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## Article

# Computer animations of color markings reveal the function of visual threat signals in *Neolamprologus pulcher*

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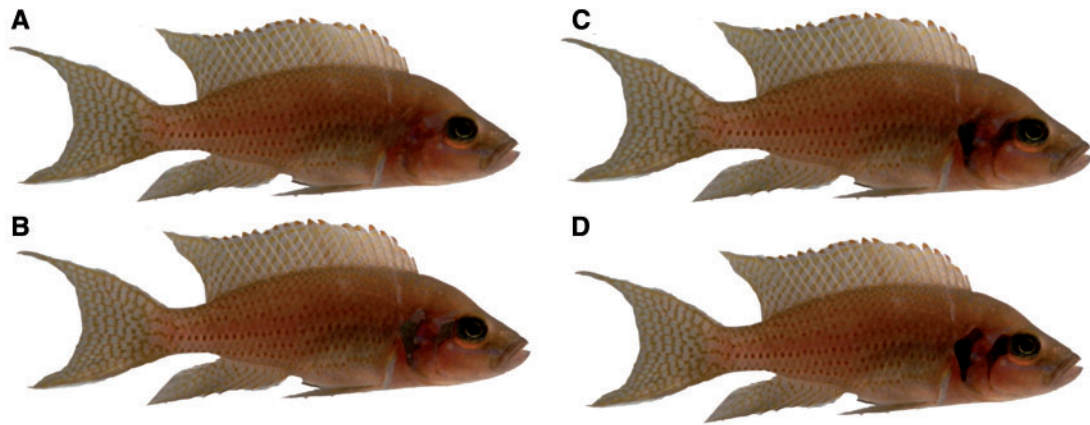
## Abstract

Visual signals, including changes in coloration and color patterns, are frequently used by animals to convey information. During contests, body coloration and its changes can be used to assess an opponent's state or motivation. Communication of aggressive propensity is particularly important in group-living animals with a stable dominance hierarchy, as the outcome of aggressive interactions determines the social rank of group members. *Neolamprologus pulcher* is a cooperatively breeding cichlid showing frequent within-group aggression. Both sexes exhibit two vertical black stripes on the operculum that vary naturally in shape and darkness. During frontal threat displays these patterns are actively exposed to the opponent, suggesting a signaling function. To investigate the role of operculum stripes during contests we manipulated their darkness in computer animated pictures of the fish. We recorded the responses in behavior and stripe darkness of test subjects to which these animated pictures were presented. Individuals with initially darker stripes were more aggressive against the animations and showed more operculum threat displays. Operculum stripes of test subjects became darker after exposure to an animation exhibiting a pale operculum than after exposure to a dark operculum animation, highlighting the role of the darkness of this color pattern in opponent assessment. We conclude that (i) the black stripes on the operculum of *N. pulcher* are a reliable signal of aggression and dominance, (ii) these markings play an important role in opponent assessment, and (iii) 2D computer animations are well suited to elicit biologically meaningful short-term aggressive responses in this widely used model system of social evolution.

**Key words:** aggression, agonistic behavior, animated pictures, cichlid fish, communication, cooperation, social, threat display.

Color patterns can be important for species recognition (Jiggins et al. 2001; Detto et al. 2006; Pauers et al. 2008), and for signaling individual quality and status, for example in a reproductive context (Houde 1987; Bakker and Milinski 1993; Seehausen and van Alphen 1998; Boughman 2001). During agonistic encounters, color patterns are typically used to convey threat (Dawkins and Guilford

1993). Aggressive interactions are often costly, due to increased injury risk and energy expenditure (Maynard Smith 1982; Neat et al. 1998, Kemp and Wiklund 2001). To avoid unnecessary costs of conflict escalation, a variety of signals and matching response rules have evolved to enable assessment of the opponents' aggressive state (O'Connor et al. 1999; Gerald 2001; Lehtonen et al. 2015). Animals



**Figure 1.** Examples of animation images. Each stimulus picture was manipulated in 4 different steps: (A) no stripes, (B) pale stripes, (C) mean stripes, and (D) dark stripes. For better illustration of the differences, the overall brightness of the image presented here was modified to be 3% brighter than how it was used in the experiment.

often use color signals to convey their fighting ability or motivation, which can reduce the probability of costly escalation (Tibbetts 2008; Van Dyk and Evans 2008). These badges of status typically convey information about an individual's social and aggressive status (Maynard Smith and Harper 2003). They are assumed to be honest signals, as the receiver response would incur high costs to the sender if cheating was detected (Tibbetts 2008). Badges of status can change in accordance with status changes, although usually at a slower rate than state-dependent signals (Laucht and Dale 2012; Rhodes and Schlupp 2012). Badges of status are used for the mutual assessment of contestants together with other signals of fighting ability, most of which can change rapidly during contests in dependence of individual state (Arnott and Elwood 2009; Mäthger et al. 2009; Ligon and McGraw 2013).

Color patterns used in aggressive contexts frequently involve the presence or modulation of melanin-based pigments. For example, darker maned lions *Panthera leo* are more aggressive and sexually more active (West 2002), and darker tawny owls *Strix aluco* show a higher level of aggressiveness (Da Silva et al. 2013). Furthermore, vertical stripe patterns are often associated with intraspecific aggression in various species (e.g., Bortolotti et al. 2006; Price et al. 2008). In fish, quickly adjustable color patterns are frequently used in aggressive contests, where they may signal either dominance or submission (Barlow 1963; De Boer 1980; Dawkins and Guilford 1993; Beeching 1995; O'Connor et al. 1999).

Here, we report on an experimental study of the importance of color markings in the cooperatively breeding cichlid fish *Neolamprologus pulcher*. Groups of these fish consist of one dominant breeding pair and up to 25 immature and mature, related and unrelated helpers of both sexes (Groenewoud et al. 2016; Taborsky 2016). Group members often show aggressive behavior against each other (Reddon et al. 2011; Riebli et al. 2011, 2012). The hierarchy in *N. pulcher* groups is strongly size dependent (Balshine et al. 2001; Heg et al. 2005; Stiver et al. 2008), with some additional influence of the sex of group members (Mitchell et al. 2009). The highest level of competition occurs among similar-sized individuals (Wong and Balshine 2011; Ligoeki et al. 2015b). In this situation, size-independent signals of fighting propensity may be beneficial to avoid costly escalation of fights (Arnott and Elwood 2009; Ligon 2014). The majority of *N. pulcher*'s aggressive encounters are settled by displaying of aggressive intent, which does not require physical contact (restrained aggression; Taborsky 1984; Schürch and Heg 2010).

*Neolamprologus pulcher* exhibits two black stripes on the operculum (Figure 1), which differ in size and shape between populations along the coast of Lake Tanganyika (Duftner et al. 2007). This operculum pattern is present already at the juvenile stage in both sexes. It is involved in the recognition of familiar conspecifics (Kohda et al. 2015) and might function as a signal during aggressive interactions (Duftner et al. 2007). *Neolamprologus pulcher* frequently uses frontal displays during which they widely spread their opercula (Taborsky 1984; Balzarini et al. 2014). This behavior increases the visibility of the operculum stripes for the opponent, facilitating its assessment. It is therefore likely that the operculum stripes convey information about the aggressive state of an individual, or about their rank or fighting ability (Duftner et al. 2007).

When studying the function of variable color signals, it is difficult to disentangle the effect each participant of an interaction has on the other. To control for the opponent's visual cues and behavior, they can be standardized by using either mirror images (Earley et al. 2000; Balzarini et al. 2014), video playbacks (Ord et al. 2002; Verbeek et al. 2007), or animated computer models of conspecifics (Mehlis et al. 2008; Thünken et al. 2013; Qin et al. 2014). Computer animated models are particularly helpful when studying visual signals, as they can be easily modified according to the respective research question (Baldauf et al. 2008; Woo and Rieucau 2011; Fischer et al. 2014; Chouinard-Thuly et al. 2017). While three-dimensional (3D) animations can be programmed to express complex series of behavior (Künzler and Bakker 1998; Rosenthal 2000; Gierszewski et al. 2017), 2D animations can be used for simpler phenotypic alterations. For instance, stationary pictures of animals can be modified with help of editing programs and then animated in front of a background (Baldauf et al. 2009; Woo and Rieucau 2011; Fischer et al. 2014). Individuals of different fish species have been shown to discriminate between pictures of different heterospecifics or generic shapes moving in a computer animation (Baldauf et al. 2009; Gerlai et al. 2010). For example, *N. pulcher* differs in behavior when confronted with moving fish images of different cichlid species, as well as with moving fish images and a moving rectangle (Fischer et al. 2014). Computer animations have been used to study agonistic behavior in a range of fish species, for example, fighting fish *Betta splendens* (Allen and Nicoletto 1997; Verbeek et al. 2007), three-spined sticklebacks *Gasterosteus aculeatus* (Mehlis et al. 2009), and *N. pulcher* (Fischer et al. 2014). These studies demonstrate the potential of animations and photo editing to

study the function of visual color signals in a diverse range of species.

In the present study we aimed at elucidating whether the darkness of the black operculum stripes of *N. pulcher* functions as signal of aggression and dominance using standardized 2D computer animations of virtual intruders (Fischer et al. 2014). The pictures were obtained from photographs of conspecifics, in which we modified the darkness of the operculum stripes in four steps (dark, mean, pale, and no stripes). The hypothesis assuming that the operculum stripes are used to signal aggressive propensity predicts a positive correlation between the darkness of stripes and the amount of aggression displayed by an individual. In terms of opponent assessment, we expected a modifying effect of the experimentally varied operculum patterns presented by our animations on the aggression of focal test fish. Furthermore, we expected the stripe darkness of focal test fish to change according to the darkness level of stripes of the animated opponents.

## Materials and Methods

Experiments were conducted in August 2013. The individuals used in this study were  $F_1$  and  $F_2$  generation laboratory-reared offspring of wild fish, caught at the Zambian coast of Lake Tanganyika close to Kasakalawe point. They were kept in aggregations of 10–30 individuals in tanks of either 50 L, 200 L, or 400 L, depending on numbers of individuals. The water temperature was kept constant at  $27 \pm 1^\circ\text{C}$  and the light regime was set to a light:dark cycle of 13:11 h. Tanks were filtered and aerated with internal biological filters, and the bottom of each tank was covered with a 2-cm sand layer. The tanks did not contain breeding shelters to prevent fish from reproducing. Surface shelters were provided to minimize potential negative effects of aggression in the tank. All fish were fed with commercial flake food (5 days/week) and with defrosted food (1 day/week).

### Computer animations

For preparing the animations, 10 fish (5 males and 5 females) between 40 and 45 mm standard length (SL) were haphazardly caught from the holding tanks. To avoid effects of familiarity impairing our results (Kohda et al. 2015), fish used to create the animations were taken from different tanks than the test subjects. Lateral pictures were taken in a small glass box with an Olympus SZ-11 camera (Olympus Lens 4.5–90.0 mm 1:3.0–6.9). Fish in the pictures had a neutral posture, with fins halfway between completely spread (an aggressive display) and totally relaxed. The photo box was illuminated with a 60 W 230 V light bulb. A Munsell color reference card with standard white color was placed in each picture. All darkness measurements were calculated in relation to this white standard, to minimize variation caused by random illumination differences (Frommen et al. 2008). The lateral pictures of the 10 fish were processed in the CIE Lab color space (Hasting and Rubin 2012) with the software “Adobe Photoshop CC”. We chose this color space because it transforms the image’s color data into device-independent data, thus preventing camera-specific biases compromising the analyses (Hill et al. 1997; Sharma et al. 2005). In the CIE Lab color space the “*L*” axis indicates the darkness (black = 0 to white = 100); the “*a*” axis ranges from green to red (negative values indicate green, positive red) and the “*b*” axis from blue to yellow (negative values indicate blue, positive yellow; Stevens and Cuthill 2005; Hasting and Rubin 2012). As our focus was on the darkness of the facial stripes, we restrict our analysis to the “*L*” value. The *L*-value

of the bars was calculated as a percentage value of the white reference to correct for illumination differences.

From each original picture we obtained 4 manipulated copies, 3 with stripes of different darkness and 1 without stripes (see Figure 1 for examples). The pictures with stripes had *L*-values of  $45 \pm 3$  (pale stripes),  $25 \pm 5$  (mean stripes), and  $5 \pm 1$  (dark stripes), respectively. For the picture with no stripes, the operculum color was adjusted to be of the same color as the surrounding head area. The values for the “mean stripes” images were obtained by averaging the *L*-values of the stripes of the 10 original pictures. After manipulation, the fish in the picture was cut out from the background using “Adobe Photoshop CC”. The cutouts were pasted into a 2D animation using “Power Point 2010”, with the same olive-green background as used in previous studies (Fischer et al. 2014; Hess et al. 2016). The size of the cutout was standardized to 41 mm SL, which was the mean size of the fish used for the animation images. The size of the animation fish was kept constant, independently of the test fish size, to control for distortion of general image characteristics, other than the operculum stripes. An HP windows computer and a COMPAQ 1520 screen ( $32 \times 23 \text{ cm}^2$ ) were used to display the animations to the test fish. The same screen model was successfully used by Fischer et al. (2014) and Hess et al. (2016). During the animation, the fish image entered the screen head first from one side and moved in a straight line at a speed of 1.3 s/cm until exiting the frame on the opposing side. Afterwards, the same movement was repeated in the opposite direction. In this way, the animated fish moved back and forth on the screen for 10 min.

### Behavioral tests

A total of 28 individuals (14 males and 14 females) between 40 and 45 mm SL were haphazardly caught from the holding tanks. After capture, a lateral picture of their head was taken under the same conditions and with the same material as for the animation pictures, to get a baseline reference for their operculum stripe darkness. This baseline measure was important because *N. pulcher* tend to darken slightly during handling. After taking the baseline picture, the fish were measured, and their sex was determined by checking their genital papilla. Then, each fish was placed into a 25 L ( $l \times w \times h$ :  $40 \times 25 \times 25 \text{ cm}$ ) experimental tank. The experiment was conducted in 6 tanks in parallel, following the procedure described in Fischer et al. (2014). The bottom of each experimental tank was covered with sand and the water was aerated with an air stone. A flowerpot half served as shelter. The tanks were visually isolated from each other using opaque partitions. Test subjects were allowed 15 h of acclimation time in the experimental tank before the first test started (Fischer et al. 2014). For each test, we placed the screen outside the tank on the short wall opposite the shelter, and let the fish acclimate to an empty background for 10 min. After this, the animation started and continued for 10 min. During this time, we continuously recorded the number of aggressive behaviors. Overt aggression refers to behaviors aimed at physical contact with the opponent, that is, biting and ramming attempts directed toward the animation (Balzarini et al. 2014; Fischer et al. 2014). Restrained aggression comprised aggressive displays, that is, fin spread, operculum spread, and fast approach (Schürch and Heg 2010; Balzarini et al. 2014; Fischer et al. 2014). All observations were recorded using the “Observer5.0” software (Noldus).

Each test subject was confronted once with each of the four animation types (“pale” stripes, “mean” stripes, “dark” stripes, “no” stripes) in a random order, resulting in 112 trials in total. To exclude that variation between stimulus fish would influence the test

subjects' behavior, all animations shown to an individual test subject used manipulated images of the same stimulus fish picture. These 4 animation presentations were spread over 2 consecutive days. On each day, 1 animation was shown in the morning and 1 in the afternoon, with a break of 4 hours in between. Half of the subjects were randomly assigned to receiving same-sex animation pictures, the other half to animation images from opposite sex fish. The 5 female animation sets (1 set equals 4 modified images of the same stimulus fish) were used in 7 male and 7 female trials, the same was the case for the 5 male animation sets. Thus, each animation set was used between 2 and 4 times in total. Immediately after each trial, we took a picture of the test fish's operculum stripes, to record potential changes in darkness depending on the kind of animation it had received. The photographs were taken in the same way as those for the baseline and the animation pictures. After the picture was taken, the fish was returned to the experimental tank.

### Statistical analysis

For all statistical analyses we used the software "R", version 3.1.2 (R Core Team 2013). Mixed models were run with the package "lme4" (Bates et al. 2015). Model selection was done by stepwise removal of non-significant terms and interactions (Engqvist 2005). All  $P$ -values were obtained with likelihood ratio tests, which follows a  $\chi^2$  distribution (for all mixed models), or  $F$ -tests (for the parametric linear model (LM); Lu and Zhang 2010). A likelihood ratio test is needed for model simplification when the factors used in the model have more than 2 levels, as it gives an overall  $P$ -value for the factor's effect by comparing the full model to one missing the specified factor (Dalggaard 2008; Zuur et al. 2009). This step is required as  $P$ -values from the lme4 package outcome do not reflect all possible data comparisons (Crawley 2007; Zuur et al. 2009). When needed, we ran Tukey post-hoc tests on the models with the package "multcomp" (Hothorn et al. 2008), and the resulting  $P$ -values were corrected for multiple testing. If Poisson-distributed models were over-dispersed (Bolker et al. 2009), we included an additional individual-based random effect (Elston et al. 2001).

### Color analyses

Darkness of the operculum stripes was calculated as the average  $L$ -value of the 2 stripes' area, with high values indicating lighter stripes and low values darker ones. Baseline values were normally distributed, so we used parametric statistics. We analyzed differences in baseline darkness among test fish with a LM, with sex and size of the test fish as explanatory variables. The interaction between the test fish's sex and size was also included.  $P$ -values were obtained with an  $F$ -test.

To analyze color change in response to the animations, we created an " $L$  difference" variable by subtracting the baseline  $L$ -values from the  $L$ -values after the animation. On this variable, we fitted a linear mixed model (LMM). The animation darkness level, the sex of the test fish and that of the animation fish were set as fixed factors. Size of the test fish and order of testing (the order in which the animations were presented within a set) were inserted as covariates. Order of testing was not treated as a factor because we were not interested in the differences between subsequent trials, but in the overall time effect. The interaction between the sex of the animation and of the test fish was also included. The identity of the test fish and of the animation fish were set as random factors and never excluded from the model. The use of post-animation to pre-animation differences and the inclusion of the test fish's identity as

random effect in the model account for different individual responses to handling. Because the residuals of the model on " $L$  difference" values were not perfectly normally distributed according to  $Q-Q$  plots and the dependent variable could not be transformed, we confirmed the robustness of the  $P$ -values obtained from the model with permutation tests with 10,000 repetitions. The permutation resulted in an adjusted  $\alpha$ -level of 0.0403. As animation type had a significant effect on darkness change, and because we were interested in the differences between animations, we ran Tukey post-hoc tests on animation type to discern the differences between animations. Finally, as the interaction between sex of the animation and of the test fish was significant, we analyzed the results for each sex separately. For each sex, we ran 1 LMM on the difference in  $L$ -values before and after animation, with sex of the animation as fixed factor, and identity of the test fish and of the animation fish as random factors.

### Behavioral observation

Counts of aggressive behavior were combined into "overt aggression" and "restrained aggression". Of the latter, operculum spread was analyzed separately, as we hypothesized this behavior being the aggressive display most related to the stripe darkness, resulting in the variables "operculum spread" and "restrained aggression without operculum spread". We will refer to the second one as "restrained aggression" for brevity.

Generalized linear mixed effect models (GLMMs) assuming Poisson distribution were conducted to analyze overt aggression, operculum spreads, and restrained aggression. The animation type, sex of the test fish and of the animation fish were set as fixed factors. The  $L$ -value of each subject's baseline pictures, the size of the test fish, and trial order were inserted as covariates. We considered also the interactions between the sex of the animation and the sex of the test fish, and between animation type and baseline  $L$ -value. For each model, the identities of the test fish and of the animation fish were set as random factors. No post-hoc tests were required.

## Results

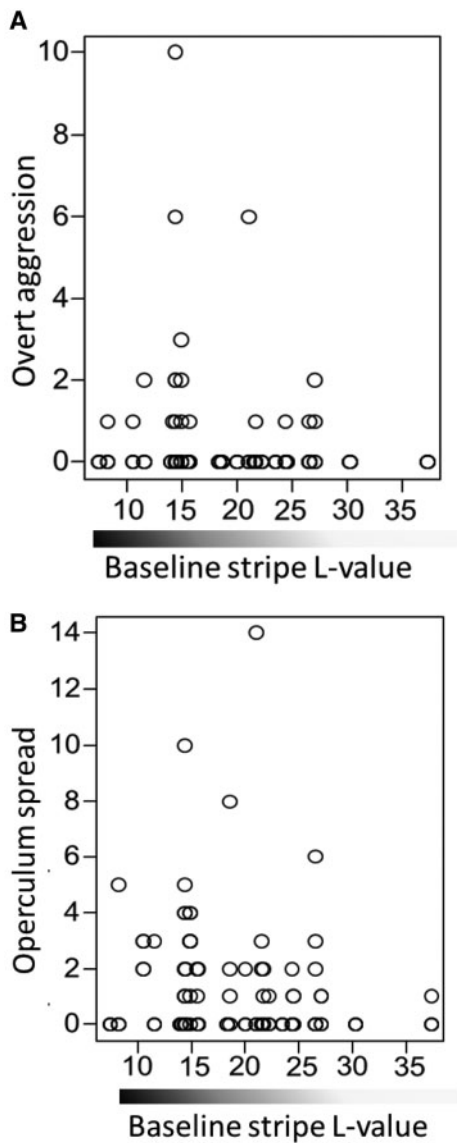
### Behavioral observations

Subjects with darker baseline operculum stripes showed significantly more overt aggression and more operculum spreads (overt aggression, GLMM,  $\chi^2 = 6.840$ ,  $P = 0.009$ ; operculum spreads, GLMM,  $\chi^2 = 4.488$ ,  $P = 0.034$ , Figure 2, Table 1) than individuals with lighter baseline stripes. There was no significant difference in restrained aggressive behavior (GLMM,  $\chi^2 = 0.061$ ,  $P = 0.805$ , Table 1). Subjects did not differ significantly in aggression shown toward the different animation types (overt aggression, GLMM,  $\chi^2 = 1.032$ ,  $P = 0.793$ ; operculum spreads, GLMM,  $\chi^2 = 0.153$ ,  $P = 0.985$ ; restrained aggression, GLMM,  $\chi^2 = 1.943$ ,  $P = 0.584$ , Table 1).

The size of focal subjects was significantly related to aggression, with smaller subjects attacking the presented animation more often (overt aggression, GLMM,  $\chi^2 = 10.058$ ,  $P = 0.002$ ), and showing more operculum spreads (GLMM,  $\chi^2 = 6.463$ ,  $P = 0.011$ ). However, they did not differ from bigger fish in restrained aggression (GLMM,  $\chi^2 = 0.516$ ,  $P = 0.473$ , Table 1).

There was a positive, non-significant trend for females to engage in more overt aggression against the animated display than males (GLMM,  $\chi^2 = 3.237$ ,  $P = 0.072$ , Table 1), but not in operculum spreads or other restrained aggressive behaviors (GLMM,  $P > 0.37$ , Table 1). Overall, restrained aggression showed a significant order





**Figure 2.** Baseline stripe darkness and aggression. The frequencies of (A) overt aggression and (B) operculum spreads and their relationship with stripe darkness (*L*-values; mind that the stripe darkness is *decreasing* from left to right).

**Table 1.** Behavioral responses to the animations

	Overt aggression		Operculum spreads		Restrained aggression (excluding Op. spreads)	
	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>
Baseline stripe darkness	6.840	<b>0.009</b>	4.488	<b>0.034</b>	0.061	0.805
Test fish size	10.058	<b>0.002</b>	6.463	<b>0.011</b>	0.516	0.473
Test fish sex	3.237	<u>0.072</u>	0.026	0.871	0.530	0.467
Animation fish sex	1.515	0.218	0.129	0.720	0.268	0.605
Order of testing	2.796	<u>0.095</u>	2.373	0.123	9.939	<b>0.002</b>
Animation type	1.032	0.793	0.153	0.985	1.943	0.584
Animation type × baseline stripe darkness	4.209	0.240	1.351	0.717	2.510	0.474
Animation fish sex × test fish sex	0.146	0.703	0.448	0.503	1.790	0.181
Marginal $R^2_{\text{GLMM}}$	0.397		0.207		0.184	
Conditional $R^2_{\text{GLMM}}$	0.604		0.409		0.182	

*Notes:* Results of GLMMs for overt aggression, operculum spreads, and restrained aggression excluding operculum spreads. *P*-values were obtained with likelihood ratio tests. Significant *P*-values are in bold, non-significant trends ( $0.05 < P < 0.1$ ) are underlined. Marginal and conditional  $R^2$  values were calculated for each full model, following Nakagawa and Schielzeth (2013).

effect, with subjects being more aggressive in the first test than in the others (GLMM,  $\chi^2=9.939$ ,  $P=0.002$ ). This effect was not significant in overt aggression (GLMM,  $\chi^2=2.796$ ,  $P=0.095$ , Table 1) and operculum spreads (GLMM,  $\chi^2=2.373$ ,  $P=0.123$ , Table 1). The other covariates did not have a significant effect on the subjects' behavior (all  $P > 0.12$ , Table 1).

### Stripe darkness

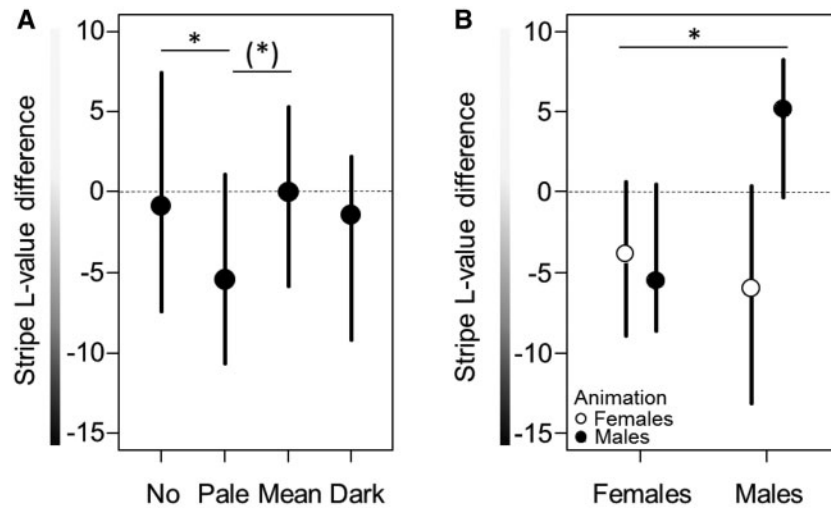
The darkness of operculum stripes in the presented animation caused a significant response in the darkness of the test subjects' stripes (LMM,  $\chi^2=8.44$ ,  $P=0.038$ , Figure 3A and Table 2 part a), which was apparently due to the fish showing darker operculum color after viewing the "pale" animation (Tukey post-hoc, no stripes vs. pale stripes,  $Z=-2.634$ ,  $P=0.042$ ; pale stripes vs. mean stripes,  $Z=2.316$ ,  $P=0.094$ ; Table 2 part b). Furthermore, there was a significant interaction between the sex of the test fish and that of the animation fish (LMM,  $\chi^2=6.12$ ,  $P=0.013$ , Figure 3B and Table 2). While the animation's sex did not elicit different responses in stripe coloration of females (LMM,  $\chi^2=0.294$ ,  $P=0.588$ , Figure 3B), males became darker when shown a female animation and paler when shown a male animation (LMM,  $\chi^2=9.161$ ,  $P=0.003$ ). The other explanatory variables in the model did not have a significant effect on stripe darkness, before or after the animation (all  $P > 0.17$ , Table 2).

We found a positive, though non-significant trend for males to exhibit a darker stripe baseline than females (LM,  $F=3.501$ ,  $P=0.073$ , Figure 4, Table 3). The other explanatory variables in the model did not have a significant effect on baseline stripe darkness (all  $P > 0.2$ , Table 3).

### Discussion

Our results show that the darkness of the operculum stripes of *N. pulcher* reflects an individual's aggressiveness. In particular, stripe darkness was strongly related to overt aggressive behaviors and the amount of operculum displays shown against the animated opponent. Furthermore, test fish responded to the stripe darkness of animated stimulus fish by changing their own operculum stripe darkness rather than by measurable changes in their behavior.

The relationship between operculum color and aggressive propensity showed up in the most intense form of aggression, ramming



**Figure 3.** Changes in stripe darkness depending on animation type and sex. (A) Changes in stripe darkness significantly differed depending on type of animation (see results part for statistics of the full model). Animation types were “no stripes” (No), “pale stripes” (Pale), “mean stripes” (Mean), and “dark stripes” (Dark). Asterisks indicate results of the Tukey post-hoc tests. (B) Changes in stripe darkness were dependent on sex of the test fish and of the animation fish. White dots represent animations of female stimulus images; black dots represent animations of male stimulus images. The Y-axis represents changes in stripe darkness difference (mind that the stripe darkness is *decreasing* from bottom to top). Significant differences are visualized with \*, while non-significant trends ( $0.05 < P < 0.1$ ) are indicated with (\*).

**Table 2.** Change in stripe darkness after exposure to an animation

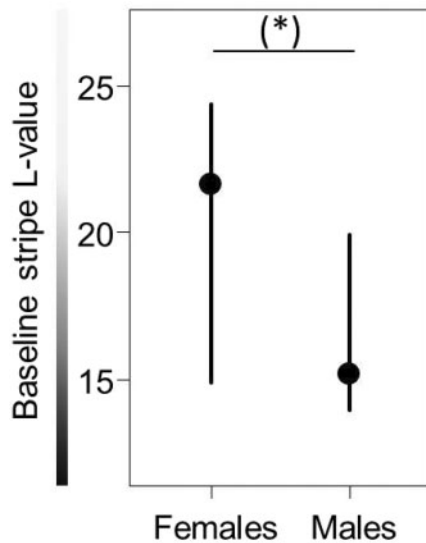
(a)		
LMM model	$\chi^2$	<i>P</i>
Test fish size	0.053	0.819
Test fish sex	4.25	0.039
Animation fish sex	1.62	0.203
Order of testing	1.83	0.176
Animation type	8.44	0.038
Animation sex $\times$ test fish sex	6.12	0.013
Marginal $R^2_{\text{GLMM}}$	0.074	
Conditional $R^2_{\text{GLMM}}$	0.52	
(b)		
Tukey post-hoc test on animation type	<i>Z</i>	<i>P</i>
No stripes vs. pale stripes	-2.634	<b>0.042</b>
No stripes vs. mean stripes	-0.344	0.986
No stripes vs. dark stripes	-1.316	0.552
Pale stripes vs. mean stripes	2.316	<u>0.094</u>
Pale stripes vs. dark stripes	1.332	0.543
Mean stripes vs. dark stripes	-0.985	0.758

*Notes:* (a) The results of the LMM on the change in stripe darkness after the respective trial. *P*-values were obtained with a likelihood ratio tests. Marginal and conditional  $R^2$  values were calculated for each full model, following Nakagawa and Schielzeth (2013). (b) The results of the Tukey post-hoc test run on animation type. Animation types are “no stripes”, “pale stripes”, “mean stripes”, “dark stripes”. Significant *P*-values are depicted in bold, non-significant trends ( $0.05 < P < 0.1$ ) are underlined.

and biting, which involves body contact, and in the frontal display involving the spreading of the opercula. During this behavior the stripes are pointedly exposed to the opponent. This aggressive behavior is energy demanding (Taborsky and Grantner 1998). Its correlation with stripe darkness indicates that the latter may serve as an honest signal of aggressive propensity, and thus as a badge of status (Grafen 1990; Price et al. 2008).

Furthermore, the exposure to a simulated opponent caused a stripe darkening in the test fish. This effect was most pronounced

when an opponent with “pale” stripes was presented. Apparently, the test subjects were able to distinguish between animated stimuli that differed only in the darkness of their stripes. The observation that opponents with pale operculum stripes caused a more intense darkening of the test subjects’ stripes than opponents with darker markings suggests that pale individuals were perceived as representing less of a threat or as being of lower social status. Showing darker operculum stripes to a less aggressive or subordinate individual could convey own dominance to the opponent, which might lead to competitive success without need for contest escalation. Conflicts between opponents that differ greatly in resource holding potential might be resolved by displays mainly based on color signaling. Comparable effects have been described in chameleons *Chamaeleo calyptratus*, where darkening skin coloration signals losing and causes a decrease in aggression received (Ligon 2014). In accordance to the hypothesized signal use during fights, our test fish did not differ in their aggressive behavior exhibited toward the different animations. Our experimental design aimed at disentangling the color pattern of stimulus fish from their behavior, to assess the response of test subjects only to the color variation of their rivals. Accordingly, the presented opponents did not behave coherently with the information contained in their color signal. As the presented stimulus fish did not respond to the test subject’s behavior, the adjustment of own color signaling might be an appropriate response to the perceived differences in aggressive propensity of opponents as expressed by their operculum darkness. In accordance with this interpretation, there was an order effect in aggressive behavior, with fish attacking more strongly the first animation of the series presented to them, than the following ones. *Neolamprologus pulcher* recognizes individual conspecifics by visual stimuli (Hert 1985; Balshine-Earn and Lotem 1998; Kohda et al. 2015). As each set of 4 animations presented to a test subject in our experiment had been created from the same original image, the test fish apparently habituated to this perceived opponent, probably because the latter was not responding to their aggressive behavior.



**Figure 4.** Sex differences in baseline stripe darkness. Baseline stripe darkness of male and female focal individuals before presentation of the animations. The Y-axis represents baseline stripe darkness (L-values; mind that the stripe darkness is *decreasing* from bottom to top). A non-significant trend ( $0.05 < P > 0.1$ ) is indicated with (\*).

At the beginning of our experiment, males tended to have darker stripes than females. This might suggest that males are more aggressive than females. However, our results suggest the opposite, with females tending to engage more in overt aggressive interactions than males. Further evidence on sex differences in aggression of *N. pulcher* is, thus far, ambiguous. Within groups containing helpers of both sexes, there were no sex effects in the aggression of dominants against subordinates (Taborsky 1985), but in groups with helpers of only one sex, dominants tended to be more aggressive toward same-sex subordinates (Mitchell et al. 2009). No sex differences were identified when analyzing the personality axis of aggression (Schürch and Heg 2010) as well as territory defense behavior under natural conditions (Groenewoud et al. 2016). Desjardins et al. (2006) found that females were more aggressive than males toward conspecific intruders, while no difference was described by Zöttl et al. (2013), and Witsenburg et al. (2010) observed the opposite effect. These divergent results point toward a strong context dependence regarding the occurrence of sex-differences in *N. pulcher* aggression. Another explanation for differences in baseline stripe darkness between sexes could be a difference in response to stress, as the stress response is known to be connected with the sexual hormone regulation axis (Handa et al. 1994; Tilbrook et al. 2000). However, hormonal studies on *N. pulcher* have so far highlighted differences in glucocorticoid levels mainly between dominant and subordinate individuals (Ligocki et al. 2015a), rather than between sexes. Similar differences between dominants and subordinates in stress hormone levels have been found also in cooperatively breeding birds and mammals, where they also appear to be independent of sex (Creel 2001). Nonetheless, we corrected for potential effects of handling stress on the response of operculum color to the experiment by analyzing the difference in darkness before and after the experimental trials.

It is interesting to note that in female test subjects, the operculum stripes became darker independently of the animation's sex, while in males the sex of the animation influenced changes in darkness. While males confronted with a female animation got darker stripes,

**Table 3.** Baseline stripe darkness

	<i>F</i>	<i>P</i>
Test fish size	1.400	0.248
Test fish sex	3.501	<u>0.073</u>
Test fish size × test fish sex	0.669	<u>0.421</u>
Adjusted $R^2_{LM}$	0.118	

*Notes:* Results of the LM on baseline stripe darkness. *P*-values were obtained with an *F*-test. A non significant trend ( $0.05 < P > 0.1$ ) is underlined. The adjusted  $R^2$  value for the full model is reported.

they became paler when confronted with a male animation. Thus, *N. pulcher* appears to be able to differentiate between sexes based on animated 2D pictures alone. The visual cues used to differentiate between the sexes remain elusive, as we manipulated the darkness of the stripes in a standardized way, not differentiating between male and female stimuli. However, stripe darkness appears to indicate an individual's aggressive propensity, which is connected to its resource holding potential (see Briffa et al. 2015). Thus, in a mating context stripe darkness could signal individual quality, a topic which should be investigated in future studies. Overall, the results show that the operculum color pattern of *N. pulcher* carries information that could be used in both competitive and mating contexts. This is in accordance with knowledge about other melanin-based signals (e.g., Barlow 1963; West 2002).

Smaller individuals showed more overt aggression and operculum spreading than larger ones, independently of their stripe darkness. Prima facie this seems surprising, as larger individuals are usually more engaged in aggressive interactions and territory defense (Taborsky and Limberger 1981; Brintjes and Taborsky 2008; Heg and Taborsky 2010; Groenewoud et al. 2016). However, field experiments revealed context dependent defense behavior of differently sized subordinates (Brintjes and Taborsky 2011; Groenewoud et al. 2016), and differential responses to intruders in dependence of relative size and rank (Ligocki et al. 2015b; Groenewoud et al. 2016), and of resource value (O'Connor et al. 2015). In our study, smaller subjects (overall range 40–45 mm) were of similar size as the stimulus fish image (41 mm). In the size-based hierarchy of *N. pulcher* groups, competition is highest between similar sized individuals, and fights between similar sized individuals are more intense (Brintjes and Taborsky 2008; Wong and Balshine 2011).

A general problem of computer animated stimuli is that the used visual devices are tailored to the human visual spectrum (Baldauf et al. 2008, 2009; Chouinard-Tuly et al. 2017). However, several fish species are tetrachromatic, being able to see in the ultraviolet (Carleton et al. 2000; Rick et al. 2006; Baldauf et al. 2008; Nava et al. 2011) or infrared range (Meuthen et al. 2012). Ambon damselfish *Pomacentrus amboinensis* for example use UV facial patterns to discern conspecifics from closely related species living in the same habitat (Siebeck et al. 2010). It remains to be investigated whether *N. pulcher* is able to perceive signals beyond the human sensitivity range and if so, in which context this ability would be used. However, in the present study we aimed to clarify the function of the darkness of operculum stripes, which could hence be studied without detailed knowledge of the color perception of this species.

In conclusion, our results reveal a clear relationship between operculum stripe darkness and aggressiveness in *N. pulcher*. Individuals with darker stripes showed more overt aggression and operculum displays. Thus, operculum stripes function as honest



signal of aggressive propensity and badge of status. Accordingly, focal fish's stripe darkness changed in dependence of the stimulus' experimentally manipulated operculum stripe darkness. Finally, our study confirms that *N. pulcher* reacts predictably to 2D computer animations, and that these fish are able to discriminate between sexes and simulated differences in aggressive state using slightly altered images.

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