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Animated images as a tool to study visual communication: a case study in a cooperatively breeding cichlid

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Abstract

Investigating the role of visual information in animal communication often involves the experimental presentation of live stimuli, mirrors, dummies, still images, video recordings or computer animations. In recent years computer animations have received increased attention, as this technology allows the presentation of moving stimuli that exhibit a fully standardized behaviour. However, whether simple animated 2D-still images of conspecific and heterospecific stimulus animals can elicit detailed behavioural responses in test animals is unclear thus far. In this study we validate a simple method to generate animated still images using PowerPoint presentations as an experimental tool. We studied context-specific behaviour directed towards conspecifics and heterospecifics, using the cooperatively breeding cichlid *Neolamprologus pulcher* as model species. *N. pulcher* did not only differentiate between images of conspecifics, predators and herbivorous fish, but they also showed adequate behavioural responses towards the respective stimulus images as well as towards stimulus individuals of different sizes. Our results indicate that even simple animated still images, which can be produced with minimal technical effort at very low costs, can be used to study detailed behavioural responses towards social and predatory challenges. Thus, this technique opens up intriguing possibilities to manipulate single or multiple visual features of the presented animals by simple digital image-editing and to study their relative importance to the observing fish. We hope to encourage further studies to use animated images as a powerful research tool in behavioural and evolutionary studies.

Keywords

video animations, behavioural rules, *Neolamprologus pulcher*, predator recognition, body size, species recognition, computer animations, fish.

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1. Introduction

Visual signals are among the most important cues used in communication within and between animal species (Darwin, 1871; Ladich et al., 2006; Baldauf et al., 2008). Many studies have successfully used live animals as stimuli to investigate the role of visual information in communication (Baerends et al., 1955; Fischer & Frommen, 2013; for a review, see Hailman, 1977 and citations therein). Still, the use of live stimuli bears a number of disadvantages as, for example, it limits the possibility to systematically manipulate specific visual features (Rowland, 1979). Additionally, presentations of live stimuli may be highly variable due to inter- and intra-individual differences in behaviour, such as activity or position of the stimulus animals (Shashar et al., 2005; Campbell et al., 2009). Moreover, using live stimuli increases the total number of animals used in an experiment, thus countering the ethical framework of the '3Rs' (Replacement, Reduction, Refinement) (Russel & Burch, 1959).

Behavioural ecologists have therefore used a variety of techniques replacing live individuals to examine the role of visual signals, including the use of mirrors (Lissmann, 1932; Tinbergen, 1951; Balzarini et al., 2014), dummies (Tinbergen & Perdeck, 1950; Rowland, 1979), still images (Sheehan & Tibbetts, 2011) and video recordings (Balshine-Earn & Lotem, 1998). In recent years, especially computer animations have received increasing attention (Künzler & Bakker, 1998; Baldauf et al., 2008, 2009b; Mehlis et al., 2008; Ioannou et al., 2012; Veen et al., 2013). Computer animations are a valuable tool when manipulating visual traits as potentially confounding factors can be kept constant (Shashar et al., 2005; Baldauf et al., 2008; Woo & Rieucau, 2011; Veen et al., 2013). Compared to still images, animated images can resolve habituation and attention problems of the test animals (Woo & Rieucau, 2011). Moreover, compared to live animals, computer animations exhibit relevant signals continuously, which can lead to more intense behavioural reactions by test individuals (Woo & Rieucau, 2011). Possible disadvantages of animated images include missing interactions between stimulus and test animals, lacking size references and the fact that the output of the video screen is tuned to the human visual system (Shashar et al., 2005; Baldauf et al., 2008). This implies the necessity to validate computer animations as an experimental tool to study behavioural responses of test animals (Baldauf et al., 2008).

Computer animations have been successfully used in several behavioural studies in fish, showing that this technique is generally useful to elicit meaningful behavioural responses in this group of vertebrates (Zbinden et al., 2004; Shashar et al., 2005; Baldauf et al., 2008, 2009b; Woo & Rieucau, 2011). In the past decades, cichlids have become a model taxon in evolutionary and behavioural research (Barlow, 2000; Seehausen, 2006; Wong & Balshine, 2011). So far, studies showing that cichlids express adequate responses towards simple animated images of conspecifics have focused mainly on mate choice preferences (Baldauf et al., 2009a, b, 2010, 2011, 2013; Thünken et al., 2013). In these studies the amount of time spent in close proximity to the corresponding stimulus image was recorded, while the actual behavioural responses of test animals were ignored. In zebrafish (*Danio rerio*), simple animated images have been shown to elicit differential fear responses towards predators and harmless heterospecifics (Ahmed et al., 2012). However, whether such simple animated images can also elicit more detailed behavioural responses (i.e., fine-tuned aggressive and attention behaviours) towards different species and differently-sized specimens has not yet been investigated. This would, for example, enable direct comparisons between responses of test individuals towards conspecifics and heterospecifics of different quality.

Thus far, most studies have used sophisticated computer animations, which either require time consuming computer programming (Zbinden et al., 2004; Baldauf et al., 2008) or precise knowledge of morphometric landmarks of the study species (Künzler & Bakker, 1998; Baldauf et al., 2008; Veen et al., 2013). Simple animated 2D-images have been used more rarely (Baldauf et al., 2009a, b, 2010, 2011, 2013; Thünken et al., 2013) despite their high potential to create fast and cheap animated stimuli using broadly available software packages such as GIMP© or Microsoft PowerPoint©. These programmes allow the animation of the respective image by user-defined animation paths. More sophisticated software of image-editing can be applied if, for example, specific visual cues such as colouration are to be manipulated. This provides researchers with a great variety of possibilities to manipulate visual information without the possession of refined programming skills or the knowledge of morphometric landmarks.

In the present study we aim to establish animated images as a tool to study context-specific behaviours of the cooperatively breeding cichlid *Neolam-*

prologus pulcher. *N. pulcher* is a model organism to study the evolution and mechanisms of cooperative breeding (Taborsky, 1985; Wong & Balshine, 2011). Social groups of these fish consist of one breeder pair and 1 to 25 related and unrelated helpers (Taborsky & Limberger, 1981). Helpers participate in territory defence, territory maintenance and alloparental brood care (Taborsky & Limberger, 1981; Taborsky, 1984; Wong & Balshine, 2011). Group members defend their territory against intruders as well as ovivorous and piscivorous predators (Taborsky & Limberger, 1981). Adequate responses towards species-specific levels of threat are a major force in shaping the evolution of the complex behavioural repertoire of *N. pulcher* (Taborsky & Oliveira, 2012), which has been studied intensively, both under natural and semi-natural conditions and in targeted experiments (Taborsky, 1982; Bergmüller & Taborsky, 2005; Desjardins et al., 2006, 2008; Bruintjes & Taborsky, 2011; Zöttl et al., 2013). A number of these previous experimental studies have put considerable effort into standardizing presentations of live stimulus fish (e.g., Bergmüller & Taborsky, 2005; Desjardins et al., 2008; Zöttl et al., 2013).

In the present study we developed and validated a test design in order to investigate detailed behavioural responses of *N. pulcher* towards 2D-animation sequences of differently sized conspecifics and heterospecifics, which vary in their degree of threat towards the test animal. We aimed to answer the following questions: (1) Do individuals of *N. pulcher* differentiate between a moving artificial object and a moving image of a conspecific or a heterospecific individual? (2) Do they differentiate between a moving image of a conspecific and that of a predator? (3) Do they use the relative size of the presented individual to distinguish between a conspecific and a predator? (4) Do they differentiate between moving images of harmless herbivores and dangerous predators when these are presented in different sizes? We measured aggression and attention behaviours as well as the distance maintained from the screen. As previous results from an experiment involving the presentation of live stimulus fish showed that *N. pulcher* directed more aggression towards herbivores than towards predators (E.O., S.F., B.T., unpubl. results, see also Zöttl et al., 2013), we predicted that images of less dangerous stimuli will provoke more aggression and attention behaviours in the test fish. Furthermore we expected test fish to keep a greater distance from the screen if more dangerous stimuli are presented.

2. Methods

2.1. Study species

All species used in this study are cichlids endemic to Lake Tanganyika (Konings, 1998). *N. pulcher* inhabits sandy to rocky habitats along the shoreline from 3 to 45 m depth (Taborsky, 1984; Duftner et al., 2007). The experimental *N. pulcher* and all specimens used to produce the animations were derived from laboratory breeding stocks kept at the Institute of Ecology and Evolution, University of Bern, under standardized housing conditions (see Arnold & Taborsky, 2010). As predator stimulus we used *Lepidiolamprologus elongatus*, a piscivorous cichlid (Hori et al., 1983) which is the main predator of *N. pulcher* (Heg et al., 2004). As a harmless stimulus species we used *Opthalmotilapia ventralis*, a herbivorous cichlid, which feeds on plankton or grazes the bio-cover of rocks (Hori et al., 1983; Konings, 1998) and which poses no threat to *N. pulcher*. Both stimulus species occur in sympatry with *N. pulcher* in several populations along the shores of southern Lake Tanganyika (Karino, 1998; Ochi & Yanagisawa, 1998). Our experiments were conducted at the ‘Ethologische Station Hasli’, Institute of Ecology and Evolution, University of Bern, Switzerland, in March 2012 and June 2012 under the licences 16/09 and 52/12 of the Veterinäramt Bern, Switzerland.

2.2. Production of animations

To create the 2D-animation sequences we took images from one *N. pulcher*, six *L. elongatus* and six *O. ventralis* individuals. Each fish was transferred to a clear plastic box where it could be laterally aligned to the front screen using a glass plate and photographed under standardized light conditions. The lighting was centred above the clear plastic box. For the images used in Experiments 1 and 2, we used a 30 W neon lamp, and for the images used in Experiment 3 we used a 20 W LED SunStrip daylight lamp. After images were taken all stimulus fish were placed back into their respective home tanks in the laboratory stock. The images were transferred to a computer and the shape of each fish was cut out using Picasa 3, Photoshop CS5 and GIMP (GNU Image Manipulation Program, v. 2.6.12) and pasted onto a white background in Experiment 1 and 2. For Experiment 3 we used a greenish background to imitate natural water conditions. Thereafter the images were transferred to Microsoft PowerPoint and animated to enter the computer screen from the right side, leaving the screen on the left side, re-entering the monitor on the left and leaving on the right, where it re-entered again. The stimulus image took 30 s (= 1 cm/s) to move from one side of the monitor to the other (following Baldauf et al., 2009b). Fish images always entered the screen with the head first. The entire presentation lasted for 3 min in Experiments 1 and 2, and 5 min in Experiment 3. The sizes of the presented images of the stimulus fish were within the natural size range of adult and subadult individuals of each species (Konings, 1998).

2.3. General experimental set-up

Each experimental tank (40 × 25 × 25 cm) was equipped with a 2 cm layer of sand, a flower pot half as shelter and an air stone for oxygen supply. One day before the experiment started the test fish were haphazardly caught from the laboratory stock. We measured their standard lengths (SL; from the tip of the snout to the posterior end of the vertebral column) to the nearest millimetre using a 1 mm grid and a binocular microscope. For Experiments 1 and 2 the sex of the test fish was noted. Thereafter the test fish were transferred to the experimental tank, where they were allowed to acclimatize to the new environment overnight. On the next day a flat screen monitor (Compaq 1520, with a 38.1 cm diagonal screen size and 1024 × 768 pixels resolution) was placed randomly on the right or left side next to the experimental tank. Thereafter the observer sat motionless for a period of 5 min in front of the tank allowing the test fish to acclimatize to the screen and the observer. Then the animation (see below) and behavioural recordings started. During the entire presentation we counted all aggressive behaviours, classified according to an established ethogram of *N. pulcher* (Taborsky, 1982; Hamilton et al., 2005; Balzarini et al., 2014). Aggressive behaviours included fin spreading (raising of dorsal and pectoral fins), head down displays (approaching the opponent with the head pointing towards the substrate), lateral displays (body arranged in a lateral position towards the animations with raised fins), frontal displays (body arranged in a frontal position towards the animations with raised fins), fast approaches (fast swimming towards the video animation without any contact of the aquarium glass) and overt attacks (approaching the video animation with contact of the aquarium glass). For the statistical analyses, these counts were combined to a single aggression variable (see Reddon et al., 2012). We also counted how often the test fish was facing the animation, which always included a change in body position (termed 'facing toward'), and the times the test fish was following the pathway of the animation without fins raised (termed 'following'). These behaviours were combined to a single variable termed ‘attention’. Furthermore we recorded the number of fright behaviours (freezing and fleeing from the screen). As fright behaviours made up only 6% of the total observed behaviours and occurred too infrequently to be statistically analysed as a distinct behavioural category, we omitted this category from our further analyses.

To investigate the anxiety level of test fish faced with different animations we recorded the distances of the test fish to the screen. In Experiments 1 and 2, we divided the test tank in 8 equally sized zones of 5 cm width, reaching from the tank bottom to the water surface. To distinguish the zones we drew vertical lines on the front glass of each experimental tank. Zones were numbered from 1 to 8, with zone 1 being closest to the screen and the shelter with its opening directed towards the screen being located in zones 4 and 5. Every 30 s we recorded the zone the test fish was in and calculated a preference index (IP) as:

$$\mathrm{IP} = \frac{(\sum_{i=1}^{8} \text{counts in zone } i)}{180}$$

with $i = \{1, \ldots, 8\}$ being the number of the respective zone (following Frommen et al., 2009). Thus larger values of IP indicate that a fish was farther away from the animation (see sketch of experimental set-up in Figure A1 in the Appendix in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x). In Experiment 3, the test aquarium was divided into three 13 cm wide zones with zone 1 being closest to the screen. The shelter was placed in zone 3, that is, the zone furthest away from the screen, with its shelter opening facing towards the observer (see Figure A2 in the Appendix). In this experiment we recorded the time test fish spent in the three zones. As in this experiment all test fish spent most of their time in the central zone of the experimental tanks, a weighted mean of all zones would not have been sufficiently sensitive to detect differences in the anxiety level of test fish. Therefore, as a measure of anxiety, we calculated the percentage of time each test fish spent in the zone closest to the screen.

After the experiments test fish were transferred back to their respective home tanks in the laboratory stock. In each experiment, test fish were exposed once to all treatments resulting in within-subject designs. Different test fish were used for the three experiments.

2.4. Experiment 1

Each of 28 test fish (SL: 3.3–4.3 cm, 14 males and 14 females) was exposed to four different displays in randomized order. In two successive displays the test fish were presented with moving images of fish: either an image of the predator *L. elongatus* (‘predator’) or that of a conspecific (‘conspecific’) was shown. To assure that possible responses were due to the recognition of a fish image, test fish were furthermore presented with a moving object control (‘object’). Here, a moving rectangle of similar colour and size as a *N. pulcher* was presented. Finally, to rule out that fish were responding towards the monitor itself we presented just the empty white background (‘white screen’). All images were sized to a standard length of 5.5 cm.

2.5. Experiment 2

To investigate how the size of the animated images influences test fish behaviour 28 test fish (SL: 3.3–3.8 cm, 16 males and 12 females) were randomly exposed to a large (SL = 4.3 cm) or a small (SL = 2.8 cm) *N. pulcher* and a large (SL = 16.7 cm) or a small (SL = 3.8 cm) *L. elongatus*. The rationale for the choice of these body sizes was to obtain different levels of perceived predation threat. The small presented *L. elongatus* would be unable to prey on fish in the size class of our test fish, whereas the large *L. elongatus* would be easily able to do so under natural conditions (Konings, 1998; Hellig et al., 2010). To rule out individual differences between the differently sized predator and conspecific we used a single image of the same individual and changed its size to create the small and the large stimulus.

2.6. Experiment 3

To investigate if *N. pulcher* differentiate between a harmless herbivore and a predator, 25 test fish (SL: 2.1–4.0 cm) were exposed to small (SL = 5.6 cm), medium (SL = 9.0 cm) and large (SL = 12.0 cm) *O. ventralis* and *L. elongatus*, respectively. Images of stones (5 cm in height and 6 cm in width) shown on the background near the bottom served as size reference relative to the predator (Zbinden et al., 2004; Baldauf et al., 2008). As the analysis of Experiment 1 revealed that test fish showed more attention behaviours towards the white background than to a moving object (see Results), we changed the background to a greenish colour, which also mimics best the natural water colouration of Lake Tanganyika (S.F., pers. obs.). To test whether fish reacted differently towards images presented on a green background we compared the per minute aggression towards an image of a predator presented in front of a white background (taken from Experiment 1) and a similar sized predator presented on a greenish background (taken from Experiment 3). Test fish directed the same amount of aggression towards the predator presented on a white background and to the predator presented on a greenish background (for details Figure A3 in the Appendix). We randomly selected images of the six available *L. elongatus* and *O. ventralis* individuals and presented them in all three size classes in randomized sequences. Consequently, test fish were exposed to individuals in all three size classes. For screenshots of displays used in the three experiments, see Figures A4–A6 in the Appendix.

2.7. *Statistical analysis*

For statistical analyses we used R 2.14.1 (R Core Development Team, 2012) with the package lme4 (Bates et al., 2011). To analyse the aggressive and attention behaviours we used generalized linear mixed models (GLMM) with loglink function to account for a Poisson error structure. The individual observations of each test fish were not independent, as each test fish was exposed to several stimulus images. To account for this within-subject design individual identity of test fish was included as a random factor in all models. For the analysis of Experiment 1 the four treatments (white screen, object, conspecific and predator) and the sex of the test fish were included as fixed effects and the SL of the test fish as a covariate. We did three orthogonal comparisons (a maximum of three independent orthogonal comparisons are possible for a four-level factor; Crawley, 2007) by setting the contrasts of the model to compare (i) white screen against (object & conspecific & predator), (ii) (white screen & object) against (conspecific & predator) and (iii) conspecific against predator (Crawley, 2007). For Experiments 2 and 3 the type of stimulus fish (conspecific, herbivore, predator), and size of the stimulus image (small, medium, large) were incorporated as fixed effects and size of test fish as a covariate. In Experiment 2, the sex of test fish was used as a further fixed effect. If models were over-dispersed (Bolker et al., 2009) an individual-based random effect was included (Elston et al., 2001). Full models for the analyses of Experiments 2 and 3 included the interactions between the size of the stimulus fish and the type of stimulus fish. To simplify all models we used stepwise backward elimination of non-significant interaction terms (Engqvist, 2005; Bolker et al., 2009).

To analyse the anxiety levels of test fish we compared the preference indices (IP) using a linear mixed model (LMM) with the four treatments (white screen, object, conspecific, predator) as fixed factor in Experiment 1 and stimulus fish (conspecific, predator) and size of stimulus fish (large, small) as fixed effects in Experiment 2. For the analysis of Experiment 1 the model contrasts were set to conduct orthogonal comparisons in the same way as described for the analysis of behaviours (Crawley, 2007). In both analyses sex was incorporated as a fixed effect and size of test fish as a covariate.

To analyse the anxiety level in Experiment 3 we calculated the percentage of time spent in zone 1, close to the monitor. We applied a folded root transformation (Williamson & Gaston, 1999) and used stimulus fish (herbivore, predator) and size class of stimulus fish (small, medium, large) as fixed effects in a LMM. Size of the test fish was included as a covariate. Full models for the analysis of Experiments 2 and 3 included the interactions between the type of stimulus fish and the size of the stimulus fish. To simplify the models we used stepwise backward elimination of non-significant interaction terms (Engqvist, 2005; Bolker et al., 2009).

Residuals and Q/Q-plots of all LMM models were visually inspected and the distributions of residuals were compared to a normal distribution using Kolmogorov–Smirnov and Shapiro tests. To obtain p-values we conducted a Markov Chain Monte Carlo (MCMC) sampling procedure in the library languageR (Baayen, 2008). Given probabilities are two-tailed throughout. The behavioural recordings for Experiments 1 and 2 were conducted by more than one person. To account for possible observer effects, the identity of the observer was included as a random effect in these models. Random effects were never removed. For exact R-equations see Appendix B in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

3. Results

3.1. Experiment 1

The test fish directed fewer attention behaviours towards animated images of a fish or a rectangle than to an empty background (Figure 1a; Table 1, attention behaviour). In contrast, aggressive behaviour and distance kept from the screen did not differ between the animated images and the empty background (Figure 1b, c; Table 1, aggression behaviour and distance to screen). When comparing the response towards an artificial display and an image of fish, the test fish showed more attention and aggressive behaviours towards

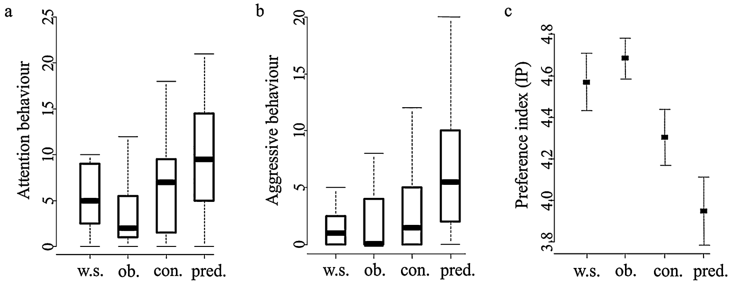

Figure 1. Comparison of (a) attention, (b) aggressive behaviour and (c) presence in the different zones between the four treatments in Experiment 1. w.s., white screen; ob., object image; con., moving conspecific image (= *N. pulcher*); pred., moving predator image (= *L. elongatus*). Boxplots of medians, quartiles and whiskers (1.5 × interquartile range) are shown in (a) and (b); means ± SE are shown in (c).

the screen, and they stayed at closer distance to it when faced with a fish display (Figure 1; Table 1). Furthermore the test fish showed more attention and aggressive behaviours towards a predator than towards a conspecific display (Figure 1a, b; Table 1, attention behaviour and aggression behaviour), and they stayed closer to the predator display (Figure 1c; Table 1, distance to screen). Size and sex of the test fish did not influence the amount of attention or aggressive behaviours shown during the displays (Table 1, attention behaviour and aggression behaviour). In general, larger test fish and female test fish stayed closer to the screen during all presentations (Table 1, distance to screen).

3.2. Experiment 2

The test fish showed more attention and aggressive behaviours towards the smaller stimulus fish (Figure 2a, b; Table 2, attention behaviour and aggression behaviour) and they stayed significantly closer to the screen when a smaller stimulus fish was presented (Figure 2c; Table 2, distance to screen). The test fish showed more attention behaviours to the conspecific than to the predator during the display of large stimulus fish, whereas they showed similar amounts of attention when the stimulus fish were small (Figure 2a; see significant interaction term stimulus species × stimulus size in Table 2, attention behaviour). Larger test fish showed less attention and aggression behaviours towards the displays (Table 2, attention behaviour and aggression behaviour), whereas the sex of the test fish did not influence the behaviours or distances kept towards the displays (Table 2).

Table 1.
Comparison of the attention behaviour, aggression behaviour and distance to screen of test fish in Experiment 1.

Factor	Estimate ± SE	Z/t-value	p-value
Attention behaviour			
Intercept	4.322 ± 1.875	2.31	0.021*
w.s. ➔ (ob., con., pred.)	0.077 ± 0.032	2.40	0.016*
(w.s., ob.) ➔ (con., pred.)	−0.367 ± 0.055	−6.73	0.001*
con. ➔ pred.	−0.211 ± 0.048	−4.36	0.001*
Sex	−0.104 ± 0.334	−0.31	0.76
SL	−0.737 ± 0.520	−1.42	0.16
Aggression behaviour			
Intercept	4.981 ± 2.785	1.79	0.07**
w.s. ➔ (ob., con., pred.)	−0.016 ± 0.053	−0.31	0.75
(w.s., ob.) ➔ (con., pred.)	−0.510 ± 0.080	−6.40	0.001*
con. ➔ pred.	−0.338 ± 0.064	−5.31	0.001*
Sex	−0.180 ± 0.494	−0.36	0.72
SL	−1.178 ± 0.773	−1.52	0.13
Distance to screen			
Intercept	1.429 ± 1.173	1.22	0.22
w.s. ➔ (ob., con., pred.)	−0.028 ± 0.032	−0.88	0.38
(w.s., ob.) ➔ (con., pred.)	0.278 ± 0.056	4.95	0.001*
con. ➔ pred.	0.178 ± 0.065	2.74	0.007*
Sex	−0.560 ± 0.209	−2.68	0.009*
SL	0.867 ± 0.325	2.67	0.009*

Intercept estimates show the grand mean of all treatments. Orthogonal comparisons of the treatments are listed (w.s., white screen; ob., object image; con., conspecific image; pred., predator image). The arrows indicate the direction of comparison within the contrast. The estimate value always refers to the treatment left of the arrow. If treatments are combined in parentheses, mean values of these are used in the comparisons. Z-values are presented for attention and aggression behaviours; t-values are presented for the distances. Reference category for estimate of factor 'sex': females; $N = 28$, $*p < 0.05$; $**0.05 < p < 0.1$.

3.3. Experiment 3

The test fish significantly increased their attention and aggressive behaviours towards the smaller stimulus images of both displayed species (Figure 3a, b; Table 3, attention behaviour and aggression behaviour). Aggression towards predators was significantly lower than towards herbivores, whereas attention behaviours did not differ between displays of predators or herbivores (Figure 3a, b; Table 3, attention behaviour and aggression behaviour). During the displays of large and of small stimulus fish, the test fish spent more time

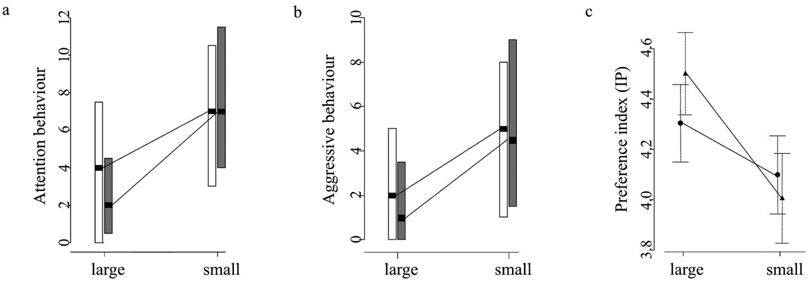

Figure 2. Comparison between (a) attention, (b) aggressive behaviour and (c) presence in the different zones, shown in the presence of large and small sized stimulus fish in Experiment 2. Circles and white bars represent the display of a conspecific image (= *N. pulcher*); triangles and grey bars represent the display of a predator image (= *L. elongatus*). Medians and interquartile ranges are shown in (a) and (b); means ± SE are shown in (c).

Table 2.
Comparison of the attention behaviour, aggression behaviour and distance to screen of the test fish in Experiment 2.

Factor	Estimate ± SE	Z/t-value	p-value
Attention behaviour			
Intercept	6.752 ± 2.556	2.64	0.008*
Stimulus species	−0.36 ± 0.143	−2.52	0.012*
Stimulus size	0.504 ± 0.116	4.34	0.007*
SL	−1.613 ± 0.728	−2.22	0.027*
Sex	0.225 ± 0.266	0.85	0.4
Stimulus species × Stimulus size	0.489 ± 0.173	2.82	0.005*
Aggression behaviour			
Intercept	9.649 ± 3.781	2.55	0.011*
Stimulus species	0.009 ± 0.097	0.1	0.92
Stimulus size	0.707 ± 0.103	6.84	0.001*
SL	−2.712 ± 1.082	−2.51	0.012*
Sex	0.533 ± 0.385	1.38	0.17
Distance to screen			
Intercept	6.300 ± 2.905	2.17	0.032*
Stimulus species	0.053 ± 0.086	0.61	0.54
Stimulus size	−0.351 ± 0.827	−4.07	0.001*
SL	−0.473 ± 0.827	−0.57	0.57
Sex	−0.483 ± 0.291	−1.66	0.1

Reference categories for estimates of factor ‘stimulus species’ (conspecific (= *N. pulcher*)), factor ‘stimulus size’ (large stimulus fish) and factor ‘sex’ (females). Z-values are presented for the attention and aggression model and t-values for the distance model. $N = 28$, $*p < 0.05$.

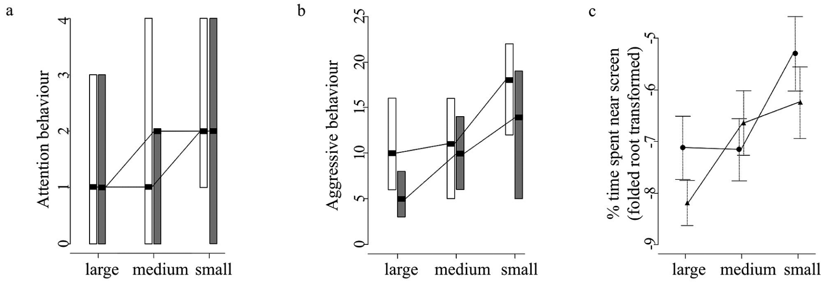

Figure 3. Comparison between (a) attention, (b) aggressive behaviour and (c) presence in the zone closest to the screen, when presented with large, medium and small sized stimulus fish in Experiment 3. Circles and white bars represent the display of herbivore images (= *O. ventralis*) and triangles and grey bars represent the display predator images (= *L. elongatus*). Medians and interquartile ranges are shown in (a) and (b); means ± SE are presented in (c).

in front of the herbivore images than of the predator images (Figure 3c; Table 3, distance to screen). In contrast, during the display of the medium-sized stimulus fish, the test fish spent more time close to the predator images (Figure 3c; see significant interaction term stimulus fish × medium in Table 3, distance to screen).

The test fish spent a similar proportion of time near displays of large and medium sized herbivores, and they spent more time closer to the display of small herbivores than to larger or medium sized displays (Figure 3c; Table 3, distance to screen). Conversely, when confronted with a display of a large predator, the test fish decreased the time close to the screen, whereas they stayed longer near the displays of small or medium sized predators (Figure 3c; Table 3, distance to screen). Larger individuals showed more attention behaviour towards both presented stimulus species, whereas aggressive behaviour and the time spent in front of the screen did not depend on the size of the test fish (Table 3).

4. Discussion

The results show that *N. pulcher* differentiate between 2D-animation sequences displaying (1) a moving artificial object and moving images of a conspecific or heterospecific, (2) moving images of a conspecific and a predator, (3) different sizes of the presented stimulus images and (4) moving images of different heterospecifics, namely of a harmless herbivore and a dangerous predator. The results of our experiments indicate that simple 2D-animation sequences of still images can be a valid, powerful tool to study

Table 3.
Comparison of the attention behaviour, aggression behaviour and percentage of time test fish spent in front of the screen in Experiment 3.

Factor	Estimate ± SE	Z/t-value	p-value
Attention behaviour			
Intercept	-2.311 ± 0.790	-2.92	0.003*
Stimulus fish	0.163 ± 0.115	1.42	0.16
Medium	0.254 ± 0.147	1.73	0.083**
Small	0.343 ± 0.144	2.39	0.017*
SL	0.798 ± 0.248	3.22	0.001*
Aggression behaviour			
Intercept	1.478 ± 0.601	2.46	0.014*
Stimulus fish	0.335 ± 0.081	4.12	0.001*
Medium	0.247 ± 0.103	2.4	0.017*
Small	0.660 ± 0.010	6.62	0.001*
SL	0.097 ± 0.192	0.51	0.61
Distance to screen			
Intercept	-8.905 ± 2.547	-3.5	0.001*
Stimulus fish	1.062 ± 0.562	1.89	0.06**
Medium	1.543 ± 0.562	2.75	0.006*
Small	1.937 ± 0.562	3.45	0.001*
SL	0.237 ± 0.814	0.29	0.77
Stimulus fish × Medium	-1.576 ± 0.794	-1.99	0.049*
Stimulus fish × Small	-0.114 ± 0.794	-0.14	0.89

Reference categories for estimates of factor 'stimulus fish' (predator (= *L. elongatus*)), factor 'medium' (large stimulus fish) and factor 'small' (large stimulus fish). Z-values are presented for the attention and aggression model and t-values for the distance model. $N = 25$, $^*p < 0.05$; $^{**}0.05 < p < 0.1$.

detailed behavioural responses towards visual stimuli of conspecifics and heterospecifics in fish. Furthermore, the results allow to draw general conclusions about the functionality of the observed behaviours using different stimulus species.

N. pulcher showed more attention and aggressive behaviours towards the fish images compared to the two control presentations of a rectangular object and a white background. This represents an adequate context-specific response, given that the content of the control presentations should not pose any threat to the fish. The test fish also differentiated between images of a conspecific, an herbivore and a predator, suggesting that they can derive information from the images about the identity of the displayed fish species, or at least about the relative threat level a displayed species poses to them. As predicted from experiments with live fish (E.O., S.F., B.T., unpubl. results; Zöttl et al., 2013), *N. pulcher* showed more aggressive behaviours towards the image of the herbivore species than towards images of predators. The herbivore species may represent a weak space competitor for *N. pulcher* under natural conditions (Karino, 1998). Attacking this harmless herbivore appears to reflect a low-cost low-benefit strategy, which has previously been reported from a more naturalistic social setting involving structured family groups of *N. pulcher* and live stimulus fish of the same species (Zöttl et al., 2013). Furthermore, the test fish spent less time in front of the screen when confronted with a large predator compared to a large herbivore, which very likely reflects an adaptive response to avoid predation risk (Hellig et al., 2010). Surprisingly, test fish showed more attention behaviours towards the empty background compared to the moving rectangle treatment. This on the first view unexpected result might be explained by the fact that the treatments were presented in a randomized order, meaning that 75% of test fish were confronted with a moving stimulus first. This might have resulted in an increase of attention behaviours towards the white screen as the fish might have been waiting for something entering the screen.

While fish are generally known to use visual as well as olfactory cues to spot predators (Smith, 1997; Ferrari et al., 2010), it is still unknown which cues are used by *N. pulcher*. Zöttl et al. (2013) provided evidence that visual cues play an important role. The result that *N. pulcher* were able to differentiate a predator from a conspecific based on visual cues alone further underpins this finding. Furthermore, as all our test fish had no predator experience previous to experiments, the response supports previous results that predator recognition has an innate component (Zöttl et al., 2013).

Opponent size is the major predictor of conflict outcome and social dominance in *N. pulcher* (Reddon et al., 2011; Taborsky et al., 2012; Dey et al., 2013). The results of Experiment 2 indicate that in *N. pulcher* absolute opponent size rather than a relative measure based on own size determines the outcome of intraspecific conflicts. In this experiment, test fish were given one night to habituate to the new test surrounding, which in the laboratory is sufficient time for test fish to establish a territory (Arnold & Taborsky, 2010). Therefore the presented conspecific was most likely perceived as a territory intruder. Because of the linear, size-dependent social hierarchy in *N. pulcher* (Dey et al., 2013), a large intruder poses a high threat towards a smaller territory owner, as it will easily succeed in evicting the small owner (von Siemens, 1990). Thus, *N. pulcher* adequately responded towards the displays of a differently-sized conspecific by decreasing aggression and increasing their distance towards the image of a large conspecific as compared to a small conspecific display.

The test fish showed less attention towards a large predator than towards a large conspecific. In a natural context, an adequate response towards a large conspecific should depend on the latter's behaviour. Thus, a careful assessment of the opponent is required, which may lead to increased attention behaviour. In contrast, the best response towards large predators should be to reduce activity (e.g., Thünken et al., 2010). Our results show that at least in terms of attention behaviour *N. pulcher* differentiate between a large conspecific and a large predator.

Our study extends previous knowledge on size-based intraspecific conflict management in *N. pulcher* and shows that size-dependent behavioural rules also apply to interspecific interactions. *N. pulche*r attacked more often and stayed closer to smaller compared to larger predator displays. This finding may be explained by the fact that *L. elongatus* of up to 8 cm feed primarily on shrimps, copepods and fish fry (Hellig et al., 2010). At this size they therefore pose no threat to adult *N. pulcher*, while *L. elongatus* become life-threatening predators when reaching sizes of 12 cm or more. Interestingly, the test fish stayed closer to intermediate predator displays than to intermediate herbivore display*s*. While this might seem surprising at first, one should bear in mind that a size increase in herbivores may linearly or even over proportionally increase the ability to act as space competitor. In contrast, the predatory species *L. elongatus* is a true risk only when much larger than 8 cm (Hellig et al., 2010), but never acts as space competitor at earlier life stages. These size dependent behavioural rules may even be more important than the discrimination between species specific levels of threat, as test fish directed similar amounts of aggression and stayed similarly close to relatively smaller or relatively larger stimulus images, irrespective of their species identity. In our experiments we manipulated the size of the stimulus image, which allowed us to draw conclusions about the actual size, independently of confounding factors such as individual identity.

Animated images are a promising technique, which allows experimenters to standardize the phenotypic appearance and movements of stimuli (e.g., Zbinden et al., 2004; Mehlis et al., 2008; Baldauf et al., 2009b). Thus far, the generation of animated sequences of stimulus images often required a substantial time effort and computing expertise (e.g., Künzler & Bakker, 1998; Zbinden et al., 2004; Veen et al., 2013). The use of simple PowerPoint presentations (Baldauf et al., 2009b, this study) provides a cheap and technically simple tool to generate animated images, where phenotypic traits such as colouration, size, movement speed and, to some degree, also the movements themselves can easily be manipulated while controlling for confounding factors. This opens possibilities to study the mechanism of visual communication by analysing the behavioural responses towards altered 'signals' sent by the animations. Our study revealed that, by using this animation technique, detailed behavioural responses towards conspecific and heterospecific stimuli can be obtained, which allowed us to extract basic behaviour rules for our study species. These can be summarised as (1) 'attack fish smaller or of similar size than yourself' and (2) 'if the opponent is larger than yourself, base your decision whether to attack on the species-specific level of risk posed by your opponent'. We hope this study will encourage more research applying 2D-animation sequences in behavioural ecology to investigate visual communication.

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Appendix A

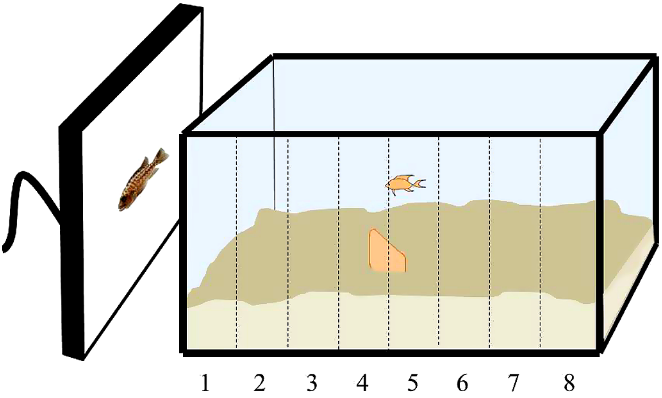

Figure A1. Observer view of the experimental set-up in Experiments 1 and 2. The test aquarium was divided in 8 equally sized zones with the shelter in zones 4–5 and the opening facing towards the screen. The screen was randomly placed left or right next to the experimental tank.

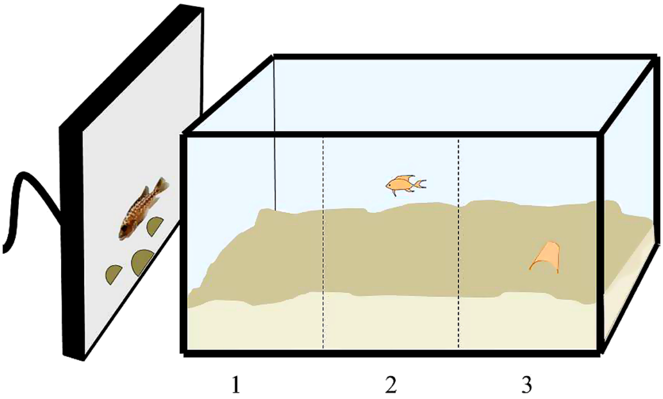

Figure A2. Observer view of the experimental set-up in Experiment 3. The test aquarium was divided into 3 equally sized zones with zone 1 close to the screen and the shelter in zone 3, with the opening towards the observer. In this experiment we used a greenish background. Stones shown onto the background near the bottom served as size references.

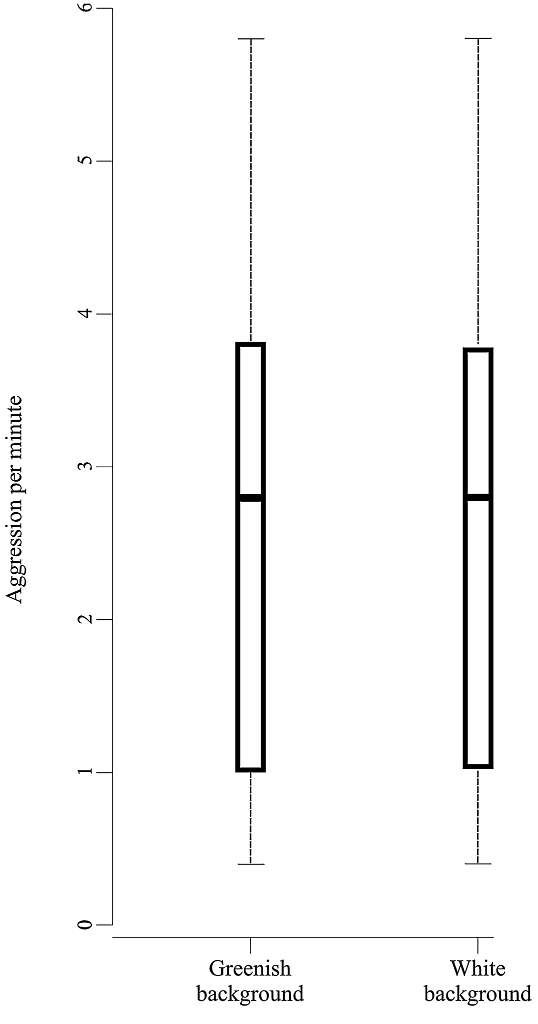

Figure A3. Comparison between the aggression towards a similar sized predator presented on a white background and a predator presented on a greenish background. We used the aggression towards the predator in Experiment 1 and the aggression towards the small sized predator in Experiment 3. As we used different observation times in both experiments we calculated per minute aggression and compared it using a Mann–Whitney U-test in R 2.14.1. Test fish directed comparable amounts of aggression towards a predator presented on a white background and a predator presented on a greenish background (Mann–Whitney U-test, $W = 415$, $p = 0.25$). Figure A3 shows per minute aggression towards the predator presented on a greenish background and the predator presented on a white background. Medians, quartiles and whiskers (1.5× interquartile ranges) are shown.

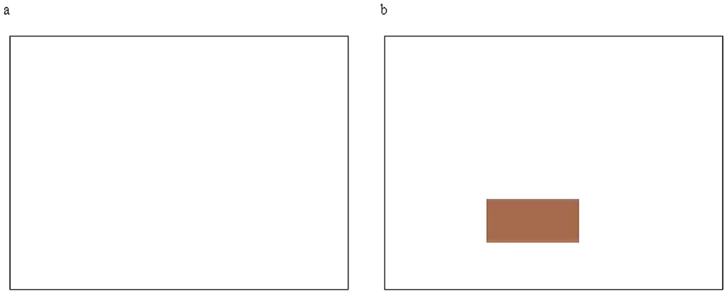

Figure A4. Screenshots of PowerPoint slides during (a) the presentation of an empty background and (b) the presentation of an animated rectangular, similar sized and coloured as *N. pulcher* in Experiment 1.

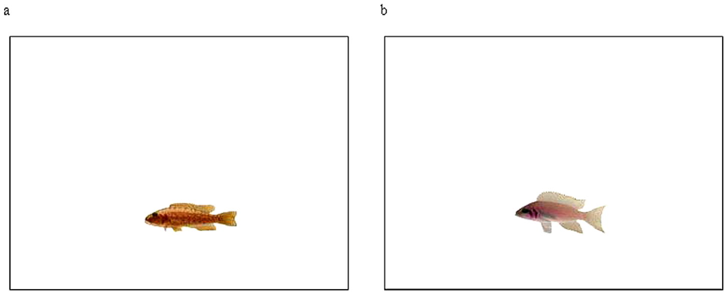

Figure A5. Screenshots of PowerPoint slides during (a) the presentation of an animated predator (*L. elongatus*) and (b) the presentation of an animated *N. pulcher* in Experiment 1. Both slides were used as well in Experiment 2 (= small size class).

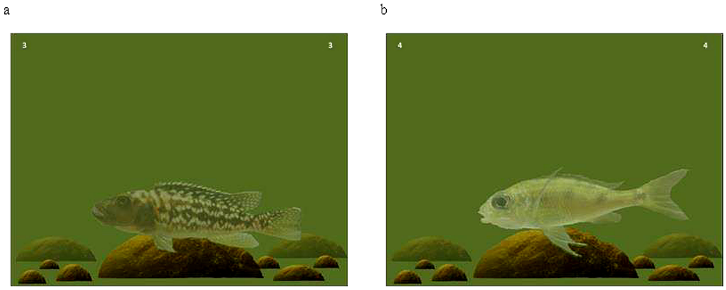

Figure A6. Screenshots of PowerPoint slides during (a) the large predator and (b) the large herbivore display in Experiment 3.

Appendix B: R-equations to fit linear mixed models

(a–c) Full models to calculate aggression and attention behaviours towards the stimulus images, as well as the preference index in Experiment 1. (d–f) Full models to calculate aggression and attention behaviours towards the stimulus images, as well as the preference index of test fish in Experiment 2. (g–i) Full models to calculate aggression and attention behaviours towards the stimulus images as well as the percentage of time spent near the screen in Experiment 3. ‘base’ refers to the individual based random effect and was only applied if models were over-dispersed.

(a) glmer(aggr ∼ Treatment + Sex + SL + (1|Fish.ID) + (1|Observer), family = poisson, data = xxx)

(b) glmer(att2 ∼ Treatment + Sex + SL + (1|Fish.ID) + (1|Observer), family = poisson, data = xxx)

(c) lmer(IP.a ∼ Treatment + Sex + SL + (1|Fish.ID) + (1|Observer), data = xxx)

(d) glmer(aggr ∼ species × size_stimulus + SL + Sex + (1|Fish.ID) + (1|Observer), family = poisson, data = xxx)

(e) glmer(attention ∼ species × size_stimulus + SL + Sex + (1|Fish.ID) + (1|Observer), family = poisson, data = xxx)

(f) lmer(IP.a ∼ species × size_stimulus + SL + Sex + (1|Fish.ID) + (1|Observer), data = xxx)

(g) glmer(aggression ∼ species × size_stimulus + SL + (1|ID) + (1|base), family = poisson, data = xxx)

(h) lmer(attention ∼ species × size_stimulus + SL + (1|ID), family = poisson, data = rec)

(i) lmer(fol.per.tim.fr ∼ species × size_stimulus + SL + (1|ID), data = xxx)