Information depends on context: behavioural response to chemical signals depends on sex and size in crayfish contests

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Abstract
Securing information about oneself or an opponent can be crucial to update the likelihood of winning a contest and the relative costs of continuing or escalating. This information can subsequently reduce costly errors. However, information encoded in signals exchanged by opponents can differ based on context. We sought to unravel these differences by pairing male and female crayfish (*Orconectes rusticus*) under varying sex and size conditions. A pre-optimized technique was used to visualize a well-studied contest signal in crayfish (i.e., urine). Behavioural responses were quantified prior to and after the release of that signal. There was a characteristic de-escalation of behavioural intensity after an opponent released urine. However, behavioural changes after the release event were dependent on the sex and the relative size of the opponents. Urine also significantly altered both sender and receiver behaviour, but lack of behavioural differences suggests urine plays a role in both opponent and auto-communication.

Keywords
agonistic behaviour, chemical communication, assessment.

1. Introduction
Animals gather information from a fluctuating environment to make decisions directly tied to survival and reproduction (Dusenberry, 1992; Dall et al., 2005). For species that engage in agonistic contests over resources, the social and physical status of individuals within the local population can be an important source of information dictating contest persistence. While intraspecific contests can provide access to resources, contests are costly, and
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engaging with superior opponents is disadvantageous. Utilizing information about oneself or an opponent can provide important information that can directly affect contest engagement, escalation, and resolution (Arnott & Elwood, 2009). These decisions, which can influence contest behaviour based on a fluctuating cost to benefit ratio, are known as assessment strategies (Parker, 1974; Arnott & Elwood, 2008, 2009).

Broadly, assessment strategies fall along a spectrum ranging from self to mutual assessment, based on the type of information available and deemed to be in use to dictate contest escalation or retreat (Fawcett & Mowles, 2013; Mesterton-Gibbons & Heap, 2014). Self-assessment strategies are associated with information regarding ‘self’ only (i.e., energetic reserves, fighting ability) and a mutual assessment strategy hinges on the ability of individuals engaged in a contest to ascertain relative fighting ability between oneself and an opponent (Taylor & Elwood, 2003; Arnott & Elwood, 2009). All animals are expected to have some amount of information about one’s own resource holding potential (RHP), or overall fighting ability, but the distinction between self- and mutual assessment appears to be determined by the type of information available regarding potential opponents (Mesterton-Gibbons & Heap, 2014). General ‘public’ information about potential contestants is approximate and more likely to facilitate a self-assessment type strategy. This type of information is gathered through prior experience or an approximated probability of where one’s RHP falls within the population distribution (Fawcett & Johnstone, 2010; Mesterton-Gibbons & Heap, 2014). In other words, how likely is an individual to win or lose a contest based on the proportion of previous contests won or lost or the proportion of conspecifics stronger than that individual in the population (Mesterton-Gibbons & Heap, 2014)? Gathering specific (and perhaps more reliable) information directly from a particular opponent would lend to a relative comparison of RHP and thus a mutual assessment type strategy (Enquist et al., 1990; Mesterton-Gibbons & Heap, 2014). To gain this type of information about an opponent, individuals are likely to exchange signals via displays or direct physical interactions (Mesterton-Gibbons & Heap, 2014). Because there is a positive selective pressure to both the sender and receiver to exchange the information efficiently (Otte, 1974), this information exchange can provide contestants with potentially more reliable information about the costs of engaging an opponent.
Exchange of information in animal contests can mitigate costs before, during, and after physical contact. Pre-contest exchanges take the form of cue assessment or signal displays and likely convey information about social status of the potential opponents (Arnott & Elwood, 2009). For example, coloration and visual displays in grey triggerfish (Cleveland & Lavalli, 2010), vocalizations (bellows) in bison (Wyman et al., 2012), or chemical badges in Iberian rock lizards (Martín et al., 2007) appear to tie directly to dominance status and influence the likelihood of engaging conspecifics carrying these badges/exchanging this information. Post contest displays are also gaining more attention as a means to convey post-fight status (i.e., dominance or submission). Post-victory stridulations in mangrove crabs (Chen et al., 2014) and submissive electric chirps in electric fish (Batista et al., 2012) are used to deter further physical contact and reduce costs after the contest has been ‘settled’. Once engaged in a contest, information can be directly tied to one’s own fighting capacity or some relative measure of competition with the opponent (Arnott & Elwood, 2009).

Several decapod crustacean species (e.g., fiddler crabs, hermit crabs, lobsters, crayfish) have been established as models for the role signals play in agonistic contest outcome and behaviour (Bergman & Moore, 2005a; Zeil & Hemmi, 2006; Aggio & Derby, 2011; Breithaupt, 2011; Mowles & Briffa, 2012). Diverse visual and tactile displays, mostly relating to the chelae, have been implicated in pre/post contest displays and assessment strategies (Zeil & Hemmi, 2006; Mowles & Briffa, 2012). However, fully aquatic species have demonstrated a heavy reliance on chemical information in most aspects of their behaviour, particularly contests (Hay, 2009; Aggio & Derby, 2011; Breithaupt, 2011). Specifically, experimental manipulation of chemical information exchange during dyadic contests has demonstrated significant effects on status recognition (Zulandt-Schneider et al., 2001; Shabani et al., 2009), winner effects (Bergman et al., 2005; Johnson & Atema, 2005), and pre-copulatory fighting bouts (Bushmann & Atema, 1997; Simon & Moore, 2007; Berry & Breithaupt, 2010) in various lobster and crayfish species. The bulk of these studies have implicated urine as the primary vehicle for this chemical information. From a metabolic point of view, urine is used for osmoregulation (Vogt, 2002). Yet, musculature and hormonal glands surrounding the nephropores (urine release sites) allow for modulation of content (i.e., chemical composition) as they appear to play a role in pheromone production and are likely linked to the controlled release
of chemical products into the urine (Bushmann & Atema, 1996). Previous research has shown that urine output is significantly increased during contest behaviour (Breithaupt & Eger, 2002), the absence of urine alters contest outcomes (Zulandt-Schneider et al., 2001; Bergman et al., 2003), and urine can influence behaviour in the absence of physical interaction (Bergman & Moore, 2005b). Taken together, we can conclude that crustacean urine is a potent chemical signal that drives contest outcomes and decisions.

However, numerous studies have demonstrated that male and female crustaceans use this information differently. Due to large population densities (Mather & Stein, 1993; Perry et al., 1997; Nystrom, 2002; Davis & Huber, 2007) and propensity for agonistic behaviours (Bovbjerg, 1953; Moore, 2007), both male and female crayfish frequently engage in inter- and intra-sexual fights for resources (Bergman & Moore, 2003; Martin III & Moore, 2007; Fero & Moore, 2014). Males and females are capable of chemical recognition of the other sex (Stebbing et al., 2003; Belanger & Moore, 2006), and they exhibit differing contest assessment strategies (Wofford et al., 2015). This led us to hypothesize that males and females respond differently to urine release during contests.

This study aimed to establish first the status of urine as a chemical signal in crayfish contests. Specifically, the release of this chemical stimulus by a sender should elicit significant behavioural changes in a receiver immediately after release. While we know that urine can determine contest outcome and dynamics, we sought to examine fine scale temporal dynamics (i.e., within contest decisions) surrounding a release event. Furthermore, we wanted to determine if the behavioural changes caused by urine release were context dependent. Specifically, do factors that change the social context of an agonistic interaction (i.e., sex of the opponents, body size of the opponents) significantly alter behavioural reaction to the release of urine during a contest? We used a previously developed method (Breithaupt & Eger, 2002) to visualize and quantify urine release. Behavioural changes were measured before and after a release event to determine if information exchange had taken place. We staged contests in which dyads varied in sex and opponent size to examine these hypothesized behavioural changes under different ecologically relevant social contexts.
2. Methods

2.1. Animals

Male and female crayfish, *Orconectes rusticus*, were collected using a kick seine method from the Portage River near Bowling Green, OH, USA (Wood County, 41°21′42″ N, 83°35′28″ W). All crayfish were measured, using calipers, for chelae length (males: mean ± SEM = 2.40 ± 0.06 cm; females: mean ± SEM = 2.58 ± 0.23 cm) and post orbital carapace length (males: mean ± SEM = 2.48 ± 0.04 cm; females: mean ± SEM = 2.63 ± 0.03 cm). Crayfish with intact walking legs, chelae, and sensory appendages were housed in a recirculating system within an environmental chamber held at a constant temperature (23°C) and light/dark cycle (12L:12D). All crayfish were visually and mechanically isolated for a minimum of seven days to eliminate effects of prior social history developed in the field (Karavanich & Atema, 1998; Zulandt-Schneider et al., 2001). Crayfish were fed an ad libitum diet of commercial rabbit food pellets three times a week. Each animal was used only once in these trials, and were frozen after a trial as required by collection permit protocols. An animal was secured in an individual plastic container (7.5 × 7.5 × 7.5 cm) and placed in a commercial freezer (approximately −15°C) until movement ceased (approximately 10–15 min). All crayfish were in non-reproductive form. Males were considered non-reproductive if their stylets were cornified and non-bifurcated while females were considered non-reproductive if glair, a white substance used for egg adherence, was absent from the ventral portion of the telson and the base of the walking legs (McLay & van den Brink, 2016).

2.2. Experimental design

The purpose of this experiment was to quantify a chemical signal and examine its use during agonistic assessment of crayfish contests. Chemical visualization was possible via the injection of a fluorescent dye (explained below). The presence of the chemical signal was correlated with the behavioural dynamics of fighting in same-sex and mixed-sex contests. Thus, we had a fully factorial 3 × 2 design with the first factor (sex of opponents) having three conditions (male only, female only, and mixed sex) and the second factor (combatant size) having two conditions (size matched and size different). Within each of these sex condition treatments, there were two size conditions in which opponents were either size matched (SM) or size different (SD). Crayfish were matched based on carapace length. Size-matched
animals were selected such that carapace length of the two contestants was within 10%. Beyond a 10% carapace length difference, larger animals begin winning significantly more often than chance (Pavey & Fielder, 1996; Daws et al., 2002). Consequently, size different animals were randomly paired to achieve carapace length differences between approximately 11 and 30% (range: 11.11–33.33%; mean ± SEM: 17.57 ± 0.55%). We decided to cap size differences at 30% to achieve more realistic pairings (Bergman & Moore, 2003; Fawcett & Mowles, 2013). In mixed sex contests, the size different treatment included equal trial numbers in which males were larger and in which females were larger.

2.3. **Injection protocol**

Both contestants used per trial were injected with a 0.05% sodium fluorescein (Sigma: F-6377, Lot 103H3412; Sigma-Aldrich, St. Louis, MO, USA) solution dissolved in Van Harreveld crayfish saline (Van Harreveld, 1936). All animals were subjected to injections at 0.01 ml/gram of body mass using a 1 ml syringe affixed with a 26 gauge (0.45 × 13 mm) needle. This protocol was based on techniques first developed by Breithaupt & Eger (2002) and modified by Bergman et al. (2005) and Simon & Moore (2007). The injection site was located on the dorsal surface of the carapace. The needle was inserted approximately 2 mm at the injection site, and the syringe contents were then slowly injected into the body cavity. The needle was quickly removed and a small dot of Loctite® gel control super glue was placed over the injection site, followed by a small strip of black electrical tape. Super glue was used to quickly adhere the tape to the animal to stop haemolymph loss. Super glue was favoured over the wax method used by Breithaupt & Eger (2002) to reduce weight additions to the animal, which could potentially skew perceived RHP. Tape strips did not exceed 0.25 square cm and thus only covered a small section of the animal’s carapace. Additionally, *O. rusticus* are primarily dark brown and green in colour, making the black tape inconspicuous; aggressive behaviours did not appear altered based on the presence of the tape patch. Animals were allowed a minimum 1-h recovery period before being used in a trial and were monitored for signs of distress.

2.4. **Fight arena**

All trials were conducted in a dark room using a specialized arena constructed of ‘2 × 4’ blocks of wood (4.44 × 9.52 cm) painted black to reduce
A schematic of the fight arena set up is shown in Figure 1. The left drawing depicts the view into the test arena from the side-view camera, while the right drawing, rotated 90 degrees, shows the set up from the side. Black lights were placed around the top of the structure to facilitate fluorescent release.

A 21 l aquarium (40.6 × 20.3 × 25.4 cm) was affixed with two opaque Plexiglas® inserts to reduce tank dimensions (22.9 × 20.3 × 25.4 cm). This reduction increased the probability of agonistic behavior and ensured that the entire arena was in view of the side camera (defined below). The aquarium was filled with 7.1 l of pre-conditioned tap water and suspended on top of two ‘2 × 4’ wood pieces in order to allow for filming from underneath. Both cameras used were Sony HDR-CX405 9.2 megapixel cameras (Sony Electronic, Novi, MI, USA). The side camera was positioned on a tripod separate from the arena structure, approximately 20 cm from the side of the aquarium. The bottom camera was affixed to a black ‘2 × 4’ suspended approximately 48 cm from the bottom of the aquarium. Both cameras were set to low lux to account for the lighting conditions. The focus and exposure were adjusted manually before each trial to achieve the clearest picture possible. Five 60-W black lights were affixed to the top and sides of the wooden structure to facilitate lighting.
of the tank and visualization of the fluorescein-laced urine. While this arrangement utilized more (and greater wattage) black lights than the methods used in Bergman et al. (2005) and Simon & Moore (2007), those experiments also used two Kodak Ekta Graphic IIIA slide projectors (with approximately 300-W bulbs) with no obvious changes to fighting behaviour. Furthermore, the crayfish contests in this study demonstrated the same behavioural repertoires performed under varied lighting conditions (Zulan<ref>zulan</ref>nt-Schneider et al., 2001; Bergman et al., 2005; Simon & Moore, 2007) consistent with behaviours expected from a well-established crayfish fight ethogram (Bergman & Moore, 2003).

2.5. Fight protocol

Crayfish were placed into the arena and were visually and physically isolated for a 15-min period to allow them to acclimate to the trial conditions (Bergman et al., 2005; Simon & Moore, 2007). Trials lasted for 20 min, and the arena was drained and rinsed between each trial. The room was darkened and the black lights were turned on prior to crayfish retrieval and placement in the test arena. Lighting conditions were then held constant across acclimation and trial periods to minimize behavioural differences. Crayfish were not blinded for use in these trials. Previous studies (Bergman et al., 2005; Simon & Moore, 2007) and preliminary trials (unpublished data) have demonstrated that social behaviours are largely unaffected by the visual presence of fluorescein.

2.6. Analysis

Only trials in which one or both crayfish demonstrated visible urine release during physical fighting behaviour (i.e., opponent to opponent contact) were considered for analysis. We obtained 92 trials in which physical contact classified as fighting behaviour between opponents occurred. Of these trials, 68.5% (63 trials) demonstrated visible urine release by one or both opponents. In order to keep trial numbers as similar as possible across treatment types, a female size matched (SM) trial in which urine release was visible during an interaction was randomly chosen to be excluded from analysis. This left us with 62 total trials that met the release criteria.

Videos obtained from the side view camera were the primary source of data for analysis. Videos obtained from the bottom view camera were used to corroborate or supplement incomplete data from the side camera videos.
Videos were scored manually by an observer blind to treatment type and included the determination of a winner and loser for each contest, identification of intensity levels throughout the contest for each opponent, and quantification of urine release events. Contest winners and losers were determined by retreat behaviours (Moore, 2007). Specifically, losers were determined as those that retreated from the interaction via backwards walking or a rapid retreat (i.e., tail flip). The contest was considered over when the two individuals were separated by more than two body lengths for at least 15 s. The intensity levels recorded were determined using a modified version of previously developed ethograms (Table 2: Bergman et al., 2005; Simon & Moore, 2007). Urine release events were recorded alongside the changes in intensity levels for both contestants. A release event was determined to have occurred when fluorescein was ejected from the nephropores. Behavioural changes and urine release events were recorded for both individuals (i.e., senders and receivers) in each trial. Senders were defined as the animal that released urine at a given time point and receivers were defined as the animal that did not release at that time point. Therefore, the sender and receiver title was assigned for each release event.

Linear mixed models (LMM) followed by analysis of deviance tables using Type II Wald Chi Square tests (Zuur et al., 2009) were used to determine the effect of opponent sex and size on behaviour pre and post urine release in the lme4 package (Bates et al., 2015) in R statistical software (version 3.3.0) (R Development Core Team, 2016). Models were constructed using sex treatment (male only, female only, or mixed sex dyads), size treatment (size matched or size different dyads), and time (pre or post release) as fixed effects and trial number and release number as nested random effects. Differences of least squares means (‘difflsmeans’) from the lmerTest package (Kuznetsova et al., 2016) in R was used as a post hoc test to discern which factors were responsible for significant differences within significant main effects detected by the ANOVAs.

Preliminary mixed model analyses revealed that sender and receiver behaviour did not significantly differ after urine release events. Therefore, we chose to focus only on sender behaviour for the remainder of our analyses. Because the number of release events per contest was inconsistent across trials, we chose to analyse only the first three release events that occurred in each trial, leaving us with 78 total observations (i.e., release events) (Table 1).
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Table 1.
Trial numbers of release events loaded into LMM.

<table>
<thead>
<tr>
<th>Sex treatment</th>
<th>Size treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size matched</td>
<td>Size different</td>
</tr>
<tr>
<td>Male versus Male</td>
<td>$N = 15$</td>
<td>$N = 6$</td>
</tr>
<tr>
<td>Female versus Female</td>
<td>$N = 9$</td>
<td>$N = 15$</td>
</tr>
<tr>
<td>Male versus Female</td>
<td>$N = 18$</td>
<td>$N = 15$</td>
</tr>
</tbody>
</table>

The table represents treatment types for the two factors of interest (opponent sex and relative opponent size) and the number of release events per treatment loaded into the LMM analysis.

An Excel macro was used to compile the appropriate response variables (described below) obtained from manual video scoring and used in the LMM. Mean behavioural intensity and mean shifts in behavioural intensity were calculated at the time of the urine release event, as well as 2, 5 and 10 s pre- and post-release. The 10 to 1 (overall behavioural intensity change over 10 s) measure was calculated by subtracting the intensity 10 s before the release event from the intensity at the time of the release event (pre 10 to 1) or by subtracting the intensity at the release event from the intensity 10 s after the release event (post 10 to 1). Intensity levels recorded were also classified into non-contact phase, non-escalated contact phase, or escalated phase (Table 2). The proportion of time spent at each of those intensity levels was calculated pre- and post-release. Finally, the sequence of behaviours was created by turning the numerical intensities into string values, denoting the numerical sequence of behaviours that took place leading up to and following a release event. The behavioural sequence was not considered in the LMM, but these values were later averaged across all contests types to create graphical representations of intensity changes over time.

We performed a test for collinearity (Zuur et al., 2009) and found a significant, positive correlation between the mean behavioural intensity at 2, 5 and 10 s. Consequently, we chose to analyse mean behavioural intensity at the 5 s time point only. The best-fit model for our response variables included the interaction of all three fixed effects (sex treatment, size treatment, and time) and the inclusion of both random effects (trial and release number). Release number was nested under trial number. We determined the best-fit model using the Akaike Information Criterion (AIC) value for each model.
### Table 2. Extended ethogram.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Intensity level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-contact phase</td>
<td>−2</td>
<td>Tailflip away from opponent</td>
</tr>
<tr>
<td></td>
<td>−1</td>
<td>Walking backwards away from opponent, no tail flip</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Ignore opponent with no response or threat display</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Approach without a threat display</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Approach with threat display using meral spread</td>
</tr>
<tr>
<td>Non-escalated contact phase</td>
<td>3</td>
<td>Touching opponent with open or closed claws; no forceful movement</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Antennal whipping</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Forcefully pushing opponent away with one closed claw</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Initial claw use by boxing or pushing with closed claws</td>
</tr>
<tr>
<td>Escalated contact phase</td>
<td>7</td>
<td>Active claw use by boxing or pushing with open claws</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Active grabbing of opponent’s claws and other appendages</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Unrestrained grabbing and tearing; attempting to rip or tear opponent’s claws or appendages</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Inversion of one or both contestants with locked claws and unrestrained grabbing and tearing</td>
</tr>
</tbody>
</table>

The table represents an extended ethogram used in this study which was based on a previously developed and utilized ethogram (Bergman et al., 2005; Simon & Moore, 2007). The numerical intensity levels represented in the middle column were used to determine behavioural intensities utilized for analyses. For proportional analyses, we broadly classified these into three separate phases (left column).

### 3. Results

#### 3.1. Qualitative analysis

The release plots (averaged across all treatments) revealed a characteristic increase in agonistic intensity leading up to the release event and then an equally characteristic drop in behavioural intensity (behavioural de-escalation) following a urine release event (Figure 2A). Release plots separated by sex treatments (Figure 2B) and relative opponent size (Figure 2C) demonstrated differences in behavioural intensity changes that were subsequently verified by statistical analyses.
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Figure 2. Behavioural changes pre and post urine release. Release plots demonstrate qualitative changes in behavioural intensity over time in relation to a urine release event. 2A shows changes in behaviour at each second averaged across all release events ($N = 78$). 2B shows differences in behavioural changes between different sex conditions (male–male ($N = 21$), female–female ($N = 24$), male–female ($N = 33$)). 2C shows differences in behavioural changes between size matched ($N = 42$) and size different ($N = 36$) conditions. The red dashed line at time point 0 denotes the release event on all graphs.
3.2. LMM analyses

3.2.1. Effect of time

Time played a significant role in several response variables (Table 3). The change in intensity level over ten s (i.e., 10 to 1) differed significantly pre- and post-release ($\chi^2 = 21.93$, df = 1, $p < 0.001$). Intensity level increased prior to urine release (positive 10 to 1 value) and decreased after the urine release event (negative 10 to 1 value). There was also a significant time effect on the second to second behavioural intensity shifts occurring at the 5 s time point ($\chi^2 = 5.96$, df = 1, $p < 0.05$) and at the 10 s time point ($\chi^2 = 29.40$, df = 1, $p < 0.001$). In both cases intensity shifted from lower to higher levels pre-release (positive values), but shifted from higher to lower intensities (negative values) post-release.

3.2.2. Effect of sex

Sex of the opponents significantly influenced the proportion of time spent in the non-contact phase behaviours ($\chi^2 = 6.36$, df = 2, $p < 0.05$). Specifically, female same sex dyads spent a greater proportion of time in the non-contact phase compared to male same sex contests and mixed sex contests (Least Squares Means: $t = 2.73$, df = 27.2, $p < 0.05$).
Table 3. LMM analyses.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Fixed effects</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of non-contact behaviours</td>
<td>Sex treatment</td>
<td>6.35</td>
<td>2</td>
<td>0.04</td>
</tr>
<tr>
<td>Proportion of non-escalated contact behaviours</td>
<td>Sex treatment $\times$ Size treatment $\times$ Time</td>
<td>5.82</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>Behavioural intensity overall change (10 to 1)</td>
<td>Time</td>
<td>21.93</td>
<td>1</td>
<td>$2.82 \times 10^{-6}$</td>
</tr>
<tr>
<td>Mean behavioural intensity shift (5 s)</td>
<td>Time</td>
<td>5.96</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Sex treatment $\times$ Time</td>
<td>9.06</td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean behavioural intensity shift (10 s)</td>
<td>Time</td>
<td>29.40</td>
<td>1</td>
<td>$5.90 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

The table represents significant findings from the LMM analyses and shows response variables significantly impacted, fixed effects responsible for changes, and reports from analysis of deviance tables.

3.2.3. Interaction effects

There was a significant interaction effect of time and sex treatment on behavioural intensity shifts at the 5 s time point ($\chi^2 = 9.06, \text{df} = 2, \ p < 0.05$). Mixed sex contests exhibited a significant difference in intensity shifts pre- and post-release (Least Squares Means: $t = -3.85, \ p < 0.001$). Specifically, there were positive behavioural intensity shifts pre-release and negative shifts post-release. Male and female same sex contests did not demonstrate a significant difference in behavioural shifts pre- and post-release. However, mixed sex contests also demonstrated significantly greater shifts pre-release (i.e., positive values) than female same sex contests (i.e., negative values) (Least Squares Means: $t = -2.11, \ p < 0.05$).

The proportion of time spent in the non-escalated contact phase was the only response variable impacted by the interaction of all three fixed effects; however, this difference was only marginally significant ($\chi^2 = 5.82, \text{df} = 2, \ p = 0.05$; Table 3). The post hoc did not reveal any significant differences.

4. Discussion

We found that the presence of a chemical signal (urine) induced a stereotypical de-escalation of aggressive behaviour in crayfish contests, regardless of the sex or relative size of the contestants (Figure 2). Behavioural intensity shifts (e.g., 10 to 1 data, shifts at 5 and 10 s) demonstrated significant
changes from lower to higher intensity levels pre-release and decreases to lower intensity levels after a release event. These data indicate an escalation phase occurs prior to urine release and de-escalation occurs after release. This is consistent with our expectation that urine is an important driver in contest dynamics in crayfish contests.

We also found that the sex and relative size of the opponents play a significant role in the magnitude of change elicited by this chemical signal. The sex of the opponents dictated the intensity of behaviour and the magnitude and direction of intensity changes seen due to a release event. For instance, while mixed sex contests followed the pre-release escalation and post release de-escalation trend we would expect, female and male same sex contests did not demonstrate an obvious change. These data suggest that urine potentially sends a different message in same and mixed sex contests, and supports previous studies that suggest differences in contest resolution for same sex and mixed sex contests in crayfish (Wofford et al., 2015). The role of opponent size requires a more complex explanation. While the post hoc test did not reveal any significant differences in the proportion of time spent at non-escalated contact behaviours, some general trends were present. Size matched and size different dyads only seemed to play a pronounced role in mixed sex contests. Regardless of opponent size, females showed little change in the proportion of time spent at non-escalated contact behaviours. Males exhibited consistent increases in proportion of non-escalated contact behaviours performed post release, but this trend was also persistent across size matched and size different trials. However, mixed sex contests experienced opposite directional changes pre- and post-release, and these trends were dependent on relative opponent size (Figure 3). Relative opponent size not only altered the proportion of time spent at certain intensities, these trends were also dependent on the sex of the opponent. These results support the long-standing hypothesis that urine functions as a signal in crayfish contests and our hypothesis that the social information contained within is dependent on the sex and relative size of the opponents.

Signals and cues play an integral role in contest behaviour by reducing the chances of escalation and potential injury (Bradbury & Vehrencamp, 1998; Arnott & Elwood, 2009). Specifically, information exchanged during physical contact can convey information about energetic state (of oneself or an opponent) or intent to retreat (i.e., Scheel et al., 2016). This could then elicit
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Figure 3. Fixed Effects Interactive Effects on Non-escalated Contact Phase. Bar plot exhibits significant interactive effect of all three fixed variables on non-escalated contact phase ($\chi^2 = 5.82, df = 2, p = 0.05$). Female same sex contests showed little to no change in non-escalated contact behaviours post-release, regardless of size treatment. Males showed a trend towards increased proportion of non-escalated contact behaviours post-release for both size matched and size different contests. Changes for these behaviours post-release in mixed sex contests were dependent on the size treatment.

des-escalation in aggressive behaviours or retreat from a contest. These anticipated behavioural changes (i.e., des-escalation) were seen in both senders and receivers and were consistent across treatment types, suggesting that urine release conveys information pertinent to contest resolution in crayfish. This information could be driving assessment strategies in use for crayfish under different social contexts that could alter dominance relationships and, consequently, hierarchy establishment.

The sex of the opponents is one social context in which we consistently find assessment based differences. Many studies have attributed sex based assessment differences to disparate information sources driving contest persistence and escalation. Some hypotheses assert differences in RHP based
signals (i.e., weaponry strength versus visual or vocal displays), as well as the role of perceived resource value (PRV) in contest resolution. Male contests tend to be heavily tied to RHP of the opponents while female contest resolution appears to rely on the PRV, especially in reproductive periods (Draud et al., 2004; Elias et al., 2010). However, in our system non-reproductive crayfish demonstrate limited sexual dimorphism (McLay & van den Brink, 2016), and non-reproductive males and females vie for the same resources (e.g., food and shelters) throughout the season (Martin III & Moore, 2010; Fero & Moore, 2014), likely diminishing (but not negating) both RHP and RV asymmetries. This limited asymmetry coupled with previous work and the data presented here suggest that deviation in chemical information is likely an important driver in sex-based assessment differences in crayfish.

Although crayfish urine release is a means of homeostatic regulation between the hypertonic haemolymph and the hypotonic external environment, amino acids have been found in urine excretions as well as higher than normal glucose levels under stressful conditions (Vogt, 2002). Consequently, the chemical composition of this signal can change depending on various physiological and dietary factors. Given the metabolic usage of urine, the chemical composition of the urine signal could relay information about one’s internal state. Nutritional status (i.e., starvation; Schirf et al., 1987), environmental stressors (i.e., hypoxia, desiccation; Buckup et al., 2008), and exposure to toxicants (Torreblanca et al., 1992) all significantly influence chemical composition of crayfish haemolymph and tissues, which can contribute to differential composition of urine and, consequently, the ‘message’ conveyed by urine release. The sex of the individual is another variable that likely contributes to the chemical composition of crayfish urine released during contests. We know that crayfish have the ability to discriminate sex (among other things) based on odour alone (Stebbing et al., 2003; Bergman & Moore, 2005b; Belanger & Moore, 2006), and the release of urine during a contest elicits varied behavioural changes based on sex. Furthermore, mounting evidence suggests that males and females behave and perhaps communicate differently when engaged in mixed sex contests (Simon & Moore, 2007; Martin III & Moore, 2010; Wofford et al., 2015). Altogether, there is the implication that urine is conveying different information based on the sex of the individuals engaged in the contest and consequently differentially influencing contest resolution for males and females.
Size differential of the opponents also played a role in behavioural changes caused by urine release. This was unsurprising as body size is an important and ubiquitous RHP proxy known to influence assessment and contest outcome (Arnott & Elwood, 2009). However, the role of relative size was less prevalent than (and modulated by) the sex of the opponents. Only mixed sex contests seemed to be affected by the size of the opponent in terms of reaction to a chemical signal. Based on the crayfish ethogram (Table 2), non-escalated contact behaviours could be classified as ‘exploratory’ behaviours. These types of behaviours (i.e., initial touch, closed claw boxing) are likely used as low cost information collection phases (Bruski & Dunham, 1987; Seebacher & Wilson, 2007; Hsu et al., 2008; Percival & Moore, 2010). In mixed sex contests, we see a trend suggesting that different sized opponents decrease the prevalence of these exploration behaviours post urine release. However, size matched opponents increase these behaviours post-release. Because chemical information is almost certainly acting in concert with other modalities (i.e., visual or tactile information; Bruski & Dunham, 1987; Smith & Dunham, 1990; Callaghan et al., 2012), these data suggest that urine might serve as a climactic or initial signal depending on what other types of information are readily available. For example, in size asymmetrical contests visual information is available to distinguish aspects of opponent RHP once the interaction begins. However, for contests in which opponents are the same size, tactile or chemical information likely plays a much larger role. These differences in information availability might explain why we see a drop in exploratory based behaviours after urine release in size different contests (climactic information) and a rise in exploratory based behaviours in size matched contests (initial information).

Perhaps the most intriguing finding was what was not significant. There was no significant difference between sender or receiver behaviour upon urine release. This suggests that chemical information is possibly acting as a source of information for both the sender and the receiver. Previous work suggests that urine released during crayfish contests contains various neurochemicals or hormones that can be used as information about dominance or individual identification (Bergman & Moore, 2005a; Moore & Bergman, 2005). For example, serotonin and its metabolites play significant roles in crustacean aggression and are hypothesized to be excreted in urine (Huber et al., 1997). The injection of serotonin has consistently shown significant effects on crayfish aggression and contest outcome (Edwards & Kravitz, 1997;
Huber & Delago, 1998), and in vivo studies of amine metabolism in crayfish and lobsters have shown the excretion of a metabolically expensive serotonin conjugate, serotonin–O–sulfate, in urine (Huber et al., 1997). The presence of serotonin and the sulfate conjugate in urine has been hypothesized as a source of cheat-proof information in a contest. Specifically, greater relative concentrations of serotonin–O–sulfate might carry the message of a socially or energetically dominant individual (Huber et al., 1997).

Recent findings suggest that both male and female crayfish are actually using a form of self-assessment during dyadic contests (Wofford et al., 2015), leading one to hypothesize that any chemical information about internal state is available to the sender as well as a receiver. Accordingly, crayfish anatomy facilitates simultaneous chemically mediated opponent and auto-communication. Urine is released from nephropores, which are located directly beneath antennules, the primary chemosensory organs (Vogt, 2002). Meaning, crayfish can potentially obtain self-referent information about their internal hormonal or energetic state (i.e., serotonin or metabolic by-product levels). Potential high stress situations (e.g., an escalated contest or attempted predation event) could also influence internal levels of serotonin or other biogenic amines and their relative concentrations in urine excretion. Similarly, handling stress (i.e., injections) could artificially elevate these levels, influencing contest dynamics surrounding a urine release event. However, the authors are confident the hour long recovery period and the fact that both opponents underwent the injection protocol negated behavioural effects of any differences in relative amine concentrations due to stress. If urine is providing self-referent information and is acting as an external ‘checks and balances’ system for an individual, it would pay for the opponent to eavesdrop on this information. Indeed, the lack of significant difference between sender and receiver behaviour in this study demonstrates that both contestants are gaining information from urine, presenting the opportunity for assessment strategy switching or a mixed strategy in crayfish.

Recent work has called for a reorganization of the way in which we think about assessment strategies, specifically, in terms of the information utilized by contestants. Understandably, the spectrum of assessment strategies ranging between self- and mutual assessment have previously been associated with a gradient of cognitive abilities (Elwood & Arnott, 2012; Fawcett & Mowles, 2013). Mutual assessment strategies are argued to be reserved for more cognitively complex organisms (Elwood & Arnott, 2012; Fawcett &
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Mowles, 2013). Fawcett & Mowles (2013) argue that ‘comparative decision making’ required for mutual assessment is already ubiquitous across non-contest related contexts (e.g., mate selection, resource preference) and is prevalent in cognitively ‘simple’ organisms (Dussutour et al., 2010; Egge et al., 2011). Furthermore, theoretical (Fawcett & Johnstone, 2010; Mesterton-Gibbons & Heap, 2014) and empirical work (Hsu et al., 2008; Garcia et al., 2012) suggest that assessment strategies vary across populations, individuals, and even throughout different stages of a single contest.

Crayfish provide us with a model system for studying the spectrum of assessment strategies possible, as their populations are a conglomeration of different sized animals of both sexes constantly vying for resources (Bergman & Moore, 2003; Martin III & Moore, 2007) or hierarchy establishment for access to resources (Fero & Moore, 2008, 2014). Because males and females have inherently different costs and resource needs (Trivers, 1972), we would also expect them to have different choice paradigms when engaging in contests. Therefore, it is not unreasonable to assume that males and females are using different information, especially during the reproductive season when RHP and RPV asymmetries can become quite large between the sexes (Martin III & Moore, 2010). However, as in the previous assessment study by Wofford et al. (2015), non-reproductive male and female same sex contests seem to behave similarly in terms of urine release and behavioural response (Wofford et al., 2015) (Figure 2). The break-down of these trends in mixed sex contests suggests that males and females are using different information to make within-contest decisions, but this only becomes apparent in mixed sex contests. Perhaps the differential information usage is based on a source other than urine (i.e., mechanical information from chelae boxing) (Gherardi, 2002). Alternatively, the message encoded in the urine may be different for males and females. Considering that the sexes differ in chelae morphology (Stein, 1976; Gherardi, 2002) and, hypothetically, in chemical signature (Bushman & Atema, 1997; Stebbing et al., 2003; Belanger & Moore, 2006) either of these explanations is plausible.

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References


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> #build mixed model
> library(lme4)
> #Mixed model
> model.lmm.1<-lmer(Response.Variable ~ Sex.Treatment * Size.Treatment * Time + (Release.Number|Trial),
> data = urinedata, REML = FALSE)
> 
> "Response.Variable" corresponds to following list
> # Proportion of Non-Contact Behaviors
> # Proportion of Non-Escalated Contact Behaviors
> # Proportion of Escalated Contact Behaviors
> # Ten to One (Overall Intensity Change)
> # Behavioral Intensity at 5 second Time Point
> # Behavioral Intensity Shift at 2 second Time Point
> # Behavioral Intensity Shift at 5 second Time Point
> # Behavioral Intensity Shift at 10 second Time Point
> 
> #Discerning Main Effects
> Anova(model.lmm.1)
>
> #Run post hocs
> library(lmerTest)
>
> difflsmeans(model.lmm.1)

**Figure A1.** R code used for data analysis. Linear mixed models (LMM) followed by analysis of deviance tables using Type II Wald Chi Square tests were used to determine the main effects of opponent sex and size on behaviour pre- and post-urine release in the lme4 package (Bates et al., 2015). Models were constructed using sex treatment (male only, female only, or mixed sex dyads), size treatment (size matched or size different dyads) and time (pre- or post-release) as fixed effects and trial number and release number as nested random effects. Differences of least squares means (‘difflsmeans’) from the lmerTest package (Kuznetsova et al., 2016) in R was used as a post hoc test to discern significant differences within significant main effects detected by the ANOVAs.