Josi, Dario and Frommen, Joachim G (2021) Through a glass darkly? Divergent reactions of eight Lake Tanganyika cichlid species towards their mirror image in their natural environment. Ethology, 127 (10). pp. 925-933. ISSN 0179-1613

DOI: https://doi.org/10.1111/eth.13207

Publisher: Wiley

Version: Published Version

Received: 21 January 2021 | Revised: 17 May 2021 | Accepted: 1 June 2021
DOI: 10.1111/eth.13207

ETHOLOGICAL METHODS

ethology WILEY

Through a glass darkly? Divergent reactions of eight Lake Tanganyika cichlid species towards their mirror image in their natural environment

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Funding information
SNF grants 31003A–166470 & 310030_185021 to Joachim G. Frommen; 31003A_156152 to Michael Taborsky

Editor: Barbara Taborsky

Abstract

Behavioural reactions towards a mirror image are frequently used to measure individual aggression in a standardized way, especially in fishes. However, this approach was criticized recently on several grounds. One point of concern is that mirror tests are often conducted under highly artificial laboratory settings, while there exists a lack of knowledge of how individuals will react towards their reflection in the wild. We measured the responses of eight sympatric lamprologine cichlid species towards their mirror images in their natural environment in Lake Tanganyika. All species reacted by showing aggression towards their mirror image. The occurrence of overt and restrained aggression varied between species, reflecting species-specific aggressive behaviours. The finding that larger species showed a higher amount of overt attacks further supports this interpretation. A commonality across all species was that aggression escalated over time, resembling behavioural patterns during prolonged aggressive encounters between life opponents with similar resource holding potential. These results shed light on the behaviour of closely related fish species towards their mirror image under natural conditions and contribute knowledge to the ongoing discussion on the suitability of mirror tests when measuring aggression.

KEYWORDS
aggression, animal personalities, behavioural syndromes, boldness, mirror test, *Neolamprologus pulcher*

1 | INTRODUCTION

The reactions of animals towards their mirror image fascinate scientists since the early days of animal behaviour research (Hediger, 1948; Lissmann, 1932; Tinbergen, 1951). Nowadays, mirrors are either used to test for an animals' ability to recognize itself (see de Waal, 2019 for a list of examples) and hence as a proof of self-awareness (Gallup, 1982; de Waal, 2019) or to simulate the presence of conspecifics and to elicit either social (Cattelan et al., 2017; Milinski, 1987; Svendsen & Armitage, 1973) or aggressive behaviours (e.g. Rowland, 1999; Svendsen, 1974; Thompson, 1964). The latter is based on the assumption that most animal species do not recognize their mirror image and treat it as a foreign animal (Gallup, 1968; de Waal, 2019). If this assumption is justified, mirror tests appear to be highly suitable to measure aggression, as they are easy to apply, minimize stress and the risk of injury for the contestants, and allow reducing the number of used animals (Rowland, 1999; Sloman et al., 2019).

In fishes, using mirrors to measure aggressive behaviour is common (e.g. Lissmann, 1932; Moretz et al., 2007; Rowland, 1999; Scherer et al., 2016). Already Tinbergen (1951) studied the response of male three-spined stickleback (*Gasterosteus aculeatus*) towards their mirror image in his ground-breaking early studies on animal instinct. However, various authors have questioned whether mirror images elicit biological meaningful aggressive behaviours on several grounds (e.g. Desjardins & Fernald, 2010; Elwood et al., 2014; Pitcher, 1979). First, the assumption that fishes generally lack self-recognition and treat the mirror image as opponents has been probed recently, with varying outcome (Ari & D'Agostino, 2016; Hotta et al., 2018; Kohda et al., 2019; Stewart et al., 2017). For example, Kohda et al. (2019) found that after a habituation phase the cleaner wrasse *Labroides dimidiatus* reacted towards its mirror image in a way that can reasonably be interpreted as passing the classical mark test. Passing this test is taken as evidence of self-recognition in birds and mammals, including primates (Gallup, 1982; de Waal, 2019). In contrast, the cooperatively breeding cichlid *Neolamprologus pulcher* failed the mark test (Hotta et al., 2018), indicating that there is considerable between-species variation in mirror self-recognition in fishes. Second, evidence is accumulating that even if fish do not recognize themselves, they still might perceive their mirror image as behaving differently from a live opponent (Desjardins & Fernald, 2010; Pitcher, 1979). The reasons for this might include that aggressive communication depends on coordinated behaviours, which often contain a head-to-tail alignment of the contestants during lateral displays. When fighting a mirror image, such alignment is not possible (Arnott et al., 2011; Elwood et al., 2014). In mangrove rivulus (*Kryptolebias marmoratus*), for example, a sophisticated set-up that allowed animals to see and interact with their mirror image in head-to-tail postures elicited behaviours that corresponded much better with the fishes' performance during real fights than a simple mirror presentation (Li, et al., 2018a). Besides lateral displays, also other interactions of the mirror opponent might not be a naturally occurring response to a given display (e.g. a lack of submission as a response to an overt attack, (Arnold & Taborsky, 2010)). Third, aggressive displays might be composed of multiple signals in different modalities that are only partly based on visual cues (Bayani et al., 2017; Frommen, 2020; Rowland, 1999). In such cases, mirror presentations might miss crucial parts of the communication occurring during aggressive encounters.

Accordingly, several studies showed diverging aggressive responses of fishes tested against mirror images or live opponents (Arnott et al., 2016; Balzarini et al., 2014; Elwood et al., 2014; Li, et al., 2018a; Li, et al., 2018b; Ruzzante, 1992; Serra et al., 2017; Verbeek et al., 2007). Furthermore, even if aggressive behaviours towards a mirror image and a live opponent are comparable, differences in hormonal response or brain activity indicate that contestants discriminate between a conspecific and a mirror image. This has been shown, for example in Mozambique tilapia (*Oreochromis mossambicus*) (Oliveira et al., 2005), Burton's mouthbrooder (*Astatotilapia burtoni*) (Desjardins & Fernald, 2010) and mangrove rivulus (Li, et al., 2018b).

A further drawback of standardized mirror tests in the laboratory might be that behaviours shown under laboratory conditions are of limited explanatory power for behaviours shown under natural conditions. For example, while habituated chimpanzees show mirror self-recognition in the laboratory (Gallup, 1982), such reactions were absent in individuals confronted with a full-length mirror in the wild over a prolonged time period (Anderson et al., 2017). Similar issues might arise when mirrors are used to measure aggression. Here, space-limited experimental set-ups provide only scant opportunities to withdraw from fighting and move away. Hence, aggressive interactions with a mirror image might exaggerate the aggressive responses, especially of inferior individuals that would quickly withdraw from fighting under natural conditions due to their low resource holding potential (RHP). Furthermore, in the laboratory animals might be regularly confronted with reflecting surfaces like glass or metal surfaces (e.g. Reiss & Marino, 2001). Their reactions when confronted with a mirror image might thus not be naïve anymore, and habituation to the stimulus might lead to changes in behaviour towards a mirror over time (Anderson et al., 2017; Kohda et al., 2019; Meliska & Meliska, 1976). These laboratory shortcomings call for careful verification of the explanatory value of mirror tests in a natural context. In the wild, the occasions where animals might be confronted with their mirror image are limited either to reflections in water surfaces (de Waal, 2019), though this is physically restricted to body parts not containing the head region in aquatic organism (Dibble et al., 2017), or to human-made reflecting devices like polished metal or glass surfaces. Hence, most knowledge about how animals react when approaching their mirror image in the wild is derived from a limited number of species (de Waal, 2019). Systematic analyses of how wild animals react to their mirror image and whether these reactions are comparable to those elicited under laboratory conditions are scarce (but see Anderson et al., 2017), especially in fishes.

In the present study, we aimed at filling this gap by confronting eight lamprologine cichlid species from Lake Tanganyika with a mirror in their natural environment. Cichlids are among the fishes showing the most diverse social and brood-care systems (Frommen & Fischer, 2021; Jordan et al., 2021), which are associated with an impressive behavioural repertoire (Barlow, 2000; Kawanabe et al., 1997; Taborsky, 2016). Aggressive encounters between cichlids frequently include visual aggressive displays (e.g. Barlow, 2000; Bayani et al., 2017; Chabrolles et al., 2017; Keeley & Grant, 1993). Accordingly, mirrors are readily used to measure aggressive propensity, even though studies verifying the explanatory potential of mirror images are limited to a few species and gained mixed results. While for some cichlid species aggressive behaviours shown towards a mirror are correlated with aggression shown towards a live conspecific, this relation is absent in others (Balzarini et al., 2014; Desjardins & Fernald, 2010; Scherer et al., 2016). We analysed the behaviour of eight substrate-breeding lamprologine cichlid species (see Table 1). For three of these species, laboratory data on mirror

TABLE 1 Summary of the eight species (scientific names, sample sizes, mean standard length, usual breeding substrate) used in this study, and the number of individuals that showed overt (OA) or restrained aggression (RA) at least once. Furthermore, the table provides an overview and example references of all lamprologine cichlids that were tested for their aggression towards a mirror image thus far. For three of the tested species (plus *L. elongatus*, which was not tested in the current study), mirror tests have already been conducted in the laboratory

		Standard length (cm)			Reacted			
Species	***N***	**mean**	**±**	**SE**	**with OA**	**with RA**	**Breeding substrate**	**Mirror test applied in the laboratory**
Neolamprologus multifasciatus	11	3.3	±	0.2	1/11	11/11	Cave (snails)	no
Telmatochromis vittatus	10	3.8	±	0.3	3/10	10/10	Cave (snails)	yes[a]
Neolamprologus savoryi	13	5.1	±	0.2	9/13	12/13	Cave	no
Neolamprologus pulcher	20	5.8	±	0.1	15/20	19/20	Cave	yes[e.g. a,b,c,d]
Telmatochromis temporalis	10	5.9	±	0.5	8/10	10/10	Cave	no
Julidochromis ornatus	10	6.4	±	0.3	10/10	10/10	Cave	yes[e]
Variabilichromis moorii	10	7.6	±	0.3	6/10	10/10	Open	no
Neolamprologus tetracanthus	10	9.4	±	0.6	9/10	9/10	Cave	no
Lepidiolamprologus elongatus	NA	NA		NA	NA	NA	Open	yes[a]

[a] Balzarini et al. (2014).

[b] Reddon et al. (2012).

[c] Riebli et al. (2011).

[d] Hotta et al. (2018).

[e] Kua et al. (2020).

tests are available, while the other five were not tested with mirrors before (see Table 1 for references). We aimed at answering the following questions: 1. Do lamprologine cichlids react with aggressive behaviour towards their mirror in their natural environment? If so, what kind of aggressive behaviours are exhibited? Based on evidence from the laboratory, we predict that if the mirror image is treated as threat, fish will show both overt attacks and restrained displays, comparable to what would be expected when confronted with a living opponent. 2. Do species differ in their use of overt attacks and restrained displays? Given that members of different lamprologine cichlids differ in their use of attacks and displays when fighting living opponents, we predict species-specific differences in aggressive behaviours. 3. Do body size differences across species predict aggressive behaviours? As the risks of being aggressive may decrease with increasing body size, we predict that larger species show more aggressive behaviours than smaller once. 4. Do aggressive behaviours escalate over time? As the mirror image is of the same size as the focal fish and fights between living opponents of the same RHP often escalate with increasing duration, we predict the same also for our mirror experiments.

2 | METHODS

2.1 | Study site and species

All experiments were carried out by SCUBA Diving at the southern tip of Lake Tanganyika, close to the city of Mpulungu, Republic of Zambia. We tested fishes at three study sites close to the villages of Kasakalawe, Chikonde and Mwina. All study sites feature a sandy bottom with interspersed patches of rubble and partly sand-covered rocks (typically 10–40 cm in size), as well as empty gastropod shells. The depth varied between 7.0 and 11.0 m. We analysed the behaviour of 10–20 haphazardly chosen individuals per species (see Table 1).

2.2 | Experimental design

All tested species are to a certain extent philopatric and defend home ranges of various sizes close to the bottom of the lake (Jungwirth et al., 2021; Kawanabe et al., 1997). These home ranges either include a shelter under a rock or in a snail shell, or a stretch of substrate that is defended against intruding fishes (Groenewoud et al., 2016; Heg & Bachar, 2006; Jordan et al., 2016; Josi et al., 2020; Jungwirth et al., 2021; Zimmermann et al., 2019). At the beginning of each observation, we followed a haphazardly chosen adult individual to determine its home range. In case of the cave-breeding species (see Table 1), we then placed a mirror measuring 19 × 15 cm (L × h) at a distance of 20 cm to the breeding shelter. For the open-breeding species (see Table 1), the mirror was placed at the centre of the home range. If fish reacted towards the mirror image within the next 5 min, we started our observations. If fish left the area instead of showing a reaction, we concluded that we frightened the fish or failed to spot the correct shelter or home range and terminated the trial. This happened in very few cases. Using a measuring stick, we indicated a zone measuring 19 × 10 cm (L × w) in front of the mirror. As soon as the focal fish entered the zone, we started the observation. We chose a 5-min observation period in order to avoid stressing the focal animal over a prolonged period of time, following studies using living, restrained lamprologines in the field (Josi et al., 2020) and laboratory (Zöttl et al., 2013). Comparable periods were also used for studies using computer animations (Balzarini et al., 2017; Fischer et al., 2014) and mirror tests on lamprologines in the laboratory (see Table 1 for references). Aggressive interactions were noted on a waterproof board with a pencil. Following established ethograms for mirror tests in lamprologine cichlids (Balzarini et al., 2014; Kua et al., 2020; Reddon et al., 2012; Riebli et al., 2011), we counted all aggressive interactions directed towards the mirror image, that is overt attacks and restrained displays (see Table 2 for details). For analyses, counts of different behaviours were combined into one overt and one restrained value (Balzarini et al., 2014). We furthermore visually estimated the size of the focal individual (standard length) by comparing it to the measuring stick.

2.3 | Statistical analyses

All data were analysed with R version 3.3.3 (R Core Team, 2017). First, we analysed whether the species differed in their levels of aggression. Overt and restrained aggression were analysed in two separate general linear models (GLM). A negative binomial error distribution (R package MASS (Venables & Ripley, 2002)) was used in both cases as these models had a better fit than models assuming a Poisson distribution. Overt or restrained aggression was set as response variable, and the respective species was set as predictor.

Second, we tested whether species show a different behavioural repertoire when facing a mirror (e.g. whether mainly overt or restrained aggression was shown). We fitted a GLM with a negative binomial error distribution and set the number of overt aggressive behaviours as response variable and the number of restrained aggressive behaviours as well as species as predictors. Additionally, we included the interaction between species and restrained aggression to investigate whether the relation between the amount of restrained aggression and overt aggression differed across species. As this interaction was significant (see Results), we ran GLMs with the same response and predictor variables for each species separately.

Third, we tested whether species' body size relates to the amount of overt attacks and restrained displays. Two generalized linear mixed models (GLMM) were fitted with one of the two aggression values as response variable and body size as predictor. To account for repeated measures of the same species, the latter was included as random effect in the analysis.

Fourth, we analysed whether the aggression changed over experimental time. We fitted overt and restrained aggressions per minute as response variables in two separate GLMMs with a negative binomial error distribution. The experimental minute (1–5) was set as predictor. To account for repeated measures of the same individual over time and for the different species, we included individual ID nested in species as random effects.

TABLE 2 The ethogram describes all overt attacks and restrained displays that were shown by at least some of the tested species

Aggression	Behaviour	Description
Overt	Biting	Biting attempt, touching the mirror, mouth open
	Ramming	Fast approach with physical contact to the mirror, mouth closed
Restrained	Fast approach	Swimming with accelerated speed towards mirror, opercula are spread
	Slow approach	Swimming towards mirror, opercula are spread
	Fin spread	All fins are maximally spread; fish is close to the mirror
	S-bend	Body held stiffly in a bent position along the longitudinal axis
	Opercula spread	Opercula spread when facing the mirror, but not combined with an approach movement
	Head down	Body inclined downwards, up to 60 degrees. Unpaired and pelvic fins are spread
	Head up	Body inclined upwards, up to 60 degrees. Unpaired and pelvic fins are spread
	Lateral display	Fish still for a while, showing lateral view to mirror, with fins spread
	Frontal display	Fish still for a while, showing frontal view to mirror, with fins spread
	Frontal quivering	Whole body quivers fast and repeatedly in front of the mirror
	Touching	Slow approach with physical contact to the mirror, mouth closed

Note: The ethogram was modified from Balzarini et al. (2014).

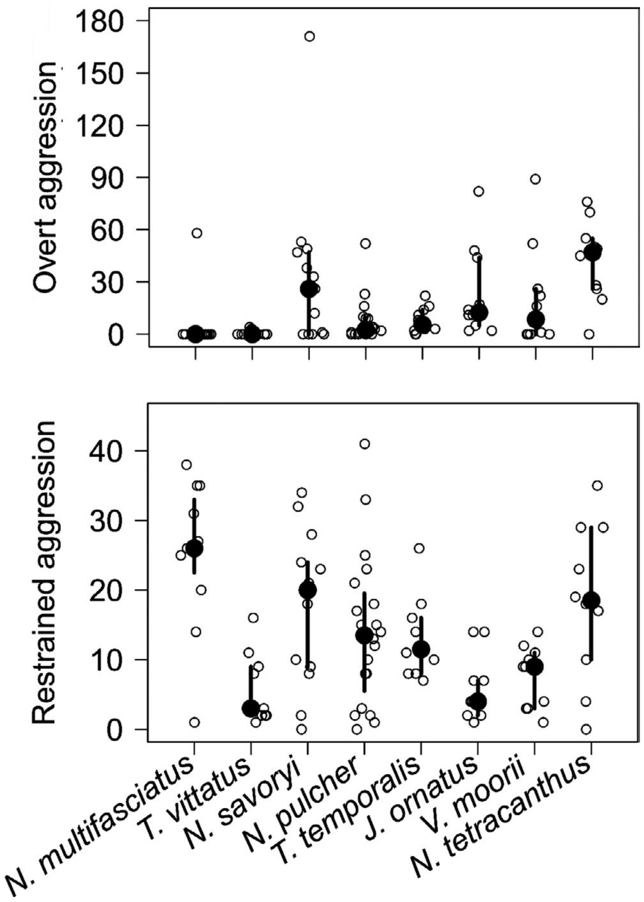

FIGURE 1 The number of (a) overt attacks and (b) restrained displays counted during 5-min observations for the eight different species tested in this study. Solid black dots indicate medians, and lines show the interquartile ranges. White circles depict single data points. Species are sorted by increasing mean body size

3 | RESULTS

The mirror image elicited overt and restrained aggressive behaviours in individuals of all tested species (Table 1). The amount of overt aggressive behaviours differed significantly between species (GLM: Df = 7; χ^2 = 29.5; *p* < .001; Figure 1); the same was true for restrained aggression (GLM; species: Df = 7; χ^2 = 39.66; *p* < .001; Figure 1). The relation between overt and restrained aggression differed across species (restrained aggression * species: Df = 7; χ^2 = 23.48; *p* = .001), indicating that species rely differently on restrained displays or overt attacks. The post hoc analyses of this interaction for each species revealed a significant positive relationship between overt and restrained aggression for *N. pulcher* (intercept: 1.75; β ± SE: 0.09 ± 0.03; Df = 1; χ^2 = 7.59; *p* = .006), and a negative relationship between overt and restrained aggression for *T. temporalis* (intercept: 1.85; β ± SE:−0.15 ± 0.07; Df = 1; χ^2 = 4.43; *p* = .035). There was no significant relation for all other species (all *p* > .25). Overall, larger species attacked the mirror more often, indicated by the positive relation between overt aggression and body size across species (GLMM: intercept: 0.34; β ± SE: 0.37 ± 0.13; Df = 1; χ^2 = 7.06; *p* = .008). There was no relation between restrained aggression against the mirror and species' body size (GLMM: intercept: 2.78; β ± SE:−0.051 ± 0.06; Df = 1; χ^2 = 0.70; *p* = .41). Finally, individuals showed more overt aggression the longer the experiment lasted (GLMM: intercept:−1.58; β ± SE: 0.30 ± 0.06; duration: Df = 1; χ^2 = 27.32; *p* < .001; Figure 2), while the levels of restrained aggression decreased over time (GLMM: intercept: 1.10; β ± SE:−0.15 ± 0.03; duration: Df = 1; χ^2 = 28.53; *p* < .001; Figure 2).

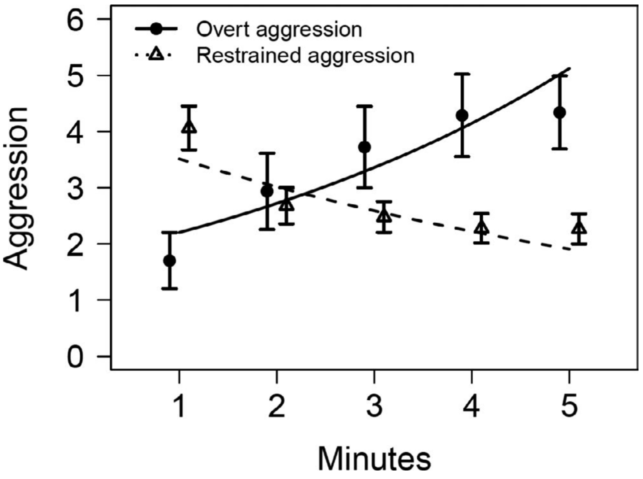

FIGURE 2 The amount of overt and restrained aggression changed over the 5-min lasting experimental trial (see results for statistics). Full circles and empty triangles represent mean values for all species per minute. Bars represent the standard error of the respective mean value. Regression lines were predicted from the respective models

4 | DISCUSSION

Verifying the use of mirrors under natural conditions is crucial to understand whether mirror images can be a useful tool to study aggressive behaviour in animals. In the present study, members of all tested species reacted towards their mirror image by showing overt and / or restrained aggression (see Table 1). Only very few individuals permanently withdrew from interacting with the mirror during the 5-min interval (D. Josi and J. Frommen, pers. obs), while most took up interactions again even when leaving the zone in front of the mirror temporarily. Taken together, these findings indicate that the mirror is not perceived as overly artificial but elicits behaviours comparable to what is shown towards mirror images in the laboratory. It will be an important aspect of future studies to determine whether mirror image stimulation also elicits behaviour that accurately reflects aggressive interactions between living conspecifics, both at the behavioural and neurobiological/hormonal levels (Balzarini et al., 2014; Li, et al., 2018b; Scherer et al., 2016).

The amount of overt attacks and restrained displays differed between species. Furthermore, different species relied to various extends on attacks and displays. This finding might reflect species-dependent aggressive strategies. For example, in the highly social *N. pulcher*, attacks and displays were correlated with each other, indicating that some individuals were generally more aggressive than others. Here, individuals that readily started a physical contest also displayed their RHP at a higher rate. Comparable findings are described from studies using computer-animated opponents in the laboratory, where individuals that showed higher levels of overt attacks communicated their aggressive propensity by an increase in aggressive displays (Balzarini et al., 2017). In contrast, in the pair breeding *T. temporalis* individuals showed either a high amount of attacks or displays, indicating that some individuals readily join in physical aggression, while others rely on prolonged periods of visual assessment. One reason for such species-specific aggressive behaviours might be differential vulnerability to predation due to different sizes. Increased predation risk is a cost of extensive aggressive interactions (Jakobsson et al., 1995), where fighting individuals may show only limited attention for potential threats (Hess et al., 2016; Satoh et al., 2021). The most common predators of lamprologines are predatory cichlids and mastacembalid eels (Groenewoud et al., 2016; Tanaka et al., 2016). As for many other fishes, the size of prey animals these predators can swallow is limited due to the size of their mouth (Freudiger et al., 2021). Hence, members of smaller species are expected to be at higher risk when involved in escalating fights, which is reflected in our finding that overt aggressive interactions became more intense with increasing mean size of the respective species. However, this effect can be influenced by other ecological factors, like the size of the species' home range. For example, the comparatively large *Variabilichromis moorii* defend large territories measuring up to 4 m^2 (Sturmbauer et al., 2008), while the territory of the small *Neolamprologus multifasciatus* might not exceed 100 cm^2 (Jordan et al., 2016). Hence, the comparatively low amount of mirror aggression in *V. moorii* might rather reflect the need to patrol continuously a large home range than an overall non-aggressive strategy, while *N. multifasciatus* seldom left the reach of the mirror at all. These findings highlight the importance of keeping in mind the specific ecology of the tested species when interpreting aggressive behaviours shown towards a mirror (see also Baran & Streelman, 2020).

Aggressive interactions became more intense over time in all species, with decreasing amounts of restrained displays and increasing numbers of overt attacks (see Figure 2). These findings are comparable to what is shown when two living contestants with comparable RHP meet (e.g. Bruintjes & Taborsky, 2008; Hsu et al., 2008) and might indicate that mirror tests elicit behaviours that can be interpreted in a biological meaningful way, at least for contestants with comparable RHP. This assumption can be further elucidated by comparing our field findings to a recent laboratory study on the explanatory value of mirror tests in two of the tested lamprologine cichlids (Balzarini et al., 2014). This comparison reveals that when presented with a mirror *N. pulcher*, a species that relies heavily on frontal visual displays (Balzarini et al., 2017), shows more restrained displays than overt attacks both under laboratory and field conditions. Importantly, in the laboratory, restrained displays and overt attacks shown towards a mirror image and live opponent behind a glass partition were correlated, making mirror tests a suitable method to simulate aggressive intrusions (Balzarini et al., 2014). This picture becomes more complicated in *Telmatochromis vittatus*, a species relying to a much larger extent on anti-parallel lateral displays (Balzarini et al., 2014). Here, low levels of overt aggression towards the mirror shown in the field correspond well with overt aggression shown towards live opponents in the laboratory (Balzarini et al., 2014). However, in the laboratory, overt attacks towards the mirror image were more common. The amount of restrained displays, in contrast, was comparable between mirror tests in the laboratory and in the wild, but was lower than, and not correlated with, the number of displays shown towards a live opponent. Finally, in a sophisticated laboratory study on the impact of water warming on aggression in *Julidochromis ornatus* (Kua et al., 2020), individuals showed more restrained displays against the mirror than overt attacks, while the opposite was the case in the present field study. Interestingly, overt attacks and restrained displays were correlated in the laboratory, while this correlation was absent in wild fish. Taken together, these findings further add to the argument that a mirror test conducted in the laboratory might not necessarily reflect aggressive behaviour shown towards live conspecifics or under natural conditions in all species, calling for the need of independent method validation for each species of interest (Chouinard-Thuly et al., 2017; Rowland, 1999).

Summarizing, our study shows that under natural conditions different lamprologine cichlids react towards their mirror image with biologically meaningful behaviours. In combination with recent phylogenomic advancements (Ronco et al., 2021), mirror presentations thus have the potential to elucidate the evolution of contest behaviour and especially the relationship between social system, body size and the use of overt attacks and restrained displays, while controlling for shared ancestry. Still, the respective species' ecology, aggressive strategies and behavioural repertoire might impair the specific validity of mirror tests, calling for careful method verification for each species of interest before drawing strong conclusions (cf. Taborsky et al., 2019).

ACKNOWLEDGEMENTS

This article is dedicated to Michael Taborsky, on his retirement as full professor in Behavioural Ecology at the University of Bern. We strongly benefitted from his support, his deep insights into the evolution of cooperative behaviour and his passion for Lake Tanganyika and its cichlids. We thank Taylor Banda, Lawrence Makasa, Harris Phiri and Danny Sinyinza from the Department of Fisheries, Ministry of Agriculture & Livestock, Republik of Zambia, for their permission and logistical support of our work. We are grateful to the late Hirokazu Tanaka for support and invaluable discussions in the field. We thank Celestine and the late Augustin Mwewa and their team for their management and care at the Tanganyika Science Lodge. Ryan Earley, Barbara Taborsky and an anonymous referee provided thoughtful comments on the first draft of our manuscript. Open Access Funding provided by Universitat Bern.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

DJ and JGF conceived the study and planned the experiments; JGF organized funding; DJ and JGF collected the data; DJ analysed the data; DJ and JGF wrote the manuscript.

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How to cite this article: Josi, D., & Frommen, J. G. (2021). Through a glass darkly? Divergent reactions of eight Lake Tanganyika cichlid species towards their mirror image in their natural environment. *Ethology*, 127, 925–933. https://doi.org/10.1111/eth.13207