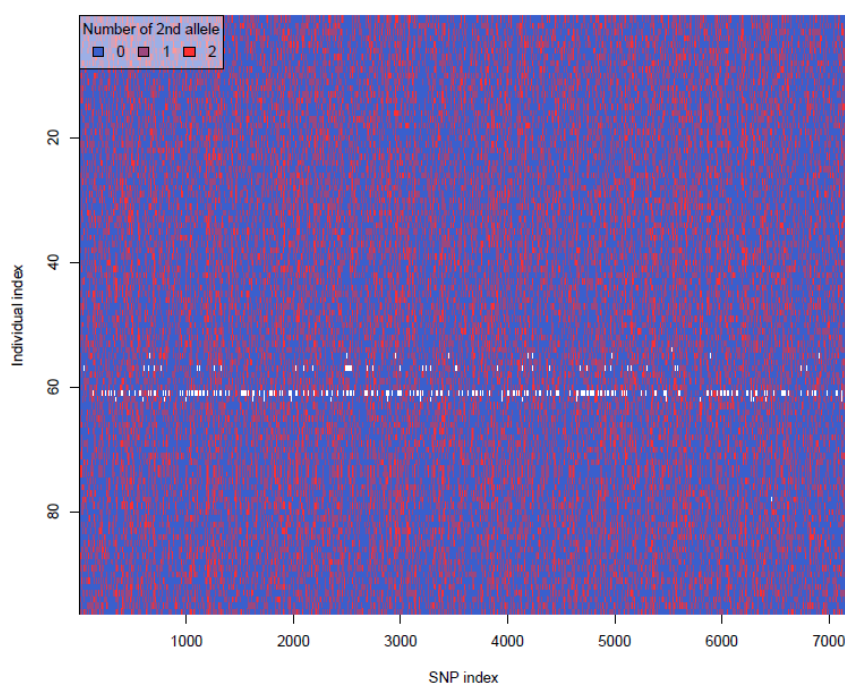
In conclusion, this study was the first to use genome-wide data to assess whether spotted grunter from two estuaries could be differentiated. The hypothesis that spotted grunter from these two estuaries could be genetically diverging due to local adaptation was investigated using a combination of different methods. While the findings suggest the presence of a single panmictic population of spotted grunter, several highly differentiated loci were also identified and among these loci, some were associated with traits that could potentially correspond to fine-scale local adaptation. This research contributes to the growing body of literature on spotted grunter population structure and demographic dynamics in South Africa. Whilst this study did not conclusively confirm whether the Western Cape is home to a novel, resident population of spotted grunter it did suggest that there may be early stages of adaptive

divergence taking place that could result in more pronounced population structure over time. A greater focus on phenotypic/ environmental association studies would increase the understanding of the genetic mechanisms that underpin local adaptation in spotted grunter.

5.6 Appendix

Appendix 5.1: Heterozygosity values for *Pomadasys commersonnii* captured in two South African estuaries: The Breede Estuary and the Kei Estuary

Measure	Value		
	Breede	Kei	Total
Heterozygosity	0.347	0.345	0.349
G _{ST}	0.007		
Gprimest	0.014		



Appendix 5.3: SNP plot for *Pomadasys commersonnii* captured from two estuaries in South Africa.

Appendix 5.2: Characterization of 37 *Pomadasys commersonnii* outlier SNPs with at least 80% match to known Zebrafish genome for gene ontology (GO) annotations obtained from Ensembl BioMart online tool.

Gene ID	Transcript ID	Gene Name	Gene Type	GO Term		
				Name	Accession	Evidence code
ENSDARG00000005948.8	ENSDART00000003674	ankmy2a	Protein Coding	Protein Binding	GO:0005515	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	DNA Binding	GO:0003677	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Nucleus	GO:0005634	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Hydrolase Activity	GO:0016787	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	ATP Binding	GO:0005524	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Nucleic Acid Binding	GO:0003676	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Nucleus	GO:0005634	IBA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Nucleotide Binding	GO:0000166	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	DNA Helicase Activity	GO:0003678	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Helicase Activity	GO:0004386	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Nucleobase-Containing Compound Metabolic Process	GO:0006139	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Damaged DNA Binding	GO:0003684	IBA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	DNA Duplex Unwinding	GO:0032508	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Cellular Response to DNA Damage Stimulus	GO:0006974	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	DNA Helicase Activity	GO:0003678	IBA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Nucleotide-Excision Repair	GO:0006289	IEA

Appendix 5.2: Characterization of 37 *Pomadasys commersonnii* outlier SNPs with at least 80% match to known Zebrafish genome for gene ontology (GO) annotations obtained from Ensembl BioMart online tool.

Gene ID	Transcript ID	Gene Name	Gene Type	GO Term		
				Name	Accession	Evidence code
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Hydrolase Activity, Acting on Acid Anhydrides, in Phosphorus-Containing Anhydrides	GO:0016818	IEA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Transcription by RNA Polymerase II	GO:0006366	IBA
ENSDARG00000021985.9	ENSDART00000141618	ercc2	Protein Coding	Positive Regulation of Mitotic Recombination	GO:0045951	IBA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	DNA Binding	GO:0003677	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	DNA-Binding Transcription Factor Activity	GO:0003700	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Regulation of DNA-Templated Transcription	GO:0006355	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Nucleus	GO:0005634	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Protein Dimerization Activity	GO:0046983	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	RNA Polymerase II Cis-Regulatory Region Sequence-Specific DNA Binding	GO:0000978	IBA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Regulation of Transcription by RNA Polymerase II	GO:0006357	IBA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	DNA-Binding Transcription Factor Activity, RNA Polymerase II-Specific	GO:0000981	IBA

Appendix 5.2: Characterization of 37 *Pomadasys commersonnii* outlier SNPs with at least 80% match to known Zebrafish genome for gene ontology (GO) annotations obtained from Ensembl BioMart online tool.

Gene ID	Transcript ID	Gene Name	Gene Type	GO Term		
				Name	Accession	Evidence code
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Protein Binding	GO:0005515	IPI
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Cytoplasm	GO:0005737	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Circadian Regulation of Gene Expression	GO:0032922	IBA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Response to Light Stimulus	GO:0009416	IGI
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Transcription Regulator Complex	GO:0005667	IEA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Photoperiodism	GO:0009648	IEP
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	Circadian Rhythm	GO:0007623	IGI
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	E-Box Binding	GO:0070888	IBA
ENSDARG00000003631.10	ENSDART00000133959	clockb	Protein Coding	CLOCK-BMAL Transcription Complex	GO:1990513	

Appendix 5.4: ddRADseq Sequence statistics for *Pomadasys commersonnii* captured from two estuaries in South Africa.

Estuary	Individual	Number of reads		Clusters		Estimation		Reads	Loci in Assembly
		Raw	Passed filter	Total	High Depth	Heterozygous	Error	Consensus	
Breede	B518	1750417	1747910	275117	89549	0.004566	0.001901	87057	38199
Breede	B543	1682457	1679869	310485	85326	0.004476	0.001671	83013	38090
Breede	B544	1796819	1794089	290839	91139	0.004155	0.001581	88729	38008

Appendix 5.4: ddRADseq Sequence statistics for *Pomadasys commersonnii* captured from two estuaries in South Africa.

Estuary	Individual	Number of reads		Clusters		Estimation		Reads	Loci in Assembly
		Raw	Passed filter	Total	High Depth	Heterozygous	Error	Consensus	
Breede	B554	1665789	1663129	284270	85307	0.004394	0.00158	82949	38388
Breede	B512	1775343	1772873	321235	93399	0.004217	0.002059	90662	37034
Breede	B549	1883927	1881115	280155	100102	0.004331	0.00181	97367	38852
Breede	B542	1824986	1822093	290419	89906	0.0045	0.001785	87259	37422
Breede	B516	1838167	1835533	291188	103001	0.004501	0.001775	100237	39139
Breede	B520	2233784	2230600	297769	107406	0.00463	0.001851	104384	39420
Breede	B522	2248035	2244861	321664	115201	0.004304	0.002015	111824	39202
Breede	B506	2029620	2026649	347630	102856	0.004524	0.001665	100068	39149
Breede	B503	1976484	1973628	338873	96855	0.004357	0.001857	94082	38863
Breede	B555	1583944	1581511	303496	87450	0.004289	0.00172	85000	38077
Breede	B541	1866737	1863561	300396	89141	0.004444	0.001674	86589	38519
Breede	B509	1890955	1887930	291788	95335	0.004512	0.001745	92731	38978
Breede	B504	1976080	1973216	315368	96423	0.00431	0.001698	93837	39080
Breede	B513	1541732	1539322	262792	84118	0.004318	0.001694	81962	38271
Breede	B539	1547370	1545113	268517	81719	0.004503	0.001681	79553	38043
Breede	B535	1724013	1721516	293294	89881	0.00439	0.001984	87199	37600
Breede	B548	1662657	1660433	278311	88057	0.0044	0.001733	85702	37795
Breede	B507	1863373	1860419	301435	96563	0.00437	0.001778	93925	38931
Breede	B534	1703328	1700791	292726	89839	0.004496	0.0016	87571	38698

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Estuary	Individual	Number of reads		Clusters		Estimation		Reads	Loci in Assembly
		Raw	Passed filter	Total	High Depth	Heterozygous	Error	Consensus	
Breede	B540	1571364	1569067	267658	88072	0.004342	0.001666	85825	38586
Breede	B510	1698857	1696359	289481	91351	0.004321	0.001661	88900	38776
Breede	B551	1593561	1591177	311399	82111	0.004453	0.001603	79990	37652
Breede	B523	1783861	1781116	309333	94688	0.004463	0.00177	92144	38766
Breede	B531	2044792	2041632	286234	98668	0.004268	0.001753	95793	39159
Breede	B533	1752912	1750320	303236	89464	0.004501	0.002075	86731	37129
Breede	B545	2136271	2133067	295152	92099	0.004714	0.001698	89381	38184
Breede	B547	1685950	1683461	281998	90477	0.004308	0.001685	88144	38486
Breede	B546	1862529	1859810	291163	95898	0.004497	0.001813	93235	38513
Breede	B536	2213838	2210574	295380	100174	0.004316	0.00184	97263	38717
Breede	B501	1887427	1884590	314582	97296	0.004199	0.001869	94650	39146
Breede	B517	2100675	2097732	285885	112507	0.004239	0.001796	109487	39464
Breede	B532	1992979	1990140	292814	102628	0.004449	0.00184	99866	39018
Breede	B514	1774836	1772140	287033	93686	0.004398	0.001757	91057	38903
Breede	B527	1915793	1912802	310301	95329	0.004562	0.001795	92648	38735
Breede	B519	1808134	1805502	281616	93534	0.004464	0.001838	90923	38499
Breede	B526	1161866	1160312	264261	60233	0.004382	0.002041	58438	28598
Breede	B529	1965190	1962304	315745	97483	0.004275	0.001765	94767	38858
Breede	B538	2370852	2367335	316026	105959	0.004316	0.001792	102970	39177

Appendix 5.4: ddRADseq Sequence statistics for *Pomadasys commersonnii* captured from two estuaries in South Africa.

Estuary	Individual	Number of reads		Clusters		Estimation		Reads	Loci in Assembly
		Raw	Passed filter	Total	High Depth	Heterozygous	Error	Consensus	
Breede	B511	1745392	1742852	275606	94441	0.004602	0.001794	91939	38996
Breede	B537	2034700	2031689	307449	103637	0.004413	0.001841	100715	39212
Breede	B521	1876363	1873513	282125	102565	0.004108	0.001788	99778	39204
Breede	B524	1931186	1928446	268303	97225	0.004507	0.001518	94675	39029
Kei	K370	1729899	1727191	307726	82779	0.00413	0.00157	80482	37742
Kei	K384	1498816	1496490	274121	77709	0.004505	0.001623	75619	37013
Kei	K383	1707164	1704540	283653	85139	0.004012	0.001646	82827	38241
Kei	K382	1797577	1794842	306603	89771	0.004448	0.001773	87248	38381
Kei	K361	1917023	1914075	298699	99400	0.004273	0.001885	96581	39242
Kei	K385	1492175	1489925	304105	74780	0.004196	0.001696	72824	36724
Breede	B525	1716665	1713933	273367	93314	0.004383	0.001738	90789	38811
Kei	K355	1704572	1702001	286902	90494	0.004295	0.001781	88054	38529
Kei	K352	1885179	1882644	340276	99171	0.004189	0.001931	96444	38832
Kei	K379	1600232	1597982	256292	86974	0.004524	0.001521	84831	38628
Kei	K372	1984290	1981438	335263	103801	0.004487	0.001852	101022	39023
Kei	K380	2073208	2070146	303241	107199	0.004315	0.001791	104231	39364
Kei	K358	1847559	1844769	319046	89104	0.004345	0.001866	86595	38694
Kei	K353	1831838	1829022	290936	91781	0.004482	0.001882	89220	38773
Kei	K386	1868076	1865596	285706	97183	0.004362	0.001681	94596	39180

Appendix 5.4: ddRADseq Sequence statistics for *Pomadasys commersonnii* captured from two estuaries in South Africa.

Estuary	Individual	Number of reads		Clusters		Estimation		Reads	Loci in Assembly
		Raw	Passed filter	Total	High Depth	Heterozygous	Error	Consensus	
Kei	K367	1544856	1542546	322157	79993	0.0042	0.001644	77857	37912
Kei	B530	1975724	1972946	301926	101143	0.004387	0.00185	98242	39151
Kei	K366	1972503	1969729	317660	98429	0.004578	0.001938	95576	38759
Kei	K371	2073829	2070917	309913	102637	0.004459	0.00175	99925	39230
Kei	K378	2008750	2005808	318093	96470	0.004424	0.001791	93594	38954
Kei	K381	2042935	2039949	383908	100026	0.00452	0.002024	97195	39033
Kei	K327	1672907	1670474	279631	89829	0.004212	0.001612	87436	38580
Kei	K321	1758900	1756251	340227	95884	0.004298	0.001791	93381	37959
Kei	K360	1296830	1294985	288484	89173	0.00402	0.002241	86630	33647
Kei	K307	787079	785753	289194	35854	0.00443	0.002374	34410	17332
Kei	K375	1544631	1542324	273220	87104	0.004276	0.001509	84926	38168
Kei	K330	1968878	1966266	308313	102219	0.004484	0.001705	99509	39075
Kei	K027	2031273	2028113	365136	104687	0.004409	0.001964	101805	38273
Kei	K336	1725911	1723396	266593	91710	0.004273	0.00143	89448	38685
Kei	K333	1889537	1886692	291568	102089	0.004297	0.001771	99336	38980
Kei	K316	972030	970646	237604	64206	0.004303	0.00221	62209	26787
Kei	K028	1818928	1815951	346265	93630	0.004315	0.001834	91155	38129
Kei	K338	2183549	2180367	337874	96729	0.004835	0.001668	93905	38464
Kei	K025	1747365	1744317	418191	89318	0.004496	0.002006	86661	36735

Appendix 5.4: ddRADseq Sequence statistics for *Pomadasys commersonnii* captured from two estuaries in South Africa.

Estuary	Individual	Number of reads		Clusters		Estimation		Reads	Loci in Assembly
		Raw	Passed filter	Total	High Depth	Heterozygous	Error	Consensus	
Kei	K374	1879893	1876981	303806	98610	0.004403	0.001674	95917	38969
Kei	K352	1701336	1699092	268712	111316	0.004172	0.002304	108376	37821
Kei	K024	821980	820587	303769	36090	0.005193	0.002293	33889	19621
Kei	K337	1717730	1715107	346098	89218	0.004502	0.001865	85443	37650
Kei	K325	1660695	1657885	310011	91430	0.004476	0.001684	89006	38006
Kei	K329	2018977	2016334	289192	105001	0.004185	0.001656	102306	39341
Kei	K331	1948661	1945864	286672	107638	0.004062	0.001645	104817	38958
Breede	B018	2148648	2144240	324528	100566	0.00462	0.001852	97517	39087
Breede	B024	2004664	2000683	322836	94324	0.00463	0.001921	91220	38614
Kei	K018	1067136	1064845	353073	45345	0.004616	0.001878	44012	19597
Breede	B013	2406825	2402216	356243	104310	0.005368	0.002148	100889	38977
Kei	K012	1645132	1642445	385795	77838	0.005364	0.001983	71966	30937
Breede	B032	2499941	2494840	367470	106961	0.004798	0.002035	103442	38952
Breede	B030	2139521	2134894	343263	97580	0.004979	0.001939	94526	38701
Breede	B012	2564467	2559468	402347	115784	0.004841	0.002345	111773	38973
Kei	K880	1656602	1653869	281999	83158	0.004256	0.001565	80931	34947
Kei	K023	1111557	1109635	295522	54974	0.004596	0.001785	53480	27066

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CHAPTER SIX

GENERAL CONCLUSION

6.1 General conclusion

Global change is inevitable, irrespective of whether it is anthropogenic or natural, and the resilience of a species is dependent on its ability to adapt to these changes. Some species respond to a changing environment by shifting their distribution and there is some evidence that this has occurred with spotted grunter (Augustyn *et al.*, 2017). In the last six decades, the subtropical spotted grunter has expanded its range into the warm temperate/cool temperate transition zone along the Western Cape Province of South Africa (Augustyn *et al.*, 2017). Initially, their presence was seasonal as they returned to warmer waters during the cooler months, however, in the last four decades they have shown a greater degree of year-round residency with empirical evidence of spawning taking place within this region (Butler *et al.*, 2022; Ziko *et al.*, 2023).

What caused spotted grunter to expand their range into the Western Cape region and what changes occurred that allowed the establishment of a viable breeding population? While there isn't a single climatic driver solely responsible for range shifts in marine species, climate change and its direct and indirect effects have recently been shown to cause major shifts in species distribution (Lenoir and Svenning, 2015; Pinsky, Selden and Kitchel, 2019). Rising sea surface temperature has been linked to several range shifts, particularly in tropical and subtropical marine species. As temperatures increase, many migratory species have shifted their spawning and overwintering grounds southwards, as has been noted in spotted grunter (Booth *et al.*, 2018). Additionally, tropical species are moving into higher latitudes as poleward-flowing currents are warming at a rate of two to three times the global mean in the past 100 years (Wu *et al.*, 2012). A change in precipitation and river-run-off has been shown to affect estuarine species in South Africa (Lamberth, Van Niekerk and Hutchings, 2008) as reduced river-run-off, due to climate change and agricultural practices, has increased/prolonged estuarine mouth closures along the South African coastline (Whitfield *et al.*, 2016). With less than 30% of South African estuaries considered permanently open, this would negatively affect several estuarine-dependent species (Augustyn *et al.*, 2017; Whitfield, 2019). If the majority of estuaries within a species range are closed, individuals will have to travel further to recruit into suitable estuaries. Therefore, the range expansion into the Western Cape, and particularly the Breede

Estuary, may have been in search of suitable, open estuaries into which they could recruit. Consequently, this range expansion was not only due to suitable environmental conditions extending towards this region but probably due to a combination of factors: habitat loss/degradation/alteration within their historical range, overfishing in their core distribution as well as possible changes in new potential habitats, such as lower predation pressure due to overfishing of dusky kob, *Argyrosomus japonicus* in the Breede area (Mirimin *et al.*, 2016). In this case, these individuals will be faced with new environmental conditions and their fate in this region is dependent on how they respond to these environmental pressures. In the case of local adaptation, one may see population divergence take place.

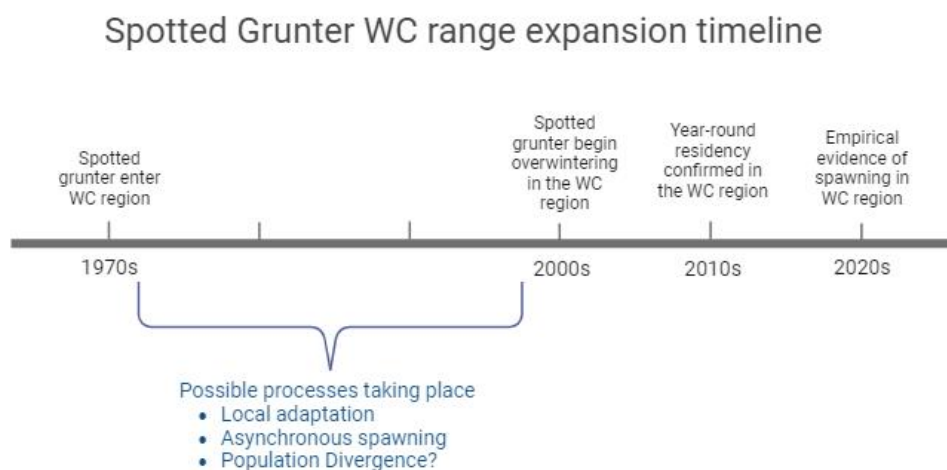


Figure 6.1: Timeline: *Pomadasys commersonnii* range expansion into the Western Cape region of South Africa.

This realization sparked a number of research questions and opportunities. *What processes occurred between the initial range expansion and the first spawning event that allowed the successful establishment of this species in this region? Do the individuals differ from those in the parent population, and if so, how?* As such, this thesis was conceived with the intent to apply current scientific methodology that could be used to differentiate spotted grunter from two estuaries and thereby contribute towards the hypothesis that the Breede Estuary is home to a newly established breeding population of spotted grunter. Given that the Breede Estuary was the focus of the investigation, it was imperative that the second study site have an established spotted grunter population as well as contrasting environmental conditions that

would increase the likelihood of identifying any differences that may exist between spotted grunter collected from each. The Kei Estuary was selected as it falls within a different biogeographic region (warm temperate/subtropical transition zone) compared to the Breede Estuary (cool/warm temperate transition zone) and is within the historical range of spotted grunter. Furthermore, the Kei Estuary is also one of the most turbid estuaries in South Africa. Whilst acknowledging a range of biotic and abiotic influences, the chosen scientific methodologies were selected based on spotted grunter traits that would be enhanced by contrasting turbidity levels. The findings from this thesis provide some evidence towards the initial conjecture that turbidity could be a driver of local adaptation for sensory systems and behaviour in spotted grunter.

Since speciation occurs slowly over extended periods of time, employing scientific methods that cover a range of timeframes was considered prudent and would have provided the best opportunity to detect speciation, if it was indeed occurring. All large speciation events would have started with small, seemingly insignificant changes. As a result, by incorporating a variety of methods, with their own unique timeframes, it may be possible to determine at which point in the divergence timeline these populations lie.

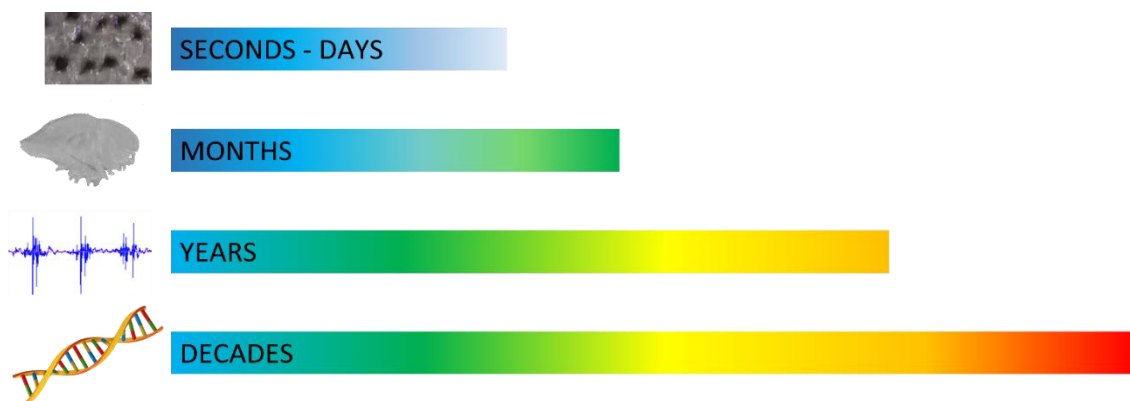


Figure 6.2. Divergence timeline: Infographic showing the expected timeframe for divergence to occur for each of the four methods used in this thesis. Spot patterns (seconds – days), otolith morphology (months), acoustic repertoire (years) and genomic differences (decades).

Changes to an animal's natural markings are one of the quickest forms of local adaptation and this can be a starting point in the path to divergence (Stevens, 2016). These changes include any modifications to the colour, pattern, size or shape of an animal's natural body markings. As

a response to local environmental conditions, small changes in spot patterns could potentially result in ethological changes and communication strategies. Over time, these behaviours can become ingrained leading to prezygotic isolation (Boughman and Wenrick Boughman, 2002; Bernardi, 2013). At this point, it is only a matter of time before genomic changes result in a speciation event. For phenotypic variation to influence population structure, it needs to provide an adaptive fitness benefit (Sköld *et al.*, 2016). This becomes complicated by plasticity, which, theoretically should impede speciation. *What is the role of plasticity in population differentiation? How does this affect migratory species, such as spotted grunter, which move between habitats with contrasting environmental conditions?* One would assume that plasticity would be favoured for these individuals, however, for populations living on the edge of their distribution there may be a stronger selection towards a specific trait (Hare and Able, 2007; Forsman, 2015; Pinsky, Selden and Kitchel, 2019).

Chapter 2, *Spot the Difference*, investigated spotted grunter spot patterns and is the first study to investigate the plasticity and the effects of turbidity on these spot patterns and to analyse and quantify consistently within these spot patterns also successfully. It thus provides several contributions towards a growing body of literature on colour and pattern change. The findings demonstrated that spotted grunter spots can be characterised by both developmental plasticity and acclimation/modification. Individuals were found to alter their spot size and lightness in response to turbidity. This level of acclimation would be beneficial for this migratory species which undergo daily movements between the estuarine and marine environments and seasonal movements along the coast (Kerwath *et al.*, 2005; Childs *et al.*, 2007). Spot size and lightness were found to be reversible and thus considered non-adaptive and therefore highly unlikely to result in the divergence of this species. However, developmental conversion can often be adaptive and this was seen in the number of spots. The individuals from the two estuaries displayed a significantly different number of spots. Developmental conversion occurs when an early environmental condition triggers a specific developmental pathway for a certain phenotype (Forsman, 2015). This is beneficial for species with high dispersal and larval phases like the spotted grunter. This developmental pathway is possibly triggered by the environmental conditions of the estuary into which they recruit. Those who recruit into less-turbid estuarine

environments may rely more heavily on spot-derived visual cues for communication or camouflage compared to those recruiting into turbid environments. In addition, turbidity is believed to be used by larvae to locate estuaries which suggests that spotted grunter may detect the level of turbidity in their surrounding environment (Blaber and Blaber, 1980). If the outcomes of these developmental pathways turn out to be adaptive, they may have an evolutionary importance (Smith-Gill, 1983; Kelly, Panhuis and Stoehr, 2012).

Population studies that deal with differences in permanent morphological traits are much easier to analyse compared to those with plastic traits. While an otolith grows consistently throughout an individual's lifetime, the layers that have been put down are permanent and will provide a record of an individual's life history (Cadrian, 2000). For this reason, otolith shape morphology can be used for stock delineation. The results from Chapter 3, *Shaping the Future*, suggest that divergence may have taken place in the Breede Estuary. Differences in otolith outlines between the Breede and Kei estuaries were expected as environmental conditions are known to influence otolith growth, however temperature and salinity ranges are very similar in these two estuaries and as such should not play an important role in the significant differences seen. Rather biotic factors such as food availability and genomic differences are expected to play a more substantial role in the observed differences. Many studies have used otolith shape morphology to distinguish between populations at a spatial scale (Vignon and Morat, 2010; Bardarson *et al.*, 2017) but this research further demonstrated the effectiveness of this method for long-term population monitoring. When population divergence occurs any differences in morphological characteristics will become more pronounced over time, which was evident in the results. The temporal differences observed in otolith shape from spotted grunter captured in the Breede Estuary pre-2002 and post-2019 may provide some evidence toward the hypothesis that this estuary is home to a newly established breeding population. The differences in the otolith outlines of spotted grunter, both spatially and temporally, suggest a shift could be occurring.

Phenotypic plasticity is most commonly studied using physical characteristics, such as the shape of otoliths. This study expanded on this and also investigated a behavioural characteristic that may be derived from phenotypic changes: sound production. The efficacy of a sound as a

means of communication is dependent on the receiver being able to receive and interpret it (Holt and Johnston, 2014). With changes to the otolith shape of spotted grunter in the Breede Estuary, it was hypothesised that it coincides with a variation in acoustic signals. The aim of Chapter 3, *Something Sounds Fishy*, was to determine if sounds produced by spotted grunter from our two study sites differed. Like most research projects, these findings lead to more questions such as whether these sounds are a learnt behaviour. This could be explored by translocating individuals and observing whether they can “learn” or adapt their sounds to their given environment. A major limitation of this chapter was the unnatural, artificial “environment” in which sounds were captured. Unfortunately, this meant that it was impossible to document the extent of spotted grunter sounds or vocabulary, but only compare and contrast the sounds recorded from each study site. Nevertheless, this still proved to be a robust method despite only scraping the surface of the potential for acoustic research in stock delineation. Furthermore, characterising acoustic signals from different species would be valuable for marine soundscape studies with the added benefit of deploying hydrophones into areas that may be difficult to sample or physically explore.

Despite the findings demonstrating differences in colouration, sound production and otolith shape in spotted grunter from the two estuaries, possibly indicating different phenotypic stocks, it would take decades to millennia for these traits to become tools for variation. Therefore, the overall genomic finding of a single panmictic stock with only subtle genomic differentiation was expected. However, there were a number of highly differentiated outliers and some of these were associated with the phenotypic traits measured above. Consequently, there is some genomic evidence to substantiate the phenotypic differences observed which could possibly indicate the very early stages of divergence (Carreras *et al.*, 2017). However, due to the low number of samples available for the phenotype-genotype association test this result should be interpreted with caution.

Sampling spotted grunter was an expensive, time-consuming and labour-intensive process and the current study only sampled spotted grunter from two estuaries in their South African distribution. Incorporating a larger number of estuaries throughout their distribution would have allowed for a more in-depth analysis of population structure. Additionally, the relative

novelty of the scientific methods, particularly applied to fish, presented an initial challenge as existing literature was scarce in general and practically absent in the South African context. The study sites and traits used were selected to optimise chances of success and as such a note of caution must be given when attempting to generalise from these results.

Each method selected for this thesis proved insightful with its own unique benefits. Spot patterns demonstrated the power of phenotypic plasticity. It was not in the scope of this research to determine the function of spot patterns but this knowledge should lend itself to a greater understanding of how and why spotted grunter might adapt their spot patterns. Most colour pattern research has been used for individual identification, which is also suitable for spotted grunter, however, this study has shown its usefulness as a trait to easily, and visually test plasticity. Linking function and plasticity will expand our knowledge of adaptation and its role in evolution (Kelly, Panhuis and Stoeck, 2012). Otolith shape morphology demonstrated how quickly environmental conditions can affect the ontogeny of a species, with differences becoming apparent within just a few years. Otolith shape morphology was the most straightforward method conducted for this thesis and can be applied to all bony fishes. Due to the effectiveness and reproducibility of this method, it is particularly useful for stock management studies. Bioacoustics demonstrated the depth of animal communication and how much there is to still discover and understand. It also highlighted the potential effects of anthropogenic noise on spotted grunter communication. Anthropogenic noise has been documented to affect acoustic signals in several species, with most species responding by increasing the amplification of their signals (Holt and Johnston, 2014). However, there must be a limit to their acoustic potential. *What happens when this limit is reached? What are the consequences for this species when acoustic communication is unviable?* These observations are important to document and investigate as the best way to mitigate the effects of anthropogenic pressures is to first identify them. Without this knowledge, there is no accountability towards better management. Protecting vulnerable, unique ecosystems is crucial for all, not just the species that inhabit these areas. Lastly, genomics revealed that some changes are occurring and that these changes may be linked to phenotypic traits.

Individually, these methods reveal a piece of the puzzle but together they build a story of how a single, environmental property (turbidity), can perhaps lead to population differentiation. First through ethology and communication modalities, which could possibly lead to reproductive isolation and unique spawning behaviour (Stratton and Uetz, 1981; Bardarson *et al.*, 2017). This in turn, could eventually derive genomic changes and overtime ultimately result in speciation (Boughman and Wenrick Boughman, 2002). Genomics alone would have demonstrated a single, panmictic population. Alone, the morphological chapters would have suggested phenotypic plasticity, but together it is possible to see that population divergence may be occurring (Tavera and Wainwright, 2019). If nothing else, this thesis demonstrates the importance of a multifaceted approach (Begg and Waldman, 1999), and these should be implemented wherever possible, particularly for vulnerable species requiring unique management strategies.

As time progresses, and for future testing, it is hypothesized that these differences will become more apparent, especially in the advent of asynchronous spawning. A few decades from now could reveal more distinct genomic differences, with more observable, unique local adaptations to local environmental conditions. Multiple decades from now we could possibly be dealing with complete stock separation (Tavera and Wainwright, 2019). This research has provided the first chapter in a story that is far from over. There are still many research opportunities and mysteries to be solved and this study has highlighted the potential of these methods in population differentiation studies. Hopefully, this research can be used to inspire researchers and policymakers to include a wider range of methods to inform and implement management strategies. Regardless, this research successfully utilised novel, scientific methods that provided some evidence toward the hypothesis that the Breede Estuary is home to a newly established breeding population of spotted grunter, *Pomadasys commersonnii*. The findings revealed that spotted grunter from the Breede and Kei Estuary differed morphologically and ethologically with subtle genomic differentiation.

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