

# To pee or not to pee: urine signals mediate aggressive interactions in the cooperatively breeding cichlid *Neolamprologus pulcher*

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## Abstract

The communication of aggressive propensity is an important component of agonistic interactions. For this purpose, animals use different sensory modalities involving visual, acoustical and chemical cues. While visual and acoustic communication used in aggressive encounters has been studied extensively in a wide range of taxa, the role of chemical communication received less attention. Here, we studied the role of chemical cues used during agonistic interactions of territory owners in the cooperative cichlid *Neolamprologus pulcher*. During staged encounters, we allowed either visual and chemical contact between two contestants or visual contact only. As chemical information in this species is most likely transferred via urine, we measured urination patterns using dye injections. Furthermore, we recorded aggressive and submissive behaviours of both contestants in response to the experimental treatment. Fish that had only visual contact with each other significantly increased their urination frequency and showed more aggressive displays compared to fish with both visual and chemical contact. Furthermore, appropriate agonistic responses appear to be dependent on available chemical information. This indicates that *N. pulcher* actively emits chemical signals to communicate their aggressive propensity via urine.

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Chemical communication thus plays a crucial role in multimodal communication of aggression in these fish, which highlights the need of studying the role of chemical communication during agonistic encounters in general, even if other signals are more obvious to the human observer.

## Significance statement

The communication of aggressive tendencies can be achieved by transmitting visual, acoustical and chemical information. In this context chemical communication received less attention than other modalities thus far. We studied the importance of chemical information released via urine during agonistic encounters in the cooperatively breeding cichlid *N. pulcher*. Using dye injections, we measured urination patterns as well as the aggressive and submissive behaviours of two contestants. We show that *N. pulcher* actively signals aggressive tendencies via altered urination patterns. Furthermore, we show that appropriate agonistic responses appear to be dependent on the availability of such chemical information. Thus, our results suggest that chemical communication plays a crucial role in multimodal communication of aggression in these fish. These findings highlight the importance of chemical communication during agonistic encounters in general, even if other signals are more obvious to the human observer.

**Keywords** Chemical communication · Agonistic behaviour · Cichlid fish · Visual signal · Chemical cues · Aggression

## Introduction

Research on animal conflict has greatly contributed to our understanding of social behaviour (Aureli et al. 2002; Hardy and Briffa 2013). Agonistic interactions can be intense (Drews 1993), especially during territorial contests, which can lead to

injuries or death of one or both contestants (Drews 1993; Galef and Giraldeau 2001). However, not all social conflicts involve actual physical aggression (Hand 1986). Instead, individuals may signal their status by showing threat displays to elicit the withdrawal of the contestant (Rowell 1974; Hurd and Enquist 2001; van Staaden et al. 2011; Balzarini et al. 2014), which underlines the importance of communication in resolving social conflicts (Derex et al. 2014).

Animals communicate by using different sensory channels (Otvic and Partan 2009). This may include combinations of visual, acoustic, chemical, electrical and/or tactile cues (Partan and Marler 1999; van Staaden and Smith 2011). Within the realm of communication, Maynard Smith and Harper (2003) proposed to distinguish between the concepts of cue and signal, which has been widely accepted in the literature (but see for example Hauser (1996) and Galef and Giraldeau (2001) for alternative definitions). According to their proposal, a signal can be ‘any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver’s response has also evolved’ (Maynard Smith and Harper 2003, p. 3), while a ‘cue is a feature of the world, [...] that can be used by an animal as a guide to future action’ (Maynard Smith and Harper 2003, p. 3; see also Hasson (1994)).

Communication through chemical cues is important in behavioural interactions (Bryant and Atema 1987; Robison et al. 1998; Frade et al. 2002). Many social behaviours in a range of animal taxa are mediated by chemical cues, such as maternal bonding (Auffarth 2013), mate preference (Heymann 2006) or kinship-based choices (Mehlis et al. 2008). Further, chemical communication is known to play a role in aggressive interactions particularly in mammals, including territory defence and marking (Heymann 2006), signalling of social rank (Huck et al. 1981) and the modulation of agonistic behaviour (East and Dehnhard 2013). For example, in cooperatively breeding meerkats (*Suricata suricatta*), chemical communication is a crucial component of territorial disputes and mate defence (Jordan 2007; Jordan et al. 2007). Male meerkats use urinary markings at high rates against females to overlay their scent marks, while females invest more in scent mark investigation, but not in overlaying other scent marks (Jordan 2007). Chemical cues play a crucial role in behavioural interactions of many fish species as well (Wisenden 2000; Keller-Costa et al. 2015; Sorensen and Wisenden 2015). Their role in fish’s aggressive interactions has been shown for example in two different tilapia species (Giaquinto and Volpato 1997; Almeida et al. 2005; Barata et al. 2008; Keller-Costa et al. 2012; Boyle and Tricas 2014). In Burtons mouthbrooder (*Astatotilapia burtoni*), chemical information influences the competition of individuals for resources (Maruska and Fernald 2012).

A common way to transmit chemical information is via urination, e.g. for marking territory borders (Gese and Ruff 1997; Pal 2003) or transmitting information about dominance

(Gese and Ruff 1997) and aggressive propensity (Pal 2003). Also in fishes, transmission of chemical information via urine plays a role in agonistic interactions (Hirschhauser et al. 2008; Maruska and Fernald 2012). However, knowledge is limited about whether cues emitted via urine are a mere by-product of the individual’s metabolism or whether they are actively used to communicate aggression (Hirschhauser et al. 2008; but see Almeida et al. 2005; Barata et al. 2007).

To unravel the importance of chemical communication during aggressive interactions of fish, we used the cooperatively breeding cichlid *Neolamprologus pulcher* as a model system. *N. pulcher* is endemic to Lake Tanganyika, where it lives in social groups consisting of a dominant breeding pair and one to 30 subordinates (Taborsky and Limberger 1981; Balshine et al. 2001; Groenewoud et al. 2016; see Taborsky 2016 for review). *N. pulcher* is highly territorial and uses a repertoire of threat displays to settle disputes, both with group members as well as with intruders from the same and other species (Taborsky 1984; Wong and Balshine 2010; Riebli et al. 2011; Balzarini et al. 2014, 2016). Thus far, studies concerning communication of aggressive propensity in *N. pulcher* have mainly focused on visual behavioural displays, although there is evidence for the excretion of urine during aggressive interactions in this species (Hirschhauser et al. 2008). Yet, it is unknown whether such cues are actively used as a signal in this context. This is an unfortunate gap in our understanding of the social behaviour of these fish.

Following the definition of Maynard Smith and Harper (2003), urine itself would be termed a cue, as it did not evolve specifically to convey information. In contrast, specific *urination patterns*, which lead to changes in the opponent’s behaviour, would match Maynard Smith and Harper’s definition of a signal. In this sense, we here use the term cue when talking about urine, but signal, when talking about opponent-directed urination behaviour.

The aim of this study was to determine whether urination behaviour is used to actively signal aggressive propensity in this cooperatively breeding cichlid. We tested in staged encounters whether the frequency, duration and latency of urination relates to agonistic interactions. We predicted that (a) the urination frequency will increase when fish are prevented from smelling each other because of a mismatch between signalling and receipt of signals from the partner; (b) fish will urinate earlier during agonistic encounters than during comparable phases of isolation, because of signalling attempts when facing a competitor; and (c) dominant fish will urinate more often than subdominant ones to demonstrate their aggressive propensity. Further, we aimed to determine whether the degree of aggressive behaviour differs based on the possibility of chemical communication. We predicted that when chemical communication is hampered, (a) contestants will show higher levels of aggression, because important information about the responses of the opponent is missing, (b)

dominant fish will be more aggressive than subordinates in order to elicit the expected (chemical) response of the opponent and (c) subordinates will show less submission due to lack of proper chemical identification of the opponent's status.

## Methods

### Study animals

All fish used in this study were sexually mature first and second generation offspring from wild caught *Neolamprologus pulcher* from the Zambian coast of Lake Tanganyika. Fish were kept in 200–400-l tanks, in mixed-sex non-breeding aggregations of 20–30 fish each. Experimental subjects originated from different holding tanks to exclude effects of familiarity and kinship on aggressive interactions (Frostman and Sherman 2004; Zöttl et al. 2013). All fish were fed the same diet of commercial flake food and defrosted fresh food to standardize the chemical signature of the fish (Bryant and Atema 1987; Giaquinto and Volpato 1997; Ward and Currie 2013). Water temperature was kept constant at  $27 \pm 1$  °C. A 13 h:11 h light-dark cycle simulated natural light conditions. The water quality was checked at the beginning of each trial with 'JBL EasyTest 6 in 1' test strips. Hardness was 7°d, pH between 6.8 and 7.6, nitrate below 10 mg/l and nitrite 0 mg/l.

### Experimental setup

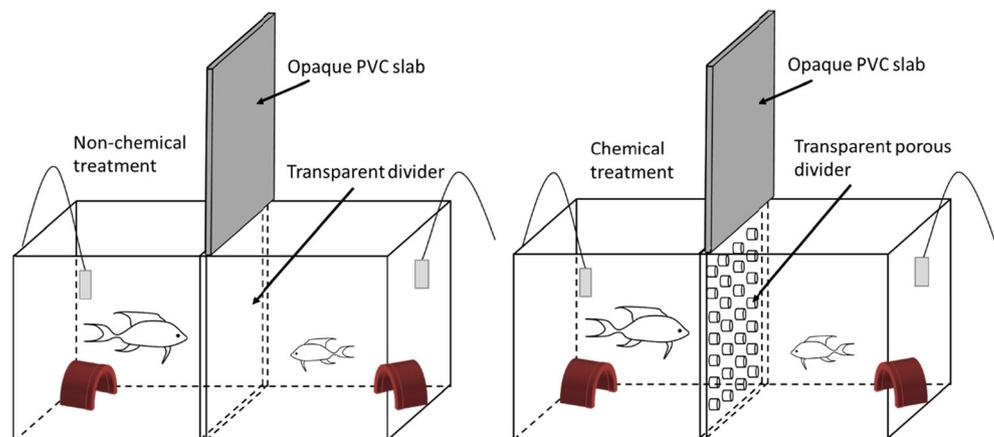
Six identical test tanks (39 cm × 24 cm × 24 cm) were covered with white paperboard on three sides to minimize disturbance from outside. The front side remained uncovered. Tanks were illuminated by fluorescent lamps (Philips MASTER TLD 18W/840) installed 20 cm above. Each tank was divided into two compartments measuring 19.5 cm × 24 cm × 24 cm each (Fig. 1) using a transparent divider as well as a removable opaque PVC slab. The transparent dividers were glued to the

side walls using silicone (JBL AquaSil transparent). They were additionally adjusted at the bottom using a guide rail to which they were glued with silicone as well. For the treatment including chemical contact, the transparent dividers were perforated with 90 holes (5 mm in diameter), allowing water exchange between the compartments. The water flow through these porous dividers was confirmed with a dye test prior to the study. For the non-chemical treatment, the transparent dividers were not perforated. The removable opaque PVC slab was attached to the transparent dividers using further guide rails and clamps, prohibiting water exchange prior to the start of the exposure phase. Dye tests were conducted in both setups to ensure that no water was exchanged prior to the start of the exposure phase. Both compartments contained half a white plastic funnel as shelter and an air stone to ensure oxygenation and water movement (Fig. 1). Video cameras (Exmor R Series, SONY) were installed approximately 50 cm in front of the tanks.

### Experimental procedure

In total, we ran 20 trials using 40 fish in a paired design. Each individual was tested only once to avoid pseudoreplication. In half of the 20 trials, chemical contact was enabled between the compartments, while in the other half it was prevented. Furthermore, in all trials, test fish differed in size to establish clear hierarchies during the agonistic encounters (Wong and Balshine 2010). Larger fish (termed 'dominants' hereafter) measured  $63 \pm 5$  mm, and smaller fish (termed 'subordinates' hereafter) measured  $47 \pm 5$  mm. The minimum size difference between members of an experimental pair was 9 mm. Test fish were caught from their storage tank, measured to the nearest millimetre and weighed with a digital balance (Mettler PM 460, DeltaRange) to the nearest 0.01 g. Afterwards, they were placed into the respective experimental compartment, where they were allowed to establish a territory during an acclimatization phase lasting at least 18 h (Balzarini et al. 2014).

**Fig. 1** The test tank was divided into two compartments, each containing a shelter and an airstone. *Left tank*: a transparent divider allowed visual contact but prevented chemical communication during the trial. *Right tank*: a transparent porous divider enabled visual and chemical contact. A removable opaque PVC slab separated fish during the non-exposure phases



### Dye injection

After acclimatization, each fish was caught and anaesthetised by immersion in a 0.5 ml/l solution of KOI MED® Sleep (KOI&BONSAI Zimmermann) for 1 min. We followed the supplier's recommended dose, which was verified to not cause any long lasting adverse effects on behaviour in extensive pilot trials using *N. pulcher* ranging between 0.5 and 7.0 cm standard length (JGF, unpublished data). Next, the fish were injected with 10 to 15 µl (depending on body size) of patent blue violet (300 mg/ml in 0.9% NaCl; Sigma-Aldrich) in the left dorsal muscle following Appelt and Sorensen (1999), using a standard insulin syringe (MYJECTOR® 0.5 ml U-100 Insulin, 29G × ½"–0.33 × 12 mm). The dye is not harmful to the fish (Appelt and Sorensen 1999). The urine of injected fish is coloured blue for at least 24 h, which allows monitoring urine pulses (Appelt and Sorensen 1999). A calibration study prior to the main experiments was conducted to estimate the amount of dye and injection volume sufficient for making urine release visible. After the injection, the fish were placed in a recovery tank for 10 min to make sure that no dye was released from the injection point prior to the start of the experiment. Over the next 15 min, injected fish turned from their natural pink-beige colour to blue. In a pilot experiment (see supplemental information), we checked whether the altered skin colour would differently change the aggressive behaviour of the fish in the respective treatments, which was not the case (see Table S1a). After 10 min of recovery from the anaesthesia, the fish were placed back into the experimental tank, where they were allowed to re-acclimatize for further 5 h.

### Experimental trial

The experimental recording lasted for 80 min, consisting of three phases: first, a pre-exposure isolation phase of 30 min, during which both fish were visually and chemically separated from each other to establish a baseline of urination patterns; second, an exposure phase of 20 min during which the opaque PVC divider was removed between the compartments. Except for two trials, aggressive interactions between both fish started within 2 min after removal of the divider. The third phase was a post-exposure isolation phase of 30 min after the opaque PVC divider was reinserted. We included this phase to observe how the urination pattern develops after an agonistic encounter, in comparison to the pre-exposure phase. Afterwards, the fish stayed in their experimental tanks until the next day, when they were placed back into their original holding tanks. After removing the subjects, the experimental tanks were emptied. Tank walls, PVC slabs, and white funnels were cleaned with ethanol and the air stones were exchanged. The tanks were newly filled with tap-water, which was stored in a supply tank not holding any fish for at least 24 h in order to allow dissolved gases to volatilize (cf. Mehliis et al. 2008,

2009). The order of the treatments (chemical contact yes/no) was randomly assigned by rolling a dice, and the trials were distributed evenly over a period of 4 weeks.

### Data assessment and analysis

The first 20 min of each phase were analysed using the Solomon Coder beta version 14.10.04 software (Péter 2015). For analyses of the videos, blinded methods were used. The urination patterns were analysed using the magnifier option of the 'Windows 7 Ease of Access Center'. Urine was excreted in consecutive pulses that were separated by periods without urine excretion. For analyses, these consecutive pulses were combined into 'blocks'. Separate blocks were defined as having a time gap of at least 10 s between consecutive pulses. We counted the total number of urine pulses for each phase (pre-exposure/exposure/post-exposure) and measured the duration of each block (sec.). Furthermore, in order to obtain a proxy for the urine volume, we calculated the mean number of urine pulses per block. Finally, we took the time until the first urine pulse in each of the three phases.

To quantify behavioural differences, we counted biting and ramming attempts of the two contestants towards each other, and combined them into one measure for overt aggression, following Balzarini et al. (2014). Furthermore, visual threat displays like raised fins, lifted opercula, fast approaches towards the opponent, head-down displays and s-shaped bending were counted as restrained aggression (Balzarini et al. 2014). Tail quivering and escape behaviour were counted as submission (Bergmüller et al. 2005). We noted if fish released urine while showing overt aggression, restrained aggression and submissive displays.

To analyse urination patterns, we fitted four generalized linear mixed-effect models (GLMMs) assuming negative binomial distributions. The number of urine pulses, block duration, pulses per block and latency to the first urine pulse served as the respective dependent variables. Fixed factors were treatment (chemical contact yes/no), rank (dominant/subordinate) and phase (pre-exposure/exposure/post-exposure). Furthermore, we added the interaction between phase and treatment. A group ID was added as a random effect to account for analysing data of both fish of a given pair. If the model revealed a significant interaction between the fixed factor treatment and phase, we analysed the three phases separately. Here, the fixed factors were treatment (chemical contact yes/no) and rank (dominant/subordinate). Furthermore, we tested the interaction between these terms. As random effect, we added group ID.

For the analyses of the behavioural data, we fitted GLMMs (assuming negative binomial distributions) with overt aggression, restrained aggression and submissive behaviour as dependent variables. These behaviours only occurred during the

exposure phase, thus we did not include the pre- and post-exposure phase in the analyses. Fixed factors were treatment (chemical contact yes/no) and rank (dominant/subordinate). Furthermore, we added the interaction between treatment and rank. A group ID was added as a random effect to account for analysing data of both fish of a given pair. If the model revealed a significant interaction between the fixed factor treatment and rank, we analysed the dominant and subordinate individuals separately. To analyse if the chance of subordinates attacking at least once differed between the respective treatments, a Barnard's unconditional test for superiority was conducted. Submissive behaviour was only displayed by subordinates (cf. Taborsky and Grantner 1998). To analyse differences in submission between the treatments, we fitted a GLMM assuming negative binomial distribution. Treatment served as fixed factor; furthermore, we included an individual-based random effect.

All models were calculated in R. We used the lme4 and R2admb statistical packages (Bates et al. 2015; Bolker et al. 2015) and RStudio (RStudio Team 2015, Version: 0.98.1062). Non-significant interactions were removed from the model (Engqvist 2005). All  $P$  values were obtained testing for two-tailed probabilities. The significance level for all tests was  $\alpha = 0.05$ .

## Results

### *Urination pattern, duration of urination and latency*

Comparing the three experimental phases combined revealed significant differences in the urination patterns between the exposure and non-exposure phases (Table 1a–c, Fig. 2a–c). Thus, we analysed the three phases separately.

The number of urine pulses during the exposure phase was significantly influenced by the presence of chemical contact (see Table 1a, Fig. 2a). Dominants and subordinates urinated more often when chemical contact was prevented (see Table 1a, Fig. 2a). The number of urine pulses during the pre- and post-exposure isolation phases did not differ from each other with regard of chemical contact (Table 1a). Throughout all three phases, dominant fish urinated more often than subordinates (Table 1a).

Analyses of the duration of urination blocks revealed that chemical contact significantly lowered the duration of urine release in the exposure phase, but not in the non-exposure phases (see Table 1b, Fig. 2b). Urination blocks were significantly longer when chemical contact was excluded (Table 1b). Additionally, there was a significant effect of rank in all phases, with dominant fish urinating over a longer time than subordinates (Table 1b).

The number of pulses per block was not significantly influenced by the treatment, but generally tended to be lower

during the exposure phase (Table S2, Fig. S3). The fish rank had no significant effect.

Investigating latencies to the first urine pulse revealed that all fish urinated earlier in the exposure phase compared to the non-exposure phases (Table 1c, Fig. 2c). The rank of fish did not affect the onset of urine release during the exposure and non-exposure phases (Table 1c). Furthermore, fish in non-chemical treatments urinated earlier than fish in chemical treatments (Table 1c). Finally, fish urinated later in the post-exposure phase compared to the pre-exposure phase (Table 1c).

Urine pulse release during the non-exposure phases occurred while fish were in the shelter or slowly moving through the tank. During the exposure phase, all urine pulses occurred during restrained aggression displays. Urine pulses did not occur during overt aggression and submissive behaviour displays or while no interactions between the fish took place.

### *Behaviours involved in aggressive encounters*

When chemical contact was hampered, changes in overt aggression differed between dominants and subordinates, as indicated by a significant interaction between rank and treatment (Table 2a, Fig. 3a). Dominants did not significantly differ in their frequency of overt aggressive attempts shown towards the subordinate (GLMM:  $N_{\text{dominants}} = 20$ ,  $z = -0.911$ ,  $P = 0.362$ ), while subordinates increased their overt aggressive behaviour. (GLMM:  $N_{\text{subordinates}} = 20$ ,  $z = -2.314$ ,  $P = 0.02$ ). Furthermore, when chemical contact was prohibited, subordinates attacked at least once in significantly more replicates than subordinates in the treatment allowing exchange of chemical cues (Barnard's test: Nuisance parameter = 0.5,  $P = 0.02$ , Fig. 3b). Restrained aggression was neither significantly influenced by the treatment nor by rank during the aggressive encounters (Table 2b, Fig. S1). Dominant individuals never showed submissive behaviours, while subordinates did (Fig. S2). The amount of submissive displays of subordinates was not significantly influenced by the possibility of chemical communication (GLMM:  $N = 20$ ,  $z = 1.329$ ,  $P = 0.184$ , Fig. S2).

## Discussion

The aim of this study was to elucidate whether urination patterns have a signalling function during aggressive interactions of the cooperative cichlid *Neolamprologus pulcher*, as suggested by a study on urinary androgen metabolites released during staged encounters in this species (Hirschenhauser et al. 2008). Our results demonstrate that urination patterns changed depending on the social context. In the course of contests, urine was released only during restrained aggressive interactions, while it was released continuously during non-contest

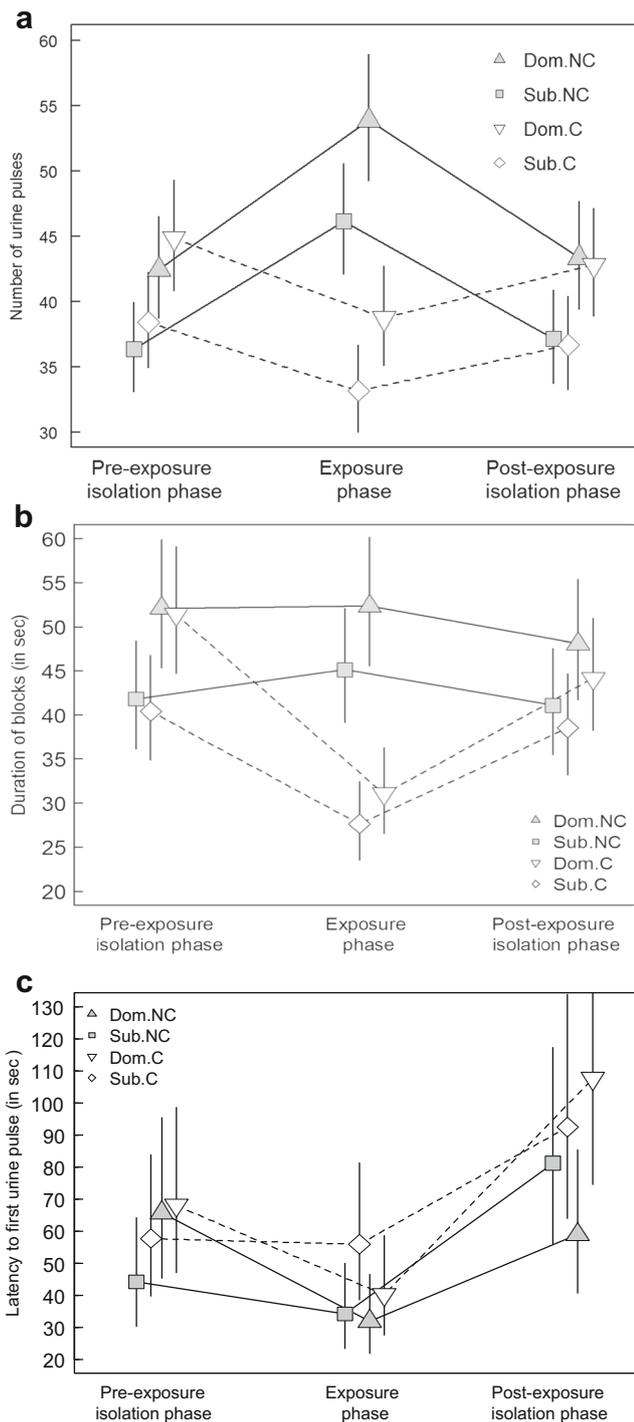
**Table 1** GLMM results for (a) the number of urine pulses, (b) the duration of urine blocks and (c) the latency to the first urine pulse during the three experimental phases. Shown are results from the full models of the combined three phases. The pre-exposure phase was set as reference (intercept). If a significant interaction between phase and treatment existed, the three phases were analysed separately. If no significant interaction occurred, the model was simplified stepwise. Significant *P* values are depicted in bold

	Estimate	Std. error	Z value	<i>P</i> value
a) Number of urine pulses, all 3 phases combined				
Intercept	3.748	0.046	80.57	< <b>0.001</b>
Exposure phase	0.238	0.044	5.34	< <b>0.001</b>
Post-exposure phase	0.021	0.047	0.45	0.653
Treatment	0.055	0.062	0.88	0.382
Rank	-0.154	0.028	-5.53	< <b>0.001</b>
Exposure phase × treatment	-0.385	0.066	-5.83	< <b>0.001</b>
Post-exposure phase × treatment	-0.067	0.066	-1.01	0.311
Pre-exposure phase				
Intercept	-0.377	0.043	86.58	< <b>0.001</b>
Rank	-0.198	0.049	-4.02	< <b>0.001</b>
Treatment	0.048	0.052	0.92	0.359
Rank × treatment	0.031	0.099	0.32	0.747
Exposure phase				
Intercept	3.985	0.051	78.12	< <b>0.001</b>
Rank	-0.151	0.048	-3.14	<b>0.001</b>
Treatment	-0.332	0.067	-4.91	<b>0.001</b>
Rank × treatment	0.002	0.098	0.03	0.980
Post-exposure phase				
Intercept	3.756	0.047	78.61	< <b>0.001</b>
Rank	-0.114	0.048	-2.34	<b>0.019</b>
Treatment	-0.018	0.058	-0.32	0.746
Rank × treatment	0.037	0.093	0.40	0.688
b) Duration of urine blocks, all 3 phases combined				
Intercept	3.921	0.065	60.02	< <b>0.001</b>
Exposure phase	0.051	0.054	0.95	0.343
Post-exposure phase	-0.036	0.054	-0.68	0.494
Treatment	-0.009	0.089	-0.11	0.921
Rank	-0.183	0.032	-5.62	< <b>0.001</b>
Exposure phase × treatment	-0.515	0.081	-6.36	< <b>0.001</b>
Post-exposure phase × treatment	-0.064	0.078	-0.82	0.411
Pre-exposure phase				
Intercept	3.960	0.065	60.10	< <b>0.001</b>
Rank	-0.247	0.067	-3.69	< <b>0.001</b>
Treatment	-0.018	0.081	-0.23	0.821
Rank × treatment	0.013	0.134	0.10	0.918
Exposure phase				
Intercept	3.958	0.073	53.79	< <b>0.001</b>
Rank	-0.151	0.064	-2.34	<b>0.019</b>
Treatment	-0.529	0.097	-5.42	< <b>0.001</b>
Rank × treatment	-0.012	0.131	-0.09	0.926
Post-exposure phase				
Intercept	3.867	0.071	54.48	< <b>0.001</b>
Rank	-0.150	0.047	-3.17	<b>0.001</b>
Treatment	-0.080	0.095	-0.84	0.401
Rank × treatment	0.030	0.090	0.33	0.739
c) Latency to first urine pulse, all 3 phases combined				
Intercept	4.059	0.136	29.650	< <b>0.001</b>
Exposure phase	-0.383	0.125	-3.053	<b>0.002</b>
Post-exposure phase	0.277	0.123	2.241	0.025
Treatment	0.319	0.156	2.044	<b>0.040</b>
Rank	0.003	0.102	0.031	0.975
Exposure phase × treatment	0.436	0.249	1.746	0.080
Post-exposure phase × treatment	0.285	0.246	1.159	0.246

conditions. Prevented chemical contact led to changes in the urination patterns of contestants, indicating that urine cues elicit responses in the contestants. Finally, when chemical communication was prevented, subordinate individuals showed increased levels of aggression, which would increase their risk of injury under natural conditions. Thus, Maynard

Smith and Harper's (2003) criteria for 'signals' appear to be fulfilled, and we conclude that urination patterns have a signalling role during aggressive interactions of *N. pulcher*.

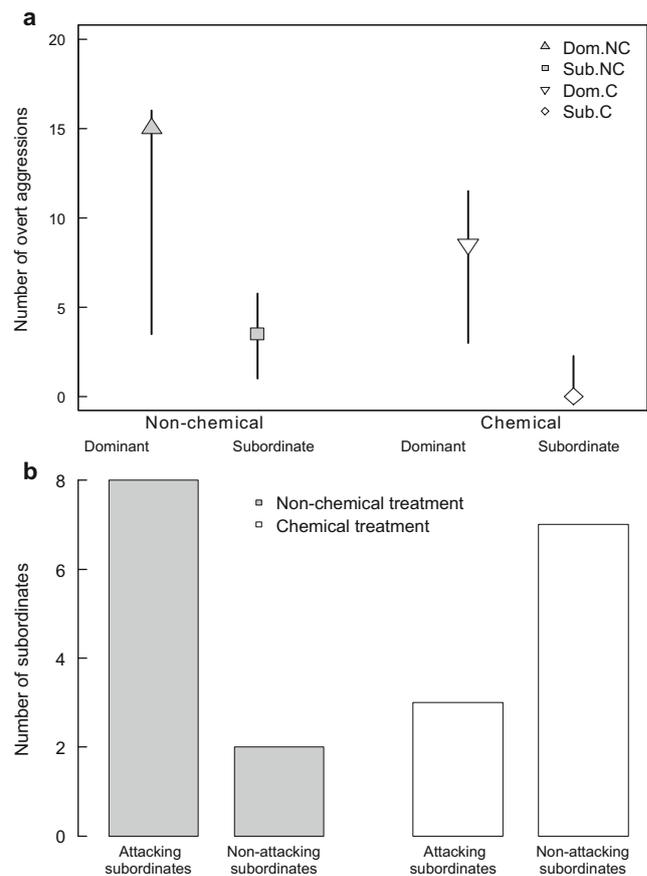
The increase in urination when chemical communication was impeded might reflect repeated attempts of test subjects to challenge the opponent. When chemical information is



**Fig. 2** Urination patterns during the pre-exposure, exposure and post-exposure phases. When chemical contact was prevented, dominant and subordinate fish showed more urine pulses (a) and longer overall urination blocks per experimental phase (b) than when chemical contact was possible. Furthermore, fish urinated earlier in the exposure phase than in the pre-exposure phase; this effect was stronger when chemical contact was prevented (c). Arithmetic means and 95% confidence intervals are depicted. *Triangles*: dominant fish; *squares*: subordinate fish; *grey symbols*: non-chemical treatment; *white symbols*: chemical treatment; *Dom NC*: dominant fish, no chemical contact; *Dom C*: dominant fish, chemical contact; *Sub NC*: subordinate fish, no chemical contact; *Sub C*: subordinate fish, chemical contact

**Table 2** GLMM results of (a) overt aggression and (b) restrained aggression during the 20 min exposure phase. If a significant interaction between rank and treatment existed, the model remained unaltered. Otherwise, the model was simplified stepwise. Significant *P* values are depicted in bold

	Estimate	Std. error	Z value	<i>P</i> value
<b>a) Displayed overt aggression during the exposure phase</b>				
Intercept	2.298	0.384	5.971	<b>&lt;0.001</b>
Rank	-0.636	0.470	-1.352	0.176
Treatment	-0.412	0.551	-0.74	0.455
Rank × treatment	-1.608	0.707	-2.274	<b>0.023</b>
<b>b) Displayed restrained aggression during the exposure phase</b>				
Intercept	5.158	0.153	33.62	<b>&lt;0.001</b>
Rank	-0.062	0.081	-0.77	0.444
Treatment	-0.319	0.208	-1.53	0.126
Rank × treatment	-0.225	0.162	-1.39	0.165



**Fig. 3** Overt aggression was significantly influenced by the treatment and the dominance status of the test fish. When chemical contact was prevented, fish showed more aggressive interactions. Medians and quartiles are depicted (a). The number of subordinate individuals attacking the dominant at least once was significantly higher when chemical communication was impossible (b). Symbols and abbreviations as in Fig. 2

available, fish can judge the opponent's agonistic propensity from their chemical cues and are thus better able to adjust their chemical and visual display behaviour. Therefore, when chemical communication was withheld, dominant fish increased their amount of urine pulses and duration of urination, as subordinates did not show appropriate behavioural responses. Subordinates, in turn, increased their urination activity as well when chemical communication was impossible, again revealing a significant influence of the mismatch between visual and chemical information they received from the opponent. These findings suggest that visual threat signals like restrained aggressive behaviours alone do not provide sufficient information to reliably judge an opponent's agonistic propensity, which highlights the importance of multimodal signalling during aggressive interactions. Finally, the number of pulses per block tended to generally decrease during aggressive encounters, further indicating the fish's ability to modulate their urination behaviour.

Both dominant and subordinate fish showed comparable effects of experimentally disabled chemical contact on urination patterns. Furthermore, regardless of the treatment, dominant fish urinated more often and over longer periods than subordinates. This is comparable to the situation in Mozambique tilapia, where dominant fish stored more urine and had a higher chemical potency of their urine than subordinates (Barata et al. 2008). Chemical cues are apparently used for estimating body size of conspecifics in three-spined sticklebacks (*Gasterosteus aculeatus*), banded killifish (*Fundulus diaphanus*) (Ward and Currie 2013) and pintado catfish, *Pseudoplatystoma coruscans* (Giaquinto and Volpato 2005). Also in *N. pulcher*, the amount of urine stored in the bladder could be a reliable proxy for body size, thus enhancing visual threat signals like opercula or fin spreads that also mainly aim to emphasize superiority in size (Beeching 1992; Balzarini et al. 2016).

It was beyond the scope of this study to investigate the chemical components transmitted via urine, but previous studies of this species showed that the amount of conjugated 11-ketotestosterone in the water was increased after a staged encounter with a competitor (Hirschenhauser et al. 2004, 2008). This androgen metabolite is excreted mainly via the urine, which makes it a strong candidate for chemical communication of aggressive propensity. Also in *Oreochromis mossambicus*, males seem to excrete a steroidal urinary compound to signal social dominance to females (Barata et al. 2008).

The opponent's sex has an influence on the aggressive behaviour in many species (Parker 1974; Bakker 1984; Balshine-Earn and McAndrew 1995; Arnott and Elwood 2009). However, in *N. pulcher*, sex differences in aggressive behaviour are rather small (Taborsky 1985; Desjardins et al. 2005; Taves et al. 2009; Dey et al. 2013; Groenewoud et al. 2016) and the sexes do not differ in visual appearance. For the

present study, we selected fish at random from their holding tanks, without regard to their sex. Thus, we cannot distinguish potential sex effects on the behavioural responses to our experimental treatment.

In summary, this study shows that urinary cues are actively used as signals during agonistic interactions of *N. pulcher*. Chemical contact strongly affected the communication of aggressive propensity, suggesting its crucial role in the assessment of an opponent's motivational state. Furthermore, in the course of a contest, urination took place only during restrained aggressive displays. These results highlight the importance of studying multimodal signals to elucidate communication in social interactions of animals.

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#### Compliance with ethical standards

**Ethical statement** The experiments adhered to the 'Guidelines for the treatment of animals in behavioural research and teaching (ASAB 2012)' and comply with the current laws of Switzerland (license number BE74/15).

**Conflict of interest** The authors declare that they have no conflict of interest.

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