

**ASSESSMENT OF THE POPULATION STRUCTURE OF THE SOUTH AFRICAN
SARDINE *Sardinops sagax* USING A MULTI-METHOD APPROACH AND THE
MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF A STOCK-
DISCRIMINATING DIGENEAN PARASITE BIOTAG OF THE GENUS
*Cardiocephaloides***

By

NWAMAKA MARY-IMMACULATA UKOMADU



Thesis presented for the degree of DOCTOR OF PHILOSOPHY

Department of Biological Sciences

UNIVERSITY OF CAPE TOWN
MARCH 2017

Supervisor: Dr. Cecile C. Reed (University of Cape Town, Biological Sciences)

Co-supervisor: Dr. Carl van der Lingen (Department of Agriculture, Forestry and Fisheries
and Marine Research Institute, University of Cape Town)

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ABSTRACT

An understanding of the population structure of commercially exploited species is essential for effective fisheries management. Fish stocks are typically identified based on the observation of differences in genetic and/or phenotypic characteristics between fish from discrete units. Recent deliberations on the management of the South African purse-seine sardine fishery recognises the likely existence of two stocks, one on the west and another on the south coasts, following studies that have documented spatial variability in several phenotypic characteristics of this species around South Africa. Those studies typically examined spatial variability in a single characteristic (e.g. gill raker morphology and meristics, body shape, otolith shape, vertebral count, parasite loads), but the application of multiple stock identification methods to the same individual fish has been recommended in order to maximize the likelihood of correctly inferring and identifying fish stocks. This study seeks to assess the population structure of South African sardine *Sardinops sagax* using a combination of stock identification methods including meristic, morphometric and parasite markers. Most of the sardine samples used in this study were obtained off the west and south coasts of South Africa during pelagic surveys conducted in 2013 and 2014 by Fisheries Branch of the Department of Agriculture, Forestry and Fisheries, with some additional samples obtained from commercial fisheries. Fish caught from the west and south coasts are presumed to be part of the putative western and southern stocks respectively. The variables examined included body shape, gill arch length, gill raker spacing, number of gill rakers, otolith shape indices (otolith circularity and form factor), the number of vertebrae, and the abundance of a 'tetracotyle' type metacercarian parasite found in the eyes of sardine; these were first analysed individually and then collectively (excluding body shape data) in a multivariate analysis to test their effectiveness in discriminating between sardine

from the putative western and southern sardine stocks. Analysis using geometric morphometrics revealed significant differences in body shape between sardine from the putative western and southern stocks. Results of GLM analyses indicated that gill arch length, the number of gill rakers, otolith form factor and circularity, and parasite abundance were effective univariate discriminators of sardine stocks. No significant difference in gill raker spacing and the number of vertebrae was found between individuals from the two stocks. A Stock Identification Index (SDI) of 0.75 derived from the univariate analyses, as well as results of the multivariate analysis of data provided strong evidence for the existence of two mixing stocks, therefore, supporting the two-stock hypothesis. The five multivariate classification models used in the study showed varying degree of allocation success. Overall classification accuracy ranged from a low 47% in the Linear Discriminant Analysis model to highs of 82% and 91% in the Classification Tree Analysis and Random Forest models, respectively. Both CTA and RF revealed the combination of variables with the strongest spatial discriminatory power to be the number of vertebrae and abundance of the 'tetracotyle' type metacercarian parasite. These results agree with those of previous studies and further support the inclusion of sardine population structure into management strategies for the purse-seine fishery for South African sardine.

Given the importance of the 'tetracotyle' type metacercaria in the discrimination of South African western and southern sardine stocks, further studies were undertaken to identify and describe this parasite using light and scanning microscopy, as well as molecular tools. Such information will assist in the definitive identification of the first intermediate host of the parasite, presently hypothesized to be a sub-tidal gastropod occurring off the west coast only. Definitive identification of the first intermediate host (or hosts) will enable the full life cycle of this parasite to be determined and the parasite endemic area to be identified; a critical knowledge gap in application of the parasite biotag approach to stock discrimination using this 'tetracotyle' type metacercarian parasite. Metacercariae collected from fresh sardine sampled from a commercial landing in Gans Bay, South Africa, were manually excysted, relaxed in warm water, fixed in 70% ethanol and stained with haematoxylin for light microscopy. The metacercarial body is oval-shaped, measuring 762 – 967 x 512 – 677µm. It is divided by transverse folds into a forebody, midbody and hindbody. Diagnostic

features include the unique, large excretory bladder lobes situated on the lateral sides of the body; two large pseudosuckers in the anterior part of the midbody; an acetabulum which is larger than the oral sucker; and a large lobulated holdfast organ in the posterior half of the midbody. These features are those of the metacercariae of the genus *Cardiocephaloides*, confirming the previous hypothesis regarding the genus of this digenean biotag. Analysis of partial 28S rDNA region sequence data showed that the metacercariae and the adult *Cardiocephaloides* found in the African penguin *Spheniscus demersus* are of the same species, likely *C. physalis*. This study is the first documentation of the morphological and molecular characterization of the stock- discriminating *Cardiocephaloides* metacercaria found in eyes of *Sardinops sagax* in South Africa.

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ACKNOWLEDGEMENTS

With special thanks:

To my sponsors: Organisation for Women in Science for the Developing World (OWSD), German Academic Exchange Service (DAAD) and University of Cape Town. I am immensely grateful for your funding and financial support. To my supervisors and mentors, Drs. Cecile Reed and Carl van der Lingen for their unparalleled love, patience, encouragement and support. I really enjoyed working under your tutelage. Thank you for the peaceful working environment you provided. Carl, thank you for the 'top-ups'. I could not have asked for better supervisors. You are the best. To A/Prof. Mike Picker. Thank you for your mentorship and fatherly presence. To my employer, University of Nigeria, Nsukka (UNN), who gave me study leave. And the staff of the Department of Veterinary Parasitology and Entomology, UNN, for keeping in touch. I appreciate the calls and the messages. To my fellow 'parasitos'. I appreciate your company and the little chit-chats we had. To Yonela and his team at DAFF, thank you for the loads and loads and loads of frozen sardine you provided for this research. Thank you Gino Jarvis for your quick response with fresh sardine. At the beginning of my research, the late Marc Hendricks was very helpful providing me with resources which aided me start up my project. May His soul rest in peace. Amen.

To Miranda Waldron and Mohammed Jaffa of the SEM Unit, University of Cape Town, who helped me with Scanning Electron Microscopy and Transmission Electron Microscopy (even though TEM did not work). To Dawit Yemane, Yahkat Barshep, Irfan Nunkoo for helping me with data analyses. To Prof. Piet King of the Department of Biology, Sefako Makgatho Health Sciences University, Medunsa. Thank you for helping me with taxonomic descriptions of parasites, morphological measurements, for teaching me how to use a drawing tube and for your input in the chapter on parasite morphology. It was an honour working with you. To Dr. Kevin Christison (DAFF research aquarium), who despite his very busy schedule, helped me in the molecular characterization of parasites and was always ready to clarify me on the areas I did not understand. Thanks so much Kevin for your input in the chapter on parasite DNA characterization. To Brett Macey, for the use of the molecular lab at DAFF Research Aquarium. To Vuyokazi (DAFF research aquarium) and Domecia, thanks for sharing your office space with me. To the staff of the Department of Biological Sciences especially Petra Muller, Liesl Phigeland, Natalie Jodamus, Soraya Abrahams. Thanks for your kindness and support.

To my husband, Ernest and beautiful daughter, Ije. I am truly blessed to have both of you in my life. Many thanks to my examiners for their corrections and inputs to the thesis. To my friends in Nigeria and South Africa ... You rock, guys. If I have missed some special people somewhere, please forgive me. I thank you too. May God bless you all abundantly. Amen

CHAPTER 1

OVERVIEW

SMALL PELAGIC FISH (SPF)

Small pelagic fish (SPF) include sardine, anchovy, herring, and sprat (Checkley *et al.* 2009). They inhabit many productive areas of the ocean including (Checkley *et al.* 2009):

- in the Pacific: the California Current (Canada, US, Mexico), the Humboldt Current (Peru, Chile) and the Kuroshio-Oyashio region (Japan);
- in the Atlantic: the Benguela Current (South Africa, Namibia), the Canary Current (Morocco, Western Sahara, Mauritania and Senegal), the European Atlantic (Portugal, Spain and France);
- in the Mediterranean and Baltic Seas;
- in the Gulf of California;
- in waters off Australia, Korea, Taiwan and China; and
- in other open ocean regions, for example western tropical Pacific.

SPF are generally microphagous planktivores and possess a flexible feeding behavior, switching between filter feeding on smaller food particles and particulate feeding on larger food particles depending on the food environment (Blaxter & Hunter 1982). In highly productive systems where there is strong upwelling, for example, off Namibia and Peru, they commonly feed on phytoplankton while in regions of weak upwelling, for example, southern California coast, zooplankton is the dominant food source (Blaxter & Hunter 1982, van der Lingen *et al.* 2009). This flexibility enhances their efficient utilization of a wide range of particle sizes in their ever-changing trophic environment, thereby making them successful inhabitants of both upwelling and non-upwelling areas (James 1988, van der Lingen *et al.* 2009).

Small pelagic fish species grow fast with populations attaining high but often fluctuating levels of abundance. This fluctuation is as a result of the large and variable natural mortality which these fish species are known to have (Barange *et al.* 2009). Consequently, they are vulnerable to over-exploitation which can lead to the collapse of fisheries. Their populations

have been described as not only being the most unreliable but also the most vulnerable to unrestrained fishing (Beverton 1983) such that their fisheries have been termed 'fragile enterprises' (Pitcher 1995).

BIOLOGY OF SARDINE (*Sardinops sagax*)

Sardinops sagax (Family Clupeidae) are small, pelagic forage fish occurring in extraordinarily dense shoals in the eastern boundary currents of the Atlantic and the Pacific oceans as well as in the western boundary currents of the Indo-Pacific ocean (Hill *et al.* 2009). Initially, the genus *Sardinops* was believed to consist of five species. However, investigations carried out on representative *Sardinops* populations in the different current systems of the world including the Agulhas, California, Humboldt, Benguela, and Kuroshio Currents as well as off New Zealand and Australia, showed that the genus is monotypic (Parrish *et al.* 1989, Beckley & van der Lingen 1999, Hill *et al.* 2009). Globally, *S. sagax* is associated with the cool temperate coastal and shelf waters of both upwelling and non-upwelling regions (Bowen & Grant 1997, Beckley & van der Lingen 1999, van der Lingen *et al.* 2009). It inhabits these regions together with other related pelagic fish species, e.g., anchovy (*Engraulis*) and herring (*Etrumeus*) (van der Lingen 2002, Agenbag *et al.* 2003, Zwolinski *et al.* 2011). *Sardinops sagax* are omnivorous microphagists, with diet consisting of copepods, fish eggs, crustacean eggs, euphausiids and phytoplankton (McFarlane & Beamish 2001, van der Lingen 2002, Emmett *et al.* 2005). *Sardinops sagax* exhibit complex schooling behavior at different temporal and spatial scales (Barange & Hampton 1997, Misund *et al.* 2003) depending on predation levels, vessel noise, and habitat heterogeneity (Fréon *et al.* 1993, Giannoulaki *et al.* 2003). Additionally, they are highly migratory species, undergoing extensive migrations in order to access conditions necessary for spawning and the survival of recruits (Ward *et al.* 2008). Furthermore, *S. sagax* serve as an essential food source for numerous pelagic predators such as piscivorous fish, birds and mammals (Crawford *et al.* 1992, Cury *et al.* 2000, Emmett *et al.* 2005), hence they are of ecological and economic importance.

Sardinops sagax are relatively short-lived with most living for less than eight years (Schwartzlose *et al.* 1999), grow quickly and are prolific breeders. Approximately two to four

days post-fertilization, eggs hatch to yolk sac larvae, about 2.2 – 2.5mm in total length, TL (Neira *et al.* 1998) which subsequently metamorphose to juveniles at 1 – 2 months of age and 35 – 40mm TL (Ward *et al.* 2008). Larvae have been reported to actively migrate to areas with favourable environmental conditions in order to enhance their chances of survival (Watanabe *et al.* 1996, Logerwell *et al.* 2001, Curtis 2004). Population size is highly dependent on oceanographic conditions such as temperature, transport, predation, food availability, and population density (Parrish *et al.* 1983, Bowen & Grant 1997), large fluctuations and collapses in sardine biomass have been reported in all regions of the world where sardine is intensively fished (Lluch-belda *et al.* 1989, Schwartzlose *et al.* 1999). *Sardinops sagax* is an important component of pelagic fisheries along the Pacific, southern African and Asian coasts with large number of fish caught in pelagic trawls or purse-seines, processed for human consumption or for by-products or as baitfish (Beckley & van der Lingen 1999, Emmett *et al.* 2005).

SARDINOPS SAGAX IN SOUTHERN AFRICAN WATERS

Sardinops sagax inhabits the temperate coastal and shelf waters from southern Angola around to KwaZulu-Natal in north-eastern South Africa (Figure 1.1; Beckley & van der Lingen 1999, Kreiner *et al.* 2001). Studies indicate that this sardine population consists of two discrete populations, namely the Namibian population and the South African population (Armstrong & Thomas 1989, Cloete *et al.* 1997). The existence of a very cold region of upwelled water, for most part of the year, off the Lüderitz coast acts as a barrier and prevents the large-scale mixing of these two sardine populations (Lett *et al.* 2007).

The range of the Namibian sardine population extends northward from the Lüderitz upwelling cell (25°S) to the warm Angola-Benguela front off the southern Angola coast (16°S - 17°S) (Boyer *et al.* 2001). The Namibian sardine stock was one of the major clupeoid stocks in the world (Boyer *et al.* 2001) and the most important pelagic fish species harvested in Namibia (Lees 1969), supporting an average annual catch of more than 700, 000 tons in the 1960s (Boyer *et al.* 2001). However, from the late 1960s, the Namibian sardine population experienced fluctuations in biomass and catches. Over-exploitation and poor recruitment led to a crash in biomass. Even with the partial increase in biomass that occurred in the mid-

1970s, as well as the reduction of Total Allowable Catch (Cram 1981), the stock never recovered. The population showed signs of recovery in the early 1990s, but abnormal environmental conditions such as the extensive hypoxic shelf waters occurring in 1993 and 1994 (Monteiro & van der Plas 2006), the 1995 Benguela Niño characterized by warm water intrusion, as well as fishing pressure in years of poor recruitment caused a further decline in biomass and catches (Boyer *et al.* 2001, Boyer & Hampton 2001). Presently, the low sardine biomass can only allow limited fishing activity that provides minimal employment and infrastructure for sardine processing factories at Walvis Bay (Hutchings *et al.* 2009).

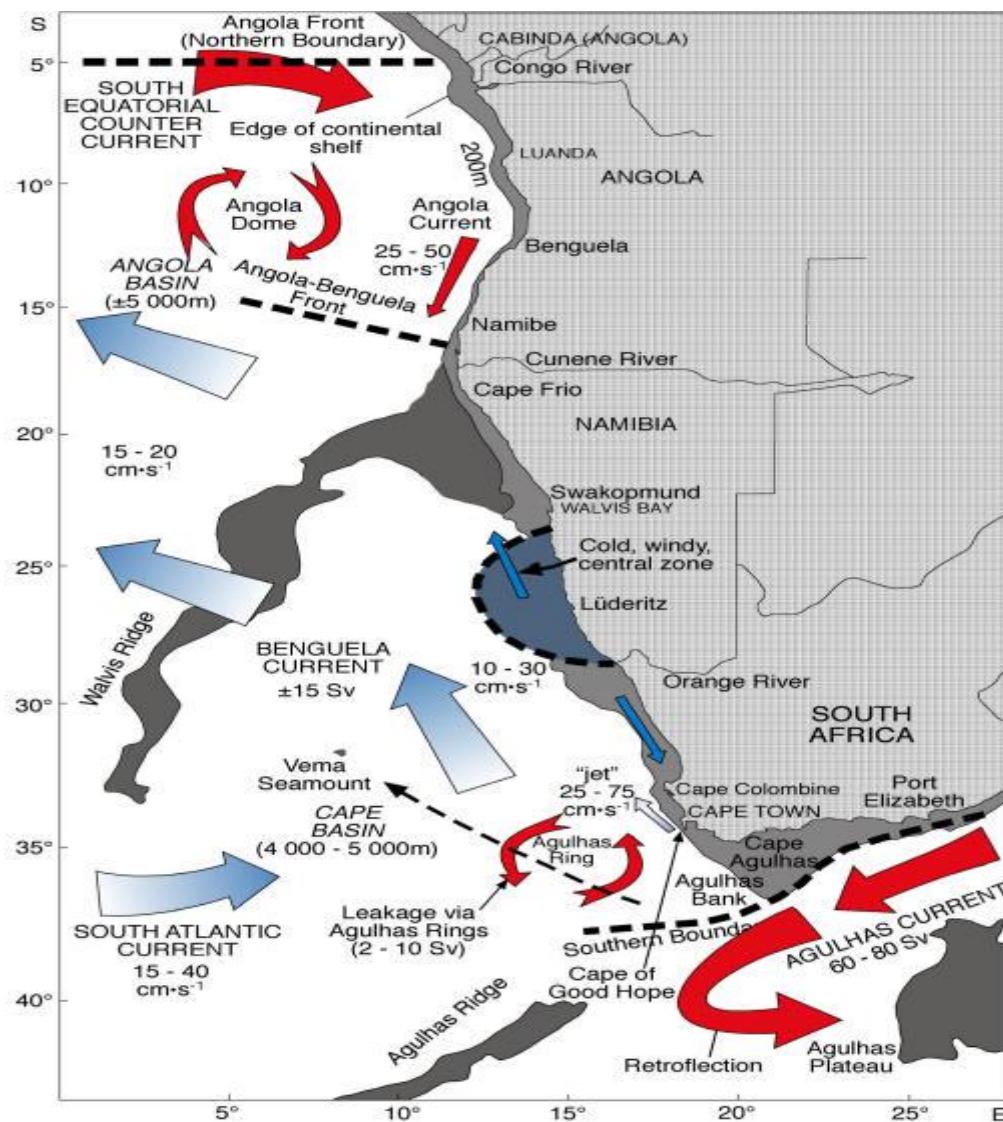


Figure 1.1. Benguela Current ecosystem: currents and boundries (from Shannon 2006)

The South African sardine population extends from the Orange River round the Cape to Richard's Bay, KwaZulu-Natal (27°S). This region is dominated by two major current systems (van der Lingen & Huggett 2003): the warm Agulhas Current off the east and south coast which flows pole-wards, and the cool Benguela Current off the west coast which flows equator-wards. Consequently, the marine systems in the east and south coasts range from subtropical to warm temperate systems which are characterized by highly diverse fish species and a low productivity. In contrast, the system in the west coast is a cool temperate upwelling system, known as the southern Benguela, which is characterized by a relatively low number of fish species and a high productivity (van der Lingen & Huggett 2003). The southern Benguela supports large populations of pelagic fish (mainly sardine and anchovy) and is separated from the northern Benguela off Namibia by the perennial Lüderitz upwelling cell (van der Lingen & Huggett 2003).

The distribution and migration patterns of the South African sardine at different life stages are assumed to be similar to the well-established patterns reported for anchovy *Engraulis encrasicolus* and as such the spatial dynamics of both fish species are assumed to be driven by the same environmental and oceanographic factors (Coetzee *et al.* 2008). Sardine distribution off South African coast has been shown to be related to fish size or age (Beckley & van der Lingen 1999, van der Lingen & Huggett 2003, Coetzee *et al.* 2008). Information, obtained from results of hydro-acoustic surveys on sardine abundance and distribution as well as commercial catch data, show that adult sardines, within the South African population, are mostly restricted to the south-western coast and Agulhas bank. However, during their major spawning season in spring and summer (September - March), they have been observed to congregate mainly on the western Agulhas Bank (Crawford 1980, Armstrong *et al.* 1987, 1991, Hampton 1992, van der Lingen & Huggett, 2003). Sardine are relatively unspecific in their selection of spawning habitats (Twatwa *et al.* 2005), hence spawning has been reported to occur in different periods in both the west and south coasts (Miller *et al.* 2006). A north-flowing jet current which accompanies a strong thermal front between cold upwelled water and warmer oceanic water, entrains and transports ichthyoplankton (fish egg and larvae) along the shelf-edge of the southwestern Cape coast

from the spawning ground to the west coast nursery ground (Bang & Andrews 1974, Nelson & Hutchings 1987, Miller *et al.* 2006). During late summer/early autumn, juveniles migrate southwards along the west coast back to the spawning ground. Subsequently, during autumn and winter, they get recruited in to the adult population on the Agulhas Bank (Barange *et al.* 1999, Coetzee *et al.* 2008), with large adults dispersing eastwards and westwards as they mature (Barange *et al.* 1999, Beckley & van der Lingen 1999). Recruitment occurs predominantly in the west coast, although a significant number of recruits have been observed in the south coast during annual winter recruitment surveys (Barange *et al.* 1999, Beckley & van der Lingen 1999, Coetzee *et al.* 2008).

Over the years, two to four year-old fish have been observed to undertake a seasonal (early austral winter) reproductive migration from the coast of the eastern Agulhas Bank to the coast of KwaZulu-Natal (KZN), as far as Durban and the north coast of KZN, in an annual phenomenon known as the 'sardine run' (Connell 2010, Fréon *et al.* 2010, van der Lingen *et al.* 2010a, b, Chiazari 2014). This sardine population is considered to be a genetically distinct sub-population whose migration is facilitated by the expansion of sardine habitat during the cooler conditions that occur in early winter. Consequently, fish migrate into the Transkei waters at roughly the same time each year. Sardine schools 'run' along the continental shelf between Port St John and Durban as a result of current reversals, upwelling of cool water into the shelf, pursuit by predators, presence of transient cyclonic eddies, and other small-scale processes (Armstrong *et al.* 1991, Fréon *et al.* 2010, Roberts *et al.* 2010). Sardine remain on the KZN coast for several months after the sardine run and continue to spawn. They are probably forced to migrate southwards in early summer due to rising water temperature, and remain hidden away in cooler zones close to the seabed (Connell 2001, 2010).

Several studies have documented the presence of sardine eggs on the KZN south coast, indicating that sardine spawn off the east coast (Anders 1975, Van der Byl 1978, Connell 2001, 2010, Miller *et al.* 2006, Fréon *et al.* 2010). Similarly, sardine larvae and juveniles have been collected off the KZN coast. Most larvae were found inshore of the main Agulhas current where transport is much slower, thus facilitating retention in enclosed bays or estuaries (Beckley & van Ballegooyen 1992, Beckley and Hewitson 1994, Beckley & Naidoo

2003, van der Lingen *et al.* 2010a). It is not known yet if these early life stages and juvenile contribute significantly to the sardine population in South Africa (Fréon *et al.* 2010).

These reports, however, indicate that the KZN coast can act as sardine nursery area for sardine eggs which subsequently replenish the stock in the east coast. And together with the occasional good survival of early life stages, the existence of this nursery area may be sufficient motivation for the continual occurrence of sardine run (van der Lingen *et al.* 2010a). The westward return migration of sardine during late winter to spring occurs at a depth away from the warmer surface waters, hence this event is not noticed most of the time (Fréon *et al.* 2010).

The sardine run supports a small-scale, seasonal beach-seine fishery with annual catches being less than 1% of the catches made by the purse-seine fishery operating off South Africa's west and south coasts (van der Lingen *et al.* 2010a). In addition, the sardine run has become more valuable as a major tourist attraction yielding economic benefits which surpasses that obtained from the fishery (van der Lingen *et al.* 2010a).

SARDINOPS SAGAX PURSE-SEINE FISHERY IN SOUTH AFRICA

Within South Africa, the small pelagic fishery is the largest commercial fishery in terms of volumes caught and the second largest in net value. The activities of South African sardine purse-seine fishery have been dominantly concentrated on the west coast since 1940 (Agenbag *et al.* 2003, Coetzee *et al.* 2008, Department of Agriculture, Forestry and Fisheries 2012). Adult sardine is the main target species fished for human consumption and for bait, while juveniles are caught as by-catch in anchovy-directed fisheries (Barange *et al.* 1999, Agenbag *et al.* 2003, Coetzee *et al.* 2008, Department of Agriculture, Forestry and Fisheries 2012).

The sardine population on the west and south coasts of South Africa has undergone wide fluctuations in abundance since the inception of industrial-scale fishing (Figure 1.2). The high sardine catches taken in the early 1960s were followed by a massive decline in the late 1960s with fluctuations in sardine catches till the 1980s. Slow recovery of population followed after the introduction of management (stock-rebuilding) strategy which included the allocation of total allowable catch (TAC) in the mid-1980s (Cochrane *et al.* 1998,

Fairweather *et al.* 2006a). Strong recruitment in the late 1990s resulted in increased biomass and consequent high sardine catches recorded between 2001 and 2005, of which majority were caught off the south coast (Department of Agriculture, Forestry and Fisheries 2012). However, poor recruitment from 2004 has led to a substantial decrease in sardine population since then (Coetzee *et al.* 2008).

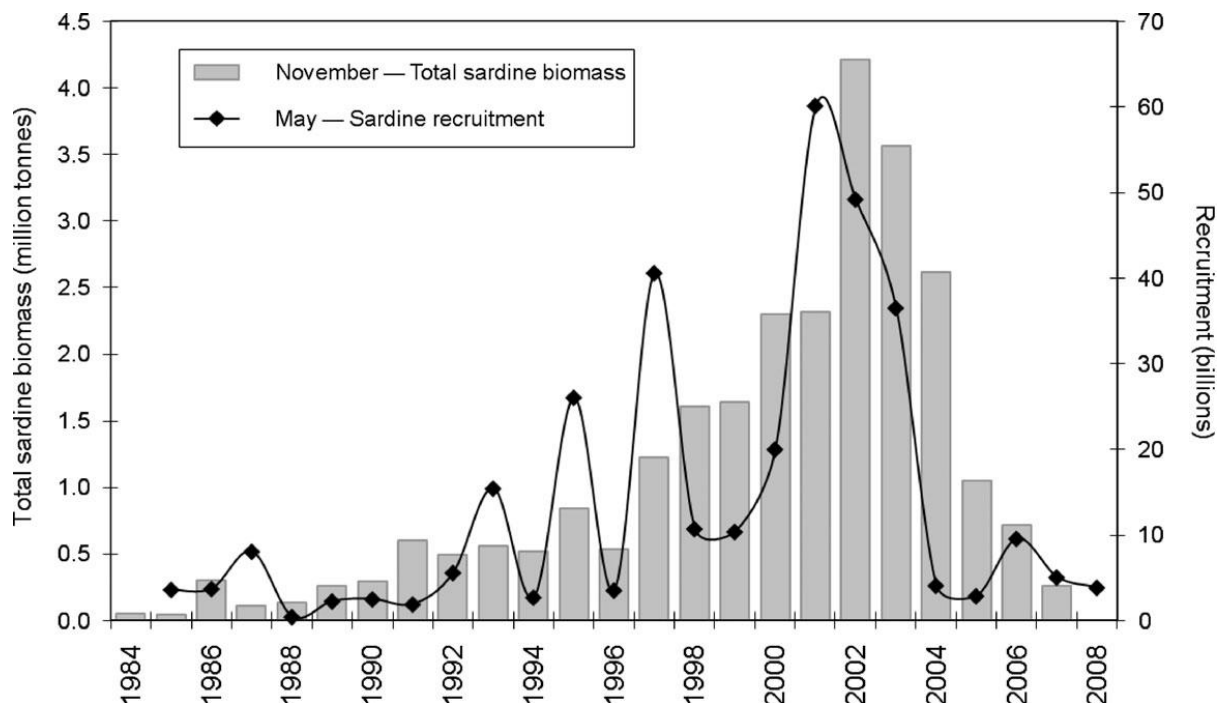


Figure 1.2. Annual acoustic estimates of sardine recruitment (measured in May) and total sardine biomass (measured in November) from 1984 to 2008 (from Coetzee *et al.* 2008).

The majority of the sardine biomass occurred on the west coast, to the west of Cape Agulhas, in the 1980s and early 1990s (Barange *et al.* 1999). However, over the years, the distribution of sardines in South Africa shown by acoustic surveys, has steadily shifted to the south and east coasts (Figure 1.3). Consequently, there has been an eastward shift in the centre of gravity of sardine catches from South Africa's west coast (Saldanha Bay) to the south coast (past Cape Agulhas) (van der Lingen *et al.* 2005, Fairweather *et al.* 2006b, Crawford *et al.* 2008).

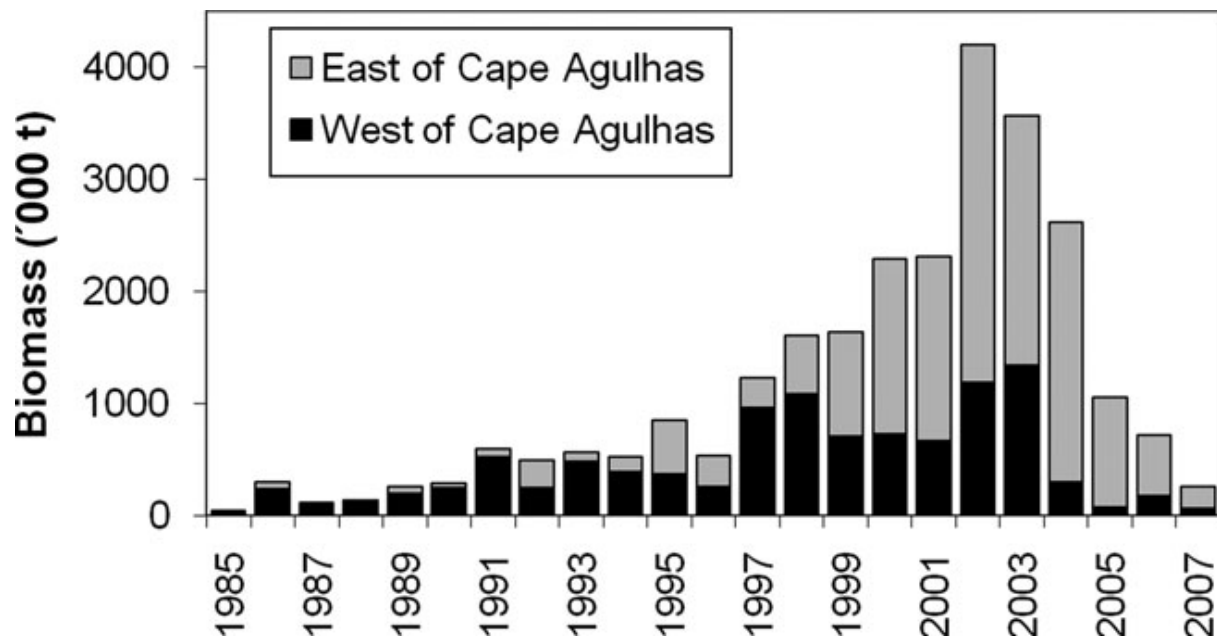


Figure 1.3. Acoustic biomass of sardine from the east and west of Cape Agulhas measured during November surveys (from Coetzee *et al.* 2008)

Several hypotheses have attributed this eastward shift in sardine population and the decrease in sardine biomass in the west coast to (van der Lingen *et al.* 2005, Coetzee *et al.* 2008):

- local depletion of fish off the west coast and Western Agulhas Bank as a result of higher exploitation rates compared to off the south coast;
- changes in the distribution of sardine spawners due to environmental changes; or
- successful spawning and recruit survivorship on the south coast compared to the west coast causing a distribution imbalance.

The resultant inequality between fish abundance and fishing effort, as well as between sardine catch location and fish processing facilities which are mostly located on the west coast has had severe financial consequences on the fishery and on management of sardine stock as well as ecological impacts (Coetzee *et al.* 2008). Because higher sardine catches were previously made on the west coast, the majority of the sardine processing plants are situated in this coast. Financially, the eastward shift in sardine distribution and catches means that fishing vessels would need to travel further east to catch sardine and then

transport them back to the west to be processed, costing more money (Coetzee *et al.* 2008, Department of Agriculture, Forestry and Fisheries 2012). Ecologically, sardine predators off the west coast have had to change their diets to less preferable alternatives (species) with lower energy content. Also, predators have to travel further to feed, leading to poor breeding performance of certain species such as the Cape Gannet *Morus capensis* (Department of Agriculture, Forestry and Fisheries 2012).

STOCK IDENTIFICATION

Various definitions of stock have evolved based on different approaches, including genotypic (e.g., Booke 1981, Carvalho & Hauser 1994), phenotypic (e.g., Bailey & Smith 1981), operational (e.g., Dahl 1909 cited in Waldman 2005) and contingent (e.g., Secor 1999) approaches.

A fish 'stock' normally denotes components of a commercial fish species exploited by fishing activities (Shaklee & Currens 2003). Of the many definitions of stock, one of the commonly accepted definition is that of Ihssen *et al.* (1981): "*an intraspecific group of randomly mating, reproductive individuals with temporal or spatial integrity*" (Waldman 2005). In view of this definition, it can be deduced that for units of individuals to be recognized and subsequently assessed, their boundaries have to be defined in relation to other units of the same species (Waldman 2005). As stocks may show differences in vital population and life history parameters compared to other stocks of the same species (Begg & Waldman 1999), there is a need to account for these differences, especially in fisheries where multiple stocks are exploited. Furthermore, the correct identification and delineation of the boundaries of an exploited fish stock with neighboring conspecifics ensures the successful implementation of policies, and the subsequent effective and sustainable management of that stock (Timi 2007).

Stock identification is the process of detection of unit stocks using various discrimination methods. It is an interdisciplinary field which forms the foundation for stock assessment, population dynamics and fishery analysis (Cadrin *et al.* 2005). Stock identification is necessary in fisheries management because it enhances the allocation of catch between competing fisheries, the recognition and protection of nursery and spawning areas, and the

development of optimal harvest as well as the monitoring of management strategies (Kutkuhn 1981, Grimes *et al.* 1987, Begg *et al.* 1999a). Because it has an extensive range of approaches, inconsistent terminologies and interpretations, it is regarded as a confusing area in fisheries science especially for non-specialists (Cadrin *et al.* 2005). Thus, information from stock structure assessment can sometimes create more uncertainty in fish management, especially when it contradicts historically established stock and management boundaries. For example, the results of Silva (2003) question the biological homogeneity of the Atlanto-Iberian stock of sardine (*Sardina pilchardus*) which was initially regarded as a 'panmictic, closed population'. Also, the findings of Abaunza *et al.* (2008) on the identification of stocks of horse mackerel *Trachurus trachurus* in the Northeast Atlantic and Mediterranean Seas implied the revision of previously defined stock boundaries. Nevertheless, ignoring such information can lead to erroneous and ineffective management which, when combined with high exploitation, has resulted in the depletion of fish stocks as seen in the cases of sardine *Sardinops sagax* (Shannon *et al.* 1993), Atlantic salmon *Salmo salar* (Cook & McGraw 1991), herring *Clupea harengus* (Dragesund *et al.* 1980) etc. (cited in Begg & Waldman 1999). Such depletion can cause loss of the total gene pool of a species (Smith *et al.* 1991). In addition, the biological characteristics of a species can change significantly as a result of disregard of stock structure information (Ricker 1981).

STOCK IDENTIFICATION METHODS

Stock identification comprises a wide range of techniques which have evolved over time from early morphological methods and life history studies through genetic methods to the detection of environmental signals in tissues (Cadrin *et al.* 2005). These methods are briefly discussed below.

Life history studies

Life history parameters such as distribution, abundance, growth, age, mortality, reproduction, recruitment reflect the underlying population dynamics of a given fish stock (Ihssen *et al.* 1981, Pawson & Jennings 1996, Begg *et al.* 1999b). Thus, differences in life history characteristics have been used in the discrimination of fish stocks (Griffiths 1996, 1997, Coetzee *et al.* 2008). Also, they serve as baseline information for the initial recognition

and delineation of geographic regions that represent individual stocks (Pawson & Jennings 1996). Although life history parameters cannot give conclusive evidence of genetic differences between fish stocks (Ihssen *et al.* 1981), they may indicate phenotypic differences among stocks which arise from different environmental conditions and/or fishing pressures (Campana *et al.* 1995, Begg *et al.* 1999a, Begg 2005). These phenotypic differences in life history parameters strongly support management of putative fish stocks as separate units (Begg & Waldman 1999). However, it should be noted that the wide intraspecific variability in life history parameters between individual fish does not enable classification of individual fish to a particular stock (Begg *et al.* 1999b, Begg 2005). If these differences exist but are not taken into consideration, and the fish population is modeled as having a single stock with the assumption that life history parameters are homogeneous, the erroneous results will misinform management decisions. This will likely lead to overfishing and depletion of less productive stocks with unknown ecological consequences (Stephensen 1999).

Morphology: Morphometrics and Meristics

Morphometrics

Morphometric analysis involves the use of two methods: landmark methods and outline methods. The former analyzes data generated from discrete morphometric points, linear distances between points, and geometric relationships among points on organisms, while the latter method evaluates perimeter shapes of organisms (Cadrin *et al.* 2005). Information on patterns of morphometric variation in fishes can be obtained from studying their body shape (Wessels 2009) or the shape of their individual features such as scale, otoliths etc. (Cadrin *et al.* 2005, Hampton 2014). These morphometric characters show changes that are associated with allometric growth and ontogeny. Also, they are influenced by genetics and prevailing environmental conditions, for example, temperature, pH, salinity, food availability etc. (Begg & Waldman 1999, Swain & Foote 1999, Cadrin *et al.* 2005), and hence can change through time. These variations are able to discriminate groups that have similar ontogenetic rates or reproductive dynamics, and thus infer phenotypic stocks (Cadrin 2000, Cadrin *et al.* 2005). Morphometric data are continuous and show strong allometric effects that should be accounted for before statistical analyses (Begg & Waldman 1999).

Meristics

Meristic characters are serially repeated morphological features of fishes (Ihssen *et al.* 1981) such as gill rakers, vertebrae, fin rays etc. They are discrete data which are influenced by genetics and environmental conditions (Tåning 1952, Barlow 1961, Lindsey 1988, Begg & Waldman 1999, Swain & Foote 1999). In addition, meristic features in fishes are fixed early in life during the larval or juvenile stages (Barlow 1961) and do not change over time.

Variability in morphometric and meristic characters has long been used in the identification of phenotypic fish stocks. Silva (2003) distinguished between sardine *Sardina pilchardus* populations in the North-eastern Atlantic and the Western Mediterranean using morphometric variation in body shape and Kinsey *et al.* (1994) identified stocks of the Spanish sardine *Sardinella aurita* based on variations in branchial morphology.

Turan (2004) used morphometric analysis of body shape and meristic counts of gill rakers and fin rays to identify different stocks of horse mackerel *Trachurus mediterraneus* in the Black, Marmara, Aegean and Mediterranean Seas; both morphometrics and meristics showed the existence of separate stocks in the Black and Marmara seas. The Aegean samples and a geographically distinct Mediterranean sample grouped together based on morphometrics, and were separated by meristics from the other Mediterranean samples suggesting some level of mixing among fish stocks in the Aegean and Mediterranean seas (Turan 2004). Again, morphometric variation in body shape, otolith shape and growth, as well as genetic methods were used by Kristoffersen and Magoulas (2008) to determine the structure of anchovy *Engraulis encrasicolus* in the Mediterranean Sea. Each of these approaches (genetics, body shape and otolith shape) detected significant differences among anchovy stocks, and thus indicated the existence of several reproductively isolated populations of anchovy in the northern Mediterranean.

Genetics

Over the years, molecular tools have been applied in fish population structure studies (e.g. in the Moroccan sardine *Sardina pilchardus*, Atarhouch *et al.* 2006, 2007; in the South African sardine, *Sardinops sagax*, Hampton 2014, Chiazzari 2014; in anchovy *Engraulis encrasicolus*, Kristoffersen & Magoulas 2008, in horse mackerel *Trachurus trachurus*,

Abaunza *et al.* 2008). This involves the use of genetic markers to determine genetic differentiation between/among stocks. The ability of these markers to detect genetic heterogeneity is dependent on time since the divergence of stocks and the degree of reproductive isolation (Adkinson 1996 cited in Begg & Waldman 1999). Therefore, a recent divergence or substantial mixing or exchange of genetic information between stocks can obscure heterogeneity in genetic markers (Clayton 1981, Carvalho & Hauser 1994, Begg & Waldman 1999). Genetic methods of stock identification include: nuclear DNA (Wirgin & Waldman 2005), chromosome morphology (Philips 2005), allozyme analysis (Koljonen & Wilmot 2005), mitochondrial DNA (Magoulas 2005, Hampton 2014), microsatellite loci (Hampton 2014), Random Amplified Polymorphic DNA (RAPD; Smith 2005), and Amplified Fragment Length Polymorphism (AFLP; Liu 2005).

Tagging

Here, marks or tags or both are applied to a fish to enable its identification and collection of information (Jacobsen & Hansen 2005). Tagging was initially applied on salmonids and subsequently to other fish species e.g. sardine *Sardinops caerulea* (Hart 1943, Clark & Janssen 1945, Clark & Marr 1955). Since the beginning of the twentieth century, its use has been extended to stock identification where putative stocks are marked in order to determine the level of mixing, if any, between these stocks (Cushing 1981, Jacobsen & Hansen 2005) and hence infer stock structure. However, for this method to be valid, it requires that a representative sample has to be tagged and successfully recaptured (Cushing 1981, Begg & Waldman 1999). This is because experimental errors have been observed due to tagging mortality, tag shedding and inefficient tag recovery especially in small fish (Clark and Marr 1955). Tags could be external, internal, electronic or thermal (Bain 2005, Jacobsen & Hansen 2005, Volk *et al.* 2005).

Detection of environmental signals in tissues

Fatty Acid Profiles

Different tissues in fish have different fatty acid profiles which are influenced by both biotic and abiotic factors (Sargent *et al.* 1989, Greene 1990, Grahl-Nielsen 2005). These differences in fatty acid profiles have been used in stock identification. Joensen *et al.* (2000)

used the composition of fatty acids in the heart tissue of reared cod *Gadus morhua* to distinguish between two reared stocks from Faroe Bank and Faroe Plateau in the Faroe Islands. Also, Joensen and Grahl-Nielsen (2004) differentiated stocks of redfish *Sebastes mentella* in the North Atlantic Ocean using the chemometric analysis of the fatty acid profile of the heart tissue.

Otolith Elemental Composition

Unlike bone, the otolith is metabolically inert (Campana & Nielson 1985, Campana 2005) and can accumulate trace elements reflective of the different physicochemical environments in which groups of fish have lived in (Gallahar & Kingsford 1996, Gillanders & Kingsford 1996). Hence, the variations in the concentrations of selected elements and isotopes in the otolith can be used in fish stock identification (Campana 2005). For example, Newman *et al.* (2000) identified distinct adult assemblages of the goldband snapper *Pristipomoides multidentis* off the waters of western and northern Australia using stable isotope ratio analysis of sagittal otolith carbonate. Also, Secor *et al.* (2001) identified three migratory (riverine, estuarine and oceanic) contingents of Hudson River striped bass *Morone saxatilis* based on their otolith elemental fingerprints. Turan (2006) differentiated Mediterranean horse mackerel *Trachurus mediterraneus* from the Black Sea from those from the Aegean Sea using sodium, barium, potassium, magnesium concentrations in their otoliths. Hampton (2014) showed small-scale differentiation in otolith microchemistry between sardine from different regions around the South African coast (discussed further below). Otolith elemental composition have been reported to successfully distinguish fish from different regions (Gillanders *et al.* 2001). However, studies have shown that it could be temporally unstable. For example, otolith microchemistry which previously differentiated between five groups of anchovy *Engraulis encrasicolus* in the Atlantic in a year, detected no difference between these groups in subsequent analyses conducted the following year (Guidetti *et al.* 2013).

Parasite 'biotags'

The use of parasites as biological tags has been successfully applied in population studies to distinguish stocks and to follow migratory movements of fish (Lester 1990, Williams *et al.*

1992, MacKenzie & Abaunza 1998) in many parts of the world. The basic principle underlying the use of fish parasites as biological tags is that fish can only become infected with a parasite if they come within the endemic area of that parasite (MacKenzie & Abaunza 1998). An endemic area is a geographical region which provides favourable environmental conditions for the existence of a parasite (that is the parasite or infection is regularly found in that area) and suitable conditions for the transmission of the parasite from one host to the next. To be useful in biotag studies, the endemic area of the parasite must be smaller than the host distribution. Therefore, finding an infected fish outside the endemic area indicates that the fish has been in this area. And in some cases, one can determine the maximum time of infection depending on the life span of the parasite (MacKenzie & Abaunza 1998). Because the distribution of marine parasites is influenced by temperature and salinity and because these conditions differ markedly around the South African coast, investigation into the use of parasite biotags in delineating sardine stocks was investigated by Reed *et al.* (2012; see below).

Parasites as biological tags have been used in the discrimination of pelagic fish species globally, often producing results at a resolution suitable for fishery management (Lester *et al.* 1985, Mackenzie 1990). Parasite biotags are more suitable in the study of smaller, delicate fish species that cannot be studied using other methods such as tagging. Even in bigger fish species, uncertainties arise due to the abnormal behavior of the fish caused by the tag, so parasite biotags offer a better option. Furthermore, parasite biotags occur naturally, therefore, they are inexpensive to use. However, the use of parasite biotags is limited in the following cases (Sindermann 1983 cited in Mackenzie & Abaunza 1998):

- where there is inadequate information in parasite biology and ecology;
- where parasite species could not be identified or taxonomic identification is inconsistent; and
- where the age (or its proxy) of individual hosts is unknown.

In the light of this understanding, it is proposed that an ideal parasite biotag should (Sindermann 1983 cited in Mackenzie & Abaunza 1998, Mackenzie 1983, 1987, Williams *et al.* 1992):

- show significantly different levels of infection in hosts from different parts of the host distribution;
- persist in the host for a long period of time;
- have relatively constant levels of infection over several years;
- be easy to detect and identify; and
- have little or no pathogenic effect on the host or on host behaviour.

In 1963, based on the understanding that parasites were directly transmitted, Kabata recommended that an ideal parasite biotag should utilize only a single host. However, the discovery that parasites require intermediate hosts (Kent *et al.* 2001), as well as reports from different studies (Boje *et al.* 1997, Mackenzie & Abaunza 1998, Moore *et al.* 2003, Mackenzie *et al.* 2008) which show the best biotags to be the larvae of three-host parasites, invalidated this recommendation (Lester & MacKenzie 2009).

Parasites from various taxonomic groups have been used as biotags for fish stocks. Speare (1995), not only distinguished groups of sailfish (*Istiophorus platypterus*) from Queensland coastal waters, Australia, but also provided information on fish movement using their cestode, trematode and copepod parasites. Timi (2003) identified four stocks of Argentine anchovy *Engraulis anchoita* in the south-west Atlantic using their parasite populations. He also reported that these parasites showed marked latitudinal gradients. Also, Mackenzie *et al.* (2008), using parasite tags, differentiated stocks of horse mackerel *Trachurus trachurus* off the coast of Morocco to south-west Norway, and within the Mediterranean Sea. Moser and Hsieh (1992) distinguished stocks of Pacific herring *Clupea harengus* off the California coast using the adult digenean *Parahemiurus merus*, the cestode *Lacistorhynchus dollfusi* and various nematodes including *Anisakis simplex* larvae.

In South Africa, the potential use of parasite biotags in discriminating sardine stocks was investigated by Reed *et al.* (2012) who identified seven parasite species from sardine. Among these species, a digenean ‘tetracotyle’ type metacercaria considered to be of the genus *Cardiocephaloides* and found in the sardine’s eyes showed the highest prevalence and met many of the criteria listed above for good biotags, and hence inferred suitability as a useful biotag (Reed *et al.* 2012). Subsequent studies by Weston (2013) and Weston *et al.*

(2015) showed a clear spatial difference in the distribution of this parasite in sardine from putative western and southern stocks, thereby discriminating between the two sardine stocks. Prevalence, infection intensity and abundance of the metacercaria were higher in sardine sampled from the west coast compared to sardine from the south coast. In addition, this parasite is easily identified in its predilection site, which is the eyes of sardine and it has a relatively long life span in sardine. Knowledge on its lifecycle is not yet complete but basic information on its hosts is attainable. Timi (2003) also used a *Cardiocephaloides* sp. biotag in the discrimination of anchovy stocks in the south-west Atlantic.

Stock identification is a continuous process which evolves as more techniques are developed. Unfortunately, reliable identification is harder to accomplish with the use of a single technique (Edmonds *et al.* 1989, Campana *et al.* 1995); thus, it is better to combine the results obtained from various techniques in order to get greater insight into the stock structure of a given fish species (Elliot *et al.* 1995) and hence make informed fishery management decisions.

STOCK IDENTIFICATION: A MULTI-DISCIPLINARY APPROACH

The aim of stock identification studies is to identify management units that are meaningful biological entities, and hence reduce the uncertainty in the assessment models and/or improve the management of fish resources (NRC 1998 cited by Abaunza *et al.* 2008). Therefore, techniques for identification of fish stocks not only help in identification and delineation of stock boundaries, but are essential for understanding and managing fish species (Baldwin *et al.* 2012). Since different methods could produce different results, it is important to utilize multiple methods to assess stock structure to maximize the likelihood of correctly defining different fish stocks (Pawson & Jennings 1996, Begg & Waldman 1999, Begg *et al.* 1999a, Turan 2004). Also, these methods have their various strengths and weaknesses. For example, morphometrics is faster and cheaper than otolith microchemistry and genetics. Again, taxonomic inconsistencies or lack of information on parasite biology can make a parasite species unsuitable as a biotag. Furthermore, approaches like otolith microchemistry can be temporally unstable. Abaunza *et al.* (2008) maintains that the most successful way of solving the problem of defining stock structure is through a holistic approach involving a broad spectrum of complementary techniques. This is exemplified by

their identification of various horse mackerel *Trachurus trachurus* stocks in the Northeast Atlantic and Mediterranean Sea by the integration of several stock identification methods including genetic markers, morphometry, parasite biotags and life-history traits (growth, reproduction and distribution). Results of morphometric and parasite studies support separation of Atlantic and Mediterranean horse mackerel stocks. Within the Northeast Atlantic population, three stocks (western, southern and North Sea stocks) were distinguished based on morphometrics, parasites and life history traits. This contrasted with the two-stock structure it was previously thought to have and called for a revision of stock boundaries. In addition, the Mediterranean horse mackerel population could be differentiated on the basis of morphometric and parasite data into western, eastern and central stocks, and the horse mackerel stock from the Mauritanian coast was differentiated by its high batch fecundity and high growth rate.

Several marine finfish species have a 'mixed stock' structure (i.e. fish stocks mix with each other) which is more complex than the structure currently recognized in management (Stephensen 1999). Therefore, a multidisciplinary approach to stock identification would be more efficient because information with multiple perspectives is obtained following the application of the different techniques (Begg & Waldman 1999, Cadrin *et al.* 2005). Application of such a method will increase the likelihood of accurate detection of discrete fish stocks.

SOUTH AFRICAN SARDINE POPULATION: MONO-STOCK OR MULTI-STOCK?

Studies on the population structure of *Sardinops sagax* from other areas of the world have reported the occurrence of distinct sardine stocks. In the Pacific, three stocks have been discriminated using monthly catch and sea-surface temperature data (Felix-Uraga *et al.* 2004), otolith morphometrics (Felix-Uraga *et al.* 2005) as well as body shape analysis (Garcia-Rodriguez *et al.* 2011).

Off the coast of Australia, initial studies by Izzo *et al.* (2012) using otolith-based techniques suggested the existence of at least three stocks. Subsequent studies on population structure by Izzo *et al.* (2017) which involved the integration of genetic, morphological, otolith, growth, reproductive and fishery data collected over 60 years using a Stock Differentiation

Index (SDI; see Chapter 2) gave a better resolution of the sardine population structure and confirmed the existence of at least four stocks which some degree of overlap in their distribution.

In South Africa, sardine have historically been managed as a single sardine population with no consideration of spatial variability in distribution or the presence of multiple stocks (de Oliveira & Butterworth 2004, Coetzee *et al.* 2008). However, recent studies have included the possibility of the existence of three phenotypically discrete stocks (Coetzee *et al.* 2008, van der Lingen 2011, Department of Agriculture, Forestry and Fisheries 2012, van der Lingen *et al.* 2015). Based on the spatial variation in body form of sardine caught around the coast of South Africa, it is hypothesized that there are three stocks of sardines (Chiazzari 2014, van der Lingen *et al.* 2015). Putative western and southern stocks occur to the west and east of Cape Agulhas respectively (Coetzee *et al.* 2008), while the eastern stock occurs off the coast of KwaZulu-Natal (Fréon *et al.* 2010, van der Lingen *et al.* 2010a). The putative western and southern stocks support the purse-seine fishery in the west and south coast of South Africa while the eastern stock supports the seasonal beach-seine fishery in the east coast of South Africa. These two fisheries have different management policies because of their size and economic importance (van der Lingen *et al.* 2015). The beach-seine fishery is managed by 25 right-holders, out of which three to seven are active (van der Lingen & Lamberth 2013).

The purse-seine fishery, on the other hand, is managed through operational management procedure which sets an annual sardine total allowable catch (TAC; de Oliveira & Butterworth 2004). Management of the purse-seine fishery was historically based on the assumption of a single sardine stock off the west and south coasts of South Africa. However, current deliberations have recognized the possibility of the existence of two mixing sardine stocks (Smith *et al.* 2011, de Moor & Butterworth 2013, de Moor & Butterworth 2015, van der Lingen *et al.* 2015, Butterworth *et al.* 2016) which have led to the review of management strategies to consider the possible multi-stock nature of the South African sardine population in order to avoid the over-exploitation of one stock.

Different studies have supported the two-stock hypothesis for sardine off the west and south coast. Coetzee *et al.* (2008) reported that at low and moderate biomass levels

individuals found to the west and individuals found to the east of Agulhas are consistently separated by an area of very low biomass or complete absence of sardine, and that sardine only occur in this area in high levels of biomass (Figure 1.4). In addition, intense spawning, in widely separated grounds, occurs off the west coast and off the east coast (Figure 1.5), with switches between the two coasts previously reported as well (Miller *et al.* 2006). This implies that ‘west coast’ sardine spawn on western Agulhas Bank and the west coast, and recruit to the west coast nursery grounds. While those in the ‘south coast system’ (‘Agulhas Bank system’) spawn on the central and eastern Agulhas Bank, and recruit mainly to the south coast nursery grounds, even though some transport occurs from east of Cape Agulhas to the west coast (van der Lingen 2011, Butterworth *et al.* 2016).

Investigation of sardine stock structure in southern Africa using morphometrics (body form) and meristics (vertebral counts) showed significant meristic and morphometric variation between sardine from South Africa and Namibia, and between sardine from around the South African coast (Wessels 2009, van der Lingen *et al.* 2010b). Namibian sardine, also known as the Northern Benguela sardine, is considered to be of a different stock (Boyer *et al.* 2001). Again, significant differences in branchial sieve morphology of sardine samples from four geographical regions of study (Namibia, South African west, south and east coasts) have been reported (Idris 2010, Idris *et al.* 2016). However, variations were not consistent across all fish size classes. Hampton (2014) discriminated between sardine from west and south coasts on a small scale using otolith elemental signatures. Magnesium concentration was higher in the west coast while barium concentration was higher in the south coast. She also reported a weak differentiation of sardine from the different regions using seven microsatellite loci and mitochondrial marker ND2. Reed *et al.* (2012) reported the potential role of a digenean ‘tetracotyle’ type metacercariae (found in the eye) and *Eimeria sardinae* (found in the testes) as parasites biotags in the identification of sardine stocks. Further investigation by Weston (2013) and Weston *et al.* (2015) using the digenean parasite as a biotag, supported the existence of western and southern sardine stock. Conversely, the results of Ssempe (2013)’s investigation using *E. sardinea* as a parasite biotag, could not distinguish between sardine from the western and southern coasts of South Africa but did support the hypothesis of an eastern stock.

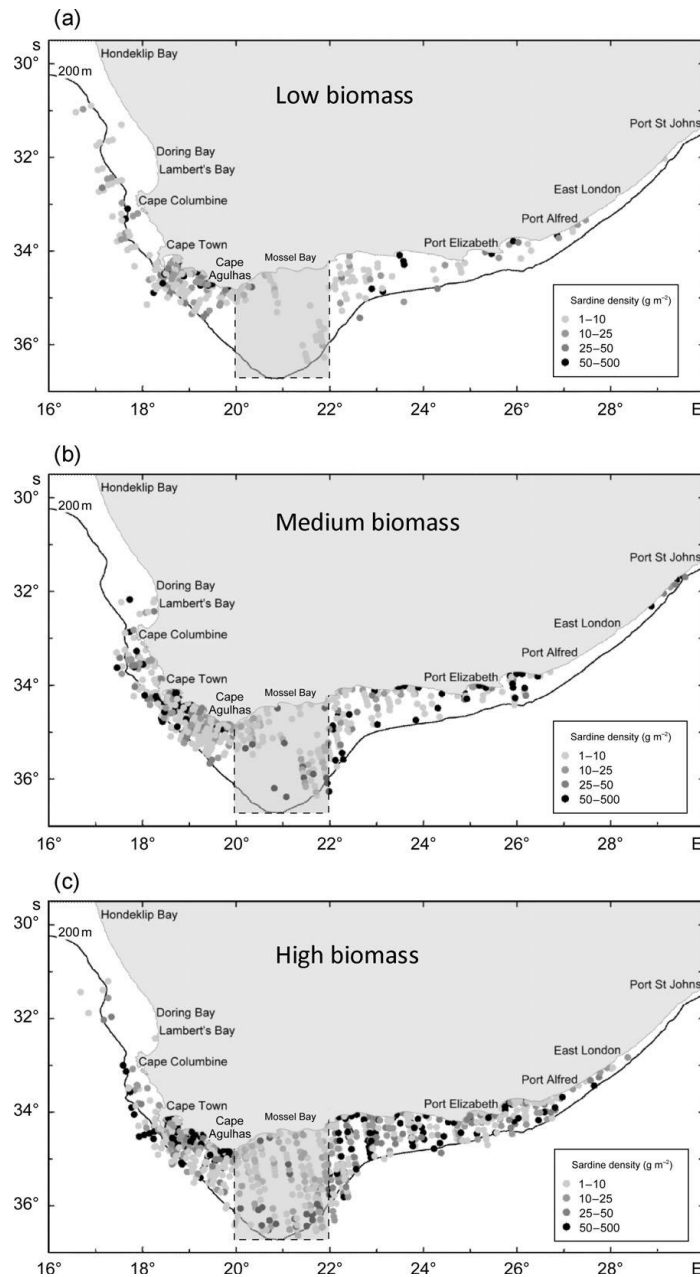


Figure 1.4. Composite density maps derived from November survey data for periods of (a) low, (b) medium, and (c) high sardine biomass. The transition zone between the west coast system and the south coast systems is represented by the block of dashed lines. The solid line indicates the continental shelf. Separation in sardine distribution is clearly shown at low and medium biomass levels (from Coetzee *et al.* 2008).

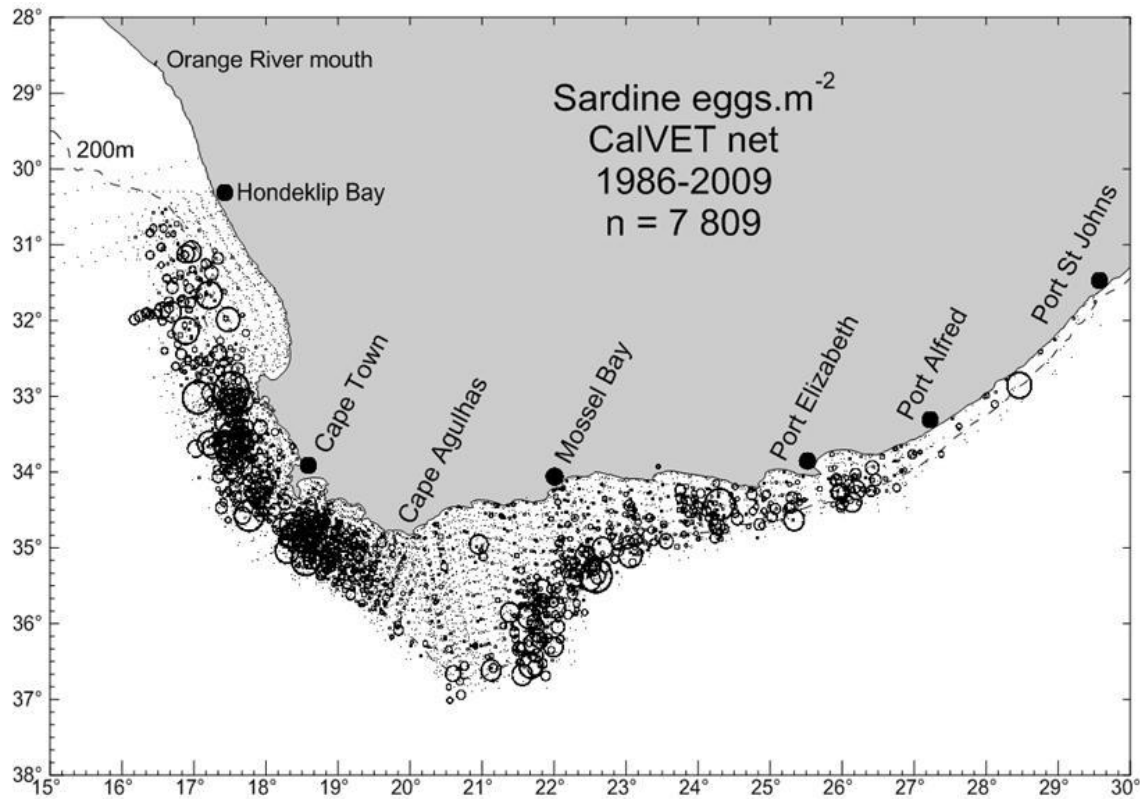


Figure 1.5. Separation in egg density at the Central Agulhas Bank indicating separate spawning grounds. Composite sardine egg density map from CalVET net samples obtained during spring from 1986 – 2009 (from van der Lingen, 2011).

Given these evidence of two interacting stocks, stock assessment models for the putative western and southern stocks were developed (de Moor & Butterworth 2012, 2013, 2015, Butterworth *et al.* 2016). Projections from these models indicate that the putative western and southern stocks are two mixing stocks, with the latter stock being less productive than the former. Also, the biomass of the southern stock depends heavily on recruitment from the western stock as fish (from 1 year old and above) are estimated to move exclusively from west to south coast each year. Some eggs spawned on the south may be transported to the west coast, but the extent to which this transportation contributes to the recruitment in the west coast is yet unknown. Further research to answer this question is needed as it would help in the better management of the South African sardine resource.

The above studies (Wessels 2009, Idris 2010, Idris *et al.* 2016, Hampton 2014, Ssempe 2013, Weston 2013; Weston *et al.* 2015) documented spatial variability in several phenotypic and

other characteristics of South African sardine. But, they typically examined spatial variability in just a single characteristic (e.g. gill raker morphology and meristics, body shape, otolith shape, vertebral count, or parasite loads) from the samples analysed. In order to maximize the likelihood of correctly inferring the population structure of the South African sardine and identifying the different stocks, a multiple stock identification method applied to the same individual fish should be done. The information obtained from the combination of techniques will provide more robust results, if sardine stocks are discriminated by several indices, and will help to correctly assess the stock structure of the South African sardine. Therefore, in this study, a novel approach to the assessment of sardine stock structure will be employed. This involves the application of multiple stock identification methods to the same fish in order to get a data set that corresponds to one another and is directly comparable. This data set will be subjected to multivariate analyses. 'Stock Differentiation Index' (Izzo *et al.* 2017; see Chapter 2) will be calculated to further aid in the stock structure assessment.

AIMS OF STUDY

1. This study aims to investigate sardine population structure off the west and south coasts of South Africa using a multi-method approach which involves the combined analyses of all data gathered from the same individual fish using different methods which include:
 - Morphometrics: body shape, otolith shape, and the branchial sieve morphology (gill arch length and gill raker spacing);
 - Meristics: number of vertebrae, number of gill rakers; and
 - Parasite biotag: digenean 'tetracotyle' type metacercariae abundance.
2. Since it may not be possible to apply all techniques of stock identification at any given time on fish samples, the 'best-fit' single technique might prove useful in the distinction of sardine stocks. Hence, the study seeks to determine the best single technique or the minimum combination of techniques that produces results which best discriminate between sardine stocks in the west and south coasts of South Africa.

3. A major limitation in the use of the 'tetracotyle type' metacercaria as biotag is the lack of information in its biology and ecology, as well as definitive taxonomic identification. This study, therefore, attempts to describe the metacercarian parasite biotag using light and scanning electron microscopy, as well as DNA sequencing. Additionally, the study contributes information to the lifecycle of this parasite by using DNA sequencing to determine its affiliation to an adult stage *Cardiocephaloides* parasite found in African penguin *Spheniscus demersus*.

THESIS CHAPTERS

Chapter 2 applies a combination of multiple stock identification techniques to the same individual fish to test the hypothesis of western and southern sardine stocks In South Africa and seeks to identify a single technique or a minimum combination of techniques (if any) that best differentiates sardine from the putative western and southern stocks.

Chapter 3 provides a morphological description of the digenean 'tetracotyle' type metacercarian parasite biotag.

Chapter 4 uses molecular techniques to identify the digenean 'tetracotyle' type metacercarian parasite from sardine and determine its relationship to an adult stage found in African penguin.

Chapter 5 comprises a general discussion, synthesis and conclusions, and recommendations based on research findings.

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CHAPTER 2

ASSESSMENT OF THE STOCK STRUCTURE OF THE SOUTH AFRICAN SARDINE *Sardinops sagax* USING A MULTIPLE METHOD APPROACH

STOCK IDENTIFICATION

Fisheries science is aimed at interpreting relevant information on exploited fish species, as well as on the records of fishing efforts and the size of catches. Such information will help in the prediction of the future size of the population so that informed decisions are made on future fishing efforts. Birth rate and death rate are factors which significantly determine population size, and methods of evaluation, modelling and management of fish stocks assume discrete populations that are driven by these internal factors and not so much by external factors such as immigration and emigration (Haddon 2001). Thus, the identification of fish populations that conform to these assumptions are important for the allocation of catches to the appropriate spawning population in the correct proportion (Campbell *et al.* 2007). This explains the need for stock identification and stock assessment studies.

Several approaches including analyses of life history parameters, phenotypic and genetic characteristics, and parasite loads, as well as the use of artificial tags have been employed in stock identification (Begg *et al.* 1999a). For example, Turan (2004) investigated the stock structure of Mediterranean horse mackerel *Trachurus mediterraneus* in the Black, Marmara, Aegean and Eastern Mediterranean Seas using morphometric and meristic characters. Zischke *et al.* (2014) examined the stock structure of wahoo *Acanthocybium solandri* in the Pacific and Indian Oceans using morphometrics and parasites. Newman *et al.* (2000) identified distinct adult assemblages of the goldband snapper *Pristipomoides multidens* off the waters of western and northern Australia based on stable isotope ratio analysis of sagittal otolith carbonate. Timi (2003) identified four stocks of Argentine anchovy *Engraulis anchoita* in the south-west Atlantic using their parasite populations.

However, there is no single 'correct' method of stock identification because the different methods are limited in one way or the other. For example, tagging studies are costly, time-

consuming (Begg & Waldman 1999), and are associated with high mortality rates (Baldwin *et al.* 2012). The use of life history parameters for stock identification is limited by the wide intraspecific variability that could occur between individuals, which could give misleading results if these differences are not taken into consideration when modelling fish population (Begg *et al.* 1999b, Begg 2005). Although parasite biotags are relatively cheaper and easy to use, the lack of biological information on the parasite is one of the limiting factors to the use of this technique (Begg & Waldman 1999). Hence, it is recommended that a wide range of methods should be applied on the same set of fish. In this way, results are verified and the likelihood of correctly identifying fish stocks is maximized (Begg & Waldman 1999, Begg *et al.* 1999a, Turan 2004, Abaunza *et al.* 2008). In view of this recommendation, stock structure studies have since been designed to comprise the integration of different stock identification methods. An example of such a multidisciplinary study is the identification of multiple stocks of horse mackerel *Trachurus trachurus* in the Northeast Atlantic and Mediterranean Sea based on integration of results of analyses of morphometry, parasite bio- tags, genetic markers and life history traits (Abaunza *et al.* 2008). Morphometrics and parasite data distinguished between horse mackerel populations in the Atlantic Ocean and the Mediterranean Sea, with some level of mixing between these populations. Based on parasites, morphometrics and life history traits, stocks in the Northeast Atlantic were further differentiated into a southern stock occupying the West Atlantic coast of the Iberian Peninsula south to Cape Finisterre (NW Spain), a western stock, distributed along the west coast of Europe from Cape Finisterre to Norway, and the North Sea stock. In the Mediterranean Sea, parasite and morphometrics data separated the horse mackerel population into western, central and eastern Mediterranean stocks. A stock from the Mauritanian coast was differentiated based on its high growth rate and high batch fecundity. Based on these findings, the previous definition of stocks into western and southern stocks was revised (ICES 2005). Another example comes from US waters, where Zemeckis *et al.* (2014) conducted an interdisciplinary review of biological information in order to assess the population structure of the Atlantic cod *Gadus morhua*. Biological information reviewed consisted of results of stock identification studies using different methods namely; genetic markers, life history parameters and artificial tagging. Their findings revealed that some stocks have been wrongly allocated and called for a review of the existing stock boundaries.

STOCK STRUCTURE OF SARDINE POPULATIONS

Over the years, stock structure and management units of several different economically important fish species have been evaluated with the aim of revising management strategies (Cadrin *et al.* 2014). Sardine have not been left out. Sardine *Sardinops sagax* inhabits marine systems off the west coast of North and South America, the west and south coast of southern Africa, in temperate and subtropical waters of Australasia, as well as around the coast of Japan (Parrish *et al.* 1989). Population structuring of *Sardinops sagax* has been reported in the four large populations off the Americas, Africa and Japan, with at least two stocks identified in each.

Three sardine sub-populations are thought to occur in the Humboldt Current: a central stock distributed from southern Peru to northern Chile), southern (occurring off Chile) and northern (occupying southern Ecuador to central Peru) sub-populations (Schwartzlose *et al.* 1999, Barange *et al.* 2009). Two sardine sub-populations are found in the Kuroshio Current: one occupying the Sea of Japan and East China Sea, and the other occurring in the Pacific coast (Ohshimo *et al.* 2009, Barange *et al.* 2009).

Studies on the stock structure of Pacific sardine off California have separately employed several methods (Smith 2005) which include monthly catch and sea-surface temperature data (Félix-Uraga *et al.* 2004), otolith morphometrics (Félix-Uraga *et al.* 2005) as well as morphological characters, artificial tags, molecular markers and parasites. However, it was difficult to resolve the population structure of *Sardinops sagax* in that system. Hence, a combination of methods that include fish and parasite data was advocated (Baldwin *et al.* 2012). There are now considered to be three sub-populations of sardine in the California Current: a northern sub-population distributed from northern Mexico to southern Alaska, a southern sub-population from Baja Peninsula to southern California, and a sub-population inhabiting the Gulf of California (Baldwin *et al.* 2012, Demer & Zwolinski 2014). The northern and southern sub-populations appear to migrate seasonally north and south along the coast in synchrony with their respective habitats, leading to segregated spawning and different identities (Demer & Zwolinski 2014). Two sardine sub-populations occupy the Benguela Current system, a northern sub-population occurring off southern Angola and the Namibian coast which is separated from the southern sub-population that occurs off the South African

west and south coasts by the Luderitz upwelling region characterized by turbulent cold water and strong offshore advection (Beckley and van der Lingen, 1999; Roux *et al.* 2013).

In addition to population structure being demonstrated for sardine in these four regions, Izzo *et al.* (2017) evaluated the population structure of sardine off Australia by combining morphological, genetic, growth, otolith, reproductive and fishery data and the epidemiology of pilchard herpes virus collected over 60 years and using a Stock Differentiation Index (SDI). They reported that sardine there also comprise a meta-population with at least four stocks: one off in the southwestern coast off western Australia; a second in the Bass Strait and Port Philip Bay; a third in the Great Australian Bight and Spencer Gulf; and a fourth off the Australian east coast.

SOUTH AFRICAN SARDINE STOCK STRUCTURE

The South African purse-seine sardine fishery was historically considered to target a single biological population and was managed as such (de Moor & Butterworth 2009a, b). However, the need for a review of the stock structure of sardine off the South African coast arose due to the occurrence of certain events such as the boom in sardine abundance in the early 21st century, and an eastward shift in the population (de Moor & Butterworth 2015). van der Lingen and Huggett (2003) documented the existence of two discrete sardine spawning areas separated by the Central Agulhas Bank, indicating distinct spawning units located on either side of Cape Agulhas, one off the west and the other off the south coast. This, and observations of changes in the distribution of spawning between these two areas, led van der Lingen *et al.* (2005) to warn on the possibility of the depletion of a west sub-population, if such existed. Coetzee *et al.* (2008) reported a consistent break in the distribution of sardine at low and medium biomass levels, with fish found to the west and to the east of Cape Agulhas but separated by the central Agulhas Bank region between Cape Agulhas and Mossel Bay. Overlap between these two components of the population was only observed at high biomass levels, and Coetzee *et al.* (2008) concluded that the South African sardine population may consist of at least two separate spawning units, one on either side of Cape Agulhas and both occupying the mid-shelf and offshore domains. This

supported the results of Miller *et al.* (2006), who used an individual based model (IBM) coupled to a 3D hydrodynamic model of the southern Benguela to investigate how spatial and temporal variability in sardine spawning might impact the transport to or retention within suitable nursery grounds of early life history stages and concluded that sardine life history strategy could be divided into two main systems, namely west coast and south coast systems separated in the region of Cape Agulhas.

Based on these reports of separate distributions and spawning grounds, further investigations of the population structure of the South African sardine commenced. These investigations focused on defining the population structure of sardine exploited by the purse-seine fishery that operates off the west and south coast, and involved separate studies on spatial variability in phenotypic characteristics (including condition factor, standardized gonad mass, body shape, otolith morphology, branchial sieve morphology and number of vertebrae), and parasite populations of sardine in the southern Benguela. Sardine samples from Namibia were included in some of these studies as a control group to test the ability of the different methods to discriminate between samples from the different part of the South African coast, since a method that could not differentiate between Namibian and South African sardine samples would not be likely to delineate sub-populations of the South African sardine should those exist.

Significant spatial variability was reported in the biological characteristics of sardine from South Africa as well as from Namibia in many of these studies. van der Lingen *et al.* (2006) reported significant spatial variation in South African sardine condition factor, with higher condition factor values observed for sardine east of Cape Agulhas compared to those from the west coast, whilst standardised gonad mass was found to be highest in fish off the west coast. In addition, annual length-at-50% sexual maturity of female sardine from the south coast has been consistently higher than those from the west coast, and whilst no significant difference in mean size-at-age and otolith length-at-age were observed, south coast sardine tended to have higher values for these parameters (van der Lingen *et al.* 2009). Wessels (2009) reported significant variability in body shape between sardine from Namibia and South Africa, and between fish from the South African west and south coasts: sardine from

the west coast sardine differed from those from the south coast in their head measurements and position of pelvic fin. Vertebral count was significantly different between Namibian sardine and South African sardine but did not differ significantly between sardine from the South African west and south coasts (Wessels 2009). Subsequent analysis by van der Lingen *et al.* (2010) showed that sardine from the east coast had significantly fewer vertebrae and a significantly different body shape to those from either the west or south coast. Hampton (2014) reported no significant differences in two indices (circularity and form factor) of otolith shape between sardine from Namibia and the South African west and south coast, but these were significantly different in sardine from the east coast of South Africa. Significant spatial variability has also been observed in branchial sieve morphometrics (gill arch length and gill raker spacing) and meristics (number of gill rakers) for sardine from southern Africa (Idris 2010; Idris *et al.* 2016). The number of gill rakers and gill arch length were significantly different for small south coast and west coast sardine, while east coast sardine differed from west and south coast sardine in their gill arch length and gill raker spacing. Weston (2013) and Weston *et al.* (2015) assessed the spatial and temporal variability in the prevalence, mean infection intensity and mean abundance of a 'tetracotyle' type metacercaria digenean parasite found in the sardine's eyes and suggested as a useful biotag by Reed *et al.* (2012). West coast sardine had significantly higher values for all three indices than did fish from the south coast (Weston *et al.* 2015), and sardine from the KwaZulu-Natal (KZN) sardine run were almost completely un-infected or showed very low levels in infection (van der Lingen *et al.* 2015). Furthermore, analysis of variability in the prevalence of *Eimeria sardinae* revealed the absence of infection in sardine from Namibia and the South African east coast, while sardine from west and south coasts were infected (Ssempe 2013). Some studies also examined sardine collected during the KwaZulu-Natal sardine run (van der Lingen *et al.* 2010; Chiazzari 2014) in order to test the hypothesis that the run represents the spawning migration of a genetically distinct sub-population of South African sardine (Fréon *et al.* 2010). van der Lingen (2010) reported significant differences in condition factor, body shape and vertebral count indicating that sardine caught off KwaZulu-Natal is morphologically different from west and south coast sardine. Chiazzari (2014) also reported significant morphological differences between sardine run stock and sardine from other regions in South Africa in head size and shape. Although

genetic analyses showed low levels of separation between adult sardine caught off KwaZulu-Natal and adult Western Cape sardine, larvae spawned off KwaZulu-Natal showed moderate levels of separation from the Western Cape stock. These results therefore support the hypothesis that the sardine run represents the reproductive migration of a genetically distinct sub-population of South African sardine.

Spatial management strategies for the purse-seine fishery are likely needed to appropriately deal with the possible existence of the two sardine stocks that it targets (Coetzee *et al.* 2008, de Moor & Butterworth 2009a, b). Subsequently, Hamman *et al.* (2012) and Pichegru *et al.* (2012) advised that a spatial management is required to help in the conservation of certain sardine predators such as the Cape cormorant *Phalacrocorax capensis* and the African penguin *Spenniscus demersus*, though their focus was more on the restriction of purse-seine fishing near the breeding areas of these predators. However, spatial management restrictions would make no difference if one fully mixed sardine population exists. On the contrary, if management strategies are based on a single sub-population when more than one sub-population actually exists, then one or more of the sub-population are likely to be over exploited if catches are not spatially and temporally spread (Kerr *et al.* 2014). In the light of this and given the increasing evidence of the existence of more than one sub-population of the South African sardine, it has become necessary to comprehensively assess if there are two interacting sub-populations – the putative western and southern stocks, in South African waters (de Moor & Butterworth 2015). In fisheries science, sub-populations are managed as units called ‘stocks’. Hence, in line with South African usage, these sub-populations will be referred to as western and southern sardine stocks for the remainder of this thesis.

Following the recognition of the possibility of the existence of western and southern sardine stocks, three main hypotheses for sardine stock structure were proposed. They included panmixia, two separate non-mixing stocks, and two interacting stocks (Smith *et al.* 2011). Initial data and assessment results not only facilitated the exclusion of the unrealistic hypothesis of two discrete stocks that do not mix, but also highlighted grey areas that needed further research. Consequently, the hypothesis of two mixing stocks - a western and

a southern stock, with the mixing component assumed to comprise recruits moving from west to south coast in appreciable numbers, was considered as the most likely (Smith *et al.* 2011, de Moor & Butterworth 2013, de Moor & Butterworth 2015, van der Lingen *et al.* 2015). Subsequent assessment of the South African sardine resource by de Moor and Butterworth (2015) compared results for the single-stock hypothesis with those that follow from the two-mixing-stock hypothesis and showed that the two-stock hypothesis was consistent with available data.

Previous investigations of the South African sardine stock structure (Wessels 2009, Idris 2010, Weston 2013, Ssempe 2013 and Hampton 2014) have so far typically involved analysis of a single or only a few different characteristics of sardine obtained from spatially and temporally disparate data sets. Some of these did not show spatial variability between the western and southern stocks (e.g. otolith shape indices, vertebral count, and gill raker spacing). This confirms that investigation of only a single characteristics may not necessarily provide evidence for the separation of fish stocks, even when true differences exist. Also, the uncertainty in these results suggest a number of possibilities namely: that there could be a weak stock separation between the regions (i.e. high overlap), or there could be no separation at all, or that the approaches used or data collected or sample size in these previous studies did not provide the power to identify differences. Hence, as recommended by Begg and Waldman (1999), in order to address this uncertainty, this chapter investigates the South African sardine multi-stock hypothesis by the integration of the results of multiple stock identification methods conducted on the same individual sardine samples using a Stock Differentiation index (SDI), as well as a multivariate analyses of data. Characteristics collected from each individual sardine analysed in this study include body shape measurements, branchial sieve (gills) morphometric and meristic data, vertebral count, otolith circularity and form factor, and parasite abundance because these approaches can be easily applied to fish samples, thereby making data collection easier. Although some of these characteristics (otolith shape indices, vertebral count, and gill raker spacing) were not able to discriminate between sardine from different regions, they were re-investigated in this study using a bigger sample size.

MATERIALS AND METHODS

Sample collection

Sardinops sagax samples were collected during research surveys conducted by the Department of Agriculture, Forestry and Fisheries (DAFF) in May 2013, November 2014, May 2014, as well as from commercial fishing vessels in 2014. Fish were bagged and labelled and stored in a freezer until they could be dissected in the laboratory. Sardine from two regions of the South African coast - the west and south coasts, were analysed for this study (Figure 2.1). Samples from the west coast were caught at locations west of Cape Agulhas and are assumed to be part of the putative western stock, while samples from the south coast were caught east of Cape Agulhas and are assumed to be part of the putative southern stock. Whilst sardine from the putative eastern stock may mix with southern stock fish following their return migration from KZN waters at the onset of summer, they were not considered to form part of this study.

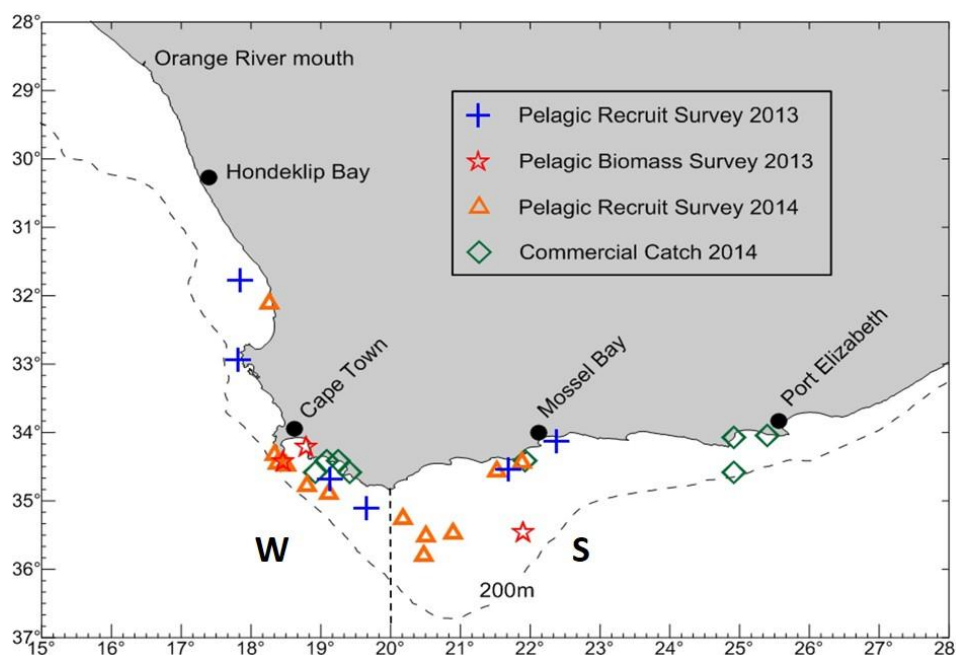


Figure 2.1. Sample locations of *Sardinops sagax* used in the present study. The dashed line at 20°E shows the separation of samples into two regions: W is the area extending from the Orange River to Cape Agulhas and S is the area east of Cape Agulhas, South Africa. The 200m depth contour is also indicated.

Processing of samples and data collection

After thawing in the laboratory, individuals were weighed to the nearest 0.01g and caudal length measurements (in cm) were taken using a measuring board.

Body shape

Each individual was photographed with the left side facing upward (Figure 2.2). Individuals damaged or broken during collection were excluded from study. A graduated rule and a waterproof label containing the fish's relevant information were placed above and below individuals respectively. Landmarks were marked with pins (Figure 2.2, Table 2.1).

Landmarks are positions of particular anatomical characters that represent discrete biological attributes (Adams *et al.* 2013). Photographs were taken using a Canon EOS 650D SLR camera mounted on a sturdy iron stand, 40cm above the photographic stage at a fixed focal length of 35mm.



Figure 2.2. Landmark locations used in the study.

Table 2.1. Description of 11 morphometric landmarks (adapted from Wessels 2009) shown in Figure 2.2 above.

Description	Landmark number
The tip of snout	1
The occipital ridge (first line on top of the head above the gill plate)	2
The front insertion point of the dorsal fin	3
The back insertion point of the dorsal fin	4
The upper insertion point of the caudal fin	5
The beginning of the caudal fin in the middle of the body (point at the end of the backbone where the expanded bones that support the caudal fin start)	6
The lower insertion point of the caudal fin	7
The front insertion point of the anal fin	8
The front insertion point of the pelvic fin	9
The front insertion point of the pectoral fin	10
The hinge of the jaw which aligns with the first line on the gill plate	11

After image acquisition, individuals were dissected to collect other organs of interest namely branchial sieve (gills), otoliths, eyes and vertebral columns. Adults were differentiated from immatures based on visual assessment of gonad stage. Immatures generally lacked gonads.

Branchial sieve morphometrics and meristics

Branchial sieves were dissected from individuals and stored in 70% alcohol. A Leica MZ9.5 dissecting microscope (Leica Microsystems, Taiwan) with a 12mm ocular micrometer fitted in the eye-piece was used for measurements. The micrometer was calibrated before use. The first gill arch from the left side of the branchial sieve was gently removed and measured under the dissecting microscope (King & Macleod 1976).

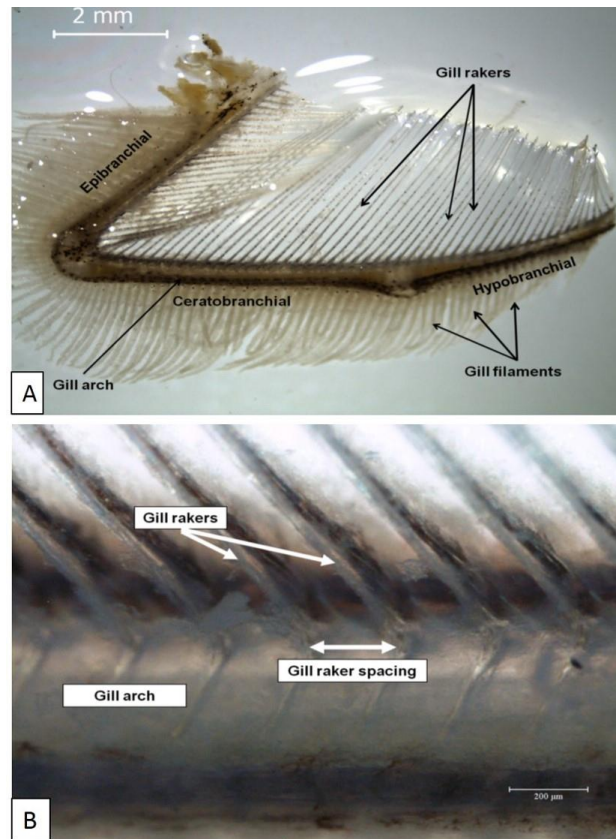


Figure 2.3. Image of the entire first gill arch of a sardine (A) and close-up of a portion of the gill arch showing how gill the various measurements were taken (B) (from Idris 2010).

The lengths of the upper and lower limbs of the gill arch were measured at 6.3x magnification and combined to give gill arch length (GAL). Data from the lower limb comprised measurements of the central (ceratobranchial) and lower (hypobranchial) sections of the gill arch while the upper limb is made up of the epibranchial section (Figure 2.3A). Gill raker spacing (GRS) was measured at 60x magnification. Five measurements of GRS from the middle of the gill arch were taken (following Tanaka *et al.* 2006) with measurements made from the mid-point of one gill raker to the next (Figure 2.3B). Mean measurements of GRS were used for analyses. The number of gill rakers (GRN) on the upper and lower limbs were counted at 10x and 20x magnifications (Figure 2.3B). The sum of the count data was used for analyses.

Otolith shape indices

Pairs of sagittal otoliths (Figure 2.4) were dissected from individuals, rinsed with fresh water, air-dried and stored in plastic tubes. Broken and vaterite otoliths were excluded from the dataset. Otoliths were positioned on a black background with their sulci facing down and rostrum to the left. Photographs were taken at 200x magnification using a Leica EZ4 HD photomicroscope (Leica Microsystems, Taiwan).

Otolith images were imported into tpsDIG version 2.17 (Rohlf 2005) which produces the outline of otoliths through an automatic outlining process. Based on a threshold defined by the user, the outline tool traces the white outline of otoliths against the contrasting dark background on which they are photographed. The otolith area and perimeter were automatically calculated from the outlines and are used to generate the shape indices - otolith circularity and otolith form factor (Tuset *et al.* 2003, Stransky & Macellan 2005).



Figure 2.4. Image of a pair of sagittal otoliths from sardine.

Otolith circularity (OCC) describes the extent to which otolith shape resembles a circle. A perfect circle is represented by the minimum value of 4π (12.57). As values increase, otolith

shape becomes increasingly elongated or oval. Otolith circularity is calculated with the formula below:

$$\text{Circularity} = \frac{\text{Perimeter}^2}{\text{Area}}$$

Otolith form factor (OF) measures how regular the perimeter of an otolith is. A value of 1 indicates a perfectly smooth edge while otoliths with irregular outlines have values less than 1. Otolith form factor is calculated with the formula below:

$$\text{Form factor} = \frac{4\pi * \text{Area}}{\text{Perimeter}^2}$$

‘Tetracotyle’ type metacercarian parasite abundance

Both eyes were dissected from each individual sardine and examined for the presence of the ‘tetracotyle’-type metacercaria parasite under a Leica EZ4D (Leica Microsystems, Taiwan) dissecting microscope at 350x magnification (Figure 2.5). Individuals were recorded as ‘infected’ or ‘non-infected’ and the number of metacercariae per individual were counted to determine parasite abundance, defined as the number of individual parasites in a single host whether infected or not (Bush *et al.* 1997).

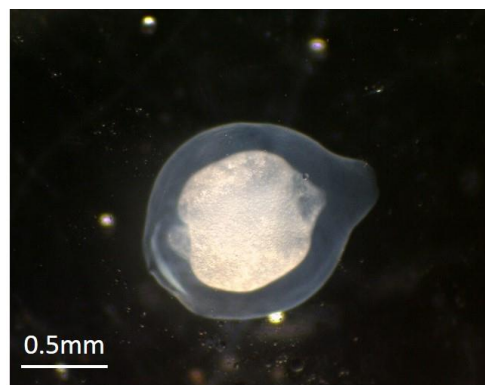


Figure 2.5. Image of a digenean ‘tetracotyle’-type metacercarian parasite from the eyes of the South African sardine, *Sardinops sagax*.

Vertebral count

Individuals were filleted to expose the vertebral column and the number of vertebrae was counted and recorded for use in meristic analyses.

DATA ANALYSES

Data was analysed in two ways in order to obtain as much information as possible. Univariate analyses were first done separately for each variable, followed by multivariate analyses to determine if one can discriminate between *a priori* defined groups of observations (that is specimens from the putative western and southern stocks) using the variables, and which variables are the best at discriminating between the two sardine stocks.

To discriminate between specimens from the putative western and southern stocks, all samples collected from each coast were combined and distinction was made between western and southern stocks. This was done to increase the total sample size because the scales of sampling in the two regions (western and southern) differed both spatially and temporally. However, samples were combined bearing in mind that season or year of collection could affect the outcome of analyses. Therefore, seasonal effect was tested for in the univariate analyses of certain variables such as otolith shape indices and tetracotyle abundance. This decision was based on previous studies (for otolith shape indices, Hampton 2014; for tetracotyle abundance, Weston 2013).

Univariate analyses

Body shape

Body shape was analysed using the geometric morphometrics (GM) technique (Corti 1993, Adams *et al.* 2013). Shape is defined as all geometric features of an object except its size, position and orientation (Dryden & Mardia 1998). Traditionally, morphometric studies involved the application of multivariate statistical analyses to sets of morphometric variables that were mostly linear distance measurements but sometimes ratios, counts, and angles (Marcus 1990, Adams *et al.* 2004). Because linear measurements are highly correlated with size (Bookstein *et al.* 1985), a lot of effort was channeled towards the

development of methods for size correction in order to extract size-free shape variables and reveal patterns of shape variation (Jungers *et al.* 1995). Even though many methods of size-correction were proposed, traditional morphometrics still experienced many challenges because of the following reasons (Adams *et al.* 2004). Firstly, different methods of size correction gave different results and did not agree with each other. Again, linear distances were not demarcated by homologous points. Also, two different shapes can yield the same set of distance measures because linear distances are not clearly defined, and as such the statistical power for differentiating shapes is greatly reduced. In addition, some aspects of shape are lost. Graphical representation of shape from linear distances is usually not possible because a set of linear distance measurements do not contain all the information needed to capture the geometry of the original object, that is, linear measurements do not preserve geometric relationships among variables.

These problems led to the development of an alternative method that captures the geometry of morphological structure known as Geometric Morphometrics (Corti 1993, Adams *et al.* 2013).

Geometric morphometrics is the statistical analysis of shape variation, and the covariation of shape with other variables, (Bookstein 1991) that combines rigorous statistical analysis with shape change visualization (Adams *et al.* 2013). Approaches in geometric morphometrics quantify shape after removing non-shape variation by analyzing the relative positions of landmarks using landmark-based methods; and outline curves and surfaces using outline-based methods (Jensen 2003, Adams *et al.* 2004, 2013). The removal of non-shape variation is achieved through a generalized Procrustes Analysis (GPA), also known as Procrustes superimposition. GPA superimposes the landmark configurations in specimens to a common coordinate system using a least-squares method (Gower 1975, Rohlf & Slice 1990, Dryden & Mardia 1998). Superimposition is achieved through an iterative procedure which removes variation in size by scaling each configuration so that it has centroid size of 1.0. Centroid size measures spread of landmark around their centroid or centre of gravity. Variation in position is removed by translating configurations to a common centre of gravity, and variation in orientation is removed by rotating configurations to minimize the total sum of squares deviation of their landmark coordinates to the average configuration.

After superimposition, Procrustes shape coordinates are produced. These shape coordinates are aligned such that they describe the location of each specimen in a curved space linked to Kendall's shape space (Rohlf 1999, Slice 2001). The aligned shape coordinates are then projected orthogonally into linear tangent space to generate Kendall's tangent space coordinates which are used for multivariate analyses of shape variation (Rohlf 1999, Kent & Mardia 2001).

All landmark configurations in a sample or dataset in a GPA are optimally aligned to the common target (average) configuration in a way that minimizes the sum of squared distances between these configurations and the average configuration. In this way, size, position and orientation are kept constant, and any remaining variation in landmark positions is attributed to shape change or shape difference which are visualized as relative displacements or shifts of landmarks. Differences in shape are evaluated based on the relative displacements of landmarks from the average configuration to any particular configuration, or relative displacements from one shape to a nearby shape in the shape space (Klingenberg 2013). All landmarks involved in Procrustes superimposition equally determine the alignment of each configuration relative to the average shape, and jointly contribute to total shape variation (Klingenberg 2013). This is because Procrustes superimposition spreads variation from landmarks with greater variation to landmarks with less variation (Chapman 1990, Rohlf & Slice 1990, Walker 2000). Thus, variation in each landmark position is in relation to the positions of the other landmarks. Also, the amounts of variation in individual landmark positions result from the superimposition of the entire landmark configurations and not the individual landmark themselves. Klingenberg (2013) pointed out that shape changes originate (in the tissue) between the landmarks, not in the landmarks themselves, and warned that one should resist the temptation of attributing shape changes to particular landmarks. Conclusively, in contrast to the traditional approach, geometric morphometrics preserves geometric information of shape difference, increases statistical power and provides a means of visualizing patterns of shape change (Rohlf & Marcus 1993).

Eleven landmarks were digitized in the same order on each sardine image using the imaging software, tpsDig version 2.17 (Rohlf 2005) in order to generate a set of two-dimensional

Cartesian coordinates which record the relative positions of landmarks. Raw coordinates were imported as TPS files into the freeware software, MorphoJ (Klingenberg 2011) for body shape analysis. After Procrustes superimposition, the resulting Procrustes shape coordinates were screened for outliers to determine if they followed a normal multivariate distribution. A covariance matrix was generated and shape coordinates were subjected to Principal Component Analysis (PCA). Shape variation in individuals from the putative western and southern *Sardinops sagax* stocks were tested using Procrustes ANOVA. To test for group differences, individuals were averaged within coasts (putative stocks) and the averaged data was used for testing differences ('stock' effect) using permutation tests and discriminant analysis. Variation in shape are visualized using wireframe graphs and thin plate spline deformation grids.

Branchial sieve morphometrics and meristics (gill arch length, gill raker spacing, number of gill rakers)

As fish size (length) has been shown to have an effect on branchial sieve variables in sardine (Idris *et al.* 2016), linear regressions were used to test for a size effect by plotting caudal length against gill arch length (GAL), gill raker spacing (GRS) and gill raker number (GRN). Additionally, a General Linear Model (GLM) that included fish length as a co-variate was used to test for differences in GAL, GRS and GRN values of fish from the putative western and southern stocks. Separate GLMs were run for GAL, GRS and GRN. Residuals were visually examined to determine normality and homoscedasticity, and data were log-transformed where necessary to normalize residuals. Significance levels were set at $P < 0.05$. The equation for the GLM for GAL had the form:

$$\text{Log (gill arch length)} = \beta_0 + \beta_1 * \text{stock} + \beta_2 * \text{fish length} + \beta_3 * (\text{stock} * \text{fish length}) + \text{error}$$

where β_0 = intercept; β_1 , β_2 , β_3 = the parameter estimates for the explanatory variables - putative stock, the covariate fish length, and their interaction, respectively.

The equation for the GLM for GRS and GRN had the form:

$$\text{Log (y)} = \beta_0 + \beta_1 * \text{stock} + \beta_2 * \text{fish length} + \beta_3 * (\text{stock} * \text{fish length}) + \text{error}$$

where y = gill raker spacing or the number of gill rakers; β_0 = intercept; β_1 , β_2 , β_3 = the parameter estimates for the explanatory variables - putative stock, the covariate fish length, and their interaction, respectively.

Otolith shape indices (form factor and circularity)

To determine if the left and right otolith differ in circularity and form factor, paired t-tests were performed on data from a subset ($n = 86$) of individuals. Both otolith shape indices are significantly correlated with fish size in sardine and other small pelagic fish off South Africa (Hampton 2014), and the relationship between fish length and each otolith shape index was tested here using linear regression. Separate multivariate General Linear Model (GLMs) that included fish length as a co-variate were used to test for differences in the form factor and circularity of otoliths of fish from the putative western and southern sardine stocks. Sex and Season (autumn or spring) were included in the GLM for adult fish only because these have been reported to have a significant effect on sardine otolith shape indices (Hampton 2014). A GLM run on immature sardine only tested the effect of putative stock and fish size on otolith shape indices. Residuals were visually examined to determine normality and homoscedasticity, and data were log-transformed where necessary to normalize residuals. Significance levels were set at $P < 0.05$. The equations for the GLMs had the forms:

Adult Sardinops sagax

$$\text{Log (y)} = \beta_0 + \beta_1 * \text{stock} + \beta_2 * \text{sex} + \beta_3 * \text{season} + \beta_4 * \text{fish length} + \text{error}$$

where y = otolith form factor or otolith circularity; β_0 = intercept; β_1 , β_2 , β_3 , and β_4 = parameter estimates for putative stock, sex, season and the covariate fish length, respectively.

Immature Sardinops sagax

$$\text{Log (y)} = \beta_0 + \beta_1 * \text{stock} + \beta_2 * \text{fish length} + \text{error}$$

where y = otolith form factor or otolith circularity; β_0 = intercept; β_1 , β_2 = parameter estimates for putative stock, and the covariate fish length, respectively.

‘Tetracotyle’ type metacercarian parasite abundance

‘Tetracotyle’ type metacercarian parasite abundance is significantly correlated with sardine size (Weston *et al.* 2015) and relationship between size and abundance was tested here using linear regression. Because the abundance data were not normally distributed and did not fulfil the assumptions of a parametric General Linear Model (GLM), a Generalized Linear Model was used to analyse metacercaria abundance in putative western and southern sardine. Putative stock and season (autumn or spring) were modelled as categorical variables while length was modelled as a continuous variable. The equation for the Generalized Linear Model had the form:

$$\text{Log (parasite abundance)} = \beta_0 + \beta_1 \cdot \text{stock} + \beta_2 \cdot \text{season} + \beta_3 \cdot \text{fish length} + \text{error}$$

where β_0 = intercept; β_1 , β_2 , β_3 = the parameter estimates for the explanatory variables - putative stock, season, and the covariate fish length, respectively.

A Poisson model was initially fitted to the data to identify over-dispersion. Results showed evidence of over-dispersion as variance was six times greater than the mean (over-dispersion = 6, $z = 4.7076$, $P = 1.254\text{e-}06$). Therefore, a negative binomial model, which is more appropriate for count data with high variance, was fitted to abundance data (Podolska & Horbowy 2003, Penston *et al.* 2008, Zuur *et al.* 2009; Weston *et al.* 2015). The final model was fitted using the log-link function:

$$\text{Log } (\mu) = x^T \beta$$

where μ = mean, $x^T \beta$ = linear function of the model coefficients and the covariates - stock, season and length (O’Neill & Faddy, 2003).

Significant levels were tested set at $P < 0.05$.

Vertebral count

The number of vertebrae a sardine has is set early in life and remains unchanged throughout the fish's life span (Barlow 1961). Hence, this parameter is not influenced by fish length. Because the vertebral count data were not normally distributed, differences in the number of vertebrae in individuals from the putative western and southern stocks was tested for using a Mann-Whitney test at significance level of $P < 0.05$.

Univariate data analyses were performed and graphs plotted using R software version 3.2.2 (R Core Team 2015) and Microsoft Excel 2013. Body shape analysis was done using MorphoJ software (Klingenberg 2011).

Integration of results of separate univariate analyses using the Stock Differentiation Index (SDI)

Based on the results of the separate univariate analyses performed on the different variables obtained from individuals from the two putative stocks, a Stock Differentiation Index (SDI) was developed. For each stock structure approach that showed significant differentiation between western and southern stock, a difference value (DV) of 1 was allocated. On the other hand, a DV of 0 was allocated to variables that did not show significant differentiation between fish from the two stocks. The SDI was calculated using the formula below:

$$\frac{\Sigma DV}{\text{count DV}}$$

where ΣDV = sum of the difference values and count DV = total number of stock structure approaches used.

SDI measures the relative difference among fish from putative stocks or different regions. A SDI value of 1 indicates maximum regional or stock difference whereas in contrast, a SDI

value of 0 indicates no difference between regions or stocks; hence, the null hypothesis of a single stock is accepted. Although sampling methodologies were the same for the two regions in the present study, the scales of sampling (spatial and temporal) as well as levels of sampling effort differed between the west and south coasts. Hence, only semi-quantitative deductions on stock structure can be made based on SDI. Welch *et al.* (2009, 2015) did not explicitly define a critical threshold for inferring separation among regions or stocks but only suggested $SDI > 0$ as evidence for the existence of separate stocks. However, Izzo *et al.* (2017) proposed thresholds that could be used to evaluate the strength of separation of groups to different stocks. Strong evidence for separation is inferred when SDI is > 0.66 , whilst a SDI ranging from $0.33 - 0.66$ is regarded as moderate evidence for separation and a $SDI < 0.33$ is considered weak evidence.

Multivariate analyses

Variables included in this analysis comprised the branchial sieve morphometrics and meristics, otolith shape indices, vertebral number and parasite abundance, all collected from the same set of fish. No body shape variables were included. As explained earlier, all landmarks contribute equally to total shape variation such that shifts or displacements at any landmark are relative to shifts or displacements at all other landmarks (Klingenberg 2013). This is because variation in shape is spread through all the landmarks during Procrustes superimposition (Chapman 1990, Walker 2000). Therefore, differences in shape are not attributed to a particular landmark or group of landmarks and hence could not be included in the multivariate analysis.

Multivariate analyses of data were performed in PRIMER version 6 (Clarke & Gorley 2006) and PERMANOVA+ for PRIMER (Anderson *et al.* 2008). To test for the effect of sampling year on data set, data was first partitioned into subsets (west 2013 vs west 2014, south 2013 vs south 2014, west 2013 vs south 2013, and west 2014 vs south 2014) and analysed.

Afterwards, the combined data set was analysed. Before analyses, raw data (measurements and counts) were log-transformed and were also normalized because they were on different measurement scales. An Euclidean distance resemblance matrix of normalized variables was generated and a PCA plot was produced in order to aid visualization of the relationship among samples. Homogeneity of multivariate dispersions of data obtained from the two

stocks were tested using the PERMDISP option in PERMANOVA+. After which, a single factor Permutational analysis of variance (PERMANOVA) was used to test the null hypothesis of no difference between individuals from the putative western and southern stocks in the variables under study. For PERMANOVA, 1000 permutations were used under a reduced model (terms in this model that are not of importance to the test are removed; Anderson *et al.* 2008).

To assess the *a priori* grouping of samples collected from the west and south coast as being representative of the putative western and southern stock respectively, classification analyses were performed using R software version 3.2.2 (R Core Team, 2015). The classification tools used were: Discriminant Analyses (Linear, LDA; and quadratic, QDA), Classification Tree Analysis (CTA), Artificial Neural Network (ANN), and Random Forest (RF). Prior to analyses, the body size effect for all variables was removed using the formula below:

$$V_{\text{std}} = \log(V) - \beta(\log(CL) - (\log(\overline{CL})))$$

where V_{std} = standardized variable; $\log(V)$ = log of the variable; β = slope of regression of the variable to the caudal length, CL; $\log(CL)$ = log of the caudal length; $\log(\overline{CL})$ = mean of the log of caudal length for all fish.

Discriminant Analyses (DA)

As explained by Zuur *et al.* (2007), DA extracts usually two axes that maximize the separation of *a priori* known groups. However, for DA to produce valid results, data must meet a number of assumptions. These assumptions include a multivariate normality of variables within each group. This means that variables must be approximately normally distributed within each group as normality of distribution is required for hypothesis testing. The homogeneity of variance across groups is also assumed. The spread (variance) of each variable should be approximately the same in each group. DA uses pooled variance, hence this assumption is essential for DA's validity. However, a quadratic discriminant function can be used in cases of violation of homogeneity. Additional assumptions include similar covariance matrices (relationship between variables must be similar for the different

groups), independent observations of variables with data collected on a continuous scale and nominal variables avoided. There should be linear relationships between variables but these should not be 100% collinear. And finally, there should be at least two observations per group but preferably four to five times the number of variables.

DA is similar to PCA because both methods aim to find the optimal rotation during projection of observations. However, PCA differs from DA by extracting a series of orthogonal axes that collectively explain most of the variation in the data.

Classification Tree Analysis (CTA)

CTA models are better at handling non-linear relationships and high-order interactions between explanatory variables than regression, GLM, GAM or discriminant analysis. CTA results are not affected by missing values in predictor and response variables, or by monotonic transformation of variables. Hence, their interpretation is relatively easy (De'ath & Fabricus 2000, Zuur *et al.* 2007, James *et al.* 2014). Based on a combination of predictor variables, variation in a response variable is examined by the repetitive partitioning of data into homogenous groups by algorithm. The model formulates splitting rules for each value of predictor variable from which it creates candidate splits. The candidate split with the smallest misclassification error rate is selected and used to split data into subgroups. This procedure is repeated until the algorithm reaches the terminal node, that is, until no substantial reduction in misclassification error rate is obtained or until the number of observations in the sub-groups are too small for splitting. To avoid over-fitting, tree-based sets of criteria are run using cross-validated error rates on the training data. Optimal size of the tree is determined through cross-validation and is selected based on the complexity parameter, *cp*, that gives the lowest cross-validation error. Details on the method are given in De'ath & Fabricus (2000), Zuur *et al.* (2007), and James *et al.* (2014).

Artificial Neural Networks (ANNs)

As explained by Dayhoff and DeLeo (2001), ANNs are biologically inspired computational methodologies used to perform multifactorial analyses. They are nonlinear statistical data modeling tools that show the complex relationships or look for patterns between inputs and outputs. ANN models resemble the biological neuron network and consists of computing nodes interconnected by weighted connection lines whose weights are adjusted by input data during a 'training' process. Using training algorithms, input data are repetitively submitted along with known output to the network. Weights are increasingly adjusted to move the network's output closer to the desired values, that is, to minimize the error between the answer given by the network and the true answer. ANNs linearly combine all the input variables in the data into a derived value which undergoes non-linear transformation in the 'hidden layer object' or node. There are multiple hidden layer objects within the ANN in which different non-linear transformations are conducted, with each node/neuron having its own linear combination and derived value. In this way, the strength of classification of ANN is increased. If training is successful, ANNs can perform analytical tasks such as predictions, classification, and function approximations, as well as pattern recognition and completion from input data. Hence, ANNs can be used to solve major problems in different application areas. Details of the method are given in Dayhoff and DeLeo (2001), and Zuur *et al.* (2007).

Random Forest (RF)

RF is an ensemble learning method used for classification, regression, ranking important variables and other tasks in predictive statistics (Ho 1995, 1998, Breiman 2001). Ensemble methods are learning methods that use multiple learning algorithms to make better predictions. Random Forest is an important classification tool for solving problems that have a large number of correlated predictors (van der Vyver *et al.* 2015). Using the training data, RF constructs multiple decision trees based on a random subset of predictors that de-correlates the multiple trees constructed. In this way, variance is reduced, trees are more reliable and classification accuracy is increased (Ho 1998, Breiman 1996, Hastie *et al.* 2009). The output of Random Forest is the class that represents the mode of the classes

(classification) or the mean prediction (regression) of the individual decision trees (Ho 1995, 1998).

Model selection and performance

Each of the five models were trained on the same subset of data (training set). Then, their performance measured by how well they classified individuals from the two putative stocks were assessed on both the training and the validation sets. The stepwise selection approach was used in model selection for all model types used in the study. Four variable selection algorithms (forward, backward, both direction and hills climbing search) implemented by the Fselector R package (Romanski & Kotthoff 2016) were used. Overall classification accuracy was set to a cut-off threshold of 1%. Improvement in overall classification accuracy which results from addition of predictors is used to determine the relative importance of predictors in the model. For each model, the best variable sets were those selected at least twice by the algorithms. The combinations of variables associated with best performance were subsequently used as variable sets for the final model.

Predictive performance of models was evaluated using the 'Leave Group Out Cross Validation' (LGOCV) which involves the repeated training of model on a subset of data and the subsequent use of the model to evaluate the remaining subset (Kuhn & Johnson 2013). Predictive performance is expressed as the mean $\pm$ SD of the predictive accuracy from the LGOCV.

For multivariate analyses, PRIMER version 6 (Clarke and Gorley 2006) and PERMANOVA+ for PRIMER (Anderson, Gorley and Clarke 2008), as well as R version 3.2.2 (R Core Team 2015) were used.

RESULTS

A total of 1567 individuals were used in this study. Of these, 950 were from the putative western stock, obtained from 17 samples, while 617 were from the putative southern stock, collected from 13 samples (Tables 2.2 and 2.3). Caudal lengths of individuals from the

putative western stock ranged from 6.0cm to 20.2cm, while those of individuals from the putative southern stocks ranged from 9.0cm to 20.5cm (Figures 2.6 and 2.7). Individuals from both putative stocks had a comparable size range, although the putative western stock had more juveniles with caudal length of < 9.0cm. After image acquisition, the removal of data from damaged and broken organs (e.g. broken otoliths, vaterite otoliths, damaged gills) and of individual fish with incomplete data, the resulting dataset consisted of 1550 individuals – 933 from the putative western stock and 617 from the putative southern stock.

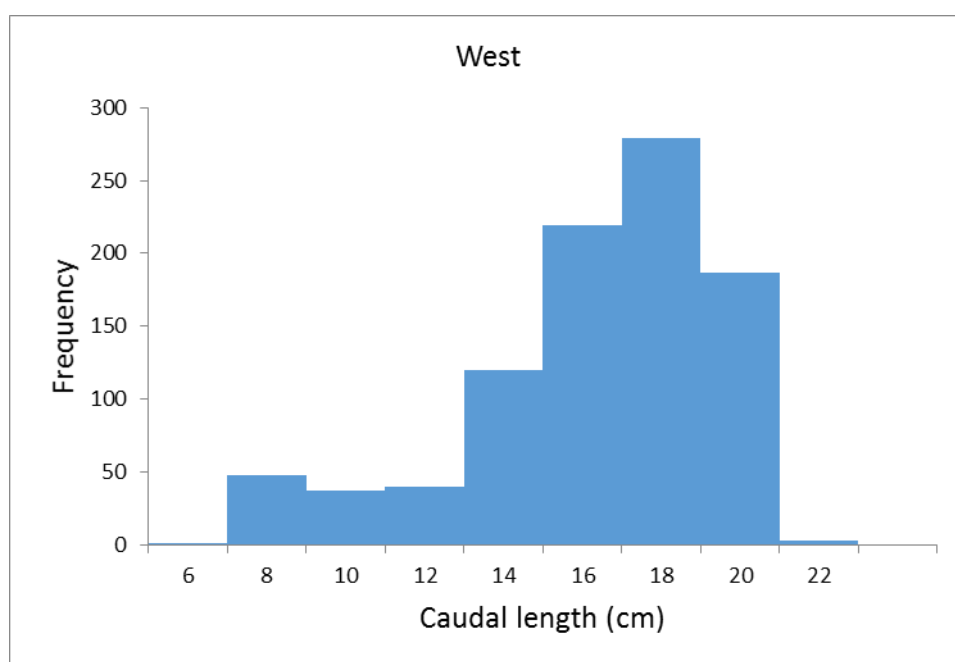


Figure 2.6. Frequency distribution of caudal length for *Sardinops sagax* from the putative western stock off the coast of South Africa in 2013 and 2014 used in this analysis.

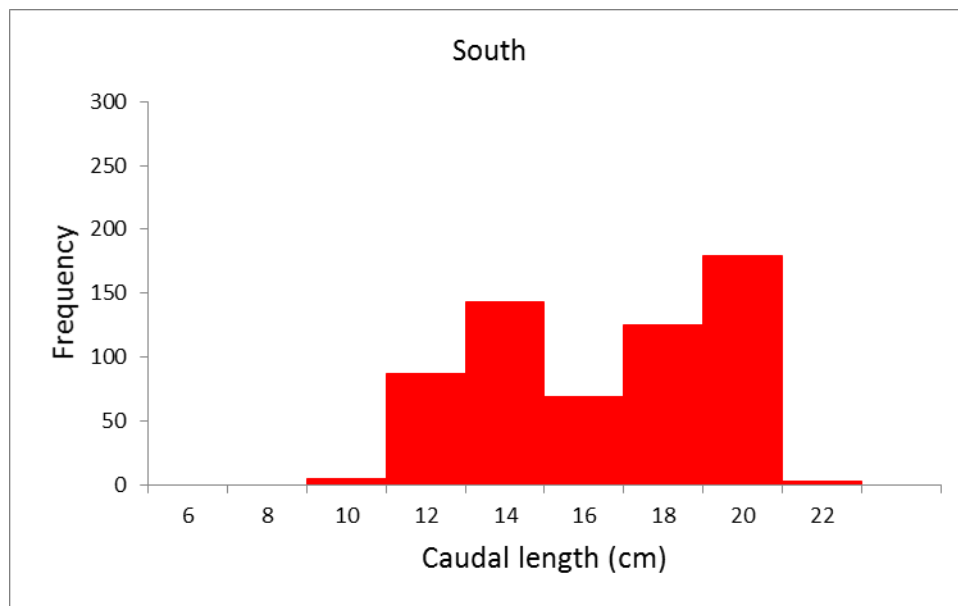


Figure 2.7. Frequency distribution of caudal length for *Sardinops sagax* from the putative southern stock off the coast of South Africa in 2013 and 2014 used in this analysis.

Table 2.2. Descriptive statistics of *Sardinops sagax* samples from the putative western stock collected in 2013 and 2014 and used in this analysis. Standard errors are shown with mean caudal length and mean weight values. Abbreviations: PRS, Pelagic Recruitment Survey; PSB, Pelagic Spawner Biomass Survey; CS, samples obtained from commercial fishing vessels; n, sample size; Lat., Latitude; Long., Longitude; Jan., January; Nov., November.

S/N	Survey	Date of sampling	Coast	Lat.	Long.	n	Mean caudal length, cm ($\pm$ SE)	Caudal length range (cm)	Mean weight, g ($\pm$ SE)	Weight range (g)
1	PRS 2013	May 2013	W	-31,7745	17,8485	56	7.10 $\pm$ 0.36	6.2 - 7.8	3.98 $\pm$ 0.71	2.0 - 6.0
2	PRS 2013	June 2013	W	-32,933	17,8215	49	9.00 $\pm$ 0.99	7.7 - 12.5	8.91 $\pm$ 3.73	5.5 - 22.5
3	PRS 2013	June 2013	W	-34,6867	19,1238	51	14.53 $\pm$ 0.67	13.4 - 16.0	39.42 $\pm$ 0.67	27.5 - 61.5
4	PRS 2013	June 2013	W	-35,1115	19,6475	192	15.86 $\pm$ 1.35	11.5 - 19.8	50.70 $\pm$ 13.79	13.5 - 91.5
5	PSB 2013	Nov. 2013	W	-34,425	18,4542	52	13.60 $\pm$ 0.71	12.0 - 15.2	30.34 $\pm$ 5.81	18.0 - 43.5
6	PSB 2013	Nov. 2013	W	-34,2147	18,7838	50	18.22 $\pm$ 0.86	16.5 - 20.2	80.32 $\pm$ 10.79	59.5 - 99.0
7	PRS 2014	May 2014	W	-32,12	18,27	65	16.99 $\pm$ 1.30	13.7 - 20.0	65.02 $\pm$ 16.01	28.0 - 105.0
8	PRS 2014	May 2014	W	-34,3283	18,34	39	15.54 $\pm$ 1.87	12.0 - 18.5	49.27 $\pm$ 17.14	18.5 - 83.5
9	PRS 2014	May 2014	W	-34,4623	18,3802	60	17.59 $\pm$ 1.33	15.4 - 20.2	70.75 $\pm$ 17.18	48.5 - 106.0
10	PRS 2014	May 2014	W	-34,4827	18,5092	32	13.93 $\pm$ 1.38	11.6 - 16.2	39.78 $\pm$ 11.80	19.5 - 60.0
11	PRS 2014	May 2014	W	-34,4827	18,5092	29	17.42 $\pm$ 0.76	15.5 - 19.0	75.86 $\pm$ 10.11	56.0 - 98.5
12	PRS 2014	May 2014	W	-34,789	18,794	31	15.22 $\pm$ 1.25	13.0 - 17.6	49.71 $\pm$ 12.87	25.5 - 84.5
13	PRS 2014	May 2014	W	-34,903	19,1165	47	12.01 $\pm$ 0.70	10.0 - 13.3	21.91 $\pm$ 3.84	13.0 - 34.5
14	CS 2014	Jan. 2014	W	-34.42	19.08	27	19.17 $\pm$ 0.61	18.8 - 20.2	89.93 $\pm$ 10.19	72.8 - 112.5
15	CS 2014	Jan. 2014	W	-34.58	18.92	53	18.77 $\pm$ 0.63	17 - 19.5	93.78 $\pm$ 8.60	72.2 - 112.2
16	CS 2014	March 2014	W	-34.42	19.42	42	18.95 $\pm$ 0.60	18.0 - 20.0	95.83 $\pm$ 8.99	75.0 - 107.5
17	CS 2014	March 2014	W	-34.42	19.42	82	16.48 $\pm$ 0.77	14.5 - 17.6	64.77 $\pm$ 9.58	44.5 - 87.5
OVERALL						950	15.41 $\pm$ 3.21	6.0 - 20.2	53.47 $\pm$ 27.71	2.0 - 119.0

Table 2.3. Descriptive statistics of *Sardinops sagax* samples from the putative southern stock collected in 2013 and 2014 and used in this analysis. Standard errors are shown with mean caudal length and mean weight values. Abbreviations: PRS, Pelagic Recruitment Survey; PSB, Pelagic Spawner Biomass Survey; CS, samples obtained from commercial fishing vessels; n, sample size; Lat., Latitude; Long., Longitude; Jan., January; Nov., November.

S/N	Survey	Date of sampling	Coast	Lat.	Long.	n	Mean caudal length (cm)	Caudal length range (cm)	Mean weight (g)	Weight range (g)
1	PRS 2013	June 2013	S	-34.539	21.6815	50	18.41 ± 0.91	15.8 - 20.0	82.42 ± 12.60	48.0 - 112.5
2	PRS 2013	June 2013	S	-34.1233	22.3695	81	17.41 ± 1.20	15.5 - 19.4	68.68 ± 13.43	43.5 - 104.5
3	PBS 2013	Nov. 2013	S	-35.47	21.8933	103	13.20 ± 1.32	11.0 - 17.5	29.69 ± 8.20	16.0 - 75.0
4	PRS 2014	June 2014	S	-35.26	20.17	33	14.41 ± 1.13	11.8 - 16.0	40.42 ± 9.75	16.5 - 55.5
5	PRS 2014	June 2014	S	-35.8046	20.4768	28	11.01 ± 0.60	9.0 - 12.2	16.59 ± 3.24	8.5 - 23.5
6	PRS 2014	June 2014	S	-35.5188	20.5032	15	10.65 ± 0.47	9.8 - 11.3	13.97 ± 2.24	10.5 - 15.0
7	PRS 2014	June 2014	S	-35.4786	20.8925	62	11.83 ± 0.90	10.0 - 14.3	21.65 ± 6.06	11.0 - 39.5
8	PRS 2014	June 2014	S	-34.4493	21.8812	30	14.75 ± 0.51	14.0 - 15.6	42.92 ± 5.80	33.5 - 53.5
9	PRS 2014	June 2014	S	-34.5697	21.5258	27	12.37 ± 0.61	11.5 - 14.0	23.89 ± 3.64	19.5 - 34.5
10	CS 2014	Jan. 2014	S	-34.58	24.92	50	18.64 ± 0.84	18.1 - 20.5	96.07 ± 12.76	75.0 - 122.2
11	CS 2014	March 2014	S	-34.08	24.92	29	18.91 ± 0.57	17.7 - 19.8	104.74 ± 9.45	81.3 - 117.7
12	CS 2014	May 2014	S	-34.42	21.92	71	18.62 ± 0.72	16.8 - 20.2	97.54 ± 13.07	72.5 - 128.5
13	CS 2014	May 2014	S	-34.04	25.39	40	16.77 ± 0.50	16.3 - 17.9	74.45 ± 7.59	62.0 - 86.5
OVERALL						617	15.56 ± 2.94	9 - 20.5	57.81 ± 32.37	8.5 - 133.5

Univariate analyses

Body shape analysis

1567 images of individual sardine were digitized using tpsDig imaging software (Rohlf 2005) and raw coordinates were imported into MorphoJ software (Klingenberg 2011) and subjected to Procrustes superimposition and Principal Component Analysis (PCA). A preliminary screening indicated the presence of ten outliers (results not shown) which were excluded from the dataset to achieve a multivariate normal distribution. The final dataset containing 1557 individuals, 940 from the putative western stock, and 617 from the putative southern stock was used for body shape analysis.

PCA scatterplots (PC1 vs PC2, PC3 vs PC4) show an overlap of individuals from the putative western and southern stocks (Figures 2.8 and 2.9). However, extremes of the principal components suggest a large amount of variation in the samples. The first four principal components (PCs) account for approximately 70% of variation in body shape, with PC1 accounting for 37.28% of the total variation. PC1 is related to changes in the curvature of the body from concave (negative extreme) to convex (positive extreme; Figure 2.8A). PC2 accounted for 14.06% of shape variation and is primarily related to the expansion and contraction of the body width on the positive ('fat' fish) and negative ('slender' fish) extremes respectively (Figure 2.8B). PC3 and PC4 are characterized by localized shape changes. The shape variation accounted for by PC3 (9.94% of total variation) indicates localized changes in the head and tail. The positive extreme shows a fish with a shorter head and a slightly longer tail and the reverse trend is reflected in the negative extreme (Figure 2.9A). PC4 is primarily related to difference in the width of the ventral aspect of the body, from the pelvic fin to the insertion of the caudal fin. This change is graphically shown as a constriction in this region, giving the fish the appearance of a 'pot belly' (Figure 2.9B).

Results of Procrustes ANOVA performed on all sardine shapes from putative western and southern stocks showed statistically significant variation ($p < 0.0001$) in body shape with 'stock' explaining a small but statistically significant 2.19% of total sum of squares (Table 2.4).

Table 2.4. Shape variation. Abbreviations: SS, sum of squares; MS, mean sum of squares; df, degrees of freedom; P(param.), p-value for parametric test.

Effect	% SS explained	SS	MS	df	F	P(param.)
Putative stock	2.19	0.032	0.001794	18	35.11	<0.0001
Residual		1.430	0.000051	27990		

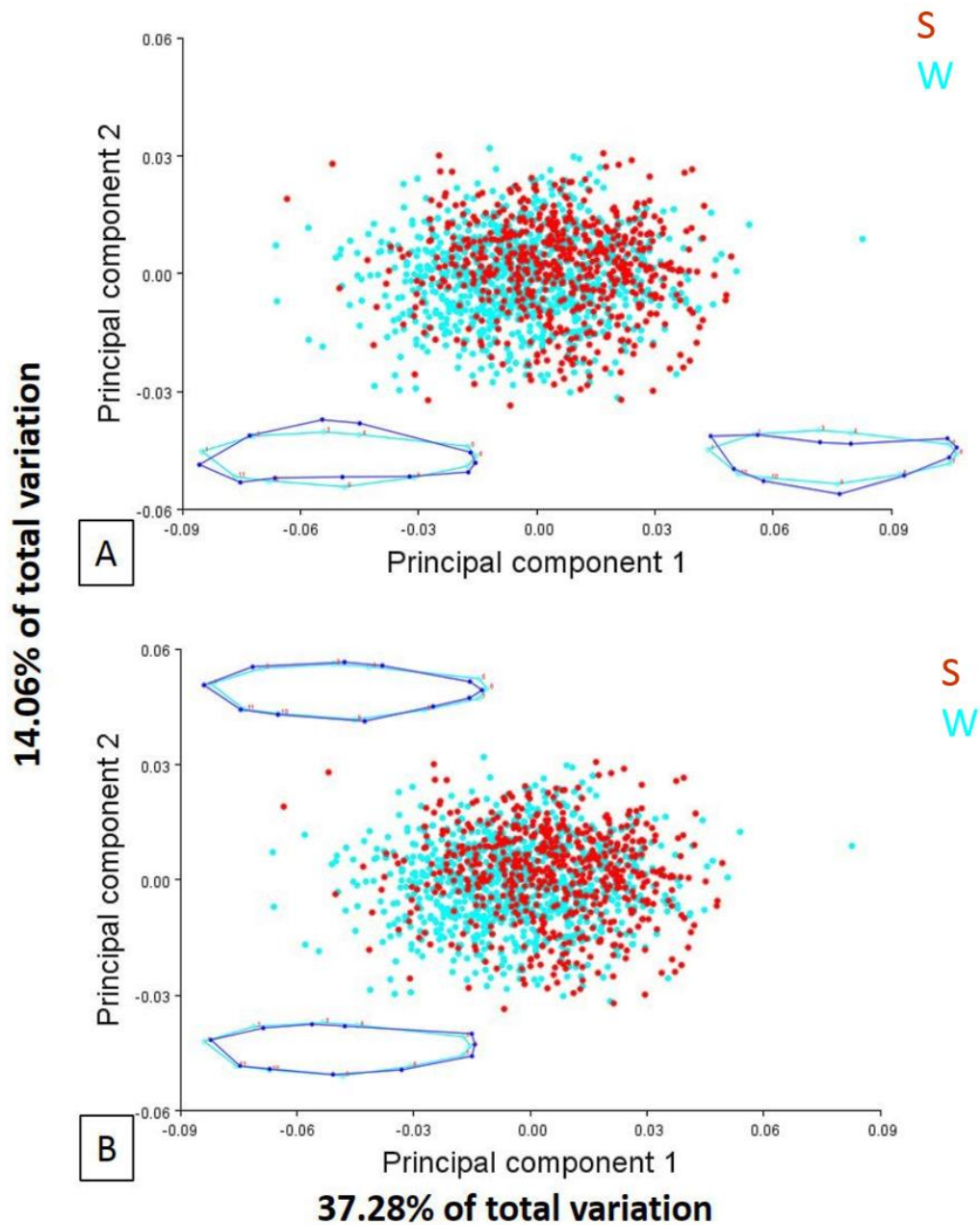


Figure 2.8. PCA plots of PC1 (A) vs PC2 (B) which jointly explain 51.34% of shape variation for sardine from the putative western (blue symbols) and southern (red symbols) stocks. Shape changes are visualized with wireframe graphs using a scaling factor/magnification (simple scalar multiplication of shape differences between a starting shape and its target shape) of 0.09 for both negative and positive extremes. Light blue colour represents the starting shape while deep blue colour represents the target (average) shape.

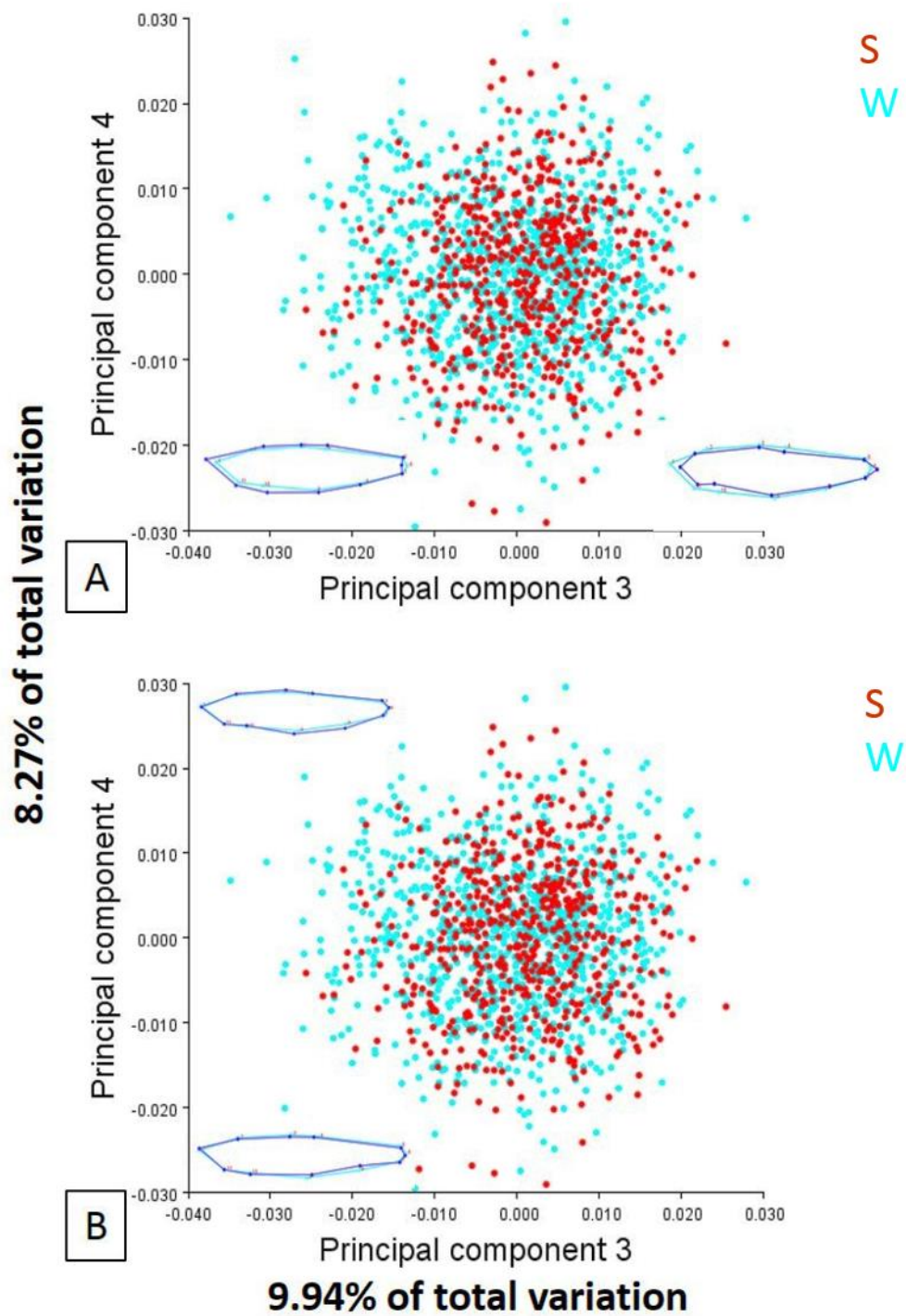


Figure 2.9. PCA plots of PC3 (A) vs PC4 (B) which jointly explain 18.21% of shape variation. Shape changes are visualized with wireframe graphs using a scaling factor/magnification (simple scalar multiplication of shape differences between a starting shape and its target shape) of 0.04 for both negative and positive extremes. Light blue colour represents the starting shape while deep blue colour represents the target (average) shape.

Similar to the PCA, the results from the discriminant function analysis reveal a lot of overlap in the range of shapes of sardine from the putative western and southern stocks (Figure 2.10). Analysis involved only two groups, hence, there is only a single axis of shape change. Discriminant scores are presented as histograms with bars plotted proportional to their frequency. The body shapes of the mean fish from the two putative stocks are significantly different from each other (Mahalanobis distance = 0.817, $P(\text{perm.}) < 0.0001$) (Table 2.5). The mean fish from the putative southern stock has a fairly convex shape compared to the concave shape of the putative western stock (Figure 2.10). Shape differences are magnified ten times to aid visualization. The cross-validation table shows that body shape is fairly accurate (62.34% and 66.13% for putative western and southern stocks respectively) in predicting sardine stock identity (Table 2.6).

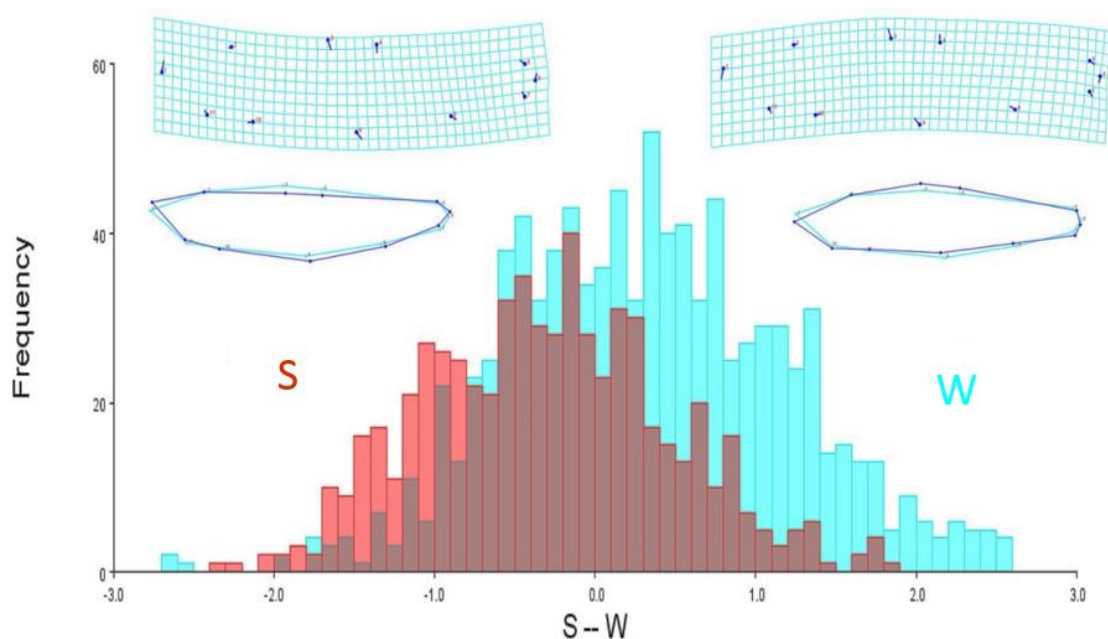


Figure 2.10. Discriminant analysis of sardine *Sardinops sagax* from putative western and southern stocks using body shape after averaging within stocks. Discriminant scores are predicted by a jack-knife (leave-one-out) cross-validation and frequencies are shown using histograms. Mean shapes of fish from the putative western and southern stocks are

visualized using wireframe graphs and transformation grids magnified 10 times. Light blue represents the starting shape while deep blue represents the target (average) shape.

Table 2.5. Differences between mean body shape after averaging individuals within stock (putative western and southern stocks). Permutation tests done with 1000 random permutations. Abbreviations: P, p-value for parametric test; P(perm), p-value for permutation tests.

	T-square	P(param.)	Mahalanobis distance	P(perm.)	Procrustes distance	P(perm.)
Shape	248.652	<0.0001	0.817	<0.0001	0.0093	<0.0001

Table 2.6. Jack-knife cross-validated classification table for all *Sardinops sagax* shapes from putative western and southern stocks used in this study.

Shape (putative stock)	West	South
Western	62.34%	37.66%
Southern	66.13%	33.87%

Branchial sieve morphometrics and meristics

S. sagax specimens from the west and south coast had similar branchial sieve mean measurements, though individuals from the putative southern stock had slightly higher mean values (Table 2.7).

Table 2.7. Mean (+ SE) measurements of branchial sieves of *Sardinops sagax* from putative western and southern stocks off the South African coast in 2013 and 2014. Abbreviation: GAL, gill arch length; GRS, gill raker spacing; GRN, number of gill rakers.

Stock	Sample Size	Range (cm) Caudal length	GAL (mm)	GRS (mm)	GRN (number)
Western	933	6.0 – 20.2	47.23 ± 10.88	0.15 ± 0.02	157.37 ± 24.50
Southern	617	9.0 – 20.5	47.55 ± 9.96	0.16 ± 0.04	158.33 ± 20.06

Gill Arch Length (GAL)

Sardinops sagax specimens from the putative western stock had gill arch lengths (GAL) ranging from 15.36mm to 70.56mm (Figure 2.11). Mean GAL per sample for 17 samples collected from the west coast ranged from 19.54 ± 1.29 mm to 62.16 ± 3.26 mm (Figure 2.13). Individuals from the putative western stock had an overall mean GAL of 47.23 ± 10.88 mm (Table 2.7). On the other hand, specimens collected from the south coast had GALs ranging from 25.28mm to 67.84mm (Figure 2.12). Mean GAL per sample for 13 samples collected from the south coast ranged from 31.97 ± 1.82 mm to 60.84 ± 3.37 mm (Figure 2.14). Individuals from the putative southern stock had an overall mean GAL of 47.55 ± 9.96 mm (Table 2.7).

The relationship between caudal length and $\log_{10}$ transformed GAL was found to be highly significant and positive in both western and southern stocks (Figure 2.15), indicating that gill arch length increases as fish get larger. The relationship between the two variables did not differ significantly between the two coasts. Significant interaction was found between stock and caudal length indicating that the relationship between fish length and GAL is different for each stock. After accounting for size, a significant difference was found in GAL between individuals from the putative western and southern stocks. GAL was longer in individuals from the southern stock ($F_{(1,1541)} = 21.98$, $P < 0.001$) compared to those from the western stock (Table 2.8).

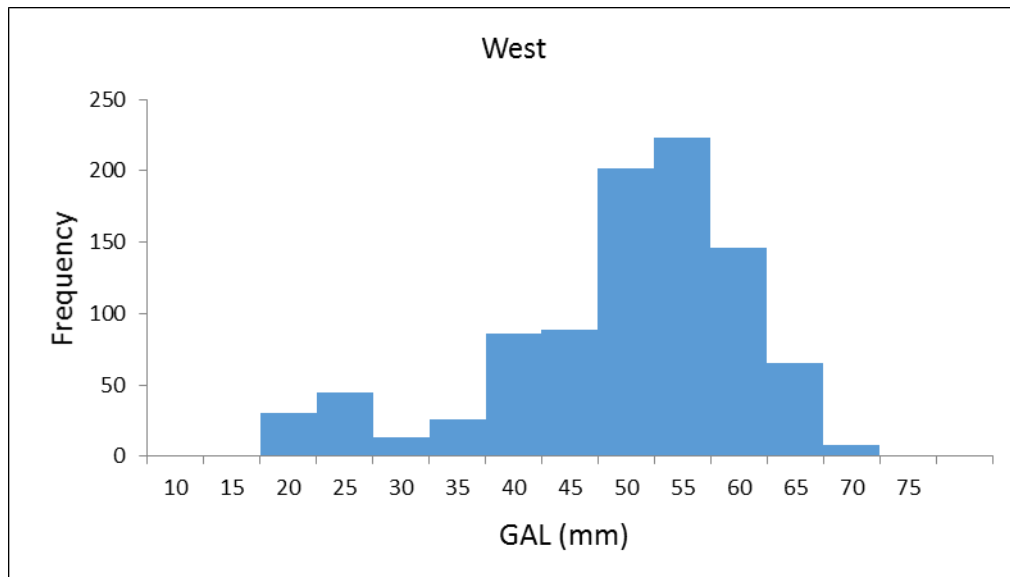


Figure 2.11. Frequency distribution of gill arch length (GAL) for *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

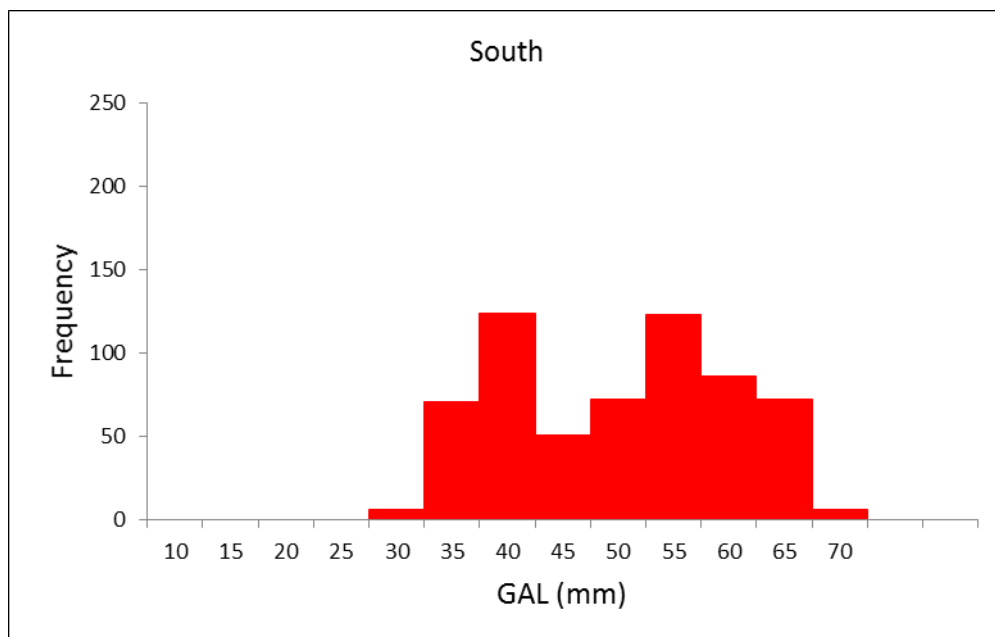


Figure 2.12. Frequency distribution of gill arch length (GAL) for *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

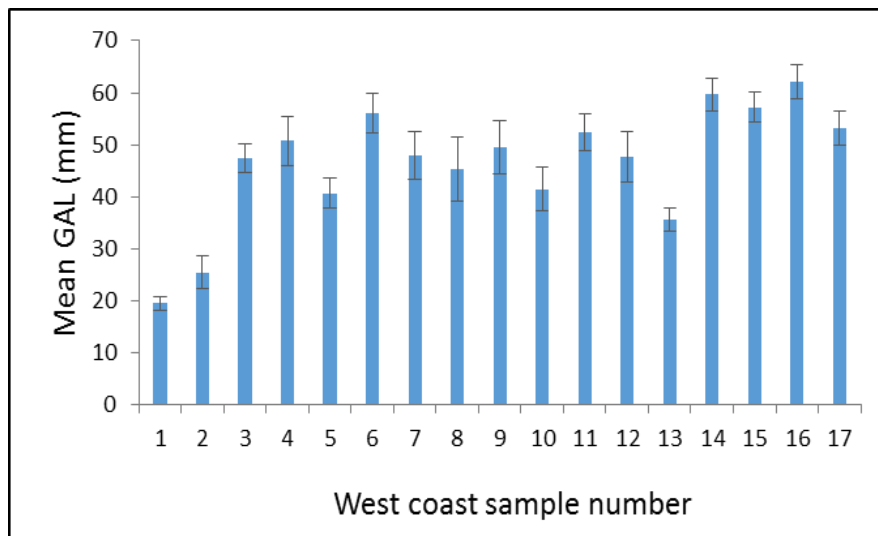


Figure 2.13. Mean gill arch length (GAL) per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

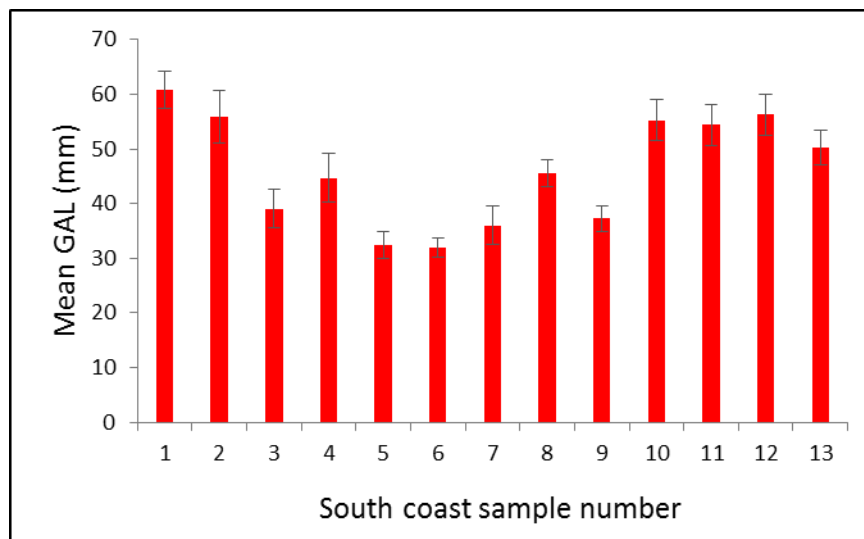


Figure 2.14. Mean gill arch length (GAL) per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

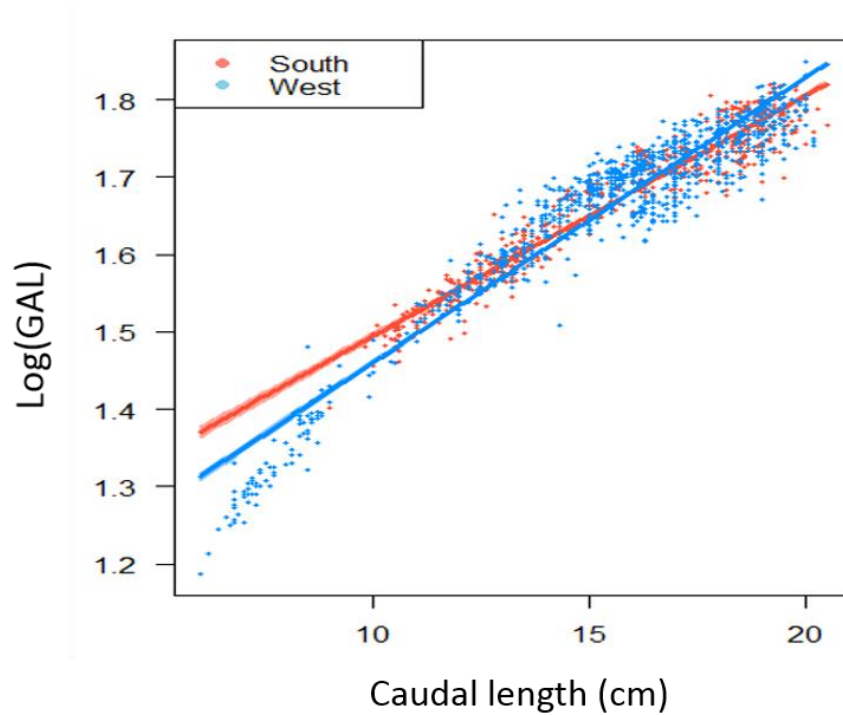


Figure 2.15. Linear relationship between caudal length and $\log_{10}$ -transformed gill arch length (GAL) of *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

Table 2.8. GLM analysis. Difference in $\log_{10}$ -transformed gill arch length (GAL) between *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock	South	1.18	0.0072	1	0.02	21.98	< 0.001
	West	1.09	0.0089				
Caudal length		0.03	0.0005	1	18.0	16647.62	< 0.001
Stock:caudal length		0.006	0.0006	1	0.1	104.75	< 0.001
Residuals				1541	1.7		

Gill raker spacing (GRS)

For *S. sagax* specimens from the putative western stock, gill raker spacing (GRS) ranged from 0.086mm to 0.23mm (Figure 2.16). Mean GRS per sample ranged from 0.13 ± 0.02 mm to 0.17 ± 0.02 mm (Figure 2.18). On the whole, specimens from the putative western stock had a mean GRS of 0.15 ± 0.02 mm (Table 2.7). For specimens from the putative southern stock, GRS ranged from 0.082mm to 0.296mm (Figure 2.17). Mean GAL per sample collected from the south coast ranged from 0.12 ± 0.02 mm to 0.23 ± 0.03 mm (Figure 2.19). An overall mean GRS of 0.16 ± 0.04 mm was observed from individuals from the putative southern stock (Table 2.7).

Caudal length was found to have highly significant and positive relationship with GRS in sardine from both western and southern stocks (Figures 2.20). This indicates that gill raker spacing increases as a fish gets larger. The relationship between gill raker spacing and fish size differed significantly between sardine from the west and south coast, with southern stock fish showing a greater rate of increase in GRS as fish size increases compared to western stock fish.

After accounting for size, no significant difference ($F_{(1,1541)} = 0.65$, $P = 0.42$) was found in GRS between individuals from the putative western and southern stocks (Table 2.9). Both caudal length and the stock:caudal length interaction had a significant impact on GRS, with the significant interaction term inferring the difference in the relationship between GRS and fish size for sardine off the two coasts.

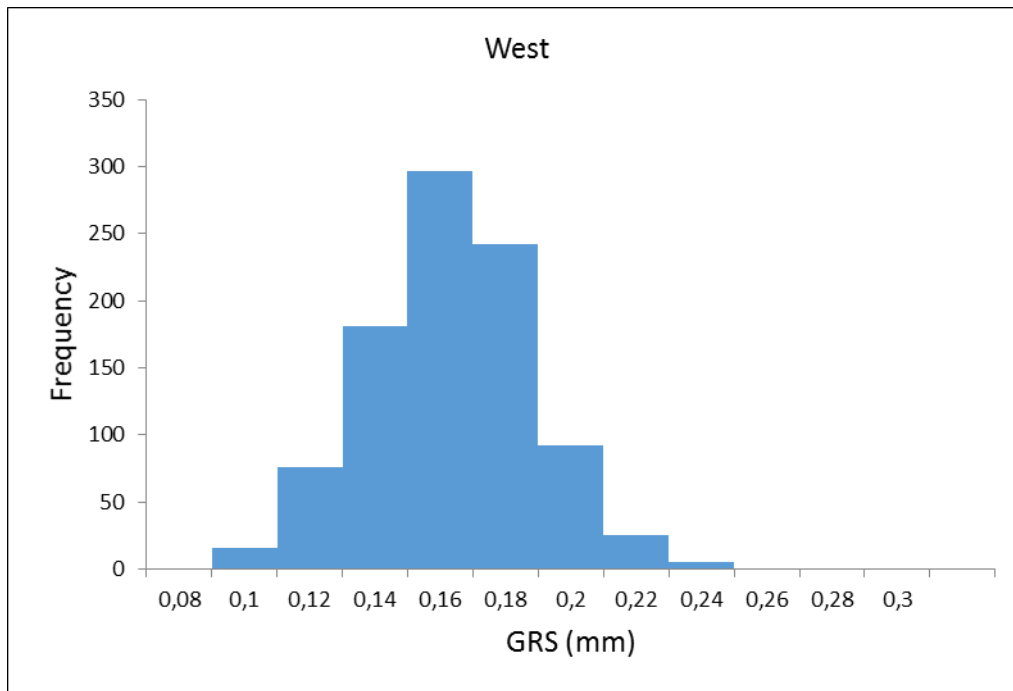


Figure 2.16. Frequency distribution of gill raker spacing (GRS) for *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

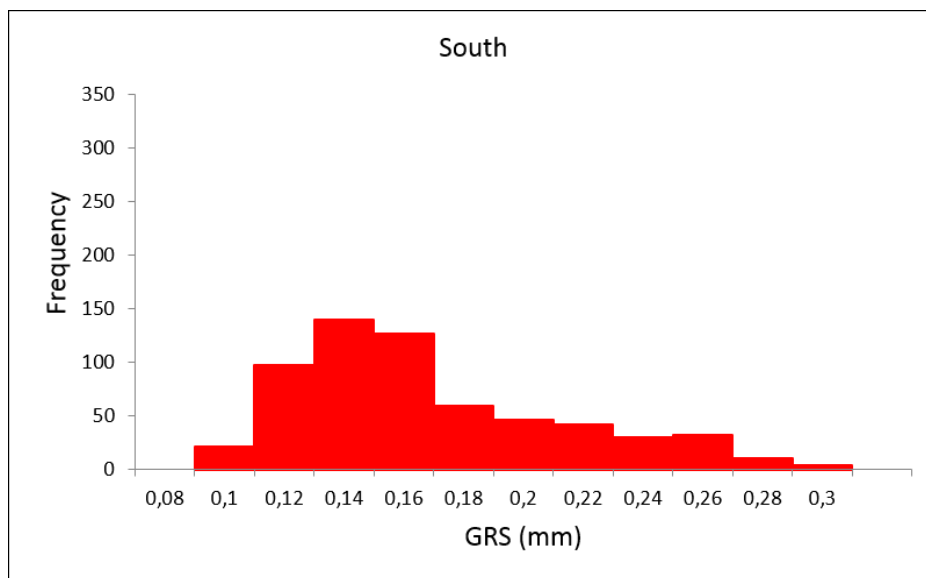


Figure 2.17. Frequency distribution of gill raker spacing (GRS) for *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

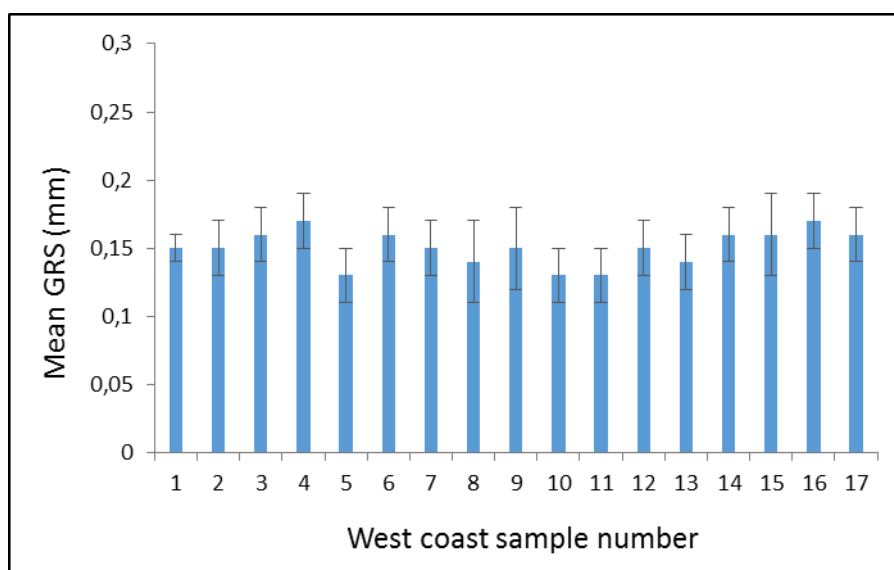


Figure 2.18. Mean gill raker spacing (GRS) per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

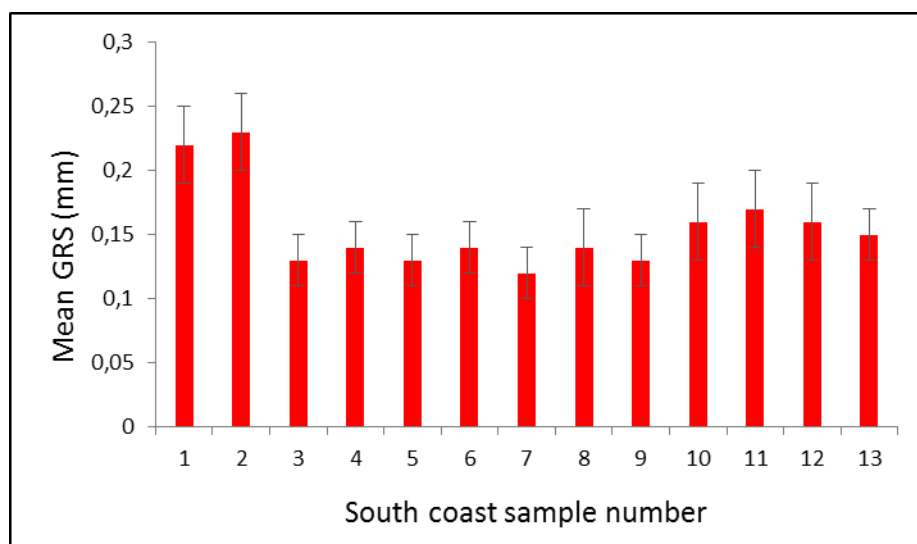


Figure 2.19. Mean gill raker spacing (GRS) per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

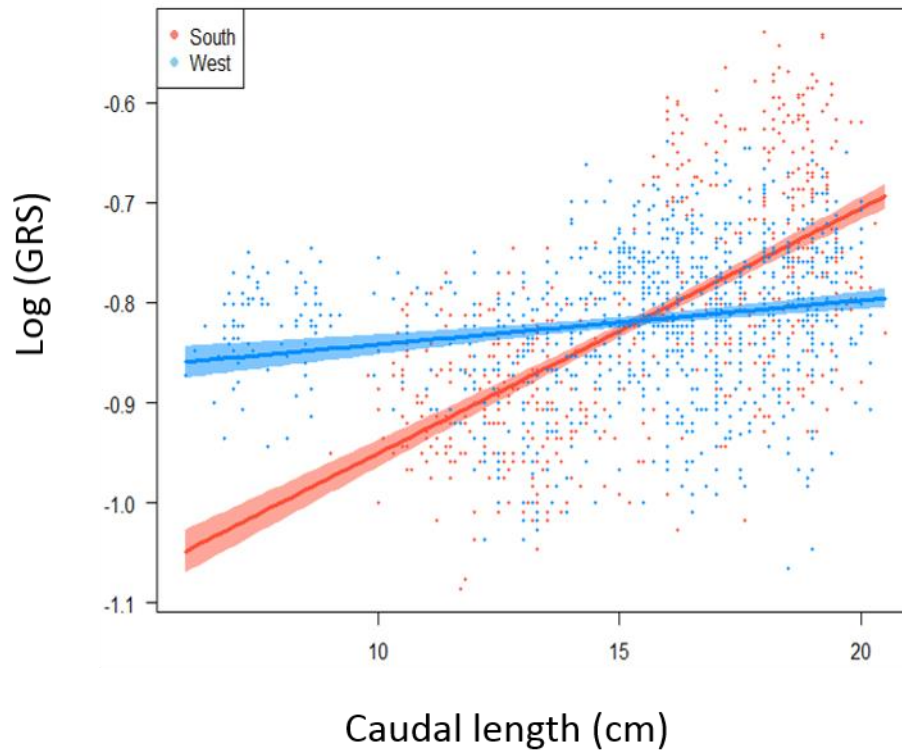


Figure 2.20. Linear relationships between caudal length and $\log_{10}$ -transformed gill raker spacing (GRS) of *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

Table 2.9. GLM analysis. Difference in $\log_{10}$ -transformed gill raker spacing (GRS) between *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock				1	0.004	0.65	0.416
	South	0.017	0.007				
	West	0.022	0.009				
Caudal length		0.001	0.003	1	1.96	310.95	< 0.001
Stock:caudal length		0.001	0.006	1	1.36	216.71	< 0.001
Residuals				1541	9.72		

Number of gill rakers (GRN)

Number of gill rakers in *S. sagax* specimens from the western putative stock ranged from 78 to 203 (Figure 2.21). Mean GRN per sample collected from the west coast ranged from 91.19 ± 5.42 to 184.56 ± 7.13 (Figure 2.23), with an overall mean GRN of 157.37 ± 24.50 (Table 2.7). Specimens from the putative southern stock had GRN ranging from 112 - 200 (Figure 2.22). The mean GRN per sample collected from the south coast ranged from 125.13 ± 6.38 to 180.04 ± 8.78 (Figure 2.24), with an overall mean of 158.33 ± 20.06 (Table 2.7).

Caudal length was found to significantly and positively influence GRN in both western and southern stocks (Figure 2.25), indicating that larger fish have more gill rakers than smaller ones.

After accounting for size, GRN in individuals from the western stock was found to be significantly different ($F_{(1,1541)} = 13.71$, $P = 0.0002$) to GRN in individuals from the southern stock, with the former having fewer gill rakers than the latter (Table 2.10). The GLM analysis confirmed the significant relationship between number of gill rakers and fish size but the significant Stock:coast interaction showed that these relationships differed for sardine off the west and south coast. Rate of increase in gill raker number with increasing fish size is higher in fish from the west compared to those from the south coast.

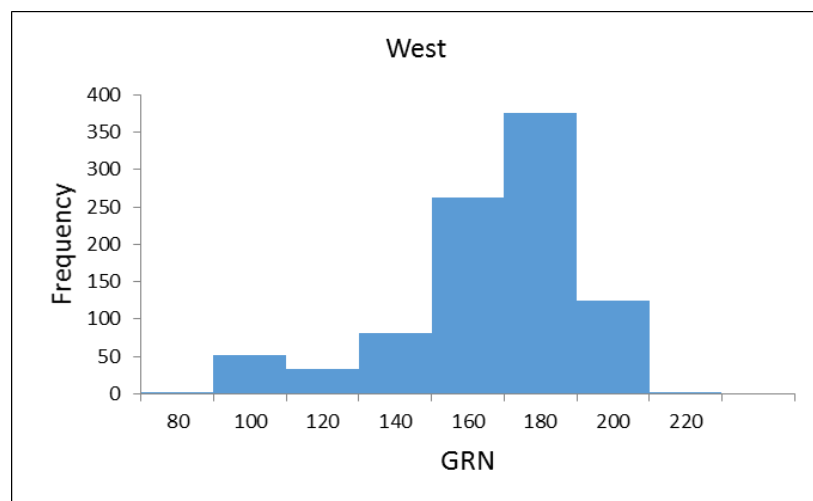


Figure 2.21. Frequency distribution of number of gill rakers (GRN) for *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

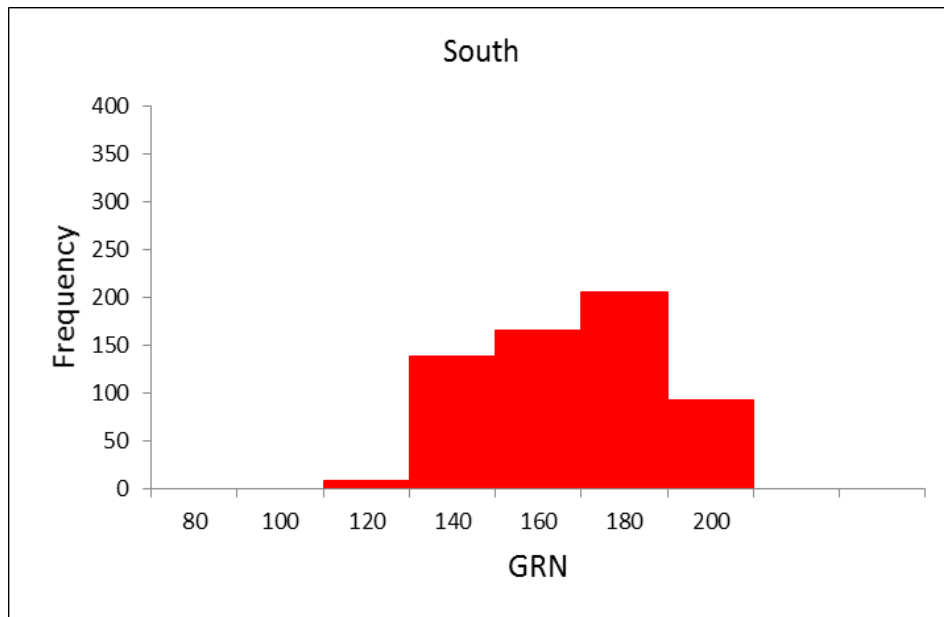


Figure 2.22. Frequency distribution of number of gill rakers (GRN) for *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

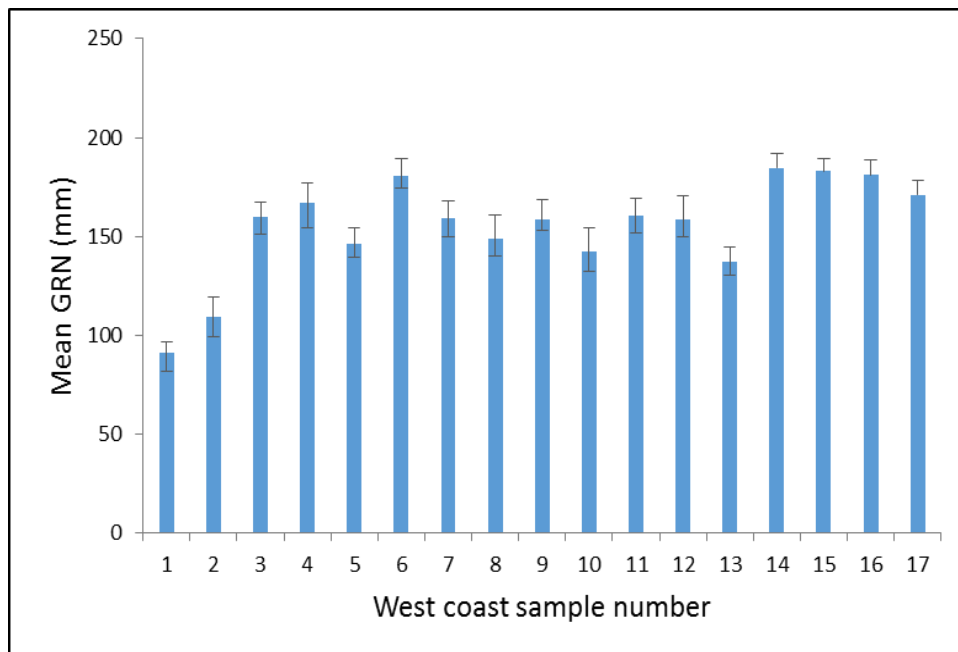


Figure 2.23. Mean number of gill rakers (GRN) per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

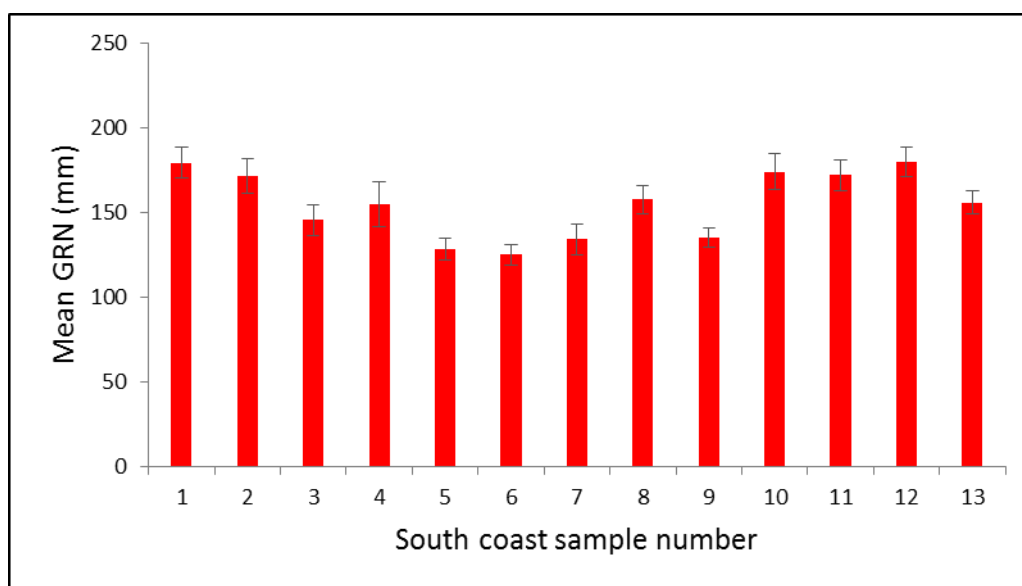


Figure 2.24. Mean number of gill rakers (GRN) per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

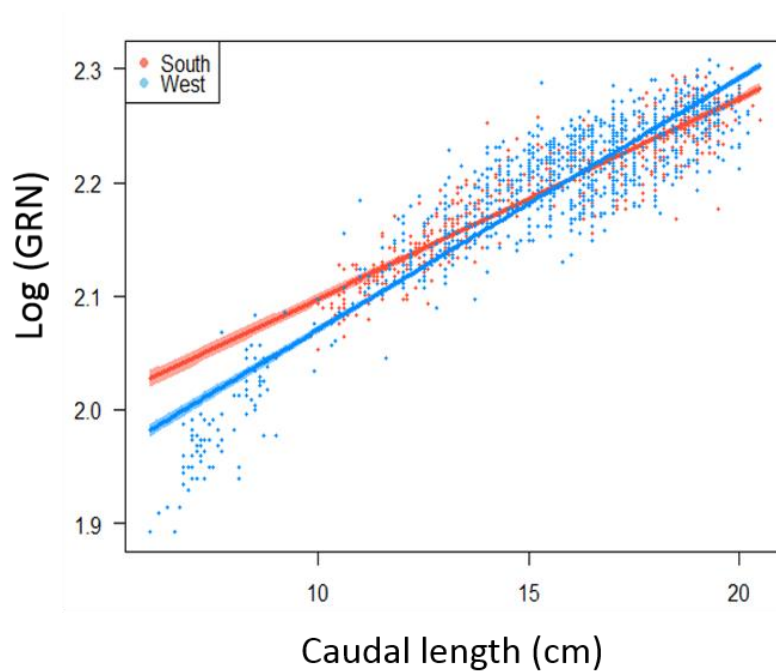


Figure 2.25. Linear relationship between caudal length and $\log_{10}$ -transformed number of gill rakers (GRN) of *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

Table 2.10. GLM analysis. Difference in $\log_{10}$ -transformed number of gill rakers (GRN) between *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock				1	0.01	13.71	< 0.001
	South	1.92	0.0061				
	West	1.85	0.0075				
Caudal length		0.02	0.0004	1	6.29	8117.89	< 0.001
Stock:caudal length		0.005	0.0005	1	0.07	89.49	< 0.001
Residuals				1541	1.19		

Otolith shape indices

Otolith form factor (OF)

Otolith form factor (OF) in all (mature and immature) *S. sagax* specimens from the putative western stock ranged from 0.31 to 0.66 (Figure 2.26). Mean OF per sample for 17 samples collected from the west coast ranged from 0.41 ± 0.04 to 0.59 ± 0.03 (Figure 2.28). *S. sagax* from the putative western stock had an overall mean OF of 0.45 ± 0.05 (Table 2.11). For all specimens collected from the southern coast, OFs ranged from 0.31 to 0.54 (Figure 2.27). Mean OF per sample for 13 samples collected from the south coast ranged from 0.4 ± 0.03 to 0.48 ± 0.03 (Figure 2.29), with an overall mean OF of 0.43 ± 0.04 . Otolith form factor for immature individuals from the putative western stock ranged from 0.42 to 0.66, with a mean otolith form factor of 0.54 ± 0.06 , while immature individuals from the putative southern stock had otolith form factor ranging from 0.37 to 0.54, with a mean otolith form factor of 0.46 ± 0.03 (Table 2.11)

A negative and highly significant relationship was found between caudal length and OF in both adult and immature individuals from the putative western and southern stocks (Figures

2.30 and 2.31). This indicates that otolith form factor (OF) decreases as fish get larger and that otoliths have more irregular outlines in larger fish compared to smaller ones.

Results of the GLM analysis that included only mature individuals show a significant difference in otolith form factor ($F_{(1,1281)} = 16.64$, $P < 0.001$) between individuals from the putative western and southern stocks (Table 2.12). South coast otoliths had more irregular outlines than west coast otoliths. Similarly, immature *S.sagax* from the putative western stock differed significantly ($F_{(1,255)} = 387.66$, $P < 0.001$) from their counterparts from the southern stock in their otolith form factor (Table 2.13). As in the adult *S. sagax* specimens, outlines for otoliths of immature fish from the southern coast are more irregular compared to those from the west coast. Otolith form factor was significantly influenced by fish length ($F_{(1,1281)} = 266.66$, $P < 0.001$) (Tables 2.12 and 2.13). Season also significantly influenced adult otolith shape with summer samples having more irregular outlines than the winter samples, although this difference was only just significant ($F_{(1,1281)} = 4.23$, $P = 0.040$). A stronger significant difference was found between samples from male individuals and female individuals ($F_{(1,1281)} = 5.77$, $P = 0.016$).

Table 2.11. Mean (+ SE) otolith form factor of *Sardinops sagax* from putative western and southern stocks off the South African coast in 2013 and 2014.

			Sample Size	Range Otolith form factor	Mean (+SE) Otolith form factor
All individuals	Stock	Western	933	0.31 – 0.66	0.45 ± 0.05
		Southern	617	0.30 – 0.54	0.43 ± 0.04
	Sex	Male	677	0.30 – 0.62	0.43 ± 0.04
		Female	612	0.31 – 0.54	0.43 ± 0.04
Immature individuals	Stock	Western	141	0.42 – 0.66	0.54 ± 0.06
		Southern	115	0.37 – 0.54	0.46 ± 0.03

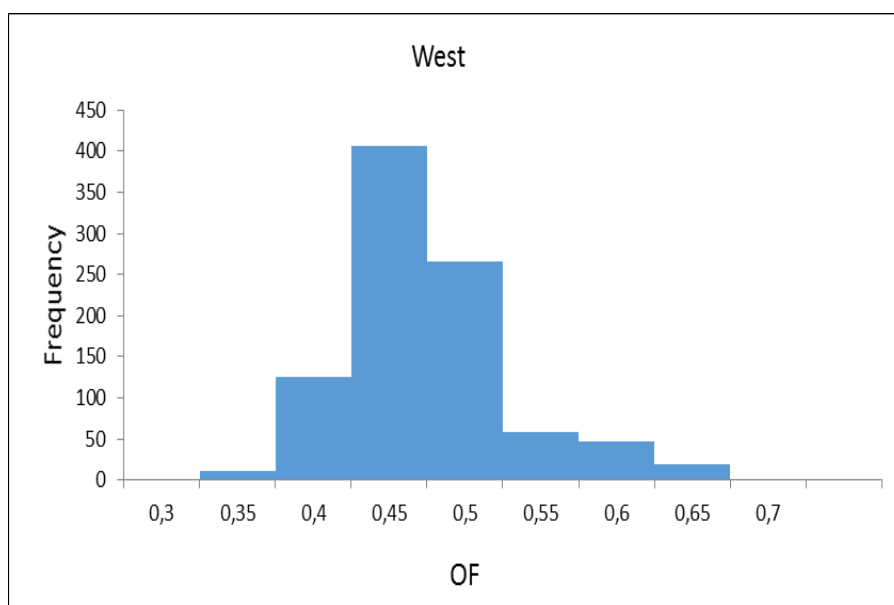


Figure 2.26. Frequency distribution of otolith form factor (OF) for *Sardinops sagax* (immature and mature fish) from the putative western stock off the South African coast in 2013 and 2014.

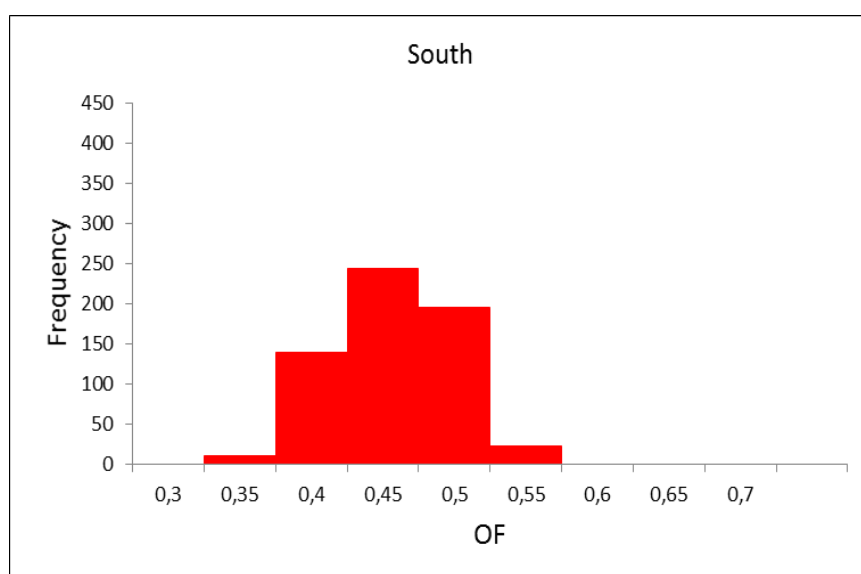


Figure 2.27. Frequency distribution of otolith form factor (OF) for *Sardinops sagax* (immature and mature fish) from the putative southern stock off the South African coast in 2013 and 2014.

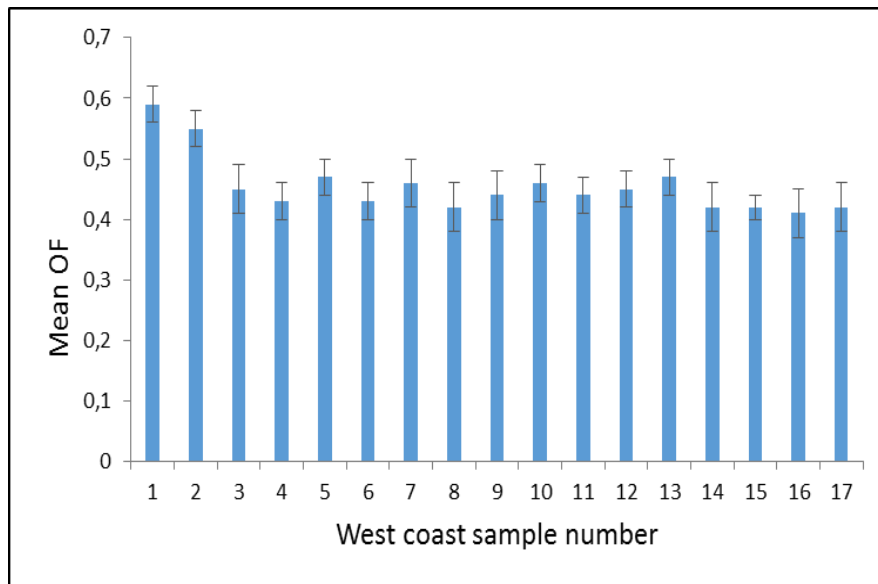


Figure 2.28. Mean otolith form factor (OF) per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

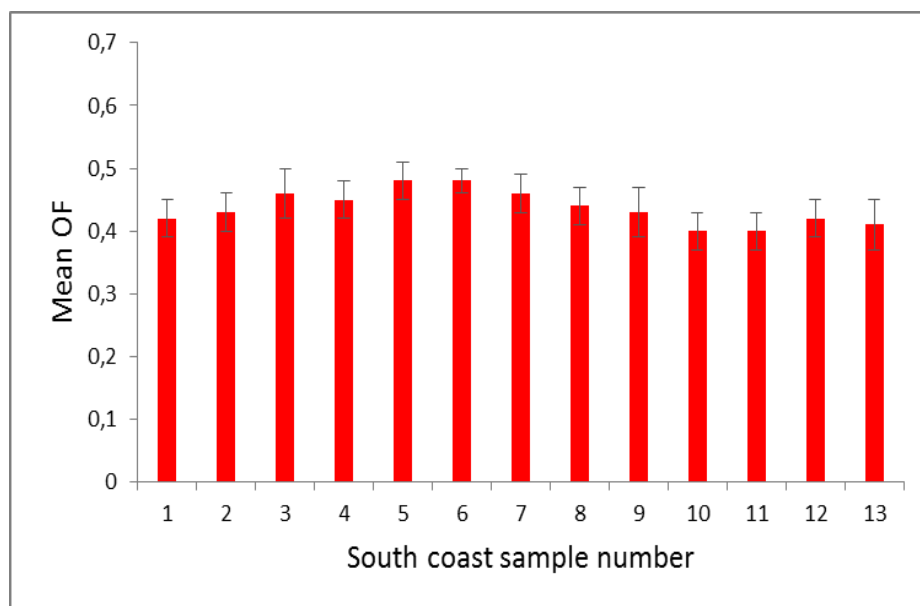


Figure 2.29. Mean otolith form factor (OF) per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

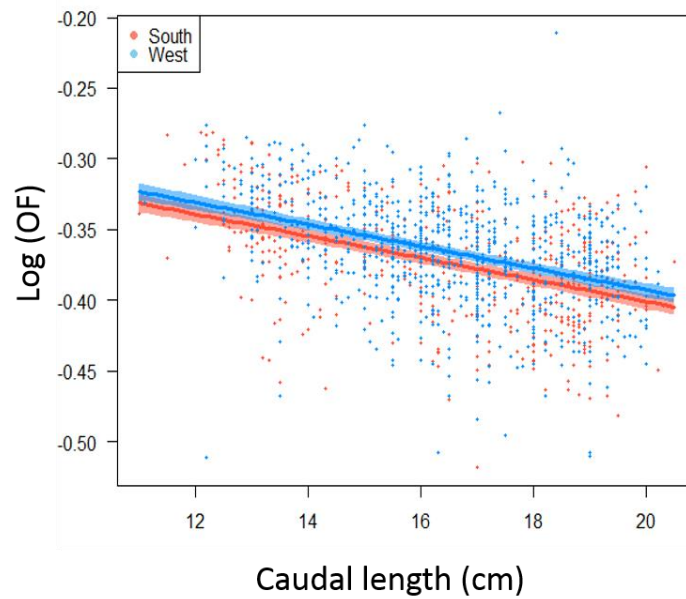


Figure 2.30. Linear relationships between caudal length and $\log_{10}$ -transformed otolith form factor (OF) of adult *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

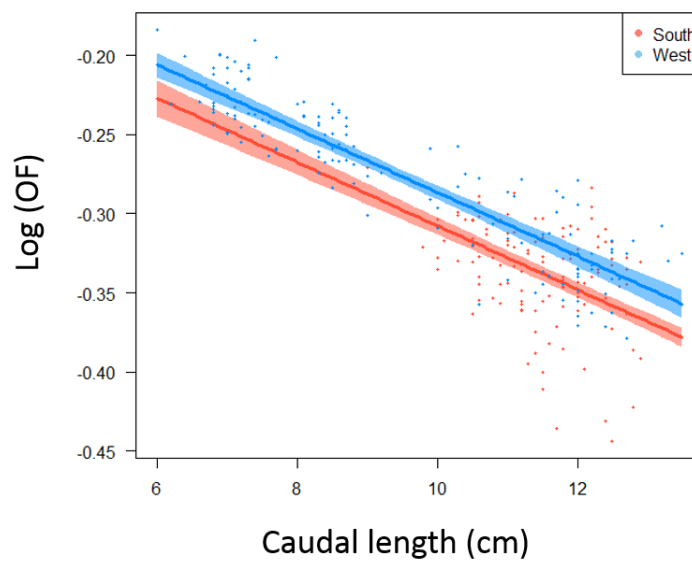


Figure 2.31. Linear relationship between caudal length and $\log_{10}$ -transformed otolith form factor (OF) of immature *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

Table 2.12. GLM analysis. Difference in log₁₀-transformed otolith form factor (OF) between adult *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock				1	0.02	16.64	< 0.001
	South	-0.37	0.002				
	West	-0.36	0.001				
Sex				1	0.01	5.77	0.016
	Female	-0.36	0.001				
	Male	-0.37	0.001				
Season				1	0.01	4.23	0.040
	Summer	-0.36	0.002				
	Winter	-0.37	0.001				
Caudal length		-0.007	0.008	1	0.34	266.66	< 0.001
Residuals				1281	1.62		

Table 2.13. GLM analysis. Difference in log₁₀-transformed otolith form factor (OF) between immature *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock				1	0.25	387.66	< 0.001
	South	-0.31	0.0026				
	West	-0.29	0.0023				
Caudal length		-0.02	0.0010	1	0.29	443.57	< 0.001
Residuals				255	0.17		

Otolith circularity (OCC)

Otolith circularity (OCC) in all *S. sagax* specimens from the putative western stock ranged from 19.18 to 40.62 (Figure 2.32). Mean OCC per sample for 17 samples collected from the west coast ranged from 21.22 ± 0.91 to 30.79 ± 3.24 (Figure 2.34). *S. sagax* from the

putative western stock had an overall mean OCC of 28.35 ± 3.26 (Table 2.14). For all specimens collected from the southern coast, OCC ranged from 23.44 to 41.50 (Figure 2.33). Mean OCC per sample for 13 samples collected from the south coast ranged from 26.02 ± 1.34 to 31.88 ± 2.68 (Figure 2.35), with an overall mean OCC of 29.29 ± 2.85 . Otolith circularity for immature individuals from the putative western stock ranged from 19.18 to 30.08, with a mean value of 23.77 ± 2.72 , while immature individuals from the putative southern stock had otolith circularity ranging from 23.45 to 34.96, with a mean otolith value of 27.40 ± 2.16 (Table 2.14)

A positive and highly significant relationship was found between caudal length and OC in adult and immature individuals from both the putative western and southern stocks (Figures 2.36 and 2.37). This indicates that larger fish have a higher OC with the otolith gradually losing its circular form and becoming more elongate or oval in larger fish.

Results of the GLM analysis performed on mature individuals shows a significant difference in otolith circularity ($F_{(1,1281)} = 16.56$, $P < 0.001$) between individuals from the putative western and southern stocks (Table 2.15). Otoliths of sardine from the south coast are more elongate than those from west coast fish. Similarly, immature *S. sagax* from the putative western stock differed significantly ($F_{(1,255)} = 387.3$, $P < 0.001$) from their counterparts from the southern stock in their otolith circularity (Table 2.16). As in the adult *S. sagax* specimens, the otoliths of immature fish from the southern coast are less circular and longer compared to those from the west coast. The GLM confirmed the strong and significant effect of fish length on otolith circularity for both adult ($F_{(1,1281)} = 266.22$, $P < 0.001$; Table 2.15) and immature ($F_{(1,255)} = 444.03$, $P < 0.001$; Table 2.16). Season significantly influenced otolith shape in adult sardine, with summer samples having a more elongate shape than the winter samples ($F_{(1,1281)} = 4.19$, $P = 0.040$). Sex was also a significant parameter, with samples from male individuals being less circular and longer than samples from female individuals ($F_{(1,1281)} = 5.73$, $P = 0.017$).

Table 2.14. Mean (+ SE) otolith circularity of *Sardinops sagax* from putative western and southern stocks off the South African coast in 2013 and 2014.

			Sample Size	Range Otolith circularity	Mean (+SE) Otolith circularity
All individuals	Stock	Western	933	19.18 – 40.62	28.35 ± 3.26
		Southern	617	23.44 – 41.50	29.29 ± 2.85
	Sex	Male	677	20.44 – 41.50	29.53 ± 2.72
		Female	612	23.44 – 40.33	29.22 ± 2.69
Immature individuals	Stock	Western	141	19.18 – 30.08	23.77 ± 2.72
		Southern	115	23.45 – 34.96	27.40 ± 2.16

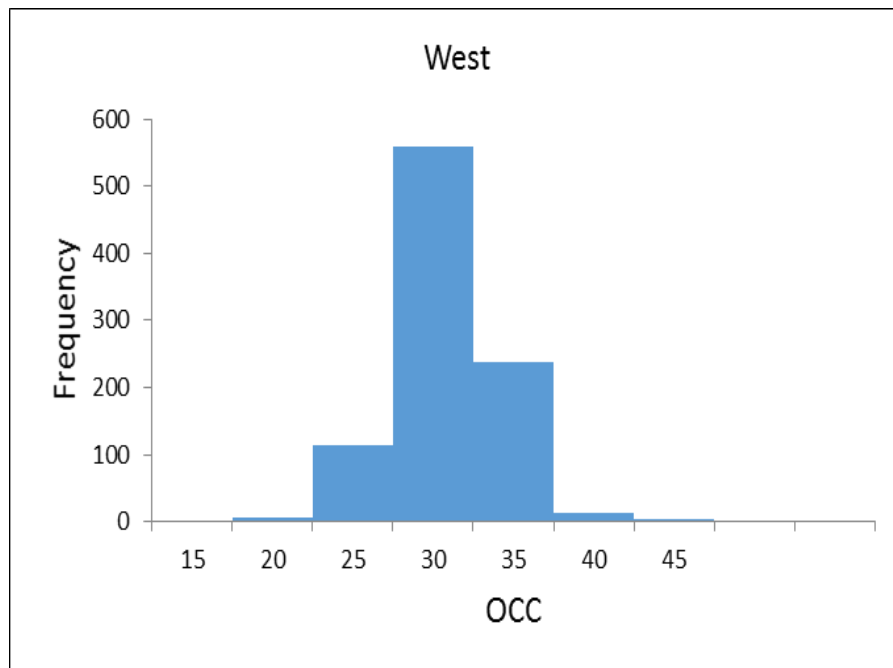


Figure 2.32. Frequency distribution of otolith circularity (OCC) for *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

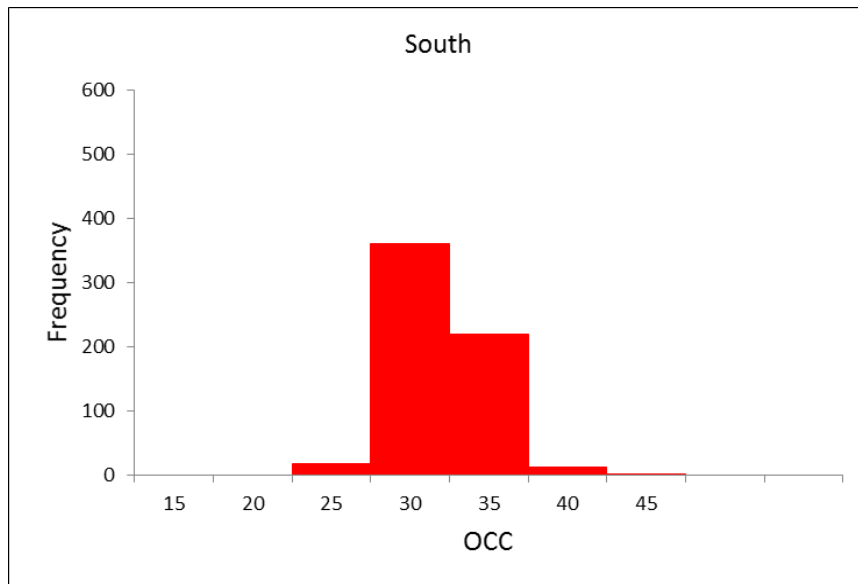


Figure 2.33. Frequency distribution of otolith circularity (OCC) for *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

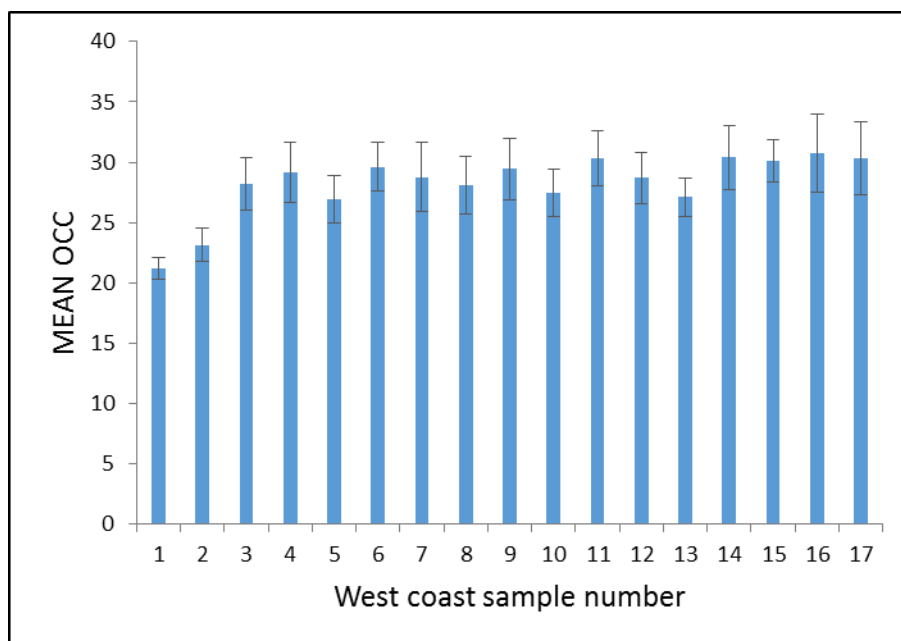


Figure 2.34. Mean otolith circularity (OCC) per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

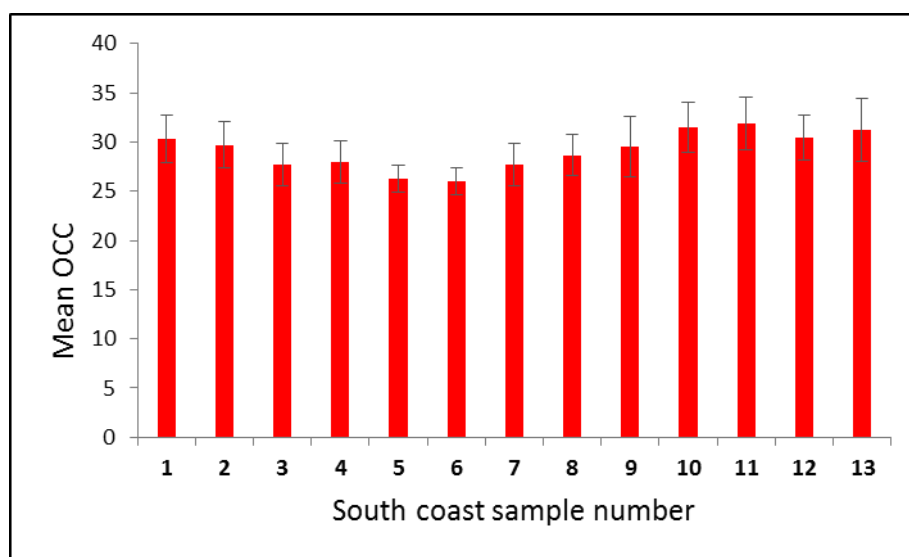


Figure 2.35. Mean otolith circularity (OCC) per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

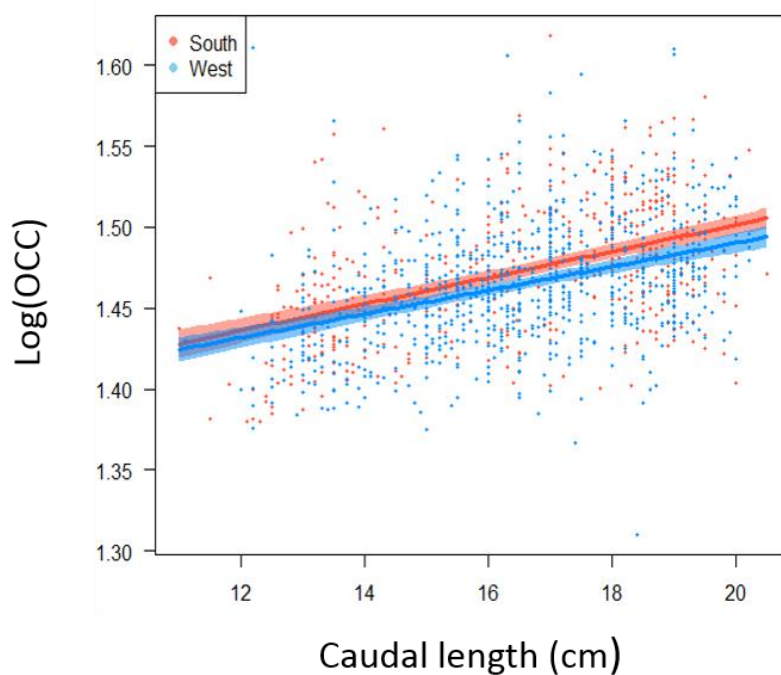


Figure 2.36. Linear relationship between caudal length and $\log_{10}$ -transformed otolith circularity (OCC) of adult *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

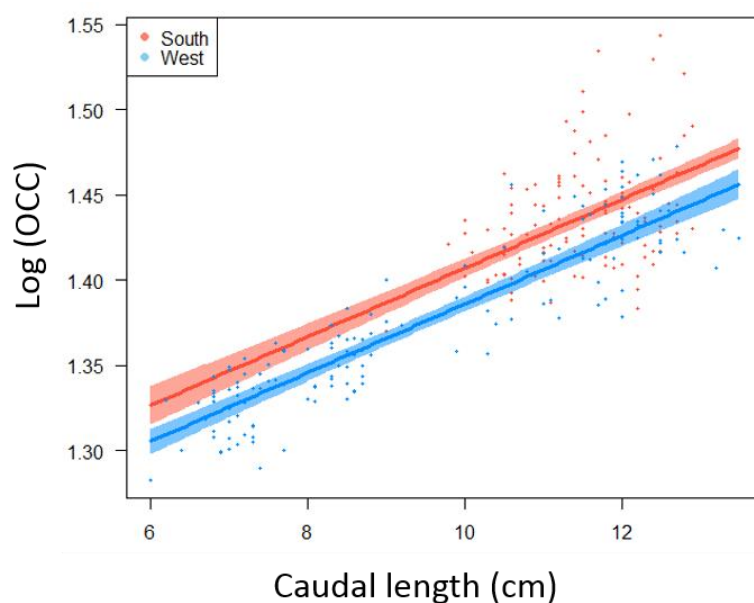


Figure 2.37. Linear relationship between caudal length and $\log_{10}$ -transformed otolith circularity (OCC) of immature *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

Table 2.15. GLM analysis. Difference in $\log_{10}$ -transformed otolith circularity (OCC) between adult *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock				1	0.02	16.56	< 0.001
	South	1.47	0.002				
	West	1.46	0.001				
Sex				1	0.01	5.73	0.017
	Female	1.46	0.001				
	Male	1.47	0.001				
Season				1	0.01	4.19	0.04
	Summer	1.47	0.002				
	Winter	1.47	0.001				
Caudal length		0.01	0.008	1	0.34	266.22	< 0.001
Residuals				1281	1.61		

Table 2.16. GLM analysis. Difference in log₁₀-transformed otolith circularity (OCC) between immature *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014.

		Estimate	SE	df	SS	F	P
Stock				1	0.25	387.3	< 0.001
	South	1.41	0.0026				
	West	1.39	0.0023				
Caudal length		0.02	0.0010	1	0.29	444.03	< 0.001
Residuals				255	0.17		

‘Tetracotyle’ type metacercaria parasite abundance

The abundance of the digenean ‘tetracotyle’ type metacercaria parasite in the eyes of *S. sagax* specimens from the putative western stock ranged from 0 to 55 parasites.fish⁻¹ (Figure 2.38). Mean abundance per sample for 17 samples collected from the west coast ranged from 0 to 8.14 ± 10.31 parasites.fish⁻¹ (Figure 2.40), with an overall mean abundance of 2.02 ± 4.43 parasites.fish⁻¹ (Table 2.17). For specimens from the putative southern stock, abundance ranged from 0 to 71 parasites.fish⁻¹ (Figure 2.39). Mean abundance per sample for 13 samples collected from the south coast ranged from 0 to 7.9 ± 11.19 parasites.fish⁻¹ (Figure 2.41), with an overall mean abundance of 1.53 ± 4.80 parasites.fish⁻¹.

No sardine <10cm CL were found to be infected, whilst infected and un-infected fish larger than 10 cm CL were recorded. A positive and highly significant relationship was found between caudal length and metacercaria abundance in individuals from both the putative western and southern stocks (Figure 2.42). Metacercaria abundance increased with increasing fish length.

The GLM analysis indicated that whilst fish length was the strongest predictor of metacercarial abundance, putative stock was also a significant predictor. Parasite abundance was significantly higher (Wald Chi-square = 35.41, P < 0.001, Table 2.18) in sardine from the west coast [$\exp^{(1.22)} = 3.41$] compared to the south coast [$\exp^{(0.77)} = 2.15$]

whilst season did not significantly influence metacercaria abundance (Wald Chi-square = 1.16, $P = 0.28$) (Table 2.18).

Table 2.17. Mean (+ SE) abundance of metacercariae in *Sardinops sagax* from putative western and southern stocks off the South African coast in 2013 and 2014.

		Sample size	Range Abundance	Mean (+ SE) Abundance
Stock	Western	933	0 – 55	2.02 ± 4.43
	Southern	617	0 – 71	1.53 ± 4.80

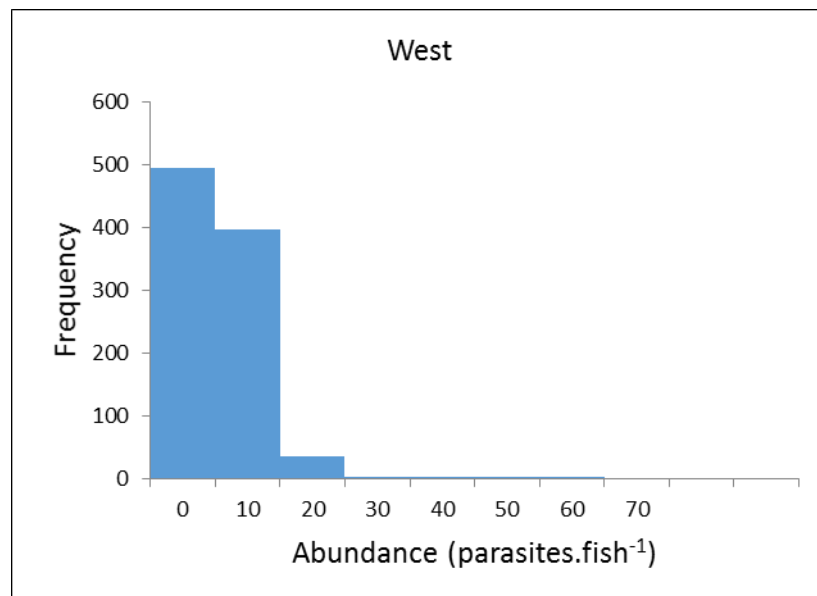


Figure 2.38. Frequency distribution of 'tetracotyle' type metacercaria parasite abundance in *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

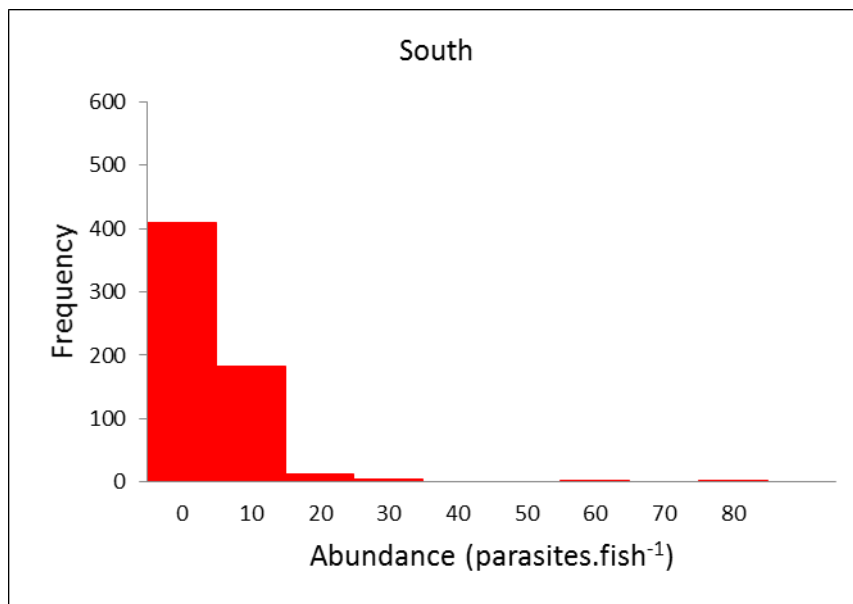


Figure 2.39. Frequency distribution of 'tetracotyle' type metacercaria parasite abundance in *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

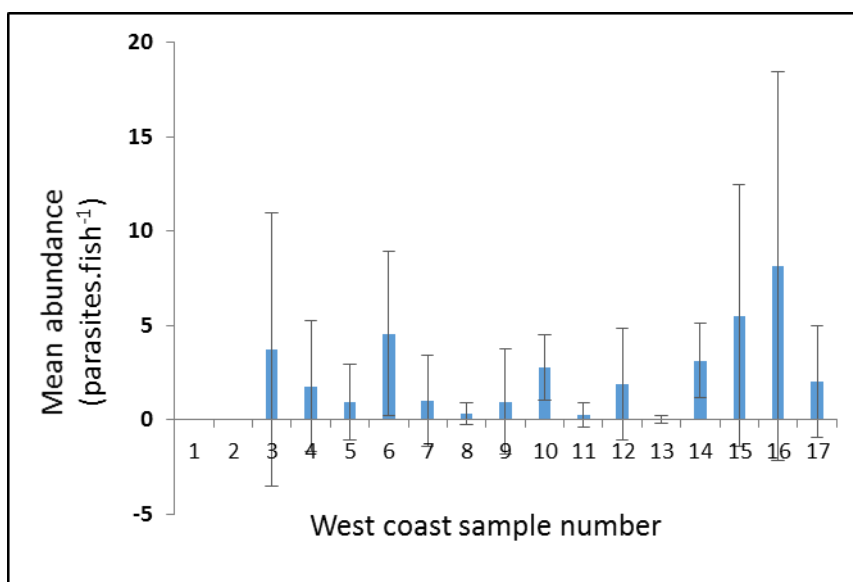


Figure 2.40. Mean abundance per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

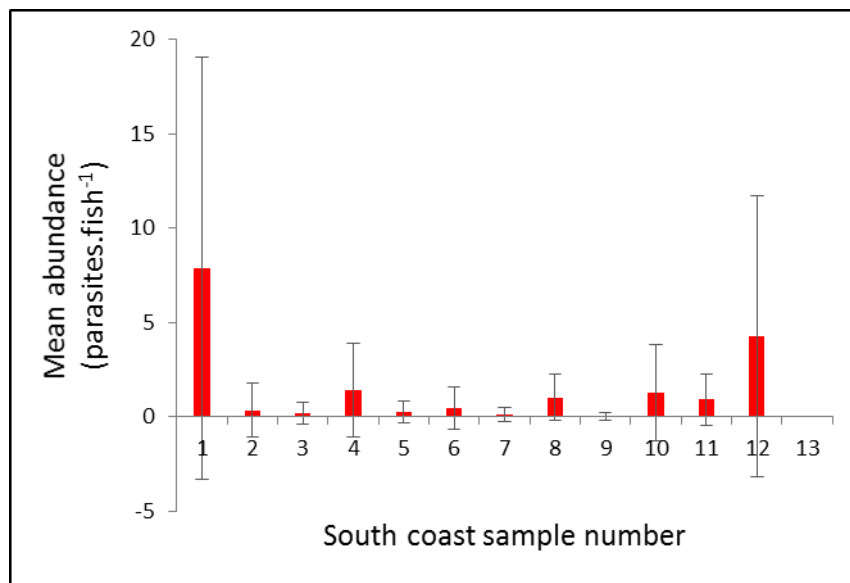


Figure 2.41. Mean abundance per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

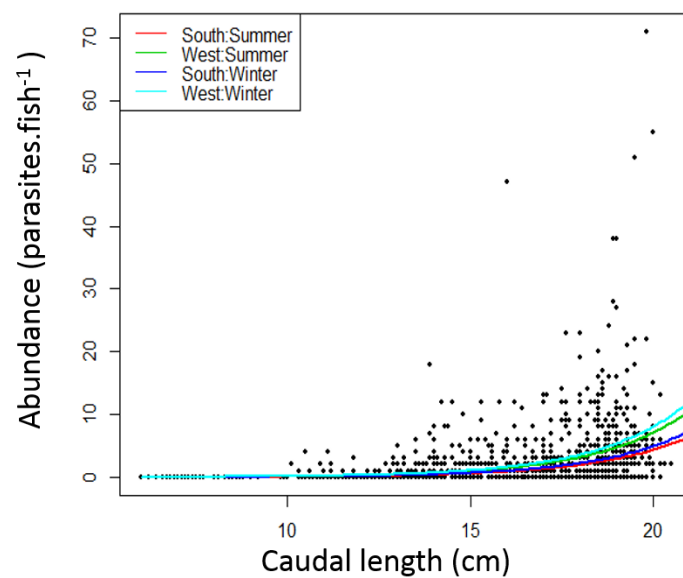


Figure 2.42. Fitted predictive polynomial regressions for relationship between caudal length and 'tetracotyle' type metacercaria parasite abundance in *S. sagax* from the putative western and southern stocks off the South African coast, and in winter and summer in 2013 and 2014.

Table 2.18. GLM analysis. Difference in ‘tetracotyle’ type metacercaria parasite abundance in *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014. Results are given on the logarithmic scale.

		Estimate	SE	df	Wald Chi-square	P
Stock	Southern	0.77	0.08	1	35.41	< 0.001
	Western	1.227				
Season	Summer	0.93	0.08	1	1.16	0.28
	Winter	1.015				
Caudal length		0.40	0.02	1	588.94	< 0.001

Vertebral count

The number of vertebrae in *S.sagax* specimens from the putative western stock ranged from 46 to 53 (Figure 2.43). Mean abundance per sample for 17 samples collected from the west coast ranged from 49.71 ± 1.6 to 51.23 ± 0.58 (Figure 2.45), with an overall mean of 50.93 ± 0.78 (Table 2.19). For specimens from the putative southern stock, abundance ranged from 46 to 52 (Figure 2.44). Mean abundance per sample for 13 samples collected from the south coast ranged from 50.3 ± 1.06 to 51.19 ± 0.48 (Figure 2.46), with an overall mean of 50.94 ± 0.73 (Table 2.19).

No significant difference in the number of vertebrae (Mann Whitney $W = 283510$, $P = 0.80$) was found between individuals from the putative western and southern stocks.

Table 2.19. Mean (+ SE) abundance of vertebrae in *Sardinops sagax* from putative western and southern stocks off the South African coast in 2013 and 2014.

Stock	Sample size	Range Number of vertebrae	Mean (+ SE) Number of vertebral bones
Western	933	46 – 53	50.93 ± 0.78
Southern	617	46 – 52	50.94 ± 0.73

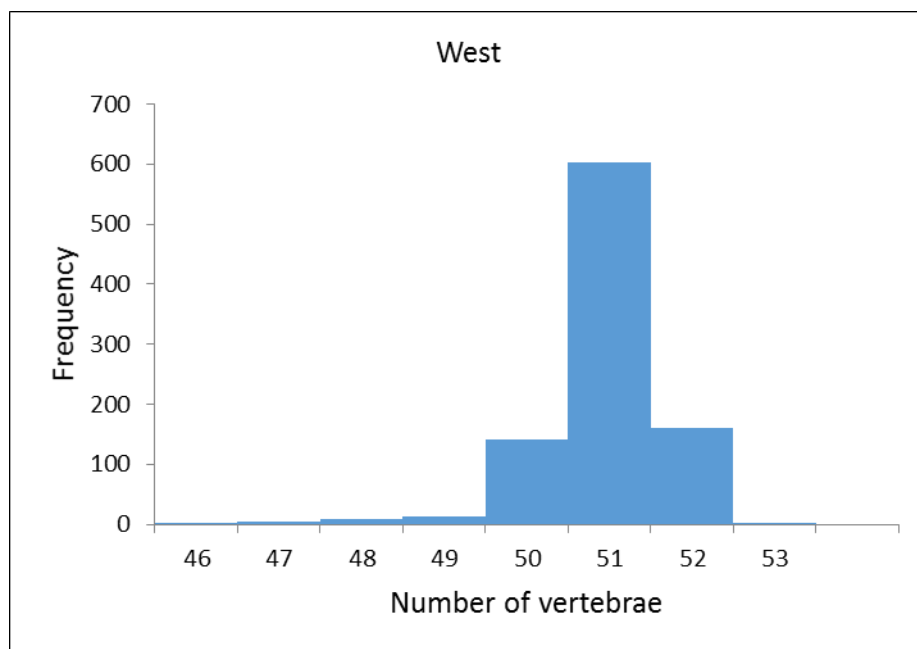


Figure 2.43. Frequency distribution of number of vertebrae bones in *Sardinops sagax* from the putative western stock off the South African coast in 2013 and 2014.

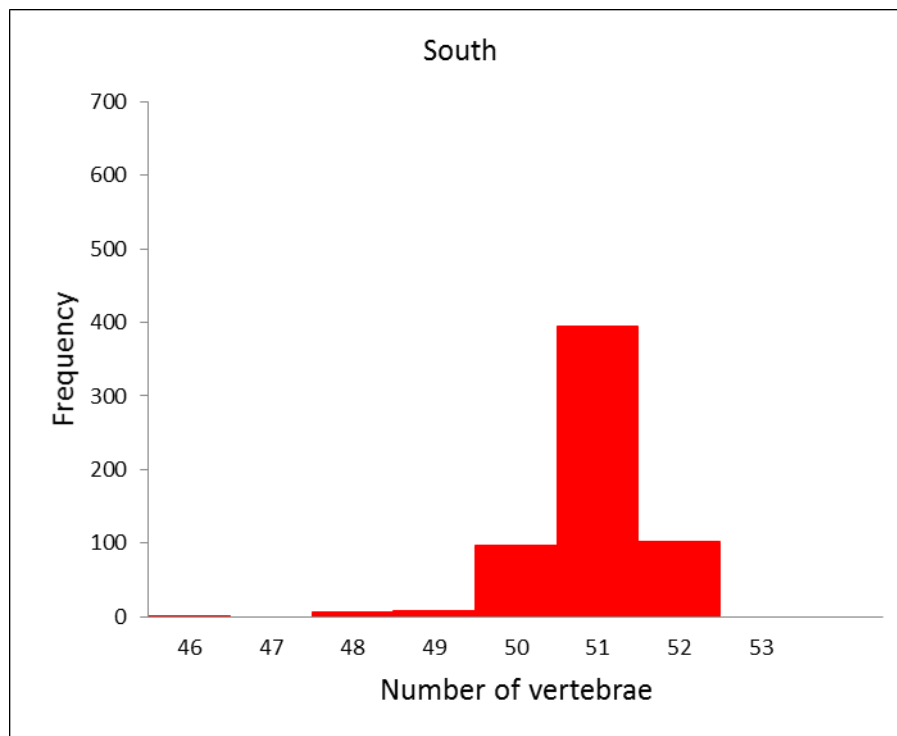


Figure 2.44. Frequency distribution of number of vertebrae in *Sardinops sagax* from the putative southern stock off the South African coast in 2013 and 2014.

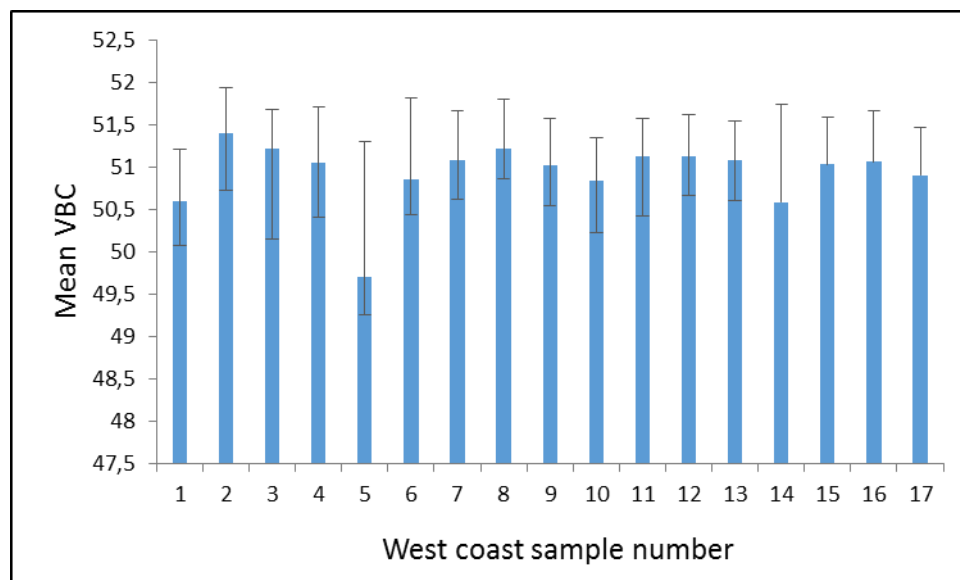


Figure 2.45. Mean number of vertebrae per sample of *Sardinops sagax* from the west coast of South Africa in 2013 and 2014.

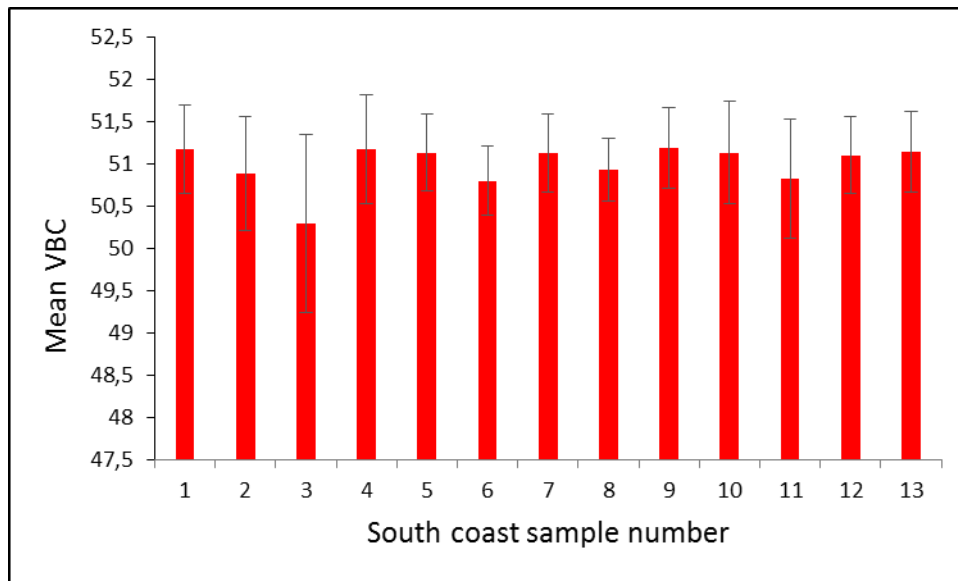


Figure 2.46. Mean number of vertebrae per sample of *Sardinops sagax* from the south coast of South Africa in 2013 and 2014.

Integration of results of univariate analyses using the Stock Differentiation Index (SDI)

The difference values (significant difference = 1, no difference = 0) for each univariate test of differences between the two putative stocks: western & southern, based on the examination of a range of stock structure approaches - morphometric, meristic and parasite data analysed in this study are shown in Table 2.20. A SDI value of 0.75 was calculated from these results, which provides strong evidence of separation of individuals from the west and south coast of South Africa and supports the hypotheses of western and southern sardine stocks.

Table 2.20. Stock Differentiation Index (SDI) for the putative western and southern sardine stocks off the South African west and south coast derived from morphometric, meristic and parasite data.

Variable (Data Source)	Difference value	SDI
Body shape	1	0.75
Gill arch length	1	
Gill raker spacing	0	
Number of gill rakers	1	
Otolith form factor	1	
Otolith circularity	1	
Metacercariae parasite abundance	1	
Vertebral count	0	
$\Sigma DV = 6$		

Multivariate Analyses

The combination of variables used in the multivariate analyses was made up of seven variables namely, gill arch length (GAL), gill raker spacing (GRS), the number of gill rakers (GRN), otolith form factor (OF), otolith circularity (OCC), vertebral count (VC) and metacercariae parasite abundance (TETRA). Individuals from the two putative stocks (western and southern) were found to have comparable values for all variables examined. However, box-and-whisker plot of the raw data shows some variation in the distribution of data in the form of skewness, the presence of outliers (plotted as dots outside the box at the end of the whiskers) and the degree of dispersion or spread (differences in the spacing between the hinges of the box). Spread (variance) of each variable was not homogenous in some of the data from sardine from the two coasts. The observed variation is due, to a large extent, the effect of fish length. Hence, variables were log-transformed and standardized to the mean to remove the effect of allometry and to ensure all variables were on the same scale.

Year of sampling significantly influenced the data set. Sardine samples caught from the west coast in 2013 (n = 236) and 2014 (n = 236) differed significantly in the multivariate suite of variables examined (PERMANOVA: Pseudo-F = 4.71, P (perm) = 0.03). Test for homogeneity of multivariate dispersion showed similar degree of dispersion for data obtained from the two years (PERMDISP: F = 0.51, P (perm) = 0.57). There was also a significant difference

between sardine from the south coast in the combination of variables studied for two years ($n = 231$ for 2013 and $n = 191$ for 2014; PERMANOVA: Pseudo-F = 73.32, $P(\text{perm}) = 0.001$). For the south coast sardine, degree of dispersion differed significantly for the two years (PERMDISP: $F = 97.09$, $P(\text{perm}) = 0.001$). Hence, dataset from the two years (west 2013 vs south 2013 and west 2014 vs south 2014) were first analysed separately, and then as a combined dataset. Results of PERMANOVA analysis showed a significant difference between sardine from the two stocks in 2013 ($n = 234$ from western stock, 231 from southern stock, Pseudo-F = 9.91, $P(\text{perm}) = 0.001$) with data from the two stocks showing dissimilar degree of dispersion (PERMDISP: $F = 196.4$, $P(\text{perm}) = 0.001$). Similarly, in 2014, a significant difference was found between sardine from the two stocks (PERMANOVA: Pseudo-F = 18.55, $P(\text{perm}) = 0.001$). However, data from the two stocks had similar degree of dispersion (PERMDISP: $F = 2.36$, $P(\text{perm}) = 0.13$). For the combined dataset, results of the PERMANOVA analysis indicated a significant difference (Pseudo-F = 15.11, $P(\text{perm}) = 0.001$) between *Sardinops sagax* from the two stocks. The test for homogeneity of multivariate dispersion (PERMDISP) showed that the degree of dispersion is similar ($F = 0.042$, $P(\text{perm}) = 0.85$) for data obtained from the two putative stocks. Figures 2.47 and 2.48 show the box-and-whisker plot of all variables (both raw and standardized values) included in the multivariate analyses.

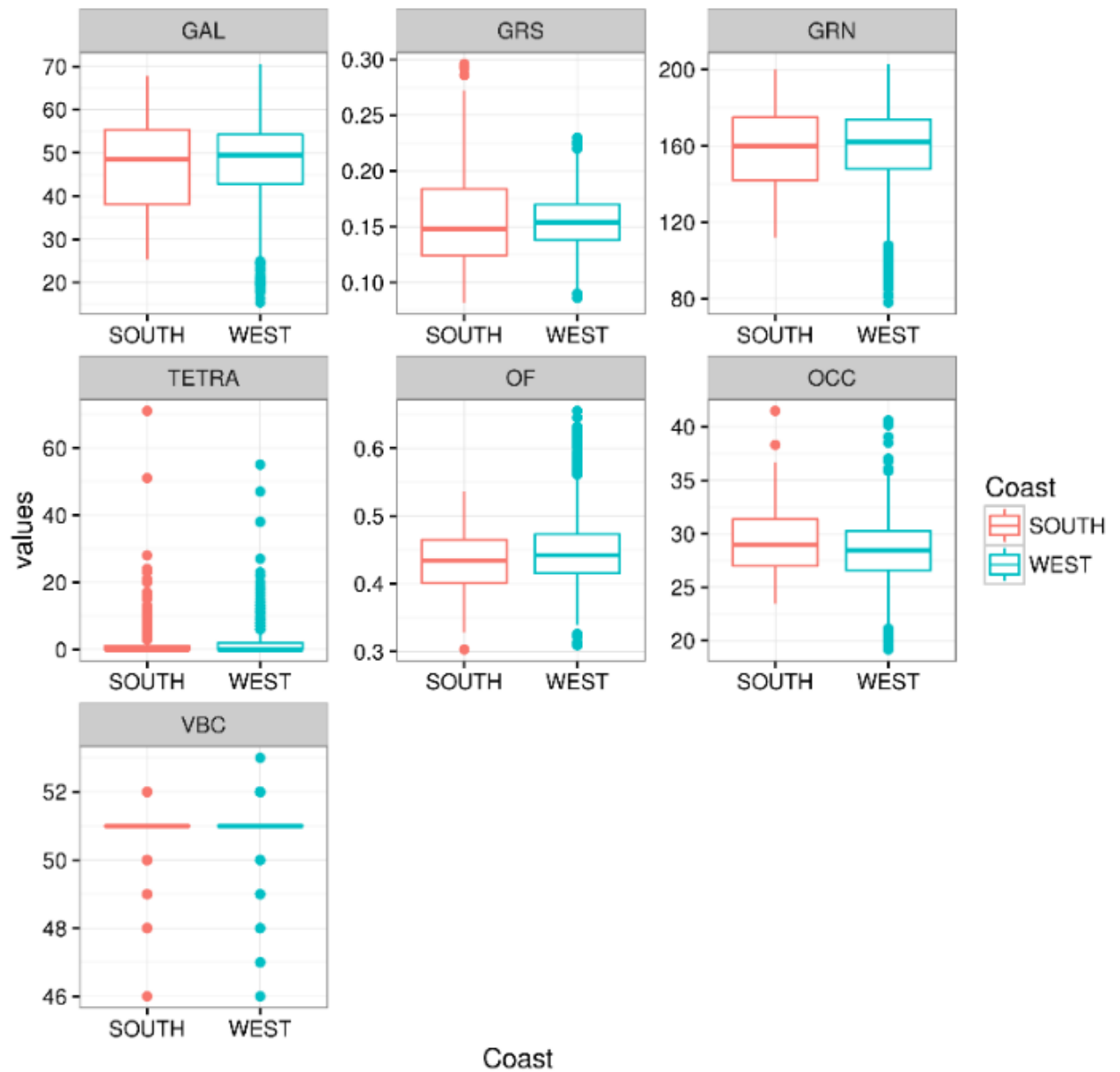


Figure 2.47. Box-and-whisker plots of raw values of variables for *Sardinops sagax* from the putative western and southern stocks off the South African coasts in 2013 and 2014.

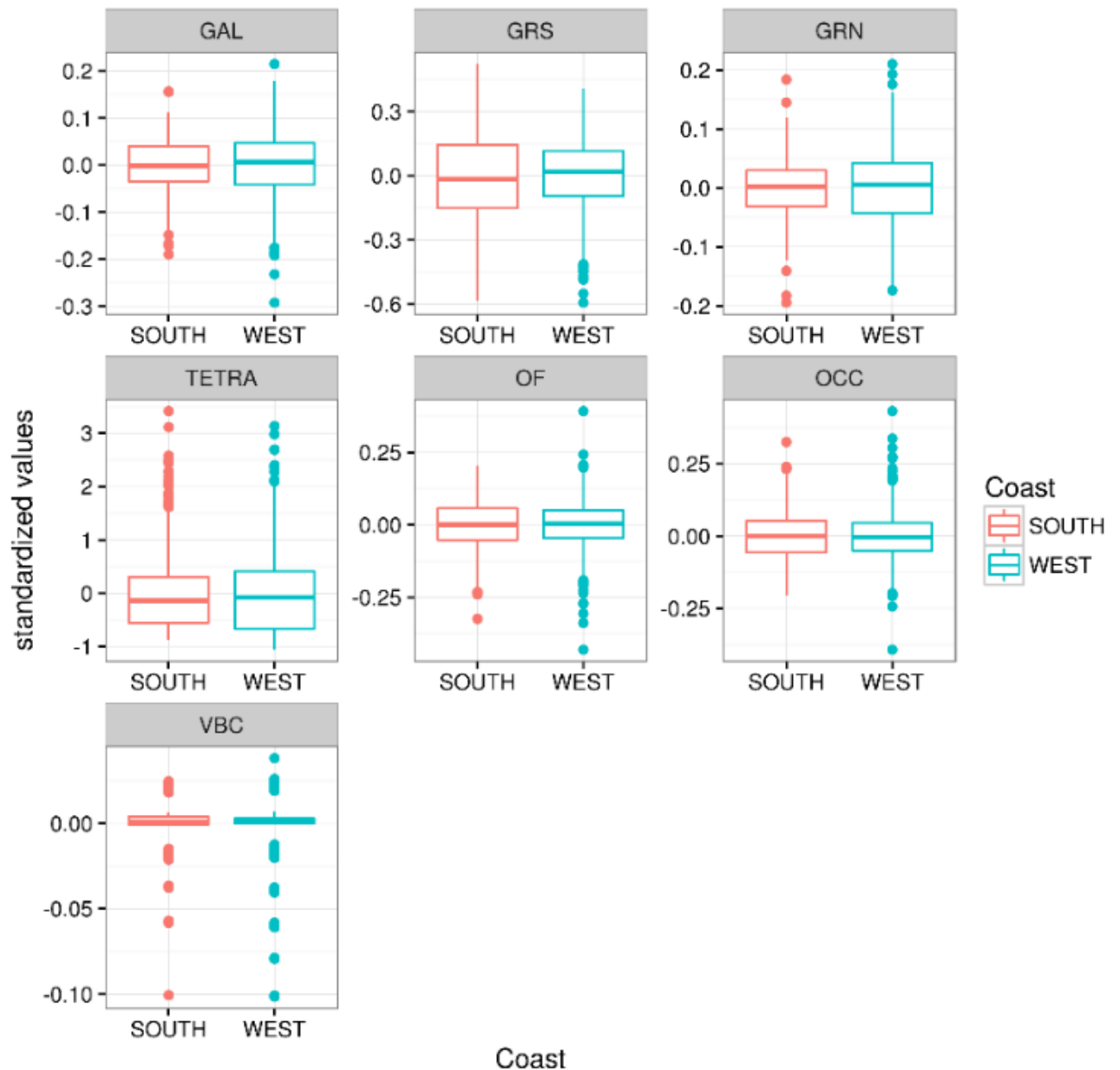


Figure 2.48. Box-and-whisker plots of standardized values of variables for *Sardinops sagax* from the putative western and southern stocks off the South African coasts in 2013 and 2014.

The PCA plot suggested a distinction between individuals from the two coasts, even though there was an extensive overlap between individuals from the two putative stocks (Figure 2.49). The first four PCs accounted for 93.0% of the total variation (Table 2.21).

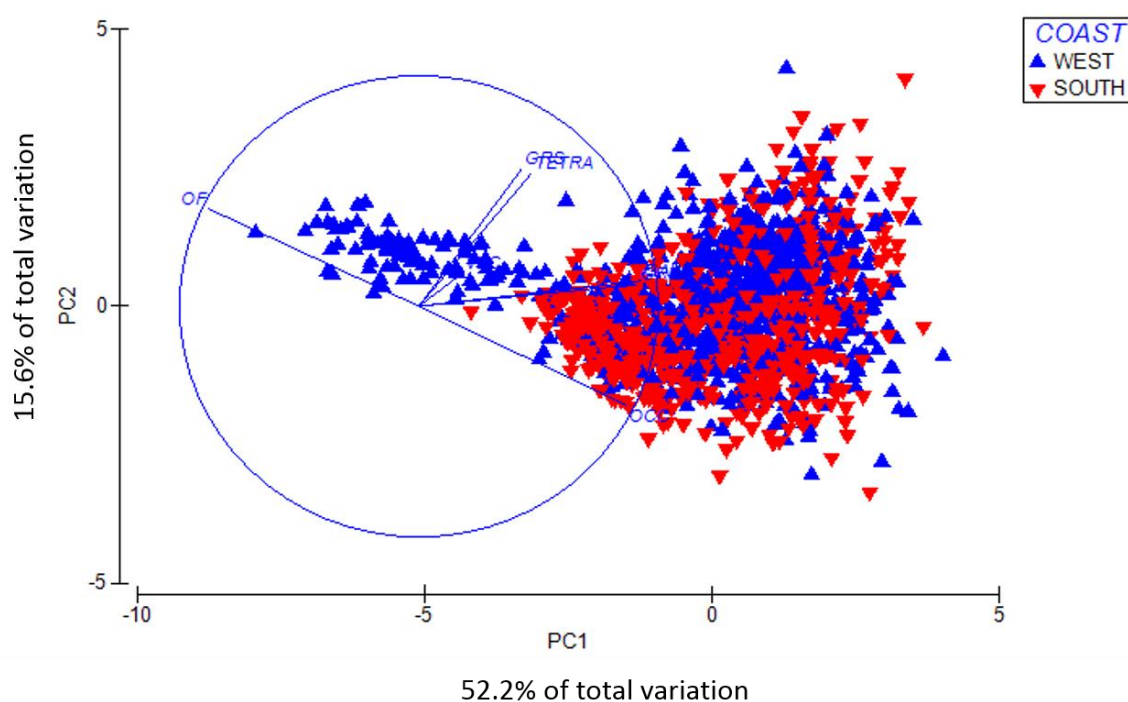


Figure 2.49. PCA ordination of *Sardinops sagax* from the putative western and southern stocks off the South African coasts in 2013 and 2014, based on the variables under study namely: GAL, gill arch length; GRS, gill raker spacing; GRN, number of gill rakers; OF, otolith form factor; OCC, otolith circularity; VBC, vertebral count; and TETRA, metacercariae parasite abundance.

Table 2.21. Eigenvalues of Principal Components and the % variation explained.

Principal component	Eigenvalues	%variation	Cumulative %variation
1	3.65	52.2	52.2
2	1.09	15.6	67.8
3	0.982	14	81.8
4	0.783	11.2	93

Looking at the correlation between these Principal Components and the original variables (Table 2.22), the first PC explained 52.2% of total variation and is strongly correlated with four of the original variables namely: gill arch length (GAL), gill raker spacing (GRS), otolith form factor (OF) and otolith circularity (OCC). This indicates that these variables vary together and that an increase in one means an increase in the others. It would follow that

individuals with a long gill arch length will most likely have a higher number of gill rakers, higher otolith circularity values (more elongated otoliths) and lower otolith form factor values (more irregular outlines). A strong negative correlation (-0.8779) of OF with PC1 is observed to drive the separation of a group of west coast fish from the rest of the individuals implying that these individuals have a lower otolith form factor than the rest.

Table 2.22. Correlation between Principal Components and original variables. Values ≥ 0.5 are considered important and are presented in bold. Abbreviation: GAL, gill arch length; GRS, gill raker spacing; GRN, number of gill rakers; OF, otolith form factor; OCC, otolith circularity; VBC, vertebral count; and TETRA, metacercariae parasite abundance.

Principal Component	Variables						
	GAL	GRS	GRN	TETRA	OF	OCC	VBC
PC1	0.9295	0.4311	0.9110	0.4756	-0.8779	0.8656	0.1794
PC2	0.1099	0.5967	0.0945	0.5762	0.4224	-0.4288	0.1411
PC3	-0.0855	0.0307	-0.1174	-0.1658	-0.0423	0.0476	0.9636
PC4	0.0008	-0.6475	0.0375	0.5862	0.0284	-0.0345	0.1291

A weak correlation of PC1 with parasite abundance and gill raker spacing was also observed, but because both correlation values were < 0.5 they were not considered as important on PC1. Nevertheless, they contributed to the direction of variation explained on this axis. PC2 accounted for 15.6% of total variation and increases with increasing gill raker spacing (GRS) and metacercaria abundance (TETRA). This suggests that individuals with wider spaced gill rakers will tend to have higher number of metacercariae.

PC3 explained 14% of the total variation and increases with increasing number of vertebrae (VBC). PC4 explained 11.2% of the total variation. Again, gill raker spacing (GRS) and metacercaria parasite abundance (TETRA) are found to be correlated with this PC axis, although this PC increases with decreasing gill raker spacing, implying that individuals with narrow gill raker spacing will most likely have higher number of metacercariae. It is interesting to observe that metacercaria abundance has the roughly same correlation value (approximately 0.6) in both PC2 and PC4. One would deduce that metacercaria abundance increases regardless of the width of gill rakers. PC2 and PC4 capture the relationship

between gill raker spacing and metacercaria abundance in both individuals with narrow and wide gill raker spacing.

Five classification models (Linear discriminant analysis [LDA], Quadratic discriminant analysis [QDA], Artificial neural network [ANN], Classification tree analysis [CTA] and Random Forest [RF]) were used to determine if one can discriminate between *Sardinops sagax* individuals sampled from the west and south coasts of South Africa (the putative western and southern stocks). Five models were used in order to see how the different models perform bearing in mind that there are assumptions that might not be met by the data under analysis.

The combinations of variables selected by each model based on the step-wise selection procedure and their respective overall classification (training and validation) accuracies are shown in Table 2.23. The combinations of variables that gave the highest overall classification accuracy (i.e. the most important predictor/s) differed among the five classification models. Generally, overall classification accuracy increased up to a point on addition of predictors to the model. Cross-validation is a model evaluation method that is used to evaluate the performance of a trained model on new data set which it has not seen. It measures how well the model will do when asked to make new predictions on a new data set. Therefore, even though overall classification accuracies of the LDA, QDA and ANN models appear to increase with the addition of more variables, their cross-validated classification accuracies which decrease on addition of more variables show that these models do not have good predictive power compared to CTA and RF.

In the LDA, the overall classification accuracy for the training set was highest (52%) in the model that included all the variables. In contrast, upon validation, the model that included only vertebral count (VBC) gave the highest overall classification rate (49%). The addition of more predictor variables in the LDA did not increase the overall accuracy in the validation dataset, rather there was a decrease. Results indicate a very low predictive power of this classification tool which is not different to a 50% random chance. In the QDA, the highest cross-validated overall accuracy (62%) was obtained using two models, namely the model which had gill raker spacing (GRS) as the only predictor, and the model which had the variable combination of gill raker spacing, vertebral count, number of gill rakers, parasite

abundance, and otolith form factor (GRS + VBC + GRN + TETRA + OF). In ANN, the variable set comprising gill raker spacing, parasite abundance, gill arch length and number of gill rakers (GRS + TETRA + GAL + GRN) gave the highest overall cross-validated classification accuracy of 71%, and further addition of variables to this model decreased classification accuracy. Both QDA and ANN had a fairly good predictive power of 60-70%. CTA and RF had similar results; they both selected the same variable set, namely vertebral count and parasite abundance (VBC + TETRA) and these gave the highest overall cross-validated accuracy (82% for CTA and 93% for RF). Further addition of variables did not increase overall cross-validated classification accuracy in either of these models.

Predictive performance differed among the five classification models (Table 2.24). Four of the five models had an overall classification accuracy of >50%, the exception being the Linear Discriminant Analysis that had a value of 47% (Table 2.24). The Random Forest model had the highest overall classification accuracy of 91%.

CTA and RF performed very well, with both models having high (>80%) classification accuracy both in the overall classification and in the classification of individuals from the two regions (west and south coasts) and hence putative stocks. Though predictive power is higher in RF compared to CTA, both were able to successfully differentiate between individuals from the western and southern sardine stocks. ANN had relatively high (70%) predictive power however it had a poor allocation success (54%) for individuals from the putative southern stock. The other classification models, QDA and LDA, had lower overall classification accuracy indicating the poor predictive power of their variable combinations in the discrimination of individual sardine from the west and south coasts.

Table 2.23. Model selection of variables from *Sardinops sagax* from the putative western and southern stocks off the South African coast in 2013 and 2014 based on step-wise selection procedure. Important predictors as selected by the different models and the overall cross-validated classification accuracies are indicated in bold.

Classification model	Combination of variables	Overall accuracy (validation)	Overall accuracy (training)
LDA	VBC	0.494	0.511
	VBC + GRN	0.486	0.509
	VBC + GRN + OCC	0.480	0.506
	VBC + GRN + GAL + TETRA	0.471	0.507
	VBC + GRN + OCC + GAL + TETRA	0.463	0.508
	VBC + GRN + GAL + TETRA + GRS + OF	0.461	0.512
	VBC + GRN + OCC + GAL + TETRA + GRS + OF	0.449	0.515
QDA	GRS	0.620	0.615
	GRS + GRN	0.616	0.621
	GRS + GRN + TETRA	0.617	0.630
	GRS + GRN + TETRA + OF	0.612	0.631
	GRS + VBC + GRN + TETRA + OF	0.620	0.630
	GRS + VBC + GRN + TETRA + GAL + OCC	0.619	0.635
	GRS + VBC + GRN + TETRA + OF + GAL + OCC	0.617	0.637
ANN	GRS	0.625	0.631
	GRS + TETRA	0.682	0.701
	GRS + TETRA + GAL	0.689	0.731
	GRS + TETRA + GAL + GRN	0.707	0.747
	GRS + TETRA + GAL + GRN + OCC	0.705	0.768
	GRS + TETRA + GAL + GRN + OCC + VBC	0.702	0.769
	GRS + TETRA + GAL + GRN + OCC + VBC + OF	0.702	0.775
CTA	VBC	0.752	0.783
	VBC + TETRA	0.822	0.855
	VBC + TETRA + OF	0.822	0.855
	VBC + TETRA + OF + GAL	0.822	0.855
	VBC + TETRA + OF + GAL + GRN	0.822	0.854
	VBC + TETRA + OCC + OF + GAL + GRN	0.821	0.855
	VBC + TETRA + OCC + OF + GAL + GRN + GRS	0.796	0.842
RF	VBC	0.905	0.904
	VBC + TETRA	0.926	0.925
	VBC + TETRA + GRS	0.880	0.878
	VBC + TETRA + GAL + OCC	0.887	0.887
	VBC + TETRA + GRS + GAL + OCC	0.862	0.858
	VBC + TETRA + GRS + GAL + GRN + OF	0.847	0.839
	VBC + TETRA + GRS + GAL + OCC + GRN + OF	0.819	0.820

Table 2.24. Comparison of the predictive performance (Mean $\pm$ SE) of the five classification models based on variables obtained from *Sardinops sagax* from the putative western and southern stocks off South African coast in 2013 and 2014. Cross-validation results are shown.

Model	Overall classification accuracy (%)	Classification accuracy (% , West)	Classification accuracy (% , South)
LDA	47 $\pm$ 0.05	47 $\pm$ 0.08	46 $\pm$ 0.08
QDA	61 $\pm$ 0.05	58 $\pm$ 0.07	66 $\pm$ 0.08
ANN	70 $\pm$ 0.05	80 $\pm$ 0.06	54 $\pm$ 0.09
CTA	82 $\pm$ 0.05	83 $\pm$ 0.09	81 $\pm$ 0.08
RF	91 $\pm$ 0.03	94 $\pm$ 0.04	87 $\pm$ 0.05

DISCUSSION

Morphometric, meristic and parasite biotag approaches were used to assess the population structure of South African sardine off the west and south coasts and the results from this study provide strong evidence for phenotypically differentiated western and southern stocks. Although analyses of mitochondrial and microsatellite DNA have indicated that the South African sardine population is genetically homogenous (Hampton 2014) due to mixing between the stocks, there is still sufficient separation to cause phenotypic variation in meristic and morphological and parasite biotag characters as observed in the present study.

Body shape

Analysis using geometric morphometrics showed that sardine from the putative western stock differed from sardine from the putative southern stock in their body shape. This could be attributed to morphological variation within populations which is influenced by local environmental factors such as temperature, salinity, food availability (Begg & Waldman 1999, Swain & Foote 1999). Hence, these variations are used in discriminating between groups that share similar ontogenic rates or reproductive dynamics, and as such infer phenotypic stocks (Cadrin 2000, Cadrin *et al.* 2005). The western and southern coast of South Africa have different temperature conditions and different levels of food availability; the former being colder and more productive than the latter (Miller *et al.* 2006, Hutchings *et*

al. 2009), are hypothesized to lead to changes in the body shape observed in this study. The current system off the west coast is characterized by the cold Benguela Current flowing up the coast from the Southern Ocean, as well as the Lüderitz upwelling cell which is caused by the southeasterly wind blowing surface water offshore, especially in summer. Conversely, the warm Agulhas Current which flows eastwards down from the Mozambican coast characterizes the southeastern coast of South Africa. The division of these two oceanographic systems is thought to be located in the region of Cape Agulhas (Barange *et al.* 1999, van der Lingen *et al.* 2006, Branch *et al.* 2010). In addition to the existing difference between the two aquatic environment, the huge amount of sediment and freshwater that flows into the west coast from the Orange River (the largest river in South Africa) further influences the oceanic conditions of the west coast (Reed *et al.* 2012).

In this study, two major shape differences between the two sardine stocks mainly related to body curvature (concave versus convex) and body width ('fat' versus 'slender') were revealed by the Principal Component Analysis (Figures 2.8A, 2.8B). Additionally, localized shape changes (Figures 2.9a, 2.9B) were shown in the head and tail region (short head, long tail versus long head, short tail), as well as in the ventral body region from the pelvic fin to the tail (constriction vs no constriction). Furthermore, Discriminant Function Analysis, incorporating all these changes, clearly discriminates between the two stocks with a moderate (> 60%) cross-validated classification accuracy (Figure 2.10, Table 2.6), supporting the evidence of the existence of two stocks.

Interestingly, results of body shape analysis supported the subjective evidence, given by fishermen, of two sardine 'species' in South Africa namely: the southeastern small-sized species with big belly which in this study, is graphically illustrated as the average shape with a 'slender', convex body, a long tail and a 'pot belly'; and the bigger west coast species graphically illustrated as the average shape with a 'fat', concave body and a short tail (Fairweather *et al.* 2006b). The findings of the present study agree with Wessels (2009) and Groenewald *et al.* (in prep), who found significant differences in body shape in sardine from the South African west and south coasts.

Similar to the findings of the present study, previous studies have discriminated between sardine and other fish stocks based on differences in the linear measurements of their heads. For sardine *Sardina pilchardus* in the Northeastern Atlantic and Western Mediterranean, two morphotypes were distinguished based on their head-to-body ratios (Silva 2003). The Mediterranean horse mackerel *Trachurus mediterraneus* populations in the Black, Marmara, Aegean and Eastern Mediterranean Seas were distinguished based on the differences in their head measurements which was attributed to habitat use and prey size (Turan 2004). Turan *et al.* (2006) reported that mouth length and body depth had the highest discriminatory power in stock structure studies of the bluefish *Pomatomous saltatrix* in the Black, Marmara, Aegean and northeastern Mediterranean Seas. In New Zealand, shape difference in stocks of orange roughy *Hoplostethus atlanticus* occupying Puysegur Bank and Lord Howe Rise was attributed to mostly head measurements (Haddon & Willis 1995).

Branchial sieve morphometrics and meristics

As expected, gill arch length and the number of gill rakers increased as fish size increased because body parts naturally increase with size (Bone *et al.* 1995). Similar trends were reported in herring *Clupea harengus* (Gibson 1988), Pacific mackerel *Scomber japonicus* (Molina *et al.* 1996), Atlantic menhaden *Brevoortia tyrannus* (Friedland *et al.* 2006) and South African sardine *Sardinops sagax* (Idris 2010, Idris *et al.* 2016). On the other hand, the number of gill rakers has been found to increase with size only up to a certain point. For example, the number of gill rakers in anchovy *Engraulis encrasicolus* did not increase beyond 80mm caudal length (King and Macleod 1976). Also, the number of gill rakers in Japanese anchovy *E. japonicus*, Pacific jack mackerel *Trachurus japonicus* and Pacific round herring *Etrumeus teres* were observed to be almost constant at fish lengths greater than 100mm, 80mm and 90mm, respectively (Tanaka *et al.* 2006).

Gill raker spacing also increased as fish length increased. King and Macleod (1976), Gibson (1988), and Idris (2010) and Idris *et al.* (2016) separately reported similar observation in anchovy *Engraulis encrasicolus*, herring *Clupea harengus* and South African sardine

Sardinops sagax respectively. Conversely, in some filter feeders like Pacific mackerel *Scomber japonicus* that have gill rakers only on the first gill arch, gill raker spacing decreased as fish length increased (Molina *et al.* 1996).

Sardine from the putative western and southern stocks differed significantly in their gill arch length and number of gill rakers, with individuals from the southern stock having longer gill arches and a higher number of gill rakers. However, no difference was observed in the gill raker spacing in the two stocks, even though gill raker spacing was smaller in sardine from the putative western stock. This phenotypic variation could be as a result of the difference in the trophic environments leading to a difference in diets in the two regions. *Sardinops sagax* is omnivorous, feeding both on phytoplankton and zooplankton. However, zooplankton (mainly small calanoid and cyclopoid copepods, anchovy eggs and crustacean eggs) is the major contributor of dietary carbon (van der Lingen 2002). On the west coast, sardine feeds mainly on the smaller cyclopoid copepods (Louw *et al.* 1998, van der Lingen 2002) while in the south coast, calanoid copepods are the second greatest contributors dietary carbon after fish eggs (Mketsu 2008). van der Lingen (2002), studying the diet of sardine from the areas off Cape Columbine to east of Port Elizabeth, reported that percentage of calanoid copepods in sardine diet increased to 96% as sampling moved eastward. Sardine from the west coast also ingests a greater amount of phytoplankton compared to south coast sardine (Davies 1957, van der Lingen 2002, van der Lingen *et al.* 2009). Results of the present study support the findings made by Idris (2010) and Idris *et al.* (2016) who also reported that gill arch length and number of gill rakers were able to differentiate South African sardine *Sardinops sagax* from the west and south coasts. Similar to the present study, they also reported no significant difference in gill raker spacing between sardine from the western and southern stocks. However, they reported small sardine from south coast to have shorter gill arch length than sardine from the west coast. The present study did not have many small fish from the two coasts in its data set, hence comparison of results cannot be made. Previous studies have discriminated between fish stocks based on variation in branchial sieve morphology. These include round sardinella *Sardinella aurita* (Kinsey *et al.* 1994), sardine *Sardina pilchardus* (Andreu 1969), orange roughy *Hoplostethus atlanticus* (Haddon & Willis 1995), Arctic char *Salvelinus alpinus*

(Malmquist 1992), European white fish *Coregonus lavaretus* (Amundsen *et al.* 2004) and Mediterranean horse mackerel *Trachurus mediterraneus* (Turan 2004).

Feeding behavior involves both filter feeding and particulate feeding, with sardine switching from filter feeding to particulate feeding at prey size >1.23mm (van der Lingen 1994). Therefore, phenotypic plasticity as well as the ability to switch feeding behavior facilitates the ingestion of different sizes and densities of prey by the South African sardine (Idris 2010).

Otolith shape indices

Phenotypic variability in otolith shape is influenced by genetic as well as environmental factors such as food availability, water temperature, depth and salinity (Ponton 2006, Vignon & Morat 2010). Individual variability has been observed, although its cause is unknown (Vignon & Morat 2010). In general, otoliths in juveniles (larvae and recruits) have a more spherical shape which develops into the characteristics shape of the species, through the gradual deposition of minerals as fishes grow (Hampton 2014). Hüsey (2008) reports that smaller immature fish show a relatively faster rate of change in otolith shape than mature fish. Otoliths are metabolically inert structures which are laid down early and grow continuously throughout a fish's life. They are species-specific, hence differences in otolith shape within a species would suggest that the individuals have spent most of their life in a different environment (Campana & Casselman 1993, Campana 1999, Turan 2006).

Otolith shape has been used to differentiate between sardine stocks elsewhere, including Pacific sardine *Sardinops sagax* (Félix-Uraga *et al.* 2005, Javor *et al.* 2011) and common sardine *Strangomera bentincki* (Curin-Osorio *et al.* 2012). Also, other fish stocks have been discriminated based on otolith shape variability, e.g. herring *Clupea harengus* (Burke *et al.* 2008), horse mackerel *Trachurus trachurus* (Stransky *et al.* 2008), and anchovy *Engraulis encrasicolus* (Kristoffersen & Magoulas 2008). Methods used in analysing otolith shapes include, but are not limited to, geometric morphometrics, Fourier analysis, as well as multivariate discriminant analysis of differences in otolith morphometrics and shape indices. The present study used otolith shape indices (otolith form factor and circularity) in discriminating between the putative western and southern stocks of South African sardine.

Tuset *et al.* (2003) identified otolith form factor and circularity as dimensionless indices which are sensitive to small differences between individuals from different coasts, with form factor being more informative. Thus, they are useful in stock identification.

In the present study, the otolith circularity value increased as fish length increased. Hence, the otoliths of larger fish tended to be more elongated than the otoliths of younger fish, which were more circular. On the other hand, otolith form factor decreased with an increase in fish length. Therefore, otoliths of older fish had irregular outlines as opposed to otoliths from the smaller individuals which had smoother edges. These relationships were also observed in South African small pelagic fish including sardine by Hampton (2014), and agrees with Capoccioni *et al.* (2011) who suggested that small fish are more likely to have smoother and rounder otoliths.

GLM analysis showed that after fish length stock is the next most important variable in discriminating between western and southern sardine stocks. West coast sardine had a significantly different shape from the south coast sardine, the former having a smoother and rounder shape than the latter. This could be as a result of the different environmental condition between the two coasts (Hutchings *et al.* 2009), such that otoliths of fish caught in the nutrient-rich west coast would be smoother and rounder while otoliths of individuals caught in the less productive south coast would be more irregular and longer. Also, significant seasonal variability was observed in the study and could be attributed to seasonal changes in food availability, water temperature and salinity (Hutchings *et al.* 2009). Again, sardine otolith shape significantly differed in male and female fish. Males tended to have longer otoliths with more irregular edges. The reason for this variability is not known but could be related to the condition factor at the time of capture. The factors that primarily determine otolith shape are not well understood (Burke *et al.* 2008). Differences in otolith shape could be as a result of past growth rate (Reznick *et al.* 1989, Ponton 2006) or biological processes (Zelditch *et al.* 2004).

The findings of the present study differed from those of Hampton (2014) who did not find any significant difference in otolith shape indices of sardine from the west and south coasts

of South Africa, even though South African east coast sardine were found to have otoliths of significantly different shape to those of fish from the South African west and south coast sardine as well as Namibia. This difference in results of the two studies could be temporally driven. Data used by Hampton (2014) could have been collected during periods of high degree of mixing between putative western and southern stocks, hence no significant difference was found in otolith shape indices of these two stocks. de Moor *et al.* (in press) reported substantial inter-annual differences in the extent of mixing between South African sardine stocks. Since phenotypic traits such as otolith shape is environmentally-mediated, the loss of difference in otolith shape indices reported by Hampton (2014) could reflect periods of small environmental variability between west and south coasts. Idris *et al.* (2016) in their study of the spatial variability in branchial basket morphology and meristics of southern African sardine, explained that temporal variability in oceanographic conditions could affect sardine development and distribution, and thus might blur signals from phenotypic characters.

Results of this study showed that otolith shape indices is an effective discriminator of individuals from the putative western and southern stocks off the coast of South Africa. However, the difference between the results of the present study and Hampton (2014) indicate that temporal instability could influence its effectiveness as a stock discriminator.

Metacercariae parasite abundance

A significant positive relationship was found between ‘tetracotyle’ type metacercariae abundance and fish length. This implies that metacercariae accumulate in sardine and fish do not ‘lose’ or ‘shed’ the parasites. Usually, parasite indices (e.g. infection intensity, abundance) are influenced by size. Timi and Poulin (2003) in their study on the relationship between parasite assemblages and fish size, observed a positive relationship between fish length and the characteristics of a parasite population such as infection intensity. A size effect is commonly observed in fish-parasite systems because older/larger fish have higher feeding rates, feed on larger particles, and have been exposed to parasites for a longer period (Timi & Poulin 2003).

The potential of the 'tetracotyle' type metacercaria which infects sardine eyes as a biotag for the South African sardine was first reported by Reed *et al.* (2012). Their results were supported by Weston (2013) and Weston *et al.* (2015) who reported significant variability in prevalence, infection intensity and abundance of this parasite in sardine from the putative western and southern stocks, with the endemic area most likely occurring off the west coast. Thus, those results indicated the existence of western and southern stocks on either side of Cape Agulhas, thereby supporting the hypothesis of two mixing stocks.

Previous studies have discriminated fish stocks using fish parasites. For example, Timi (2003) identified stocks of Argentine anchovy *Engraulis anchoita*. McClelland and Melendy (2011) delineated stocks of Atlantic cod *Gadus morhua* using parasite biotags. Mattiucci *et al.* (2008) delineated stocks of Atlantic horse mackerel *Trachurus trachurus* using *Anisakis* larvae. The present study found significant spatial variability in the abundance of 'tetracotyle' type metacercaria in the putative western and southern sardine stocks. This agrees with the findings of Reed *et al.* (2012), Weston (2013), van der Lingen *et al.* (2015) and Weston *et al.* (2015), and provide further strong evidence for the existence of western and southern sardine stocks off the South African coast.

The distribution of marine parasites, especially in their endemic area, is mainly determined by temperature and salinity gradients of the system (Timi 2003, Timi & Poulin 2003, MacKenzie 2005, Timi 2007), as well as the distribution of the intermediate hosts (for parasites that have indirect life cycle; Janovy *et al.* 1997, MacKenzie 2005). The observed difference in the abundance of 'tetracotyle' type metacercariae around the South African coast may directly or indirectly be as a result of the different aquatic conditions in the west and south coasts. Given the higher abundance of metacercariae observed in the west coast, as well as the suitable environmental conditions provided by this region for both the metacercaria and the first intermediate host, it would follow that the endemic area of this parasite is most likely off the west coast. Thus, the metacercaria transmission from the first intermediate host to the second intermediate host (sardine *Sardinops sagax*) is facilitated (Weston 2013). However, if the endemic area is restricted to the west coast as

hypothesized, one would expect to observe zero abundance levels in fish on the south coast. However, sardine has a much greater distribution compared to the metacercaria. This implies that metacercariae get transported to the south coast by infected sardine from the west coast, thus explaining the gradient in metacercaria abundance observed in this study. This transportation of metacercariae across coasts could occur during the movement of western stock recruits and adults to the southern stock (de Moor & Butterworth 2013, van der Lingen & Hendricks 2014; de Moor & Butterworth 2015; de Moor *et al.* in press). And seasonal visits to the west coast by south coast individuals could also result in infection of southern stock fish by metacercariae. High levels of parasite abundance (as high as 71 parasites in fish (19.8cm CL) from Mossel Bay) found in the putative southern stock in the present study indicates fish that have spent a long time on the west coast before moving to the south coast where it was caught. Because of the cumulative infection of fish by metacercariae and the inability of fish to 'lose' the infection, a reduction in parasite abundance would be caused by changes in fish population level (such as fishing and predation), especially for sardine from the putative western stock which inhabit the endemic area of the parasite. Both fishers and predators target larger fish, hence removal of these fish may affect parasite overall abundance values in both putative stocks. Additionally, the parasite itself may have a negative impact on the sardine and cause mortality at high infection, as has been suggested for infection of seabream *Diplodus vulgaris* by a parasite of the same genus, *Cardiocephaloides longicollis* (Born-Torrijos *et al.* 2016).

Timi (2003) reported that the Argentinian anchovy *Engraulis anchoita* inhabiting the cold southern region of the southwest Atlantic is characterized by infection with 'tetracotyle' type metacercaria of *Cardiocephaloides physalis* which occurs mainly in colder waters supporting the hypotheses that the endemic area of this parasite is in the west coast. The metacercaria examined in the present study is thought to be the same species (Reed *et al.* 2012). The life cycle of this parasite is not completely understood. *Cardiocephaloides physalis* adults have been reported from the duodenum of African penguin, *Spheniscus demersus* (Randall & Bray 1983, Horne *et al.* 2011). Since sardine is one of the main dietary item of this bird species (Randall & Randall 1986), one would deduce that penguins got infected by eating fish infected with metacercariae.

Knowledge of the identity and distribution of the first intermediate host is essential in the determination of the location and distribution of the parasite endemic area (Janovy *et al.* 1997, MacKenzie 2005). Digenetic trematodes commonly use molluscs as their first intermediate hosts (Roberts & Janovy 2009). Thus, it is assumed that the metacercaria in present study utilizes a gastropod as its first intermediate host (Reed *et al.* 2012). The metacercaria of the congener *Cardiocephaloides longicollis* uses the sea bream *Diplodus annularis* as its second intermediate host and marine birds are its definitive hosts. Its 'tetracotyle' type metacercaria infects the brain of sea breams (Osset *et al.* 2005) and has preference for mainly larval demersal and benthic fish (Born-Torrijos *et al.* 2016). Iannotta *et al.* (2009) reported that the first intermediate host for this parasite is the marine gastropod *Nassarius corniculus* which is a common scavenging whelk that occurs in marine and brackish waters of the Mediterranean. It is hypothesized that an intertidal whelk, *Burnupena papyracea*, could be the first intermediate host of the *Cardiocephaloides* metacercarian parasite in this study. *Burnupena papyracea* is a cooler water species, inhabiting the west coast of South Africa. It is very abundant in the subtidal zone (Orr 1956, Branch *et al.* 2010) and its distribution fits the endemic area hypothesized for the 'tetracotyle' type metacercariae (Weston 2013). Therefore, its presence off the west coast only would explain the higher abundance levels of the parasite in the putative western stock. Definitive identification of the first intermediate host of this parasite is critical to gaining a full understanding of its life cycle.

Weston *et al.* (2015) analysed sardine sampled from commercial catches collected throughout the year and reported significant seasonal and interannual effects on infection prevalence and intensity and the abundance of metacercarian parasites, but there was no significant difference in parasite abundance between seasons observed in this study. Most of the samples in the study were collected during summer and winter research surveys by the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa, in 2013 and 2014, with a few commercial samples added to increase the sample size. The reason why no seasonal pattern in abundance was found in the present study may be due to this limited temporal coverage.

Previous studies have reported the effect of season on parasite populations of many fish species. Zander and Kesting (1998) reported an increase in parasite abundance in gobiid fishes in the southwestern Baltic Sea during summer. Harrod and Griffiths (2005) reported a summer increase in infection intensity of the strigeid *Ichthyocotylurus erraticus* in the host *Coregonus autumnalis*. Timi (2003) also found seasonal patterns in prevalence and abundance of parasites of the Argentine anchovy *Engraulis anchoita*. The seasonal patterns were attributed to the geographic origin of samples, with significantly higher values in prevalence and abundance observed in spring than in autumn.

Vertebral count

No significant difference was found in the number of vertebrae in sardine from the putative western and southern stocks. The number of vertebrae, being a meristic character, is fixed early in the larval stage of the fish and as such is dependent on the environmental conditions at that time (Barlow 1961, Florence *et al.* 2002). One would expect to see a significant difference in this parameter since the west and south coast have spawning habitats and nursery areas influenced by different environmental conditions. It is assumed that fish spawned on the west coast and western Agulhas Bank mostly develop in cooler waters influenced by upwelling and the cold Benguela Current, while fish spawned on the eastern Agulhas Bank develop in the warmers influenced by the warm Agulhas Current (Miller *et al.* 2006). de Moor & Butterworth (2015) developed a two-mixing-stock assessment model for South African sardine that assumes that some west-stock recruits move to and join the south stock each year. Movement is estimated to be appreciable, and as a result of mixing individuals within the southern stock could have a more variable number of vertebrae, making stock identification difficult (Begg & Waldman 1999, Murta *et al.* 2008). This could be the reason for vertebral bone count not being an effective tool in the discrimination of sardine from the west and south stocks.

Integration of data using a Stock Differentiation Index (SDI)

Apart from vertebral count and gill raker spacing, other methods of stock identification analysed on the univariate platform supported the two-stock hypothesis. Previous studies on the population structure of the South African sardine *Sardinops sagax* recommended that data from these individual methods should be integrated into a multidisciplinary study in order to get conclusive information on the stock structure (van der Lingen *et al.* 2015). Integrated approaches combine information obtained from a range of temporal and spatial scales such that stocks could be identified with a high degree of confidence (Begg & Waldman 1999, Waldman 1999, Abaunza 2008, Welch *et al.* 2009, 2015).

In the present study, the SDI of 0.75 indicated that western and southern sardine stocks are two interacting stocks. SDI, as a semi-quantitative approach (Welch *et al.* 2009, 2015), has been used in the identification of regional differences among sardine stocks, as well as in the establishment of semi-quantitative measures of stock separation in Australia (Izzo *et al.* 2017). However, similar to individual methods of stock identification, the conclusions drawn from this approach are limited by the extent to which samples represent the population or sub-group, especially if data was collected only from commercial fisheries that operate at discrete locations and seasons. In other words, conclusion is limited by sample size. Hence, relying only on samples from commercial fishing affects the results of any stock identification methods because results generated from such samples are most likely to be biased towards maintaining traditional stock management units (Izzo *et al.* 2017). Samples used in the present study were obtained from mainly research surveys and comprised 73.3% of total samples used in the study. Therefore, samples collected for this study may be considered to provide more accurate representations of the population and stocks from which they were drawn.

Since the SDI approach recognises differences and similarities between regional groups, patterns of connectivity among adjacent groups may not be picked up. This limits the application of this approach to the highly migratory pelagic species. However, SDI provides a good starting point for holistic studies of the stock structure of pelagic species, especially in

the absence of other quantitative methods for combining multi-disciplinary data (Izzo *et al.* 2017). Nominal SDI value thresholds proposed by Izzo *et al.* (2017) helped in detection of mixing among stocks. Nominal SDI values < 0.33 indicates weak separation of stocks, $0.33 - 0.66$ indicates a moderate separation while > 0.66 indicates a strong separation. It would follow that as SDI value increases, the level of mixing decreases; such that the value of 1 would indicate no mixing between stocks (an unlikely event) and the value of 0 would indicate the presence of a single stock. The combination of SDI method with multi-criteria decision analyses (Stewart 2008) may improve future application of this method and overcome some limitations associated with drawing conclusions from spatially and temporally disparate data sets (Walmann 1999).

Multivariate analyses of data

Multivariate analyses of data confirmed the existence of two mixing stocks off the coast of South Africa. Analyses of subsets of data indicated inter-annual variation in the data. Hence, dataset was partitioned and analysed by sampling year (2013 and 2014). A combined dataset was also analysed as well. Results of the PERMANOVA for all datasets (2013, 2014, combined) supported the two-stock hypothesis. A high degree of correlation was observed between principal components and the original variables. Important variables on PC1 were gill arch length, number of gill rakers, otolith form factor, and otolith circularity. On PC2 and PC4, gill raker spacing and parasite abundance were important discriminators, while vertebral count was the only discriminator on PC3. Spatial discriminatory power differed among the five classification models used in the study (Table 2.21). Random Forest (RF) and Classification Tree Analysis (CTA) had the highest overall allocation success, followed by Artificial Neural Network (ANN). Quadratic Discriminant Analysis (QDA) and Linear Discriminant Analysis (LDA) had the least success. These varying degrees of classification success are explained by the differential ability of the models to detect non-linear relationship between variables in the data sets from the two stocks. Random Forest, Classification Tree Analysis and Artificial Neural Network are statistical tools used in modelling non-linear relationships and interactions in variables and were therefore able to capture the variability in the data sets. Comparing the performance of the different models

will help to decide on the model(s) to be used in future classification analysis. Based on the results, Random Forest and Classification Tree Analysis have shown to be the most suitable models for analysing data of this type.

It was interesting to observe that for all models, except Linear Discriminant Analysis, parasite abundance consistently contributed to the variable combinations that gave the highest overall classification accuracy (Table 2.22). Important variables were reduced to two (VBC + TETRA) in CTA and RF, which were the two models with the highest classification accuracy. This indicates that vertebral count and metacercaria parasite abundance were the two strongest discriminators between western and southern stocks. It also agrees with van der Lingen *et al.* (2015) that the parasite biotag approach is a powerful and efficient technique compared to other methods for discriminating between stocks of the South African sardine. Vertebral count was not significant in the univariate analysis, but was found to be a strong discriminator in the multivariate analysis. This could be because of the non-linear and interacting differences between the two coasts that are only picked up by multivariate methods such as Classification tree analysis and Random Forest, but not by univariate methods (Zuur *et al.* 2007). This result has equally provided convincing evidence for the two-stock hypothesis and contributed to the stock assessment studies and development of management strategies for the South African sardine resource (de Moor & Butterworth 2013, 2015), including using parasite prevalence-at-length data to estimate the movement of sardine from the western stock to the southern stock (de Moor *et al.* in press). Similarly, in the Principal Component Analysis, parasite abundance was weakly correlated with PC1, and strongly correlated with PC2 and PC4, indicating also that it is a strong predictor of stock. The identification of parasite species is necessary not only for effective diagnosis, treatment and control of parasitic diseases; but also for the success of future taxonomic and ecological studies. This is because it provides the framework for future experimental study, and enhances our understanding of parasitism (McManus & Bowles 1996). Given the observed importance of the 'tetracotyle' type metacercaria in this study, it is necessary that its identity is confirmed and life cycle is known. The subsequent chapters seek to develop morphological and molecular characterizations of the metacercariae, laying

the foundation for future investigations into this parasite and, importantly, the identification of its first intermediate host.

In conclusion, a thorough understanding of population structure is essential for the delineation of stock boundaries and the development of appropriate management regulations (Kutkuhn 1981, Grimes *et al.* 1987), especially when populations are made up of multiple stocks (Altukhov 1981) that have variable levels of productivity and are exploited at different levels (Ricker 1981, Hunt & Neilson 1993).

The multidisciplinary approach is an efficient method of investigating stock structure, as information from multiple perspectives is obtained in such studies which results in less uncertainty in interpretation (Abaunza *et al.* 2008). In the present study, multiple methods allowed for the collection of multiple stock descriptors (from the same set of individual fish) which when analysed multivariately gave strong support for the existence of two sardine stocks: western and southern stocks.

With the integration of available information from a range of stock identification methods, a generalised and stable pattern of stock structure is developed, which forms the scientific basis for sustainable stock management (Stransky *et al.* 2008). However, in practice, some of these techniques are costly and time consuming, therefore it is better to choose the most practical and useful approaches. The selection of a suitable technique depends on the relative cost (per sample) of data generation and analysis, the technical expertise, sampling and processing equipment, as well as the levels of stock resolution, accuracy and precision required (Beacham *et al.* 1998). Data on vertebral count (though not a strong discriminator of sardine stocks when tested independently, and thus not an effective discriminator when used alone) and parasite abundance have been found to be useful in the discrimination of sardine from the putative western and southern stocks. These two methods are relatively cheap to process and analyse samples, require low level of expertise (the 'tetracotyle' type metacercaria is relatively easy to identify), and hence require less time and simpler equipment to process samples than for other data.

The results of this study strongly supports the sardine multi-stock hypothesis and provides evidence of the presence of two stocks, one on the South African west coast (the western stock) and on the south coast (the southern stock), with some mixing between them as indicated by the overlap in some morphometric (gill raker spacing) and meristic (vertebral count) data. According to the two-mixing-stock assessment models of de Moor and Butterworth (2015) and de Moor *et al.* (in press), the west stock is more productive than the south stock, and the south stock recorded a peak in its biomass in the early 21st century as a result of the movement of a substantial number of recruits from the western stock. Currently, west stock abundance and recruitment is below average, and is a cause of concern because the west stock spawner biomass and recruitment are the key feeders to the sardine population on the south as well as west coasts. Therefore, the findings in the present study have management implications for the South African sardine resource, because the management of fisheries as separate units off the west and south coasts of South Africa should be considered. A management recommendation for the sardine fishery would be the development of operational management procedures (de Moor *et al.* 2011) that consider population structure and the spatial pattern of fishing, as is presently being done. This process also requires the assessment of future management options for the sardine resource, e.g. the spatial allocation of total allowable catch (TAC) in order to achieve balanced levels of exploitation on the west and south coasts. In this way, the need for and the extent of the amendment of the single stock hypothesis can be assessed in order to ensure that sardine management strategies are robust enough to cater for the underlying stock structure (de Moor & Butterworth 2015).

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CHAPTER 3

MORPHOLOGY OF A STRIGEID METACERCARIA FOUND IN THE EYES OF SARDINE, *Sardinops sagax* FROM GANSBAAI, SOUTHWESTERN COAST OF SOUTH AFRICA.

SUBCLASS DIGENEA

The class Trematoda is divided into two subclasses, namely Aspidogastrea and Digenea (Littlewood *et al.* 1999a, b, Gibson 2002a). The Aspidogastrea (also known as Aspidobothria and Aspidocotylea) is a small group of parasitic worms which infects freshwater and marine molluscs, fish and freshwater turtles (Gibson & Bray 1994, Gibson 2002a, Rohde 2002). Aspidogastreans are characterized by the presence of an attachment organ comprising many suckerlets, rugae or alveoli. Also, their simple life cycle does not include larval multiplication, i.e. asexual reproduction (Cribb *et al.* 2001, Rohde 2005). Furthermore, the life cycle of aspidogastreans uses molluscs as obligate intermediate and/or final hosts, and vertebrates as facultative or obligate definitive hosts (Rohde 2002, 2005).

The Digenea, on the other hand, is a very large and diverse group of endoparasitic flukes which parasitises all classes of vertebrates (Cribb *et al.* 2001, Cribb *et al.* 2003, Gibson 2002b, Robert & Janovy 2009). Hence, classification and identification of digeneans, especially at higher taxonomic level, is not only difficult but also evolving rapidly (Gibson & Bray 1994, Cribb *et al.* 2003, Rohde 2005), such that Gibson and Bray (1994) described it as a 'conundrum'. Early classifications by different workers were based on various criteria (Gibson 1987) namely adult morphology, life history patterns (Pearson 1972, Bozhkov 1982 cited by Gibson 1987), cercarial morphology (La Rue 1957, Cable 1974), daughter-parthenita i.e. redia and daughter-sporocysts (Odening 1961 cited by Gibson 1987) and life history stages (Odening 1974 cited by Gibson 1987, Brooks *et al.* 1985, Brooks 1989, Brooks *et al.* 1989). However, these classifications were not acceptable as a result of taxonomic discrepancies. Gibson (1987) explained that these inconsistencies occurred because of the absence of recognisable non-homoplasious morphological characters as well as the group's complex life history patterns. In spite of the observed discrepancies, these early

classifications provided the foundation for future phylogenetic studies on the interrelationships of digenetic flukes. With time, additional resolution of evolutionary relationships within the subclass Digenea was achieved, though not completely, by subsequent researchers (Barker *et al.* 1993, Blair & Barker, 1993, Blair *et al.*, 1998, Fernández *et al.*, 1998a, b, Tkach *et al.*, 2000, 2001a, b, c, Cribb *et al.* 2001, Olsen *et al.* 2003, Tkach *et al.* 2003). Based on phylogenetic analyses of gene sequences obtained from 163 digenean taxa, Olsen *et al.* (2003) proposed a revised classification of digeneans consisting of the creation of new taxa and the amendment of membership of currently recognized superfamilies, where necessary. In their classification, digenetic trematodes were divided into two orders (Diplostomida and Plagiorchiida), 14 suborders and 22 superfamilies. Although the study was carried out using a large number of representatives from over 140 digenean families, some important taxa (Allocreadiidae, Gymnophallidae, Lilopidae, Mesotretidae, Paramphistomidae, Ptychogonimidae, Rhytidodidae and Urotrematidae) which could have helped in the full interpretation of digenean phylogeny were missing; thus, limiting this classification. It is hoped that molecular techniques will eventually help to resolve the remaining taxonomic inconsistencies in levels where morphology has failed.

Digenetic trematodes parasitise almost all organs of the vertebrate body either as adult or juvenile (Robert & Janovy 2009). They are mainly gut parasites, but in tetrapods they also occur in extra-intestinal sites including the circulatory system, lungs, air sacs, oesophagus, urinary bladder, liver, eye and ovary. In fish, they are found in the swim bladder, body cavity, urinary bladder, gall bladder, flesh, ovary, circulatory system, as well as under the scales, and on the gills (Cribb *et al.* 2003).

Sexual adult digeneans have varying sizes, shapes as well as internal anatomy. Sizes range from 0.16mm in length as seen in the microphallid *Levinseniella minuta*, found in the small intestine of the scaup duck *Nyroca affinis* (Stunkard 1958), to over 12m as described from the didymozoid *Nematobibothrioides histoidii* in the muscle of sunfish *Mola mola* (Noble 1975). Usually, their shapes range from elongate to oval, but other forms do exist (Gibson 2002b). For example, the Transversotrematids are transversely elongate, the Hemiurids are

telescopic (posterior part telescopes into the anterior part) and the Didymozoids can be helical, threadlike or roughly spherical (Rohde 2005). Also, digenetic flukes have smooth or spinous living teguments which bear sensory receptors (Rohde 2005, Robert & Janovy 2009). In addition, digeneans have an oral sucker, on occasion a ventral sucker and occasionally none. Generally, their gut consists of a short prepharynx, a muscular pharynx, an oesophagus and a pair of blind caeca (occasionally single). A network of flame cells and excretory ducts drain into an excretory vesicle with an excretory pore at the posterior end of the body. Apart from the Schistosomes (blood flukes) and some Didymozoids (tissue parasites of fish), digeneans are hermaphrodites. Sperm is produced in the testes (there may be one or many, often two testes) and gets to the cirrus sac through ducts. The cirrus sac, though absent in some digeneans, comprises a seminal vesicle and an ejaculatory duct/cirrus. The male system opens into a male genital pore. Eggs are produced in the ovary, fertilized in a chamber within the oviduct called the ootype and extruded to the exterior through the uterus. A small genital atrium encloses the male and female pores, and opens into the exterior through a common genital pore. Vitelline follicles, which are sometimes scattered throughout the body, produce vitelline cells which release eggshell precursors upon stimulation by Mehlis' glands. Other anatomical structures include the seminal receptacles (canalicular and uterine receptacles) for storing sperm, and the Laurer's canal which can serve both as a drain for reproductive wastes and an opening for fertilization (Gibson 2002b, Rohde 2005, Roberts & Janovy 2009).

DIGENEAN LIFE CYCLE

The life cycle of digenetic trematodes is different from that of its sister taxon, Aspidogastrea. It makes use of at least two hosts (Robert & Janovy 2009):

- a molluscan intermediate host (usually a gastropod) in which one or more generations are produced asexually. However, many digeneans include a second and third intermediate host (Galaktionov & Dobrovolskij 2003); and
- a vertebrate final host in which the sexual adults live.

There are exceptions to this arrangement. Some digenetic flukes have non-gastropod hosts (Pearson 1972). For example, bivalves are hosts to members of the Plagiorchiid

superfamilies - Allocreadiidae, Bucephalata, Faustulidae, Gorgoderidae and Monorchiidae (Cribb *et al.* 2003). Some Sanguinicolidae infect polychaete annelids e.g. developmental stages of the fish blood fluke *Aporocotyle simplex* has been described from the annelid *Artacama proboscidea* (Køie 1982). The Xiphidiata and Hemiuroidea are frequent parasites of arthropods (Cribb *et al.* 2003) and the Ptychogonimids use scaphopods (Gibson & Bray 1994). Again, there are a few species that lack vertebrate final hosts. This is exemplified in *Proctoeces maculatus* (Superfamily Gymnophalloidea) which reaches sexual maturity in its molluscan intermediate host, the slipper limpet *Crepidula convexa* (Aitken-Ander & Levin 1985). Similarly, *Proterometra dickermani* (Family Azygiidae) completes its life cycle in the tissues of a single host species, the snail *Goniobasis livescens* (Anderson & Anderson 1963). Furthermore, some trematodes mature in invertebrate final hosts. For example, sexual adults of macroderoidid trematodes have been found in hirudinid leeches (Timmers 1979, Vande Vusse *et al.* 1981). The adult dicrocoeliid fluke, *Allocorrigia filiformis* has also been described from the antennal glands of the crayfish *Procambarus clarkii* (Turner 1984).

The digenean life cycle is highly complex and variable, and as such is regarded as the most striking characteristics of digeneans (Cribb *et al.* 2001). This complexity and variability can be attributed to two significant aspects of the life cycle (Cribb *et al.* 2001, Robert & Janovy 2009), namely:

- alternation of asexual and sexual generations between different hosts, and the asexual reproduction which occurs within the first intermediate host; and
- plasticity of life history patterns and life stages. This could occur as a morphological convergence of life history stages (e.g. the cercarial tails of opecoelids and the lepocreadid *Lepidapedon elongatum*) or as a secondary reduction of stages depending on differing environmental conditions (Gibson & Valtonen 1984, Gibson 1987). Due to the fact that the different life stages exist in different forms, digeneans have been able to parasitize numerous vertebrate and invertebrate hosts.

In a typical life cycle, as described by Cribb (2005), eggs produced by sexual adults are extruded to the external environment where they hatch into short-lived, motile, ciliated miracidia. The miracidium swims to and penetrates the molluscan first intermediate host,

usually a gastropod but in some cases bivalves, annelids or scaphopods, wherein it develops into a mother sporocyst. In contrast, the very small eggs of Hemiuroidea, Opisthorchioidea and Pronocephaloidea only hatch in the molluscan gut after ingestion, from where the miracidia gets to the haemocoel of the host. The mother sporocyst is a simple sac which feeds through its tegument. It is actually an adult and better referred to as the first (intramolluscan) generation. It asexually produces the second (intramolluscan) generation, which consists of daughter sporocysts or rediae. Unlike the daughter sporocyst which resembles its mother, the redia has a mouth, pharynx, and a short saccular gut. Both redia and daughter sporocysts asexually reproduce cercariae. However, daughter sporocysts and rediae can reproduce themselves, instead of cercariae, if transplanted from an infected to an uninfected host. Among the digeneans, the cercarial morphology is highly variable and is related to their mode of host infection (i.e. by penetration or ingestion). It may or may not resemble the sexual adult. Most cercariae have tails which are used for swimming. The cercaria actively emerges from the mollusc in a process called 'shedding'. After emergence, it reaches the host through one of the following ways (Cribb *et al.* 2003, Cribb 2005):

- direct penetration of the definitive hosts as shown by the blood flukes (Schistosomatids of birds and mammals, Spirorchids of turtles and Sanguinicolids of fishes). The cercaria develops to the adult stage in the circulatory system;
- direct ingestion by the definitive fish host as observed in some members of Bivesiculidae, Azygiidae, Fellodistomidae and Tandanicolidae. The cercaria develops to the adult stage in the host's gut;
- direct attachment to the body surface of the final host. Only the Transversotrematids (parasites of teleost fishes) exhibit this behaviour. The cercaria develops to the adult stage on the body surface;
- encystment on food of the definitive host as metacercaria, which waits to be eaten along with food by the final host, hence it expends little energy and survives longer than a motile cercaria. In a two-host cycle, the definitive host gets infected immediately after ingestion of the metacercaria. This occurs in important marine digenean groups such as Paramphistomoidea, Pronocephaloidea, Haplosporinoidea and Haploporidae;

- direct ingestion of the cercaria by the second intermediate host as observed in a three-host life cycle. The cercaria encysts to form a metacercaria in the gut or body cavity of its host. The metacercaria undergoes development towards the adult stage while still in the second intermediate host, and infects its final host when the former is eaten by the latter. In the final host, the metacercaria develops into a sexual adult. This behaviour is seen in all Hemiuroidea and some Azygiidae, Fellodistomidae and Gorgoderidae;
- direct penetration by the cercaria of second intermediate hosts and ensuing development to the metacercaria. Metacercariae develop into sexual adults in the definitive host following ingestion of the infected second intermediate host. This life cycle pattern is the most common form in the Digenea. This behaviour commonly occurs in Bucephaloidea, Diplostomoidea, Echinostomatoidea, Lepocreadioidea and Opisthorchioidea and in Xiphidiata (Allocreadioidea, Gorgoderoidea, Microphalloidea and Plagiorchioidea); and
- no cercarial emergence and the final hosts eat the intermediate host as observed in Heronimidae, Cyclocoelidae (Echinostomatoidea), Eucotylidae (Microphalloidea) and Hasstilesiidae (Cribb *et al.* 2003).

Digeneans can also have shortened or abbreviated life cycle when there is progenesis (selfing) (Cribb 2005). In rare cases, the life cycle can be completed in the first intermediate host e.g. *Proctoeces maculatus* (Aitken-Ander & Levin 1985) and *Parahemiurus bennettiae* (Jamieson 1966 cited by Cribb 2005). Progenesis is a situation in which the metacercariae skips the final host, reaches maturity and starts producing eggs in the haemocoel of the intermediate host (Lagrué *et al.* 2001). This phenomenon occurs when there is a risk of dead-end transmission, i.e. absence of final hosts, or where transmission opportunities are limited (Lagrué & Poulin 2007, 2008). As an illustration, the freshwater fish trematode *Coitocaecum parvum* lives in the intestine of its final hosts – the common bully, *Gobiomorphus cotidianus* and upland bully, *Gobiomorphus breviceps* in New Zealand. Its first intermediate host is the New Zealand mud snail, *Potamopyrgus antipodarum* while the second intermediate host for its metacercariae is the amphipod *Paracalliope fluviatilis*. In the absence of *Gobiomorphus*, *C. parvum* adopts the progenetic life cycle where its

metacercariae produces viable eggs, which upon the death of *P. fluviatillis*, are released into the environment (Lefebvre *et al.* 2005). In this way, the life cycle is successfully completed, even though the inbreeding associated with selfing is disadvantageous. Additionally, a progenetic life cycle is triggered when there is intraspecies competition among larvae for resources (space and energy) within the second intermediate host and/or for transmission routes. For instance, unlike other digeneans which have one conspecific parasite per host, up to six *C. parvum* can share a host. In this situation, there is bound to be competition for resources among the parasites, thus making them progenetic. Similarly, interspecies co-infection causes progenesis especially where one of the competitors can alter the host's behaviour to its advantage, e.g. co-infection of *P. fluviatillis* by *C. parvum* and the manipulative avian parasite, *Microphallus* sp. (Lefebvre *et al.* 2005, Lagrue & Poulin 2008). The cercaria, metacercaria and sexual adult constitute the third generation in the life cycle of digenetic trematodes.

METACERCARIA

The metacercaria is a stage between the cercaria and sexual adult. However, the blood flukes lack the metacercarial stage while members of the strigeid genera like *Alaria*, *Strigea* have a unique stage between the cercaria and metacercaria – the mesocercaria. The metacercarial stage serves as a link for transmission to definitive hosts which do not eat the first intermediate host or which do not occur in the environment of the mollusc. It also serves as a 'medium' for surviving unfavourable conditions (Robert & Janovy 2009). Not all digeneans have metacercaria in the encysted form; some species have free-moving metacercariae e.g., *Acanthatrium oregonense* (Knight & Pratt 1955, Burns 1961) and some *Diplostomum* species (Grobelaar 2011). Usually, the metacercaria encysts in or on their intermediate hosts except for some (e.g. the Fasciolidae, Paramphistomidae) which encyst on vegetation and inanimate substrates. Consequently, the latter group has an elaborate cyst wall unlike the former which has a thin cyst wall formed by parasite and host components (Robert & Janovy 2009). Based on the degree of development which a metacercaria must attain before it can be infective to its definitive host, three broad groups are recognised (Dönges 1969 cited by Robert & Janovy 2009) namely:

- Group 1 – the metacercaria encysts on vegetation or inanimate objects. This group requires no growth and is infective immediately after encystment, e.g. *Fasciola* sp.
- Group 2 – the metacercaria does not grow in the intermediate host but rather undergoes physiological development for several days to be infective to the final hosts. This group is exemplified in the echinostomes.
- Group 3 – the metacercaria undergoes growth and development for weeks in the intermediate host before going into a resting stage, e.g. members of superfamily Diplostomoidea.

METACERCARIAL TYPES WITHIN THE DIPLOSTOMATOIDEA

Members of the superfamily Diplostomatoidea include Diplostomidae, Proterodiplostomidae, Cyathocotylidae, Brauninidae, and Bolbocephalodidae (Niewiadomska 2002a). These trematodes are parasites of birds, reptiles and mammals. Both sexual adults and metacercariae of this group are uniquely characterized by the presence of a holdfast organ situated posterior to the acetabulum (ventral sucker) on the ventral surface of the body. It plays both adhesive and digestive roles (Niewiadomska 2002a). Other anatomical structures of the metacercaria are:

- a digestive system which consists of an oral sucker which is situated terminally or sub-terminally, pre-pharynx (absent in some species), a muscular pharynx, and an oesophagus which bifurcates into blind-ending intestinal caeca. Nutrients are absorbed through this system as well as through the tegument (Erasmus 1958, Ashton *et al.* 1969, Graczyk & Fried 2001);
- pseudosuckers, also known as lateral suckers or lappets, located laterally on either side of or below the oral sucker. Different authors have speculated on their function. They could be organs of taste (Mataré 1910 cited by Grobbelaar 2011) or attachment (Rees 1955). Szidat (1969) suggested they aid metacercarial penetration into the host's tissues. These are understandably inconspicuous in young metacercariae, and in a retracted form (Brown 1899, Bibby & Rees 1971). Thus, under these conditions, recognition of this structure and morphological measurements will be difficult. However, they is a diagnostic feature in identification of metacercarial species especially in well-developed specimens (Grobbelaar 2011);

- a ventral sucker or acetabulum which serves as an organ of attachment (Brown 1899). It is located in the middle of the body and is a circular opening surrounded by muscles, just like the oral sucker;
- an excretory system made up primarily of flame cells (which are connected by excretory ducts), the reserve bladder system and the excretory pore (Agrawal & Khan 1982). The pattern of excretory ducts is important in metacercarial classification (Ashton *et al.* 1969) (Table 3.1); and
- an undeveloped reproductive system. Gonad primordia may or may not be visible. When visible, it is seen as a small dark mass of stained cells located posterior to the holdfast organ (Chakrabarti 1967). The reproductive system only develops in the adult worm within the final host.

Development of pseudosuckers, adhesive organs and reserve bladder system commences as the metacercaria grows within the second intermediate host. The reserve bladder is fully developed and functional by the time the metacercaria reaches full maturity. Thus, morphological and physiological continuity between the metacercaria and adult is ensured (Erasmus 1958, 1967, Pearson 1961).

Some metacercarial structures, at times, may not be visible under light microscopy and are omitted from morphological descriptions. This results in variability in the morphometrics and morphological appearance of a metacercaria being described. The nature of intermediate host species, i.e. whether natural, paratenic or experimental, can also influence the size of the metacercaria at maturity (Chandler 1942, Ferguson 1943, Chibwana & Nkwengulia 2010, Peoples & Poulin 2011). Metacercariae found in unnatural hosts may be smaller in size or undeveloped when compared with their counterparts found in their natural hosts. Other factors which may cause morphological variations in metacercarial identification are: intensity of infection (Graczyk 1992, Saldanha *et al.* 2009), degree of deformation caused during specimen preparation (cover slip pressure, fixation and preparation protocols etc.) (Rees 1955, Hoffman & Hundley 1957, Timi *et al.* 1999), and age of the metacercariae (Cort *et al.* 1941, Chandler 1942, Erasmus 1958, Timi *et al.* 1999). Cort *et al.* (1941) and Ulmer (1957), in their separate studies on the life cycle of the strigeid

trematode *Cotylurus flabelliformis*, described three age groups of ‘tetracotyle’ type metacercaria namely:

- ‘developing’ forms – all developing stages including those beginning to undergo reorganization;
- ‘pre-cysts’ – stages with a developed hind body but lack a cyst wall; and
- ‘cysts’- fully developed ‘tetracotyles’ with a distinct cyst wall.

Morphological variations can also arise from technical issues such as narrow magnification range, inadequate resolution, limited depth of focus and difficulties in obtaining photomicrographs (Zagon 1971). Given the limitations associated with light microscopy, scanning electron microscopy (SEM) has been included as an additional technique in the study and elucidation of metacercarial structures and tegumental features to aid morphological description (Font & Wittrock 1980, Køie 1987, Krejci and Fried 1994, Ursone & Fried 1995, King & Van As 1997, Desclaux *et al.* 2006). Nevertheless, specimens can also undergo some degree of deformation during preparation for SEM.

Just like other higher taxa within the digenea, taxonomic classification of the Diplostomoidea has its own share of inconsistencies. This is shown by the different versions of systematics of the Diplostomoidea (Dubois 1968, Szidat 1969, Yamaguti 1971, Shoop 1989, Niewiadomska 2002a). Nevertheless, the Diplostomoidea is divided into families based on the following criteria (Niewiadomska 2002a):

- host-specificity of adults; and
- morphological characters such as structure and shape of the forebody and holdfast organ, distribution of the vitellarium, presence or absence of bi-segmentation of the body, presence or absence of the cirrus-sac, and the structure of the copulatory apparatus.

Occasionally, life cycle arrangement and morphological characters of the developmental stages were used in classifying diplostomatoids (Dubois 1968, Ginetsinskaya & Dobrovolsky 1963, Szidat 1969, Shoop 1989). One of such developmental stages is the metacercaria.

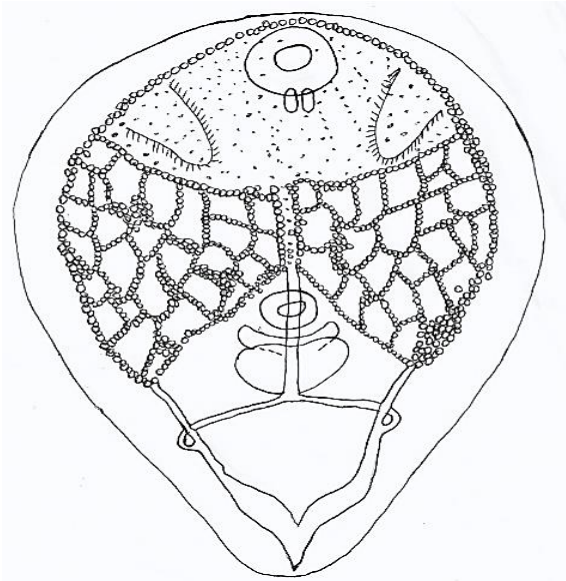


Figure 3.1. 'Tetracotyle' type metacercaria (redrawn from Niewiadomska 2002a).

Table 3.1. Characteristics of the different metacercarial types within the Diplostomatoidea (as described by Niewiadomska, 2002a).

‘Diplostomulum’	‘Neascus’	‘Prohemistomulum’	‘Tetracotyle’
Examples of adult genera: <i>Alaria</i> , <i>Diplostomum</i>	Examples of adult genera: <i>Uvulifer</i> , <i>Bolbophorus</i>	Examples of adult genera: <i>Cyathocotyle</i> , <i>Holostephanus</i>	Examples of adult genera: <i>Strigea</i> , <i>Cardiocephaloides</i>
May or may not be encapsulated	Encapsulated. Syncysts (clusters of cysts) may occur	Encapsulated	Encapsulated
Cyst wall may or may not be of parasite origin	Cyst wall of parasite origin	Thick-walled cysts of parasite origin	Distinct cyst wall of parasite origin
Round, oval or elongate forebody, ventrally concave	Foliaceous or oval forebody	Round or oval body	Ventrally concave forebody, may be cup-shaped or flattened
Small, conical hindbody	Well-developed hindbody		Hindbody may be small or well-developed
Pseudosuckers present or absent	Pseudosuckers present or absent	Pseudosuckers absent	Pseudosuckers present
Reserve bladder composed of three longitudinal canals connected by only anterior and posterior commissures, or with additional commissures. Systems of ramifications have terminal enlarged pockets filled by round or oval excretory bodies	Reserve bladder in the forebody is made up of a network formed by ramified median and two lateral canals. In the hindbody, excretory bodies are free (not enclosed). Median canal may or may not be connected by commissures	Reserve bladder consists of four main canals form two loops connected to acetabulum anteriorly. Excretory bodies are enclosed by a system of blind-ending ramifications originating from the main canals	Reserve bladder presents as a network of excretory ducts filling the entire body. Free excretory bodies in canals
Found in mammals, fishes, reptiles, and amphibians,	Found in fishes and oligochaetes	Found in snails, leeches, amphibians, fishes	Found in fishes, snails, leeches, oligochaetes, reptiles, amphibians, rarely in birds and mammals

Based on the structure of the reserve bladder and excretory system in the metacercaria, four main metacercarial types within the Diplostomatoidea were described and distinguished by larval generic names (Dubois 1968, Shoop 1989, Niewiadomska 2002a). However, these larval generic names were not consistently used for all the larval genera described, resulting in taxonomic discrepancies in Diplostomoidea systematics which are yet to be resolved. The metacercarial types are 'Diplostomulum', 'Neascus', 'Prohemistomulum' and 'Tetracotyle' (Table 3.1). Additional larval genera *Tylodelphys* and *Tetracotyloides* were suggested by Szidat (1969) but this was based only on the morphology of the metacercaria. However, *Tylodelphys* and *Diplostomulum* have a similar biology (Szidat 1969), such that Grobbelaar (2011) believes that the former is a transitional form of the latter and hence should be regarded as a subgenus.

The 'tetracotyle' type metacercaria (Figure 3.1) is characteristic of the Family Strigeidae (Niewiadomska, 2002b). Adults of the Family Strigeidae are distinguished by a cup-shaped forebody and bilobed (dorsal and ventral lobes) holdfast organ. Sexual adults are mainly avian parasites while their metacercariae are parasites of the eyes (La Rue *et al.* 1926) and brain (Brown 1899, Hunter & Vernberg 1960, Moravec *et al.* 1997) of marine teleost fish. They have also been reported from eyes of certain amphibians (Kelley 1934). 'Tetracotyle' type metacercariae have been found in various hosts by different authors from various parts of the world. These include: Cort *et al.* (1941), Ulmer (1957), Hunter and Vernberg (1960), Moravec *et al.* (1997) from North America; Szidat (1969), Timi *et al.* (1999) from South America; Parukhin (1975) (cited by Reed *et al.* 2012), Prevot & Bartoli (1980) (cited by Timi *et al.* 1999) from Europe and Agrawal & Khan (1982) from India.

Metacercariae have been reported from the eyes and brain of freshwater fish from different parts of southern Africa. These include work done by: Mashego and Saayman (1989) from Lebowa (South Africa), Barson and Avenant-Oldewage (2006) from Reitvlei Dam (South Africa), Madanire-Moyo *et al.* (2010) from Nwanedi-Luphephe dams, Limpopo River System (South Africa), Grobbelaar (2011) from Okavango (Botswana) and Orange-Vaal (South Africa) River Systems, Jansen Van Rensburg *et al.* (2013) from Okavango Delta (Botswana), Moema *et al.* (2013) from Tshwane, Guateng Province (South Africa). However these

metacercariae are 'diplostomulum' type metacercariae (Family Diplostomidae). Presently, there is no documentation on the taxonomic description of the 'tetracotyle' type metacercariae from hosts in South African aquatic system. This chapter describes the 'tetracotyle' type metacercariae found in the eyes of South African sardine *Sardinops sagax* using light and scanning electron microscopy. This is the first description of the 'tetracotyle' type metacercariae from a marine host - sardine sampled from Gansbaai on the southwestern coast of South Africa.

MATERIALS AND METHODS

Sample collection

Fresh sardine landed in Gansbaai by commercial fishing vessels were collected and taken to the laboratory at the Department of Biological Sciences, University of Cape Town (UCT) for dissection. Sardine eyes were examined using a Leica MZ9.5 dissection microscope (Leica Microsystems, Taiwan) and encapsulated metacercariae (cysts) were removed. Photographs and measurements (in micrometers) were taken of cysts using a Leica EZ4D (Leica Microsystems, Taiwan) light microscope. Metacercariae were identified using light and scanning electron microscopy.

Light microscopy

Metacercariae were manually excysted and relaxed in warm water to facilitate intravital staining of internal structures. They were fixed in 70% ethanol and stained with Van Cleave's haematoxylin. Slides were viewed and photographs of specimens were taken using a Leica DM500 compound microscope (Leica Microsystems, Taiwan) at 100x and 400x magnification. Microscope projection drawings were made at 200x magnification using a drawing tube (Nikon drawing tube x1.25) attached to a Nikon Optiphot-B microscope (Nikon, Japan). Morphometric characters were measured with a Nikon Eclipse Ni microscope (Nikon, Japan) using the NIS-Elements AR imaging software programme (Nikon, Japan). Measurements are presented in micrometers as range (mean $\pm$ standard deviation) (Table 3.2). Slides were deposited in the collection of the Department of Biology at the Sefako Makgatho Health Sciences University.

Scanning Electron microscopy

Both encysted and excysted metacercariae were fixed in cacodylate buffered 3% glutaraldehyde at 4°C, rinsed in 0.1M cacodylate buffer and postfixed in 1% osmium tetroxide for one hour. Samples were rinsed again with distilled water to ensure the removal of all fixatives. They were then dehydrated through a series of graded ethanol concentrations (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%), critical-point dried using a CPD 020 critical point dryer (Balzers Union, Balzers, Germany) and mounted on stubs. The stubs were sputter-coated with gold-palladium alloy using a Polaron vacuum evaporator (Quorum Technologies, East Sussex, UK) and scanned in a NOVA NANOSEM 230 scanning electron microscope (FEI, Oregon, USA) for the visualization of images.

PARASITE DESCRIPTION

HOST: South African sardine, *Sardinops sagax*

SITE OF INFECTION: Eyes. Cysts are attached to the suspensory apparatus (ligaments and retractor lentis muscle) of the lens (Figures 3.2C, D, 3.4, 3.8A).

PREVALENCE OF INFECTION: 41.49% (n = 1545, infected hosts = 641)

MEAN INTENSITY OF INFECTION: 4.39 ± 6.27

DIAGNOSTIC AND MORPHOMETRIC FEATURES: Figures 3.2 – 3.8. Description based on 20 whole mounts and 57 live cysts. Range of different measurements are given in micrometers. Mean $\pm$ standard deviation are shown in parentheses (Table 3.2).

Table 3.2. The range (**mean** $\pm$ standard deviation) of different measurements (μm) of strigeid metacercariae collected from the eyes of sardine, *Sardinops sagax* landed in Gansbaai, South Africa. Morphometrics were obtained from whole mounts (sample size, n = 20).

METACERCARIAL MEASUREMENTS	μm
Body length	762.06 – 967.42 (874.86 $\pm$ 61.69)
Body width	511.80 – 676.52 (604.74 $\pm$ 57.62)
Fore body length	145.13 – 222.90 (188.27 $\pm$ 26)
Fore body width	223.44 – 300.74 (270.60 $\pm$ 25.05)
Mid body length	475.32 – 592.86 (535.90 $\pm$ 42.39)
Mid body width	511.80 – 676.52 (612.28 $\pm$ 55.79)
Hind body length	120.38 – 197.59 (154.70 $\pm$ 22.79)
Hind body width	214.86 – 338.59 (271.93 $\pm$ 33.38)
Pre-oral sucker length	6.07 – 11.88 (8.49 $\pm$ 1.86)
Oral sucker length	87.37 – 112.31 (103.70 $\pm$ 6.67)
Oral sucker width	92.11 – 116.45 (106.88 $\pm$ 6.24)
Pharynx length	48.28 – 65.43 (55.35 $\pm$ 4.60)
Pharynx width	39.47 – 50.90 (45.68 $\pm$ 3.70)
Oesophagus length	20.65 – 41.13 (29.16 $\pm$ 5.04)
Oesophagus width	15.17 – 29.10 (18.90 $\pm$ 2.15)
Caecum length	586.30 – 721.31 (658.67 $\pm$ 33.16)
Caecum length at start	15.57 – 32.17 (21.57 $\pm$ 4.96)
Caecum length at end	30.76 – 49.74 (42.23 $\pm$ 4.79)
Distance: Anterior body end to pseudosuckers	138.75 – 250.70 (191.10 $\pm$ 29.68)
Distance: Oral sucker to pseudosuckers	46.18 – 109.9 (75.31 $\pm$ 18.35)
Pseudosucker 1 length	183.14 – 233.20 (206.70 $\pm$ 17.52)
Pseudosucker 1 width	80.2 – 108.44 (95.16 $\pm$ 10.70)
Pseudosucker 2 length	164.83 – 221.72 (192.77 $\pm$ 15.04)
Pseudosucker 2 width	79.38 – 116.33 (95.56 $\pm$ 9.13)
Distance: Oral sucker to acetabulum	194.09 – 324.55 (252.38 $\pm$ 33.20)
Acetabulum length	122.15 – 146.47 (135.14 $\pm$ 7.40)
Acetabulum width	121.04 – 147.03 (134.99 $\pm$ 7.70)
Pre-acetabular space (length)	249.47 – 385.71 (336.01 $\pm$ 39.02)
Post-acetabular space (length)	369.54 – 439.94 (405.18 $\pm$ 23)
Distance: Anterior body end to holdfast lateral lobe	322.43 – 441.28 (391.92 $\pm$ 29.36)
Distance: Anterior body end to holdfast middle lobe	397.38 – 520.75 (465.22 $\pm$ 40.59)
Distance: Acetabulum to holdfast	0.00 – 16.62 (1.56 $\pm$ 4.82)
Holdfast length (posterior to acetabulum)	127.32 – 199.80 (172.24 $\pm$ 17.59)
Holdfast width	216.24 – 321.20 (281.19 $\pm$ 32.35)
Holdfast right lobe length	180.03 – 278.97 (208.76 $\pm$ 26.69)
Holdfast right lobe width	81.98 – 136.83 (102.28 $\pm$ 14.75)
Holdfast middle lobe 1 length	130.88 – 169.79 (147.33 $\pm$ 11.20)
Holdfast middle lobe 1 width	80.91 – 134.78 (106.94 $\pm$ 11.33)
Holdfast middle lobe 2 length	134.77 – 174.58 (157.30 $\pm$ 10.76)
Holdfast middle lobe 2 width	92.79 – 127.10 (109.60 $\pm$ 9.94)
Holdfast left lobe length	160.32 – 239.92 (202.41 $\pm$ 20.98)
Holdfast left lobe width	76.27 – 133.62 (100.28 $\pm$ 16.70)
Excretory bladder left length	568.48 – 675.44 (626.24 $\pm$ 30.84)
Excretory bladder left width	100.77 – 147.89 (126.74 $\pm$ 14.02)
Excretory bladder right length	569.97 – 685.15 (638.74 $\pm$ 32.22)
Excretory bladder right width	97.24 – 157.90 (128.29 $\pm$ 16.08)
Excretory bladder duct length	138.40 – 202.62 (174.17 $\pm$ 19.05)
Excretory bladder duct width	34.33 – 61.87 (45.82 $\pm$ 8.90)
Cyst length	685 – 1530 (940 $\pm$ 0.20)
Cyst width	635 – 1220 (830 $\pm$ 0.16)
Cyst wall thickness	4.11 – 5.23 (4.88 $\pm$ 0.34)

Encysted metacercariae: Body (Figure 3.2A) 685 - 1530 (940 ± 0.20) x 635 - 1220 (830 ± 0.16). Not free-moving, lying longitudinally flat, not coiled. White in colour, body filled with numerous refractile excretory granules (Figures 3.2A, B, 3.5E, F). Two cyst walls of parasite origin. Outer cyst wall thin, 4.11 - 5.23 (4.88 ± 0.34), fragile and easily ruptured. Inner cyst wall, a tough, gel-like substance (Figure 3.2E), difficult to remove without injury to worm. Hence, manual excystation was challenging, especially in fixed specimens. Cysts present as single individuals or in clusters (Figure 3.3, 3.4). Metacercariae occur in varying sizes and show different degrees of development depending on stage of infection (Figure 3.2F).

Excysted metacercaria: Figures 3.6, 3.7. Oval-shaped body (Figures 3.2B, 3.6, 3.7, 3.8H), 762.06 - 967.42 (874.86 ± 61.69) x 511.8 - 676.52 (604.74 ± 57.62), divided by transverse folds into 3 regions: a short fore body, 145.13 - 222.9 (188.27 ± 26) x 223.44 - 300.74 (270.6 ± 25.05); a large, round mid body, 475.32 - 592.86 (535.9 ± 42.39) x 511.8 - 676.52 (612.28 ± 55.79); and a small, conical hind body, 120.38 - 197.59 (154.7 ± 22.79) x 214.86 - 338.59 (271.93 ± 33.38) (Figures 3.2B, 3.8B, 3.8H). Sub-terminal oral sucker (Figures 3.5A, 3.8B, C, D), 87.37 - 112.31 (103.7 ± 6.67) x 92.11 - 116.45 (106.88 ± 6.24), followed by pharynx, 48.28 - 65.43 (55.35 ± 4.60) x 39.47 - 50.9 (45.68 ± 3.70) (Figure 3.5A). Pre-pharynx absent. Short oesophagus, 20.65 - 41.13 (29.16 ± 5.04) x 15.17 - 21.9 (18.9 ± 2.15), bifurcates into intestinal caeca, 586.3 - 721.31 (658.67 ± 33.16) x 15.57 - 32.17 (21.57 ± 4.96) at the level of anterior end of pseudosuckers (Figures 3.5A, B, D). Within the hind body, caeca widen to 30.76 - 49.74 (42.23 ± 4.79) and end bluntly (Figure 3.5B, 3.6, 3.7). Anterior to the acetabulum, at each side of the caeca lie two large, well-developed, bi-lobed, pseudosuckers, 183.14 - 233.2 (206.70 ± 17.52) x 80.2 - 108.44 (95.16 ± 10.70) and 164.83 - 221.72 (192.77 ± 15.04) x 79.38 - 116.33 (95.56 ± 9.13) (Figures 3.5C, D).

In the mid body, circular acetabulum, 122.15 - 146.47 (135.14 ± 7.40) x 121.04 - 147.03 (134.99 ± 7.70), situated just above the lobulated holdfast organ (Figure 3.5B, 3.8G). Holdfast organ has four lobes – two large lobes lateral to the acetabulum and two small medial lobes posterior to the acetabulum. Lateral lobes measure 180.03 - 278.97 (208.76 ± 26.69) x 81.98 - 136.83 (102.28 ± 14.75) and 160.32 - 239.92 (202.41 ± 20.98) x 76.27 - 133.62 (100.28 ± 16.70). Medial lobes measure 130.88 - 169.79 (147.33 ± 11.20) x 80.91 - 134.78 (106.94 ± 11.33) and 134.77 - 174.58 (157.3 ± 10.76) x 92.79 - 127.1 (109.6 ± 9.94).

Laterally located large excretory bladder lobes (Figure 3.5B, C), 568.48 - 675.44 (626.24 ± 30.84) x 100.77 - 147.89 (126.74 ± 14.02) and 569.97 - 685.15 (638.15 ± 32.22) x 97.24 - 157.9 (128.29 ± 16.08). Excretory ducts extend posteriorly from bladder into hind body to form a vesicle 138.4 - 202.62 (174.17 ± 19.1) x 34.33 - 61.87 (45.82 ± 8.9) which opens into an excretory pore. Genital primodium situated anterior to excretory pore, in between the blunt ends of the caeca, within the hind body (Figure 3.5B, 3.6, 3.7).

Tegument smooth but leathery (Figures 3.8E, F). Sensory receptors on and around the anterior region of fore body. Cavity of oral sucker is surrounded by sensory receptors (Figures 3.8C, D).

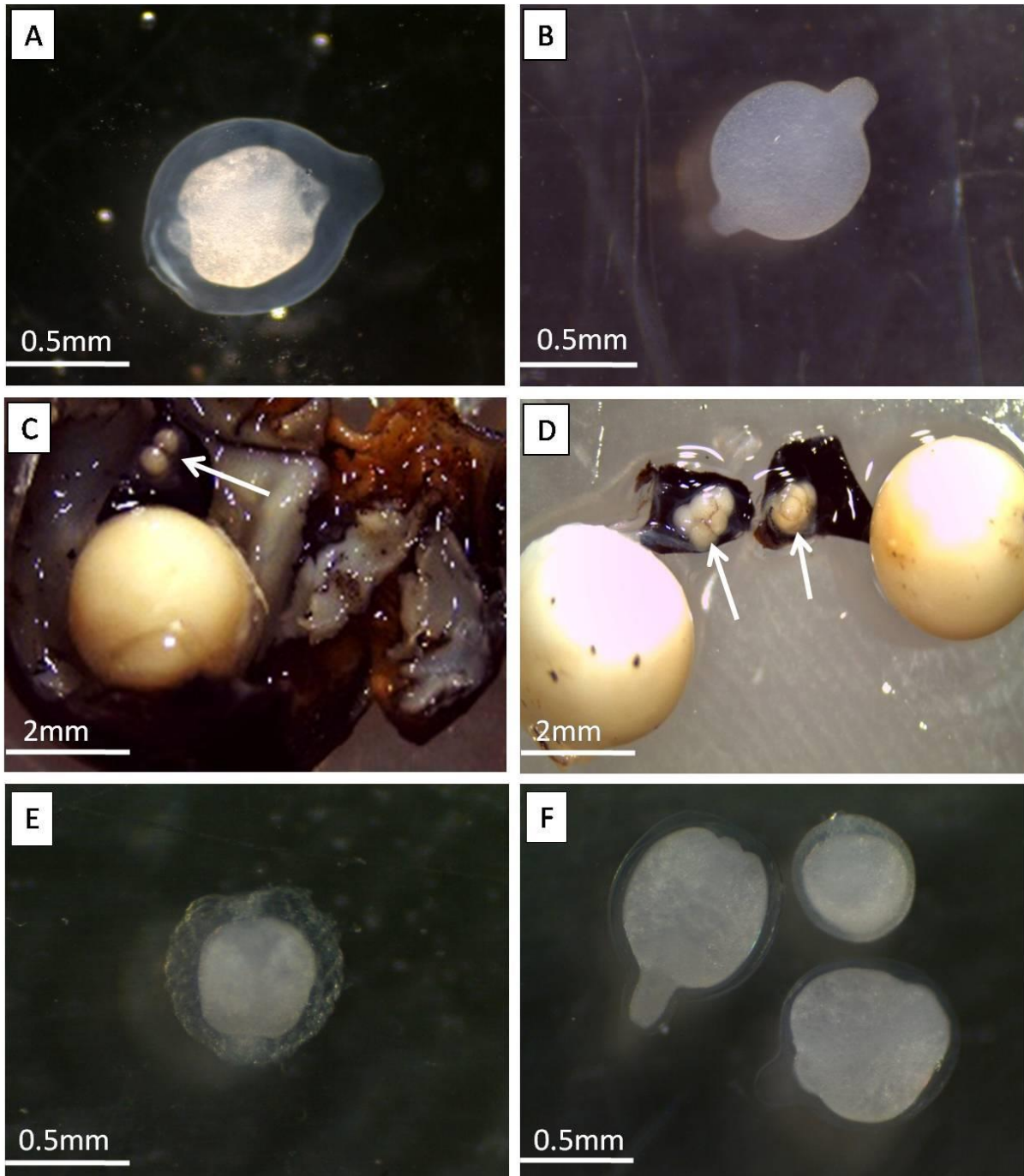


Figure 3.2. Micrographs of strigeid metacercariae from the eyes of sardine, *Sardinops sagax* landed in Gans Bay, southwestern coast of South Africa showing: **(A)** Encysted metacercaria; **(B)** Excysted metacercaria; **(C)** Cluster of two cysts (shown by an arrow) in the eye; **(D)** Clusters of cysts (shown by arrows) attached to eyeballs; **(E)** Inner cyst wall surrounding metacercaria; and **(F)** Cysts in different stages of development.

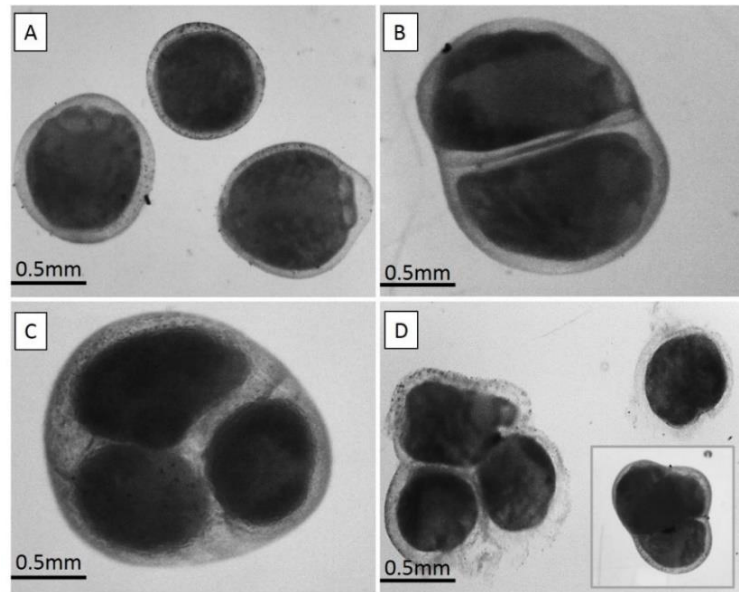


Figure 3.3. Micrographs of strigeid metacercariae from the eyes of sardine, *Sardinops sagax* landed in Gans Bay, southwestern coast of South Africa showing: **(A)** Single cysts; and Clusters of **(B)** two cysts; **(C)** three cysts; and **(D)** four cysts (inset: intact cluster).

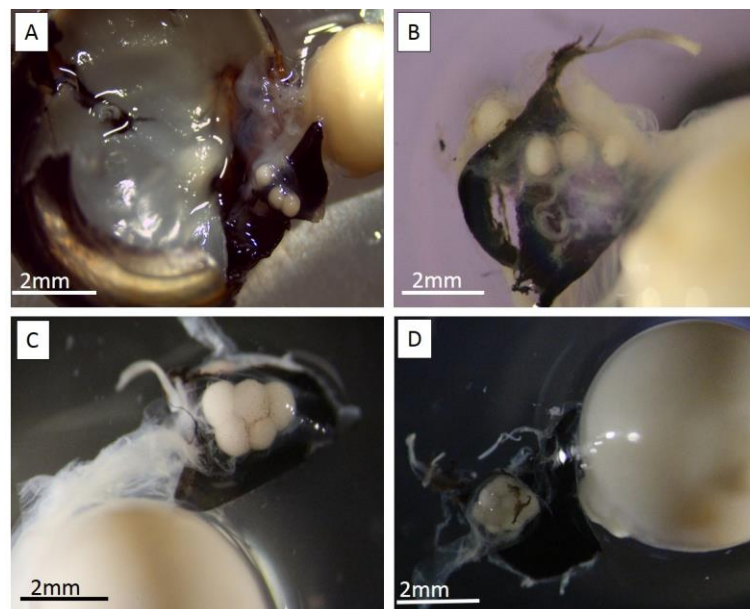


Figure 3.4. Metacercarial cysts attached to suspensory apparatus of the lens.

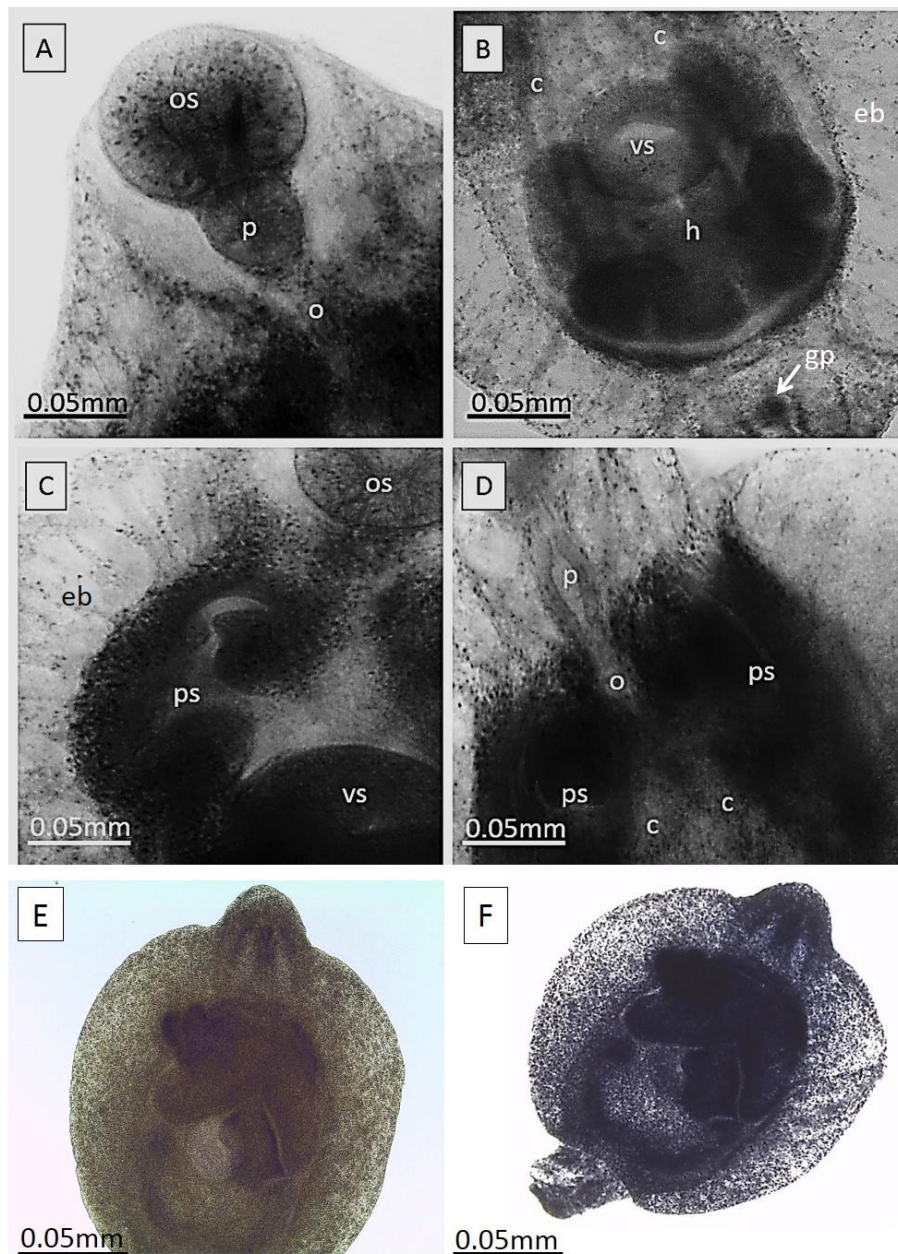


Figure 3.5. Light micrographs of anatomical structures of strigeid metacercariae from the eyes of sardine, *Sardinops sagax* landed in Gans Bay, southwestern coast of South Africa showing: **(A)** Oral sucker, **os**, followed by pharynx, **p**, and oesophagus, **o**; **(B)** ceaca, **c**; ventral sucker, **vs**; holdfast organ, **h**; flanked by excretory bladder, **eb**. Genital primodium, **gp**, shown by arrow; **(C)** Oral sucker, **os**, followed by pseudosucker, **ps** and ventral sucker, **vs**; flanked by excretory bladder, **eb**; **(D)** Pharynx, **p**; oesophagus, **o**; pseudosucker, **ps**, and ceaca, **c**; and **(E), (F)** Numerous refractile excretory granules obscuring internal structures.

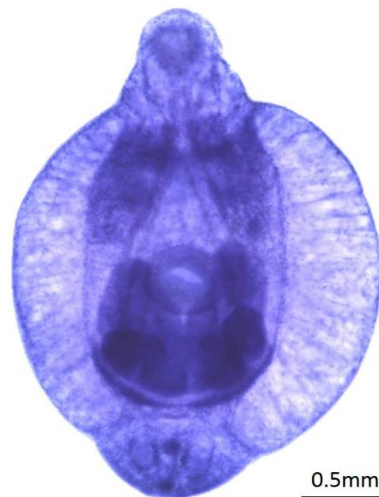


Figure 3.6. Light micrograph showing the general morphology of a strigeid metacercaria from the eyes of sardine, *Sardinops sagax* landed in Gans Bay, southwestern coast of South Africa.

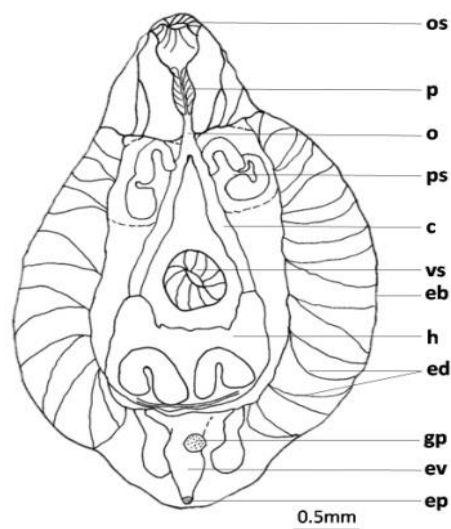


Figure 3.7. Microscope projection drawing of a strigeid metacercaria from the eyes of sardine, *Sardinops sagax* landed in Gans Bay, southwestern coast of South Africa. **os**: oral sucker; **p**: pharynx; **o**: oesophagus; **ps**: pseudosucker; **c**: ceacum, **vs**: ventral sucker, **eb**: excretory bladder; **h**: holdfast organ; **ed**: excretory ducts; **gp**: genital primodium; **ev**: excretory vesicle; **ep**: excretory pore.

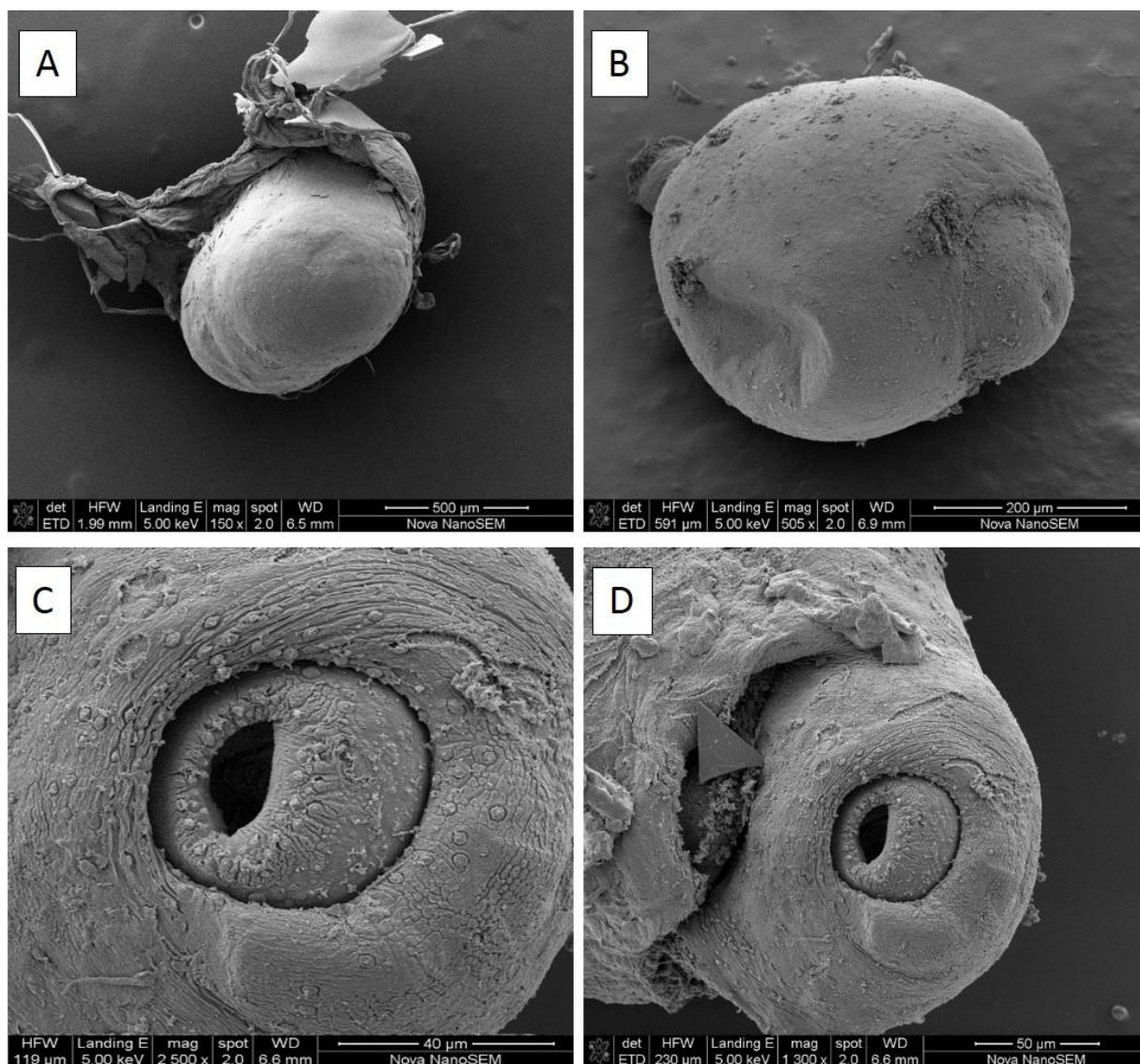


Figure 3.8. Scanning electron micrographs of strigeid metacercariae from the eyes of sardine, *Sardinops sagax* landed in Gans Bay, southwestern coast of South Africa, showing: **(A)** Encysted metacercaria attached to a part of suspensory apparatus of the lens, scale bar = 500µm; **(B)** Excysted immature metacercaria showing distinct fore body and mid body separated by transverse folds, as well as a closed oral sucker, scale bar = 200µm; and **(C), (D)** Anterior region of fore body showing sub-terminal oral sucker and sensory receptors on and around it. Cavity of oral sucker is surrounded by sensory receptors, scale bar = 40µm and 50µm, respectively.

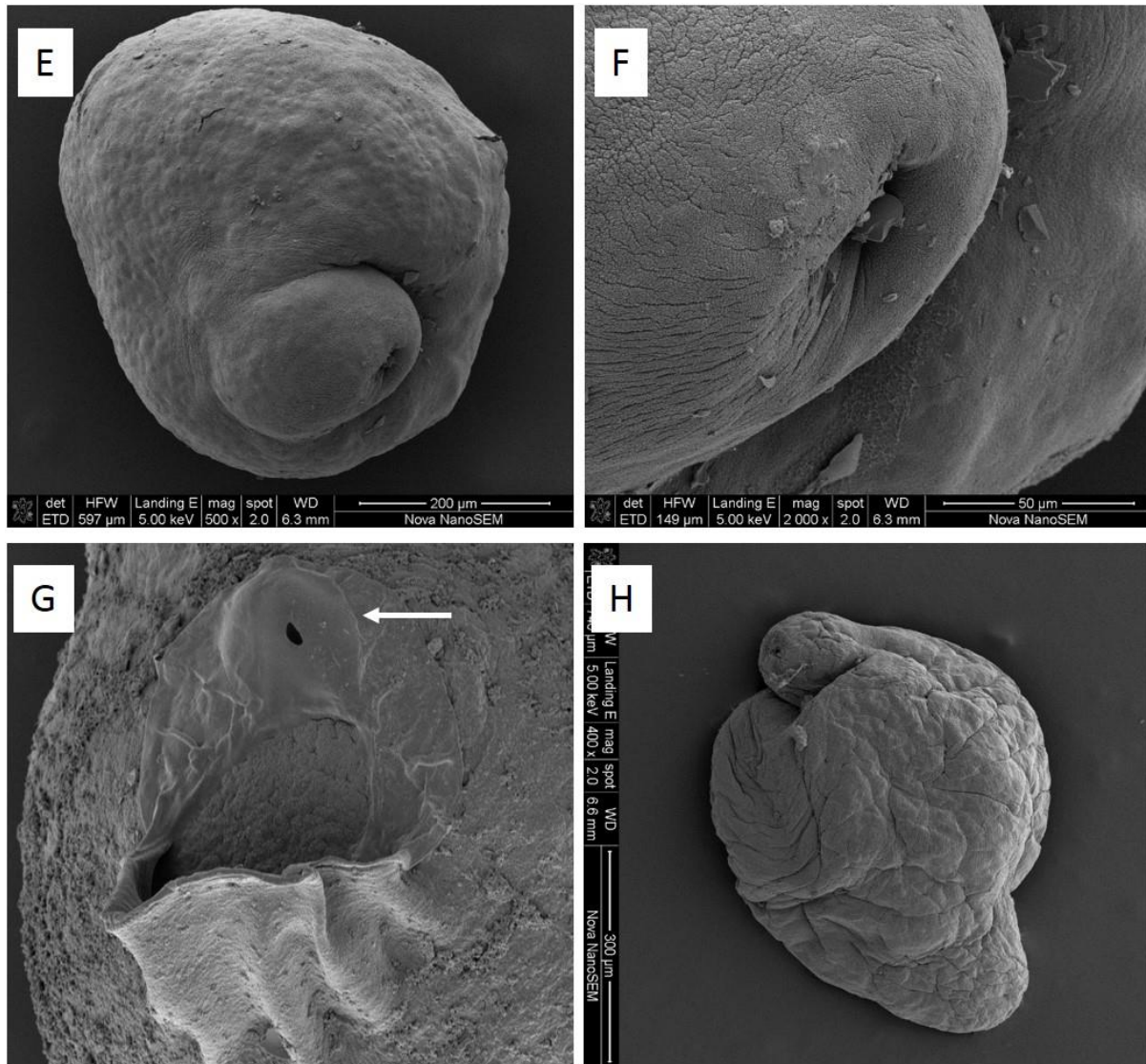


Table 3.3. Comparison of mean measurements in μm (ranges in parenthesis) of specimens from present study with metacercariae reported by Moravec *et al.* (1997) and Timi *et al.* (1999).

	present study	Moravec <i>et al.</i> (1997)	Timi <i>et al.</i> (1999)
Host	Sardine (<i>Sardinops sagax</i>)	Red grouper (<i>Epinephelus morio</i>)	Anchovy <i>Engraulis anchoita</i>
Host locality	Gansbaai, South Africa	Yucatan Peninsula, Gulf of Mexico	Argentina & Uruguay
Site of infection	Eyes	Brain	Eyes
State of metacercariae	Encysted	-	Encysted
Cyst	830 (635 - 1220)	-	600 (450 - 780)
Body length	874.86 (762.06 - 967.42)	1520 - 2080	656 (550 - 860)
Body width	604.74 (511.8 - 676.52)	1020 - 1520	468 (360 - 620)
Oral sucker length	103.7 (87.37 - 112.31)	136 - 150	93 (70 - 110)
Oral sucker width	106.88 (92.11 - 116.45)	190 - 204	97 (80 - 120)
Pharynx length	55.35 (48.28 - 65.43)	-	50 (40 - 60)
Pharynx width	45.68 (39.47 - 50.9)	68	36 (30 - 40)
Oesophagus length	29.16 (20.65 - 41.13)	272 - 340	-
Oesophagus width	18.9 (15.17 - 21.9)	-	-
Forebody length	188.27 (145.13 - 222.9)	272	-
Forebody width	270.6 (223.44 - 300.74)	272 - 381	-
Midbody length	535.9 (475.32 - 592.86)	-	-
Midbody width	612.28 (511.8 - 676.52)	-	-
Hindbody length	154.7 (120.38 - 197.59)	408 - 499	-
Hindbody width	271.93 (214.86 - 338.59)	462 - 721	-
Acetabulum length	135.14 (122.15 - 146.47)	190 - 245	132 (100 - 150)
Acetabulum width	134.99 (121.04 - 147.03)	204 - 260	133 (110 - 148)
Pre-acetabular space (length)	336.01 (249.47 - 385.71)	-	194 (210 - 420)
Post-acetabular space (length)	405.18 (369.54 - 439.94)	-	244 (185 - 310)
Holdfast length	172.24 (127.32 - 199.80)	367 - 476	140 (100 - 200)
Holdfast width	281.19 (216.24 - 321.2)	1006 - 1088	276 (240 - 310)

REMARKS

'Tetracotyle' type metacercariae have been previously reported from the eyes of sardine *Sardinops sagax* (Parukhin 1975 cited by Reed *et al.* 2012). The features observed on specimens in the present study are diagnostic for metacercariae of the genus *Cardiocephaloides* (Family Strigeidae). *Cardiocephaloides* was initially regarded as a synonym of *Cardiocephalus* by Dubois (1968), whereas Yamaguti (1971) considered both genera to be valid. However, Baer (1969) explained that the genus *Cardiocephalus* has already been preoccupied by a fossil amphibian and is no longer available for use. In the light of this understanding, *Cardiocephaloides* was recognized as the valid generic name.

Descriptions for *Cardiocephaloides* metacercariae have been reported from the northern hemisphere namely: *Cardiocephalus brandesii* from the brain and eyes of flathead grey mullet *Mugil cephalus* and Atlantic silverside *Menidia menidia* (Hunter & Vernberg 1960); an unidentified strigeid metacercaria from the brain of mummichog *Fundulus heteroclitus* from the Atlantic coasts of the United States (Abbott 1968 cited by Timi *et al.* 1999); *Cardiocephalus longicollis* from the eyes of sea breams *Diplodus annularis*, *D. vulgaris*, salem *Boops salpa*, sand steenbras *Pagellus mormyrus* and garfish *Belone belone* from Europe (Prevot & Bartoli 1980 cited by Timi *et al.* 1999); and an unidentified species of *Cardiocephalus* from the brain of red grouper *Epinephelus morio* from Mexico (Moravec *et al.* 1997). In the southern hemisphere, Timi *et al.* (1999) described *Cardiocephaloides* metacercariae found in the eyes of anchovy *Engraulis anchoita* from South America.

Only brief descriptions were given by Hunter and Vernberg (1960) and Abbott (1968), hence specimens from the present study could not be compared with theirs. Metacercarial morphology reported by Moravec *et al.* (1997) and Timi *et al.* (1999) are similar to the morphology of specimens in the present study. Although *Cardiocephalus* metacercaria (Moravec *et al.* 1997) have a larger body size, the caeca extend into the posterior end of the body, similar to the caeca of metacercariae in the present study. Also, both descriptions (Moravec *et al.* 1997 and present study) report the presence of gonadal primordia in the hindbody. On the other hand, *Cardiocephaloides* metacercariae described by Timi *et al.*

(1999) differ from specimens described in the present study by having shorter ceaca (i.e. ceaca ends in the middle of the body), even though both metacercariae have roughly the same body sizes. In addition, gonadal primordia were not observed in the description reported by Timi *et al.* (1999). These variations observed in metacercarial morphology could be as a result of differences in the age of the metacercariae under study or due to specimen preparation (fixation protocol, cover slip pressure etc.) (Table 3.3).

Adult *Cardiocephaloides* are parasites of larids and penguins (Niewiadomska 2002b). In South Africa, *Cardiocephaloides physalis* adults were previously reported from jackass penguin *Spheniscus demersus* by Randall & Bray (1983), and recently by Horne *et al.* (2011). Although the life cycle of *C. physalis* is unknown, it is suggested that its first intermediate host is a gastropod and the second intermediate host is a clupeid fish (Lutz 1926 and Baer 1969 cited by Randall & Bray 1983). Prevot & Bartoli 1980 (cited by Horne *et al.* 2011), in their studies on the life cycle of the congener *Cardiocephaloides longicollis*, found the first and second intermediate hosts to be a gastropod mollusc and sparid fish respectively, whereas the definitive hosts are gulls, *Larus argentatus* and *Larus ridibundus*. Randall and Bray (1983) pointed out that sparids have not been recorded as part of a penguin's diet. Thus, it is unlikely that sparids are intermediate hosts of *C. physalis*. Clupeids (anchovy, sardine and round herring, in order of importance) form the main component of the penguin's diet (Randall & Randall 1986). Therefore, penguins get infected after eating fish harboring *C. physalis* metacercariae (Timi *et al.* 1999). Given these points, specimens reported in the present study are probably *C. physalis* metacercariae. However, experimental infection of animals in the laboratory would be useful in order to confirm the metacercarial species (Bosma 1934). On the South American Atlantic coast *C. physalis* has also been reported from the Magellanic penguin *Spheniscus magellanicus*, which has a feeding behavior similar to its South African counterpart, the jackass penguin. *Spheniscus magellanicus* eats the argentine anchovy as its main dietary item (Scolaro & Badano 1986, Timi *et al.* 1999) and higher *C. physalis* metacercaria indices (prevalence and intensity of infection) were recorded in areas (in South American coast) where there are penguin colonies (Timi *et al.* 1999).

This study is the first description of strigeid metacercariae found in the eyes of *Sardinops sagax* sampled from Gansbay on the southwestern coast of South Africa. Over the years, parasites have been identified using traditional methods such as morphology, host and geographical distribution, epidermiology and behavioural characteristics, cross-breeding studies etc. However, it is now obvious that taxonomic relationships which were centered on traditional approaches need to be reviewed. Hence, molecular techniques and phylogenetic studies are currently employed as additional tools of parasite identification in order to achieve better taxonomic resolution (Steussy & Thomson 1981). In many cases, DNA characterization studies have produced outcomes which agree with results of traditional methods. But still in some cases, taxonomic inconsistencies have occurred creating confusion (Mcmanus & Bowles 1996). In view of this, the next chapter presents a molecular characterization of *Cardiocephaloides* metacercariae sampled from different locations around the South African coast.

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CHAPTER 4

MOLECULAR CHARACTERIZATION OF *Cardiocephaloides* sp. Sudarikov 1959 (DIGENEA: STRIGEIDAE) METACERCARIA FROM THE EYES OF SOUTH AFRICAN SARDINE, *Sardinops sagax*.

INTRODUCTION

Parasite identification typically involves the use of traditional methods such as optical microscopy that aid the visualization of diagnostic morphological features. However, these diagnostic features are difficult to identify in some parasite specimens, leading to the incorporation of molecular tools into taxonomic studies of parasites (Tavares *et al.* 2011). Hence, all developmental stages of parasites, including those with complex life cycles, can now be identified to species level (Criscone *et al.* 2005, Locke *et al.* 2010).

The use of molecular techniques to characterize and identify parasites has not only aided better taxonomic resolution, but also has helped to detect and describe parasite diversity that exists within and between species (Bickford *et al.* 2007). DNA-based methods are particularly useful in identification of distinct species, subspecies and strain groups. Morphological and molecular identification methods each have their own advantages (Shoshani 1986). Morphology remains the primary method of identification. It can be applied to various material ranging from museum and fossil material to live specimens. Morphological methods are less costly and easy to perform (Tavares *et al.* 2011). Although genetic methods are relatively expensive and require a high degree of skill in order to achieve consistent results, they are highly sensitive and detect even the slightest variations in species DNA (McManus & Bowles 1996). The molecular approach yields a highly extensive data set which can provide a detailed life history record (Hillis 1987, McManus & Bowles 1996). In addition, in studies involving parasites with complex life cycles, DNA sequences can be used to identify species at all developmental stages (Criscone *et al.* 2005), especially the poorly differentiated ones (Jousson *et al.* 1999). Thus, both approaches are able to answer questions which the other alone cannot address, thereby complementing each other (Hillis 1987). Some DNA characterization studies have produced outcomes which agree with

results of morphological methods (e.g. Bell *et al.* 2001, Pina *et al.* 2009, Athokpam & Tandon 2014), while molecular studies have revealed inconsistencies in taxonomic classification which was based on morphology (e.g. Bell & Sommerville 2002, Chen *et al.* 2007, Curran *et al.* 2013). Hence, the combination of morphological and molecular studies yields a more accurate identification than the use of a single method alone (Shaklee & Tamaru 1981, Duellman & Hillis 1987, Nolan & Cribb 2005, Olsen & Tkach 2005, Bickford *et al.* 2007, Blasco-Costa *et al.* 2016a, b).

Since the 1990s, phylogenetic studies have helped to shed some light into poorly known areas of digenean phylogeny using sequence information obtained from molecular markers, including the 18S small subunit rDNA, 28S large subunit rDNA, internal transcribed spacers (ITS) 1 and 2, mitochondrial DNA, and cytochrome c oxidase subunit 1 (Nolan & Cribb 2005, Olsen & Tkach 2005). Such areas include:

- phylogenetic relationship between families (Littlewood *et al.* 1999a, b, Fernández *et al.* 1998a, b, Cribb *et al.* 2001, Tkach *et al.* 2000, Tkach *et al.* 2001a, b, c, Olsen *et al.* 2003, Tkach *et al.* 2003, Olsen & Tkach 2005, Lapierre 2011, Curran *et al.* 2012, Curran & Pulis 2014);
- species description and delineation as well as examination of biogeographical and evolutionary relationship among species (Galazzo *et al.* 2002, Chen *et al.* 2007, Nolan & Cribb 2005, Blasco-Costa *et al.* 2010, Locke *et al.* 2010, Caffara *et al.* 2011, Locke *et al.* 2011, Nguyen *et al.* 2012, Galaktionov *et al.* 2012, Chibwana *et al.* 2013, Curran *et al.* 2013, Blasco-Costa *et al.* 2016b, García-Varela *et al.* 2016);
- life cycle evolution (Cribb *et al.* 2003); and
- life cycle elucidation: linking larval stages to sexual adults. Molecular data has been shown to serve as an alternative to the traditional approach of experimental infection to elucidate life cycles (Cribb *et al.* 1998, Jousson *et al.* 1998, 1999, Overstreet *et al.* 2002, Pina *et al.* 2009, Al-Kandari *et al.* 2011, Caffara *et al.* 2011, Eydal *et al.* 2011, Locke *et al.* 2011, Blasco-Costa *et al.* 2016a, García-Varela *et al.* 2016).

The digenean life cycle is complex (see Chapter 3), comprising two or three hosts and several developmental stages (Jousson *et al.* 1998, 1999). This plasticity of life history patterns and life stages has greatly contributed to their success as parasites in both vertebrates and invertebrates (Cribb *et al.* 2001, Roberts & Janovy 2009). Identification of larval stages is essential in the elucidation of digenean life cycles (Jousson *et al.* 1998), but the different larval stages bear little resemblance to adults. Therefore, the taxonomic keys that compare variations in diagnostic features for a particular family, genus or species cannot be used in identifying larval stages. This has made taxonomic identification and classification of these larval stages very challenging because digenean systematics is primarily based on the morphology of sexual adults. Also, the existence of these larval stages in different forms within different hosts has made it difficult to identify host species used in the different life cycle phases, thereby hampering life cycle elucidation studies (Locke *et al.* 2011).

The genus *Cardiocephaloides* Sudarikov, 1958 (Synonym *Cardiocephalus* Szidat, 1928) belongs to the superfamily Diplostomatoidea, and metacercariae within this superfamily are difficult to identify to species level (Locke *et al.* 2010, Locke *et al.* 2011, Moema *et al.* 2013). Locke *et al.* (2011) explained that this difficulty is as a result of the allocation of metacercariae to larval types or 'genera' namely 'Tetracotyle', 'Neascus', 'Diplostomulum' and 'Prohemistomulum' instead of identifying them to their adult genera based on diagnostic morphological characters as described by Niewiadomska (1970) and Shoop (1989). Adult *Cardiocephaloides* are gut parasites of seabirds and utilize fish as their second intermediate host (Niewiadomska 2002b). Species in this genus include; *Cardiocephaloides longicollis* Rudolphi, 1819; *C. hilli* S. J. Johnston, 1904; *C. musculosus* S. J. Johnston, 1904; *C. physalis* Lutz, 1926; *C. brandesi* Szidat, 1928; *C. medioconiger* Dubois and Vigueras, 1949 (syn. *megaloonus* Cable, Connor and Balling 1960); and *C. szidati* Hartwich, 1954 (Dubois 1968).

Molecular techniques have been used to identify the metacercariae of different digeneans (e.g. Anderson 1999, Jousson *et al.* 1999, Francisco *et al.* 2010, Moema *et al.* 2013, Athokpam & Tandon 2014, Blasco-Costa *et al.* 2016a, b) but there is no documentation on

the identification of *Cardiocephaloides* metacercaria using molecular tools. However, adult stages of *C. longicollis* (Olson *et al.* 2003, Born-Torrijos *et al.* 2016) and *C. medioconiger* (Hernandez-Mena *et al.* 2014) have been characterized using different genetic markers.

This chapter represents the first molecular characterization (which will enable definitive identification) of the metacercariae of *Cardiocephaloides* obtained from South African sardine, *Sardinops sagax*. In addition, it links the metacercariae to sexual adults using the sequence information of these life stages, thereby contributing information that would assist in life cycle studies.

MATERIALS AND METHODS

Fish sampling and collection of parasites

South African sardine *Sardinops sagax* were sampled from different sites off the west and south coasts of South Africa (Figure 4.1) during research surveys conducted by the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa in 2013 and 2014, and also from commercial catch samples taken in those years. Metacercariae were collected from the eyes and fixed in absolute ethanol. *Cardiocephaloides* adults (identified as *Cardiocephaloides physalis* by Horne *et al.* 2011) were collected from post-mortem jackass penguins *Spheniscus demersus* at the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) Seabird Centre, Cape St Francis, South Africa. Also, unidentified *Cardiocephaloides* adults were obtained from post-mortem penguins at SANCCOB Seabird Centre, Table View, South Africa. Adult specimens were also fixed in absolute ethanol. Both metacercariae and adult specimens were taken to the laboratory at the Department of Agriculture, Forestry and Fisheries (DAFF) Research Aquarium, Sea Point, Cape Town, South Africa for molecular work.

DNA extraction and PCR amplification

Prior to extraction, individual metacercariae and adult worms were rehydrated by placing them in 1.5ml Eppendorf tubes containing 500µl of distilled water and centrifuging at 14,000 rpm (Spectrafuge 16M, Labnet International, Inc. North America) for five minutes, after which the supernatant was discarded. This process was repeated thrice. DNA was extracted

from single individuals using QIAamp® DNA mini-kit (Qiagen, USA) following manufacturer's protocol and stored at -20°C.

PCR amplification of 28S large subunit (LSU) region was performed in a total volume of 25µl using a MULTIGENE™ thermal cycler (Labnet International, Inc. North America) and KAPA Taq ReadyMix (Kapa Biosystems, USA) containing Taq buffer, dNTPs, Taq DNA polymerase, MgCl₂ and stabilizers. 28S rDNA genes were amplified using the forward primer C1, 5'-ACCCGCTGAATTTAAGCAT-3' and the reverse primer D2, 5'-TGGTCCGTGTTTCAAGAC-3' (Hassouna *et al.* 1984, Chisholm *et al.* 2001). Cycling parameters consisted of denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds; annealing at 52°C for 30 seconds; extension at 72°C for 1½ minute; and a final stage of extension at 72°C for 5 minutes. Visualization of PCR products was done on 1% agarose gel stained with ethidium bromide to determine DNA quality and quantity.

DNA sequences were analysed on an ABI PRISM™ 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) using the BIG DYE™ Terminator v3.0 cycle sequencing premix kit at the Central Analytical Facilities (CAF), University of Stellenbosch, Western Cape. Sequencing was done in both directions for all amplifiable fragments using the C1-D2 primers for the 28S LSU rDNA region. 28S LSU sequences were cleaned in ChromaxPro version 1.7.5 (Technelysium Pty Ltd, Australia) and edited by eye. Contiguous sequences were assembled using CLC Main Workbench version 7.7.3 (Qiagen Aarhus A/S, Denmark).

Molecular analyses

Newly generated partial LSU rDNA sequences (see Table 4.1 for accession numbers) were aligned to appropriate sequences, retrieved from GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the MUSCLE algorithm in MEGA v7.0.14 (Edgar 2004). The 28S LSU rDNA dataset comprised species of Diplostomatidae and Strigeidae as both families are closely related and include: representative sequences for two species each of *Alaria* Schrank 1788, *Apharyngostrigea* Ciurea 1927, and *Diplostomum* von Nordmann 1832; one species each of *Apatemon* Szidat 1928, *Australapatemon* Sudarikov 1959, *Cardiocephaloides* Sudarikov 1959, *Ichthyocotylurus* Odening 1969, *Posthodiplostomum* Dubois 1936, and *Tylodelphys* Diesing 1850. One species of *Pleorchis*

Railliet 1896, that has a similar sequence to the newly generated sequences, but from a different family Acanthocolpidae, was used as an outgroup.

Alignments were edited manually and trimmed to match the shortest sequence. Only unambiguously aligned positions were used to construct phylogenetic trees. Analysis involved 23 nucleotide sequences with a total of 682 positions in the final dataset. Gaps and missing data were discarded from datasets (Complete Deletion Option). Evolutionary divergence between sequences and across all sequence pairs was calculated as the number of base differences per sequence and *p*-distance on raw data, respectively (Appendix 1).

All phylogenetic analyses were run on Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0.14 (Kumar *et al.* 2016). Phylogenetic reconstructions were inferred using Neighbour-joining (NJ) (Saitou & Nei 1987) and Maximum Likelihood (ML) methods (Kumar *et al.* 2016). For NJ analyses, evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004). For ML analyses, maximum likelihood fits of different substitution models were conducted and the model with the lowest Bayesian Information Criterion (BIC score) was selected. Computations for ML analyses were based on the Hasegawa-Kishino-Yano (HKY; Hasegawa *et al.* 1985) +G model predicted as the best estimator by the Bayesian Information Criterion (BIC) (Appendix 2). To obtain the initial tree(s) for the heuristic search, Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and the topology with superior log likelihood value was selected. Bootstrap nodal support values (1000 replicates) for branch nodes on strict consensus trees (Felsenstein 1985) were estimated and branches supported by less than 10% bootstrap values were collapsed.

Table 4.1. Taxa used for phylogenetic analysis. Abbreviations used: A, adult; C, cercaria; M, metacercaria; NG indicates not given. Isolate codes are indicated in bold.

Species	Life cycle stage	Host species	Locality	GenBank Accession number	Reference
<i>Alaria alata</i>	NG	NG	NG	AF184263	Tkach <i>et al.</i> 2001b
<i>Alaria mustelae</i>	M	<i>Lithobates sylvaticus</i>	Nelson County, North Dakota, USA	JF820607	Pulis <i>et al.</i> 2011
<i>Apatemon</i> sp. ApaPan1	C	<i>Potamopyrgus antipodarum</i>	Tomahawk lagoon, New Zealand	KT334166	Blasco-Costa <i>et al.</i> 2016a
<i>Apharyngostrigea cornu</i>	A	<i>Ardea cinerea</i>	Kherson Region, Ukraine	AF184264	Tkach <i>et al.</i> 2001b
<i>Apharyngostrigea pipientis</i>	A	<i>Nycticorax nycticorax</i>	Nelson County, North Dakota, USA	JF820597	Pulis <i>et al.</i> 2011
<i>Australapatemon niewiadomski</i> n. sp. AusApl4	A	<i>Anas platyrhynchos</i>	Balclutha, New Zealand	KT334165	Blasco-Costa <i>et al.</i> 2016a
<i>Cardiocephaloides longicollis</i>	A	<i>Larus ridibundus</i>	Kherson Region, Ukraine	AY222171	Olson <i>et al.</i> 2003
<i>Diplostomum phoxini</i>	M	<i>Phoxinus phoxinus</i>	Wales, United Kingdom	AY222173	Olson <i>et al.</i> 2003
<i>Diplostomum</i> sp. SE-2012	C	<i>Lymnaea stagnalis</i>	Nelson County, North Dakota, USA	JX262945	Tkach <i>et al.</i> 2012
<i>Ichthyocotylurus erraticus</i>	A	<i>Coregonus autumnalis</i>	Lough Neagh, Northern Ireland, United Kingdom	AY222172	Olson <i>et al.</i> 2003
<i>Posthodiplostomum</i> sp. VT-2013	M	<i>Channa punctata</i>	Manipur, Lamphel, India	KF738450	Athokpam & Tandon 2014
<i>Tylodelphys</i> sp. IBC-2016	M	<i>Gobiomorphus cotidianus</i>	Otago, South Island, New Zealand	KU588151	Blasco-Costa <i>et al.</i> 2016b
<i>Pleorchis uku</i>	A	<i>Aprion virescens</i>	Lizard Island, GBR, Australia	DQ248216	Bray <i>et al.</i> 2005

RESULTS

DNA extraction from metacercariae obtained from most sardine hosts was unsuccessful as metacercariae had disintegrated in their frozen hosts before dissection. Out of the 19 28S LSU ribosomal DNA (rDNA) sequences (17 metacercarial sequences and two adult sequences) obtained, nine sequences were removed from the dataset because they were very poor and failed to assemble. Consequently, only ten sequences (two adult sequences, one each from the south and west coast; one metacercarial sequence from the south coast and seven metacercarial sequences from the west coast) were used for comparisons of isolates (Figure 4.1).

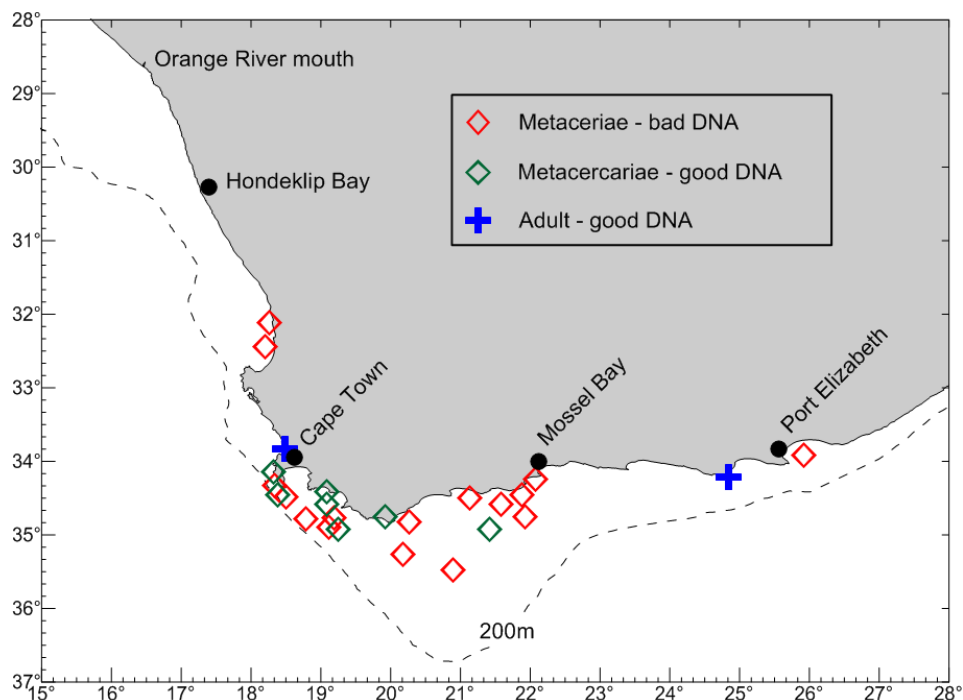


Figure 4.1. Map showing sampling locations of *Sardinops sagax* and adult penguins *Spheniscus demersus* from which *Cardiocephaloides* metacercariae and adults, respectively, were collected for molecular characterization.

Phylogenetic reconstructions derived from the dataset using NJ (see Appendix three) and ML methods had similar topology and showed congruent sister relationships among the taxa represented in the present study. Hence, only the ML tree is presented in the results (Figure 4.2). Trees were rooted in *Pleorchis uku*. Taxa grouped into two major clades with all the isolates generated in the present study (two adults and eight metacercariae) clustering into one of these clades. Both analyses show *Cardiocephaloides longicollis* from a black-headed gull *Larus ridibundus* from Ukraine, as a sister taxon to the isolates in the present study. All *Cardiocephaloides* sequences formed a strongly supported monophyletic clade which is related to the clade formed by other strigeid and diplostomid sequences. In addition, the *Cardiocephaloides* clade is illustrated as the earliest divergent clade in the ML phylogram. The analyses included only *Cardiocephaloides longicollis* sequence because of the lack of comparative sequences in GenBank.

The newly sequenced specimens did not show any base pair difference in their 28S sequences. However, their 28S sequences differed from *Cardiocephaloides longicollis* sequence in six sites, resulting in a 0.9% (6/682) nucleotide difference. Table 4.2 shows the locations of the nucleotide differences. The sequences from adult stages collected from penguin *Spheniscus demersus* from the west and south coast corresponded to each other with 100% similarity. Likewise, sequences from metacercariae isolated from sardine *Sardinops sagax* sampled from the west coast and the south coast were 100% identical to each other, as well as to the adult sequences.

Table 4.2. Nucleotide differences across 28S sequences of *Cardiocephaloides* isolates in the present study and *Cardiocephaloides longicollis* isolate from Olson *et al.* (2003).

Nucleotide position	419	425	450	463	495	660
<i>Cardiocephaloides</i> isolates in present study	C	G	A	G	C	T
<i>Cardiocephaloides longicollis</i>	T	T	G	A	T	C

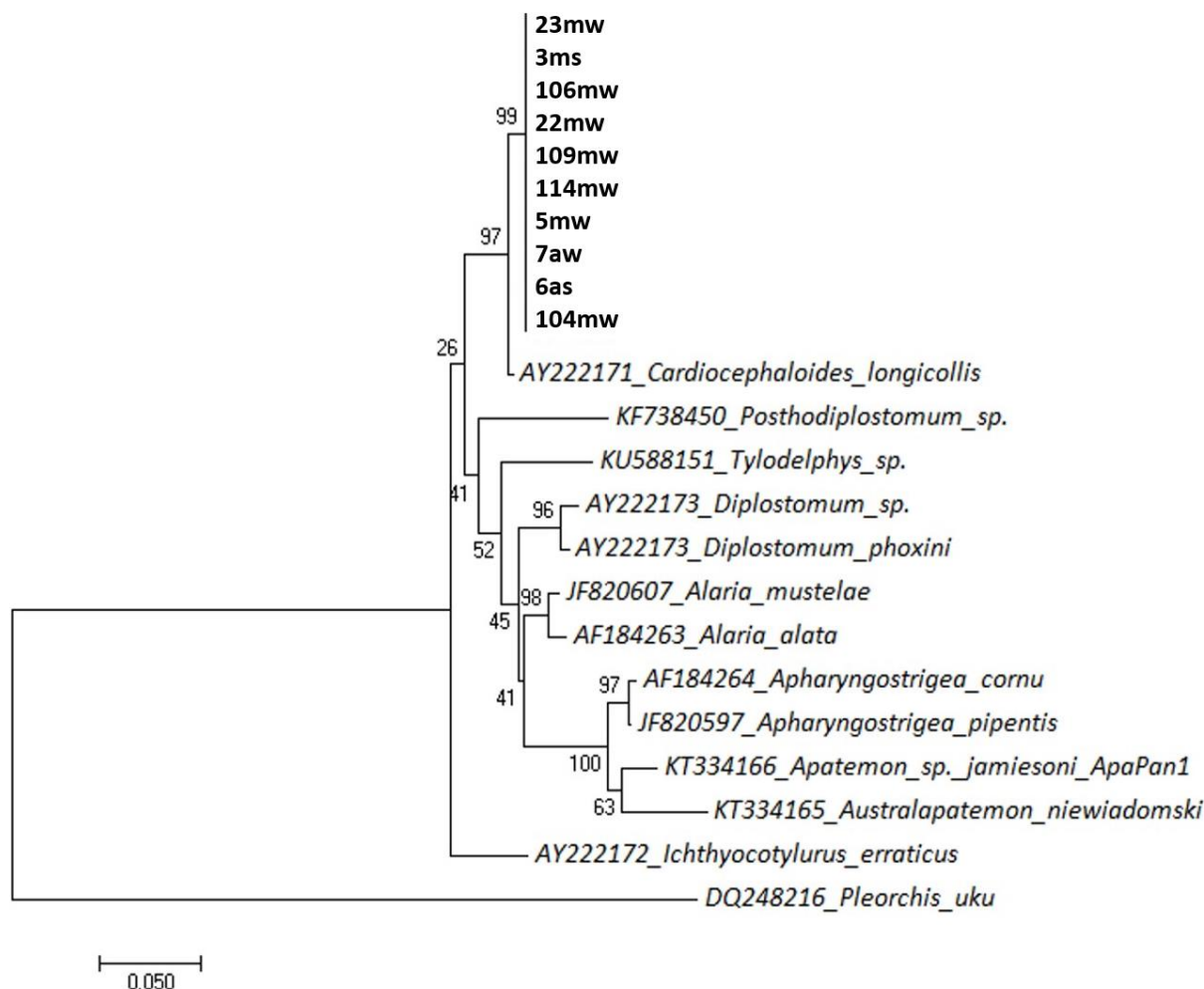


Figure 4.2. Phylogram based on Maximum Likelihood analysis of partial 28S rDNA sequence data. The tree is rooted in *Pleorchis uku* and numbers at nodes are bootstrap percentages of 1000 replicates (bootstrap values <10 not reported). GenBank accession numbers and taxon names are specified at branch tips and isolates collected in the present study are indicated in bold. The scale bar represents the number of substitutions per site. Abbreviations used: mw, isolate from metacercaria collected from sardine sampled off the west coast; ms, isolate from metacercaria collected from sardine sampled off the south coast; as, isolates from adult collected from jackass penguin host off the south coast; aw, isolates from adult collected from jackass penguin host off the west coast. Isolate number is given before the abbreviation for each isolate.

DISCUSSION

With the advent of DNA-based PCR amplification techniques, the accurate identification of morphologically similar but genetically distinct species has been greatly facilitated, which has enabled the resolution of several taxonomic inconsistencies (McManus & Bowles 1996, Olson & Tkach 2005). Species identification has involved the comparison of ribosomal DNA (rDNA) sequences because of their structural characteristics. The rDNA group is highly repeated and comprises the highly variable, fast-evolving ITS regions flanked by the highly conserved, slow-evolving 18S and 28S regions (Adlard *et al.* 1993).

Various studies have utilized molecular techniques to characterize digenean metacercarial stages (e.g. Nguyen *et al.* 2012, Moema *et al.* 2013, Athokpam & Tandon 2014, Blasco-Costa *et al.* 2016a, b). In this study, partial 28S rDNA sequence analyses showed 100% similarity among isolates, therefore indicating that metacercariae found in South African sardine *Sardinops sagax* and adult stages of the *Cardiocephaloides* found in jackass penguins *Spheniscus demersus* from South Africa are of the same species. Traditionally, studies to elucidate parasite life cycles involve the experimental infection of animals in the laboratory (Locke *et al.* 2011). However, the use of molecular data for linking larval stages to adults, and as such inferring the life cycle of digenetic trematodes, is rapidly increasing (Cribb *et al.* 1998, Jousson *et al.* 1998, Locke *et al.* 2011, Cribb *et al.* 2011, Galaktionov *et al.* 2012, Blasco-Costa *et al.* 2016a). Also, molecular techniques are currently being used in detecting cercarial stages in snail hosts, and hence used to estimate prevalence of trematode infections in these gastropods (Born-Torrijos *et al.* 2014a, b, Koprivnikar & Poulin 2009a, b).

Phylogenetic analysis showed the clading of query sequences close to *Cardiocephaloides longicollis*. Nucleotide differences in six sites (0.9%) were observed between the 28S sequences of *Cardiocephaloides longicollis* and the isolates (Table 4.2). The 28S rDNA region is highly conserved, and as such should be common to organisms of the same species (Moema *et al.* 2013). Furthermore, Sonnenberg *et al.* (2007) explained that even a single nucleotide difference in rDNA genes suggest genetic separation. Also, the sequence generated from an adult worm, identified as *Cardiocephaloides physalis* by Horne *et al.*

(2011), was 100% identical to other query sequences in the present study. Thus it is probable that the *Cardiocephaloides* isolates in the present study is a different species, probably *Cardiocephaloides physalis*. These results corroborate the morphology-based identification of the metacercarial stage described in Chapter three.

Cardiocephaloides is a genus of strigeid digeneans found in larids and penguins (Niewiadomska 2002). Adult members of this genus have the following diagnostic features: relatively small, cordiform, pear-shaped forebody; long hindbody (4 – 8.5 times longer than forebody) with a clavate or cylindrical neck region; pocket-shaped copulatory bursa; and vitellarium confined to the hindbody (Niewiadomska 2002). Their metacercariae are ‘tetracotyle’ type metacercariae which parasitize the eyes (La Rue *et al.* 1926) and brain (Brown 1899, Hunter & Vernberg 1960, Moravec *et al.* 1997) of their second intermediate hosts - marine teleost fish and certain amphibians (Kelley 1934). Although the life cycle of the *Cardiocephaloides* sp isolated in the present study is not yet fully known, it uses clupeoid fish as second intermediate hosts and penguins as definitive hosts.

Cardiocephaloides physalis has been reported as the only *Cardiocephaloides* sp that uses penguins *Spheniscus* sp as its definitive hosts (Dubois 1968, Randall & Bray 1983, Clarke & Kerry 2000, Diaz *et al.* 2010), thus supporting the hypothesis that the *Cardiocephaloides* sp in the present study is *C. physalis*. Conversely, life cycle studies on its congener *Cardiocephaloides longicollis* recorded the second intermediate hosts of this species to be sparid fish and the definitive hosts to be gulls, *Larus argentatus* and *Larus ridibundus* (Prevot & Bartoli 1980 cited by Horne *et al.* 2011). In addition, *C. longicollis* has been reported to show preference for mainly demersal and benthic fish species, and not pelagic fish species (Born-Torrijos *et al.* 2016).

This study is the first documentation of the genetic characterization of metacercariae and adults of *Cardiocephaloides* from sardine *Sardinops sagax* and penguin *Spheniscus demersus* from South Africa using 28S rDNA sequences. Results of this study have strongly indicated that metacercariae and adults are of the same species, likely *C. physalis*, and this information will improve our understanding of the transmission pathways and roles in the ecosystem (Poulin *et al.* 2016) of the parasite of interest – *Cardiocephaloides* sp. Also,

information obtained in the present study will also be of use in documenting the identity of the first intermediate host, as well as the larval stages (miracidium, sporocyst, redia, cercaria) found in this host. The first intermediate host has been hypothesized to be the gastropod *Burnapaena papyracea* (Weston 2013; van der Lingen *et al.* 2015) which is distributed along the west coast. Using molecular tools, future studies can test for the presence of larval stages including cercariae in this snail species and in a variety of inter-tidal and sub-tidal gastropods. Identification of the first intermediate host will help to confirm the location of the endemic area and whether it is only off the west coast as hypothesized.

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CHAPTER 5

DISCUSSION AND CONCLUSION

Understanding the population structure of a fish species, as well as the distribution of fishing effort and mortality rate, is essential for effective fishery management (Grime *et al.* 1987). This is especially important in fisheries where fishing effort is localized relative to the overall population distribution, which is usually the case, and even more so if effort is spatially un-balanced. In this situation multiple stocks, should they exist, could be exploited at different rates that may not be biologically sustainable. Hence management decisions should be based on knowledge of the existing population structure (Ricker 1981).

Depending on the research question posed, the availability of resources, and the improvements in scientific understanding and technology, different methods have been used to identify fish stocks based on differences in fish characteristics (Begg & Waldman 1999). These differences can be as a result of genetic isolation between stocks; or due to phenotypic variation which is driven by local environmental factors and indicates the prolonged separation of post-larval fish in different environmental conditions (Begg *et al.* 1999). Swain and Foote (1999) explained that genetic factors can contribute to phenotypic variations, such that differences between stocks are dependent on how the genotypes that determine phenotypic characters co-vary with the environments that influence these characters. In addition, they suggested that phenotypic variation, whether genetically- or environmentally-induced, may be sufficient enough to enable the identification of different stocks. However, they emphasized the need for the separation of the influence of these two 'arms of variation', if the management procedure is aimed at the identification of genetically distinct stocks. The absence of evidence of genetic isolation between stocks does not indicate true panmixia because it may take a long time for genetic markers to reveal any sub-division in fish populations (Grosberg & Cunningham 2001). In addition, the rate of evolution may have occurred over too short a time to allow for the evolution of structural proteins or other genetic markers (Clayton 1981). Thus, it would then follow that 'stock' is not strictly a genetic concept, but also represents a semi-discrete group of fish that have some definable characteristics which provide the information needed by fisheries managers to delineate fish stocks (Abaunza *et al.* 2008, Begg & Waldman 1999).

The South African marine environment is dominated by two current systems namely the warm Agulhas Current off southeast coast and the cold Benguela Current off the west coast, separated from each other in the region of Cape Agulhas (Barange *et al.* 1999, van der Lingen *et al.* 2006, Hutchings *et al.* 2009, Branch *et al.* 2010). The west coast is a wind-driven coastal upwelling system whereas the south coast is a warm temperate shelf system, and these contrasting conditions result in the existence of temperature and salinity gradients around the South African coast, with the west coast being colder and more productive than the warmer south coast (van der Lingen & Huggett 2003, Miller *et al.* 2006, Hutchings *et al.* 2009). Since the phenotypic variation between stocks is usually associated with the geographical region inhabited by a species (Begg & Waldman 1999), and in the absence of a strong evidence for genetic isolation and given these contrasting conditions around the South African coastline, one would expect spatial variability in morphological and meristic characteristics of sardine occupying the west and south coast of South Africa. Initial investigations were conducted to determine if there is spatial variability in phenotypic characteristics of west coast and south coast sardine. These phenotypic characteristics were: body shape, otolith shape indices (otolith circularity and form factor), branchial sieve morphology (gill arch length, gill raker spacing and number of gill rakers) and number of vertebral bones. With the exception of vertebral bone count, otolith shape indices and gill raker spacing, previous studies indicated that there could be two stocks around the South African coast (Wessels 2009, Idris 2010, Idris *et al.* 2016, Hampton 2014). In the present study, results of separate univariate analysis showed significant spatial variability between west coast and south coast sardine for all phenotypic characteristics except for vertebral count and gill raker spacing. In contrast to the findings of no significant variability in otolith shape indices (Hampton 2014), results of this study showed that these descriptors significantly discriminated between sardine from the west and south coasts of South Africa. Results of the investigation on spatial variability of body shape Wessels (2009) suggested some degree of variability in sardine shape between the two coasts, which was confirmed in the present study. Investigations of the spatial and temporal variability in the 'tetracotyle' type metacercaria infection in the South African sardine, both in a previous study by Weston (2013) and in the present study, revealed the existence of western and southern sardine

stocks. The observed discrepancies between some of the results of previous studies (eg. otolith shape indices) and the present study confirmed Begg and Waldman (1999)'s view that 'true' stock differences may not be revealed by investigating only a single characteristics, hence confirming the need for a multidisciplinary approach in the assessment of South African sardine stock structure.

The use of multiple methods allowed for the collection of multiple stock descriptors on the same individuals, thus it provided the opportunity for a comprehensive quantification of the South African sardine *Sardinops sagax* population structure in a common statistical framework and without the attendant limitations of comparing different data sets obtained at different times (Cadrin *et al.* 2014). Integration of data from multiple approaches using both the Stock Differentiation Index (SDI) and quantitative analysis in a multivariate framework gave strong and consistent evidence of the existence of two interacting sardine stocks around the South African coast. Presently, the sardine purse-seine fishery has been hypothesized to consist of two mixing stocks - western and southern stocks, as evidenced by the existence of two main spatially separated spawning areas, by the differential parasite loads between sardine from the western and southern putative stocks, and by variations in phenotypic features of sardine from the two stocks (Butterworth *et al.* 2016). Therefore, the findings from this study provide additional support for deliberations on the two-stock hypothesis of the South African sardine population.

Although a multidisciplinary approach involving the integrating of data from a wide range of techniques is regarded as the method of choice in population structure studies, some of these techniques are costly, and much time and effort are required to generate data. Morphometric characters change over time and show strong allometric effects which could confound spatial variability while meristic traits do not change over time and are fixed at birth. The use of parasites as biotags is limited by lack of adequate knowledge of parasite biology and ecology, as well as by inaccurate taxonomic identification. Hence, Abuanza *et al.* (2008b) advised it would be better to choose the most useful single methods or the important descriptors of stocks where feasible. In this study, the combination of vertebral count and parasite ('tetracotyle' type metacercaria) abundance were found to be the most

useful predictors of sardine stock, even though vertebral count did not differentiate between sardine stocks when examined independently, making it an ineffective discriminator on its own. These methods require simple sample collection and storage procedures, and do not require much time and expertise for data collection. Analysis of the metacercaria biotag has been demonstrated to provide sufficient discriminatory power between sardine stocks, and as such a strong evidence of the existence of two stocks (Weston 2013, van der Lingen *et al.* 2015). In general, parasites biotags used in the discrimination of fish species have often produced results at a resolution suitable for fishery management (Lester *et al.* 1985, Mackenzie 1990, Moore *et al.* 2003, Moore *et al.* 2011, Feki *et al.* 2015).

Major constraints in the use of parasite biotags in stock identification are the lack of adequate information in the complex biology and ecology of parasites, as well as the inaccurate taxonomic identification of parasites (Sindermann 1983). In view of this, morphological and molecular identifications of the 'tetracotyle' type metacercaria were carried out in order to provide a framework for future ecological and parasitological studies. Morphological features and molecular characterization using 28S rDNA genes placed the metacercaria in the strigeid genus *Cardiocephaloides*. Adult members of this genus are gut parasites of larids and penguins while their metacercariae infect the eyes of clupeid fish (Niewiadomska 2002b), as observed in sardine *Sardinops sagax* in the present study. The *Cardiocephaloides* metacercariae, in this study, was found to be entangled in the suspensory apparatus of the fish eye lens, an area which is devoid of any immunological response. This could be because the host body recognizes that the eye is a sensitive area and would be adversely affected by any immunological response against the parasite. It is equally possible that the parasite recognizes this fact, and therefore chooses this predilection site, enhancing the successful completion of its life cycle. This implies that the higher the number of sardine eaten by the final host, the higher the chances of getting infected. It is possible that the presence of the metacercariae in the eye affects sardine behavior and survival by increasing the susceptibility of sardine hosts to predation, as previous studies have demonstrated alteration of host behavior either directly or indirectly by parasites.

Cardiocephaloides metacercaria is considered to be endemic to the west coast of South Africa where the cold oceanographic, as well as the ecological (presence of intermediate hosts) conditions are conducive for its life cycle and successful transmission of infection to intermediate and definitive hosts (Weston 2013). However, the presence of the *Cardiocephaloides* metacercariae was not restricted only to the endemic zone in the west coast. As observed in the present study, sardine from the south coast were also infected, though in lesser magnitude. Metacercariae would have been transferred to the south coast by post-recruitment fish who either visit the south coast or remain in the stock permanently (de Moor and Butterworth 2013, Weston 2013, van der Lingen & Hendricks 2014, de Moor & Butterworth 2015, van der Lingen *et al.* 2015). As fish are unable to 'lose' the metacercaria, and as such there is cumulative infection as they (sardine hosts) age. This implies that more metacercariae would be found in older fish in the west coast. However, there could be occasional high parasite abundance from an individual in the south coast as illustrated, in this study, by the presence of 71 parasites in the eye of a south coast fish.

The present study also linked 'tetracotyle' type metacercariae to adult *Cardiocephaloides physalis*, previously reported from the African penguin *Spheniscus demersus* (Randall & Bray 1983, Horne *et al.* 2011) indicating that both adult and metacercarial life stages are of the same species. Comparison of 28S LSU rDNA sequences from isolates (adult and metacercarial life stages) in the present study with the congener, *Cardiocephaloides longicollis* revealed nucleotide differences in six sites, indicating *C. longicollis* is a different species. This finding is supported by the differences in their host preferences, even though the life cycle of *C. physalis* is not yet fully known. Although, both *C. physalis* and *C. longicollis* use gastropods as their first intermediate hosts, adult *Cardiocephaloides longicollis* are parasites of gulls *Larus argentatus* and *Larus ridibundus*, and utilize sparid fish as their second intermediate hosts (Prevot & Bartoli 1980 cited by Horne *et al.* 2011). On the other hand, *C. physalis* infects penguins and uses clupeid fish as its second intermediate host (Lutz 1926 and Baer 1969 cited by Randall & Bray 1983, Horne *et al.* 2011). Again, the composition of the penguin's diet makes it unlikely for them (penguins) to be infected by *C. longicollis*, as the preferred diets for penguins are sardine and anchovy, not sparid fish

(Randall & Randall 1986). It would follow that mode of transmission of metacercariae is by ingestion of infected fish by the penguin host.

The use of molecular data in inferring the life cycle of digenetic trematodes is fast replacing the traditional method of experimentally infecting animals in the laboratory (Cribb *et al.* 1998, Jousson *et al.* 1998, Locke *et al.* 2011, Cribb *et al.* 2011, Galaktionov *et al.* 2012, Blasco-Costa *et al.* 2016a). The identification of the adult stage of the ‘tetracotyle’ type metacercariae from African penguins, carried out in the present study, forms the foundation for future investigations into the life cycle (identification of the first intermediate host, description of other larval life stages – miracidium, sporocyst, redia, cercaria as well as a description of adults) and ecology of *C. physalis* in South Africa. It is speculated that the first intermediate host could be *Burnupena papyracea*, an intertidal whelk that occupies the cool waters of the west coast of South Africa in abundant numbers (Orr 1956, Branch *et al.* 2010), and occurs in the same endemic region hypothesized for the *Cardiocephaloides* metacercariae (Weston 2013). Identification of the first intermediate host is commonly based on the detection of larval trematode infections through the process of cercarial shedding. However, molecular detection of trematode infection has been shown to be more accurate because cercarial emergence is dependent on time, environmental conditions and parasite species (Koprivnikar & Poulin 2009a, b, Born-Torrijos *et al.* 2014a, b). Hence, future studies which involve the confirmation of the first intermediate host of the *Cardiocephaloides* metacercariae, by the detection of larval trematodes, and the estimation of infection, in snail tissues using molecular techniques, are recommended.

Most of the metacercariae disintegrated within their frozen sardine hosts which were collected from different locations around the South African coasts, during research surveys by the Department of Agriculture, Forestry and Fisheries (DAFF). As observed from the study, freezing of sardine hosts was not a suitable method of preservation of the fragile, soft-bodied metacercariae for molecular analyses. A better method would be the collection- and immediate preservation in absolute or 70% ethanol, of metacercariae from fresh hosts. However, due to lack of finances and time, resampling of more sardine hosts was not possible. For future studies, if possible, an arrangement should be worked out such that

fresh sardine samples from areas far away from the processing laboratory, are brought in at the shortest possible time to avoid degradation of materials.

Successful identification of parasites to genus level was achieved using 28S large subunit ribosomal genes which is a highly conserved region within the ribosomal DNA group. The internal transcribed (ITS1 and ITS2) genes serve as diagnostic markers for species discrimination and identification of larval stages (Nolan & Cribb 2005, Littlewood 2008). Due to time and financial constraints, other molecular markers could not be used. Future studies which involve the collection of sardine hosts from additional areas in the southwestern coast, as well as DNA sequencing of the ITS region, and/or the 18S small subunit ribosomal region is recommended in order to achieve species-level resolution and confirm the species of the *Cardiocephaloides* metacercariae described in the present study.

In conclusion, the study presents a strong evidence for the existence of two interacting sardine stocks off the western and southern coast of South Africa. It provides support for the on-going discussions on the 'two-stock' structure of the South African sardine resource, the best operational management procedure to adopt for efficient fisheries management, as well as the development of stock-specific assessment models. Also, the study provides the first documentation of the morphological and molecular identification of the 'tetracotyle' type metacercariae which was found to be a strong descriptor of sardine stocks. In addition, the adult life stage of this parasite was successfully identified using molecular characterization, thereby providing essential information on the life cycle of this parasite.

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APPENDIX 1

Table showing estimates of evolutionary divergence between sequences. Standard error estimates are shown above in red ink. Average evolutionary divergence over all sequence pairs (p -distance) = 0.069 ± 0.008 . Nos 1 to 10, *Cardiocephaloides* isolates from present study; 11, *Cardiocephaloides longicollis*; 12, *Apharyngostrigea cornu*; 13, *Tylodelphys sp*; 14, *Apatemon sp jamiesoni* ApaPan1; 15, *Australapatemon niewiadomski*; 16, *Posthodiplostomum sp*; 17, *Alaria mustelae*; 18, *Apharyngostrigea pipentis*; 19, *Pleorchis uku*; 20, *Diplostomum sp*; 21, *Diplostomum phoxini*; 22, *Ichthyocotylurus erraticus*; 23, *Alaria alata*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1		0	0	0	0	0	0	0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
2	0		0	0	0	0	0	0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
3	0	0		0	0	0	0	0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
4	0	0	0		0	0	0	0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
5	0	0	0	0		0	0	0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
6	0	0	0	0	0		0	0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
7	0	0	0	0	0	0		0	0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
8	0	0	0	0	0	0	0		0	0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
9	0	0	0	0	0	0	0	0		0	2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
10	0	0	0	0	0	0	0	0	0		2.34	6.20	6.11	6.40	6.89	6.18	5.38	6.10	9.88	6.07	5.81	5.68	5.64
11	6	6	6	6	6	6	6	6	6	6		6.15	6.09	6.28	6.75	6.22	5.31	6.07	9.90	6.02	5.75	5.50	5.55
12	50	50	50	50	50	50	50	50	50	50	48		6.31	4.35	5.38	6.52	5.79	1.72	10.44	6.17	6.21	6.68	6.19
13	44	44	44	44	44	44	44	44	44	44	42	48		6.49	7.16	6.38	5.96	6.20	10.02	5.91	5.63	6.08	6.08

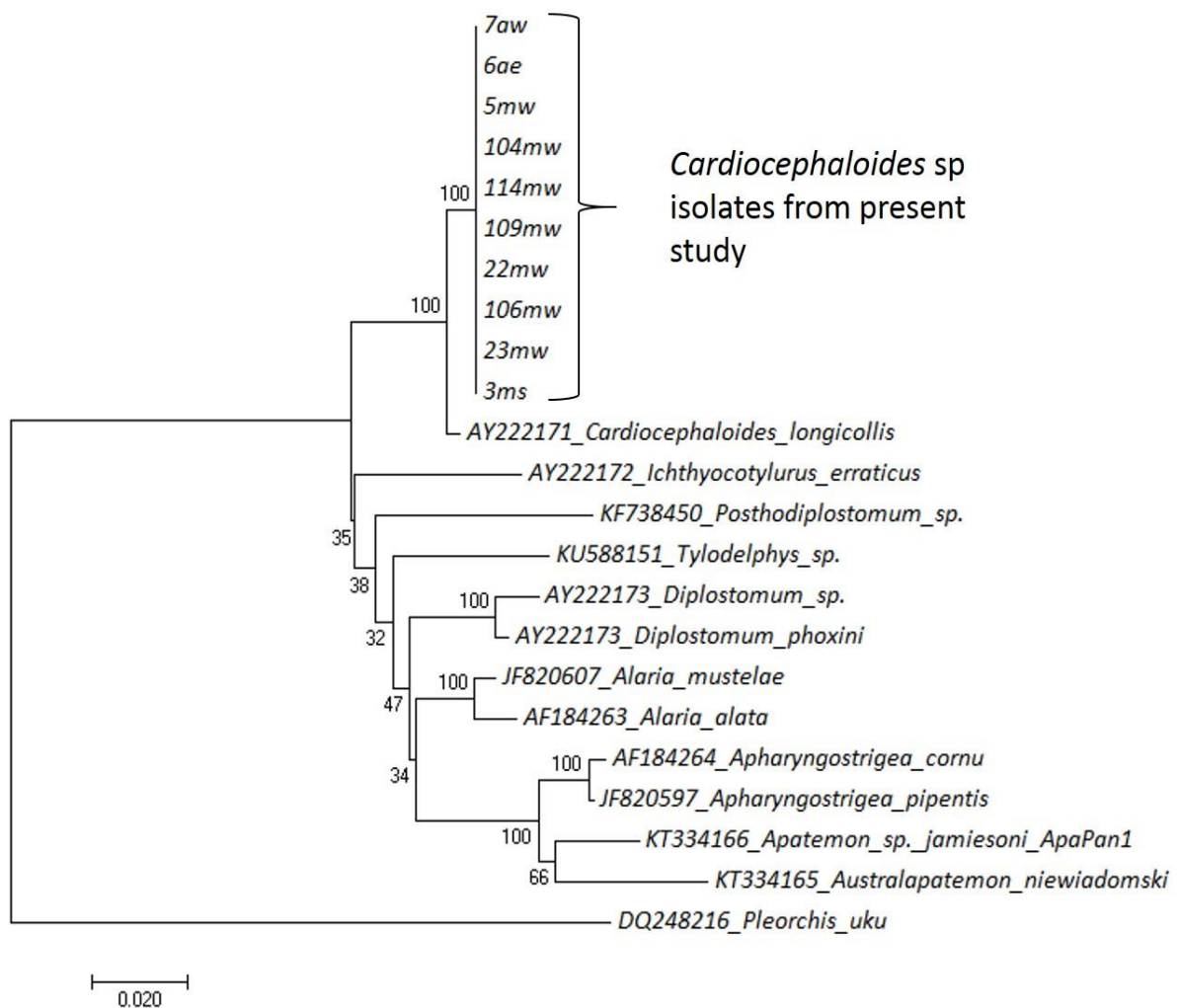
14	55	55	55	55	55	55	55	55	55	55	53	22	52		5.38	7.25	6.32	4.11	10.42	6.32	6.11	6.37	6.34
15	62	62	62	62	62	62	62	62	62	62	60	33	62	32		7.78	6.77	5.48	10.72	7.16	6.94	7.21	6.69
16	49	49	49	49	49	49	49	49	49	49	48	50	51	58	68		6.14	6.56	10.13	6.77	6.86	6.96	6.29
17	35	35	35	35	35	35	35	35	35	35	33	35	38	43	50	45		5.80	10.13	5.14	5.18	6.20	2.81
18	49	49	49	49	49	49	49	49	49	49	47	3	45	19	33	49	34		10.23	6.13	6.16	6.44	6.24
19	124	124	124	124	124	124	124	124	124	124	123	142	130	144	156	133	131	139		10.14	10.14	10.08	10.34
20	41	41	41	41	41	41	41	41	41	41	39	44	40	49	57	54	28	43	134		2.64	6.45	5.20
21	37	37	37	37	37	37	37	37	37	37	35	41	36	44	54	52	26	40	130	8		6.20	5.22
22	40	40	40	40	40	40	40	40	40	40	36	52	42	50	57	57	45	49	129	48	44		6.45
23	38	38	38	38	38	38	38	38	38	38	36	39	41	43	50	48	9	38	134	29	27	48	

APPENDIX 2

Table showing Maximum Likelihood fits of 24 different nucleotide substitution models. Model with the lowest Bayesian Information Criterion (BIC scores) is regarded as the best descriptor of nucleotide substitution pattern. Abbreviations used: GTR, General Time Reversible; HKY, Hasegawa-Kishino-Yano; TN93, Tamura-Nei; T92, Tamura 3-parameter; K2, Kimura 2-parameter; JC, Jukes-Cantor; + I, invariable sites; + G, gamma-distributed sites.

MODEL	#PARAMETERS	BIC
HKY + G	48	5340.148
GTR + G	52	5344.471
HKY + G + I	49	5348.598
TN93 + G	49	5348.907
HKY + I	48	5356.169
GTR + I	52	5356.307
TN93 + G + I	50	5357.331
TN93 + I	49	5361.409
GTR + G + I	53	5376.075
K2 + G	45	5379.336
K2 + G + I	46	5387.852
T92 + G	46	5392.183
K2 + I	45	5392.860
T92 + G + 1	47	5400.735
T92 + 1	46	5406.097
GTR	51	5504.846
HKY	47	5507.524
TN93	48	5516.705
K2	44	5529.212
T92	45	5540.118
JC + G	44	5645.969
JC + I	44	5653.438
JC + G + I	45	5655.105
JC	43	5776.384

APPENDIX 3



Phylogram based on Neighbour-joining analysis of partial 28S rDNA sequence data. The tree is rooted in *Pleorchis uku* and numbers at nodes are bootstrap percentages of 1000 replicates (bootstrap values <10 not reported). GenBank accession numbers and taxon names are specified at branch tips and isolates collected in the present study are indicated in bold. The scale bar represents the number of substitutions per site.

Abbreviations used: mw, isolate from metacercaria collected from sardine sampled off the west coast; ms, isolate from metacercaria collected from sardine sampled off the south coast; as, isolates from adult collected from jackass penguin host off the south coast; aw, isolates from adult collected from jackass penguin host off the west coast. Isolate number is given before the abbreviation for each isolate.