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Long-lasting generalization triggered by a single trial event in the invasive crayfish *Procambarus clarkii*

Andrea Dissegna, Andrea Caputi, Cinzia Chiandetti*
Department of Life Sciences, University of Trieste, Trieste, Italy

Address correspondence to:

* Cinzia Chiandetti
University of Trieste
Department of Life Sciences
Via L. Giorgieri 5, I-34127 Trieste
Italy
Email: cchiandetti@units.it

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Abstract

Behavioural flexibility allows to adapt to environmental changes, a situation that invasive species have often to face when colonizing new territories. Such flexibility is ensued by a set of cognitive mechanisms among which generalization plays a key role, as it allows to transfer past solution to solve similar new problems. By means of a habituation paradigm, we studied generalization in the invasive crayfish *Procambarus clarkii.* Once crayfish habituated their alarming response to a specific water jet, we tested whether habituation transferred to a new type of water jet. Although habituation did not generalize when the new stimulus was initially presented, it surprisingly emerged 15 and 45 days later. Hence, remarkably, in *P. clarkii* a single presentation of a new event was sufficient to trigger a long-lasting form of learning generalization from previous similar stimuli, a cognitive ability that may concur in providing adaptive advantages to this invasive species.

Keywords: Generalization, Habituation, Learning, Invasive species, Crayfish

Summary statement: A single presentation of a new event is sufficient to elicit a long-lasting form of learning generalization from previous similar stimuli in *Procambarus Clarkii*

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Introduction

Humans may unintentionally spread invasive species while transporting animals, for economic or decorative reasons, outside their native geographical range (Blackburn *et al.*, 2011). Invasive species damage the new ecosystem (Ehrenfeld, 2010) and harm human health and activities (Pejchar and Mooney, 2009), affecting the local economy as well (Olson, 2006). Investigating the common characteristics of invasive species, therefore, may contribute to anticipate future human-mediated invasions, and to reduce their occurrence and negative impact (Kolar and Lodge, 2001; Blackburn *et al.*, 2011). In particular, increasing evidence attests that many invasive species share similar behavioural traits (Chapple, Simmonds and Wong, 2012; Sih *et al.*, 2012). Many of them, for example, show enhanced inter- and intraspecific aggressiveness, which in turn is positively associated with their boldness, their foraging ability and exploratory activity (e.g., Rehage and Sih, 2004; Duckworth and Badyaev, 2007; Pintor, Sih and Bauer, 2008). Hence, being aggressive may facilitate invasive species when exploring the new environment, discovering and collecting resources and displacing locals.

Behavioural flexibility is another key trait of invasive species (Sol, 2003; Pintor, Sih and Kerby, 2009; Wright *et al.*, 2010). It attests animals' capacity to accommodate behaviour to environmental changes, therefore assisting invasive species to disperse and establish outside their ecological niche (Wright, 2010). Wright and colleagues (2010) suggested that behavioural flexibility arises from two mechanisms: animals can "copy" an adaptive behaviour from others, *via* social learning; or animals can "invent" an adaptive behaviour, *via* innovation. While both these mechanisms have been linked to behavioural flexibility (Audet and Lefebvre, 2017), so far their presence have been demonstrated in few species (e.g, see Caldwell and Whiten, 2002; Brosnan and Hopper, 2014). Furthermore, innovation and social learning may represent complex behavioural traits entailing other simpler processes (Heyes, 2012; Griffin and Guez, 2014). Hence, other basic mechanisms may have a more direct effect on behavioural flexibility. In invasive crayfish, for example, these mechanisms include a broader stimulus-sensitivity to heterospecifics alarm cues, and a superior memory capacity in associative learning tasks than native species (Hazlett, 2000; Hazlett, Acquistapace and Gherardi, 2002; Hazlett *et al.*, 2003).

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Generalization could be another basic mechanism underlying behavioural flexibility. It attests the animal's capacity to use past solutions in present situations regarded as similar. In the context of predation, for example, animals generalize their antipredator response to a new predator exploiting the recurrent cues that anticipate a threat across different situations (like the sudden appearance of an odour, a noise or a looming shadow) (Ferrari *et al.*, 2007). Generalization can be considered a basic form of learning because it is universal across animals and is independent from the context, the stimulus-modality or the type of response (learned or innate) (Ghirlanda and Enquist, 2003). But, to the best of our knowledge, it has never been studied in relation to the invasive potential of a species.

With this hypothesis in mind, we tested individuals of *Procambarus clarkii* (Girard, 1852), an invasive decapod that has been introduced in Spain from the southern regions of the USA and Mexico and spread quickly all over Europe (Gherardi, 2006, 2010; Souty-Grosset *et al.*, 2016). Specifically, it arrived in Italy in 1987 for breeding purposes and the first reproductive population has been documented in 1989 in Piedmont region (Del Mastro, 1992; Aquiloni *et al*., 2010). Hence, it represents an ideal model to study the relationship between behavioural flexibility and the invasive potential of a species. One previous study (Chiandetti and Caputi, 2017) addressed generalization ability in the visual domain in *P. Clarkii*, but without providing conclusive evidence. In the present experiment, we used a similar habituation paradigm to test whether in crayfish this ancestral form of learning generalizes between a familiar and a novel water jet. Furthermore, we tested the duration of habituation for up to 45 days.

Methods

Subjects. Red swamp crayfish (*P. clarkii*) (n = 14, males = 3) were collected from an artificial pond called "Bonifica del Brancolo" (45°46' N, 13°30' E, GO, Italy) and transported to our laboratory. On their arrival, Crayfish were housed in individual plastic tanks (10 x 14 x 12 cm) filled with clean water. The walls of the tanks were opaque to limit their visual experience with the external environment. Illumination was provided following a 12:12 dark:light cycle and water temperature was kept constant at 21°C. Animals' rostrum to telson length ranged between 9.06 cm and 11.05 cm (mean = 9.94 cm, S.E.= 0.09 cm).

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Apparatus

Crayfish were tested within an opaque dry rectangular arena (as the one used in Chiandetti and Caputi, 2017). An LCD flat screen illuminated the inside of the arena with a white diffused light. On the opposite side, two water sprayers were attached to the wall at about 20 cm from the floor and 2 cm apart from each other. The exploratory space where the crayfish could freely walk was surrounded by a poliplack partition in a semi-circular shape. The whole arena was covered by a black curtain hanging from above that further isolated the tank from the external environment and the movements of the experimenter. The experiment was recorded at a frame rate of 29 fps by a camera placed above the arena.

Procedure

Crayfish were tested in 3 individual sessions (Session 1, Session 2 and Session 3) at 1, 15 and 45 days. At the beginning of each session, one crayfish at a time was placed at the centre of the arena and confined in a clear cylinder; once released, the crayfish could freely explore the new environment for 10-minutes. Then, the test started: whenever the crayfish faced the wall with the monitor, a stimulus was administered. After the reaction to the stimulus, the crayfish started to explore again the environment and, after about 2 minutes and whenever in front of the wall with the monitor again, another stimulus was administered. The unrestrained conditions allowed an ecological administration of the stimuli and prevented the animal to associate a specific place within the arena or a side of its body axis (left or right) with the stimulation. The habituation test consisted of 5 repetitions of the same stimulus - i.e., the habituation stimulus (trials 1 to 5, 7 to 11 and 13 to 17) - followed by a novel stimulus (trials 6, 12 and 18). The habituation stimulus was a jet of vaporized water (VAP); the novel stimulus was a direct jet of water (JET). The stimulation was manually delivered by the experimenter and never directed toward the crayfish's body, although of course droplets of water could fill in the whole testing area. We scored the time to reach the defense posture by counting the number of frames *per* second from the moment in which the crayfish reacted to the stimulus by lifting the claws until the moment of freezing (Kelly and Chapple, 1990; Glantz, 1974). Stimulus administration started only while the animal was engaged in locomotor activity with the claws close to the ground, independently whether bent or outstretched and still or in motion. The fact that crayfish increased their exploratory activity (i.e., they continued to walk) while learning to ignore the irrelevant water stimulus guaranteed that what we

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observed (i.e., the decrement of the response) was the outcome of true habituation and not the result of motor fatigue.

We presented all animals with a VAP-JET and not a JET-VAP sequence of stimulation to optimize the testing procedure to the number of available animals on the basis of previous literature. If the VAP stimulus elicited a response that habituated over time, then the recovery of the response for the JET would have been even stronger. Also, if we could observe a spontaneous recovery of the response for the weaker stimulus (i.e., VAP), we should have expected a recovery also for the more intense one (i.e., JET), as proposed by Thompson and Spencer (1966). We acknowledge the lack of a measurement of the baseline response to JET stimulus, although it is well established that habituation also occurs in response to both stronger stimuli - within the same stimulus modality, but at a slower rate (see for instance Davis and Wagner, 1968), and relevant stimuli (see for instance Daniel et al., 2019).

Statistical analyses

We analysed both habituation and generalization, scored by counting the fps needed to show the defensive response, using non-parametric statistics. We used an overall *Friedman* test and a pairwise *Wilcoxon signed rank* test to demonstrate the presence of habituation to the VAP stimulus. We used a *Wilcoxon signed rank* test to demonstrate the presence of generalization of the habituation to the JET stimulus and the response recovery between consecutive sessions. We provided effect sizes (Cohen's *d*) for all statistical comparisons, nonsignificant as well. We also provided the Bayes factor for the alternative hypothesis being true compared to the null hypothesis to support that our study had enough power to detect all the effects, instead of simply having too few subjects to be sensitive. Data were analyzed in Jamovi (v.1.1.9, https://www.jamovi.org/) and are available in the Open Science Framework (OSF): DOI 10.17605/OSF.IO/FX67G (https://osf.io/fx67g/).

Results

Results are shown in Fig. 1A.

Session 1: Crayfish' response to the VAP stimulus changed from trial 1 to 5: $\chi^2(4) = 15.8$, $p = .003$, $BF_{10} = 4.70$. In particular, the response was shorter on trial 5 than on trial 1: *mean difference* = 13.3±3.24 frames, $W = 96$, $p = .007$, $d = 1.07$, $BF_{10} = 27.42$. Stimulus specificity was attested by a significant increment in crayfish response to the JET stimulus:

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trial 5 *vs* trial 6: *mean difference* = -11.7±5.97 frames, W = 15, p = .036, d = -.60, BF_{10} =3.83.

Session 2: Crayfish recovered their response to the VAP stimulus when tested after 15 days, trial 5 *vs* trial 7: *mean difference* = -10.8±3.94 frames, W = 18, p = .033, d = -.61, BF_{10} = 3.86. They decreased their response to the following repetitions of the stimulus: $\chi^2(4)$ = 14.4, p = .006, BF_{10} = 10.44; trial 7 *vs* trial 11: *mean difference* = 13.7±4.17 frames, W = 67, p = .031, d = .65, BF_{10} = 2.37. This time, crayfish generalized the response decrement to the JET stimulus: trial 11 *vs* trial 12, *mean difference* = 3.33±5.20 frames, W = 58, p = .40, d = .09, BF_{10} = .28.

Session 3: Crayfish' response to the VAP stimulus significantly recovered after 30 days, trial 11 *vs* trial 13: *mean difference* = -20.30±3.42 frames, W = 1, p < .001, d = -1.58, BF_{10} = 523. The decrement of their response from trial 13 to trial 17 was significant: χ^2 (4) = 21.3, p < .001, BF_{10} = 2759.15; trial 13 *vs* trial 17: *mean difference* = 21.2±4.69 frames, W = 89, p = .003, d = 1.16, BF_{10} = 48.20. Again, crayfish generalized the response decrement to the JET stimulus: trial 17 *vs* trial 18, *mean difference* = -1.16±3.68 frames, W = 46.5, p = .73, d = -.01, BF_{10} = .27.

Crayfish's response to the JET stimulus changed across the trials 6, 12 and 18 (Fig. 1B): $\chi^2(4)$ = 21.3, p < .001, BF_{10} = 32.44. Their response to trial 6 was greater than their response to trial 12 and 18 (*post-hoc Durbin-Conover*: T = 5.29, p < .001; T = 3.44, p = .002).

Discussion

We demonstrated that *P. clarkii* habituated the alarming response to a repeated vaporized water jet, but then the response recovered when a direct water jet was introduced. However, whereas the response to the vaporized jet showed spontaneous recovery across the different sessions, with time they were able to generalize the habituated response between the two types of stimuli. In addition, it is remarkable that the generalization occurred after one learning trial and resisted for up to 45 days.

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This is the first experiment demonstrating a generalization ability in *P. clarkii*. Indeed, a previous study by Chiandetti and Caputi (2017) addressed a similar question in this species by adopting the same paradigm but without providing conclusive evidence of generalization. When looming shapes were used to elicit an alarming response, crayfish showed discrimination between “curvy” and “spiky” shapes, as they responded stronger when the “curvy” shape was presented following habituation to the “spiky” shape. But when a rotated version of the “spiky” shape was presented, the habituated response did not recover, showing either generalization or, more parsimoniously, a failure to discriminate between the rotated versions of the same shape due to low visual acuity. In the present study, however, results are clear-cut in showing that *P. clarkii* discriminated between the two water jets (session 1). Hence, the lack of response recovery to the water jet observed in sessions 2 and 3 can reliably be attributed to a learning process, namely to a long-lasting form of generalization.

Whether this phenomenon is prototypical for crayfish or distinctive of invasive species like *P. clarkii* is an open question. The lack of investigations on generalization abilities in other crayfish hinders any comparison of performance across different species of crayfish. *Procambarus cubensis* was shown to increase the retention time of the testing chamber characteristics following longer exposure times (Shuranova et al., 2005) when locomotor exploration was recorded. However, the retention time tested in that study (24 hours), can be barely compared with that used in the current study. On the other hand, invasive species living in changing environments might benefit from behavioural flexibility more than species living in stable environments because the risk of facing unknown stimuli is higher. Therefore, a mechanism could have evolved in these species to transfer innate or learned behaviours to new situations. Accordingly, Hazlett and colleagues (2000, 2002, 2003) suggested that invasive species have developed the ability to recognise and respond to a wider range of stimuli than species living in isolation because they have had the opportunity to experience a greater range of habitats in their evolutionary history, which points to rooted flexibility in invasive species. On the other hand, invasive species could learn through experience how to cope with the challenges posed by the current environment. In such case, behavioural flexibility may entail the capacity to invent new solutions (innovation) or learn these solutions from others (social learning). But animals can also exploit past solutions that had worked in similar situations and that can be generalized to the present one. This solution may be more efficient when innovations and

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social learning cannot be accomplished rapidly. Furthermore, crayfish do possess some core social abilities, but they seem more a solitary species in which social forms of learning occur in a limited range of situations (Gherardi, Aquiloni and Tricarico, 2012). Therefore, we propose that crayfish transferred their alarming response between two different stimuli through a generalization mechanism, obtaining, by means of such ability, an evident adaptive advantage to solve problems also in a new and unknown areal. Indeed, the distances they can cover, even on land, expose them to mutable contexts and environmental clutter in which they have the chance to exploit such ability more than native species (Barbaresi, Santini, Tricarico and Gherardi, 2004, and see also Mugan and MacIver 2020). Whether invasive crayfish have a superior generalization capacity than native species remains unexplored, but our habituation paradigm can be reliably used to address this question in future research.

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Conflicts of Interest: No competing interests declared.

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Figures

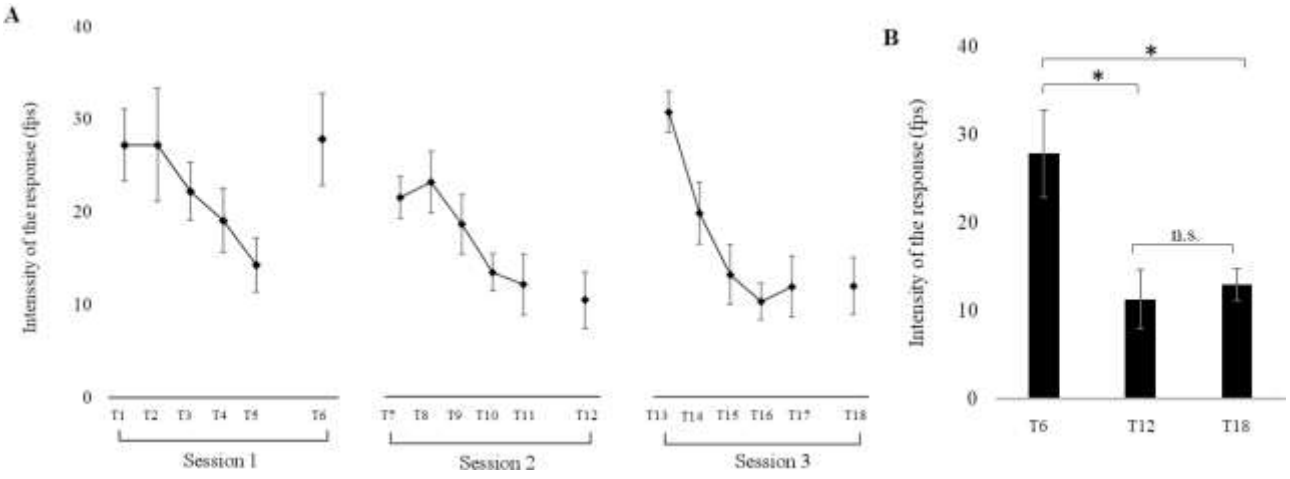

Figure 1

Panel A depicts the average defensive response of the 14 crayfish to the VAP and the JET stimuli in the 3 Sessions. Wilcoxon signed-rank tests revealed that crayfish decreased the duration of their defensive response to the VAP stimulus in each session (T1 > T5; T7 > T11; T13 > T17; $p < .05$). But they recovered their response to the JET during the first session only (T5 < T6; $p < .05$).

Panel B depicts the same response to the JET stimulus only. A set of *post-hoc Durbin-Conover* tests showed that the response to the JET was higher during the first session than in the second and third sessions (T6 > T14; T6 > T18; $p < .05$).

All the *p-values* are from two-tailed tests with $\alpha = 0.05$. Bars represent $\pm$ 1 S.E.M. Data were collected from animals housed in our laboratory. This longitudinal study was run for the first time in our laboratory.

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