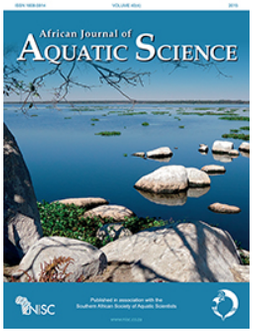

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An assessment of the reproductive biology of the Marico barb *Barbus motebensis* from the upper Groot Marico catchment, South Africa

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Barbus motebensis **is endemic to the Groot Marico River catchment in the North West province, South Africa, and is considered of particular conservation importance due to being Red-Listed as Vulnerable. Due to its vulnerable conservation status, the aim of this study was to provide information on its condition factor, breeding season, gonadosomatic index, fecundity range and length at maturity from specimens collected by means of electrofishing and seine-netting between November 2012 and May 2014 from the Draaifonteinspruit and Kaaloog se Loop. Length at maturity was estimated as 37.5 mm TL for males and 44.5 mm TL for females. Microscopic gonad staging and gonadosomatic index demonstrated that *B. motebensis* has an extended breeding season from spring to summer. Relative (578 eggs g^{-1}) and absolute (3 728 eggs) fecundity were relatively high for small barbs.**

Keywords: condition factor, fecundity, gonadosomatic index, histology, length at maturity, spawning season

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Introduction

Fish are a freshwater conservation priority and knowledge on their biology and ecology is needed to guide conservation action (Marriott 1998; Whitehead et al. 2007; Tweddle et al. 2009). Of particular concern in South Africa are the small barbs of the genera *Barbus* and *Pseudobarbus*, which have been extirpated from many of the lower reaches of rivers as a result of habitat degradation and predation by alien fishes, and now persist as small isolated populations (e.g. Tweddle et al. 2009; Weyl et al. 2014). Although most existing case studies are from the Cape Floristic Region (Ellender and Weyl 2014), recent research on the Marico barb *Barbus motebensis* Steindachner, 1894 has demonstrated similar impacts on this species (Kimberg et al. 2014).

Barbus motebensis is endemic to the Limpopo River system where it inhabits slow-flowing sections and shallow pools of small streams in the catchments of the Marico and Crocodile rivers (Engelbrecht and Bills 2007). The species is typically associated with the upper foothills of mountain streams where it is usually found in association with banks and marginal vegetation (Engelbrecht and Bills 2007). *Barbus motebensis* is considered of particular conservation importance as it is listed as Vulnerable in the International Union for Conservation of Nature (IUCN) Red List (Nel et al. 2011). This is because of its limited natural distribution, small estimated population size, and isolation in headwater refugia (Skelton 2001; Smith-Adao et al. 2006; IUCN 2014; Kimberg et al. 2014).

The upper Groot Marico River (Figure 1) is one of the strongholds of the *B. motebensis* population (Kimberg et al. 2014). This river was identified as a freshwater ecosystem priority area (FEPA) because of its free-flowing nature, its ecological category of A or B, and because it would act as a fish sanctuary for *B. motebensis* (Nel et al. 2011). In this FEPA, the primary threat to *B. motebensis* appears to be predation by the alien largemouth bass *Micropterus salmoides* (Kimberg et al. 2014). Kimberg et al. (2014) recommended that *M. salmoides* eradication could result in habitat gains for *B. motebensis*, but that careful planning of such interventions would be necessary. An important aspect of planning restoration projects is an estimate of the potential recovery rates of the target species. This requires knowledge of their life history, particularly of their reproductive biology. As there is currently no information on the reproductive biology of this species, the aim of the present study was to undertake an assessment of the reproductive biology of *B. motebensis* to: (1) provide histologically validated macroscopic staging criteria; (2) determine length at maturity, spawning season and spawning frequency; and (3) provide estimates of total and relative fecundity.

Materials and methods

The study was conducted in the upper Groot Marico River, North West province, South Africa. The tributaries of the Groot Marico River are characterised by relatively neutral pH values (7.3–8.1) and low hardness (TDS: 159–323 mg l^{-1}) (Kimberg et al. 2014). The area lies in a summer rainfall region and therefore, temperature and river flows follow a seasonal pattern with winters characterised by low water flow and low temperature (9.6 °C), while summers are characterised by high temperatures (22.7 °C) and increased

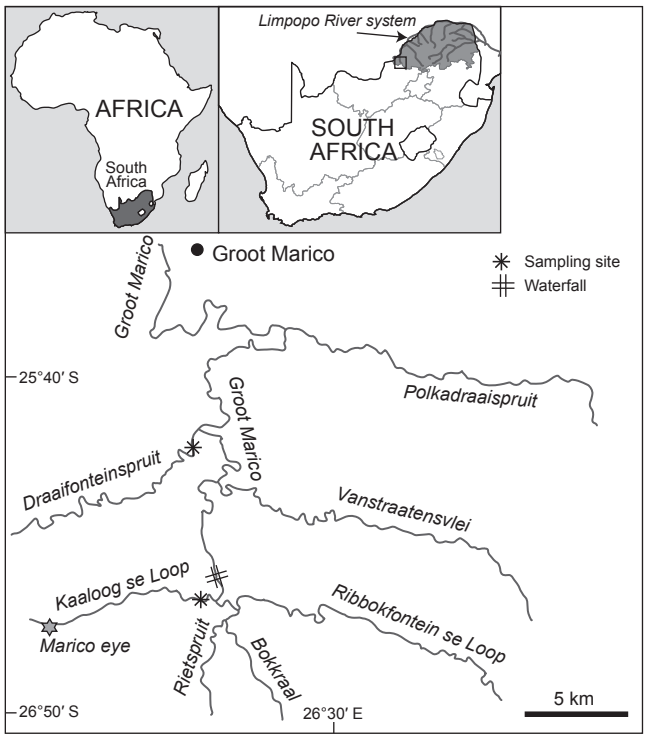

Figure 1: Map of the upper Groot Marico catchment showing its seven tributaries, two sampling sites and natural barrier waterfall (adapted from Kimberg et al. 2014)

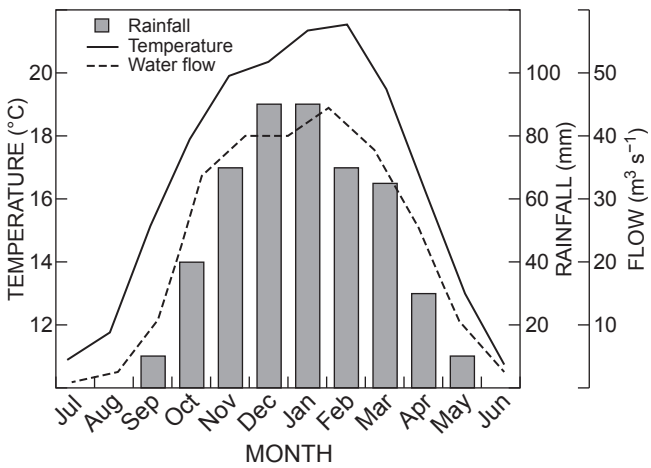

Figure 2: Average monthly flow at weir A3H008 Renosterfontein in 1925–1932, and mean monthly *in situ* water temperature and rainfall in the Draaifonteinspruit and Kaaloog se Loop from July 2013 to June 2014

water flows (Figure 2). Fish were sampled from two of the seven perennial, spring-fed headwater tributaries, the Draaifonteinspruit (25°41′54″ S, 26°25′45″ E) and Kaaloog se Loop (25°46′35″ S; 26°26′02″ E). Due to the vulnerable conservation status of this species, sampling sites were chosen based on areas of higher abundance of *B. motebensis* determined during distribution surveys conducted by Kimberg et al. (2014). To minimise the impact of repeated sampling on populations, while allowing for seasonal comparisons, samples were collected by means of electrofishing and seine-netting on single days in July 2013 (winter), September 2013 (spring), November 2013 (summer) and April 2014 (autumn). The total sample comprised 122 individuals, or approximately 15 individuals per tributary population per season. On collection, fish were euthanised using clove oil (1 mg l^{-1}), and placed in 10% neutrally buffered formalin solution to fix and preserve specimens for further analysis. After fixation, all fish were examined macroscopically for bodily abnormalities such as lesions, haemorrhages or any other deformities. After blotting the fish dry, they were weighed to the nearest 0.001 g and measured to the nearest 1 mm for standard length (SL), fork length (FL) and total length (TL). The fish were then dissected and the developmental stage of the gonads was scored using macroscopic staging criteria (Whitehead et al. 2007) (Table 1). Testes and ovaries were removed, blotted dry and weighed to the nearest 0.001 g. Morphometric relationships between SL, FL, TL and weight (*W*) were determined by regression analysis.

Validation of macroscopic staging criteria

To validate macroscopic staging criteria histologically, testes and ovaries that had been scored macroscopically were processed according to standard protocol (Humason 1962). The protocol involved the gonads being dehydrated in graded ethanol, cleared with xylene, embedded in paraffin wax, sectioned at 5 µm with a Leica rotary microtome (RM2125RT), positioned on a microscope slide, stained with haematoxylin and eosin and a coverslip was mounted with Entellan. Sections were viewed under 10–40× magnification and their histological appearance was compared to histological criteria for each developmental stage (i.e. regressed, developing, ripe and spent) as described for *Pseudobarbus phlegethon* by Whitehead et al. (2007). Female gonads were also examined to determine whether there was evidence for repeated spawning (i.e. presence of ova in various developmental stages in the same ovary).

Maturity

As is common practice (Whitehead et al. 2007; Ellender et al. 2012; Winker et al. 2012), length at 50% sexual maturity $L_{m_{50}}$ was estimated by fitting a two-parameter logistic ogive to the proportion of reproductively active (developing, ripe, spent) fish in 5 mm TL size classes according to the equation: $P_L = 1/1 + e^{-(L - L_{m_{50}})\delta}$, where P_L is the percentage of mature fish (gonads containing spermatozoa in males and primary yolk vesicle oocytes in females) at length L and δ describes the width of the logistic ogive.

Spawning period

Spawning period was determined by the proportion of ripe individuals in the population and from the progression of the gonadosomatic index (GSI) over time [GSI = gonad mass (g) × 100 ⁄ total mass of fish (g)]. The GSI values were grouped by sampling season and compared using a Kruskal–Wallis non-parametric ANOVA.

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Table 1: Developmental stage criteria describing the macroscopic and microscopic appearance of preserved male and female gonads of *Barbus motebensis* from the upper Groot Marico catchment (adapted from Whitehead et al. 2007)

Stage	Macroscopic appearance	Microscopic appearance
Regressed	Ovaries translucent in colour	Oogonia, chromatin nucleolar, early and late perinucleolar-stage
	Ovaries occupy half or slightly more than half the length of the abdominal cavity	Oocytes dominate ovary
	Testis thin and thread-like, whitish. Slightly more than half the length of the abdominal cavity	Spermatogonia dominate sections
		Immature states of spermatogenesis (largely spermatocytes); no spermatozoa observed
Developing	Ovaries appear grainy. Eggs yellowish. Larger eggs clearly discernible and opaque. Ovaries occupy about two-thirds of the abdominal cavity	Ovary contains primary and secondary yolk vesicle oocytes
	Testes thicker and whiter than Stage 1. Occupy two-thirds of the abdominal cavity	Testes contain cells in various stages of spermatogenesis, dominated by primary and secondary spermatocytes, small amounts of spermatozoa present
Ripe	Eggs round, appear yellow in colour. Ovaries fill the abdominal cavity	Ovary dominated by secondary and tertiary yolk vesicle oocytes
	Testes thicker, white-yellow in colour and fill the abdominal cavity	Testis lobules filled with spermatozoa – the dominant sperm cell type
Spent	Ovaries reduced in size, appear as deflated sacks, sometimes with a few remaining eggs	Post-ovulatory follicles and atretic oocytes visible
	Testes reduced in size, appear as deflated sacks	Residual spermatozoa and increasing numbers of spermatogonia present in testes

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Fecundity

Fecundity was determined by direct counts of the number of yolked eggs in the gonads of a sample of ripe female fish collected in spring and summer only. As a result of sample size, limitations and the requirement of ovarian tissue for histological analysis, fecundity was determined from one ovary. After weighing the whole ovary, one (either the left or right ovary) was chosen haphazardly and dissected. After weighing this single ovary, ova were then carefully dissected out and counted. To correct for potential bias resulting from asymmetrical gonad development, total fecundity (FT) was then determined by the product of the number of ova per gram of the inspected ovary and the total mass of both ovaries. Relative fecundity was total fecundity expressed as a function of body mass.

Results

The total sample (122 fish) comprised 70 fish sampled from the Draaifonteinspruit (27 males and 43 females) and 52 fish from the Kaaloog se Loop (20 males and 32 females), resulting in a total of 47 males (34–83 mm TL) and 75 females (37–91 mm TL). Morphometric relationships are summarised in Table 2.

Table 2: Relationships between total length (TL), standard length (SL), fork length (FL), weight (*W*) and fecundity (*F*) in *Barbus motebensis* from the upper Groot Marico catchment

Relationship	Regression
TL:SL (mm)	$TL = 1.533 + (1.077 \times FL)$ ($r^2 = 0.99$, df = 1, 123)
SL:FL (mm)	$SL = -1.104 + (0.914 \times FL)$ ($r^2 = 0.98$, df = 1, 123)
FL:TL (mm)	$FL = -1.024 + (0.921 \times FL)$ ($r^2 = 0.98$, df = 1, 123)
TL (mm):*W* (g)	$W = 0.00000249\ TL^{3.353}$ ($r^2 = 0.98$, df = 1, 116)
F (ova):TL (mm)	$F = 0.0034\ TL^{3.15}$($r^2 = 0.71$, df = 1, 24)
F (ova):*W* (mm)	$F = 232.3 + (510.4 \times W)$ ($r^2 = 0.71$, df = 1, 20)

Gonadal development

Histological assessments were consistent with macroscopic staging criteria (Table 1). Regressed testes contained spermatogonia, primary and secondary spermatocytes and spermatids, but were devoid of spermatozoa (Figure 3a). Primary and secondary spermatocytes dominated sections of testes staged as developing, but traces of spermatozoa were visible in the intercellular lumen (Figure 3b). Spermatozoa were dominant in ripe testes, with the intercellular lumen filled with large amounts of spermatozoa (Figure 3c). Spent testes contained few spermatozoa, with the posterior intercellular lumens as well as the sperm duct appearing empty in some males. Additionally, spent testes contained spermatogonia, primary and secondary spermatocytes and spermatids, with spermatogonia numbers dominating (Figure 3d). Intersex was recorded in two males in which each testis contained a small number of oocytes within their testes tissue. One of these males, of 44 mm (TL) from autumn, was collected from Kaaloog se Loop, while the other, of 61 mm (TL) was collected from Draaifonteinspruit in spring. The area of their testes occupied by ovarian tissue did not affect the accuracy in macroscopically determining the sex of the fishes or staging of the gonads. No intersex was recorded in females during this study.

Ovaries microscopically staged as regressed had predominantly early and late perinuclear oocytes (Figure 4a). Developing ovaries consisted of early and late perinuclear oocytes as well as primary and secondary yolk vesicle oocytes (Figure 4b). Some ovaries that had been scored as developing contained atretic oocytes. Tertiary

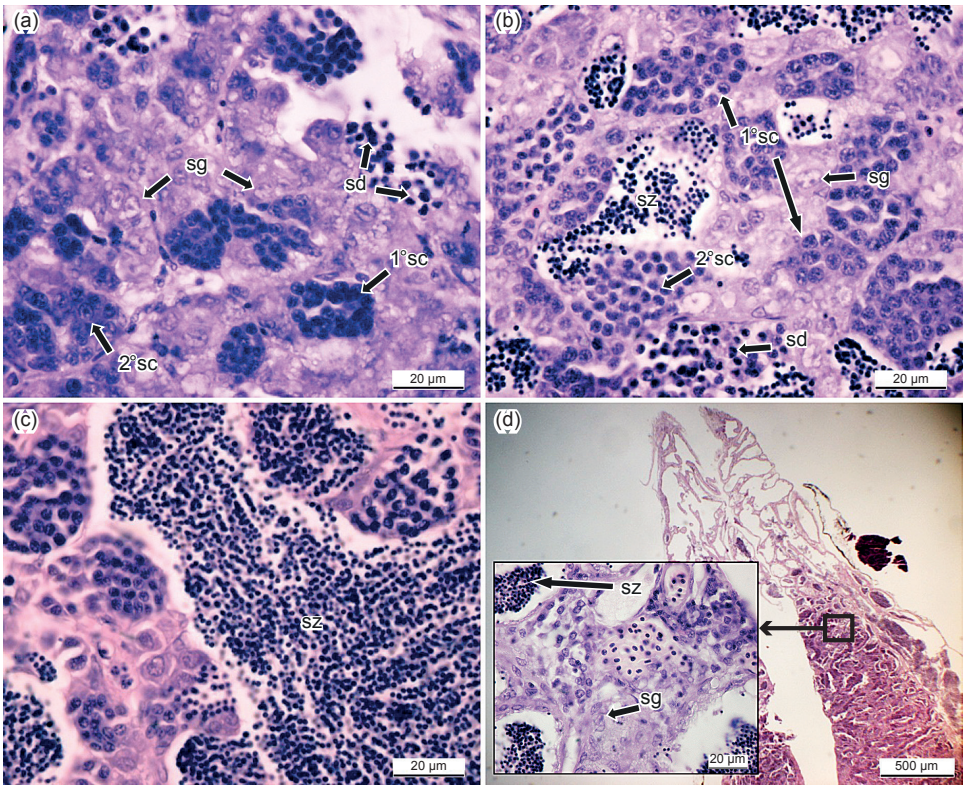

Figure 3: Micrographs of representative *Barbus motebensis* testes: (a) Stage 1: regressed; (b) Stage 2: developing; (c) Stage 3: ripe; and (d) Stage 4: spent; sg = spermatogonia; 1°sc = primary spermatocytes; 2°sc = secondary spermatocytes; sd = spermatids; sz = spermatozoa

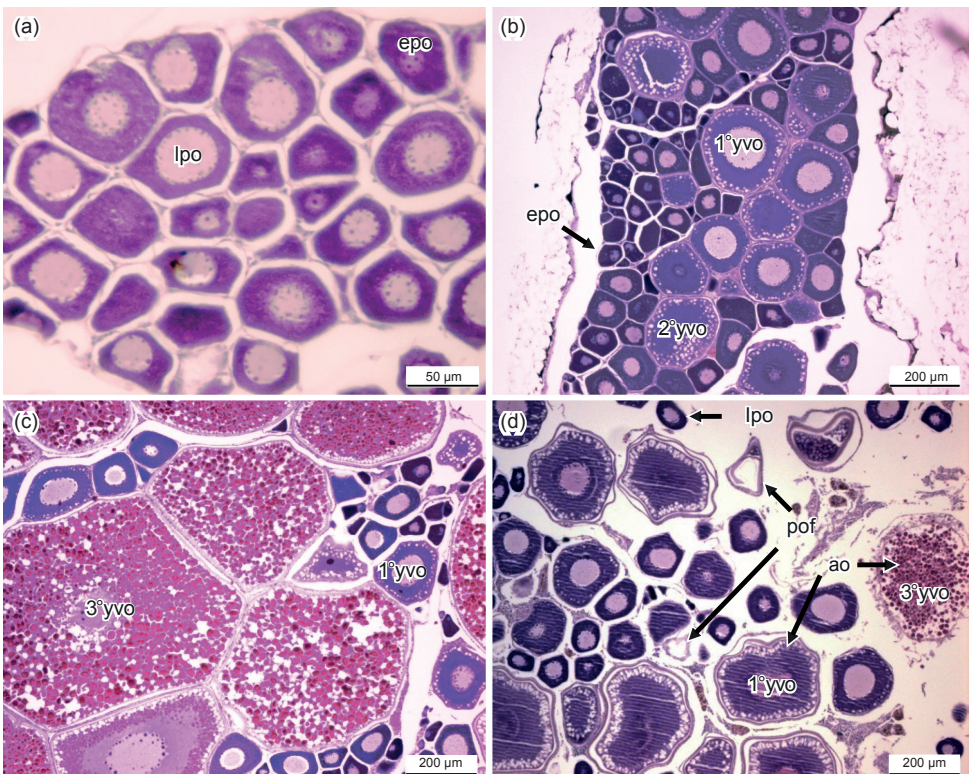

Figure 4: Micrographs of representative *Barbus motebensis* ovaries: (a) Stage 1: regressed; (b) Stage 2: developing; (c) Stage 3: ripe; and (d) Stage 4: spent; epo = early perinuclear oocytes; lpo = late perinuclear oocytes; 1°yvo = primary yolk vesicle oocytes; 2°yvo = secondary yolk vesicle oocytes; 3°yvo = tertiary yolk vesicle oocytes; pof = post-ovulatory follicles; ao = atretic oocytes

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yolk vesicle oocytes dominated sections of ripe ovaries (Figure 4c), which also contained early and late perinuclear oocytes, primary and secondary yolk vesicle oocytes, and few post-ovulatory follicles. The presence of ova in various stages of development in ripe ovaries was indicative of multiple spawnings over the reproductive season. Spent ovaries (Figure 4d) contained many loosely-spaced atretic oocytes and melanomacrophage centres, as well as early and late perinuclear oocytes, primary and secondary yolk vesicle oocytes, post-ovulatory follicles, and very few remaining tertiary yolk vesicle oocytes.

Maturity

The smallest mature male sampled during this study measured 37.5 mm TL (46% of maximum length L_{max}) whereas at 50 mm TL all males were mature. It was noted that *B. motebensis* displayed sexual dimorphism, with the heads of mature males being covered with tubercles and those of females being without tubercles during the breeding season. The smallest mature female fish was 42.5 mm TL (47% of L_{max}), $L_{m_{50}}$ was 44.5 mm TL, and at 55 mm TL all females were mature (Figure 5).

Breeding season

Macroscopic staging data and the progression of GSI for male and female fish are shown in Figure 6. For male fish, neither macroscopic staging nor the GSI were able to determine a clear spawning season, with macroscopically staged ripe fish being present throughout the year and no significant difference ($p > 0.05$) in mean GSI. Ovaries showed a clear reproductive season, with a large proportion of ripe fish in the spring (September) and summer (November) samples, while spent and developing fish were dominant in the autumn and winter samples. This trend was also supported by GSI, which differed significantly between sampling events ($p < 0.05$), and increased from GSI = 3 in winter to GSI >7 in spring (Figure 6). Similar increased values were maintained into the summer season.

Fecundity

Total fecundity ranged from 582 to 3 728 eggs, with a mean fecundity calculated as 1 614 (SD 843) eggs per fish. Overall, relative fecundity was estimated at 578 (SD 168) eggs g^{-1}. Relationships between TL, *W* and fecundity are summarised in Table 2.

Discussion

This study demonstrated that *B. motebensis* has an extended spawning season, with spawning starting in spring and lasting until the end of summer in March. The spawning season coincided with changes in a variety of environmental variables, i.e. increasing day-length, increasing temperature and increasing water flow (Figure 2). Environmental cues such as changes in day length, water flow rate and water temperature are important determinants of reproduction in many freshwater fishes (Cambray 1982; Marriott 1998; Booth and Weyl 2000; Weyl et al. 2009), but because these variables are strongly auto-correlated in the Groot Marico River, it was not possible to determine which of these factors ultimately triggers spawning. The

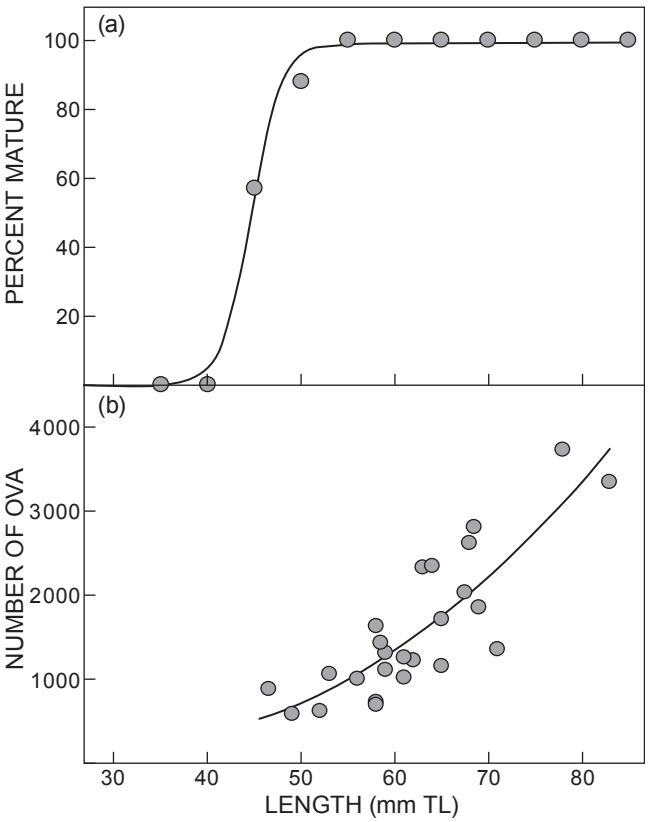

Figure 5: (a) Maturity ogive and (b) length:fecundity relationship for *Barbus motebensis* females from the upper Groot Marico River in 2012–2014

asynchronous pattern of oocyte development observed in ripe female gonads is, however, typical of partial spawners that produce multiple batches of eggs over an extended breeding season (Cambray 1982; West 1990; Cambray 1994; Booth and Weyl 2000; Whitehead et al. 2007).

Partial spawning, and a protracted spawning season, is a reproductive strategy employed by many African cyprinid fishes, including *Barbus anoplus*, *Labeo capensis* and *Labeo umbratus* (Munro et al. 1990) and *Labeobarbus batesii* (Tiogué et al. 2013), and is likely to have evolved as a response to high environmental variability. Although little is known about the spawning behaviour of *B. motebensis*, males were observed guarding territories among marginal vegetation (OLFW pers. obs.), and the low GSI of males in comparison to females is indicative of a pair-spawning reproductive strategy and low sperm competition (Langen et al. 2013). Observations of spawning behaviour are, however, necessary to validate this hypothesis.

Early maturity (<50% of maximum length) and relatively high fecundity (3 728 eggs) is consistent with an opportunistic life-history style, as is generally employed by small southern African barbs (Table 3), and suggests adaptation to environmental perturbations. In an assessment of habitat preferences of fishes in the Groot Marico system, Kimberg et al. (2014) demonstrated that *B. motebensis* is a rhithronic pool specialist typically associated with the upper foothills of mountain streams. In comparison with other small barbs,

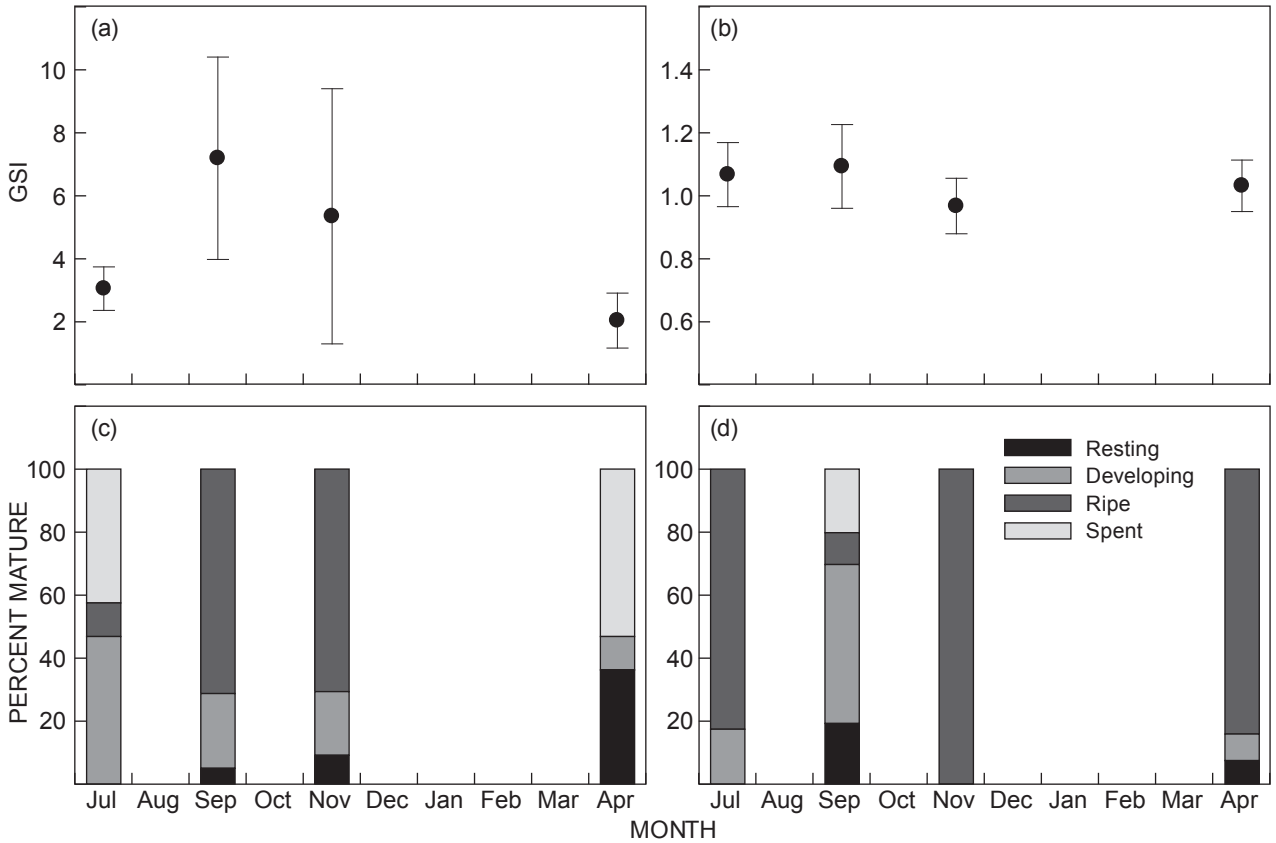

Figure 6: Mean GSI values for mature (a) female and (b) male *Barbus motebensis*, and microscopic staging for (c) females and (d) males from the upper Groot Marico River in 2012–2014. Error bars denote SD

Table 3: Comparative life histories of small barbs; – denotes no data

Species	Standard length (mm)	Length at maturity (mm)	Fecundity maximum	Relative fecundity	Breeding season	Reference
Barbus motebensis	80	F: 44.5, M: 37.5 (FL)	3 728	578	Spring and summer	Present study
Barbus anoplus	120	40 (FL)	3 000	–	Spring and summer	Cambray (1982)
Barbus treurensis	95	–	2 040	–	Spring	Kleynhans (1984)
Barbus brevipinnis	45	40.4 (FL)	1 341	–	Spring and summer	Schulz and Schoonbee (1999)
Barbus neefi	70	–	759	–	Year round	Vlok (2005)
Barbus erubescens	95	F: 42, M: 45 (SL)	400	–	Summer (winter rainfall area)	Marriott (1998)
Pseudobarbus burchelli	135	–	10 678	–	Spring and summer	Cambray and Stuart (1985)

the protracted spring/summer spawning season of *B. motebensis* is similar to that of other headwater specialists including *Barbus erubescens* Skelton, 1974 (Marriott 1998; Skelton 2001; Impson et al. 2007), *Barbus brevipinnis* Jubb, 1966 (Schulz and Schoonbee 1999), *Barbus trevelyani* Günther, 1877 (Skelton 2001), *Pseudobarbus asper* Peters, 1864 (Cambray 1994) and *Pseudobarbus phlegethon* Barnard, 1938 (Whitehead et al. 2007). Multiple spawning over an extended breeding season is most likely an adaptation employed by these fish to the relatively unstable flow conditions in headwater streams, which respond rapidly to rainfall (Ellender and Weyl 2015). In comparison, barbs that inhabit lower foothill streams and lowland rivers, such as *Barbus anoplus*, *Barbus pallidus* A.Smith, 1841, *Barbus paludinosus* and *B. unitaeniatus* Günther, 1866, are all summer breeders (Table 3). Within this group, *Barbus neefi* Greenwood, 1962, which breeds year-round with no resting phase (Vlok 2005), appear to be the exception.

With regard to conservation implications, the opportunistic life history (extended breeding season, early maturity and relatively high fecundity) implies that, from a reproductive perspective, *B. motebensis* is likely to have the capacity to rapidly recolonise restored habitat if the *M. salmoides* removals suggested by Kimberg et al. (2014) are undertaken. The rate of recovery will, however, depend on its dispersal ability.

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