Can we control the invasive cane toad using chemicals that have evolved under intraspecific competition?

GREGORY S. CLARKE, MICHAEL R. CROSSLAND, AND RICHARD SHINE

School of Life and Environmental Sciences A08, University of Sydney, Sydney, New South Wales 2006, Australia

Abstract. Many invasive species experience intense intraspecific competition, because they are abundant in anthropogenically disturbed habitats where few native species persist. Species-specific competitive mechanisms that evolve in this context may offer novel, highly targeted means to control invasive taxa. We conducted laboratory experiments to evaluate the feasibility of this method of control, based on waterborne cues that are produced by tadpoles of the cane toad (Rhinella marina) to suppress the development of conspecific embryos. Our trials examined the nature and species-specificity of the effect, the robustness of the cue to freezing and storage, and the amounts required to suppress toad embryos. Our results were encouraging. The cue appears to be chemical rather than a biological organism, and may well be species-specific; the four species of native anurans that we tested were not influenced by toad larval cues. The cue retains its effectiveness after being frozen, but not after being dried, or after 7 d in water. It is effective at very low concentrations (the amount produced by three tadpoles within 750 L of water). Overall, the cane toad's suppressor pheromone may offer an effective new way to control invasive toads.

Key words: Adelaide River; Australia; biocontrol; Bufo marinus; cane toad; invasive species; larval communication; pheromone; Rhinella marina.

INTRODUCTION

Invasive species have severe impacts both ecologically and economically (Mack et al. 2000, Pimentel et al. 2005, Reaser et al. 2007). Increasing rates of introduction of invasive species, and their consequent impacts, have spawned a search for novel methods to control invaders. Progress has been slow in most systems because of the difficulty of finding a method of control that reduces invader numbers without also affecting native taxa. For example, colonies of invasive fire ants can be killed with general-purpose insecticides, but in the process, native ants are also killed, and as a result, control efforts ultimately may enhance rather than reduce rates of fire ant invasion by eliminating the fire ant's native competitors (Eubanks 2001). The key to minimizing collateral damage is to identify species-specific traits of the invader (traits that are not shared by other sympatric native taxa) that can be targeted for selective control. Attempts to deploy species-specific biological control have sometimes succeeded (Hajek and Delalibera 2010) but often failed (Louda 2000, Messing and Wright 2006). Given these low overall success rates (Saunders et al. 2010), extensive testing of potential impacts is crucial prior to implementation.

One renowned example of biological control gone wrong is the introduction of the cane toad (Rhinella marina) to over 40 countries in a futile attempt to control insect pests in agriculture (Lever 2001). The rapid spread of cane toads through tropical Australia has had devastating effects on native predator populations (Shine 2010). Substantial efforts to control adult toads via hand-collecting, trapping, and fences have had little effect on toad abundance or dispersal rate (Peacock 2007); and the expenditure of more than AUD$110 000 000 on an attempt to construct a toad-killing virus did not result in any usable weapon (Shanmuganathan et al. 2010). Clearly, we need new approaches based on a deeper understanding of the animal's biology. One such approach may be via manipulation of the invader’s intraspecific communication systems (Wassersug 1997).

Early life stages of anurans interact with each other via complex mechanisms (Brönmark and Hansson 2000), including developmentally plastic responses to chemical cues both during the embryonic stage (Crossland and Shine 2012) and the tadpole stage (Petranka 1989, Relyea 2002, 2004, Schalk et al. 2002). Previous studies have highlighted the potential of cane toad pheromones for controlling this invasive species in Australia (Hagman and Shine 2009a, Hagman and Shine 2009b, Crossland et al. 2012). In particular, waterborne cues from conspecific tadpoles powerfully suppress the development and viability of newly laid toad eggs, such that the larvae that hatch from such eggs exhibit low rates of growth, development, and survival (Crossland and Shine 2012). These waterborne pheromones may provide a targeted way to control invasive cane toads, but we need to know more about this phenomenon to evaluate its potential as a toad-control weapon.
We explore the feasibility of using the suppression pheromone as a control for cane toads in Australia. We conducted laboratory trials to investigate the following questions: (1) Is the suppression effect due to chemicals or to pathogenic organisms such as algae and microorganisms? (2) What is the risk of collateral damage to native anurans? (3) In practical terms, how could we store this weapon (can it be frozen or dried)? (4) How long does the cue remain potent at room temperature? (5) What concentrations are required to elicit the suppression effect?

**Methods**

**Study species and animal husbandry**

The cane toad *Rhinella marina* is native to Mexico, Central and South America. In 1935, this large anuran was introduced into Australia and has since spread widely (Lever 2001). Cane toads have aquatic eggs and larvae, and terrestrial adults. They breed in shallow water bodies and produce large clutches of eggs that hatch within 72 h (Hearnden 1991). Tadpoles typically metamorphose in 14–28 d (Cohen and Alford 1993, Child et al. 2008).

**Collection of eggs and tadpoles**

Adult cane toads collected from the Adelaide River floodplain, Northern Territory (NT; 12°34′ S, 131°18′ E) were induced to spawn by subcutaneous injection of leuprolin acetate (Lucrin, Abbot Australasia, Botany, New South Wales, Australia) diluted in amphibian ringers’ solution (0.25 mg/mL). Males were given 0.25 mL and females 0.75 mL, and pairs of animals were then left overnight in a 1 × 1 m enclosure with a pool of water at one end. Eggs were collected the following morning and held in a shallow tray containing aerated bore water at room temperature prior to use in the trials. Cane toad tadpoles to be used as stressors were collected from ponds on the Adelaide River floodplain and kept in 70 L bins with a pool of water or (2) newly laid clutches of frog eggs collected in the field. Frog eggs were only collected from ponds that did not contain toad spawn or larvae. Prior to the experiments, eggs were kept in aerated 70 L tanks filled with bore water.

**Water quality**

Larval viability might be impaired by reduced water quality rather than specific cues. To rule out such an effect, we measured levels of ammonia and pH (API test kits, Chalfont, Pennsylvania, USA) at the end of the exposure period, when tadpoles reached developmental stage 25 (Gosner 1960). For experiments that included treatments with differing exposure periods, we tested the water quality at the time when stressor organisms were removed (when differences are likely to be greatest).

**Is suppression caused by a chemical or a micro-organism?**

To answer this question, we passed water containing tadpole cues (and control water) through filters fine enough to exclude algae (Wong and Beebee 1994), bacteria (Holt et al. 1994), fungi (Carter et al. 2004) and most viruses (Maclachlan and Dubovi 2010). One-liter plastic containers filled with 750 mL bore water were allocated to control or live tadpole treatments. Live tadpole treatments contained three mid-development cane toad tadpoles (snout–vent length [SVL] 8.22–9.39 mm, Gosner stage 34–38), whereas control treatment contained only bore water. After 72 h, we removed the tadpoles from the live tadpole treatment to obtain water with tadpole cues. Of the total of 16 replicates of each treatment, eight were haphazardly allocated to filtered treatments, leaving eight as unfiltered. The water from filtered treatments was syringed through a 0.45-μm pre-filter, followed by a 0.2-μm sterile filter (Ministart, Sartorius Stedim Biotech, Goettingen, Germany) into new uncontaminated 1-L containers. Unfiltered treatments were also syringed into new uncontaminated 1-L containers. This created four treatments: filtered tadpole cue, unfiltered tadpole cue, filtered control, and unfiltered control (N = 8 replicates per treatment). Containers were placed in a fully randomized grid, with five toad embryos (Gosner stage 16–17) randomly assigned to each container.

When hatchlings reached Gosner stage 25 (free swimming, feeding stage), we recorded their survival and tested water quality. Groups of five tadpoles were haphazardly chosen per treatment and transferred to new containers with 750 mL of fresh bore water. Tadpoles were fed blended Hikari Algae Wafers ad libitum and water was changed daily. After 5 d, tadpole body length and body size were photographed then measured (ImageJ, National Institutes of Health, Bethesda, Maryland, USA) and developmental stage was assessed.

**Are native anurans vulnerable to toad larval cues?**

To examine the effects of the toad suppression chemical on native species, we exposed two clutches of each of four species of native frog embryos to the suppression cue.

**Exposure period and methods.**—Plastic containers filled with 750 mL bore water with either five or ten (Appendix Table S1) frog embryos (Gosner stages 8–13) were randomly allocated to an exposed or control treatment. Exposed treatments contained three live cane toad tadpoles (stressors) separated from the embryos by a 1 × 1 mm mesh partition, permeable to waterborne cues...
but preventing physical contact between the eggs and larvae. Control treatments were identical except for the lack of stressor tadpoles. After 30 h, when frog eggs had developed into hatchlings (stage 23–24), the stressor tadpoles were removed from the exposed treatment. This exposure time frame is sufficient to cause highly significant suppression effects in cane toads (Clarke 2014).

When hatchlings reached Gosner stage 25 (free swimming, feeding stage), we recorded their survival and tested water quality. Groups of tadpoles were then haphazardly chosen from each treatment and allocated to plastic tanks containing 60 L of water and a 2 cm layer of sediment collected from a local pond. Tanks were set up in a paired block design, with each group of tadpoles being randomly allocated to a block, and located in a covered area exposed to ambient temperature, and protected with flyscreen to exclude predatory aquatic insects. Additional nutrients (0.1 g blended Hikari Algae Wafers/tadpole) were added (total of 1 g or 0.5 g/tank) when tadpoles were transferred, and again at 14 d and once every 7 d thereafter. After a 21–25 d period, we recorded tadpole body size (mass to 0.001 g, SVL), developmental stage, and survival.

Is the suppression chemical still effective after it is frozen or dried?

Macerated toad tadpoles suppress toad embryos to the same degree as do the cues produced by live tadpoles (Clarke 2014). We exploited this result to determine if freezing or drying macerated tadpoles affected their ability to induce the suppression effect.

Preparation of dried tadpoles.—We macerated 1.5 g of toad tadpoles (SVL 6.87–7.68 mm, Gosner stages 30–34) in 10 mL of water, which was then passed through a fine mesh cloth to remove any large particles. Maceration of these tiny (<0.1 g) larvae causes instant death and is thus humane. We placed 1 mL of this solution onto a single filter paper (110 mm diameter; Ashless No. 41; Whatman, Maidstone, UK) and allowed it to dry for 18 h in a darkened room. For exposure, one filter paper (containing dried tadpole) was placed in each dried tadpole replicate and one (uncontaminated) filter paper was placed in each replicate of control and frozen tadpole treatments.

Preparation of frozen tadpoles.—A total of 1.5 g of toad tadpoles (SVL 7.00–8.29 mm, Gosner stages 31–34) were frozen then defrosted and macerated in 10 mL of fresh water. This material was passed through a fine mesh cloth, and 1 mL of this solution was added to frozen tadpole treatments.

Exposure period and methods.—Using plastic containers filled with 750 mL bore water, groups of 10 tadpole eggs (developmental stage 17) were exposed to frozen tadpole cues or dried tadpole cues in the laboratory for 30 h (N = 9). A control treatment containing only bore water was included, as well as a live tadpole treatment (containing three toad tadpoles: SVL 6.72–8.96 mm, developmental stages 30–34, separated from the embryos by a 1 × 1 mm mesh partition).

When hatchlings reached Gosner stage 25 (free swimming, feeding stage), we recorded their survival and tested water quality. Groups of 10 hatchlings haphazardly chosen from each treatment were transferred to larger plastic tanks (N = 5 per treatment) set up in a randomized block design with five replicates per treatment. Each tank contained 15 L of water and a 2 cm layer of sediment collected from a local pond. These tanks were located in a covered outdoor area, exposed to ambient temperature, and protected with flyscreen to exclude predatory insects. Additional nutrients (1 g blended Hikari Algae Wafers) were added to each tank; thereafter, tadpoles were left to grow and develop.

After 5 d, tadpoles were photographed to measure body size (SVL, body surface area; ImageJ, National Institutes of Health) and survival was recorded. Tanks were checked daily for metamorphs (defined as emergence of at least one front limb), which were then kept individually until tail resorption was complete, at which time they were measured (snout–urostyle length [SUL] using digital calipers) and weighed to the nearest 0.001 g. Under these conditions, toad tadpoles typically metamorphose within 16 d (Cabrera-Guzmán et al. 2011); we concluded our experiments after 30 d.

How long does the waterborne suppression cue remain potent?

To explore the stability of the suppression cue if left in water, we compared the effects of embryonic exposure to fresh tadpole cues vs. cues that had been kept for 7 d at room temperature after being produced.  

Stimulus preparation.—Plastic containers were set up with 750 mL bore water. Three live toad tadpoles (SVL 8.12–9.45 mm, developmental stage 32–37) were placed in exposure treatments (N = 9), whereas controls (N = 18) contained only water. After 72 h, tadpole tadpoles were removed and all containers were left to sit for 7 d. Containers were kept at room temperature in the laboratory and covered with flyscreen to exclude insects. After 4 d, three live toad tadpoles were added to each of nine randomly selected containers from the control treatment for 72 h (thus providing the fresh tadpole cue). At the end of the 7-d period, groups of 10 toad embryos were randomly allocated to one of the following treatments: control (water that had been standing for 7 d), 7-d old tadpole cue (water containing tadpole cues that were 7 d old), and fresh tadpole cue (water containing tadpoles that had just [<1 h] been removed).

Embryos were exposed to these cues until they reached stage 25, at which time we recorded survival and haphazardly transferred groups of 10 tadpoles into larger tanks (15 L of water, as described previously). Tadpole survival
and body size were recorded after 5 d, after which the larvae were left to develop for 30 d.

What concentrations are required to elicit the suppression effect?

This experiment was designed to identify the minimum concentration of the toad tadpole cue needed to suppress development, size, and survival of toad embryos, a critical issue for implementation of the chemical as a control mechanism.

Stimulus preparation.—Previous studies on the suppression effect have used high concentrations of the suppression cue (the amount produced by three toad tadpoles in 750 mL of water; Crossland and Shine 2012, Clarke 2014). We prepared 1 L plastic containers \((N = 12)\) containing three toad tadpoles in 750 mL of bore water. After 72 h, we removed the tadpoles and combined the remaining water to form a stock solution that we then serially diluted by adding measured amounts of bore water to produce concentrations of 100%, 10%, 1%, 0.1%, 0.01%, and 0.001% of the original concentration.

Exposure period and methods.—Groups of 10 toad eggs (developmental stage 16) were randomly allocated to each concentration treatment \((N = 9\) per treatment). A control treatment contained bore water only. When hatchlings reached Gosner stage 25 (free swimming, feeding stage), we recorded their survival. Groups of 10 tadpoles were then haphazardly chosen per treatment and allocated to larger tanks (15 L of water, as described previously) kept in ambient conditions. We recorded tadpole survival and body sizes at 5 d of age and checked daily for metamorphs. Survival was recorded at 30 d of age, at which time the experiment ended.

Statistical methods

To avoid pseudoreplication, we used tank mean values for analysis of treatment effects on tadpole response variables (SVL, body surface area, and mass). Treatment effects were analyzed using ANOVA with block and clutch as random factors where appropriate. Where more than one clutch was included in an analysis, we included the clutch \(\times\) treatment interaction, and then removed it if non-significant. If a significant interaction term was found, the effects of treatment were analyzed separately for each clutch. Data were log-transformed where necessary to meet assumptions of normality and equal variance. Significant ANOVA results were followed by post-hoc Student’s \(t\) multiple comparisons tests. Gosner stage was analyzed using ordinal logistic regression. Analyses were conducted using JMP 9.0 (SAS Institute, Cary, North Carolina, USA), with an alpha level of \(P = 0.05\). The effect of treatment on survival was analyzed as a binomial response via logistic regression (logit link function) in R (R Development Core Team 2012). If the data showed overdispersion, analyses were based on the quasi-binomial distribution rather than the binomial distribution. We have refrained from applying corrections for multiple testing because of subjectivity in defining the sets of related tests among which such corrections should be made (García 2004, Nakagawa 2004). In practice, application of sequential Bonferroni corrections within each experiment did not affect any of our major conclusions.

Results

Water quality

In all of the experiments we conducted, there was no significant effect of treatment on water quality (pH or NH3 levels) at the end of the exposure period.

Is suppression caused by a chemical or a micro-organism?

Survival of hatchlings and tadpoles.—All toad embryos survived to developmental stage 25 (free-swimming) in all treatments, and 138 of 140 tadpoles survived to day 5 (99%). It is clear that survival was not affected by these treatments over a 5-d post-hatching period.

Tadpole development and growth rates.—Treatment affected the body size of tadpoles at 5 d of age (SVL \(F_{3,27} = 88.12, P < 0.01\); body surface area \(F_{3,27} = 76.86, P < 0.01\)). Post-hoc tests showed that tadpoles from treatments exposed to tadpole cues were smaller than were those from control treatments, regardless of filtration (Fig. 1a,b). That is, filtration did not reduce the suppressive effects of embryonic exposure to toad tadpoles. Developmental stage at 5 d of age differed among treatments \((\chi^2 = 43.00, 3\ df, P < 0.01)\), with tadpoles from both sets of controls (filtered and unfiltered) more developed than were tadpoles from eggs that had been exposed to cues from live tadpoles (either filtered or unfiltered; Fig. 1c).

Are native anurans vulnerable to toad larval cues?

Tadpole survival at 14–25 d of age.—All eggs hatched successfully. Tadpole survival was high with little effect of treatment (Fig. 2a). For *Litoria caerulea*, survival was high both in Clutch 1 (46 of 50; 92%) and Clutch 2 (100%). In *Litoria nasuta*, survival was 100% in Clutch 1 and 82% (41 of 50) in Clutch 2 (no significant effect of exposure to tadpole cues: \(t = 0.34, P = 0.74\)). For *Litoria rothii*, Clutch 1 exhibited high survival in both treatments (48 of 50; 96%). In Clutch 2, survival was moderate (34 of 50; 68%), with no significant effect of treatment \((t = 0.92, P = 0.39)\). In *Litoria tornieri*, survival was high both in Clutch 1 (114 of 120; 95%) and Clutch 2 (110 of 120; 92%). Exposure to toad tadpole cues did not reduce survival either in Clutch 1 \((t = 1.42, P = 0.19)\) or Clutch 2 (100% survival in toad tadpole treatment).
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Tadpole body size and development at 14–25 d of age.—Because we found a significant interaction term between clutch and treatment for tadpole mass in *Litoria caerulea* ($F_{1,11} = 5.93$, $P = 0.045$) but not for SVL ($F_{1,11} = 2.53$, $P = 0.16$), we analyzed the effects of exposure on Clutch 1 and 2 separately for all response variables. Despite the interaction, exposure to toad tadpole chemicals did not affect the phenotypic traits of tadpoles after 21 d in either Clutch 1 (SVL $F_{1,3} = 0.01$, $P = 0.93$; mass $F_{1,3} = 1.04$, $P = 0.38$; developmental stage $\chi^2 = 3.45$, 1 df, $P = 0.06$) or Clutch 2 (SVL $F_{1,4} = 4.15$, $P = 0.11$; mass $F_{1,4} = 5.94$, $P = 0.07$; developmental stage $\chi^2 = 1.31$, 1 df, $P = 0.25$; Fig. 2b,c,d).

After 21 d, clutches of *Litoria nasuta* did not differ significantly in their response (for body length or mass) to embryonic exposure treatments (SVL $F_{1,8} = 0.23$, $P = 0.65$; mass $F_{1,8} = 0.01$, $P = 0.91$), so we removed this interaction term from the analysis. Treatment did not significantly affect mean body size of tadpoles after 21 d (SVL $F_{1,9} = 0.62$, $P = 0.45$; mass $F_{1,9} = 1.08$, $P = 0.33$; Fig. 2b,c), although tadpoles from Clutch 1 were larger than those from Clutch 2 (SVL $F_{1,8} = 28.68$, $P < 0.01$; mass $F_{1,8} = 17.75$, $P < 0.01$). The developmental stage of tadpoles after 21 d was not significantly affected by embryonic exposure to toad tadpoles either in Clutch 1 ($\chi^2 = 0.49$, 1 df, $P = 0.48$) or Clutch 2 (all tadpoles at developmental stage 26; Fig. 2d).

Tadpoles of *Litoria rothii* were similarly unaffected by embryonic exposure to toad tadpole chemicals. There was a significant interaction term between clutch and treatment for tadpole body mass ($F_{1,8} = 5.32$, $P = 0.05$) but not for SVL ($F_{1,8} = 3.28$, $P = 0.11$). Embryonic exposure to toad tadpole chemicals did not significantly affect the body length or mass of tadpoles after 21 d either in Clutch 1 (SVL $F_{1,9} = 0.90$, $P = 0.40$; mass $F_{1,9} = 1.18$, $P = 0.34$; Fig. 2b,c) or Clutch 2 (SVL $F_{1,4} = 3.19$, $P = 0.15$; mass $F_{1,4} = 4.55$, $P = 0.10$; Fig. 2b,c). At day 21, all surviving tadpoles from both clutches were at Gosner stage 26 (Fig. 2d).

As for the other species, *L. tornieri* were largely unaffected by embryonic exposure to toad tadpole chemicals. Clutches did not differ significantly in their responses (in larval body length or mass) to embryonic exposure treatments after 21 d (SVL $F_{1,10} = 0.30$, $P = 0.60$; mass $F_{1,10} = 0.58$, $P = 0.46$). Body size did not differ significantly between clutches at this time (SVL $F_{1,10} = 3.52$, $P = 0.09$; mass $F_{1,10} = 2.16$, $P = 0.17$), nor was there a significant effect of treatment on tadpole body size (SVL $F_{1,11} = 0.48$, $P = 0.50$; mass $F_{1,11} = 0.49$, $P = 0.50$; Fig. 2b,c). Despite a difference in mean developmental stages between clutches ($\chi^2 = 9.53$, 1 df, $P < 0.01$), embryonic exposure to toad tadpole chemicals did not affect larval development in either clutch ($\chi^2 = 0.78$, 1 df, $P = 0.38$; Fig. 2d).

Is the suppression chemical still effective after it is frozen or dried?

Survival rates of toad eggs and tadpoles.—Rates of survival were high across all treatments. In total, 275 of 280 embryos (98%) survived to stage 25, and 196 of 200 survived to day 5 (98%). We did not formally analyze these data. However, at 30 d of age, survival decreased in the experimental animals exposed to dried tadpole ($t = 2.87$, $P = 0.01$), frozen tadpole ($t = 3.53$, $P < 0.01$), and live tadpole ($t = 4.07$, $P < 0.01$) cues, compared to the controls (Fig. 3a).
Rate of metamorphosis within 30 d of age.—No metamorphs emerged from the 50 eggs exposed to the frozen tadpole treatment, and only 1 of 50 emerged from the live tadpole treatment. In contrast, rates of metamorphosis were similar in the dried tadpole and control treatments (comparing these two, $t = 0.24, P = 0.82$; Fig. 3b).

Tadpole body sizes at 5 d of age.—There was a significant effect of treatment on tadpole body length and tadpole body area ($\text{SVL } F_{3,12} = 166.30, P < 0.001$; surface area $F_{3,12} = 150.09, P < 0.001$); tadpoles from the dried tadpole treatment were similar in size to controls, whereas tadpoles exposed to frozen tadpole and live tadpole were smaller (Fig. 3c).

Metamorph body sizes.—Low sample sizes (eight metamorphs from the controls, 10 from the dried tadpole treatment, and none from either frozen tadpole or live tadpole exposure treatments) meant that we could only
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How long does the waterborne suppression cue remain potent?

Survival of eggs and tadpoles.—High survival across all treatments to stage 25 (237 of 240, 98%, embryos survived) and at 5 d (146 of 150 tadpoles, 97%) precluded formal analysis. At 30 d, 91 of 150 tadpoles were alive (61%). Tadpoles exposed to 7-d-old tadpole cues survived at similar rates to the controls ($t = 1.34, P = 0.21$), whereas exposure to the fresh tadpole cue induced lower survival rates (vs. controls, $t = 2.34, P = 0.04$; Fig. 4a).

Rate of metamorphosis within 30 d of age.—No metamorphs emerged from the fresh tadpole cue treatment, but rates were similar between the 7-d-old tadpole cue and controls ($t = 1.34, P = 0.21$), whereas exposure to the fresh tadpole cue induced lower survival rates (vs. controls, $t = 2.34, P = 0.04$; Fig. 4a).

Metamorph body sizes.—Only eight of 50 (16%) larvae metamorphosed in controls and another eight (16%) from the 7-d-old tadpole cue treatment. Body sizes of these two groups were similar ($SUL F_{1,7} = 0.39, P = 0.55$; mass $F_{1,7} = 0.55, P = 0.48$).

What concentrations are required to elicit the suppression effect?

Survival rates of toad eggs and tadpoles.—Survival rates to hatching were high overall (448 of 490, 91%), with all treatments similar to controls ($P > 0.05$; Appendix Table S2a). However, only 158 of 350 (45%) survived to 5 d of age. Survival rates of tadpoles from eggs that had been exposed to 0.001% tadpole cue were similar to those of controls, whereas all other treatments (100% through to 0.01%) showed lower survival (vs. controls, $P < 0.05$; Fig 5a; Appendix Table S2b). At 30 d of age, overall survival was 34% (118 of 350). Survival rates of tadpoles from eggs that had been exposed to 0.001% and 0.01% were similar to controls ($P > 0.05$), whereas exposure to 10% and 100% showed lower survival (vs. controls, $P < 0.05$; Fig. 5b; Appendix Table S2c). No tadpoles from 0.1% and 1% treatments survived to 30 d of age.

Tadpole body sizes at 5 d of age.—Mean body sizes differed among treatments (SVL $F_{6,14} = 26.36, P < 0.01$; surface area $F_{6,14} = 26.00, P < 0.01$). Post-hoc analysis showed that tadpoles from 0.001% were larger than controls, whereas tadpoles from 0.01% were similar in size to the control tadpoles (Fig. 5c). In contrast, tadpoles from 100%, 10%, 1%, and 0.1% treatments were smaller than controls, 0.01%, and 0.001% dilutions (Fig. 5c). Thus, the body sizes of tadpoles from 100%, 10%, 1%, and

Fig. 3. Phenotypic traits and rate of survival of cane toad (Rhinella marina) tadpoles as a function of embryonic exposure to conspecific larvae cues. Embryos were exposed to cues from frozen (tadpoles that were frozen then macerated), dried (tadpoles that were macerated, then dried), or live tadpole (cues from free-swimming tadpoles). Controls (exposed to water only) were also included. At 30 d post-hatching, we recorded rates of (a) survival (tadpoles + emerged metamorphs) and (b) metamorphosis. We also recorded (c) tadpole body length at 5 d post-hatching. Graphs mean ± SE.

compare metamorph sizes between controls and dried tadpole exposure. No significant difference was apparent ($SUL F_{1,6} = 0.24, P = 0.64$; mass $F_{1,6} = 2.35, P = 0.18$).
0.1% were affected by suppression, whereas tadpoles from 0.01% and 0.001% were not.

Rate of metamorphosis within 30 d of age.—Few metamorphs emerged, with only 12 (three from control, nine from 0.001%) recorded by day 30. Those two groups did not differ significantly in mean body sizes (SUL $F_{1,6} = 1.62, P = 0.25$; mass $F_{1,6} = 2.05, P = 0.20$).

**DISCUSSION**

Broadly, our results are encouraging for the use of conspecific tadpole pheromones as a control method to prevent effective breeding by invasive cane toads. The suppression cue appears to be a chemical not a micro-organism, is potentially toad-specific (having no observable effects on the four native species that we tested) and, although the waterborne chemical loses its potency within 7 d, it can be frozen and thus, transported to places where fresh tadpoles are unavailable. Importantly, the chemical works at very low concentrations (suppression can be induced by the output of approximately four tadpoles per 1000 L of water), suggesting that it may be effective even in large water bodies.

The experiments in which we filtered water containing the suppressor pheromone showed that the active component is very small (<0.2 μm), and thus probably a chemical rather than a living organism. Treatment did not affect survival rates in these short-term experiments, and other work shows that such effects take longer than 5 d to manifest (Clarke 2014). Ruling out micro-organisms clarifies the mechanism underlying this phenomenon. Tadpoles of some other anuran taxa produce substances that encourage the growth of pathogenic organisms, such as algae (Wong and Beebee 1994) and water mold (Kiesecker and Blaustein 1999), that may act as agents of interference competition. In practical terms, a chemical is liable to provide a more straightforward weapon for toad control than would a living organism.

One fundamental requirement for an effective control agent is that it strongly affects the target species and no other. In particular, native fauna should not be negatively influenced by the attempts to control invaders. That criterion is easier to satisfy in Australia than in many other parts of the world, because the most troublesome invasive species in Australia tend to be taxa with no close phylogenetic relatedness to any component of the endemic biota (Ratcliffe et al. 1952, Lever 2001). Importantly, our data thus far suggest that the chemical cues produced by larval cane toads are unlikely to affect the growth, development, or survival of native Australian frogs. Although we only tested four species, and our experiments were short-term, a lack of effect on native species accords with previous work on other communication chemicals produced by cane toad tadpoles. The alarm pheromone and the attractant pheromone powerfully influence toad tadpoles, but do not affect any of the native anuran species tested to date (15 species tested by Hagman and Shine 2008a, b, 2009b, five species tested by Crossland et al. 2012). The same specificity may well be true of the suppression pheromone. Nonetheless, the critical assumption of minimal collateral impact warrants additional research both on other anuran taxa and on other pond-dwelling species (fishes, insects,
crustaceans, etc.) in areas with and without invasive cane toads. Ideally, such work should be conducted in the field, as well as the laboratory, and should focus on broadening the range of anuran taxa tested (to include a much greater number of genera and localities, and additional phylogenetic lineages) to account for variation within and between different species across the cane toad’s potential invasion range. Also, future work should examine collateral impacts over broader time periods, encompassing the entire larval life of potentially vulnerable native taxa.

As expected if the suppression cue is chemical (rather than a living organism), the cue remains potent even after being frozen. However, it breaks down within 7 d (perhaps much sooner) in water and loses effectiveness if dried (still reducing survival, but not tadpole body size, metamorphosis rate, or size of metamorphs). The effectiveness of frozen tadpoles may enable toad control even in areas where tadpoles are difficult to access, such as at the invasion front. Ultimately, the most effective way to deploy the suppressor cue will be to identify and synthesize (or extract) the specific chemicals involved; but until that is accomplished, it may be possible to use fresh or frozen tadpoles to generate the cue in high-priority conservation areas.

Our dose–response trials showed a clear pattern, whereby the suppression effect was strong (and similar in magnitude) at a wide range of concentrations and negligible below a threshold of 0.01% dilution. Tadpoles exposed to the lowest concentration (0.001%) were actually larger than controls at 5 d of age, but survival rates and metamorph body sizes were similar to those of controls. Importantly, the minimum effective concentration in our experiments (0.1%) was very low, equivalent to the amount produced by three tadpoles in 750 L of water. Even tiny concentrations of pheromones may have

**Fig. 5.** Rates of survival and body sizes of cane toad (*Rhinella marina*) tadpoles as a function of embryonic exposure to concentrations of conspecific larvae cues. We prepared concentrations by taking serial dilutions of tadpole cue 100% (water containing three toad tadpoles for approximately 72 h). Controls (no cues) were also included. At 30 d post-hatching, we recorded rates of (a) survival (tadpoles + emerged metamorphs) and (b) metamorphosis. We also recorded (c) tadpole body length at 5 d post-hatching. Graphs show mean ± SE.
powerful effects (Sleeper et al. 1980, Zeeck et al. 1998), suggesting that successful toad control may be possible with very low concentrations of the relevant chemical, and thus with minimal risk of untoward collateral effects on native fauna. The number of tadpoles required to produce suppression of embryos is so low that it might even be possible to prevent toad recruitment by placing an escape-proof cage with live tadpoles in a water body. Toad tadpoles survive and grow under such conditions; and high densities reduce developmental rates and thus would prolong the production of the suppressor cue (Cabrera-Guzmán et al. 2013). Until we can identify the specific chemical responsible for inducing the suppression effect, a cageful of live tadpoles might offer a simple way to prevent toad recruitment in water bodies of particular conservation concern. Clearly, such a method would require careful field-testing prior to implementation.

The persistence of the suppression effect in frozen tadpole tissue, and its absence from dried material and 7-d-old tadpole-exposed water, suggests that the active chemical responsible for suppression may be volatile in nature (i.e. have a high vapor point at room temperature). Volatile chemicals form a major part of olfactory communication systems in many taxa (Jackson and Morgan 1993, Hofer et al. 2001, Brennan and Zufall 2006) and have previously been tested for control of pest tadpoles in fishery production (Helms 1967, Carmichael and Tomasso 1983). Further studies of volatile chemicals in this respect are a high priority.

In summary, the suppression cue offers considerable promise as a management tool to curtail recruitment of invasive cane toads in Australia. Further studies are needed to identify the specific chemicals responsible and test the effects of those chemicals on larger, more ecologically relevant scales (such as natural ponds). Such studies could simultaneously evaluate the effects of toad breeding on natural systems and the potential side effects both of removing toad tadpoles (using attractant pheromones; Crossland et al. 2012) and of inducing high rates of tadpole mortality (using the suppression cue; Clarke 2014). Even if the suppression cue has no direct effect on other life forms in the pond, the mortality of thousands of tadpole exposure at an early (and toxic; Hayes et al. 2008) stage of development may have other effects. For example, native tadpoles are more likely to scavenge dead toad tadpoles than to feed on live ones (Crossland 1997); and thus, may be killed by poisoning if we induce high mortality in toad tadpoles. Such effects are unlikely to be significant at a whole-pond level, but warrant careful analysis.

By focusing on the early life stage of toads, we are not only targeting the most vulnerable life stage, but also the one that is most concentrated spatially, and hence where control efforts can influence a high proportion of all individuals in that life stage within a given area. Additionally, cane toads breed in only a small (and distinctive) proportion of the water bodies available (Williamson 1999, Hagman and Shine 2006, Semeniuk et al. 2007). Hence, at some sites, it may be feasible for managers to deploy suppression cues in all of the local water bodies that are suitable for toad breeding. Prevention of recruitment is the key to controlling a high-fecundity invader like the cane toad. Unless breeding can be stopped, no other control method (for example, targeted at adults) can have any long-term impacts on toad abundance (Shine 2010). Encouragingly, pheromone-based methods may be capable of massively reducing recruitment rates of cane toads in local areas. We will never exterminate cane toads from Australia, but we may be able to reduce their densities in areas where water bodies are accessible and where the benefits of toad control outweigh the costs of implementation.

More generally, our results suggest that we may indeed be able to use larval pheromones for control of invasive anurans, as first suggested by Richard Wassersug many years ago (1997). Intraspecific communication systems may provide highly species-specific weapons that do not affect native taxa. Pheromones may prove useful for the control of many problematic species, including lampreys (Johnson et al. 2009), beetles (Ritter and Persoons 1974, Töth et al. 2003), invasive snakes (Mason and Greene 2001), invasive fish (Elkins et al. 2009), ectoparasites (Sonenshine 2006) and rodents (Kamalakkannan et al. 2006). We may one day be able to add anurans to that list. Because many invasive species thrive in anthropogenically disturbed habitats that contain few other taxa, we doubt that cane toads are unique in having evolved mechanisms for intraspecific competitive suppression. Future research could usefully explore the possibility that invasive species have evolved weapons with the potential to provide novel, highly targeted mechanisms for the control of troublesome introduced taxa.

Acknowledgments
This work was supported by the Australian Research Council. We thank Mark Fisher and Madeleine Sanders for their assistance, and Melanie Elphick for aiding in manuscript preparation.

Literature Cited
Clarke, G. S. 2014. Can we control cane toads by using their own weapons against them? Thesis. School of Biological Sciences, University of Sydney, Sydney, NSW Australia.


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