

## CORRECTED PROOF

## Research Article

**Anti-predator strategies of the invasive African clawed frog, *Xenopus laevis*, to native and invasive predators in western France**Natasha Kruger<sup>1,2,\*</sup>, John Measey<sup>1</sup>, Anthony Herrel<sup>3</sup> and Jean Secondi<sup>2,4</sup><sup>1</sup>Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Western Cape, South Africa<sup>2</sup>UMR 5023 Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, ENTPE, CNRS, Université de Lyon, Université Lyon 1, Villeurbanne, France<sup>3</sup>UMR 7179 C.N.R.S./M.N.H.N., Département Adaptations du Vivant, Bâtiment d'Anatomie Comparée, Paris, France<sup>4</sup>Faculty of Sciences, University of Angers, Angers, FranceAuthor e-mails: [krugernatasha9@gmail.com](mailto:krugernatasha9@gmail.com) (NK), [john@measey.com](mailto:john@measey.com) (JM), [anthony.herrel@mnhn.fr](mailto:anthony.herrel@mnhn.fr) (AH), [jean.secondi@univ-angers.fr](mailto:jean.secondi@univ-angers.fr) (JS)

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**Co-Editors' Note:** This paper is a contribution to the **Behaviour in Aquatic Invasions** Special Issue of *Aquatic Invasions*. Papers in this Special Issue explore how behaviour contributes to invasion success; native species' behavioural strategies that reduce the impacts of invasions; how knowledge of behaviour can enhance management of invasive species; and potential effects of climate change on the behavioural impacts of aquatic invasive species.

**Citation:** Kruger N, Measey J, Herrel A, Secondi J (2019) Anti-predator strategies of the invasive African clawed frog, *Xenopus laevis*, to native and invasive predators in western France. *Aquatic Invasions* 14 (in press)

**Received:** 10 December 2018**Accepted:** 23 May 2019**Published:** 3 July 2019**Handling editor:** Amy Deacon**Thematic editor:** Kit Magellan**Copyright:** © Kruger et al.

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**Abstract**

When species are translocated to a novel environment, individuals become exposed to new predators against which they may not express very efficient defences at least at an initial stage. The strength of anti-predator defence is an important parameter that may determine the ability of local communities to control the expansion of invasive populations. The African clawed frog, *Xenopus laevis*, is a globally invasive amphibian that has successfully established invasive populations on four continents. In its invasive distribution in western France, *X. laevis* encounters novel aquatic predators. Some may be related to the predators in the native range but others may belong to different taxonomic groups and not be functionally or ecologically equivalent. We tested whether naïve *X. laevis* tadpoles from the invasive French population exhibit anti-predator response to local predators, and whether the response depends on the degree of relatedness with predators encountered in the native range of the frog, or whether individuals may express generic neophobia to any cue they are not familiar with. We exposed naïve lab-reared tadpoles to a native non-predatory water snail, *Planorbarius corneus*, a native predatory beetle, *Dytiscus dimidiatus*, and an invasive predatory crayfish, *Procambarus clarkii*. We found that *X. laevis* tadpoles innately reduce their activity when exposed to beetle and crayfish stimulus cues, but not to snails. Reducing activity can decrease the probability of being detected by predators. This demonstrates that invasive tadpoles respond to known and novel predators regardless of the evolutionary history. Whether the produced response is always effective against a totally novel predator remains to be tested.

**Key words:** diving beetle, crayfish, tadpole, activity, predation, amphibian**Introduction**

Biological invasions often fail through lack of preadaptation to the new climate, enemies, and competitors encountered in the novel environment (Lodge 1993; Moyle 1986; Newsome and Noble 1986). The presence of unknown predators can prompt the development or adaptation of traits in a similar manner as density, food availability, and temperature (Relyea and Hoverman 2003; Gomez-Mestre et al. 2010; McCoy et al. 2011). For instance,

the presence of predators induces deep tail fins and short bodies in larval gray treefrogs, *Hyla versicolor* LeConte, 1825. These morphological responses to larval predators increase the probability of a tadpole surviving predation (Relyea and Hoverman 2003). Predators can also prompt behavioural adaptations such as vigilance or a decrease in activity in prey species (Nunes et al. 2013, 2014; Ferrari et al. 2014). For example, leopard frogs, *Rana pipiens*, reduce their activity in the presence of mudminnows, *Umbra limi*, and dragonfly larvae, *Anax* spp. (Relyea 2001). Such behavioural traits have recently been shown to be strongly affected, with performance consistently lower in the presence of alien species (Nunes et al. 2019). In aquatic environments, individuals acquire an abundance of information about the presence of predators via chemical cues (Wisenden and Chivers 2006). Early detection of predator cues plays a key role in antipredator behaviour. If a prey animal detects the predator first, it can evade an attack or simply avoid areas of higher predation risk. Numerous types of chemical cues give prey advance notice of the presence of the predator. In particular, the odours from injured conspecifics can accompany predator odours and act as an alarm cue for naïve prey (Ferrari et al. 2010).

Organisms with complex life cycles, such as amphibians, can show stage-dependent responses to predator exposure. Earlier tadpole stages are smaller and more vulnerable to different types of predators than older, larger stages (Relyea 2003). Phenotype, including body size, can directly determine an individual's response to predators (Langerhans 2009; Hettyey et al. 2010; Nunes et al. 2013). To evade possible fatal encounters with unknown predators, tadpoles may react in a neophobic manner. This is a simple, but costly, mechanism to control ecological plasticity in a novel environment due to avoidance of novel stimuli. Neophobic responses towards unfamiliar predators may offer naïve prey an adaptive mechanism to avoid the initial encounters by reacting to all new chemical stimuli associated with conspecific injury and alarm cues (Brown et al. 2013). However, prey species can also exhibit antipredator responses toward a new predator type upon their first encounter if the predator species is related to predators present in the native range. For instance, both predators in the native and invasive ranges may release similar chemical cues that trigger the same behaviour in the prey. In amphibians, spontaneous predator avoidance may also result from learning of predator cues from the egg stage (Brown et al. 2013) allowing individuals to be responsive to potential predators at hatching.

The African clawed frog, *Xenopus laevis* (Daudin, 1802) originates from southern Africa. It is a globally invasive amphibian that has successfully established invasive populations on four continents (Measey et al. 2012). *Xenopus laevis* was introduced into the Deux-Sèvres department in western France from a breeding facility near Bouillé-Saint-Paul at the beginning of the 1980s (Fouquet 2001; Fouquet and Measey 2006). Currently, *X. laevis* is

present in five French departments (JS, *unpublished data*; Louppe et al. 2017). Some local predators in the novel environment may be functionally and phylogenetically similar to predators found in the native range. Alternatively, novel predators may be neither phylogenetically or functionally similar, nor otherwise ecologically equivalent, to predators they evolved with. We tested whether naïve *X. laevis* tadpoles from the invasive French population exhibit anti-predator responses to local predators, and whether the response depends on the degree of relatedness with predators encountered in the native range of the frog. Alternatively, naïve tadpoles may express neophobia to any cue they are not familiar with. We exposed naïve lab-reared tadpoles to a non-predatory water snail, *Planorbarius corneus*, a native diving beetle, *Dytiscus dimidiatus*, and an invasive crayfish, *Procambarus clarkii*. In western France *X. laevis* and the native diving beetle *D. dimidiatus* have co-occurred since the introduction of *X. laevis* into France. Species of the genus *Dytiscus* are also present across southern Africa while no related species of crayfish occur in the native range of *X. laevis*. We predict innate antipredator responses of *X. laevis* tadpoles to phylogenetically “familiar” predators as a result of coevolutionary history. A decrease in activity is expected as it is one of the most common and effective behavioural anti-predator responses that reduces detection and thus vulnerability (Nunes et al. 2019). We predict no response to the invasive crayfish due to the short interaction period between the two invasive species (~ 30 years). The red swamp crayfish, *P. clarkii*, is native to north-eastern Mexico and south-central USA (Hobbs et al. 1989). This species was introduced into Europe (southern Spain) in 1973 and is currently a widespread and abundant invasive species all over Europe (Habsburgo-Lorena 1979). The first population in France was recorded in 1988 in the Charente-Maritime department. Since then the distribution has expanded into many different departments in metropolitan France, including four of the departments (between 1995 and 2001) where *X. laevis* is present (Changeux 2003; Collas et al. 2007). The crayfish was not present in the Maine-et-Loire department in 2007, but was recorded in this department in 2014 due to their rapid distribution throughout the Loire basin (Collas et al. 2007, 2015). The species is not common in ponds across the study area. Yet, *X. laevis* and *P. clarkii* can be found in the same aquatic habitats. The frequency of sympatric ponds is difficult to assess, but it seems to be higher close to some river courses. The snail was used as a non-predator control. Water snails are present in southern Africa and France and co-occur with *X. laevis*.

## Materials and methods

### *Collection, care, and feeding of individuals*

Baited funnel traps were distributed in four different sites to collect *X. laevis*: site 1 (47°20'38"N; 0°45'46"W); site 2 (46°54'40"N; 0°21'11"W);

site 3 (47°01'27"N; 0°19'10"W); and site 4 (47°00'38"N; 0°21'29"W) within the invaded area in western France (De Busschere et al. 2016). This experiment was conducted between 06/2017 and 07/2017. One breeding pair per site was collected. Breeding was induced using human chorionic gonadotropin hormone (hCG: Ovidrel®/Ovitrelle® 250 micrograms/0.5 mL). The males were injected with 250 IU of hCG for the first, second and third day of breeding and the females with 50 IU on the second day and 500 IU on the third day of breeding. The male and female of a breeding pair were kept separate and were only joined on the third day. After egg laying, adults were removed the following morning from the aquaria, and the eggs were counted and assessed for viability. Tadpoles were selected for the trials at pre-metamorphic stages 42–53 according to Nieuwkoop and Faber (1994). A naïve tadpole at an early and vulnerable stage was selected as when tadpole size increases, vulnerability to predation decreases, reducing the need for antipredator responses (e.g. Nunes et al. 2013). Due to the selection of tadpole stage, we did not expect body size to display an effect. Individuals from each clutch were raised in tanks with a density of one tadpole per litre at 20 °C. Tadpoles were fed SERA Plankton tabs® (Sera GmbH, Heinsberg, Germany).

Experimental stimuli were collected using dip nets in outside ponds. Three species were collected: adults of a native water beetle predator, *D. dimidiatus* (n = 5; 1.66 ± 0.15 cm; 0.29 ± 0.12 g); juvenile invasive crayfish, *P. clarkii* (n = 6; 6.52 ± 1.20 cm; 5.61 ± 2.45 g); and a non-predatory snail, *P. corneus*, (n = 5; 2.01 ± 0.22 cm; 2.59 ± 1.90 g). These species are common although the crayfish is rarely caught with *X. laevis* during trapping. Captures were conducted outside the invasive range of *X. laevis* to prevent the potential release of conspecific alarm cues during tests. Dietary or conspecific cues were intentionally not added here to test whether tadpoles display an innate response. Snails, beetles, and crayfish were kept separately in 10 L tanks and fed every second day with pond invertebrates for beetles and crayfish, and lettuce leaves for the snails. Water in tanks for stimuli and tadpoles were changed every second day before the experiment, and for tadpoles every day during the experiment. Individuals of the same kind were placed together after measuring each individual. For the duration of the experiment, water containing stimulus cues was sampled from the stimulus tank water.

### *Behavioural test*

An adaptation of the protocol to measure activity of tadpoles in response to predators designed by Ferrari et al. (2010) was used. The experimental protocol consisted of two types of tests with two phases. For the type-1 test, tadpoles were exposed to water only during the first phase, as a control, and to the olfactory cues of one stimulus type during a second exposure phase. For the type-2 test, tadpoles were presented with olfactory cues of

the same stimulus type on the first and second exposure phases. Type-1 tests were conducted as control to assess whether exposure phase rather than cue has an effect on response. Type-2 tests were conducted to assess the initial and second response of *X. laevis* tadpoles to stimulus cues.

Tadpoles from the same clutch were raised together at a density of ~ 10 tadpoles per litre. When tadpoles reached the desired stage for the experiment, 60 tadpoles from each clutch ( $n = 4$ ) were removed and divided randomly into 3 groups of 20 for each stimulus type. Tadpoles were further divided into type-1 test ( $n = 40$  per snail, beetle, and crayfish) and type-2 test ( $n = 40$  per snail, beetle, and crayfish). All tadpoles from every group for each stimulus type were placed individually in 1 L plastic cups (5 cm radius) filled with aged tap water, where they were left to acclimate for 24 h. The test was structured as a five minute pre-stimulus period, the one minute injection period when the cue was added, and a five minute post-stimulus period (Ferrari et al. 2010). In the first exposure phase, we injected 5 ml of water only as a control for all tadpoles for the type-1 test and 5 ml of water containing the olfactory stimulus cue for the type-2 test. Tadpoles exposed to the stimulus cue from type-2 tests are referred to as “experienced” and those exposed to water only from type-1 tests as “naïve”. After the first phase trials for each type of test, tadpoles were moved to new plastic cups with new water that contained no olfactory stimulus cues. Tadpoles were fed and left 24 h to acclimate between phases. For the second phase, we followed the same protocol as for type 1 test (naïve) and type 2 test (experienced) tadpoles (5 min pre-stimulus, 1 min injection, 5 min post-stimulus period). Cups containing experienced tadpoles were re-exposed to the same type of olfactory cues again, and the set of “naïve” tadpoles were exposed to olfactory cues for the first time.

Two lines were drawn under the cup to form four quadrants. The number of lines crossed by moving tadpoles was recorded for each period. We considered that a tadpole crossed a line when its entire body (tip of nose to the tip of the tail) was on the other side of the line (Ferrari et al. 2010). We recorded simultaneously sets of 10 tadpoles using a video camera (recorded at 30 fps) placed above the cups. Tadpoles were euthanized by immersion in MS-222 (ethyl 3-aminobenzoate methanesulfonate) after the completion of the tests. Linear measurements of snout-vent-length, tail length, full length (head to tail tip), head width, head depth, and tail depth were measured using ImageJ analysis software (v 1.52a, NIH, Washington, D.C., USA).

### *Statistical analysis*

To obtain a synthetic measurement of body size, we conducted a principle component analysis on the six measurements of tadpole morphology. The first principal component accounted for 79.4% of the variance between

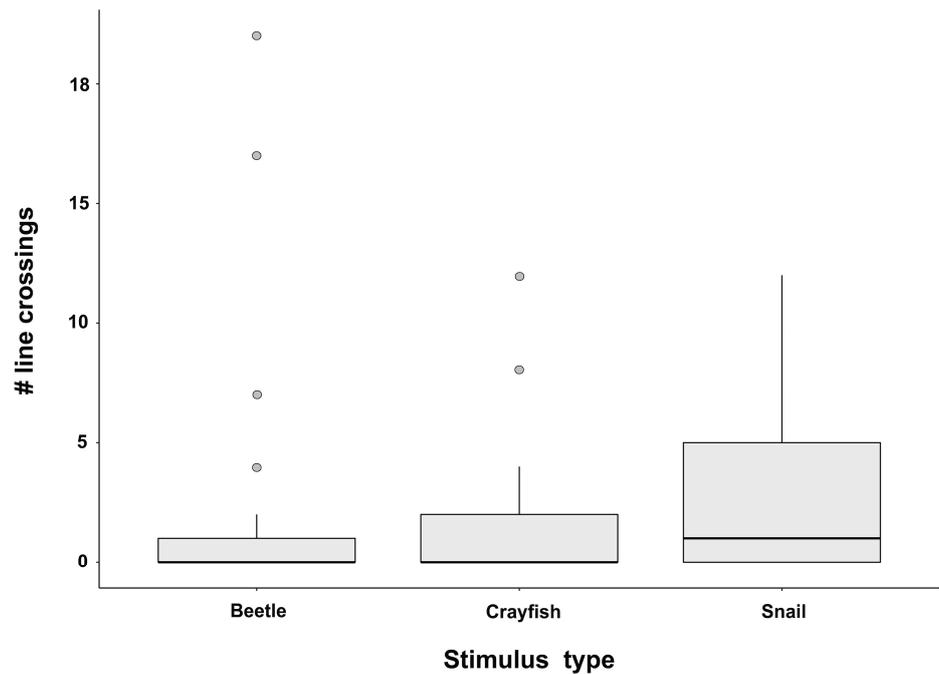
individuals, and all variables had negative loadings. Thus, the first axis is a global measurement of body size. We compared tadpole activity with snail, crayfish and beetle after the introduction of the olfactory cues and considered the pre-stimulus injection period as habituation. Some individuals did not cross any line during both testing sessions ( $n = 19/120$  naïve tadpoles;  $n = 18/120$  experienced tadpoles). We discarded them from further analyses and only kept tadpoles that moved at least once during one period. Generalized mixed models were used with post-stimulus line crosses as a response variable with stimulus type and body size (PC1) as fixed effects. Site, *i.e.* tadpole pond, was considered as a random effect. We compared different models including zero inflated, and hurdle models with different distribution using the package *glmmTMB*. Models with a generalized poisson distribution and a log link had the lowest AIC values and were selected thereafter. All analyses were carried using the statistical software R 3.4.1 (R Core Team, 2018).

## Results

No effect of stimulus type, body size, and their interaction was observed for naïve tadpole activity. The minimum model with only the intercept and the random effect presented the lowest AIC for the response after the first (water only:  $\Delta\text{AIC} = 1.4$ ) and second tests (first exposure to an olfactory stimulus:  $\Delta\text{AIC} = 2.0$ ) phase and was thus the best model. For experienced tadpoles, the lowest AIC value was observed for the minimum model ( $\Delta\text{AIC} = 1.4$ ) for the first exposure phase. In contrast, for the second exposure phase, *i.e.* “experienced” tadpoles, the best model included stimulus type only. The second best model ( $\Delta\text{AIC} = 1.7$ ) additionally included body size. Following the principle of parsimony, we retained the first model, that also differed from the null model that displayed no effect of body size ( $\Delta\text{AIC} = 3.4$ ). The number of line crossings was the highest in tadpoles exposed to snail olfactory cues (Figure 1). Activity was significantly reduced for individuals exposed to olfactory cues of beetle ( $n = 32$ ,  $z = -2.514$ ,  $p = 0.012$ ) and crayfish ( $n = 37$ ,  $z = -2.072$ ,  $p = 0.038$ ). No significant difference of activity between tadpoles exposed to beetle and crayfish were observed ( $n = 69$ ,  $z = 0.588$ ,  $p = 0.556$ ).

## Discussion

The variation in tadpole activity following exposure to different stimulus types was only observed on the second test in individuals already exposed to the same stimulus. A single exposure to predator cues without association with conspecific cues was insufficient to detect an anti-predator response. Previous studies have used predator cue accompanied by conspecific cues when testing the antipredator response of organisms (e.g. Relyea 2001; Relyea and Hoverman 2003; Ferrari et al. 2014; Lucon-Xiccato



**Figure 1.** Boxplot of activity expressed as the number of line crossings by naïve *Xenopus laevis* tadpoles exposed to the olfactory cues of a dytiscid beetle, *Dytiscus dimidiatus*, a crayfish, *Procambarus clarkii*, or a water snail, *Planorbarius corneus*.

et al. 2018). Their association induced the learning of predation risk in tadpoles (Ferrari et al. 2010). In our protocol, the absence of conspecific cues may have prevented conditioning to occur. The lack of initial response to predator cue alone has been previously reported for tadpoles (e.g. Marquis et al. 2004). Most likely, habituation to the experimental conditions was not complete before the first exposure, and behavioural differences could only be detected on the second test. Nevertheless, we observed behavioural differences in the second exposure phase of experienced tadpoles. Olfactory cues of the two predators were enough to reduce the activity of “experienced” individuals. Such a decrease in locomotor activity is consistent with the literature on tadpoles of other species exposed to aquatic predators regardless of invasion status of either predator or prey (e.g. Nunes et al. 2019). Reduction in activity is a widespread and important form of anti-predator response in the presence of predators (Lima and Dill 1990). Moving and being active increases probability of being detected by predators. Beetles and crayfish were captured outside the colonised area which discounts the possibility that tadpoles perceived dietary cues of conspecifics consumed earlier by the predator. Thus, our results support the presence of innate anti-predator behaviours to dytiscid beetles and crayfish in *X. laevis* tadpoles.

*Dytiscus dimidiatus* belongs to a predator lineage with which *X. laevis* shares a long evolutionary history. This species is not present in southern Africa but other diving beetle species from the family Dytiscidae, which are functionally similar to *Dytiscus* species, can be found (Alarie et al. 2017).

The response to *D. dimidiatus* may indicate pre-adaptation to dytiscid predation in any part of the world where frogs and beetles may come into contact. Alternatively, *X. laevis* may have developed a response to this species, absent from southern Africa, since its introduction into France. This may not be the most plausible explanation.

The innate response of tadpoles suggests that they were not naïve to the crayfish. This result was unexpected because of the lack of coevolutionary history; the first contact between these two lineages in the African clawed frog's native range dates back less than 30 years, and sympatry between *P. clarkii* and *X. laevis* still appears uncommon today. Yet, the crayfish seems to be recognized as a threat by tadpoles which suggest three hypotheses. First, *X. laevis* specifically evolved an evolutionary anti-predator response to *P. clarkii*, due to the recent invasion of the crayfish into the invasive distribution of *X. laevis*. Regardless of coexistence time, the presence of *P. clarkii* can induce an immediate response in native Perez's frog, *Pelophylax perezii*, tadpoles in Portugal, where both naïve tadpoles and those from long-term invaded populations displayed reduced activity at initial exposure (Nunes et al. 2014). Second, the odours that *P. clarkii* releases are similar to the cues released by freshwater crabs of the genus *Potamonautes*, which are predators of *X. laevis* in southern Africa (Gutsche and Elepfandt 2007). This cue type may be shared by a broader taxonomic range of species than initially expected. Third, *X. laevis* tadpoles exhibit an innate generic response to any predator cue. For instance, crayfish and beetles may have consumed other amphibian larvae in their pond of origin which provided alarm cues for *X. laevis* tadpoles. However, previous studies investigating the effect of the presence of unfed invasive *P. clarkii* on the behaviour of a native naïve amphibian species showed an initial decrease in activity, a possible neophobic response (Nunes et al. 2013). The release of cross-species alarm and dietary cues accompanied by predator cues is especially effective when alarm cues are from the same prey guilds, as they share similar predators (e.g. Adams and Claeson 1998; Fraker et al. 2009). Careful effort was made to ensure tadpoles were not exposed to cues from items predators had consumed before their capture. Therefore, tadpoles may have used more general cues indicating the presence of an organism with a carnivorous diet. The hypothesis of a generic response is only partly consistent with the neophobia hypothesis (Ferrari et al. 2010) as we did not expect to detect differences between stimulus types if *X. laevis* reduced its activity in response to any unknown cue. We cannot currently select one hypothesis but our results raise questions about the mechanisms by which invasive populations may express anti-predator behaviours to unknown predators, and reduce predation rate in novel environments. This property is probably desirable for a successful invader such as the African clawed frog.

## Conclusion

Invasive *X. laevis* tadpoles reduce their activity in the presence of diving beetles. We cannot conclude yet whether the response is due to a long-term evolutionary process in the native range, a rapid *in situ* response that evolved in the colonised range, or a generic anti-predator recognition system. Regardless of the mechanism, our results suggest that such an anti-predator behaviour may limit the ability of this predator type to control *X. laevis* populations at the larval stage. Predicting the consequences of expressing an analogous response to crayfish is not straightforward. The possible generic anti-predator responses displayed by tadpoles may not be adaptive against *P. clarkii* due to the different foraging behaviours observed between crayfish and diving beetles. For instance, a mud snail expressed the same anti-predator response against invasive crayfish as that displayed for fishes, which resulted in a higher predation rate on the snail (Sih et al. 2010).

*Procambarus clarkii* is known to impact amphibian populations where it is introduced (e.g. Cruz et al. 2006; Souty-Grosset et al. 2016), and invertebrates (especially crayfish) were found to have the biggest effect on native tadpoles (Nunes et al. 2019). However, the presence of *P. clarkii* in Zhoushan Archipelago, China, was shown to mitigate the effect of invasive *Lithobates catesbeianus* on native amphibian populations, thus highlighting the importance of considering complex interactions between co-invaders (Lui et al. 2018). Altogether, this study shows that *X. laevis* tadpoles tend to behave in a similar way to most other amphibian larvae towards sympatric predators. This may not confer strong control of invasive African clawed frog populations by local predators. The actual interactions between *X. laevis* and native predators or invasive crayfish deserve further attention to understand how the pond trophic network maybe reshaped by the accumulation of these different major invaders.

## Acknowledgements

The authors would like to thank the editor and anonymous reviewers for their contribution to improve the manuscript. NK & JM would like to thank the DST-NRF Centre of excellence for Invasion Biology (South Africa). NK would like to acknowledge the Ambassade de France en Afrique du Sud (France). The work was funded by the Life CROAA (Life15 NAT/FR/000864). The utilisation of the frogs and the research protocols were approved by Stellenbosch University Research Ethics Committee: Animal Care and Use (ethics number: 1535).

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