Piscivory does not cause pansteatitis (yellow fat disease) in *Oreochromis mossambicus* from an African sub-tropical reservoir

J. Dabrowski*[1, 2], G. Hall[3], N. Lübcker[4], P.J. Oberholster[1, 5], D.L. Phillips[6], S. Woodborne[7]

* Corresponding Author

[1]Department of Paraclinical Sciences,

Faculty of Veterinary Science,

University of Pretoria, Private Bag X04,

Onderstepoort 0110, South Africa

[2]Council for Scientific and Industrial Research (CSIR),

Natural Resources and the Environment,

P.O. Box 395, Pretoria 0001,

South Africa

[3]Stable Isotope Laboratory,

Mammal Research Institute,

Department of Zoology and Entomology,

University of Pretoria, Private Bag X20,

Hatfield, Pretoria 0028,

South Africa.

[4]Department of Zoology and Entomology,

University of Pretoria, Private Bag X20,

Hatfield, Pretoria 0028,

South Africa.

[5]Council for Scientific and Industrial Research (CSIR),

Natural Resources and the Environment,

P.O. Box 320, Stellenbosch 7599

South Africa

[6] U.S. Environmental Protection Agency

National Health and Environmental Effects Research Laboratory

200 SW 35th Street

Corvallis, Oregon 97333

USA

[7]iThemba Laboratories

Private Bag 11, WITS 2050

South Africa

Running title

The diet of pansteatitis-affected *Oreochromis mossambicus* from a sub-tropical reservoir

Summary

1. Pansteatitis (yellow fat disease) is ubiquitous in the free-ranging population of *Oreochromis mossambicus* from Loskop Reservoir (LR), South Africa. The disease is nutritionally mediated and associated with a diet high in polyunsaturated or rancid fats, frequently of fish origin. While piscivory has never been reported in dietary studies of *O. mossambicus* in their native range, their opportunistic and omnivorous feeding habits mean that piscivory cannot be ruled out as a cause of the disease.

2. The diet of *O. mossambicus* from LR (n=91) was compared to a population from Flag Boshielo Reservoir (FBR; n=81) located less than 100 km downstream, where no pansteatitis occurs. The stomach contents and stable isotope signatures ($\delta^{15}N$ and $\delta^{13}C$) of fish and food sources were evaluated across four seasons. Isotope signatures

were also compared over various time scales from historic samples and mortalities collected from LR.

3. There was no evidence of piscivorous feeding behaviour in fish from either location, or from historic LR samples. The results of the SIAR mixing model and stomach contents analysis showed that the dinoflagellate, *Ceratium hirundinella*, was the dominant food source followed by zooplankton, detritus and *Microcystis aeruginosa* in LR. The diet of fish from FBR was less diverse than fish from LR, and was dominated by sediment and detritus.

4. The distinguishing feature of the dietary comparison between reservoirs was the abundance of planktonic food items dominated by *C. hirundinella* in the diet of fish from LR. The lack of evidence for piscivory among *O. mossambicus* from LR suggests that the classic aetiology of pansteatitis does not apply. This highlights the need to further explore direct (environmental exposure to pollutants) and indirect (dietary exposure) links to pansteatitis. This study identified the major dietary constituents for *O. mossambicus*, which enables future research to focus on their nutritional and chemical composition.

Introduction

Oreochromis mossambicus (Mozambique tilapia; Peters 1852) is one of the most widely distributed exotic fish species globally (Costa-Pierce, 2003) mainly due to their high value as an aquaculture species. The species is endemic to south-eastern Africa where it is found in a range of habitats including lakes, rivers, and estuaries from the lower Zambezi River in Mozambique, to the Bushmans River in South Africa (Skelton, 2001). Their distribution includes the Olifants River, one of the main river systems in South Africa, which is beset by increasing demands for water, discharge of effluents from coal-mining, urban, industrial and agricultural activities, and variable rainfall (Ashton & Dabrowski, 2011).

The adverse effects of deteriorating water quality culminated in the deaths of large numbers of Nile crocodiles, *Crocodylus niloticus*, at two distinct locations along the river between the years 2005 and 2009 (Ashton, 2010). Mortalities were attributed to pansteatitis (also known as yellow fat disease), and occurred in Loskop Reservoir (LR) in the upper catchment (Oberholster *et al.*, 2010), and approximately 350 km downstream in the Olifants River gorge in the Kruger National Park (KNP) (Osthoff *et al.,* 2010; Ferreira & Pienaar, 2011). The Nile

crocodile population at LR has since declined to ca. 6 individuals (Botha *et al.*, 2011), while at KNP, South Africa's premier conservation area, the population is in decline after ca. 200 mortalities (Ashton, 2010; Ferreira & Pienaar, 2011). Pansteatitis was also described in *Clarias gariepinus* (Sharptooth catfish) in the Olifants River gorge at KNP (Huchzermeyer *et al.*, 2011), and associated symptoms were observed in the majority of *O. mossambicus* examined from LR during this study. The simultaneous occurrence of the disease at two distinct locations on the same river system, at various consumer trophic levels and in different fish species is remarkable, and represents the first documented case of this type.

Pansteatitis has infrequently been reported in other free-ranging animals associated with aquatic environments. These include several species of egrets and herons in Japan (Neagari *et al.*, 2011), Mediterranean striped dolphins, and a loggerhead turtle off the coast of Spain (Soto *et al.*, 2010; Orós *et al.,* 2013), and great blue herons in Chesapeake Bay in the USA (Nichols *et al.*, 1986). However, it is more commonly reported in various captive-bred or farmed animals including several species of marine and freshwater fish (Roberts *et al.*, 1979; Herman & Kircheis, 1985; Bricknell *et al.*, 1996; Guarda *et al.,* 1997; Goodwin, 2006*;* Roberts & Agius, 2008), alligators (Larsen *et al.*, 1983), and crocodiles (Huchzermeyer, 2003). The disease is characterised by an inflammatory reaction to necrotic fat cells, and is a nutritionally mediated condition. In captive-bred animals it is recurrently attributed to consumption of a diet high in rancid or unsaturated fats, frequently of fish origin, and deficient in antioxidants such as vitamin E (Roberts *et al.*, 1979; Fytianou *et al.,* 2006; Roberts & Agius, 2008). Consumption of a diet of this nature can further deplete reserves of vitamin E and other antioxidants, compounding the effects of oxidative stress, which include an accumulation of reactive peroxides in fat tissues (Fytianou *et al.*, 2006).

The specific aetiology of pansteatitis in *O. mossambicus* from LR, and in most other cases involving free-ranging animals, is unknown. Several theories have been suggested and are mainly linked to known causes of oxidative stress. These include exposure to the hepatotoxin, microcystin, produced by *Microcystis* spp. blooms (Rattner & McGowan, 2007). However, no evidence of intake or exposure to microcystin was detected when more than 70 pansteatitis-affected egrets and herons died concurrent to a bloom of *Microcystis aeruginosa* (Neagari *et al.*, 2011). Inorganic pollutants have not been measured in LR, but warrant further investigation because high concentrations of polychlorinated biphenyls (PCBs) have been found in the fat of a pansteatitis-affected loggerhead sea turtle (Orós *et al.,* 2013). In

4

LR, elevated levels of aluminium and iron have been detected in the water (Oberholster *et al.*, 2010; Dabrowski *et al.*, 2013), the fat of *O. mossambicus*, and a potential food source, *Spirogyra* spp. (Oberholster *et al.*, 2012). High levels of ingested iron have been linked to lipid peroxidation in fish (Baker *et al.*, 1997; Elbaraasi *et al.*, 2004), suggesting a possible connection between bioaccumulation of metals and oxidative stress associated with pansteatitis. Recent work in the KNP has implicated altered trophic relationships resulting from back-flooding in the Olifants River gorge, when the Massingir Reservoir dam wall was raised (Woodborne *et al.*, 2012). These include the proliferation of invasive silver carp (*Hypophthalmichthys molitrix*) as a possible food source rich in polyunsaturated fat for pansteatitis-affected *C. gariepinus* (Huchzermeyer *et al.*, 2013). Studies of the lipid profiles of *C. gariepinus* and *C. niloticus* from the KNP have shown that pansteatitis-affected animals have a higher n-3 to n-6 fatty acid ratio than healthy animals, indicating an increased intake of polyunsaturated fats which is prone to oxidation (Osthoff *et al.*, 2010; Huchzermeyer *et al.*, 2013). Given the dietary links established in captive-bred animals, the results of this research highlight the need to determine the diet of free-ranging animals with pansteatitis.

Crocodile mortalities in LR occurred concurrent to several fish kills of various species (including *O. mossambicus*), the culmination of an extended dry period (Dabrowski *et al.*, 2013), and escalating blooms of the cyanobacterium *M. aeruginosa,* and the dinoflagellate *Ceratium hirundinella* due to eutrophication (Oberholster *et al.,* 2010). Although *O. mossambicus* examined during fish kills were diagnosed with pansteatitis post-mortem, the cause of death may have been related to fluctuating environmental conditions that affected several fish species. The algal blooms are indicative of bottom-up changes in the food web, and have significantly altered physico-chemical parameters such as pH and dissolved oxygen levels in the reservoir (Dabrowski *et al.*, 2013). While the consumption of rancid (rotting) fish following fish kills may explain the occurrence of pansteatitis in crocodiles, persistent symptoms of the disease observed in mature *O. mossambicus* in LR are not easily explained. In their native range their diet usually consists of detritus, phytoplankton, periphyton, macroalgae, diatoms and zooplankton (de Moor *et al.*, 1985; Bowen, 1979; Zengeya *et al.*, 2011; Dyer *et al.*, 2013). However, *O. mossambicus* are known to exhibit trophic plasticity under different environmental conditions (Bowen & Allanson, 1982; Dyer *et al.,* 2013). In Australia, they have been shown to prey on juvenile indigenous fish in both laboratory and field situations (Doupé *et al.*, 2009), and to completely digest fish prey in as short as one hour leaving little evidence of this food source in stomach contents (Doupé & Knott, 2011). In Sri

Lanka, De Silva *et al*. (1984) reported *O. mossambicus* diets ranging from detritivory, to herbivory, to complete carnivory in different reservoirs.

Assumptions about their diet are thus limited by their omnivorous feeding habits. It is possible that *O. mossambicus* from LR are piscivores, or may scavenge on dead fish associated with fish kills, causing them to develop pansteatitis. This shift in trophic level may be apparent in their δ^{15}N values. If there is no evidence of piscivory, then it is important to establish the specific and dominant constituents of their diet to facilitate further research into their chemical and nutritional composition. The aims of this study were to determine whether there is any evidence of piscivory in pansteatitis-affected fish from LR, and how their diet compares to healthy fish in the same river system. We used the combined approach of stomach contents analysis and stable isotopes incorporating seasonal variation to assess the fish diet. This was applied to the *O. mossambicus* population in LR as well as a reference population in Flag Boshielo Reservoir (FBR). The latter reservoir is located approximately 100 km downstream from LR, and was selected because pansteatitis has never been reported at this location. The reservoir has a large population of *O. mossambicus* and the highest concentration of crocodiles in the Olifants River system outside of the KNP (Botha, 2010). Muscle samples of *O. mossambicus* collected from LR during large-scale fish kills in 2007, and a fish health study undertaken in 2010, were analysed to determine whether there was any evidence of historic variation in isotopic signatures relative to the situation in 2011.

Methods

Reservoir characteristics

The Olifants River is the main inflow for both LR and FBR, which are located in the Mpumalanga and Limpopo provinces of South Africa respectively (Fig. 1). An extensive assessment of the current state and historic trends in water chemistry, physiography and limnology was undertaken concurrent to fish sampling at both LR (Dabrowski *et al*., 2013) and FBR (Dabrowski, 2012). Regular algal blooms at LR resulted in higher maximum chlorophyll-*a* values than FBR, where no algal blooms were observed during the study period. The trophic state of LR was meso- to eutrophic (Oberholster *et al*., 2013), while FBR was oligotrophic. Dominant vegetation types differ markedly between the catchments of both reservoirs. The catchment of LR is dominated by Highveld grassland in the upper reaches,
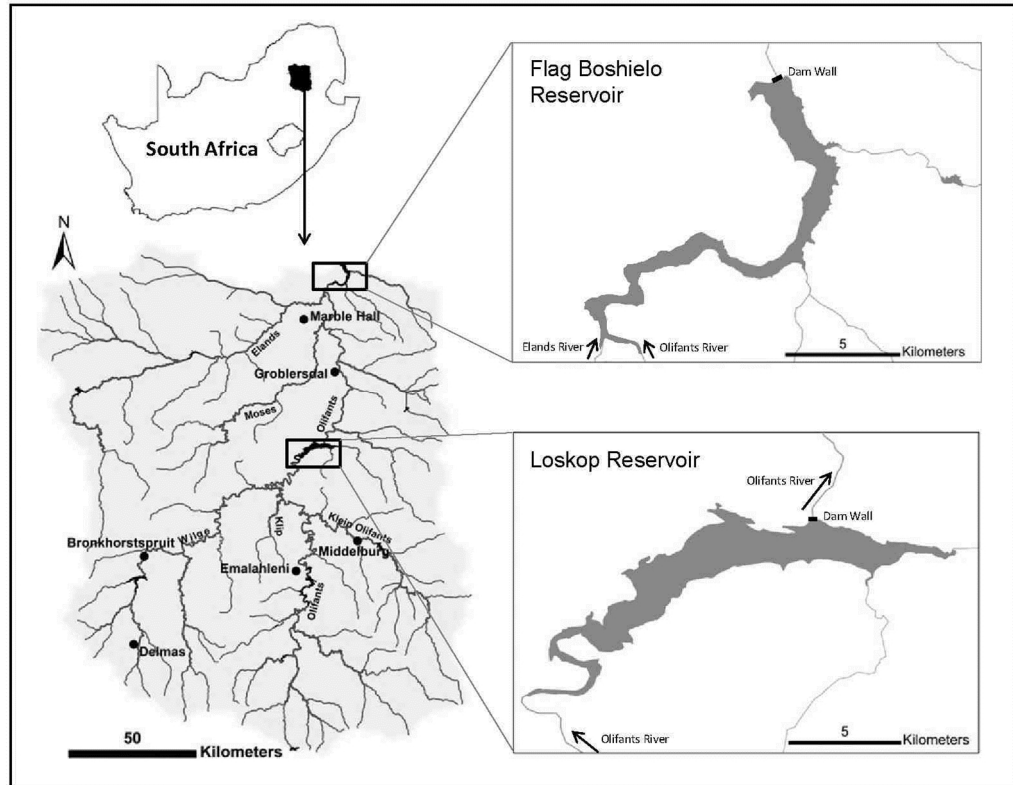
Fig. 1 Location of Loskop (LR) and Flag Boshielo Reservoirs (FBR) in the Olifants River catchment, South Africa.

and mixed Thornveld and Bushveld (semi-arid savanna dominated by grassland and *Acacia* spp. trees and various shrubs) in the lower reaches around the reservoir. The catchment of FBR is dominated by mixed Thornveld and Bushveld, much of which has been degraded (Mucina & Rutherford, 2006).

Sample collection

In order to study the dietary composition of *O. mossambicus* from both reservoirs, samples of approximately 20 fish per season were collected during April (Autumn), June (Winter), October (Spring) and December (Summer) in 2011 to represent an annual cycle. To reduce variation associated with ontogenetic dietary shifts, only fish > 200 mm total length (TL) were collected (Table 1). Gill nets were set during daylight hours using three 25 m panels with 70, 90 and 130 mm multi-filament stretched-mesh nets. After collection, fish were weighed and their TL was recorded. A section of approximately 10 g of muscle tissue was dissected from the left flank of each fish for analysis of $\delta^{15}N$ and $\delta^{13}C$ isotopes. Tissue samples were frozen for subsequent isotopic analysis.

7

Table 1 Summarised catch statistics for *Oreochromis mossambicus* sampled from Loskop (LR) and Flag Boshielo Reservoirs (FBR) in 2011 including the sex ratio (M : F), total length (TL) and weight (Mean ± SD)

Site	Season	n	M : F	Empty stomachs (%)	TL (mm)	Weight (g)
LR	Autumn	31	17 : 14	0	372 ± 6.5	1207 ± 597
	Winter	20	7 : 13	50	421 ± 3.9	1725 ± 468
	Spring	20	11 : 9	25	420 ± 3.4	1683 ± 337
	Summer	20	6 : 14	30	395 ± 4.8	1353 ± 448
	Total	91	41 : 50	23	398 ± 5.4	1458 ± 532
FBR	Autumn	21	16 : 5	19	365 ± 6.3	876 ± 404
	Winter	20	15 : 5	20	353 ± 4.8	906 ± 312
	Spring	20	7 : 13	20	321 ± 5.2	662 ± 252
	Summer	20	4 : 16	45	283 ± 3.8	474 ± 223
	Total	81	42 : 39	26	325 ± 5.7	730 ± 348

Potential food sources for *O. mossambicus* fluctuated seasonally and were sampled when abundant throughout 2011 at both reservoirs. Filamentous green algae (Chlorophyceae) were handpicked from various substrates including rocks and submerged trees. Sediment organic matter (SOM) samples were collected at all sites using an Ekman grab lowered to the reservoir bottom at various depths. The top 5 cm of sediment was collected from the grab sample. Near-shore detrital material was collected by hand in polycarbonate containers from above the sediment in areas where it accumulated, and was distinct from the fine material in SOM. All samples were stored on ice in the field and subsequently frozen prior to laboratory processing. Plankton were sampled from both reservoirs by using a plankton net (30 μm mesh, 25 cm diameter) lowered to the reservoir bottom at each study site and brought to the surface in order to obtain an integrated sample of the water column. An amount of 10 ml was sub-sampled and preserved in 10% buffered formalin for identification and sorting of zooplankton. Zooplankton replicates consisted of > 50 whole individuals, predominantly *Daphnia* spp. and copepods. Samples of *C. hirundinella*, *M. aeruginosa* and the pelagic diatom *Fragilaria crotonensis* were collected by water filtration using 3μm filters in LR.

Dominance of these taxa was confirmed by inspection of samples with a compound microscope at 1250 x magnification.

Historic <u>Oreochromis mossambicus</u> samples

Isotopic signatures were determined for samples of muscle tissue from *O. mossambicus* that were collected and frozen on several occasions prior to this study. In 2007, around the time when several large fish kills occurred, three pansteatitis-affected fish were collected by a veterinary pathologist. During an unpublished fish health study in 2010, 24 fish were collected using gill nets. Concurrent to that study, samples were collected from isolated mortalities of large *O. mossambicus* displaying severe symptoms of pansteatitis. All fish measured greater than 200 mm TL.

Dietary composition: stomach contents analysis

Fish stomachs from *O. mossambicus* were removed and preserved in 10% buffered formalin. Dietary composition was assessed by frequency of occurrence (Hyslop, 1980) as follows:

$$\%F_i = (N_i / N) \times 100$$

Where $\%F_i$ is the percentage frequency of occurrence of prey item i, N_i is the number of fish with prey i in their stomachs, and N is the total number of fish stomachs containing food examined. Stomach contents were divided into the fore-, mid- and hindgut and three subsamples of each region were spread on a Sedgwick rafter counting cell to determine the proportional contribution (% number) of each dietary item to the gut contents of each fish (Hyslop, 1980). Items in the stomach contents were identified to the lowest taxonomic level possible, and then assigned to the following categories: diatoms; *Ceratium hirundinella*; green algae; *Microcystis aeruginosa*; zooplankton; sediment; detritus. The proportion of fish with empty stomachs was recorded.

Dietary composition: stable isotope analysis

All samples were analysed by the environmental isotope facility at the Council for Scientific and Industrial Research (Pretoria, South Africa). Lipids were extracted from fish muscle

tissue using a 2:1 chloroform:ethanol mixture following the method of Logan *et al.* (2008). All other samples were treated with 1% HCl to remove possible biogenic carbonates, and then repeatedly rinsed with distilled water. Samples were oven-dried at 70°C overnight before being homogenised. Aliquots of each sample were weighed (0.8 – 1 mg) into tin capsules and combusted at 1020°C in an elemental analyser (Flash EA, 1112 series, Thermo Fisher Scientific, Bremen, Germany). A continuous flow isotope ratio mass spectrometer (CF-IRMS, Delta V Plus, Thermo Finnigan, Bremen, Germany) coupled to the EA via a Conflo IV interface, was used to measure the $\delta^{13}C$ and $\delta^{15}N$ isotopic composition of the samples. Isotope ratios were expressed as parts per thousand (‰) relative to the reference standards of air for ^{15}N, and Vienna Pee Dee belemnite for ^{13}C (Coplen, 1994). Isotope ratios were expressed relative to standards as follows:

$$X = [(R_{sample} / R_{standard})-1] \times 1000$$

where $X = \delta^{13}C$ or $\delta^{15}N$ (‰) and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{12}N$ respectively.

An in-house laboratory standard (Merck gelatine) and a blank were run after every 12 samples. To ensure reproducibility of the results, approximately every 11th sample was measured in duplicate. The variance from replicates was 0.09‰ for $\delta^{13}C$ and 0.14‰ for $\delta^{15}N$ which was very close to the instrumental reproducibility achieved from the standards. Fish muscle $\delta^{13}C$ was not adjusted for lipid content because of recent uncertainties about lipid-normalisation methods (Fagan *et al.*, 2011). Zooplankton values were adjusted because preservation in formalin has been shown to marginally affect isotopic signatures of freshwater zooplankton, with $\delta^{15}N$ increasing by 0.25‰ and $\delta^{13}C$ decreasing by 0.2‰ on average (Rennie *et al.*, 2012).

Trophic Position

The trophic position of *O. mossambicus* was calculated using the formula outlined by Post (2002):

$$\text{Trophic position} = \lambda + (\delta^{15}N_{consumer} - \delta^{15}N_{base}) / \Delta_n$$

Where λ is the trophic level of the base, $\delta^{15}N_{consumer}$ is the nitrogen isotope signature of *O. mossambicus*, $\delta^{15}N_{base}$ is that of the organism used to estimate the baseline, and Δ_n is the average trophic enrichment of nitrogen (3.4‰). The species used to estimate $\delta^{15}N_{base}$ should share the same habitat as the target species and be relatively long-lived so as to minimise short-term variation in isotopic signatures of the food web (Post, 2002). Redbreast tilapia (*Tilapia rendalli*) is abundant at both reservoirs, shares a similar habitat to *O. mossambicus*, and was used to estimate $\delta^{15}N_{base}$. As a primary consumer that also feeds on invertebrates and crustaceans, they were assigned the trophic level of 2.5. The previously described gill nets, and a seine net (5 m length, 1 mm mesh) were used to capture *T. rendalli* during summer which limited the calculation to this season only.

Statistical Analyses

A one-way analysis of similarity (ANOSIM) was used to detect differences in the stomach contents (% number) of *O. mossambicus* between reservoirs (Primer-E, ver.5.2.9, Primer-E Ltd., Plymouth, UK). This test produces the statistic *R* which measures the effect size, where *R* = 1 indicates that samples within groups are more similar than between groups, and *R* = 0 indicates that within-group similarity is equal to between group similarity. A non-metric multidimensional scaling (NMDS) plot of the data from both reservoirs, accounting for four seasons, was constructed from a similarity matrix calculated using the Bray-Curtis similarity coefficient. In a two-dimensional ordination plot, samples that are grouped more closely together represent more similar assemblages than samples spread further apart.

Isotopic composition and trophic position of *O. mossambicus* was evaluated using Analysis of Covariance (ANCOVA), with $\delta^{15}N$, $\delta^{13}C$ and trophic position as dependent variables, reservoir, season and sex as independent variables, and fish length as the covariate. The assumption of homogeneity of slopes was tested and there were significant interactions between several independent variables and fish length. Therefore a separate slopes model was used. A one-way ANOVA was used to detect differences in the isotopic signatures of *O. mossambicus* collected from LR in 2007, 2010 and 2011. Data were square root transformed where necessary to ensure the assumption of normality was met for all parametric tests. Statistical analyses were completed in Statistica Version 11 (StatSoft. Inc, Tulsa, USA).

The proportional contribution of potential food items to the diet of *O. mossambicus* in both reservoirs was assessed with an isotopic mixing model in Stable Isotope Analysis in R (SIAR) version 4.2 according to Parnell *et al.* (2010). Given the isotopic ratios in a range of possible food sources and consumers, the model uses Bayesian inference to provide probability distributions of dietary proportions. In contrast to the stomach analyses which are based on presence/absence of prey *ingested* at a single point in time, the stable isotope mixing model results reflect the proportional mass contributions of various prey to consumer tissues *after assimilation*, and integrated over a longer period of time (Phillips, 2012). Trophic enrichment factors (mean ± SD) of 3.4‰ (± 1.0) for $\delta^{15}N$ and 0.4 (± 1.3) for $\delta^{13}C$ were applied to food sources in order to account for isotopic shifts between consumer and diet (Post, 2002), and uninformative priors were used. Comparisons between food sources were made using the 95% Bayesian credibility intervals which were considered significantly different when they did not overlap.
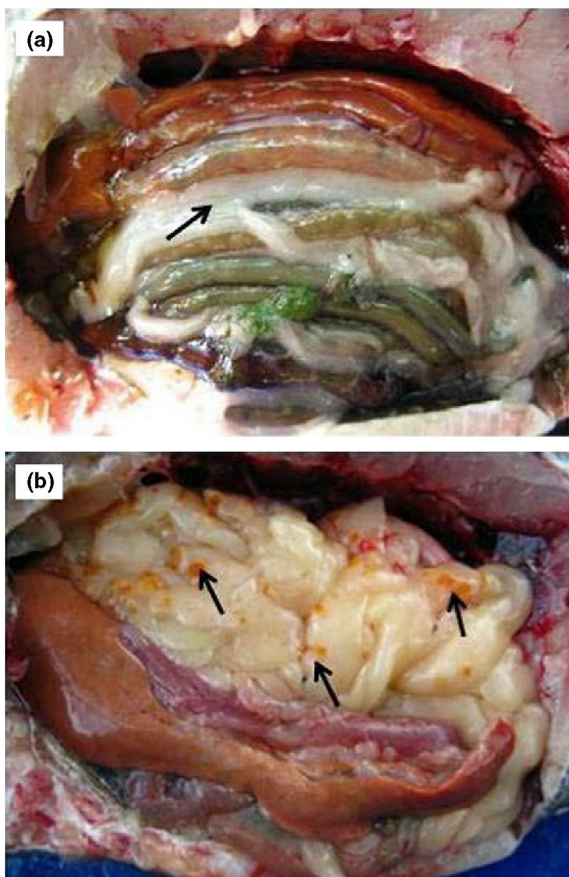

Fig. 2 Macroscopic comparison of mesenteric fat typical of healthy Oreochromis mossambicus from Flag Boshielo Reservoir (a), and pansteatitis-affected fish from Loskop Reservoir (b). Arrows indicate fat tissue and highlight concentrations of yellow-brown ceroid pigment in pansteatitis-affected fish.

Results

Oreochromis mossambicus catch summary

Oreochromis mossambicus from LR were characterised by abundant mesenteric fat containing distinct yellow, orange and brown spots (ceroid pigment) which varied in intensity and hardness, and are typical lesions of pansteatitis. In contrast, fish from FBR had little to no mesenteric fat, which when present, was white with no discolouration (Fig. 2). The mean TL in fish from LR (398 mm) was significantly longer than fish from FBR (325 mm; T-test, *P* < 0.0001). The fish from LR were also significantly heavier (T-test, *P* < 0.0001), with a mean weight of 1458 g which was almost double the mean weight of fish from FBR (Table 1).

Dietary composition: stomach contents analysis

The highest proportion of empty fish stomachs at LR was during winter, while at FBR it was during summer (Table 1). Stomach contents analysis revealed a more diverse range of prey items in fish from LR with seven categories compared to five in FBR (Fig. 3). At LR, *C.*
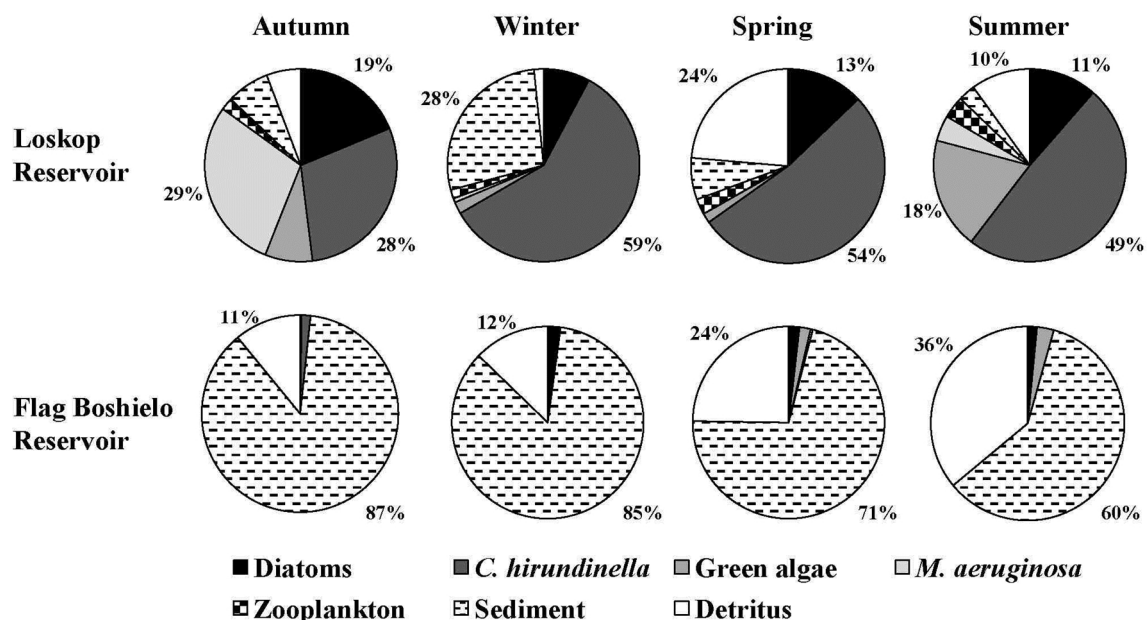

Fig. 3 Collective proportional contributions (% number) of major dietary items (shown for items > 10%) in the stomach contents of *Oreochromis mossambicus* collected over four seasons from Loskop and Flag Boshielo Reservoirs, South Africa. Sample sizes as per Table 1 excluding fish with empty stomachs.

hirundinella was the most frequently consumed prey, occurring in more than 80% of the fish stomachs (Table 2), with a substantial contribution to stomach contents during all seasons

(Fig. 3). In autumn *M. aeruginosa* occurred in 87.1% of the fish stomachs, and contributed 26% of the % number in stomach contents of fish from LR, but was not abundant during other sampling periods. Despite occurring in more than 60% of the fish stomachs from LR, zooplankton contributed a low biomass, and consisted predominantly of *Daphnia* spp. and copepods. Diatoms also occurred at a high frequency but made a relatively low proportional contribution, and were dominated by the pelagic species *Fragilaria crotonensis*. An increase in the occurrence and % number of sediment was evident in winter, while detritus increased in spring.

Seasonal variation in the stomach contents of fish from FBR was low with the highest contribution from sediment in 100% of the fish stomachs examined in all seasons (Table 2).

Table 2 Dietary composition by frequency of occurrence (% F) for *Oreochromis mossambicus* from Loskop and Flag Boshielo Reservoirs, South Africa

	Loskop Reservoir				Flag Boshielo Reservoir			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Diatoms	100	100	87	100	6	25	50	45
Ceratium hirundinella	100	100	87	100	29	–	–	9
Green algae	87	90	40	100	–	6	88	18
Microcystis aeruginosa	87	10	–	7	–	–	–	–
Zooplankton	65	70	60	71	–	–	–	–
Sediment	61	100	47	50	100	100	100	100
Detritus	74	20	100	64	94	56	100	100

Secondary dominance by detritus was evident and contributions increased over the duration of the sampling period. Diatoms, predominantly the benthic species *Fragilaria ulna,* mad a low proportional contribution, and ranged in occurrence from 5.88% in autumn to 50% in spring. Despite a high occurrence in spring, green algae never contributed more than 10% to stomach contents. In contrast to LR, *C. hirundinella* was never detected at high frequencies or in large quantities from the fish stomachs. Zooplankton and *M. aeruginosa* were never recorded from the fish stomach contents in FBR.

The NMDS showed clear separation in the stomach contents of fish from both reservoirs, which was supported by a low stress value of 0.09 (Fig. 4). This was reinforced by the results of the ANOSIM which indicated a significant difference in dietary composition between reservoirs ($R = 0.929$). The NMDS showed a high degree of seasonal overlap within each reservoir, except in LR in autumn when *M. aeruginosa* (29%) and diatoms (19%) increased in dominance (Fig. 3) resulting in a level of separation of these fish in the NMDS (Fig. 4).
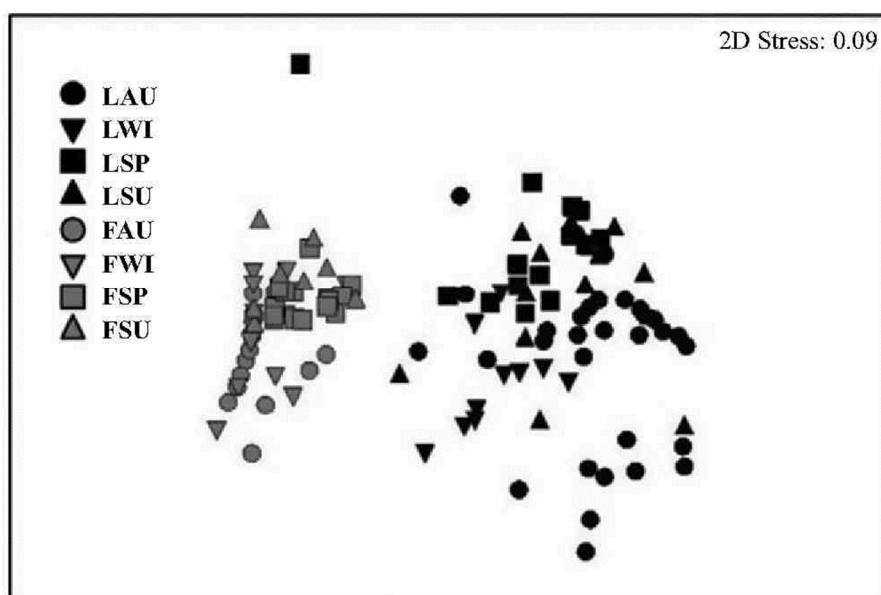
Fig. 4 Non-metric multidimensional scaling (NMDS) plot of stomach contents (% number) from *Oreochromis mossambicus* sampled in two reservoirs. L, Loskop Reservoir; F, Flag Boshielo Reservoir; AU, autumn; WI, winter; SP, spring; SU, summer.

Table 3 Carbon and nitrogen stable isotope values (Mean ‰ ± SD) and sample sizes for *Oreochromis mossambicus* dietary sources and *Tilapia rendalli* (baseline organism) collected from Loskop Reservoir and Flag Boshielo Reservoir on the Olifants River, South Africa

Species/group	Loskop Reservoir			Flag Boshielo Reservoir		
	$\delta^{15}N$	$\delta^{13}C$	n	$\delta^{15}N$	$\delta^{13}C$	n
Ceratium hirundinella	7.3 ± 0.1	−11.1 ± 1.3	2	–	–	–
Detritus	7.4 ± 0.9*	−15.4 ± 1.9*	4	3.2 ± 1.1*	−27.0 ± 2.0*	4
Diatoms[1]	18.2 ± 1.3	−26.3 ± 2.7	2	–	–	–
Green algae	12.6 ± 2.8*	−13.7 ± 3.6*	4	7.4 ± 0.5*	−21.5 ± 4.1*	4
Microcystis aeruginosa	16.2 ± 0.1	−18.1 ± 0.4	3	–	–	–
Oreochromis mossambicus[†]	15.4 ± 0.4	−13.8 ± 0.6	91	13.9 ± 0.6	−26.3 ± 0.7	81
Oreochromis mossambicus[‡]	15.2 ± 0.5	−13.7 ± 0.5	24	–	–	–
Oreochromis mossambicus[§]	15.7 ± 0.5	−13.3 ± 0.4	3	–	–	–
Oreochromis mossambicus[¶]	14.6 ± 0.4	−14.3 ± 0.5	3	–	–	–
Sediment organic matter	8.9 ± 1.4*	−19.8 ± 1.4*	9	6.1 ± 1.1*	−22.9 ± 0.9*	7
Tilapia rendalli	18.7 ± 0.5*	−14.3 ± 0.3*	7	12.8 ± 1.1*	−22.3 ± 2.8*	13
Zooplankton[2]	21.4 ± 0.7	−12.5 ± 0.1	2	–	–	–

[1]Diatoms consisted primarily of the pelagic species *Fragilaria crotonensis*.
[2]Zooplankton consisted primarily of *Daphnia* spp. and copepods.
*Indicates significant difference ($P < 0.05$) in isotopic signatures between reservoirs using a *t*-test.
Oreochromis mossambicus symbols: [†]collected in 2011; [‡]collected in 2010; [§]mortalities in 2010; [¶]collected in 2007.

Variation in isotopic signatures of <u>Oreochromis mossambicus</u>

The ANCOVA showed that isotope signatures of *O. mossambicus* differed significantly between both reservoirs. The $\delta^{15}N$ values of fish from LR were significantly higher ($F_{1,149} = 61.3$, $p < 0.0001$), and $\delta^{13}C$ values were significantly less depleted ($F_{1,149} = 365$, $p < 0.0001$), compared to fish from FBR (Table 3). At LR the $\delta^{15}N$ values were significantly higher in winter (ANCOVA, $F_{3,78} = 5.7$, $p = 0.001$) with an average of 15.6‰ compared to other

sampling periods, which ranged from 15.2 to 15.4‰. At FBR the δ^{15}N values were significantly lower in autumn with a mean of 13.6 compared to other seasons which ranged from 13.8 to 14.1‰. (ANCOVA, $F_{3,67} = 8.1$, $p < 0.001$). Although statistically significant, the seasonal differences in means were very low and unlikely to be of great biological significance. There was no effect of season on δ^{13}C values at either reservoir, and sex had no influence on δ^{15}N or δ^{13}C signatures. There was a positive relationship between δ^{15}N and length in fish from FBR (ANCOVA, $F_{16,149} = 3.4$, $p < 0.001$), but not fish from LR. The δ^{13}C values were significantly more depleted as fish increased in length at both LR and FBR (ANCOVA, $F_{16,149} = 2.1$, $p < 0.001$).

All food items identified in the stomach contents of fish from LR were collected and analysed, however, several comparable food sources could not be collected from FBR. *Microcystis aeruginosa* was never detected in the stomach contents or water column, and *C. hirundinella*, diatoms and zooplankton were so scarce that they could not be sampled in quantities sufficient for analysis. The only zooplankton detected in water samples from FBR were rotifers and there were less than 10 individuals collected from all samples combined. All fish species and food sources from LR were significantly enriched with δ^{15}N and less depleted in δ^{13}C when compared to the same taxonomic group in FBR (Table 3).

Trophic Position

The mean trophic position of *O. mossambicus* was significantly lower at LR (mean 1.5 ± 0.1) than FBR (mean 2.8 ± 0.1; ANCOVA, $F_{1,33} = 266$, $p < 0.0001$) which was over a full trophic position higher. Trophic level was not significantly related to fish length in either reservoir or in males and females.

Variation in historic isotopic signatures of <u>Oreochromis mossambicus</u>

The one-way ANOVA showed that δ^{15}N and δ^{13}C values of fish collected in 2007 differed significantly to various other time periods (Fig. 5). The mean δ^{15}N value in 2007 was significantly lower (approximately 1‰) than mortalities collected in 2010 ($p = 0.02$) and samples collected in 2011 ($p = 0.02$; Table 2). The mean δ^{13}C value in 2007 was more

depleted than mortalities ($p = 0.04$) and samples collected in 2010 ($p = 0.03$), but only differed by 0.5‰ compared to samples from 2011 (Table 2).
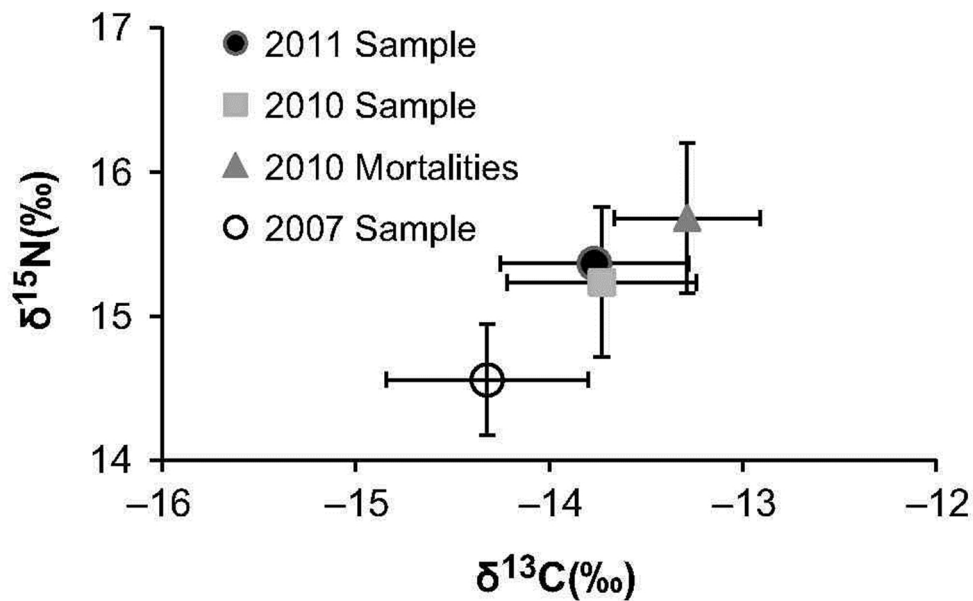

Fig. 5 Stable isotope biplot showing the mean ‰ (±SD) δ^{15}N and δ^{13}C values for *Oreochromis mossambicus* collected during various time periods from Loskop Reservoir.
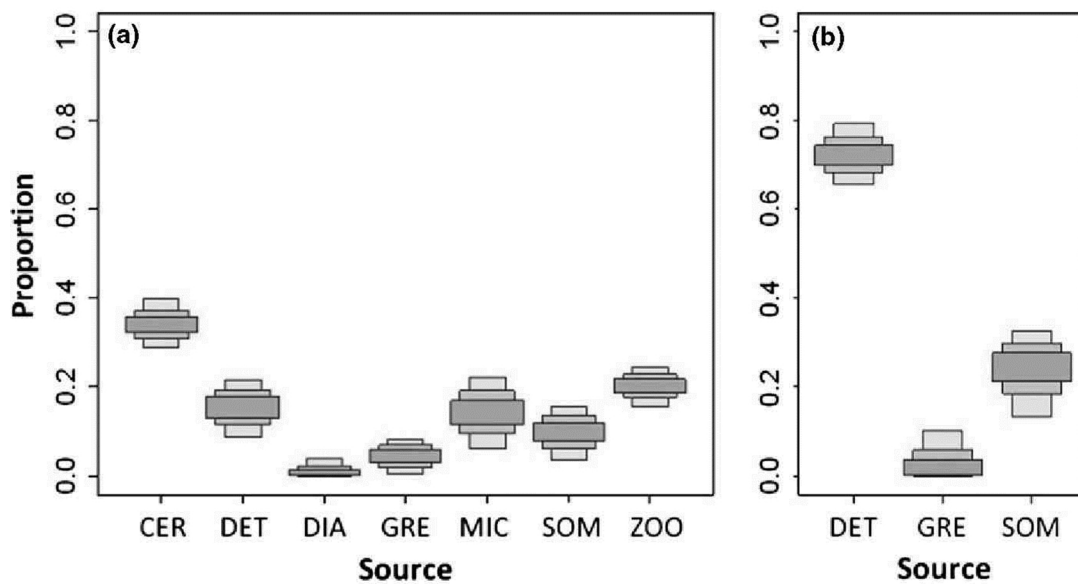

Fig. 6 Proportional contributions of dietary sources for *Oreochromis mossambicus* from Loskop Reservoir (a) and Flag Boshielo Reservoir (b) pooled across four seasons. Dietary sources are *Ceratium hirundinella* (CER); detritus (DET), diatoms (DIA), green algae (GRE), *Microcystis aeruginosa* (MIC), sediment organic matter (SOM) and zooplankton (ZOO). The boxes indicate 50, 75 and 95% Bayesian credibility intervals based on the stable isotope analysis in R mixing model.

Dietary composition: stable isotopes

No seasonal variation was evident in the range of solutions provided by the mixing model, so results were pooled for each reservoir. In LR the SIAR model indicated that *C. hirundinella* constituted the majority of the diet (29-40%; 95% credibility interval) which was in agreement with the stomach contents analysis (Fig. 6a). This was followed by zooplankton despite the low frequency of this food item in the fish stomachs. Lower contributions were indicated from *M. aeruginosa* which was similar to detritus. Diatom δ^{13}C values were approximately 13‰ more depleted than *O. mossambicus* (Table 3), and although they frequently contributed greater than 10% to stomach contents, the SIAR model indicated that they were the least important food source. The contributions of *M. aeruginosa* and zooplankton were negatively correlated in the mixing model (-0.88) meaning that proportional increases in the contribution of one would be at the expense of the other.

The SIAR model indicated that detritus was the dominant food source for fish in FBR (Fig. 6b), followed by sediment organic matter and green algae. However, the mean δ^{15}N for *O. mossambicus* (13.9‰) was enriched by between 6.5‰ (compared to green algae) and 10.7‰ (compared to detritus; Table 3). The trophic enrichment factor incorporated into the SIAR model for δ^{15}N was 3.4‰ (± 1) which means that the δ^{15}N values were highly enriched relative to these items. No combination of these three food sources explains the isotopic values for *O. mossambicus* in FBR, and the system remained undetermined despite the distribution of solutions provided by the mixing model.

Discussion

The results of this study showed no evidence of current or historic piscivory in *O. mossambicus* from LR that may explain pansteatitis. The 2011 δ^{15}N values for *O. mossambicus* were very similar when compared to samples collected in 2010, and were slightly higher than samples collected in 2007. Their trophic level indicated their position as between a primary and secondary consumer, which was consistent with the dietary analysis. Their calculated trophic level also confirmed that although their δ^{15}N values were significantly enriched compared to fish from FBR, this was purely due to differences in the trophic baseline at each reservoir. Their diet was herbivorous / detritivorous and the dominant food items identified indicated that they mostly fed in the pelagic zone. Based on the stomach

contents and mixing model results, their diet in LR is very much what would be expected according to previous studies in their native range (de Moor *et al.*, 1985; Bowen, 1979; Zengeya *et al.*, 2011; Dyer *et al.*, 2013). Both analytical methods showed that *C. hirundinella* was the dominant food source. Secondary dominance by zooplankton in the mixing model was contrary to the stomach contents where this food group accounted for less than 10%. However, the relatively large body size of cladocerans and cyclopods relative to phytoplankton species clearly makes a significant nutritional contribution. The stomach contents reflected seasonal variation in the abundance of different food sources such as *M. aeruginosa* which made a higher contribution during late summer to autumn. However, the stable isotope analysis indicated little to no seasonal variation, which is probably due to the longer integration time of muscle isotopic values compared to stomach contents.

Although our results gave no indication that *O. mossambicus* from LR were regularly feeding at a higher trophic level, we cannot exclude the possibility of a brief period of opportunistic piscivory associated with a fish kill that went undetected due to the confounding effect of tissue turnover rates. Muscle tissue half-lives of adult fish can range from 116 to173 days (Weidel *et al.,* 2011) and no major fish kills were observed at LR during 2010 or 2011. There were however, at least three large-scale fish kills in 2007 at LR. If *O. mossambicus* had been opportunistically feeding on fish during these events it seems unlikely that this would not have reflected in the δ^{15}N values of fish we analysed from that time. These fish were sampled in September of that year, and displayed severe symptoms of pansteatitis, but their δ^{15}N values were actually slightly lower than fish collected in subsequent years. It is also not known whether the lesions typical of pansteatitis in fish from LR are indicative of ongoing oxidative stress, or are the remnants of a historic event related to feeding or other causes. Observations of farmed crocodiles have shown that while pansteatitis may cause acute mortalities, some animals survive, continue growing, and the lesions are only discovered at slaughter (Huchzermeyer, 2003).

There were vast differences in the stomach contents and isotopic signature of pansteatitis-affected *O. mossambicus* from LR compared to unaffected fish from FBR. Compared to LR, the isotopic signatures of *O. mossambicus* from FBR are located very differently, being approximately less enriched on the δ^{15}N axis and utilising a distinctly depleted δ^{13}C source. The stomach contents of fish from FBR were dominated by sediment and detritus with very little seasonal variation, and the mixing model indicated that detritus was the most important

food source. However their high levels of $\delta^{15}N$ enrichment relative to the food sources we measured showed that their diet was still undetermined to an extent. One explanation is that we only measured sediment organic matter and detritus from the benthic zone, and did not include suspended particulate matter which may have been a significant food source. The presence of the benthic diatom *Fragilaria ulna* in the stomach contents of fish suggests that they were feeding in the benthic zone at least periodically. In addition to a missing food source, it is speculated that the fish in FBR may be experiencing a degree of nutritional stress. A study of carp (*Cyprinus carpio*) has shown that when feeding levels were experimentally manipulated, $\delta^{15}N$ values increased as feeding level decreased, introducing an error of up to 1‰ for both $\delta^{13}C$ and $\delta^{15}N$ values (Gaye-Siessegger *et al.*, 2004). This provides one possible explanation for their elevated trophic position compared to LR. In addition, the positive relationship between fish length and $\delta^{15}N$ values for fish from FBR was opposite to that predicted from previous research which demonstrated ontogenetic dietary shifts of *O. mossambicus* from zooplankton to phytoplankton, detritus and algae as they mature (de Moor *et al.,* 1985; Zengeya *et al.,* 2011). This could indicate increasing nutritional stress as fish mature, which has been observed in a previous study which showed how habitat segregation between juvenile and adult *O. mossambicus* resulted in variable protein content of detrital food resources, with the consequence that the adult population was stunted and malnourished (Bowen, 1979).

Eutrophication in LR has resulted in blooms of *C. hirundinella* and *M. aeruginosa* (Oberholster *et al.*, 2010), providing a year-round abundant food source for consumers such as *O. mossmabicus*. The larger range of dietary items in LR compared to FBR indicated more trophic levels and trophic diversity. The baseline enrichment with $\delta^{15}N$ at LR is probably influenced by effluent originating from wastewater treatment works in the catchment of LR. In contrast, the oligotrophic, nitrogen-limited status of FBR provides limited food sources for *O. mossambicus* as shown by the low diversity of their stomach contents, low chlorophyll-*a* concentrations, and the scarcity of phyto- and zooplankton in the water column.

The $\delta^{13}C$ values of the food web at FBR were significantly more depleted than at LR. These differences are probably a reflection of vegetation types in the respective catchments and within the reservoirs themselves. The catchment of LR is predominantly grassland, consisting of C_4 grasses while the catchment of FBR is predominantly bushveld consisting of C_3 pathway shrubs and trees. In addition, the dam wall of FBR was raised by 5 m in 2006 and

vegetation was purposely not cleared in order to provide habitat for waterfowl. As a result, submerged dead trees are scattered throughout the littoral zone providing a large source of C_3 carbon to the food web. In contrast, the littoral zone of LR is largely sand or rocky substrate with little to no vegetation.

Previous research has demonstrated that the aetiology of pansteatitis is exclusively dietary (it is not a contagious disease) and is linked to a diet rich in unsaturated or rancid fats frequently of fish origin (Roberts *et al.*, 1979; Fytianou *et al.,* 2006; Roberts & Agius, 2008). The dietary reconstruction for *O. mossambicus* from LR, where pansteatitis is ubiquitous, indicated that fish made no observable contribution to their diet. This is a distinctive feature of pansteatitis in LR, as elevated levels of piscivory have been associated with the disease in *C. gariepinus* in the KNP (Woodborne *et al.*, 2012). If piscivory is not associated with the aetiology of pansteatitis in LR, then one distinguishing feature of the dietary comparison between LR and FBR is the presence of abundant planktonic food sources dominated by *C. hirundinella*. While the food sources identified are not unique to LR, the numerous pollutants originating from various land uses in the catchment may affect their nutritional quality in some way. Given the well established dietary aetiology of pansteatitis, an investigation of the fatty acid composition of dominant food sources for *O. mossambicus* at LR would make a significant contribution to further unravelling the complex aetiology of this disease.

Acknowledgements

References

Ashton P. (2010) The demise of the Nile crocodile (*Crocodylus niloticus*) as a keystone species for aquatic ecosystem conservation in South Africa: The case of the Olifants River. *Aquatic Conservation: Marine and Freshwater Ecosystems, 20, 489-493.*

Ashton P.J. & Dabrowski J.M. (2011) *An overview of surface water quality in the Olifants River catchment*. WRC Report No. KV293/11. Water Research Commission, Pretoria, South Africa.

Baker R.T.M., Martin P. & Davies S.J. (1997) Ingestion of sub-lethal levels of iron sulphate by African catfish affects growth and tissue lipid peroxidation. *Aquatic Toxicology,* **40**, 51-61.

Botha P.J. (2010) *The distribution, conservation status and blood biochemistry of Nile crocodiles in the Olifants River system, Mpumalanga, South Africa*. Unpublished PhD thesis, University of Pretoria, South Africa.

Botha H., Van Hoven W. & Guillette L.J. (2011) The decline of the Nile crocodile population in Loskop Dam, Olifants River, South Africa. *Water SA,* **37,** 103-108.

Bowen S.H. (1979) A nutritional constraint in detritivory by fishes: the stunted population of *Sarotheradon mossambicus* in Lake Sibaya, South Africa. *Ecological Monographs*, 17-31.

Bowen S.H. & Allanson B.R. (1982) Behavioural and trophic plasticity of juvenile *Tilapia mossambica* in utilization of the unstable littoral habitat. *Environmental Biology of Fishes,* **7**, 357-362.

Bricknell I.R., Bruno D.W., Bowden T.J. & Smith P. (1996) Fat cell necrosis syndrome in Atlantic halibut, *Hippoglossus hippoglossus* L. *Aquaculture,* **144**, 65-69.

Coplen T.B. (1994) Reporting of stable hydrogen, carbon, and oxygen isotopic abundances. *Pure and Applied Chemistry,* **66**, 273-276.

Costa-Pierce B.A. (2003) Rapid evolution of an established feral tilapia (*Oreochromis* spp.): the need to incorporate invasion science into regulatory structures. *Biological Invasions*, **5**, 71-84.

Dabrowski J. (2012) Water quality in Loskop Dam. In: *Final Report: Risk Assessment of Pollution in Surface Waters of the Upper Olifants River System: Implications for Aquatic Ecosystem Health and the Health of Human Users of Water.* (Eds P.J. Ashton, J.M. Dabrowski & L. Hill). CSIR report number CSIR/NRE/WR/ER/2013/0053/B, Pretoria.

Dabrowski J., Oberholster P.J., Dabrowski J.M., Le Brasseur J. & Gieskes J. (2013) Chemical characteristics and limnology of Loskop Dam on the Olifants River (South Africa), in light of recent fish and crocodile mortalities. *Water SA*, **39**: 675-686.

De Moor F.C., Wilkinson R.C. & Herbst H.M. (1985) Food and feeding habits of *Oreochromis mossambicus* (Peters) in hypertrophic Hartbeespoort Dam, South Africa. *South African Journal of Zoology*, **21**, 170-176.

De Silva S.S., Perera M.K. & Maitipe P. (1984) The composition, nutritional status and digestibility of the diets of *Sarotherodon mossambicus* from nine man-made lakes in Sri Lanka. *Environmental Biology of Fishes*, **11**, 205-219.

Doupé R.G., Knott M.J., Schaffer J. & Burrows D.W. (2009) Investigational piscivory of some juvenile Australian freshwater fishes by the introduced Mozambique tilapia *Oreochromis mossambicus*. *Journal of Fish Biology*, **74**, 2386-2400.

Doupé R.G. & Knott M.J. (2011) Rapid digestion of fish prey by the highly invasive 'detritivore' *Oreochromis mossambicus*. *Journal of Fish Biology*, **76**, 1019-1024.

Dyer D.C., Perissinotto R. & Carrasco N.K. (2013) Post-flood dietary variation in the Mozambique tilapia *Oreochromis mossambicus* in the St Lucia Estuary, South Africa. *Marine Ecology Progress Series*, **476**, 199-214.

Elbaraasi H., Mézes M., Balogh K., Horváth L. & Csengeri I. (2004) Effects of dietary ascorbic acid / iron ratio on some production traits, lipid peroxide state and amount / activity

of glutathione redox system in African catfish *Clarias gariepinus* (Burchell) fingerlings. *Aquaculture Research* **35**, 256-262.

Fagan K-A., Koops M.A., Arts M.T. & Power M. (2011) Assessing the utility of C:N ratios for predicting lipid content in fish. *Canadian Journal of Fisheries and Aquatic Sciences*, **68**, 374-385.

Ferreira S. & Pienaar D. (2011) Degradation of the crocodile population in the Olifants River gorge of Kruger National Park, South Africa. *Aquatic Conservation: Marine and Freshwater Ecosystems,* **21,** 155-164.

Fytianou A., Koutinas A.F., Saridomichelakis M.N. & Koutinas C.K. (2006). Blood α-Tocopherol, selenium and glutathione peroxidase changes and adipose tissue fatty acid changes in kittens with experimental steatitis (yellow fat disease). *Biological Trace Element Research,* **112,** 131-143.

Gaye-Siessegger J., Focken U., Muetzel S., Abel H. & Becker K. (2004) Feeding level and individual metabolic rate affect δ^{13}C and δ^{15}N values in carp: implications for food web studies. *Oecologia*, **138**, 175-183.

Goodwin A.E. (2006) Steatitis, fin loss and skin ulcers of channel catfish, *Ictalurus punctatus* (Rafinesque), fingerlings fed salmonid diets. *Journal of Fish Diseases,* **29,** 61-64.

Guarda F., Bertoja G., Zoccarato I., Tartari E. & Biolatti, B. (1997) Spontaneous steatitis of epicardial fat in farmed white sturgeon (*Acipenser transmontanus*). *Aquaculture*, **158**, 167-177.

Herman R.L. & Kircheis F.W. (1985) Steatitis in Sunapee trout, *Salvelinus alpines oquassa* Girard. *Journal of Fish Diseases*, **8**, 237-239.

Huchzermeyer F.W. (2003) *Crocodiles: biology, husbandry and diseases*, pp. 219, CABI Publishing, Wallingford, UK.

Huchzermeyer K.D.A., Govender D., Pienaar D. & Deacon A.R. (2011) Steatitis in wild sharptooth catfish, *Clarius gariepinus* (Burchell), in the Olifants and lower Letaba Rivers in the Kruger National Park, South Africa. *Journal of Fish Diseases,* **34,** 489-498.

Huchzermeyer K.D.A., Osthoff G., Hugo A. & Govender D. (2013) Comparison of the lipid properties of healthy and Pansteatitis-affected African sharptooth catfish, *Clarias gariepinus* (Burchell), and the role of diet in Pansteatitis outbreaks in the Olifants River in the Kruger National Park, South Africa. *Journal of Fish Diseases*, **36**, 897-909.

Hyslop E.J. (1980) Stomach contents analysis – a review of methods and their application. *Journal of Fish Biology,* **17**, 411-429.

Larsen R.E., Buergelt C., Cardeilhac P.T. & Jacobson E.R. (1983) Steatitis and fat necrosis in captive alligators. *Journal of the American Veterinary Medical Association,* **183**, 1202-4.

Logan J.M., Jardine T.D., Miller T.J., Bunn S.E., Cunjak R.A. & Lutcavage M.E. (2008) Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modelling methods. *Journal of Animal Ecology*, **77**, 838-846.

Mucina L. & Rutherford M.C. (2006) *The vegetation of South Africa, Lesotho and Swaziland.* Strelitzia 19. South African National Biodiversity Institute (SANBI), Pretoria, South Africa.

Neagari Y., Arii S., Udagawa M., Onuma M., Odaya Y., Kawasaki T., Tenpaku M., Hayama H., Harada K-I., Mizukami M. & Murata K. (2011) Steatitis in egrets and herons from Japan. *Journal of Wildlife Diseases*, **47**, 49-55.

Nichols D.K., Campbell V.L. & Montali R.J. (1986) Pansteatitis in great blue herons. *Journal of the American Veterinary Medical Association*, **189**, 1110-1112.

Oberholster P.J., Myburgh J.G., Ashton P.J. & Botha A-M. (2010) Responses of phytoplankton upon exposure to a mixture of acid mine drainage and high levels of nutrient pollution in Lake Loskop, South Africa. *Ecotoxicology and Environmental Safety,* **73:** 326-35.

Oberholster P.J., Myburgh J.G., Ashton P.J., Coetzee J.J. & Botha A-M. (2012) Bioaccumulation of aluminium and iron in the food chain of Lake Loskop, South Africa. *Ecotoxicology and Environmental Safety*, **75**, 134-141.

Oberholster P.J., Dabrowski J. & Botha A-M. (2013) Using modified multiple phosphorus sensitivity indices for mitigation and management of phosphorus loads on a catchment level. *Fundamental Applied Limnology*, **182**, 1-16.

Orós J., Monagas P., Calabuig P., Luzardo O.P. & Camacho M. (2013) Pansteatitis associated with high levels of polychlorinated biphenyls in a wild loggerhead sea turtle *Caretta caretta. Diseases of Aquatic Organisms,* **102,** 237-242.

Osthoff G., Hugo A., Bouwman H., Buss P., Govender D., Joubert C.C. & Swarts J.C. (2010) Comparison of the lipid properties of captive, healthy wild, and pansteatitis-affected wild Nile crocodiles (*Crocodylus niloticus*).*Comparative Biochemistry and Physiology, Part A*, **155**, 64-69.

Parnell A.C., Inger R. Bearhop S. & Jackson A.L. (2010) Source partitioning using stable isotopes: Coping with too much variation. *PLoS ONE,* **5**, e9672.

Phillips D.L. (2012) Converting isotope values to diet composition: the use of mixing models. *Journal of Mammalogy*, **93**, 342-352.

Post D.M. (2002) Using stable isotope methods to estimate trophic position: models, methods, and assumptions. *Ecology*, **83**, 703-718.

Rattner A.B. & McGowan C.P. (2007) Potential hazards of environmental contaminants to avifauna residing in the Chesapeake Bay estuary. *Waterbirds*, **30**, 63-81.

Rennie M.D., Ozersky T. & Evans D.O. (2012) Effects of formalin preservation on invertebrate stable isotope values over decadal time scales. *Canadian Journal of Zoology,* **90**, 1320-1327.

Roberts R.J., Richards R.H. & Bullock A.M. (1979) Pansteatitis in rainbow trout *Salmo gairdneri* Richardson: a clinical and histopathological study. *Journal of Fish Diseases,* **2,** 85-92.

Roberts R.J. & Agius C. (2008) Pansteatitis in farmed northern bluefin tuna, *Thunnus thynnus* (L.), in the eastern Adriatic. *Journal of Fish Diseases,* **31,** 83-88.

Skelton P.H. (2001) *A complete guide to the freshwater fishes of Southern Africa.* Struik, Cape Town.

Soto S., Fondevila D., Gonzalez B., Gomez-Campos E. & Domingo M. (2010) Multifocal granulomatous panniculitis with ceroid pigment in two Mediterranean Striped Dolphins (*Stenella coeruleoalba*). *Journal of Wildlife Diseases,* **46**, 320-325.

Weidel B.C., Carpenter S.R., Kitchell J.F. & Vander Zanden M.J. (2011) Rates and components of carbon turnover in fish muscle: insights from bioenergetics models and a whole-lake ^{13}C addition. *Canadian Journal of Fisheries and Aquatic Sciences*, **68**, 387-399.

Woodborne S., Huchzermeyer K.D.A., Govender D., Pienaar D.J., Hall G., Myburgh J.G., Deacon A.R., Venter J. and Lübcker N. (2012). Ecosystem change and the Olifants River crocodile mass mortality events. *Ecosphere*, **3**: 1-17.

Zengeya T.A., Booth A.J., Bastos A.D.S. & Chimimba C.T. (2011) Trophic interrelationships between the exotic Nile tilapia, *Oreochromis niloticus* and indigenous tilapiine cichlids in a subtropical African river system (Limpopo River, South Africa). *Environmental Biology of Fishes,* **92**, 479-489.