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Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*

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Abstract
The ultimate functions of sociality, or the tendency to associate with conspecifics and to live within a social group, are increasingly well understood. However, the proximate mechanisms that mediate this behaviour have received less attention. The oxytocin family of nonapeptide hormones (including isotocin in teleost fish) is thought to play an important role in regulating social behaviour across a wide range of taxa and social contexts. In the current study, we investigated the influence of exogenous administration of isotocin and an oxytocin receptor antagonist on sociality in a cooperatively breeding fish, *Neolamprologus pulcher*. In our first experiment, we found that a high (and a low) dose of peripherally administered exogenous isotocin decreased the time spent associating with conspecifics in *N. pulcher*, while an intermediate dose had no effect relative to control. In our second experiment, we found that a peripheral administration of an oxytocin receptor antagonist increased grouping preference in male *N. pulcher*. The results of both experiments suggest that IT may inhibit grouping behaviour in this species. These results contribute to a growing body of literature suggesting that the broad generalization that the oxytocin family of nonapeptides facilitate grouping behaviour is overly simplistic, and that specific behavioural effects depend the study species and testing conditions.

Keywords
grouping behaviour, oxytocin, nonapeptide, social decision-making.

1. Introduction

Sociality, or the tendency for conspecifics to group together in space and time (Alexander, 1974; Wilson, 1975), is one of the most widely observed

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forms of social behaviour and one of the fundamental building blocks of social complexity (Krause & Ruxton, 2002, 2010; Earley & Dugatkin, 2010; Soares et al., 2010). Sociality varies both within and among species (Alexander, 1974; Cote et al., 2010) and is dictated by the trade-off between the advantages and disadvantages of living in a group (Hamilton, 1971; Alexander, 1974; Wilson, 1975; Krause & Ruxton, 2002, 2010). While considerable progress has been made in explaining the function of sociality (Krause & Ruxton, 2002, 2010; Earley & Dugatkin, 2010), a comprehensive understanding of the causes and consequences of sociality necessitates an integrative perspective including an appreciation for the proximate mechanisms that underlie grouping behaviour (Goodson, 2008, 2013; Goodson et al., 2009; Soares et al., 2010; Goodson & Kingsbury, 2011).

One promising potential proximate mediator of sociality is the highly conserved nonapeptide hormone oxytocin (Insel & Young, 2001; Goodson, 2005, 2008, 2013; Donaldson & Young, 2008; Lee et al., 2009; Ross & Young, 2009) and its non-mammalian homologues (e.g., isotocin in teleost fishes; mesotocin in amphibians, non-avian reptiles, birds and some non-eutherian mammals; Hoyle, 1999). Oxytocin and its homologues are produced primarily in the hypothalamus where they are released throughout the brain and excreted to the periphery via the pituitary gland (Norris, 2007). Oxytocin and its homologues have numerous functions both centrally and peripherally (Lee et al., 2009) and represent an evolutionarily ancient signalling system dating back to a duplication of the vasotocin gene in early-jawed fish (Hoyle, 1999). Oxytocin and its homologues appear to modulate a wide variety of behaviours and play a role in the stress response. In particular, there is growing evidence that the oxytocin family of nonapeptides are key regulators of social behaviour including pair bonding, affiliation, and parental care (for reviews see: Donaldson & Young, 2008, 2013; Lee et al., 2009; Ross & Young, 2009; Goodson & Thompson, 2010; MacDonald & MacDonald, 2010; Goodson & Kingsbury, 2013). More generally, oxytocin and its homologues may be important in coding the valence and salience of social stimuli, regulating social motivation and attention, and hence are likely a critical element of the social decision-making system (Ross & Young, 2009; O'Connell & Hofmann, 2012; Reddon et al., 2012).

Despite the vast and growing body of literature on the social functions of oxytocin and its homologues, surprisingly few studies have explicitly examined their role in modulating sociality (Goodson, 2013). In general, oxytocin

seems to increase sociality in mammals (e.g., Smith et al., 2010; Lukas et al., 2011; Liu et al., 2012), as does mesotocin in birds (e.g., Goodson et al., 2009, 2012; Goodson & Kingsbury, 2011). However, many ecological and life history factors influence the function of nonapeptide hormones (Goodson, 2013), and given the small number of studies in a restricted number of taxa, it is not currently possible to arrive at general conclusions about the role of oxytocin and its non-mammalian homologues in regulating sociality.

The teleost fish homologue of oxytocin is isotocin (IT), a highly similar nonapeptide in both structure and function (Hoyle, 1999; Godwin & Thompson, 2012). While IT has received far less research attention than has oxytocin, existing data suggest that IT plays a role in the regulation of social behaviour in fishes similar to the role of other oxytocin-family nonapeptide hormones (Godwin & Thompson, 2012). For example, in zebrafish (*Danio rerio*), treatment with exogenous IT increased or decreased sociality depending on dose (resulting in an inverted u-shaped dose–response curve; Braida et al., 2012). Thompson & Walton (2004) found that exogenous isotocin increases the tendency to approach conspecifics in goldfish (*Carassius auratus*), although only in individuals that showed a low sociality tendency prior to treatment. Convict cichlid fish (*Amatitlania nigrofasciata*) upregulate endogenous production of IT in preparation for parental behaviour, and treatment with a specific IT receptor antagonist interferes with parental care behaviour (O'Connell et al., 2012). Injection with a non-specific nonapeptide antagonist delayed but did not prevent pair bonding in the convict cichlid, although this result cannot be conclusively attributed to IT as the antagonist used in this study also blocks the closely related vasotocin system (Oldfield & Hoffman, 2011). Given that fish are by far the most species-rich group of vertebrates and there are so few studies looking at the role of IT in regulating social behaviour, more studies in a greater diversity of fish species are warranted.

Neolamprologus pulcher is a cooperatively breeding cichlid fish endemic to Lake Tanganyika, East Africa (Konings, 1998). *N. pulcher* exhibit a remarkably complex social system and demonstrate an impressive diversity of social behaviours and communicative signals (Taborsky, 1984, 1985; Balshine-Earn et al., 1998; Balshine et al., 2001; Sopinka et al., 2009; Wong & Balshine, 2011a; Dey et al., 2013). *N. pulcher* have recently emerged as a promising model system for the integrative study of social behaviour both because of their highly social nature and because they are small bodied,

short-lived, and highly amenable to both controlled laboratory experimentation and field study in their natural habitat (Wong & Balshine, 2011a). *N. pulcher* groups consist of a single dominant breeding pair along with 1–20 non-breeding subordinates, including both individuals from previous reproductive bouts and immigrants from other social groups (Heg et al., 2005; Stiver et al., 2006, 2007; Wong & Balshine, 2011a). Subordinate group members may actively assist the breeding pair in their reproductive efforts, serving as helpers-at-the-nest by joining in broodcare, territory maintenance and defence (Taborsky & Limberger, 1981; Taborsky, 1984, 1985; Balshine et al., 2001; Wong & Balshine, 2011a; Zöttl et al., 2012, 2013a, b). Previous work on sociality in *N. pulcher* has shown that *N. pulcher* are highly motivated to associate with conspecifics (Jordan et al., 2010; Reddon et al., 2011a; Dey et al., 2013). *N. pulcher* prefer to associate with relatives over non-relatives (Le Vin et al., 2010), familiar social partners to unfamiliar ones, and prefer large-bodied group mates to small ones (Jordan et al., 2010). Male *N. pulcher* strongly and consistently prefer to join with large groups over small ones, whereas females consider their social rank when deciding which group to join, preferring to join large groups only when they can join at a high rank (Reddon et al., 2011a). One previous study examined the effects of IT manipulations on *N. pulcher* behaviour (Reddon et al., 2012) and found that exogenous IT increased sensitivity to social information. Specifically, Reddon et al. (2012) found that *N. pulcher* treated with exogenous IT were more attentive to the characteristics of their opponent during an aggressive interaction and more responsive to aggressive social challenges from dominant individuals within their social groups.

In the current study, we explored the role of IT in modulating sociality in *N. pulcher*. Specifically, we conducted a pair of controlled laboratory experiments manipulating the IT system to determine if this nonapeptide hormone exerts a modulating influence on grouping behaviour in this highly social species. In the first experiment, we gave individual *N. pulcher* an injection of IT at one of three different doses or a vehicle-only control injection, and then provided the injected fish with a choice between interacting with a single stimulus fish or with a group of three stimulus fish. In a second experiment we examined whether endogenous isotocin was playing a role in modulating sociality in *N. pulcher* by injecting study animals with one of three doses of an oxytocin receptor antagonist (OTA) that has been shown to

alter behaviour in other fish species, or a vehicle-only control, and then subjecting them to the same behavioural test as in the first experiment. Based on previous research (Reddon et al., 2011a), we predicted that males in both experiments would show a stronger preference to associate with the group of three fish over the lone stimulus individual than would females. We did not have a specific prediction for how our IT or OTA manipulations would affect the previously demonstrated sex difference in the strength of preference for large groups. Sex differences in nonapeptide effects are commonly reported (e.g., Goodson & Bass, 2000; Liu et al., 2001; Liu & Wang, 2003; Klatt & Goodson, 2012), however, previous research on IT in *N. pulcher* did not uncover any sex-specific effects (Reddon et al., 2012).

2. Experiment 1 — exogenous isotocin

2.1. Methods

2.1.1. Study animals

The fish used in this experiment were laboratory-reared descendants of *Neolamprologus pulcher* collected from Lake Tanganyika, Africa. Focal fish were housed in a mixed-sex 183 × 48 × 60 cm (527 l) communal aquarium. The housing aquarium contained a 2-cm layer of coral sand substrate, three water filters, two electric heaters and one thermometer. A total of 50 stimulus fish were also housed in a separate but identical communal aquarium. Fish were fed daily ad libitum with dried prepared cichlid food (Hagen Nutriafin Basix). Water temperature was held constant at 26 ± 2°C within chemical parameters that mimicked the natural habitat of *N. pulcher*. Groups were housed under a 13L:11D light cycle. Focal fish were all ⩾ 3.5 cm standard length (SL, measured from the tip of the mouth to the caudal peduncle) because *N. pulcher* of this size are sexually mature and can be sexed by examination of their external genitalia (Taborsky, 1985; Stiver et al., 2005).

2.1.2. Testing apparatus

We tested the focal fish in a social choice apparatus (Reddon et al., 2011a) consisting of two 40 × 20 × 25 cm (20 l) stimulus chambers placed inside at either end of a 90 × 44 × 38 cm (150 l) glass aquarium (Figure 1). Each of the aquaria contained 2 cm of coral sand substrate and one thermometer, air stone and electric heater. The stimulus aquaria were chemically isolated from each other and from the focal fish.

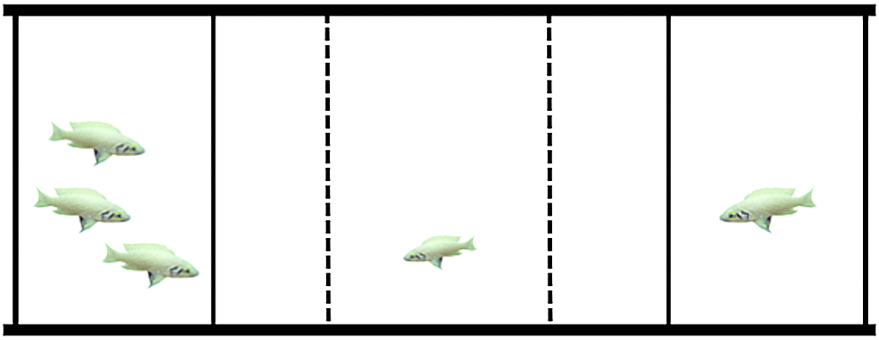


Figure 1. A schematic representation of the social choice apparatus as viewed from the front of the aquarium. The dashed lines delineate the preference zone for each stimulus chamber (10 cm). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

2.1.3. Treatment

We gave each focal fish an intraperitoneal injection of 25 μl/g body mass with a 31-gauge 0.3 ml insulin syringe. To prepare the treatments, we dissolved isotocin ([Ser^4, Ile^8]-oxytocin; Bachem, Torrance, CA, USA) in 0.9% saline and used this to create three different doses: (1) a low dose of 0.1 μg/g body mass, (2) an intermediate dose of 1 μg/g body mass and (3) a high dose of 5 μg/g body mass. The intermediate dose corresponded to a dose that has previously been shown to have effects on social behaviour in this species (Reddon et al., 2012) and is similar to nonapeptide doses that have been used in other fish species (Semsar et al., 2001; Lema & Nevitt, 2004; Santangelo & Bass, 2006; Filby et al., 2010). The fourth, control, treatment consisted of an injection of the vehicle only (0.9% saline). All fish recovered immediately from the injections and handling, exhibiting no obvious signs of distress.

2.1.4. Procedure

We tested a total of 80 focal fish (40 of each sex) in this experiment. We selected focal fish from the communal housing aquarium and sexed them by examination of their external genitalia. We recorded the standard length (to the nearest 0.1 mm) and body mass (to the nearest 0.01 g) of each focal fish. We then selected four stimulus individuals of the same sex as the focal fish from the stimulus-housing aquarium. The four stimulus fish were separated into a group of three fish and a single fish that were placed into one of the two stimulus chambers randomly based on a coin flip. Past research on other fish species and with *N. pulcher* has shown that fish possess the numerical

abilities make this type of discrimination (Agrillo et al., 2007; Dadda et al., 2009; Reddon et al., 2011a). Same-sex fish were used as stimuli so that association preference decisions represented social partner choice rather than mate choice (Dugatkin & Sih, 1995; Reddon et al., 2011a). All of the stimulus fish that we used were larger than the focal fish. Because *N. pulcher* show a rigid size-based dominance hierarchy (Reddon et al., 2011b; Wong & Balshine, 2011b; Dey et al., 2013) the focal fish would therefore assume the lowest dominance rank while associating with either the lone individual or the group of three fish (Reddon et al., 2011a). Stimulus fish were unrelated to the focal fish.

All trials were conducted in the afternoon between 12:00 and 17:00 to control for the possibility of diurnal effects on sociality. Focal fish were injected with one of the 3 IT treatments or with the saline control by an experimenter blind to the treatment condition. Following the injection, focal fish were immediately introduced into the middle section of the social choice apparatus (Figure 1) and allowed to acclimate to the novel aquarium for 5 min. After this 5 min, the entire aquarium was filmed from the front for 10 min. The fish were then left undisturbed and not filmed for 30 min. During this 30 min period, the focal fish was free to inspect each of the stimulus groups and swim freely around the central compartment of the social choice apparatus. Finally, the focal fish was filmed again for 10 min to ascertain the stability of its grouping preference over time and for the time course of the effect of the IT manipulation. The time course of the effect of exogenous IT in fish is not well known. However, mammalian oxytocin has a short half-life in the blood (on the order of minutes; Norris, 2007). Previous studies on *N. pulcher* found that behavioural effects of IT manipulations lasted for at least half an hour (Reddon et al., 2012), that grouping preferences are consistent over time (Reddon et al., 2011a) and that short-term grouping preferences reflect the eventual decision to join a group (Jordan et al., 2010). Focal fish were used only once. Stimulus fish were used only once per day but were replaced into their housing aquarium and were reused in different combinations across days.

2.1.5. Behavioural scoring

A single trained observer, blind to both the sex and the treatment group of the focal fish, scored all of the video recordings. During each 10 min observation period, we recorded the time that the focal fish spent with the majority of its body including its head within 10 cm (corresponding to approximately 2

body lengths of a typical focal fish) of each stimulus chamber. This measure of association has previously been used in this species (Reddon et al., 2011a). We also recorded the number of times the focal fish touched its head against the wall, swam up and down along the glass separating one the stimulus chambers from the central compartment, or displayed to the stimulus fish. We considered these interactions to be attempts by the focal fish to access the stimulus fish, and therefore indicative of motivation to interact with those individuals (Kelly et al., 2011; Lindeijer, 2012). Previous research in fish and birds has shown that it is worthwhile to consider association time and interactions separately and that interaction rate may be a more sensitive measure of motivation to affiliate than association time (Kelly et al., 2011; Lindeijer, 2012).

For the purposes of analysis and data presentation, we subtracted the time spent in the choice zone of the single stimulus individual from the time spent in the choice zone of the group of three stimulus individuals to produce a single grouping preference score for each focal fish. Likewise, we subtracted the number of interactions initiated across the barrier with the single stimulus individual from the number of attempted interactions with the group of three stimulus fish to produce a single social interaction score for each focal fish in this experiment. We also examined whether sex or our IT manipulation had an effect on the tendency to associate and/or interact with conspecifics in general regardless of group size. We summed both the time spent in association with either of the two stimulus groups and the number of interactions with either of the stimulus groups to produce overall association time and overall interaction rate scores for each fish during each observation period.

2.1.6. Statistical analyses

We used a 2-way analysis of variance (ANOVA) model with IT treatment, sex and their interaction as independent factors for each observation for each of our two behavioural measures of sociality (time and interaction rate) as well as our two measures of general social motivation (total association time and total interactions with either group). When we found a main effect of IT treatment, we conducted Fisher's LSD post-hoc tests to determine which treatment groups differed. We tested the residuals from our statistical models for adherence to parametric assumptions and found no violations. Data are represented in figures as mean $\pm$ SEM. All analyses were conducted using SPSS 20 (IBM, Chicago, IL, USA) for Macintosh OS X.

2.2. Results

2.2.1. Association time

During the first 10-min observation period, there was a significant effect of both IT treatment (2-way ANOVA; $F_{3,72} = 2.75$, $p = 0.049$) and sex ($F_{1,72} = 13.262$, $p = 0.001$) on the preference to associate with the group of three fish versus the lone fish (Figure 2A). There was no interaction between IT treatment and sex on this preference ($F_{3,72} = 0.09$, $p = 0.97$). Males had a stronger preference for associating with the group of three fish than did females. In both sexes, the fish that received the low (Fisher's LSD; $p = 0.025$) or the high dose ($p = 0.036$) of IT showed a reduced preference for associating with the group of three fish compared to the fish that received the saline control injection (Figure 2A). The dose also influenced the total time focal fish spent in any association zone ($F_{3,72} = 4.12$, $p = 0.009$). Again fish that received the high dose or the low dose spent less time in association with conspecifics (low: $p = 0.013$; high: $p = 0.003$) compared to those that

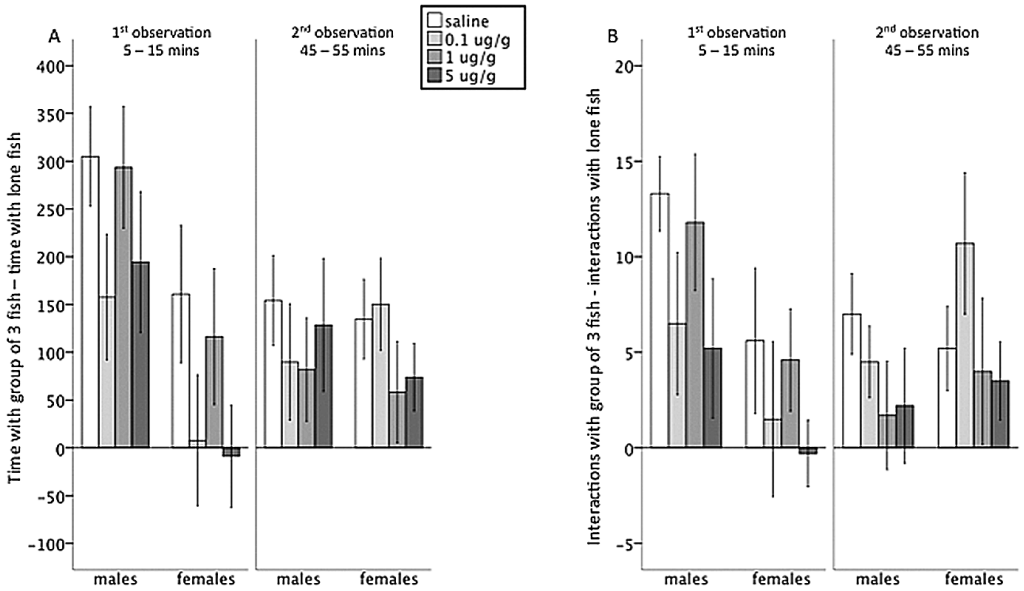


Figure 2. (A) Mean ± SE time focal fish spent associating with the large group minus the time spent associating with the lone fish during each of two observation periods when individuals were treated with one of three experimental doses of isotocin or a vehicle only control. There were significant effects ($p < 0.05$) of isotocin dose and sex during the first observation. (B) Mean ± SE number of focal fish interactions (through the glass barrier) with the large group minus the number of such interactions with the lone fish during each of two observation periods. There was a significant effect ($p < 0.05$) of sex during the first observation. $N = 10$ fish per sex per treatment.

received saline. Males tended to spend more time than females associating with conspecifics in either group, however this trend did not reach significance (2-way ANOVA, effect of sex: $F_{1,72} = 3.51,\ p = 0.065$). There was no significant interaction between sex and treatment on the tendency to associate with either of the social stimuli ($F_{3,72} = 0.44,\ p = 0.73$).

During the second observation period, there were no effects of IT treatment, sex or an IT treatment by sex interaction on the preference for the group of three fish versus the lone fish (2-way ANOVAs; all $F < 0.72$, all $p > 0.54$; Figure 2A). Across treatments, both males and females showed a preference to associate with the group of three fish (Figure 2A). During this second observation period, there was no significant effect of sex, IT treatment or a treatment by sex interaction on the tendency to associate with conspecifics in general (all $F < 1.56$, all $p > 0.21$).

2.2.2. Social interactions

During the first observation period, we found a significant effect of sex (2-way ANOVA; $F_{1,72} = 7.60,\ p = 0.007$; Figure 2B) on the number of interactions with the group of three fish compared to the lone stimulus individual. On average, males interacted more with the group of three fish than did females. There was no significant effect of the IT treatment on the preference to interact with the group of three fish compared to the lone individual ($F_{3,72} = 2.09,\ p = 0.108$; Figure 2B), nor was there any statistically significant interaction between dose and sex ($F_{3,72} = 0.08,\ p = 0.97$; Figure 2B). The data did, however, mirror the pattern we observed for association time, whereby fish that received either the low or the high dose of isotocin tended to interact less with the group of three fish when compared to the lone stimulus fish (Figure 2B).

During the second observation period there was no significant effect of sex, IT treatment or the interaction between IT treatment and sex (2-way ANOVAs; all $F < 1.46$, all $p > 0.23$; Figure 2B) on the tendency to interact with the group of three fish compared to the lone fish. Both males and females attempted to interact with the group of three stimulus fish more often than with the lone stimulus animal (Figure 2B) but the IT treatment did not appear to affect this preference.

During both observation periods, there was no significant effect of IT treatment, sex or a treatment by sex interaction on the tendency for the focal fish to interact with any of the stimulus fish in general (all $F < 0.14$, all $p > 0.13$).

3. Experiment 2 — oxytocin receptor antagonist

3.1. Methods

3.1.1. Study animals

The study animals used in this second experiment were drawn from the same population as in experiment 1 and were housed in the same way. However, no focal fish from experiment 1 was reused as a focal fish in experiment 2. We tested a total of 40 focal fish in the second experiment, 20 of each sex. Some of the stimulus fish from experiment 1 may have been reused in experiment 2 in novel combinations.

3.1.2. Testing apparatus

The testing apparatus was identical to that used in experiment 1 (Figure 1).

3.1.3. Treatment

We acquired a selective oxytocin receptor antagonist (OTA; desGly-NH^2-d(CH^2)5[$_D$-Tyr_2,Thr_4]OVT; Manning et al., 2008) as a generous gift from Professor M. Manning. This antagonist was designed for use in mammals (Manning et al., 2008) but has been successfully used to alter behaviour in fish (Braida et al., 2011; O'Connell et al., 2012). We dissolved the OTA into 0.9% saline and produced three different treatment doses of OTA in addition to a saline control. We based our intermediate dose (0.5 μg/g body mass) on the antagonist dose that has been used to alter parental care behaviour in another cichlid fish (O'Connell et al., 2012) and on the dose of a similar antagonist that has been successfully used to alter sociality in birds (Goodson et al., 2009). We also prepared treatment doses that were half (0.25 μg/g body mass; low dose) and double (1 μg/g body mass; high dose) the previously used dose to determine if the response to this antagonist is dose-dependent. As in experiment 1, focal fish received an intraperitoneal injection of one of the four treatments from an experimenter who was blind to the treatment group, immediately prior to being introduced into the social choice apparatus. The fish showed no ill effects of the injection or handling and quickly resumed typical behaviour.

3.1.4. Procedure

The procedure of experiment 2 was similar to that use in experiment 1 except that the focal fish was recorded during four 5-min blocks every 10–15 min starting 5 min after injection and introduction into the social choice apparatus (observation 1 = 5–10 min post-injection; observation 2 = 20–25 min post-

injection; observation 3 = 35–40 min post-injection; observation 4 = 55–60 min post-injection). We implemented this minor change in procedure to obtain finer scale data on the time course of the effects of the antagonist, which has only been used in fish in two previous published reports, neither of which includes detailed time course data (Braida et al., 2012; O'Connell et al., 2012). We do not know the effective time course of this OTA's effects, however, previous research has shown effects lasting at least an hour in another species of cichlid (O'Connell et al., 2012). The focal fish were free to swim about the social choice apparatus observing and interacting with both stimulus groups prior to the first observation period and between each successive observation period.

3.1.5. Behavioural scoring

Behavioural scoring was identical to experiment 1.

3.1.6. Statistical analysis

As with experiment 1, we used a 2-way ANOVA model with OTA treatment, sex and their interaction as independent factors for each observation, for each of our two behavioural measures of sociality. Where we found a main effect of OTA treatment, we conducted Fisher's LSD post-hoc tests to determine which treatment groups differed. When we found a significant interaction between OTA treatment and sex we decomposed the interaction and ran separate 1-way ANOVAs with OTA treatment as a factor for each sex with $\alpha = 0.025$ to account for the additional comparison. We tested the residuals from our statistical models for adherence to parametric assumptions and log-transformed and retested the residuals when violations were detected. Log transformation was successful in normalizing our residuals in all cases. Data are represented in figures as mean ± SEM of the untransformed data. All analyses were conducted using SPSS 20 (IBM) for Macintosh OS X.

3.2. Results

3.2.1. Association time

During the first observation period (5–10 min post-injection) there was no significant main effect of either OTA treatment (2-way ANOVA; $F_{3,32} = 0.90$, $p = 0.45$) or sex ($F_{1,32} = 0.02$, $p = 0.90$) on time the focal fish spent with the group of three stimulus fish compared to the lone stimulus fish (Figure 3A). However, there was a marginally non-significant interaction between OTA treatment and sex ($F_{3,32} = 2.49$, $p = 0.078$), whereby males

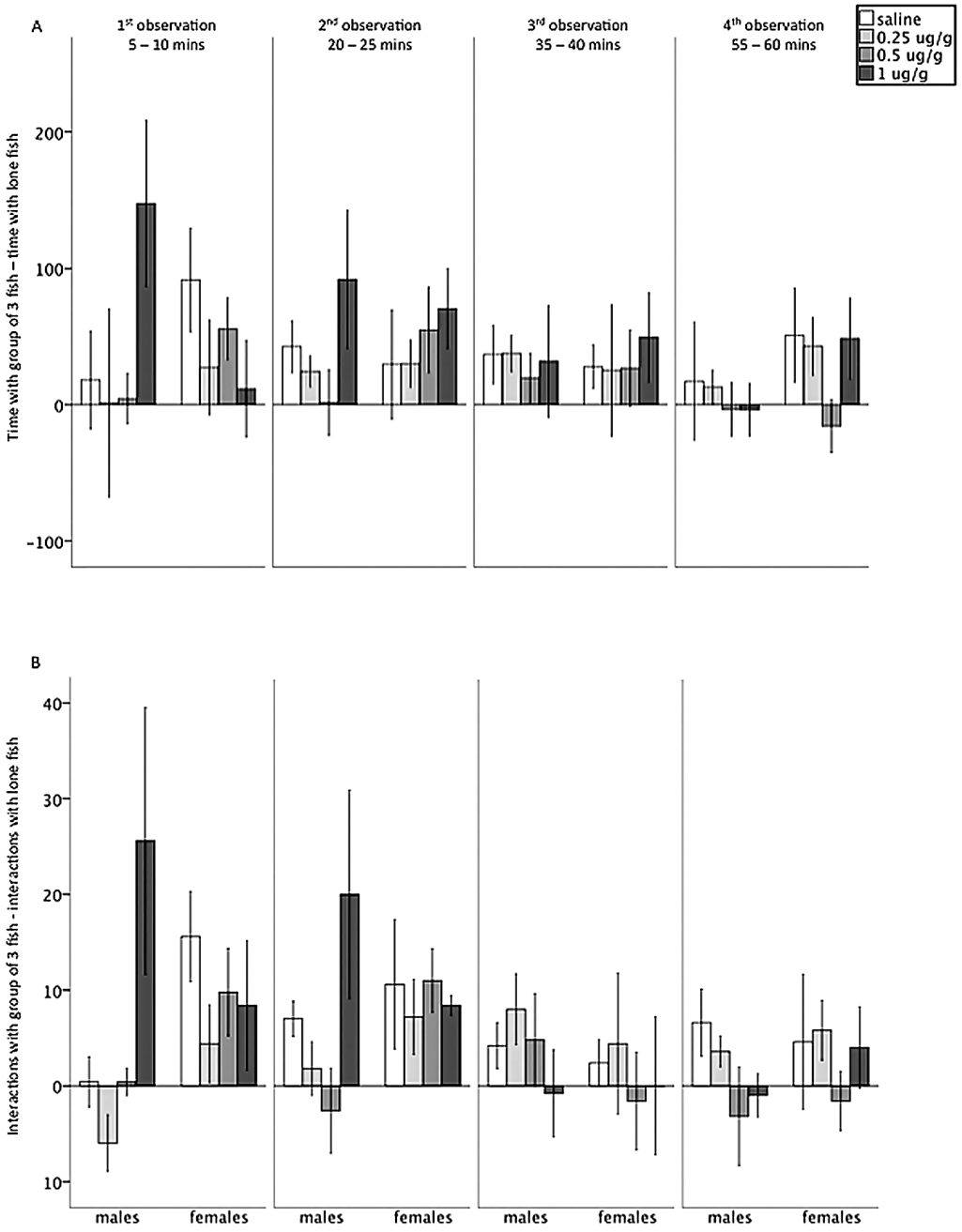
but not females that were given the high dose (1 μg/g body mass) of OTA tended to prefer the group of three fish over the lone fish (Figure 3A).

During the first observation period there was a sex by OTA treatment interaction on the time spent associating with either of the stimuli ($F_{3,32} = 3.33$, $p = 0.032$). However, when we broke this interaction down by sex, the treatment effect did not reach significance for either males ($F_{1,16} = 1.91$, $p = 0.17$) or females ($F_{1,16} = 2.64$, $p = 0.09$) separately.

During the second, third and fourth observation periods, we did not find any effect of sex, dose nor a sex by OTA treatment interaction on the preference for *N. pulcher* to associate with the group of three fish compared to one fish (2-way ANOVAs; all $F < 1.9$, all $p > 0.17$; Figure 3A). Likewise, during the latter three observation periods, there was no significant effect of OTA treatment, sex or a sex by treatment interaction on the time spent associating with either of the stimuli (all $F < 2.82$, all $p > 0.06$).

3.2.2. Social interactions

During the first observation period (5–10 min post-injection) we found a significant main effect of the OTA treatment (2-way ANOVA; $F_{3,32} = 3.86$, $p = 0.018$; Figure 3B) on the number of attempts to interact with the group of three fish versus the lone fish whereby fish given the highest dose of OTA showed the most interactions with the group of three fish compared to the lone fish. However, there was also a significant sex by treatment interaction ($F_{3,32} = 3.39$, $p = 0.03$). In order to unpack this interaction, we re-ran the analysis separately for males and females. Male *N. pulcher* showed a significant OTA treatment effect (1-way ANOVA; $F_{1,16} = 6.17$, $p = 0.005$; Figure 3B). Specifically, males that received the highest dose of OTA (1 μg/g body mass) showed a pronounced tendency to interact with the group of three stimulus fish more than the lone stimulus individual, interacting significantly more with the group of three fish than males given a control injection of saline (Fisher's LSD; $p = 0.016$), the low OTA dose ($p = 0.001$), or the intermediate OTA dose ($p = 0.018$). Females, by contrast, did not show any significant effect of treatment during the first observation period (1-way ANOVA; $F_{1,16} = 0.91$, $p = 0.46$; Figure 3B). There was a sex by OTA treatment interaction on the total number of interactions with either of the stimulus groups during the first observation period ($F_{3,32} = 302$, $p = 0.044$). However, when we broke this interaction down by sex, the treatment effect did not reach significance for either males ($F_{1,16} = 2.06$, $p = 0.15$) or females ($F_{1,16} = 0.98$, $p = 0.43$).



We did not find any effect of sex, OTA treatment nor a sex by OTA treatment interaction on the preference to interact with the group of three fish compared to the lone stimulus individual during any of the three latter observation periods (2-way ANOVAs; all $F < 2.03$, all $p > 0.13$; Figure 3B).

During the second observation period, there was a significant OTA dose by sex interaction on the total number of interactions across stimulus groups ($F_{3,32} = 3.36$, $p = 0.031$). However, when we separated this analysis by sex to decompose this interaction, we did not find a significant treatment effect in either sex (males: $F_{1,16} = 1.83$, $p = 0.18$; females: $F_{1,16} = 2.21$, $p = 0.13$). During the third and fourth observation periods there was no effect of sex, OTA treatment or an OTA treatment by sex interaction on the total number of interactions with any of the stimulus fish across stimulus types (all $F < 2.62$, all $p > 0.07$).

4. Discussion

A complex picture of the role of oxytocin and its homologues in regulating social behaviour is emerging, and it is becoming increasingly clear that blanket predictions across taxa are currently not possible (Goodson, 2013). Supporting this contention, in this study, we detected relatively weak evidence that IT is an important proximate regulator of sociality in *N. pulcher*. While we did document differences in behaviour at certain doses of both IT and OTA, these differences tended to be fairly subtle, and were inconsistent with the idea that IT has a prosocial effect. Our study joins an increasing number of reports that challenge the naïve prediction that the oxytocin family of nonapeptides is universally prosocial across species and contexts (see reviews by Churchland & Winkielman, 2012; Goodson, 2013).

Consistent with a previous study (Reddon et al., 2011a) we found that male *N. pulcher* showed a stronger preference for the group of three fish that did females, suggesting that females and males value on different characteristics of the stimuli when making this sort of social decision. We found that both a high and a low dose of IT reduced the preference for a group of

Figure 3. (A) Mean ± SE time focal fish spent associating with and (B) mean ± SE number of interactions (through the glass barrier) with the group of three conspecifics minus the time/interactions with the lone individual during each of four observation periods following treatment with one of three experimental doses of an oxytocin receptor antagonist or a vehicle-only control. There was no significant effect of sex or treatment on association time during any of the observation periods, but a significant effect ($p < 0.05$) of treatment on number of interactions in males but not females during the first observation period, whereby males that received the high dose showed a greater number of interactions with the group of three fish. $N = 5$ fish per sex per treatment.

3 conspecifics over a lone individual in *N. pulcher* of both sexes, while an intermediate dose of IT did not have any effect relative to a saline control injection. Furthermore, high and low dose of IT reduced the tendency for *N. pulcher* to associate with conspecifics in general, suggesting these treatments had an anti-social effect. This unexpected dose–response pattern, whereby an intermediate dose had no effect while high and low doses altered behaviour, is difficult to interpret. Perhaps there exists a dose of IT that increases large group preferences in *N. pulcher*, but that dose is between either our low and intermediate dose or between our intermediate and our high dose. If the IT dose–response curve crosses the no-effect line twice in an inverted u-shape, then our intermediate dose may have coincidentally aligned with one of those crossing points, while our high and low dose are both below the no effect line on either arm of the inverted-u. In our second experiment, we administered one of three doses of an oxytocin receptor antagonist or a saline control and found that a high dose of OTA increased rather than decreased sociality in males, whereas females were unaffected by any dose. The fact that the OTA treatment affected males but not females may stem from the fact that the sexes seem to be value different social parameters in this test (Reddon et al., 2011a), although the male typical preference for the group of three fish was not observed in the control group of the second experiment. The effect of our treatments appeared to wear off rapidly, and in both experiments we saw treatment differences in behaviour only during the first observation period.

Surprisingly, the control treatments in each of the two experiments, particularly for males, had different results. In the second experiment, the saline treated males unexpectedly showed no preference for the group of three fish contrary to the results of experiment 1 and to previous findings in this species using the same experimental setup (Reddon et al., 2011a). The control females also showed a greater preference for the group of three fish in the first experiment. We are uncertain why the saline treatment produced this unexpected pattern in the second experiment. Given that we had only 5 fish per sex per treatment in the second experiment, it is possible that the lack of preference for the group of three fish in the control group, particularly for males, is an artefact and should be interpreted cautiously. If the saline treated males had shown the expected preference for the group of three fish over the lone individual in the second experiment it would suggest that perhaps it is the low and intermediate doses of OTA that are suppressing sociality in males.

N. pulcher are an obligately grouping species and are never found alone or in breeding pairs without subordinates (Taborsky & Limberger, 1981; Balshine et al., 2001; Wong & Balshine, 2011a). Perhaps because social behaviour is such an integral part the behavioural biology of this species, the grouping response may be very resistant to disruption. A previous study did find behavioural effects of exogenous IT on more subtle aspects of *N. pulcher* social behaviour (Reddon et al., 2012), so it is possible that IT predominantly regulates more fine-scale and context-specific aspects of *N. pulcher* behaviour, while the motivation to associate with conspecifics is too strong in *N. pulcher* to observe a large effect of acute IT manipulations.

Another possible explanation for our results stems from the fact that our manipulations were delivered peripherally, with the assumption that both IT and OTA pass sufficiently into the brain to have a centrally-mediated effect on behaviour. The majority of the vast literature on exogenous oxytocin effects on human behaviour is based on the premise that peripherally administered oxytocin is reaching the brain in adequate quantities to produce centrally mediated effects (see MacDonald & MacDonald, 2010 for a review and Churchland & Winkielman, 2012 for a critique), and our study joins a growing literature that has reported behavioural effects from peripherally administered nonapeptides and blockers in non-human animals (e.g., Propper & Dixon, 1997; Semsar et al., 2001; Lema & Nevitt, 2004; Santangelo & Bass, 2006; Mennigen et al., 2008; Goodson et al., 2009; Filby et al., 2010; Madden & Clutton-Brock, 2011; Braida et al., 2012; Reddon et al., 2012). Thus, there is good evidence that peripheral nonapeptide treatments can generate behavioural effects. However, it is possible that one or more of our treatments did not pass into the brain at a sufficient quantity to have an effect on central receptors and instead exerted its effects through an indirect pathway involving peripheral receptors. It is also possible that the high doses passed into the brain, while the lower doses did not. This seems particularly possible for OTA, for which we saw effects of the high dose only. The blood-brain barrier generally has low permeability to nonapeptides (Ring et al., 2006; Norris, 2007; Ring, 2011; Churchland & Winkielman, 2012), although, fish blood-brain barriers may have greater permeability to neuropeptides than in birds or mammals (Bernstein & Streicher, 1965; Olson et al., 1978). There is also evidence that peripheral and central actions of nonapeptides are tightly integrated (Ross & Young, 2009; Ross et al., 2009a, b;

Goodson & Thompson, 2010) and the activation of peripheral receptors may therefore result in downstream effects on behaviour mediated ultimately by central nonapeptide systems. The additional step added by the peripheral pathway may explain some of our unexpected results. There is a growing appreciation that peripheral receptors may play an important role in regulating social behaviour (Churchland & Winkielman, 2012; Klatt & Goodson, 2012; Pedersen & Tomaszycki, 2012; Goodson, 2013). The mounting evidence demonstrating behavioural effects from peripheral nonapeptide manipulations, sometimes with unexpected results, suggests that further research on peripheral nonapeptide effects is needed.

In summary, we explored the effects of manipulations to the isotocin system on sociality in a highly social cichlid fish, *N. pulcher*. We found some support for the hypothesis that the isotocin is a regulator of sociality, namely, that both exogenous isotocin and an oxytocin receptor antagonist altered grouping behaviour. However, the effects we observed tended to be weak, transient and not in the predicted direction. This study joins a small number of published reports explicitly investigating the role of oxytocin and its homologues in regulating sociality and a small number of studies looking at the effects of experimental isotocin manipulations on social behaviour in fish. Our results highlight the need for additional research on a greater diversity of taxa exhibiting a variety of social systems. Without these additional data, it is not currently possible to make strong directional predictions about the role that the oxytocin family of nonapeptides plays in regulating sociality and social behaviour in general across the diversity of animal life.

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