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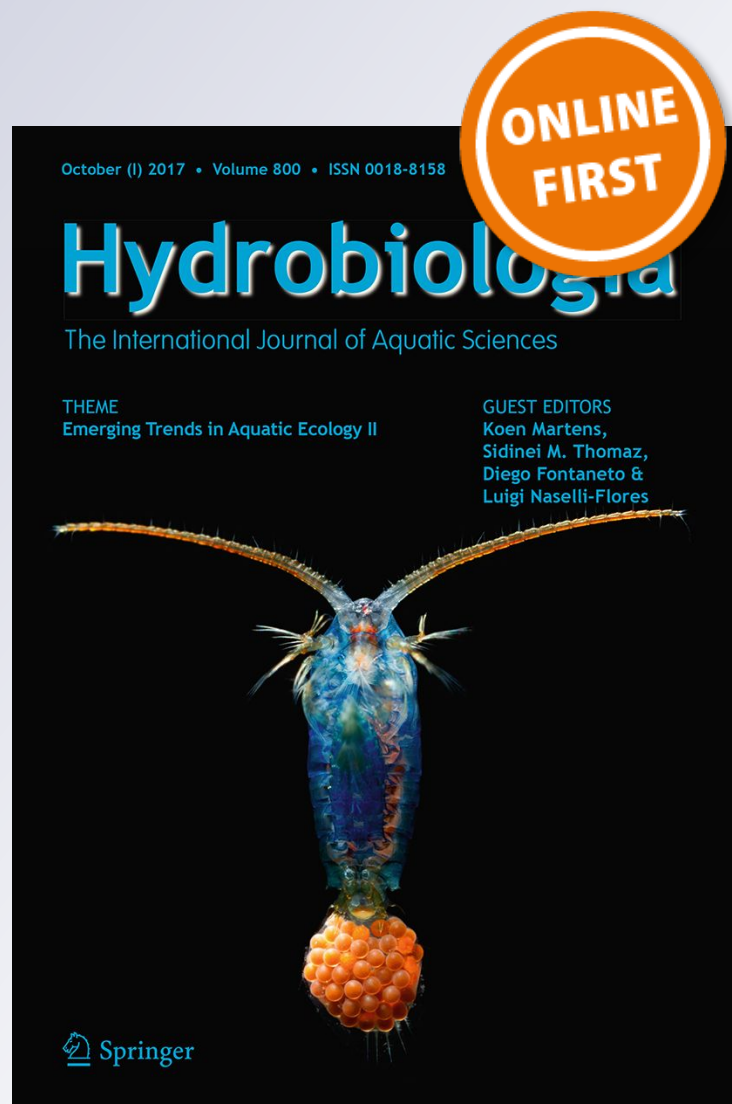
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# The origin and future of an endangered crater lake endemic; phylogeography and ecology of *Oreochromis hunteri* and its invasive relatives

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**Abstract** Cichlids of the genus *Oreochromis* (“Tilapias”) are intensively used in aquaculture around the world. In many cases, when “Tilapia” were introduced for economic reasons to catchments that were home to other, often endemic, *Oreochromis* species, the loss of native species followed. *Oreochromis hunteri* is an endemic species of Crater Lake Chala on the slopes of Mount Kilimanjaro and is part of a small species flock in the upper Pangani drainage system of Tanzania. We identified three native and three

invasive *Oreochromis* species in the region. Reconstructing their phylogeography, we found that *O. hunteri* is closely related to, but distinct from the other members of the upper Pangani flock. However, we found a second, genetically and phenotypically distinct *Oreochromis* species in Lake Chala whose origin we cannot fully resolve. Our ecological and ecomorphological investigations revealed that the endemic *O. hunteri* is currently rare in the lake, outnumbered by each of three invasive cichlid species. It is mitochondrially, phenotypically and trophically distinct from all others. The occurrence of the formerly abundant *O. hunteri* in such small numbers, its narrow habitat restriction and its limited morphological variability suggest recent population decline and loss of niche breadth in this critically endangered endemic cichlid species.

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## Introduction

Biodiversity provides a multitude of ecosystem services (Watson et al., 2005), and for sustainable development, it is therefore essential to conserve biodiversity. The cichlids in the East African Lakes provide a biodiversity hotspot in freshwater

ecosystems with several hundred endemic species in each of the big lakes Tanganyika, Malawi and Victoria (Genner et al., 2004) and many more endemic species in numerous smaller lakes (Wagner et al., 2014). While most of the species richness consists of the smaller haplochromine cichlids, most of the interest in fisheries and aquaculture is with the larger oreochromine cichlids, often referred to as “Tilapia”. The oreochromine cichlids nonetheless made several small radiations, especially in some of the smaller lakes (Trewavas et al., 1972; Schliewen et al., 1994; Ford et al., 2015) that do not have haplochromines (Seehausen, 2007). They also produced several endemic, often allopatric species in Eastern African rivers and small lakes (Trewavas, 1983).

In tropical Africa, fish is one of the most important protein sources and in Eastern Africa millions of people depend heavily on fishery-related activities for their daily food or income (Ogutu-Ohwayo et al., 1997; FAO, 2007; DeGraaf & Garibaldi, 2014). As most East African aquatic ecosystems are dominated by cichlid fish, they provide important economic and food resources. Since the 1950s, the optimization of fishery yields led to the introduction of Nile Tilapia (*Oreochromis niloticus*, Linnaeus, 1758) in many places of Africa outside the species' distribution range, with negative impacts on the native *Oreochromis* species reported in many cases through competition and hybridization (Genner et al., 2013; Deines et al., 2014). *Oreochromis niloticus* is one of the world's most important species for food provisioning from aquaculture. It has been distributed for aquaculture not only within Africa, but worldwide (Casal, 2006; Deines et al., 2016) and has established numerous wild populations in tropical and subtropical latitudes, including in China (Shuai et al., 2015), in the USA (Lowe et al., 2012), in India (Singh et al., 2013) and in Brazil (de Azevedo-Santos et al., 2011). Detrimental impacts on the local ecosystems due to resource competition with the native fish or habitat alterations due to the grazing behaviour have been reported from various countries (Crutchfield, 1995; Singh et al., 2013; Daga et al., 2016; Zhang et al., 2017). If introduced into catchments that have their own native *Oreochromis* species, *O. niloticus* causes threats additionally through hybridization with the local species (D'Amato et al., 2007; Angienda et al., 2011; Firmat et al., 2013; Deines et al., 2014).

In this study, we investigate the status of a little known endemic crater lake species, *Oreochromis hunteri* (Günther, 1889) of Lake Chala. We investigate aspects of its origins and distinctiveness within the endemic species flock of the upper Pangani drainage, its distinctiveness versus several invasive cichlids, and its ecology in the face of the presence of other cichlid species that have invaded the lake in recent decades. *Oreochromis hunteri* is thought to be part of a small species flock of three or four taxa in the upper Pangani drainage system. Lake Chala is a small (4.2 km<sup>2</sup>) but deep (90 m) crater lake with habitats of rock and lava sand. It is positioned at the eastern flank of Mount Kilimanjaro (3.317°S, 37.699°E) at 877 m above sea level, shared between Kenya and Tanzania. Inflow and outlet are both subterranean, and the water in the lake is very clear (Secchi depth ~ 6.6 m). Four different cichlid species occur in Lake Chala: the endemic *Oreochromis hunteri*, an undescribed *Oreochromis* species (sometimes erroneously referred to as *O. korogwe* (Lowe, 1955), here referred to as *O. sp.* “blue head”), the widely distributed eastern redbreast tilapia *Coptodon rendalli* (Boulenger, 1897) and a haplochromine cichlid of the riverine *Astatotilapia bloyeti* (Sauvage, 1883) complex. The latter three most likely were introduced between 1951 and 1977 (Lowe, 1955; Dadzie et al., 1988; Moser et al., 2018). Due to the co-occurrence of an endemic cichlid species that had several thousand years to explore and exploit the different ecological niches within Lake Chala, and one closely and two more distantly related species that only arrived about 40–70 years ago, Lake Chala provides a system for investigating impacts of introduced species on endemic cichlid communities of isolated lakes.

We reconstructed the phylogeography of the native and the recent colonizations, and investigated ecology and ecomorphology of all four cichlid species in Lake Chala, as well as the oreochromine cichlids of the upper Pangani/Ruvu drainage. We wanted to know if and how niches are partitioned between the endemic and the invasive species to make predictions for the future of this unique little ecosystem and its endemic cichlid species. We experimentally fished all habitats and several depth gradients in Lake Chala and combined studying habitat distribution of cichlids, analysis of 14 morphological traits and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  stable isotopes.

## Materials and methods

### Fish sampling

We sampled *Oreochromis* from four locations in North-Eastern Tanzania, in Lake Chala, in the Nyumba ya Mungu Reservoir (dammed Pangani River), in the Ruvu River (the main inflow into the Nyumba ya Mungu Reservoir) and in Lake Babati in November 2014 (Fig. 1). In Lake Chala, gill nets with mesh sizes from 12 to 22 mm were set at various depths in the benthic habitat in Lake Chala. Mesh sizes of the nets were chosen to catch fish of comparable size of all species present in Lake Chala. This might have led to a slight bias towards smaller individuals in the larger *Oreochromis* and *Coptodon* species. Nets set below 47 m and nets set in the limnetic habitat (4 times three joined nets with 16, 19 and 22 mm mesh size) remained empty. Fish were carefully removed from the net and photographed from their left side in a

custom designed photo-cuvette with a colour reference bar, a scale bar and a standardized grey background. The fish were afterwards euthanized with an overdose of phenoxyethanol diluted in lake water. From the right side of each fish, we removed the pectoral fin and fixed it in pure analytical ethanol (100%) for DNA extractions, and we removed a piece of the epaxial muscle dorsal of the lateral line, removed the skin and dried the muscle tissue in an oven (60°C for 24–48 h) for stable isotope analysis. Whole fish were subsequently fixed in 4% formalin (buffered with borax) and were later rinsed with water and stepwise transferred to 75% ethanol. Additionally, we bought several larger individuals of *O. hunteri* from a local fisherman on the lake, who was fishing with hook and line. With the same methods (gill netting), we caught and processed the fish from Lake Babati. In the Ruvu River, the fish were caught with hook and line, and the fish from the Nyumba ya Mungu were bought from the local fish market. Like this, we



**Fig. 1** Map of the upper Pangani River drainage with the sampling sites used in this study: Lake Chala, the Pangani Reservoir Nyumba ya Mungu and its main inlet, the Ruvu River, and Lake Babati



obtained samples from 4 sites and taxonomically identified 3 native and 3 invasive *Oreochromis* species.

#### DNA extraction, sequencing and phylogenetic reconstruction

DNA was extracted from fin clips of *Oreochromis* from Lake Chala (18 *O. sp.* “blue head”, 13 *O. hunteri*), Nyumba ya Mungu (6 *O. jipe* (Lowe, 1955), 2 *O. esculentus* (Graham 1928), 1 *O. niloticus*), the Ruvu River (14 *O. sp.* “blue head”, 3 *O. pangani* (Lowe, 1955), 1 *O. niloticus*) and Lake Babati (2 *O. niloticus*, 4 *O. sp.*), and *Coptodon rendalli* from Lake Chala ( $N = 4$ ) and Nyumba ya Mungu ( $N = 2$ ). We used the Qiagen DNeasy Blood&Tissue-Kit and sequenced 830 bp of the mitochondrial D-loop region.

We aligned our mitochondrial sequences in BioEdit v7.2.5 together with mitochondrial D-loop sequences of most other *Oreochromis* species occurring in Eastern Tanzania from GenBank (Nagl et al., 2001; D’Amato et al., 2007; Nyingi et al., 2009; Kinaro et al., 2016). A Templeton, Crandall and Sing (TCS) network was built and visualized in PopART (Bandelt et al., 1999), to investigate and visualize the relationship among the different populations and species.

#### Morphology and trophic ecology

For 27 *Oreochromis hunteri*, 74 *O. sp.* “blue head” and 10 *Coptodon rendalli* from Lake Chala, 20 *O. jipe*, 5 *O. esculentus*, 5 *O. niloticus*, 2 *C. rendalli* and 10 *Astatotilapia* from the Nyumba ya Mungu Reservoir, 6 *O. pangani*, 16 *O. sp.* “blue head” and 1 *O. niloticus* from the Ruvu River, we measured 15 linear morphometric distances that have proven powerful for quantifying taxonomically and ecologically relevant morphometric variation in cichlids (Barel et al., 1976, Witte, 1984) using digital callipers: standard length (SL), head length (HL), head width (HW), body depth (BD), lower jaw length (LJL) and width (LJW), snout length (SnL), snout width (SnW), cheek depth (ChD), preorbital depth (POD) and width (POW), interorbital width (IOW), eye length (EyL) and depth (EyD), and premaxillary pedicel length (PPL). The same measurements for 274 *Astatotilapia* from Lake Chala were taken from Moser et al. (2018). All measurements were log-transformed and size-corrected by using the standardized residuals of the

pooled linear regression of each log-transformed trait against log-SL. The largest *O. hunteri* that we bought from the fisherman at Lake Chala (SL > 19 cm) were excluded as they were four to eleven cm larger than the next smaller fish. All analyses, if not stated differently, were performed in R 3.2.1 (R-Core-Team, 2015).

To quantify morphological variation and differentiation, we performed principal component analyses (PCA). To prevent that the PCA axes are mainly driven by the taxa with larger sample sizes, we used only ten individuals of the species where sample sizes exceeded ten and all individuals for all other species for the calculation of PCA axes. All other individuals were subsequently projected into the morphospace. As an estimate of morphological differentiation, we calculated Bhattacharyya distances between the different species in the PC1-PC2-morphospace and tested for significance with Hotelling’s T-squared test. To test for differences in morphological variation, we calculated morphospace occupation for each species. For this purpose, and to exclude sample size effects, we subsampled 1000 times ten individuals per species and calculated the morphospace occupation using the R-function `chull`. We report the mean of these 1000 permutations and used the permutations to assess whether morphospace occupation is significantly different between species. To investigate which traits are most distinct between *O. hunteri*, *O. sp.* “blue head”, *O. jipe* and *O. pangani*, we additionally performed pairwise linear discriminant analyses (LDA).

For all fish from Lake Chala, we performed stable isotope analyses. Dried muscle tissue was ground into fine powder using a QIAGEN TissueLyzer II with five-millimetre stainless steel beads (QIAGEN). Of this powder, 0.25–0.35 mg was used to analyse  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in a ThermoFisher Scientific Flash 2000 elemental analyser coupled through a ConFlo IV interphase to a Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS). To quantify ecological variation and differentiation, we calculated Bhattacharyya distances between the different species based on the stable isotope ratios and tested for significance with Hotelling’s T-squared test.

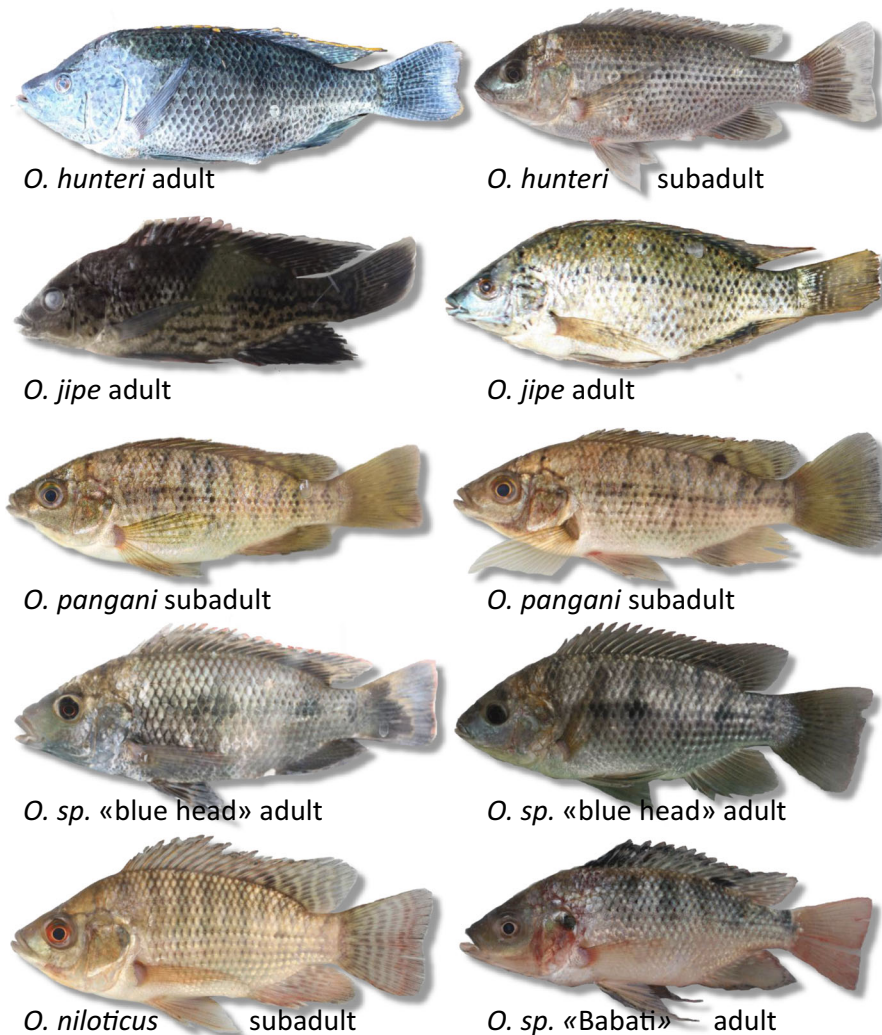
**Results**

Taxonomy, diversity and phylogeography of *Oreochromis* in Lake Chala and the nearby Ruvu/Pangani system

We found evidence for three native (*Oreochromis hunteri*, *O. jipe* and *O. pangani*) and three non-native (*O. niloticus*, *O. esculentus* and *O. sp.* “blue head”) oreochromine species in the Pangani/Ruvu drainage, including Lake Chala (Fig. 2). The D-loop haplotype network revealed that *O. hunteri* is most closely

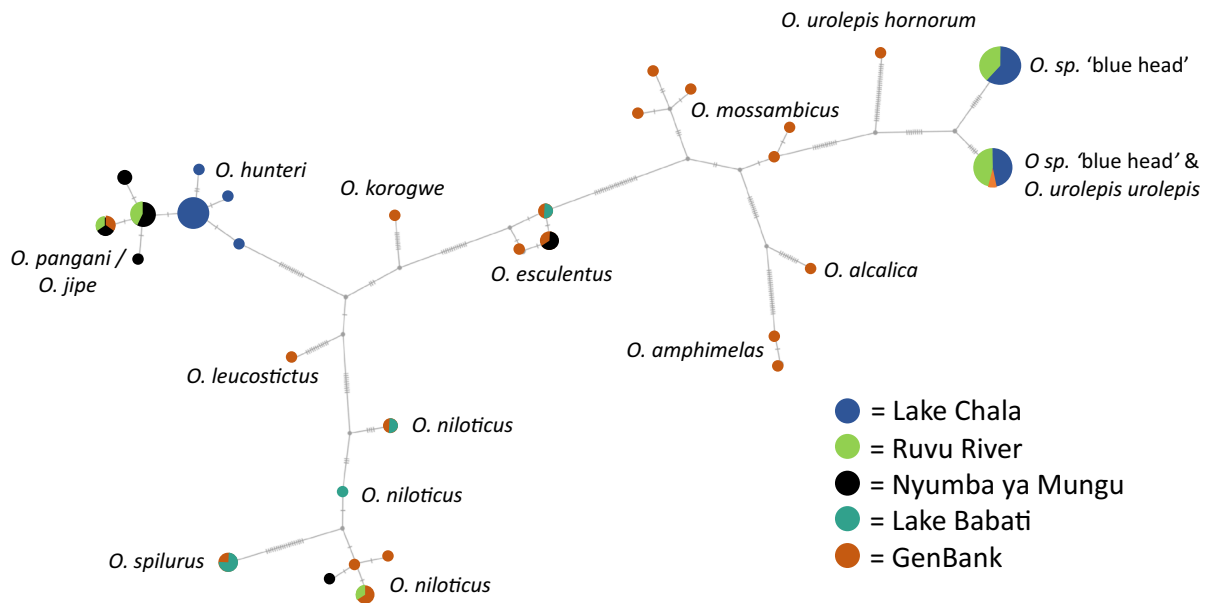
related to the geographically nearby Pangani system endemics, *O. jipe* and *O. pangani*, but does not share any haplotypes with either of these (Fig. 3). This confirms earlier morphology-based suggestions that these three species form a “Pangani system flock” of closely related species (Trewavas, 1983). We did not encounter any *Oreochromis hunteri* outside Lake Chala, neither did we encounter either of the other native Ruvu/Pangani taxa in Lake Chala.

However, we found a second, genetically distinct *Oreochromis* species in Lake Chala whose origin we could not fully resolve. This species, which we refer to



**Fig. 2** Oreochromine cichlid species of the upper Pangani system. From left to right and top to bottom: *Oreochromis hunteri* from Lake Chala, adult male and subadult male; *O. jipe* from Nyumba ya Mungo, two adult males; *O. pangani* from the

Ruvu River, two subadults; *O. sp.* “blue head” from Lake Chala, adult male with enlarged jaws; adult individual without enlarged jaws; *O. niloticus* from the Ruvu River (subadult); *O. sp.* adult male from Lake Babati



**Fig. 3** Phylogeographic relationships of the *Oreochromis* spp of Lake Chala, the upper Pangani and Lake Babati. TCS haplotype network of 347 bp of mitochondrial D-loop. We only used 347 bp because most sequences used from GenBank were restricted to this shorter fragment. The size of the circles indicates the number of individuals with a given haplotype in our data, ticks between the circles indicate single point mutations. Dark blue are individuals collected from Lake Chala, black individuals collected from the Nyumba ya Mungu Reservoir, green individuals collected from the Ruwu River

as *O. sp. "blue head"*, does not belong to the Ruwu/Pangani radiation in its mitochondrial lineage but is more closely related to *O. urolepis* from the Tanzanian East Coast rivers (Fig. 3) and also shared the distinctive large midlateral blotches with the latter (Fig. 2). In *O. sp. "blue head"*, we found two very distinct haplotype groups that differ from each other by 12 mutations (in 347 bp), suggesting that the population may be a mix of introductions from two distinct source populations. We identified one individual from Lake Chala phenotypically as *O. niloticus* or an *O. niloticus*-hybrid, but its mitochondrial haplotype was one of the two *O. sp. "blue head"* haplotypes. We also found two non-oreochromine cichlids in Lake Chala, *Coptodon rendalli* and *Astatotilapia cf. bloyeti*. Both also occur in the Ruwu/Pangani system, where *Astatotilapia cf. bloyeti* is native and *C. rendalli* has been introduced.

In the Ruwu River, we found matching phenotypic and genetic evidence for the presence of three *Oreochromis* species: the native *O. pangani*, the introduced *O. niloticus* and the unidentified *O. urolepis*

(upper Pangani, inflow to the Nyumba ya Mungu Reservoir), turquoise individuals collected in Lake Babati, brown individuals were taken from GenBank (Nagl et al., 2001; D'Amato et al., 2007; Nyangi et al., 2009; Kinari et al., 2016). Whereas *O. hunteri* is very closely related to the upper Pangani endemics *O. pangani* and *O. jipe*, we observed two only distantly related haplotype groups within *O. sp. "blue head"*, the other *Oreochromis* from Lake Chala and the Ruwu River. One of these is shared with the Rufiji-Tilapia (*O. urolepis urolepis*)

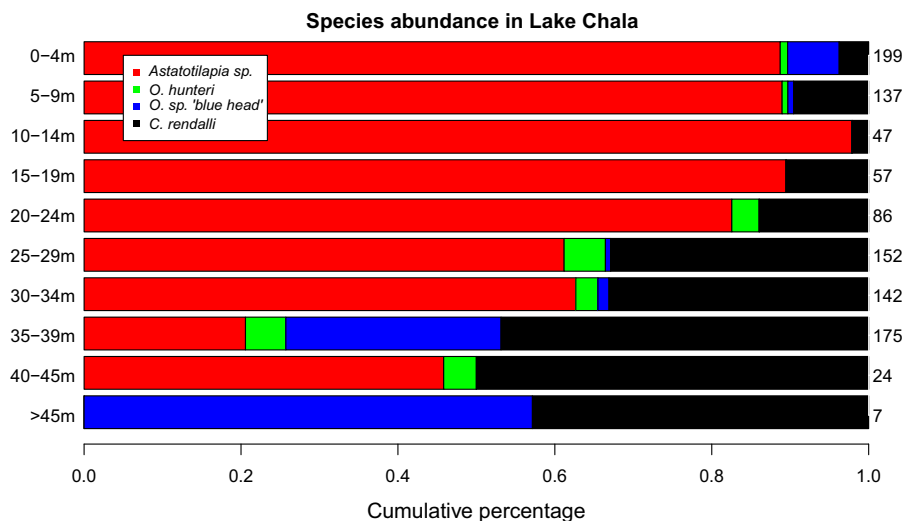
clade taxon that we also found in Lake Chala (*O. sp. "blue head"*, both haplotypes). In the Nyumba ya Mungu Reservoir finally, we found matching phenotypic and genetic evidence for the presence of three *Oreochromis* species too, but here these were *Oreochromis jipe* (supposedly introduced from Lake Jipe further upstream in the catchment) and introduced *O. niloticus* and *O. esculentus*.

In Lake Babati, we found phenotypic and genetic evidence for the presence of *O. niloticus*. Further we found mitochondrial haplotypes belonging to *O. spilurus* and *O. esculentus* (Fig. 3), but specimens of these two haplogroups could not be distinguished from each other phenotypically.

#### Ecological and phenotypic species differentiation and niche partitioning

We find that in Lake Chala each of the invasive species, *O. sp. "blue head"*, *C. rendalli* and *A. cf. bloyeti*, are more abundant than the native *O.*





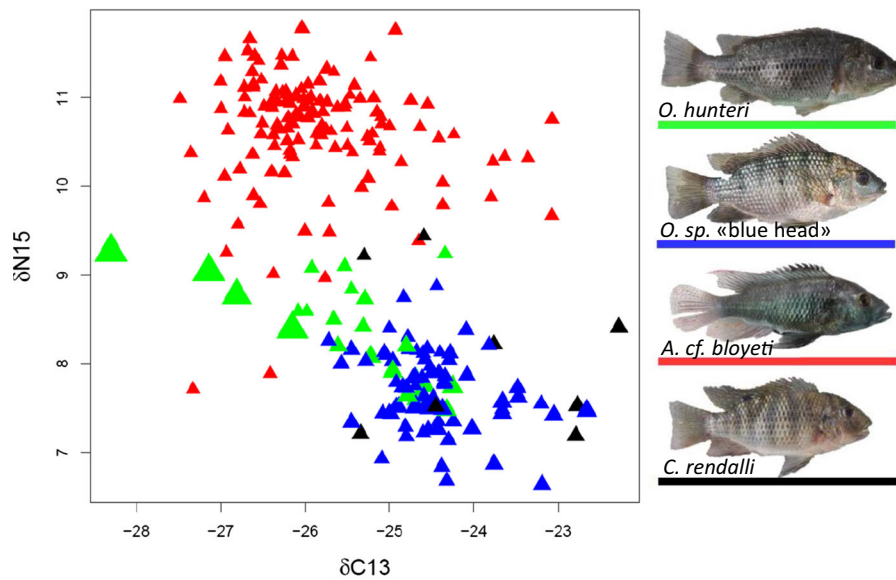
**Fig. 4** Relative abundance of the different cichlid species at different water depths in Lake Chala. All four cichlid species were present at all three geographical sampling locations. All species occurred from the very shallow areas down to a water depth of at least 40 m, but none was found deeper than 47 m. *Astatotilapia* were the most abundant taxon in the shallow habitat, whereas *Coptodon rendalli* was most abundant in the deeper habitats. The two *Oreochromis* species were both caught in the deep and in the shallow habitat, but were more common in

*hunteri* (Fig. 4). With the exception of a few fish caught shallower, *O. hunteri* seems to be restricted to a depth range between 20 and 45 m, i.e. the deeper half of the oxygenated habitat. Even at those depths, it was outnumbered between 3 and 20 times by other cichlid species (Fig. 4). However, our stable isotope data suggest significant feeding niche partitioning between *O. hunteri* and all other species (Fig. 5). In fact, all four species in Lake Chala differ significantly from each other in their stable isotope signatures (Fig. 5, Table 1). *O. hunteri* feeds on slightly more depleted carbon sources than *O. sp. "blue head"* and *C. rendalli* but is fully overlapping with *A. cf. bloyeti*. On the other hand, *O. hunteri* feeds at a slightly higher trophic level than *O. sp. "blue head"* and *C. rendalli* and lower than *A. cf. bloyeti*. The latter species feeds predominantly on invertebrates (insect larvae, zooplankton) and small fish (Moser et al., 2018) and its mean  $\delta^{15}\text{N}$  value is 10.6. *Coptodon rendalli* is known as a macrophyte eater elsewhere but probably grazes mostly on dead wood and organic debris in Lake Chala where macrophytes are nearly absent. Its mean  $\delta^{15}\text{N}$ -value in Lake Chala is about 8.0, slightly higher than *O. sp. "blue head"* with a mean of 7.7, and nearly one

the deeper habitat and virtually absent from intermediate water depths. The endemic *Oreochromis hunteri* was clearly the least abundant species in our catches. Abundance data are probably slightly biased towards the smaller *Astatotilapia* and subadult *Oreochromis* and *Coptodon*, because we used a maximal mesh size of 22 mm and therefore likely missed very large individuals of *Oreochromis* and *Coptodon*. Sample sizes are given to the right of each depth category

trophic level below *Astatotilapia*, as expected if it feeds mainly on plant matter. The mean  $\delta^{15}\text{N}$ -value of *O. hunteri* in our data is about 8.4, with the largest fish slightly but not much higher. This suggests that *O. hunteri*, despite no evidence of a unique habitat, may have a unique feeding niche that includes both vegetable and animal matter.

Our morphological analysis reveals that *O. hunteri* is clearly distinct eco-morphologically from all other species in the system too. Within Lake Chala, all four species are morphologically distinct from each other (Table 1), but the strongest overlap in morphospace is between *Oreochromis hunteri* and the invasive *Oreochromis sp. "blue head"* (Fig. 6a). The main difference between these species is a longer head, a less deep body and a narrower snout in *O. hunteri* compared to *O. sp. "blue head"* (Table 2). A corresponding pattern is detected outside Lake Chala, where all species except *Oreochromis jipe* and *O. pangani* can be morphologically distinguished in the PCA (Table 3, Fig. 6b) with the highest PC1 and PC2 values for the native *Oreochromis* species (*O. pangani* and *O. jipe*). Whereas PC1 (-ChD and -LJL) mainly separates *Oreochromis* (small cheek depth and short



**Fig. 5** Ecological variation and differentiation among the four cichlid species in Lake Chala represented by the stable isotope plot of the four species: the endemic *Oreochromis hunteri*

(green), *O. sp.* “blue head” (blue), *Coptodon rendalli* (black) and *Astatotilapia cf. bloyeti* (red). The size of the symbols corresponds to the size of each specimen

**Table 1** Differentiation between the different species within Lake Chala in morphology (lower left) and trophic ecology (upper right), measured as the Bhattacharyya distance in the

morphospace spanned by PC1 and PC2 and the stable isotope space, respectively

	<i>Astatotilapia</i>	<i>O. sp.</i> “blue head”	<i>O. hunteri</i>	<i>C. rendalli</i>
<i>Astatotilapia</i>		3.79**	1.98**	1.59**
<i>O. sp.</i> “blue head”	4.98**		0.28**	0.36**
<i>O. hunteri</i>	12.90**	0.90**		0.44**
<i>C. rendalli</i>	1.56**	4.97**	21.34**	

Significant differentiation is indicated by asterisks (\*\* $P < 0.01$ )

lower jaws) from *Astatotilapia* and *Coptodon*, PC2 (-IOW, -POD, -SnW, -BD) separates the Pangani *Oreochromis* (*O. hunteri*, *O. jipe* and *O. pangani* with comparably narrow interorbital width and snout width, and low body depth) from the introduced *Oreochromis* and *Coptodon* (comparably wide interorbital width and snout width, and deep body depth) from *Astatotilapia*. Together, these two axes explain 74.5% of the overall variation (45.7 and 28.7%, respectively).

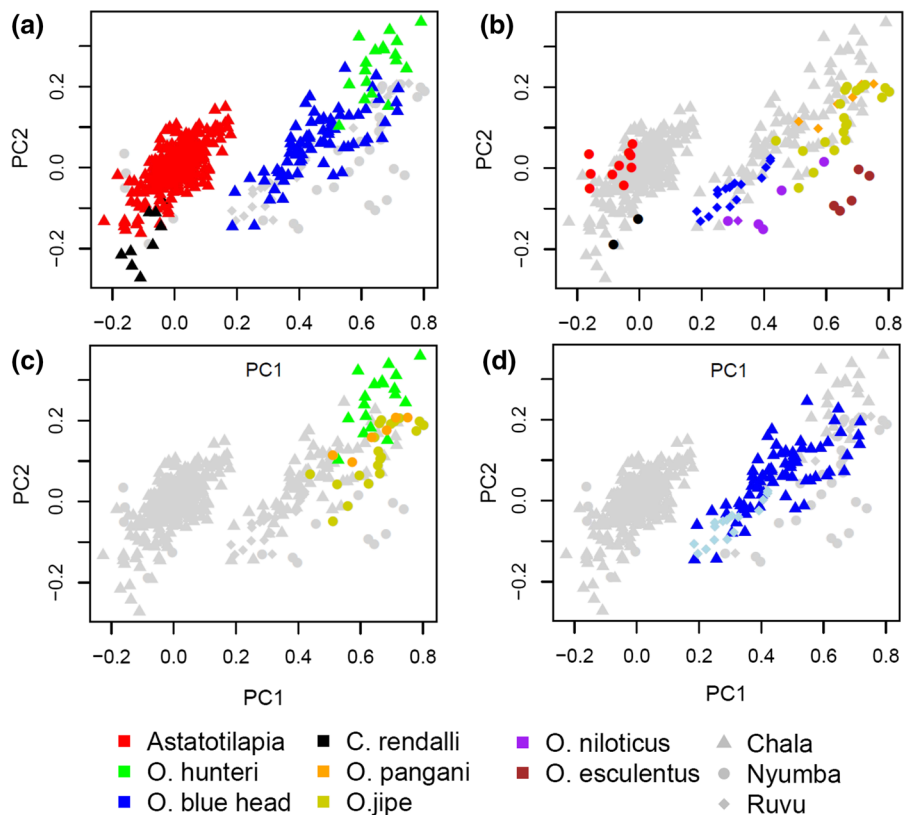
Within the endemic Pangani species flock, *O. hunteri* is morphologically distinct from both *O. jipe* and *O. pangani* (with a longer and wider head, shorter lower jaws and smaller eyes in *O. hunteri*; Table 2), whereas we could not detect significant differentiation between the latter two (Table 4, Fig. 6c). We notice

that whereas in the Pangani River (Nyumba ya Mungu Reservoir), the native species (*O. jipe*) occupies significantly more of the morphospace than the newly arrived *O.* “blue head”, the latter occupies more (though not significantly more) of the morphospace than *O. hunteri* in Lake Chala (Table 5, Fig. 6a, b).

### Discussion

*Oreochromis hunteri* and the oreochromine cichlids of the Pangani/Ruvu drainage

*Oreochromis hunteri* (Günther, 1889) is one of the least known African cichlids, despite it being the type



**Fig. 6** Morphological variation and differentiation among the cichlid species in Lake Chala and the Pangani drainage. **a** PC-plot on linear morphological traits with all individuals from Lake Chala (triangles) highlighted. **b** Same morphospace with all fish from the Ruvu River (diamonds) and the Nyumba ya Mungu Reservoir (dots) highlighted. **c** Morphospace occupation of the endemic species of the Pangani drainage and Lake Chala: *O. jipe* (yellow) from the Nyumba ya Mungu, *O. pangani* (orange) from the Ruvu River and *O. hunteri* from Lake Chala

(green). **d** Morphospace occupation of the introduced *Oreochromis* sp. “blue head” in Lake Chala (blue) and in the Ruvu River (light blue). The colour of the dots represent the different species with *Astatotilapia cf. bloyeti* in red, *O. hunteri* in green, *O. sp.* “blue head” in blue, *C. rendalli* in black, *O. pangani* in orange, *O. jipe* in yellow, *O. niloticus* in purple and *O. esculentus* in brown. The symbols of the dots represent the catching location with triangle for Lake Chala, dots for the Nyumba ya Mungu Reservoir and diamonds for the Ruvu River

species of the genus that contains the world’s most important aquaculture fish. When Lowe (1955) described a small flock of *Oreochromis* species endemic to the upper Pangani River drainage of Tanzania, she was not sure if *O. hunteri* belonged to this species flock. Trewavas (1983) suggested, based on morphological grounds, that *O. hunteri* belonged to the Pangani flock and that it was closely related to *O. jipe*. Trewavas characterized the members of the flock by high numbers of vertebrae, scales in the lateral line and dorsal fin rays and suggested that four species belonged to it: *O. pangani* of rivers Ruvu and Pangani, *O. girigan* of Lake Jipe, *O. jipe* of Lake Jipe and possibly the Ruvu/Pangani River, and *O. hunteri* of Lake Chala. This little flock had not been studied since

and never in a phylogeographic context until recently (Dieleman et al., 2018).

We collected three endemic and three invasive *Oreochromis* species in the upper Pangani drainage including Lake Chala. We find strong support for the proposed monophyly of the upper Pangani flock in the mitochondrial lineage (Fig. 3), confirming the results of Dieleman et al. (2018), who sequenced two mitochondrial markers. Their proximity in morphospace further supports their close relationship (Fig. 6c). Among the three endemic species that we obtained, *O. hunteri* is genetically and phenotypically distinct and indeed restricted to Lake Chala. *O. jipe* appears to be common in the Nyumba ya Mungu Reservoir. This was already reported by Trewavas

**Table 2** Coefficients for the morphological traits in the pairwise LDA

	HUN versus BLU	HUN versus JIP	HUN versus PAN	PAN versus JIP
Head length	<b>22.6</b>	<b>38.1</b>	<b>40.2</b>	<b>24.9</b>
Head width	5.4	<b>17.8</b>	7.9	5.1
Body depth	– <b>10.5</b>	– 9.8	<b>29</b>	– 7.6
Lower jaw length	3	2.4	9.6	1.6
Lower jaw width	1.7	– 1.0	– 2.1	5.5
Snout length	– 3.5	– 9.4	– <b>23.3</b>	– <b>10.3</b>
Snout width	– <b>19.6</b>	– 2.9	– <b>44.1</b>	<b>47.6</b>
Cheek depth	– 3.5	1.6	0.2	3.9
Preorbital depth	5.4	4.7	<b>11.3</b>	0.9
Interorbital width	– 0.6	– 9.6	– <b>11.7</b>	– <b>35.3</b>
Eye length	1.9	– <b>11.8</b>	5.5	– <b>19.2</b>
Eye depth	– 0.1	– <b>14.8</b>	– <b>28</b>	6.4
Preorbital width	– <b>14.1</b>	– <b>11.9</b>	– 0.9	– <b>19.2</b>
Positive LD1	HUN	HUN	HUN	PAN

Coefficients higher than 10 or lower than – 10 are highlighted bold (HUN = *O. hunteri*, BLU = *O. sp.* “blue head”, JIP = *O. jipe*, PAN = *O. pangani*). The last row indicates which species has the more positive LD1

**Table 3** Morphological differentiation between the different sampled *Oreochromis* species in the Nyumba ya Mungu Reservoir and its inflow, the Ruvu River, measured as the Bhattacharyya distance in the morphospace spanned by PC1 and PC2

	<i>Astatotilapia</i>	<i>O. sp.</i> “blue head”	<i>O. jipe</i>	<i>O. pangani</i>	<i>O. esculentus</i>
<i>Astatotilapia</i>					
<i>O. sp.</i> “blue head”	14.37**				
<i>O. jipe</i>	18.96**	2.63**			
<i>O. pangani</i>	16.44**	3.13**	0.59		
<i>O. esculentus</i>	53.94**	16.26**	2.93**	11.11**	
<i>O. niloticus</i>	18.98**	2.11**	1.20**	5.46**	3.54**

Significant differentiation is indicated by asterisks (\*\* $P < 0.01$ )

**Table 4** Differentiation between the different endemic species of the Pangani/Ruvu drainage including Lake Chala in morphology, measured as the Bhattacharyya distance in the morphospace spanned by PC1 and PC2

	<i>O. hunteri</i>	<i>O. jipe</i>
<i>O. hunteri</i>		
<i>O. jipe</i>	0.76**	
<i>O. pangani</i>	0.98*	0.59

Significant differentiation is indicated by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ )

(1983), but it has never been completely clear whether its original range comprised of the Pangani/Ruvu River and Lake Jipe or was confined to Lake Jipe (Lowe, 1955; Trewavas, 1983). Finally, we found *O. pangani* in the Ruvu River, the main inlet to the

reservoir, but we did not find *O. jipe* there. We cannot rule out that some *O. pangani* were among our samples from Nyumba ya Mungu, but all our larger individuals had 4 anal fin spines, a defining trait of this species according to Lowe (1955) and Trewavas (1983), and the typical colouration of *O. jipe*, whereas all our Ruvu River samples had 3 anal spines (Lowe, 1955; Trewavas, 1983).

Beside the introduced *O. niloticus* that we found in the Ruvu River and the reservoir, and *O. esculentus* which we found in the reservoir and that were already reported in the Ruvu/Pangani by Lowe (1955), we found a third most likely non-native oreochromine cichlid species in the system that we refer to as *O. sp.* “blue head”. *O. sp.* “blue head” occurs in two very divergent mitochondrial haplotypes. Both were found in Lake Chala as well as in the Ruvu River. One of

**Table 5** Morphospace occupation after correcting for differences in sample sizes for the three focal species of this study and *P* values for the difference in morphospace occupation between species

	Morphospace occupation	<i>O. hunteri</i>	<i>O. sp.</i> “blue head” (Chala)	<i>O. jipe</i>
<i>O. hunteri</i>	2.19			
<i>O. sp.</i> “blue head” (Chala)	3.23	> 0.20		
<i>O. jipe</i>	2.32	> 0.20	> 0.20	
<i>O. sp.</i> “blue head” (Ruvu)	0.80	< 0.01	< 0.01	< 0.01

these two haplotypes suggests identity with or a close relationship to *O. urolepis urolepis* (Fig. 3). As the second haplotype is divergent by twelve mutations and not closely related to any known *Oreochromis* species, the origin and the evolutionary relationship of *O. sp.* “blue head” remain partly unresolved but it is likely that it emerged from admixture of two divergent lineages in the *O. urolepis* clade.

#### The origin of *Oreochromis hunteri*

*Oreochromis hunteri* appears to have evolved a more limnetic life style when compared to typical *Oreochromis* including its close allopatric relatives *O. jipe* and *O. pangani*, but also the distant relative *O. sp.* “blue head” with which it is now sympatric in Lake Chala. This is apparent both from its body shape and stable isotopic signatures. *O. hunteri* evolved in isolation in the crater lake for at least 19,000 years (Dieleman et al., 2015). Cichlids are known for undergoing sympatric speciation into distinct ecomorphs with different habitat and/or feeding adaptations. This has involved divergence into limnetic and benthic forms in several oreochromine cichlids and divergence into shallow and deep benthic forms in several haplochromine cichlids. There is no evidence of such divergence within the contemporary population of *O. hunteri*. Analyses of fossil teeth from Lake Chala sediment cores and comparison with contemporary teeth revealed that *O. hunteri* and *O. sp.* “blue head” together show higher morphological disparity in tooth shape than the fossil assemblage. However, the fossil assemblage occupies a larger volume in morphospace than the contemporary *O. hunteri* alone, with some fossil teeth overlapping in morphospace with *O. sp.* “blue head” (Dieleman et al., 2015). This might indicate that *O. hunteri* was ecologically more diverse historically than it is now. In particular, more

robust teeth that may facilitate scraping of benthic algae are absent in the contemporary *O. hunteri* but dominate the introduced species. To conclude whether *O. hunteri* has been more diverse in the past or whether there was perhaps a second more littoral native species, more fossil data would be required.

#### The future of *Oreochromis hunteri*

Our data suggest major risks for the persistence of *O. hunteri* arising from the recent introductions of four other cichlid species to Lake Chala. While currently very rare in the lake, and perhaps only present as hybrids, the potential presence of *O. niloticus* is worrying given its invasiveness across large parts of the world and its known impact on native *Oreochromis* elsewhere through genetic introgression coupled with competitive advantages (Angienda et al., 2011; Deines et al., 2014). More immediate impacts on *O. hunteri* are likely to come from the presence of the three other introduced cichlid species, all of which are more abundant in Lake Chala now than is *O. hunteri*. Our data suggest massive habitat overlap with all three species, and dietary overlap mainly with *O. sp.* “blue head”. The latter species is probably able to hybridize with *O. hunteri* (as probably is *O. niloticus*), whereas *C. rendalli* and *A. cf. bloyeti* most likely are too distantly related to *O. hunteri* to hybridize. Our stable isotope data, our and recently published (Dieleman et al., 2018) morphological data and published tooth shape data (Dieleman et al., 2015) all suggest that *O. hunteri* is phenotypically and ecologically distinct from *O. sp.* “blue head”. Although we had a few fish in our data that we could not clearly identify, we did not detect mismatches between phenotype and mitochondrial haplotype in the individuals that we identified with certainty. However, the extent of introgression and



associated threats to the persistence of *O. hunteri* need now to be analysed using microsatellite DNA or next generation sequencing.

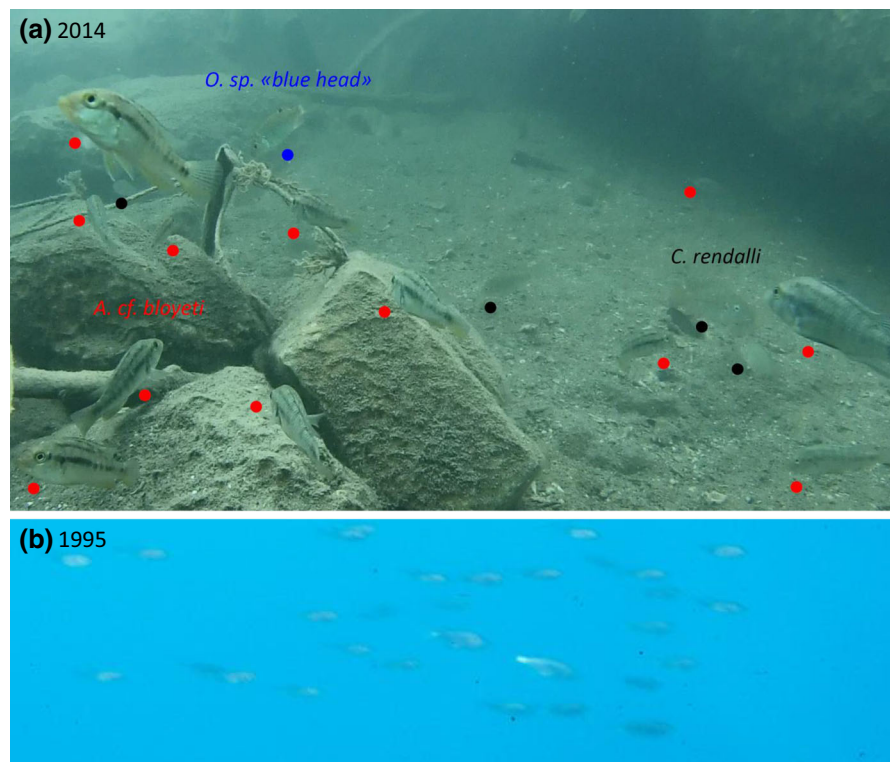
The complementarity in stable isotope signatures between *O. hunteri* and the invasive species in Lake Chala is remarkable. We do not know whether the current feeding and habitat niche of *O. hunteri* is its original range, or whether its niche has shrunk or shifted in response to the arrival of the invasive species. However, several observations point to possible recent change:

First, in 1951 Lowe observed shoals of young feeding on algae and debris from the bottom between the rocks near the shore (Lowe, 1955). We have not seen any *O. hunteri* in the shallow waters despite many diving hours, but we encountered all three invasive species very abundantly. In 1995, one of us (OS) photographed a pelagic shoal of subadult *O. hunteri* several metres above the ground (Fig. 7). We have not seen any such shoals in 2014.

Second, the *Oreochromis* teeth in the fossil assemblage studied by Dieleman et al. (2015) occupied a

larger volume in morphospace than the contemporary *O. hunteri* teeth, with some fossil teeth overlapping in morphospace with the invasive *O. sp. blue head*.

Third, the relative morphospace occupation between the introduced and the native species is much less favourable to the native *Oreochromis hunteri* in Lake Chala than for the native species *O. jipe* in the Nyumba ya Mungu Reservoir. Given that *O. hunteri* had several thousand years of evolution in Lake Chala (Dieleman et al., 2015), and therefore the possibility to adapt to the different niches within the lake without competing with any other fish species, we would have expected *O. hunteri* to be phenotypically more variable than the invasive species. This is indeed what we see in *O. jipe* compared to *O. sp. "blue head"*. However, we see the opposite in Lake Chala where the invasive *O. sp. "blue head"* is phenotypically significantly more diverse than *O. hunteri*. This again might indicate that *O. sp. "blue head"* displaced *O. hunteri* from parts of its morphospace and likely also its ecological niche.



**Fig. 7** Underwater picture of Lake Chala. **a** Picture from 2014 of all three invasive species at 6 m water depth. Note the high density of *Astatotilapia cf. bloyeti* (red) and *Coptodon rendalli* (black) in this habitat. **b** A school of *O. hunteri* swimming in the open water in 1995

## Conclusions

We provide evidence for the presence of three endemic and three invasive *Oreochromis* species in the upper Pangani drainage (Nyumba ya Mungu Reservoir, Ruvu River and Lake Chala). The three native species that we collected in the Pangani/Ruvu and in Lake Chala have retained their phenotypic and mitochondrial distinctiveness despite their current sympatry with several non-native species that arrived since the 1950s in the Ruvu/Pangani and since the 1970s in Lake Chala. However, there is serious concern for the future persistence at least of the endemic *O. hunteri* which is currently the least abundant cichlid species in Lake Chala, phenotypically less diverse than its allopatric sister species and the introduced sympatric species in Lake Chala, and missing from habitats where it was historically recorded. Nuclear genetic marker studies are required to investigate potential introgressive hybridization with the numerically dominant invasive species.

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**Author contributions** FM carried out fieldwork, participated in the design of the study, helped generating stable isotope, morphological and genetic data, analysed the data and wrote the manuscript together with OS. JvR and BN carried out fieldwork. SM helped in the laboratory and generated genetic data. OS designed and coordinated the study, made the sampling design, identified the samples and wrote the manuscript together with FM.

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**Data availability** Mitochondrial sequences are available on GenBank (Accession Numbers MH717378 - MH717444). Phenotypic data are available on Dryad (<https://doi.org/10.5061/dryad.v24tb57>).

## Compliance with ethical standards

**Conflict of interest** We have no competing interests.

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