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Testing for the occurrence of pilchard herpesvirus (PHV) in South African sardine *Sardinops sagax*

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Abstract

Catches of South African sardine *Sardinops sagax* have declined in recent years from about 200 000 t harvested annually during the period 2002–2006 to less than 100 000 t. Consequently, some companies are now importing sardine from sources elsewhere in the world to meet local demand for canned sardine and bait. This importation has the potential for the introduction of sardine pathogens, in particular the pilchard herpesvirus (PHV), which could have a negative impact on the currently small South African sardine population. The aims of the current study were to determine whether PHV is present in the local sardine population and to assess the extent to which sardine is being imported into the country and whether imported fish are from countries where the virus is known to be endemic. Fish sampled from South Africa's western ($n = 150$), southern ($n = 182$) and eastern ($n = 96$) putative stocks of *S. sagax* were analysed for the presence of PHV using real-time quantitative polymerase chain reaction (qPCR). The origin and amount of potentially infected material imported into South Africa during the period 2010–2014 was also assessed. None of the South African sardine collected during this study tested positive for PHV, suggesting that active PHV was not prevalent in the local population of *S. sagax* at the time of this study. Between 56 000 and 71 000 t of frozen sardine was imported annually into South Africa from countries where *S. sagax* occurs, including some from areas (Australia and New Zealand) where sardine infection by PHV is known to be endemic. Hence, it is plausible that the PHV pathogen, capable of perpetuating infections in local sardine populations, could be imported into South Africa along with the importation of frozen sardine. Should local sardine be naïve to the virus, as suggested by this study, then the population is at risk of infection and precautions against such must be taken.

Introduction

Sardine or 'pilchard' *Sardinops sagax* is a small pelagic fish species, the global distribution of which includes southwestern Africa, where it is the main target species of the South African purse-seine fishery and has been harvested since the late 1940s (Beckley and van der Lingen 1999). Annual sardine catches have ranged from 16 000 to 410 000 t, with catches in excess of 200 000 t per year taken during the periods 1959–1965 and 2002–2006. Since 2008, however, annual sardine catches have declined to below 100 000 t, following a reduction in the sardine population size (DAFF 2014). Mass mortalities of *S. sagax* and other clupeid species have been recorded globally from as early as the 1900s (Jones et al. 1997).

The causes of some of these mass mortalities remain unknown, but are of concern regarding the potential for accidental introduction and spread of disease to naïve populations elsewhere. The most recent mass-mortality events occurred off Australia and New Zealand in 1995 and then again in 1998/1999, where an estimated 70% of the spawning biomass of sardine off Australia was lost (Crockford et al. 2005, 2008; Whittington et al. 2008). In both instances, the causative agent was identified as a herpesvirus, the pilchard herpesvirus (PHV), which is postulated to have been introduced into Australian waters through the importation of PHV-infected sardine to feed sea-caged southern bluefin tuna *Thunnus maccoyii* in South Australia (Gaughan 2002; Crockford et al. 2008). Pilchard herpesvirus is now considered to be endemic in Australian sardine (Whittington et al. 2008). The recent reduction in South African sardine catches has resulted in the importation of frozen sardine into South Africa by local fish-processing companies and the processing of those fish to meet local demand for canned sardine and bait and to maintain market share of their brand (Attwood 2008). Frozen sardine might also be imported into South Africa for sea- and shore-based aquaculture operations in the future, given the expected expansion of the marine aquaculture sector in South Africa (RSA 2013). This could potentially have very serious consequences for the local sardine population, especially if it is shown that local sardine are naïve to PHV and frozen sardine are being imported from suppliers in countries with a known history of the virus. The aims of the current study were, therefore, to determine whether PHV is present in the South African sardine population and to assess the extent to which sardine is being imported into the country and whether these fish are imported from countries where the virus is known to be endemic.

Material and methods

Determination of PHV presence in South African sardine

Three putative ecologically or functionally discrete stocks or subpopulations of *S. sagax* occur off the coast of South Africa, namely western, southern and eastern subpopulations (van der Lingen 2011; van der Lingen et al. 2015). For the purposes of this study, sardine were collected from all three subpopulations and, in accordance with Whittington et al. (2008), it was assumed that if the virus was present it would be found in at least 10% of adult fish, would be uniformly distributed among shoals of fish within a subpopulation and among schools within shoals, and fish sampled for PHV testing would be representative of the subpopulation from which they were caught. Fish from the western ($n = 150$) and southern ($n = 182$) sardine subpopulations were collected from midwater trawls conducted during pelagic biomass surveys by the Department of Agriculture, Forestry and Fisheries (DAFF) in 2009 and 2011, with those in 2011 collected from a single trawl off Mossel Bay (Figure 1 and Table 1). Fish from the eastern ($n = 96$) subpopulation were collected from a single beach-seine net catch made during the 2012 KwaZulu- Natal sardine run (a phenomenon described in van der Lingen et al. 2010).

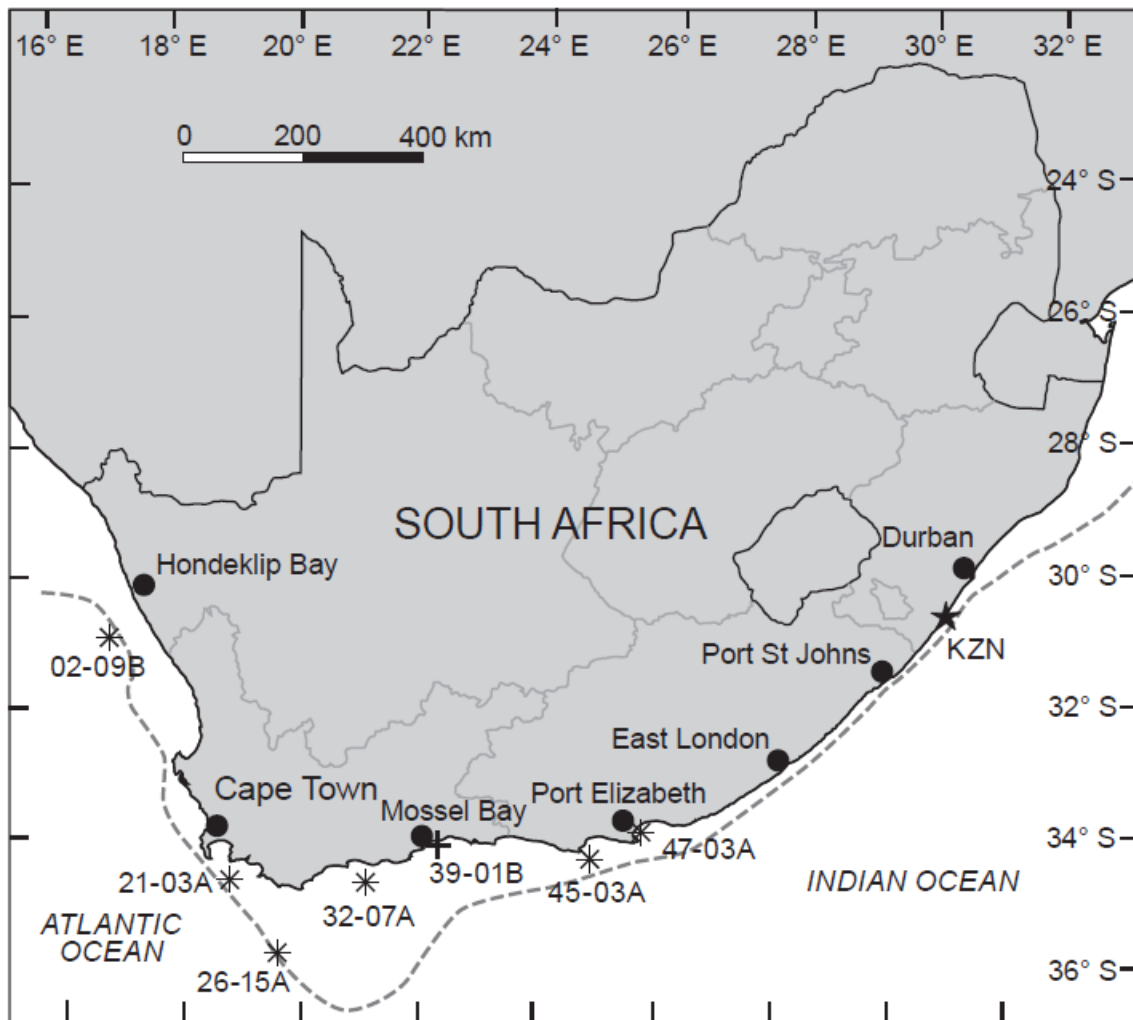


Figure 1: Location of midwater trawls from which sardine were collected for pilchard herpesvirus testing during the 2009 (asterisks) and 2011 (cross) pelagic biomass surveys and from a beach-seine net catch off the east coast during the sardine run in 2012 (star). Codes represent sample identifiers (see Table 1). Some important coastal towns and cities are shown and the 200-m depth contour is indicated by a dashed line

The fish from the Mossel Bay trawl were selected because of their extremely poor condition (sardine from that station were approximately 65% of their expected weight) and probable resultant high stress levels and suppressed immune function, which could have increased the likelihood of viral replication should latent PHV infections reside in South African sardine populations. In all, 428 sardine from eight sampling sites around the South African coast between Hondeklip Bay and Durban were tested for PHV presence. Sardine selected comprised both subadults and adults, with most fish >15 cm caudal length (Table 1). All tissue samples were blast-frozen and stored in an ultra-low freezer (−80 °C) until subsequent

laboratory processing and analysis. DNA extractions for real-time quantitative polymerase chain reaction (qPCR) from frozen sardine gill-tissue samples (25–50 mg tissue per sample) were performed using the modified DNAzol extraction method described by Crockford et al. (2005). Real-time PCR analysis was conducted using a Bio-Rad CFX96™ real-time PCR detection system on a C1000™ thermal cycler. Real-time PCR primers and cycling conditions were as described by Crockford et al. (2008). Data collection and real-time analysis occurred at the annealing step of each cycle and melt-curve data collection and analysis occurred at each increment in the latter 40 cycles. Any samples that recorded a quantification cycle (Cq) value after setting the PCR cycle number and baseline threshold values to 40 and 150, respectively, on the amplification graph and with a melt peak of 81 ± 1 °C on the melt-curve graph, were considered to be positive. Samples that failed to record a Cq value or failed to produce a specific melt peak were considered to be negative. For each PCR, a standard curve was constructed by spiking known quantities of plasmid DNA, purified from a PHV clone obtained from the Department of Fisheries in Western Australia (see Crockford et al. 2008), into the homogenised gill tissues prepared from a PHV-negative sardine. DNA added for sensitivity testing was spiked into tissues in order to achieve final concentrations that ranged from $13 \text{ ng } \mu\text{l}^{-1}$ to $1.3 \times 10^{-11} \text{ ng } \mu\text{l}^{-1}$ in the reaction tubes.

Table 1: Sampling details for sardine tested for PHV, showing sample identifier, subpopulation, collection date, number of fish and their mean caudal length (CL)

Sample identifier	Subpopulation	Collection date	<i>n</i>	Mean CL (SD) (cm)
02-09B	Western	12 October 2009	50	19.2 (0.6)
21-03A	Western	27 October 2009	50	12.7 (0.7)
26-15A	Western	30 October 2009	50	17.1 (1.9)
32-07A	Southern	05 November 2009	50	14.8 (0.3)
39-01B	Southern	29 November 2011	50	17.0 (2.4)
45-03A	Southern	14 November 2009	32	16.7 (0.8)
47-03A	Southern	16 November 2009	50	15.0 (0.8)
KZN	Eastern	15 July 2012	96	15.2 (0.6)

This method of generating a standard curve is believed to provide a more accurate measure of viral load should the virus be present in the samples tested, because it accounts for loss of DNA and/or presence of inhibitors arising from the process of extracting DNA from tissue samples. Aliquots of tissue from a PHV-negative sardine, along with non-template controls (PCR-grade H₂O), were analysed as negative controls during each PCR to ensure that there was no amplification, except for the PHV DNA. Furthermore, aliquots of tissue spiked with a

known amount of PHV DNA (positive controls) were also analysed during each PCR to ensure consistency between real-time PCRs.

Assessment of the quantities and origins of imported sardine

The origin and amount of potentially infected material imported into South Africa during the period 2010–2014 was assessed using data provided by the South African Revenue Service. The data included country of export and country of origin, import location, importer name, bill-of-entry date and number, purpose and quantities of 'Frozen Sardines' (Code TH030371; note that this category includes sardine *S. sagax* and *Sardina pilchardus*, sardinellas *Sardinella* spp. and brisling or sprat *Sprattus sprattus*.)

Results

DNA extraction and real-time PCR analysis

DNA extracted from the 428 sardine tissue samples was subjected to qPCR using PHV-specific primers (Crockford et al. 2008). Using standard curves generated from known amounts of viral (plasmid) DNA spiked into PHV-negative sardine-gill-tissue homogenates, we established that qPCR could be used to quantify as little as 1.3×10^{-10} ng μl^{-1} DNA per 15- μl reaction volume. The C_q values of replicate samples (standards and controls) differed by no more than one cycle and the efficiency of all reactions was >90%. The samples included as positive controls for the real-time PCR were the only samples that produced a positive result. No amplification occurred in the negative-control samples, nor was there amplification from any of the samples tested from the eight sampling sites ($n = 428$), representative of the three subpopulations of sardine around the South African coast. Analysis of these results using the FreeCalc software program (Cameron 2002) suggests that they are adequate to conclude that all three sardine subpopulations could be regarded as free from PHV at the minimum expected prevalence of 10% and at a confidence level >99%.

Assessment of the scale of imported sardine

The quantities of frozen sardines imported into South Africa from countries off which *S. sagax* occurs (www.Fishbase.org; Froese and Pauly n.d.) are given in Table 2. Analysis of the scale of the proposed risk posed by the importation of frozen sardines showed that 56 000–71 000 t per annum had been imported into South Africa between 2010 and 2014. Although the majority (almost 75%) of this was recorded as coming from Namibia, South Africa's neighbour, much of the remainder was imported from countries where sardine infection by PHV has been confirmed (Australia and particularly New Zealand) or suspected (USA; see below).

Discussion

None of the South African sardine collected during this study tested positive for PHV by real-time PCR, suggesting that the local population of *S. sagax* could conservatively be regarded as PHV-free. Whittington et al. (2008) used the FreeCalc software program (Cameron 2002) to estimate the sample size required for detection of PHV in a population/subpopulation of Australian pilchard (sardine) *S. sagax neopilchardus* using a variety of diagnostic tests, and

concluded that for real-time PCR a sample size of 191 is required with a minimum of seven positive tests for a population to be designated as truly positive.

Table 2: Annual importation (rounded to the nearest whole number) into South Africa of frozen sardines (a category which includes sardine *Sardinops* spp. and *Sardina pilchardus*, sardinella *Sardinella* spp. and brisling or sprat *Sprattus sprattus*) from countries off which *Sardinops sagax* occurs (from www.Fishbase.org), 2010–2014

Country of origin	Quantity of imported frozen sardines (t)					Country total
	2010	2011	2012	2013	2014	
Australia	24	115	270	220	115	758
Canada	53	99	28	50	130	437
China	1 328	1 630	1 402	1 937	2 212	10 323
Chile	602	531	746	714	72	2687
Ecuador		270	267	240	1 503	2 883
Hong Kong	4	2	690	87	25	860
Japan	1 096	1 094	1 100	1 250	1 561	7 564
Korea	467	917	419	268	324	2 450
Mauritius	232	263	55	115	133	858
Mexico			27	60		87
Mozambique	187	134	167	227	183	924
Namibia	40 813	43 754	53 594	56 643	52 615	268 763
New Zealand	9 530	7 831	6 217	4 542	8 017	40 604
Peru	105	464	304	88	221	1 240
Russian Federation		118	434	392		944
Taiwan	1 038	1 594	1 450	1 329	1 591	11 240
USA	673	3 030	832	2 189	1 018	9 832
Yemen		11			2	13
Annual total	56 152	61 857	68 002	70 351	69 722	

191 is required with a minimum of seven positive tests for a population to be designated as truly positive. These estimates assumed a population size >10 000 individuals, a minimum expected prevalence of PHV >10%, a confidence level of 95% and very conservative estimates for the most likely values for sensitivity (50%) and specificity (98%) for the real-time PCR test. Based on these assumptions and using an identical real-time PCR protocol to our study, Whittington et al. (2008) sampled 200 fish from each of the four subpopulations of sardine in Australia and reported that PHV is endemic in Australian subpopulations. Fish sampled and analysed for PHV in the current study originated from western ($n = 150$), southern ($n = 182$) and eastern ($n = 96$) South African *S. sagax* subpopulations. These sample sizes, particularly from the western and southern subpopulations, are only marginally lower than the required sample size proposed by Whittington et al. (2008) for determining whether a subpopulation is truly positive for PHV, after accounting for population size and the limitations of the diagnostic test. These findings suggest that South African sardine are PHV-free/naïve and would consequently be susceptible to infection by PHV, should infected material make contact with local populations, either through the release of waste/refuse water directly into coastal waters from the processing of infected frozen sardine, or through the use of infected sardine as bait by local fishers, or as aquafeed. Given this potential susceptibility to infection, the importation of untreated frozen sardine from other countries into South Africa has the potential for introducing sardine pathogens that could have a negative impact on the local sardine population, as appeared to have happened in Australasian waters in the mid- to late 1990s, when two mass-mortality events of Australian sardine were observed. Similar concerns over the risks to local fish populations of imported fish as aquafeed have also been raised in the Mediterranean, where large quantities of

imported frozen clupeiform species are used as the main source of feed in bluefin tuna *Thunnus thynnus* farms in the region (Tudela 2005). The origin and amount of potentially infected material imported into South Africa during 2010–2014 showed that approximately 65 000 t of frozen sardines were imported annually into the country from countries off which *Sardinops* occurs, primarily Namibia, New Zealand, Taiwan, China, the USA and Japan. Although neither *Sprattus* nor *Sardina* co-occur with *Sardinops*, *Sardinella* does so in some instances (e.g. off Namibia, Taiwan and Japan), so it is unlikely that all of the frozen sardines imported from those countries consisted of *Sardinops*. Nonetheless, it appears highly likely that considerable amounts of this species are imported from locations where sardine infection by PHV is either known (Australia and New Zealand; Whittington et al. 2008) or suspected (USA, Peru and Japan; Jones et al. 1997; Whittington et al. 2008). Hence, it is plausible that the PHV pathogen, capable of perpetuating infections in local sardine populations, could be imported into South Africa in association with frozen sardine. Given the import volumes concerned, such imports could represent a realistic risk. Furthermore, the use of imported sardine as bait, as well as the probability of infective sardine material entering the sea from processing establishments, provides plausible pathways for infection of the potentially susceptible local sardine population. Should local sardine be naïve to PHV, as suggested by this study, then the population is at risk of infection should this pathogen be introduced to the South African marine system, by whatever means, and precautions to avoid such must be taken. Further resolution regarding the likelihood and scale of the risks currently being posed by the importation of frozen sardine could be obtained through the assessment of the PHV status of imported consignments from countries where sardine populations are suspected or known to be infected. An expanded pathogen-import risk assessment is recommended to provide a qualitative assessment or estimate of the likelihood of the pathogen being imported into South Africa in association with frozen sardine (release assessment) and its potential to spread to local sardine populations (exposure assessment), in accordance with Gaughan (2002). *Acknowledgements* — We thank DAFF staff who collected sardine during the 2009 and 2011 pelagic biomass surveys and Mr Brent Chiazzari (University of KwaZulu-Natal) who collected and supplied the 2012 sardine run samples. Thanks are due to Dr Melanie Crockford for providing the PHV-positive DNA (plasmid) sample required for the quantitative real-time PCR assays.

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