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Egg temperature modifies predator avoidance and the effects of the insecticide endosulfan on tadpoles of an Australian frog

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Summary

1. Attention is shifting from simplistic explanations of global amphibian declines that posit a single cause (such as climate change, pesticide contamination or disease) to more complex scenarios that involve interactive effects. Temperature is a pervasive influence on frog development, particularly during the egg and larval stages. However, the effect of temperatures experienced early in ontogeny on later larval behaviour or response to agrochemicals is little known.

2. Eggs of the Australian frog *Limnodynastes peronii* were reared at two temperatures that simulate naturally occurring cool and warm temperature regimes (14 ± 3 °C and 20 ± 3 °C). Tadpoles were then exposed to sublethal concentrations of the organochlorine insecticide endosulfan, at a common temperature. Endosulfan often contaminates aquatic environments, yet its effects on Australian frogs are unknown. Tadpoles reduced feeding after 48 h of exposure to endosulfan concentrations that occur in the field (both 0·03 μg l^{-1} and 1·3 μg l^{-1}). Feeding remained depressed at 1·3 μg l^{-1} endosulfan up to 9 days after tadpoles were transferred to endosulfan-free water.

3. Egg-rearing temperature and endosulfan interacted to affect tadpole length. Further, tadpoles exposed to endosulfan were significantly shorter than control tadpoles. They were also more vulnerable to capture by an invertebrate (odonate) predator than controls of the same size when tested 9 days after transfer to clean water. While warm egg-rearing temperatures significantly decreased vulnerability to capture, tadpoles were proportionally more adversely affected by endosulfan. Thus, egg-rearing temperature altered predator avoidance and changed the way in which endosulfan affected growth. Moreover, endosulfan significantly decreased feeding, growth and predator avoidance.

4. *Synthesis and applications*. Not only can short-term exposure to endosulfan at levels within regulatory guidelines and frequently reported in natural waterbodies influence tadpole viability, but the sensitivity of the tadpoles to this effect depends upon the thermal regimes that they encounter over their first few days of life. These data therefore suggest that existing water quality prescriptions may not provide adequate protection, while also illustrating how aspects of climate and thermal regimes might interact with pesticides to have cumulative effects on amphibian fitness.

Key-words: Australia, climate, fitness, odonate, pesticide, sublethal, temperature.

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Introduction

Chemical contamination continues to be implicated as a possible factor in amphibian population decline (Bishop 1992; Carey & Bryant 1995; Bridges & Semlitsch 2000; Semlitsch, Bridges & Welch 2000; Mann & Bidwell 2001; Marco *et al*. 2001). The effects of contamination by organic and inorganic chemicals on amphibians may depend on other biotic and abiotic variables at the time of exposure, such as photo-enhancement by ultraviolet (UV)-B radiation (La Clair, Bantle & Dumont 1998; Zaga *et al*. 1998; Crump, Lean & Trudeau 2002), presence of other species (Lefcort *et al*. 1999) and the presence of predators (Relyea & Mills 2001). Therefore, in order to understand the impact

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of agricultural chemicals on ecological communities, more complex but biologically more realistic experimental designs are required that incorporate interactive effects between stressors acting at different phases of amphibian life cycles.

Anuran embryos and larvae generally occupy thermally heterogeneous environments, and thermal regimes can strongly influence anuran development (Duellman & Trueb 1986; Ultsch, Bradford & Freda 1999). Temperature or temperature variability can alter the potency of some pesticides to tadpoles (Boone & Bridges 1999; Broomhall 2002). Less expected is the possibility that, because different life-history stages may respond differently to a given stress (Bridges 2000), temperature changes during early life-history stages may have substantial influences on later development.

The eggs of many anuran species develop over relatively short time periods, approximately 1–10 days (Duellman & Trueb 1986). Despite the shortness of the embryonic period, embryonic conditions can modify development. For example, *Rana temporaria* larvae that had received enhanced levels of UV-B as embryos subsequently metamorphosed later, and at a smaller size, than larvae that had not (Pahkala, Laurila & Merilä 2001). Egg temperature can also influence locomotor performance of ectotherms (Elphick & Shine 1998). Given these data, embryonic thermal history may change the way in which later pesticide exposure influences tadpole growth and behaviour. However, this possibility has not attracted any study to date.

Endosulfan is an organochlorine cyclodiene insecticide that is used extensively throughout the world. In 2001, endosulfan was among the 10 pesticide residues most frequently found on fruits and vegetables from the 15 member states of the European Union (European Commission 2003), and in the USA more than 625 000 kg of endosulfan active constituent is used annually (United States Environmental Protection Agency 2002). In Australia, endosulfan is sprayed on cotton crops, stone fruits, vegetables, tobacco, cereals, legumes, oilseeds, citrus, tropical fruits, ornamentals and others (NRA 1998). Cotton is cultivated in very large continuous areas and approximately 700 tonnes of technical endosulfan (95% active ingredient) are sprayed across cotton crops during the early part of the spring season every year (NRA 1998). Endosulfan sulphate, the predominant oxidation product, is frequently transported into riverine environments during storm events (Leys *et al*. 1998; Raupach & Briggs 1998; Leonard *et al*. 1999).

Many Australian frog species breed in spring (Tyler 1994) at a time when rivers and waterbodies exhibit strong short-term fluctuations both in levels of agrochemical residues (Cooper 1996; Muschal & Cooper 1998) and thermal regimes. Furthermore, toxic endosulfan degradates may remain in soil and sediments throughout the year (Nowak & Julli 1991), suggesting that even after periodic direct exposure tadpoles may be continuously exposed to sublethal levels of endosulfan via suspended particulate matter or ingestion of sediment.

The effects of endosulfan on Australia anurans remain unstudied despite their substantial phylogenetic distance from species tested in other parts of the world (Mann & Bidwell 1999), and a high risk of endosulfan contamination in spring breeders. This study tested the hypothesis that egg-rearing temperature might modify the effects of endosulfan on tadpoles of a frog species *Limnodynastes peronii* (Duméril and Bibron) from south-eastern Australia. The experiment was designed to mimic a complication that occurs in the real world variations in egg-rearing temperature. The study also attempted to ascertain if exposure to biologically relevant concentrations of endosulfan (in the commercial pesticide Thiodan®, AgrEvo, Sydney, Australia) affected survivorship or fitness-related traits in tadpoles. Sublethal response to toxicants is an important component of pesticide exposure in a broader ecological sense (Buckler *et al*. 1995). For example, glaucous gulls *Larus hyperboreus* with high blood concentrations of organochlorines exhibit increased absenteeism from the nest (Bustnes *et al*. 2001), perhaps leading to decreased reproductive success. Although sublethal reactions are rarely considered in amphibian research, recent papers highlight the importance of testing endpoints such as growth rates, swim speed and vulnerability to predation (Semlitsch, Foglia & Mueller 1995; Bridges 1997, 1999a,b).

Methods

STUDY SPECIES AND EGG COLLECTION

Limnodynastes peronii occurs throughout eastern Australia, inhabiting dams, ponds, flooded grassland and lentic parts of streams, and is often found in suburban and disturbed sites (Anstis 2002). The species has a floating foam egg mass and the tadpoles are mostly bottom-dwellers (Anstis 2002). The larval life span can be as long as 12 months and tadpoles can overwinter (Anstis 2002).

Three floating egg masses at Gosner stage 7 (Gosner 1960), collected from ponds at Darkes Forest, south-eastern New South Wales, on 20 September 2001, were brought back to the laboratory at the University of Sydney, Sydney. Six samples of water at the site were tested for organochlorine and organophosphate pesticides and phenoxy acid herbicides by the Australian Governmental Analytical Laboratories, Sydney; none were detected (S. Broomhall, unpublished data). Therefore animals from the site were presumed to have no recent pesticide exposure. The egg masses were immediately separated into halves and each half was allocated to 1·5-l glass jars filled with 1·3-l of aerated, aged tap water.

EGG TREATMENT

Three jars of eggs (each containing half an egg mass) were placed into each of two diel-cycling incubators (a total of six jars) that were set to 14 ± 3 °C (cool) and

20 ± 3 °C (warm), temperatures which reflected natural variability within the same study site (S. Broomhall, unpublished data). Incubators were programmed in eight gradual heating and cooling steps over a 24-h period that had been fitted to a sinusoidal daily curve. Replacement water was also kept in the incubators and used to refresh jars containing eggs every second day. Eggs from the 20 ± 3 °C (warm) treatments hatched into free swimming larvae in 2 days, whereupon they were removed and placed in 2-l glass jars filled with aerated, aged tap water, in a room held at 17 ± 1 °C with a 14-h light : 10-h dark photoperiod. Tadpoles remained separated by clutch. Eggs at 14 ± 3 °C (cool) hatched after 6 days and were treated in the same manner as the tadpoles from warm eggs.

TADPOLE TREATMENT

As hatching was temporally displaced (by 4 days), tadpoles from eggs reared at cool temperatures (cool eggs) were allocated to endosulfan treatments 4 days after tadpoles from eggs reared at warm temperature (warm eggs), in order to control for the effects of developmental stage. All behavioural testing was also separated by 4 days for the same reason. In each case, tadpoles were exposed to nominal concentrations of 0 (control), 0·03 μg l^{-1} (low) and 1·3 μg l^{-1} (high) active ingredient endosulfan (in the commercial formulation Thiodan®) 11 days after hatching (Stage 24; Gosner 1960). The concentrations for this experiment represent conservative estimates of actual field exposure. The Australia and New Zealand guidelines for the fresh and marine water quality trigger value for endosulfan is 0·03 μg l^{-1} (ANZECC & ARMCANZ 2000a). In Australia, 65–70% of tested off-target locations within areas of irrigated agriculture exceeded 0·01 μg l^{-1} total endosulfan during spring and summer for 3 years running (Muschal & Cooper 1998) and 32% exceeded 0·05 μg l^{-1} (Cooper 1996). Concentration spikes up to 1·75 μg l^{-1} (Muschal & Cooper 1998) and 8·6 μg l^{-1} occur after storm events (Muschal 1997).

Seven 2-l glass jars were filled with 1·8 l of endosulfan solution (solutions comprised aerated, aged tap water spiked with endosulfan) for each concentration, for a total of 21 jars. pH was measured with a Piccolo2 pH meter (Hanna Instruments, Woonsocket, RI, USA), and varied between pH 6·9 and 7·1. The clutches were mixed in equal numbers, and five tadpoles at Gosner stage 24 were randomly selected and allocated to each of the 21 jars. In addition, 34 tadpoles were killed in MS222 (Sigma, St Louis, MO), preserved and later measured. After 96 h, the solutions were completely replaced from fresh stock solutions (thus animals were exposed for a total of 192 h). The same procedure was followed for animals from cool eggs. Exposure regimes thus mimicked flushes of contamination. Tadpoles from cool eggs were 11·73 ± 0·23 mm (mean ± SE), those from warm eggs were 11·46 ± 0·07 mm (no differences: $F_{1,65} = 1·26$, $P = 0·267$).

Nominal endosulfan concentrations were used to calculate all endpoints in this experiment. Nominal endosulfan concentrations have been found to approximate closely measured endosulfan concentrations (see below). Because endosulfan breaks down in water, concentrations would have declined during the 96 h until solutions were refreshed, and then declined again (see Endosulfan analysis). For this reason, actual exposure would have been less than reported.

FEEDING

After exposure to endosulfan for 48 h, approximately 20 mg of frozen-then-thawed endive lettuce was added to each jar. After 15 min, each jar of five tadpoles was inspected for 5 seconds and the number of tadpoles feeding, swimming or motionless was scored. Feeding was defined as any active tail movements whilst the mouth was on the food. This observation was repeated after another 15 min and the mean of the two values calculated. All food was removed 2 days later, when solutions were replaced at 96 h. Again, food was added and feeding was scored after a further 48 h in fresh solutions. At the end of the second 96-h period all animals were transferred to fresh, aged tap water, and the food was removed. Once again, food was added and feeding was scored after 48 h in clean water and again after 9 days (water was refreshed every 3 days). The data for feeding fitted a normal distribution after arcsine transformation and were analysed using ANOVA. All data were analysed using the statistics program Statview (SAS Institute 1998). For all results, main effects are only discussed when interactions were non-significant. Where interactions were significant, values are reported and the treatments were split for further analysis.

LENGTH

Tadpole survivorship after exposure to endosulfan was 100% and there was no post-exposure mortality. After 9 days in clean water (tadpoles were 28 days old), all tadpoles were successively placed in a transparent container that was positioned over grid paper and filled with water to a depth of 1 cm. Tadpoles were videotaped against the grid paper, the videos downloaded to computer, and tadpoles measured on-screen at a later date. This procedure was non-intrusive and allowed measurement of tadpoles without anaesthesia or removal from water. The data for length fitted a normal distribution and did not require transformation before ANOVA.

PREDATION

Tadpoles from cool-reared eggs and tadpoles from warm-reared eggs were run in two identical trials. In each trial, 14 tadpoles (Gosner stage 25), were selected from each of the control and two endosulfan treatments (two from each jar, for a total of 42 tadpoles), 9 days after removal from control and treated water. Similar sized tadpoles were used to control for size-dependant susceptibility to capture by an odonate

naiad predator. They were allocated to separate rectangular plastic containers, 22 cm × 13 cm × 8 cm, that had been acid washed, solvent rinsed and then filled with aerated, aged tap water. Tap water had no detectable pesticide or herbicide residues (S. Broomhall, unpublished data). The outside of the containers had been wrapped in dark grey duct tape in order to prevent test tadpoles and predators seeing movement from other containers, and the bottom was covered in a layer of washed aquarium pebbles. Tadpoles were allowed to settle for 30 min before odonate predators [*Hemianax papuensis* (Burmeister): headwidth 8·47 ± 0·04 mm] were allocated (one per container). Predators had been fed a *L. peronii* tadpole every 2–3 days and then starved for 1 day prior to testing to promote consistent hunger between individuals. A stopwatch was used to record when an odonate was seen to capture a tadpole to the nearest 30-second interval. Times to capture were log transformed to normalize the data and standardize variances, and analysed using ANOVA.

BEHAVIOURAL RESPONSES TO PREDATOR CUES (ACTIVITY)

In these trials, both warm-reared and cool-reared tadpoles were tested at the same time, after 29–33 days in clean water. Thirty-four round polyethylene plastic containers, 16 cm in diameter, were filled with aerated, aged tap water to a depth of 9 cm. Round containers were chosen to preclude preferences for corners. Plastic weed and washed aquarium pebbles were placed in the container to provide cover, in a line from the edge of the container to the centre. The edge or centre of containers was thus available both with and without cover, but the pebbles and weed occupied ≤ 15% of the available space in the container. A small glass vial 5·5 cm × 2·7 cm diameter, with its opening covered in mesh, was placed in the centre of each of the containers and predatory odonates were subsequently added to half of them. Odonate naiads were collected from Darkes Forest at the same time as parent frogs and fed a *L. peronii* tadpole every 2–3 days. Odonates were fed the same tadpole species because some tadpoles modify their behaviour according to perceived risk; this is in turn affected by diet of the predator (Laurila, Kujasalo & Ranta 1997; Lefcort *et al*. 1999). Odonates were allowed to swim freely in the containers for roughly 30 min before being placed into the vial, in order to ensure that any chemical predator cues were evenly distributed throughout the container.

Eighteen tadpoles (Gosner stage 25) from each of the control, 0·03 and 1·3 μg l^{-1} endosulfan groups (taken directly from the 2-l jars) were randomly allocated to polyethylene containers, three tadpoles per container, within predator treatments (predator and no predator), for a total of three replicates in each predator–chemical combination (2 predator × 3 chemical). Tadpoles were allowed to acclimate for about 30 min and then each container was inspected for 5 seconds to determine how many tadpoles were (i) active in the open (activity was taken as any kind of tail movement), (ii) inactive in the open, (iii) active while hiding (among the plastic weed and pebbles) and (iv) inactive while hiding. This observation was repeated twice, each time after waiting 15 min to allow the tadpoles scope to change behaviours, for a total of three observations for each container. The proportion of tadpoles in each of the behaviour categories was calculated as a mean of the three separate observations, arcsine transformed to normalize the data and analysed using ANOVA. Reanalysis as a repeated-measures ANOVA with observations as the repeated factor gave the same results.

ENDOSULFAN ANALYSIS

Nominal concentrations were calculated and mixed in the same manner as described for tadpole treatment, using the same equipment and jars; however, tadpoles were not present in the solutions. There were nine replicate jars of 1·5 l at each concentration. The Thiodan was solvent-extracted from solution using dichloromethane, separated, and passed through anhydrous sodium sulphate to remove water. They were concentrated to 1 ml in 200-ml evaporating tubes that were placed in a 35 °C water bath under nitrogen atmosphere in a Zymark TurboVap II Concentration Workstation (Zymark Corp., Hopkinton, MA, USA). Solvent extractions of three replicate jars were carried out after 1 h, 48 h and 96 h, for each concentration. The extracts were analysed by gas chromatography using a Hewlett-Packard (HP) 5890 Series II Plus (Palo Alto, CA, USA) coupled with an autosampler 7673 that was equipped with an electron capture detector and a capillary HP-5 column (30 m × 0·32 mm internal diameter × 0·25 μm film thickness). The detection limit was 0·2 μg l^{-1}. The internal standard (p,p′-dichlorodiphenyldichloroethylene) indicated an extraction efficiency of 90%. All samples were run twice. Retention time was used to identify endosulfan alpha and beta isomers, and endosulfan sulphate. The values were determined by the increase in peak height and area from standard additions, using an HP 3365 Series II Chemstation software package. Actual concentrations were 85% of nominal values, probably due to adsorbtion to the glass vessels, and concentrations decreased by 50% over 96 h (S. Broomhall, unpublished data).

Results

FEEDING

Tadpoles from cool eggs had lower feeding rates than tadpoles from warm eggs after 48 hours of exposure to endosulfan ($F_{1,18}$ = 5·998, P = 0·019), 48 h in clean water ($F_{1,18}$ = 8·917, P = 0·005) and 9 days in clean water ($F_{1,18}$ = 8·688, P = 0·006), although egg-rearing temperature did not exert an effect after 144 h of exposure to endosulfan ($F_{1,18}$ = 0·286, P = 0·596). The presence

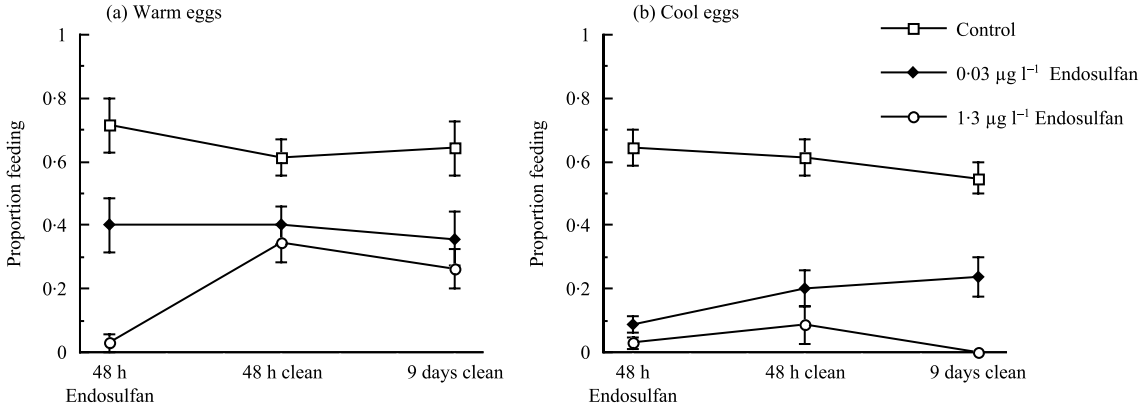

Fig. 1. Proportion of *Limnodynastes peronii* tadpoles feeding after 48 h exposure to endosulfan, 48 h in clean water and 9 days in clean water, for (a) tadpoles reared as warm eggs and (b) tadpoles reared as cool eggs. Lines are means ± 1 SE.

of endosulfan (both low and high concentrations) significantly reduced the proportion of tadpoles feeding after 48 h compared with controls ($F_{2,18}$ = 39·305, P < 0·0001; Fig. 1). The effect of endosulfan remained significant after 144 h in endosulfan ($F_{2,18}$ = 62·672, P < 0·0001), after 48 h in clean water ($F_{2,18}$ = 26·382, P < 0·0001; Fig. 1a), and remained highly significant after 9 days in clean water ($F_{2,18}$ = 44·669, P < 0·0001; Fisher's PLSD, control vs. high, P < 0·0001; control vs. low, P < 0·0001; low vs. high, P = 0·0022; Fig. 1).

LENGTH

The data for length fitted a normal distribution and did not need transformation. Prior to exposure to endosulfan (just after hatching), tadpoles from cool eggs did not differ in length ($F_{1,65}$ = 1·255, P = 0·267) or mass ($F_{1,65}$ = 2·784, P = 0·100) from tadpoles hatched from warm eggs. Nine days after endosulfan exposure (tadpoles were 28 days old), endosulfan significantly affected length ($F_{2,36}$ = 32·974, P = 0·0001) while egg-rearing temperature did not ($F_{1,36}$ = 0·0004, P = 0·984). However, the interaction between endosulfan and egg temperature was significant ($F_{2,36}$ = 6·07, P = 0·005) and thus the results were split by egg temperature and analysed separately.

Tadpoles reared as cool eggs and exposed to endosulfan were significantly shorter than control tadpoles ($F_{2,18}$ = 29·163, P < 0·0001). This was also the case for tadpoles reared as warm eggs ($F_{2,18}$ = 6·414, P = 0·008). Tadpoles reared as cool eggs and exposed to 1·3 µg l^{-1} (high) concentrations were shorter than those exposed to 0·03 µg l^{-1} (low) concentrations, which were in turn shorter than controls (Fisher's PLSD, control vs. low, P < 0·0001; low vs. high, P = 0·032; control vs. high, P < 0·0001; Fig. 2). However, tadpoles reared as warm eggs differed from this pattern, in that those tadpoles in either low or high endosulfan concentrations were shorter than controls, but not shorter than each other (Fisher's PLSD, control vs. low, P = 0·0464; low vs. high, P = 0·173; control vs. high, P = 0·002; Fig. 2).

Tail lengths followed the same significant pattern as total length; however, because tail length may vary as a function of total length, ANCOVA with total length as the covariate was performed. There were no significant differences (cool eggs, $F_{2,17}$ = 0·212, P = 0·811; warm eggs, $F_{2,17}$ = 2·676, P = 0·098), indicating that shorter tails caused by endosulfan simply reflected an overall decrease in length.

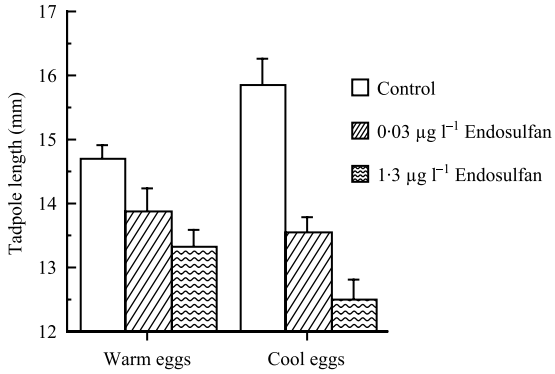

Fig. 2. Length of *Limnodynastes peronii* tadpoles, according to endosulfan exposure and egg-rearing temperature (warm, 20 ± 3 °C; cool, 14 ± 3 °C). Columns are means ± 1 SE.

PREDATION

Tadpoles that had been reared at cool temperatures as eggs were captured significantly sooner than tadpoles that had been reared as warm eggs ($F_{1,78}$ = 31·541, P < 0·0001). Exposure to endosulfan significantly shortened time to capture by an odonate predator ($F_{2,78}$ = 4·246, P = 0·018). Tadpoles exposed to low and high endosulfan were both consumed significantly faster than control tadpoles (Fisher's PLSD, control vs. high, P = 0·012; control vs. low, P = 0·016; low vs. high, P = 0·903; Fig. 3).

BEHAVIOURAL RESPONSES TO PREDATOR CUES (ACTIVITY)

The proportion of tadpoles hiding and inactive (no tadpoles were ever observed to be active while hiding)

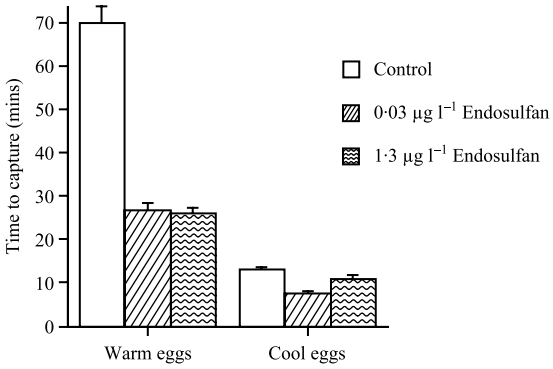

Fig. 3. Time to capture by an odonate predator of *Limnodynastes peronii* tadpoles exposed to endosulfan, according to egg-rearing temperature (warm, 20 ± 3 °C; and cool, 14 ± 3 °C). Columns are means ± 1 SE.

did not change according to egg-rearing temperature ($F_{1,24}$ = 1·326, P = 0·261), endosulfan exposure ($F_{2,24}$ = 0·506, P = 0·609) or odonate cues ($F_{1,24}$ = 2·699, P = 0·114). The proportion of those tadpoles observed to be inactive in the open did not change in relation to egg-rearing temperature ($F_{1,24}$ = 1·098, P = 0·305), endosulfan exposure ($F_{2,24}$ = 0·406, P = 0·671) or odonate cues ($F_{1,24}$ = 0·606, P = 0·444).

Discussion

Egg-rearing temperature affected feeding rate and predator avoidance by *L. peronii* tadpoles (when tested 28 days later). Endosulfan significantly decreased feeding, growth and predator avoidance in tadpoles, but did not alter behaviour. Moreover, the effects of egg-rearing temperature and endosulfan on tadpole length were interactive. Consequently, these data indicate that egg-rearing temperature may alter the later effects of endosulfan on growth, and that short-term exposure to endosulfan can influence tadpole viability either immediately or over an extended period.

The lowered feeding rate in tadpoles hatched from cool eggs may reflect a fixed lower metabolic rate associated with cool egg-rearing temperatures (because all tadpoles were maintained at the same temperature). While existing data on thermal acclimation in amphibians are extremely variable across species and studies, Rome, Stevens & John-Alder (1992) concluded that, in general, physiological functions are slower if acclimated to low temperatures than they would be if acclimated to high temperatures. Unfortunately, there are so few data on the effect of embryonic developmental temperatures on subsequent metabolism and performance in anurans, it is difficult to explore potential reasons for this result.

Regardless of egg-rearing temperature, exposure to both concentrations of endosulfan continued to reduce the proportion of tadpoles feeding even on the last day tested, nine days after tadpoles were no longer exposed to endosulfan. Thus, reductions in feeding may have potentially continued longer than the 9 days in clean water used in this experiment. Decreases in feeding rate can limit energy gain and consequently have repercussions on growth rate. For example, lower food availability reduced size at metamorphosis in tadpoles of the spadefoot toad *Scaphiopus couchii* (Newman 1994). The same trend was evident in the data reported here, with tadpoles exposed to even the lowest concentration of endosulfan shorter than control tadpoles. Larger size at metamorphosis and earlier metamorphosis can enhance survivorship to maturity, hasten first reproduction and enable larger size at first reproduction (Smith 1987; Berven 1990). Thus, pulsed contact with low concentrations of endosulfan may affect metamorphosis and subsequent survivorship of this study species.

Tadpole behaviour did not appear to change in response to predator cues, egg-rearing temperature or previous endosulfan exposure. However, as tadpoles were tested 29–33 days after cessation of endosulfan exposure, potential short-term alterations in behaviour would not have been detected. It is none the less interesting to note that this species does not appear to respond to predator cues, in contrast to many northern hemisphere species (Relyea & Werner 1999; Eklov 2000; Laurila 2000; Relyea 2000). As tadpoles had been reared in laboratory conditions and thus had never previously experienced predator cues it may be possible, albeit unlikely, that behavioural responses are partially learned in this study species. Learned predator avoidance has been shown in lizard species (Marcellini & Jenssen 1991). On the other hand, anti-predator behaviour between naive and experienced tadpoles of *Bufo americanus* (American toad) did not differ (Gallie, Mumme & Wissinger 2001), arguing that in some species predator responses may be genetically determined. Alternatively, the methodology used in this study would not have detected other forms of response, such as reductions in the distances travelled (Anholt, Werner & Skelly 2000), or long-term morphological changes, such as deeper tail fins (McCollum & Van Buskirk 1996; McCollum & Leimberger 1997; reviewed by Chivers & Smith 1998; Van Buskirk & Saxer 2001).

The temperature that an egg experienced affected the resulting tadpole's susceptibility to a predator 28 days later, despite experiencing a common (and different) temperature in those intervening days. Furthermore, endosulfan exposure increased vulnerability to a common predator when tested 9 days after cessation of pesticide exposure. Tadpoles hatched from warm eggs appeared to be better able to avoid predators than those hatched from cool eggs, yet they were also more adversely affected by endosulfan (Fig. 3). Consequently, it appears that both egg-rearing temperature and exposure to endosulfan can potentially cause long-term or perhaps permanent alterations in a tadpole's physiology. As no behavioural change in the tadpoles according to endosulfan or egg-rearing temperature was detected, the mechanism by which this effect was manifested may have been a physiological one, such as a change in burst speed or a change in body shape.

Anax dragonfly larvae preferentially kill *Pseudacris triseriata* tadpoles with wider and deeper bodies, narrower tail muscles and smaller tail fin depth (Van Buskirk, Mccollum & Werner 1997). Although the results reported here indicate that relative tail and body lengths did not differ between treatments, measures of body width and depth were not taken. Tail length can alter sprint speed and vulnerability to predation (although the direction of the change appears to be different in different species and situations; cf. Van Buskirk & Mccollum 2000; Parichy & Kaplan 1995). Because tail length did not differ between treatments, a more likely possibility for the differences in predation rates is a change in burst speed (as mediated by changes in muscle phenotype) rather than sprint speed.

Larval developmental temperature strongly affected burst speed in tadpoles of *Hyla regilla* Pacific treefrog regardless of acclimation or testing temperature (Watkins 2000). Tail myofibrillar ATPase activity, which is associated with the maximum shortening velocity of a muscle, was higher in cool-reared larvae (Watkins 2000). It may therefore be possible that egg-rearing temperature can also alter muscle phenotype, although this remains to be tested.

Whether the mechanism to explain the observed differences in predation lies with body shape or burst speed, predation is a major force structuring tadpole communities (Anholt & Werner 1995). Indeed, predators greatly influence the density and distribution of other organisms (Ormerod 2002). Thus, an increased predation risk over and above alterations in size may have substantial impacts on future survival in the current study species.

Although amphibians can often operate at suboptimal temperatures (reviewed by Rome, Stevens & John-Alder 1992), additional stresses imposed by exposure to agrochemicals (especially if they differ according to the thermal history of the tadpole, as is the case here with length) may substantially alter anuran population dynamics. The implications of changes in temperature, perhaps associated with global climate change, are therefore serious and warrant further repetition and investigation. This work has been repeated in the treefrog *Litoria peronii*, which, although it has a similar name, is from an entirely different family group, the Hylidae. Although there were subtle behavioural differences between species, egg-rearing temperature and endosulfan altered predator avoidance (S. Broomhall, unpublished data). In this way, changes in temperature regimes due to climate warming may potentially combine with agricultural practices to affect anuran populations.

Many standard toxicity tests do not address the possibility that pesticides may compromise future components of fitness, nor how pesticide exposure may interact with other factors present at the time of exposure. For example, the effects of a stressor differed at different population densities in the marine copepod *Tisbe battagliai* (Sibly, Williams & Jones 2000). An endosulfan concentration of 20·3 mg l^{-1} inhibited cortisol secretion in cells of rainbow trout *Oncorhynchus mykiss* by 76–82%, yet cell viability only decreased by 5% (Leblond, Bisson & Hontela 2001). This confirms that deleterious effects may occur at levels many times lower than those commonly used as endpoints. Similarly, Buckler *et al*. (1995) found that feeding activity (prey strike rates) was the most sensitive indicator of acidity-induced stress (and also aluminium-induced stress at pH 5·5) in Atlantic salmon *Salmo salar*, while mortality was a much less sensitive endpoint. Consideration of correlates of fitness such as those reported here are an important step towards gaining an understanding of the mechanisms by which exposure to agricultural chemicals may impact upon frog populations in a broader ecological context.

Of particular concern is that tadpole fitness was decreased by short-term exposure to an endosulfan concentration listed as the ANZZEC trigger value to protect 99% of species. Trigger values are generally expected to provide a greater than threefold protection from acute toxicity (ANZECC & ARMCANZ 2000b). Nevertheless, the current data show that this trigger concentration of 0·03 μg l^{-1} significantly (and persistently) impaired feeding, and also resulted in an increased risk of predation and significantly shorter tadpoles. This result has implications for natural resource management decisions, as existing water quality prescriptions may not provide adequate protection for populations over the long-term. This study demonstrates that extremely low concentrations of endosulfan have the potential to affect natural frog communities adversely and illustrates how interacting stressors may have cumulative effects on tadpoles. Furthermore, this study indicates that minor, short-term shifts in temperature may alter the impacts of agrochemical contaminants on anuran populations over the long-term.

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References

Anholt, B.R. & Werner, E.E. (1995) Interaction between food availability and predation mortality mediated by adaptive behavior. *Ecology*, **76**, 2230–2235.

Anholt, B.R., Werner, E. & Skelly, D.K. (2000) Effects of food and predators on the activity of four larval ranid frogs. *Ecology*, **81**, 3509–3521.

Anstis, M. (2002) *Tadpoles of South-Eastern Australia: A Guide with Keys*. Reed New Holland, Sydney, Australia.

ANZECC & ARMCANZ (2000a) *Australian and New Zealand Guidelines for Fresh and Marine Water Quality*. *I. The Guidelines*. National Water Quality Management Strategy Paper No. 4. Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand, New Zealand.

ANZECC & ARMCANZ (2000b) *Australian and New Zealand Guidelines for Fresh and Marine Water Quality*. *II. Aquatic Ecosystems: Rationale and Background Information*. National Water Quality Management Strategy Paper No. 4. Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand, New Zealand.

Berven, K.A. (1990) Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). *Ecology*, **71**, 1599–1608.

Bishop, C.A. (1992) The effects of pesticides on amphibians and the implications for determining causes of declines in amphibian populations. *Declines in Canadian Amphibian Populations: Designing a National Monitoring Strategy* (ed. C. A. Bishop), pp. 67–70. Occasional Paper 76. Canadian Wildlife Service, Ontario, Canada.

Boone, M.D. & Bridges, C.M. (1999) The effect of temperature on the potency of carbaryl for survival of tadpoles of the green tree frog (*Rana clamitans*). *Environmental Toxicology and Chemistry*, **18**, 1482–1484.

Bridges, C.M. (1997) Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. *Environmental Toxicology and Chemistry*, **16**, 1935–1939.

Bridges, C.M. (1999a) Effects of a pesticide on tadpole activity and predator avoidance behavior. *Journal of Herpetology*, **33**, 303–306.

Bridges, C.M. (1999b) Predator–prey interactions between two amphibian species: effects of insecticide exposure. *Aquatic Ecology*, **33**, 205–211.

Bridges, C.M. & Semlitsch, R.D. (2000) Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. *Conservation Biology*, **14**, 1490–1499.

Broomhall, S.D. (2002) The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability to predation in *Litoria citropa* tadpoles. *Aquatic Toxicology*, **61**, 243–250.

Buckler, D.R., Cleveland, L., Little, E.E. & Brumbaugh, W.G. (1995) Survival, sublethal responses, and tissue residues of Atlantic salmon exposed to acidic pH and aluminium. *Aquatic Toxicology*, **31**, 203–216.

Bustnes, J.O., Bakken, V., Erikstad, K.E., Mehlum, F. & Skaare, J.U. (2001) Patterns of incubation and nest-site attentiveness in relation to organochlorine (PCB) contamination in glaucous gulls. *Journal of Applied Ecology*, **38**, 791–801.

Carey, C. & Bryant, C.J. (1995) Possible interrelationships among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspectives*, **103**, 13–17.

Chivers, D.P. & Smith, R.J.F. (1998) Chemical alarm signalling in aquatic predator–prey systems: a review and prospectus. *Ecoscience*, **5**, 338–352.

Cooper, B. (1996) *Central and Northwest Regions Water Quality Program: 1995/96 Report on Pesticides Monitoring*. NSW Department of Land and Water Conservation, Technical Services Directorate, Australia.

Crump, D., Lean, D. & Trudeau, V.L. (2002) Octylphenol and UV-B radiation alter larval development and hypothalamic gene expression in the leopard frog (*Rana pipiens*). *Environmental Health Perspectives*, **110**, 277–284.

Duellman, W.A. & Trueb, L. (1986) *Biology of Amphibians*. McGraw-Hill, New York, NY.

Eklov, P. (2000) Chemical cues from multiple predator–prey interactions induce changes in behavior and growth of anuran larvae. *Oecologia*, **123**, 192–199.

Elphick, M.J. & Shine, R. (1998) Longterm effects of incubation temperatures on the morphology and locomotor performance of hatchling lizards (*Bassiana duperreyi*, Scincidae). *Biological Journal of the Linnean Society*, **63**, 429–447.

Gallie, J.A., Mumme, R.L. & Wissinger, S.A. (2001) Experience has no effect on the development of chemosensory recognition of predators by tadpoles of the American toad, *Bufo americanus*. *Herpetologica*, **57**, 376–383.

Gosner, K.L. (1960) A simplified tale for staging anuran embryos and larvae with notes on identification. *Herpetologica*, **16**, 183–190.

La Clair, J.J., Bantle, J.A. & Dumont, J. (1998) Photoproducts and metabolites of a common insect growth regulator produce developmental deformities in *Xenopus*. *Environmental Science and Technology*, **32**, 1453–1461.

Laurila, A. (2000) Behavioural responses to predator chemical cues and local variation in antipredator performance in *Rana temporaria* tadpoles. *Oikos*, **88**, 159–168.

Laurila, A., Kujasalo, J. & Ranta, E. (1997) Different antipredator behaviour in two anuran tadpoles: effects of predator diet. *Behavioral Ecology and Sociobiology*, **40**, 329–336.

Leblond, V.S., Bisson, M. & Hontela, A. (2001) Inhibition of cortisol secretion in dispersed head kidney cells of rainbow trout (*Oncorhynchus mykiss*) by endosulfan, an organochlorine pesticide. *General and Comparative Endocrinology*, **121**, 48–56.

Lefcort, H., Thomson, S.M., Cowles, E.E., Harowicz, H.L., Livaudais, B.M., Roberts, W.E. & Ettinger, W.F. (1999) Ramifications of predator avoidance: predator and heavy-metal-mediated competition between tadpoles and snails. *Ecological Applications*, **9**, 1477–1489.

Leonard, A.W., Hyne, R.V., Lim, R.P. & Chapman, J.C. (1999) Effect of endosulfan runoff from cotton fields on macroinvertebrates in the Namoi River. *Ecotoxicology and Environmental Safety*, **42**, 125–134.

Leys, J.F., Larney, F.J., Muller, J.F., Raupach, M.R., Mctainish, G.H. & Lynch, A.W. (1998) Anthropogenic dust and endosulfan emissions on a cotton farm in northern New South Wales, Australia. *Science of the Total Environment*, **200**, 55–70.

Mccollum, S.A. & Leimberger, J.D. (1997) Predator-induced morphological changes in an amphibian: predation by dragonflies affects tadpole shape and color. *Oecologia*, **109**, 615–621.

Mccollum, S.A. & Van Buskirk, J. (1996) Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution*, **50**, 583–593.

Mann, R.M. & Bidwell, J.R. (1999) Toxicological issues for amphibians in Australia. *Declines and Disappearances of Australian Frogs* (ed. A. Campbell), pp. 185–201. Environment Australia, Commonwealth of Australia, Canberra, Australia.

Mann, R.M. & Bidwell, J.R. (2001) The acute toxicity of agricultural surfactants to the tadpoles of four Australian and two exotic frogs. *Environmental Pollution*, **114**, 195–205.

Marcellini, D.L. & Jenssen, T.A. (1991) Avoidance learning by the curly-tailed lizard, *Leiocephalus schreibersi*: implications for anti-predator behavior. *Journal of Herpetology*, **25**, 238–241.

Marco, A., Cash, D., Belden, L.K. & Blaustein, A.R. (2001) Sensitivity to urea fertilization in three amphibian species. *Archives of Environmental Contamination and Toxicology*, **40**, 406–409.

Muschal, M. (1997) *Central and Northwest Regions Water Quality Program: 1996/97 Report on Pesticides Monitoring*. Department of Land and Water Conservation, Sydney, Australia.

Muschal, M. & Cooper, B. (1998) Regional level monitoring of pesticides and their behaviour in rivers. *Minimising the Impacts of Pesticides on the Riverine Environment: Key Findings from Research with the Cotton Industry, 1998 Conference* (eds N. Schofield & V. Edge), pp. 44–50. Occasional Paper 23/98. Land and Water Resources Research and Development Corporation, Canberra, Australia.

Nowak, B. & Julli, M. (1991) Residues of endosulfan in wild fish from cotton growing areas in New South Wales, Australia. *Toxicological and Environmental Chemistry*, **33**, 151–167.

NRA (1998) *Existing Chemicals Review Program for Endosulfan*. Report No. 0 642 49016 3. National Registration Authority for Agricultural and Veterinary Chemicals, Commonwealth of Australia, Canberra, Australia.

Ormerod, S.J. (2002) Applied issues with predators and predation: editor's introduction. *Journal of Applied Ecology*, **39**, 181–188.

Pahkala, M., Laurila, A. & Merilä, J. (2001) Carry-over effects of ultraviolet-B radiation on larval fitness in *Rana temporaria*. *Proceedings of the Royal Society of London, Biological Sciences*, **268**, 1699–1706.

Parichy, D.M. & Kaplan, R.H. (1995) Maternal investment and developmental plasticity: functional consequences for locomotor performance of hatchling frog larvae. *Functional Ecology*, **9**, 606–617.

Raupach, M.R. & Briggs, P.R. (1998) Integrative assessment of endosulfan transport from farm to river by multiple pathways. *Minimising the Impacts of Pesticides on the Riverine Environment: Key findings from Research with the Cotton Industry, 1998 Conference* (eds N. Schofield & V. Edge), pp. 51–57. Occasional Paper 23/98. Land and Water Resources Research and Development Corporation, Canberra, Australia.

Relyea, R.A. (2000) Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. *Ecology*, **81**, 2278–.

Relyea, R.A. & Mills, N. (2001) Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proceedings of the National Academy of Science, USA*, **98**, 2491–2496.

Relyea, R.A. & Werner, E.E. (1999) Quantifying the relationship between predator-induced behavior and growth performance in larval anurans. *Ecology*, **80**, 2117–2124.

Rome, L.C., Stevens, E.D. & John-Alder, H.B. (1992) The influence of temperature and thermal acclimation on physiological function. *Environmental Physiology of the Amphibians* (eds M.E. Feder & W.W. Burggren), pp. 183–205. University of Chicago Press, Chicago, IL.

SAS Institute (1998) *Statview*. SAS Institute, Carey, IN.

Semlitsch, R.D., Bridges, C.M. & Welch, A.M. (2000) Genetic variation and a fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl. *Oecologia*, **125**, 179–185.

Semlitsch, R.D., Foglia, M. & Mueller, A. (1995) Short-term exposure to triphenyltin affects the swimming and feeding behaviour of tadpoles. *Environmental Toxicology and Chemistry*, **14**, 1419–1423.

Sibly, R.M., Williams, T.D. & Jones, M.B. (2000) How environmental stress affects density dependence and carrying capacity in a marine copepod. *Journal of Applied Ecology*, **37**, 388–397.

Smith, D.C. (1987) Adult recruitment in chorus frogs: effects of size and date at metamorphosis. *Ecology*, **68**, 344–350.

Tyler, M.J. (1994) *Australian Frogs*. Reed, Sydney, Australia.

Ultsch, G.R., Bradford, D.F. & Freda, J. (1999) Physiology: coping with the environment. *Tadpoles: The Biology of Anuran Larvae* (eds R. W. Mcdiarmid & R. Altig), pp. 189–215. University of Chicago Press, London, UK.

United States Environmental Protection Agency (2002) *Reregistration Eligibility Decision for Endosulfan*. Case No. 0014. Office of Prevention, Pesticides and Toxic Substances, Washington, DC.

Van Buskirk, J. & Mccollum, S.A. (2000) Influence of tail shape on tadpole swimming performance. *Journal of Experimental Biology*, **203**, 2149–2158.

Van Buskirk, J. & Saxer, G. (2001) Delayed costs of an induced defence in tadpoles? Morphology, hopping, and development rate at metamorphosis. *Evolution*, **55**, 821–829.

Van Buskirk, J., Mccollum, S.A. & Werner, E.E. (1997) Natural selection for environmentally induced phenotypes in tadpoles. *Evolution*, **51**, 1983–1993.

Watkins, T.B. (2000) The effects of acute and developmental temperature on burst swimming speed and myofibrillar ATPase activity in tadpoles of the Pacific Tree Frog, *Hyla regilla*. *Physiological and Biochemical Zoology*, **73**, 356–364.

Zaga, A., Little, E.E., Rabeni, C.E. & Ellersieck, M.R. (1998) Photoenhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. *Environmental Toxicology and Chemistry*, **17**, 2543–2553.

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