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Intergenerational effects of manipulating DNA methylation in the early life of an iconic invader

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In response to novel environments, invasive populations often evolve rapidly. Standing genetic variation is an important predictor of evolutionary response but epigenetic variation may also play a role. Here, we use an iconic invader, the cane toad (*Rhinella marina*), to investigate how manipulating epigenetic status affects phenotypic traits. We collected wild toads from across Australia, bred them, and experimentally manipulated DNA methylation of the subsequent two generations (G1, G2) through exposure to the DNA methylation inhibitor zebularine and/or conspecific tadpole alarm cues. Direct exposure to alarm cues (an indicator of predation risk) increased the potency of G2 tadpole chemical cues, but this was accompanied by reductions in survival. Exposure to alarm cues during G1 also increased the potency of G2 tadpole cues, indicating intergenerational plasticity in this inducible defence. In addition, the negative effects of alarm cues on tadpole viability (i.e. the costs of producing the inducible defence) were minimized in the second generation. Exposure to zebularine during G1 induced similar intergenerational effects, suggesting a role for alteration in DNA methylation. Accordingly, we identified intergenerational shifts in DNA methylation at some loci in response to alarm cue exposure. Substantial demethylation occurred within the *sodium channel epithelial 1 subunit gamma* gene (*SCNN1G*) in alarm cue exposed individuals and their offspring. This gene is a key to the regulation of sodium in epithelial cells and may help to maintain the protective epidermal barrier. These data suggest that early life experiences of tadpoles induce intergenerational effects through epigenetic mechanisms, which enhance larval fitness.

This article is part of the theme issue ‘How does epigenetics influence the course of evolution?’

1. Introduction

Epigenetic modifications (e.g. histone modifications, small RNA activity, DNA methylation) have important influences on development and can be induced by changes in the environment, including the environment provided by the parent [1]. Such changes can alter an individual’s phenotype in an adaptive fashion but, because epigenetic marks are mostly cleared during early development in some taxa, the heritability of environmentally induced epigenetic

modifications is controversial and their importance to evolution is unclear [2]. In addition to taxa-specific effects on heritability, epigenetic inheritance may depend on the environment and the mechanism of inheritance [3].

Invasive species provide ideal systems in which to study rapid responses to environmental change and the potential role of epigenetics in mediating these responses [4,5]. Small founding populations often have low levels of genetic diversity, limiting the amount of standing genetic variation available to selection. However, invasive populations with lowered genetic diversity often have increased epigenetic diversity compared with their native-range populations [4]. Further, introduced populations are likely to encounter environmental conditions different from those that exist in their native range, exposing these individuals to new selection regimes.

One intensively studied invasive species that has been shown to exhibit rapid evolutionary change is the cane toad (*Rhinella marina*; formerly *Bufo marinus*). Translocated from its native range in South America to sugar-cane plantations in many other countries, in an unsuccessful attempt at insect control, toads were released in north-eastern Australia in 1935 [6]. Over the course of their subsequent spread though that continent, toads have accumulated substantial phenotypic changes in a diverse suite of morphological, physiological and behavioural traits [7]. At least some of those changes are heritable [8,9]. However, phenotypic variation also occurs in response to environmental cues. For example, as for tadpoles of many anuran species [10], cane toad tadpoles often use inducible defences in response to predation risk. In cane toads, these defences can be induced by exposure to alarm cues produced by injured conspecifics [11]. Exposure to alarm cues causes tadpoles to modify their phenotype and behaviour in ways that can reduce predation risk, but producing a defence is also associated with fitness costs [12]; tadpoles exposed to alarm cues exhibit reduced rates of growth, development and survival [13–15], as well as altered glucocorticoid responses post-metamorphosis [16]. In addition, exposure to alarm cues alters tadpole DNA methylation [16], suggesting that this may provide a mechanistic basis through which the environment can alter phenotype. Chemical defences are a principal way in which cane toad tadpoles defend against Australian predators; the chemical cues and toxins produced by this phylogenetically distinct invader are repulsive or lethal to a wide variety of tadpole predators and thereby reduce predation risk [17,18]. Predator-induced plasticity in the potency of chemical cues could, therefore, be a highly effective strategy for dissuading predation [19], and may be an important component of the inducible defences produced by cane toad tadpoles. However, this possibility has not been assessed. In addition, the degree to which exposure to predation cues during the larval stage may influence offspring defences or performance remains unknown.

Intergenerational plasticity in inducible defences has been demonstrated in a variety of species and can improve offspring performance by preparing offspring for environmental conditions experienced by their parents [20–22]. In the current study, we investigate whether changing DNA methylation affects tadpole phenotype and whether these effects are intergenerational. We predicted that responses to tadpole alarm cues are mediated by changes to DNA methylation and that these effects would be transmitted to the next generation [21]. To test this hypothesis, we used two approaches known to affect DNA methylation in cane toad tadpoles: exposure to alarm cues from injured conspecifics and exposure to the

DNA methylation inhibitor zebularine [16,23]. We predicted that within-generation exposure to alarm cues would increase the potency of the chemical cues produced by cane toad tadpoles, resulting in defensive costs such as reduced rates of tadpole growth, development and/or survival. We, therefore, predicted that the offspring of toads that had been exposed to predation cues during the tadpole stage may inherently exhibit stronger defences or reduced defensive costs (relative to the offspring of toads raised in the absence of these cues) [20,24].

2. Methods

(a) Sampling, experimental setup and animal husbandry

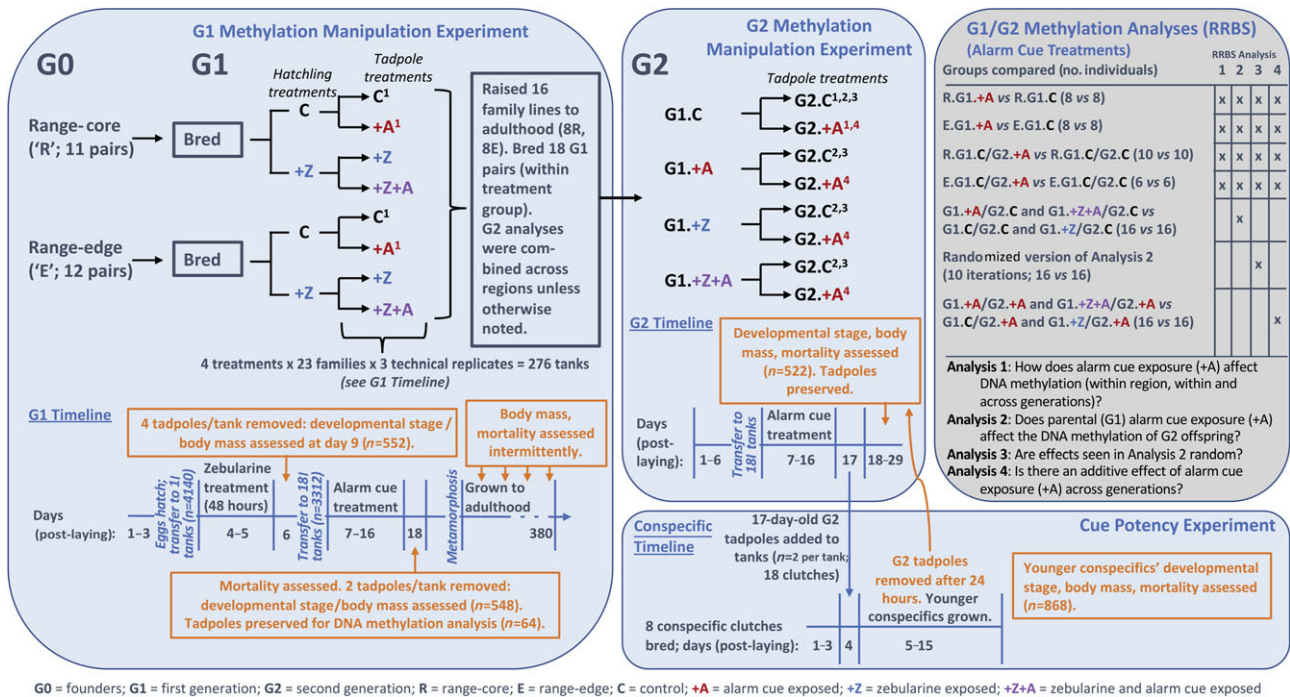
We collected wild cane toads from three locations each in the Australian range-core (R) and range-edge (E) and used them as founders (G0) for a common-garden experiment (see electronic supplementary material for locality details and full methods; figure 1, electronic supplementary material, table S1). We injected toads with leuprorelin acetate to induce spawning and collected Generation 1 (G1) egg clutches the following morning. Eggs were aerated prior to the start of experiments. In total, we produced 11 clutches from range-core pairs and 12 clutches from range-edge pairs. Because the sex of larval cane toads cannot be distinguished morphologically, all experiments described below contain mixed-sex tadpoles.

(b) G1 DNA methylation manipulation experiment (zebularine and alarm cue treatment)

Seventy-two hours after egg deposition, we randomly selected a total of 180 hatchlings per clutch, and divided them evenly between zebularine (+Z) (100 μ M; following [16]) and control (C) treatments, where they remained for 48 h (30 hatchlings per replicate; three replicates each treatment). Although little is known about developmental changes to DNA methylation in this species, zebularine treatment at days 4–5 post-laying has been shown to demethylate DNA in larval cane toads; on a global level, this treatment slightly decreases CpG methylation but has large locus-specific effects that may reduce or increase CpG methylation [16]. Post zebularine treatment, we removed four tadpoles from each tub, and at day 9 we euthanized, weighed and assessed them for development (Gosner) stage [25]. Of the remaining hatchlings, 72 from each zebularine treatment were allocated into 18 l tanks (density: 12 tadpoles per tank), which we randomly assigned to either alarm cue (A+) or control (C) treatments (3 replicates per treatment). This resulted in four treatment groups in a factorial design (C, +Z, +A, +Z+A; figure 1). We exposed tadpoles in each +A tank to approximately 4 ml bore water containing two crushed conspecifics strained through a fine mesh, at the same time each day (14.00–15.00 h) on 10 consecutive days (days 7–16 post-laying). We exposed control tanks to approximately 4 ml non-chlorinated water delivered in the same manner but without crushed conspecifics. On day 17, we assessed mortality in each tank and then removed two individuals per tank. On day 18, we euthanized, weighed, and assessed these two tadpoles per tank for developmental stage and preserved them in RNA Later. We left the remaining tadpoles to metamorphose before transferring them to outdoor bins (see electronic supplementary material for details). We assessed mortality at the metamorph stage, and subsequently body mass and mortality at regular intervals until the toads were 380 days old.

(c) Second-generation DNA methylation manipulation experiment

After G1 toads reached adulthood, we bred a second generation (G2) from G1 toads paired by treatment group ($n = 18$ pairs),



G0 = founders; G1 = first generation; G2 = second generation; R = range-core; E = range-edge; C = control; +A = alarm cue exposed; +Z = zebularine exposed; +Z+A = zebularine and alarm cue exposed

Figure 1. Experimental design of common-garden experiment exploring intergenerational effects of methylation manipulation of cane toads. Left panel depicts the founders (G0) and first generation (G1), including the methylation manipulation experiment. Centre (upper) panel depicts the second generation (G2) manipulations, including parents' (G1) treatment. Bottom right panel depicts the cue potency experiment, in which conspecific tadpoles were exposed to tadpoles from the G2 methylation manipulation experiment (blue arrow). Top right panel describes which individuals were included in each DNA methylation analysis, including the geographic region where applicable, and treatment (G2 comparisons include their parents' treatment codes). Timelines for each experiment are given, indicating the timepoints of assays (orange). Superscripts following treatment codes refer to the corresponding reduced representation bisulfite sequencing (RRBS) RRBS Analysis number in the methylation analyses table. Treatment codes are defined at the bottom of the figure.

avoiding full-sibling pairings. Owing to low survival to adulthood of G1 toads in some treatment groups, region of origin (R and E) was ignored for G2 analyses. We split clutches and exposed G2 tadpoles either to alarm cues or to a sham control from days 7 to 16 post-laying, as described in the G1 DNA methylation manipulation experiment. G2 tadpoles were not directly exposed to zebularine (i.e. any zebularine effects on G2 tadpoles result from parental exposure in G1). On day 17, we assessed mortality in each tank and then removed two individuals per tank for the cue potency experiment (below). On day 18, the two G2 tadpoles that we removed from each tank were euthanized, weighed, staged and preserved in RNALater. The remaining G2 tadpoles in experimental tanks were euthanized, weighed and staged on days 18–29 for logistical reasons (see electronic supplementary material for further details).

(d) Cue potency experiment

To determine whether exposure to +A or +Z affected the potency of the chemical cues produced by cane toad tadpoles, we exposed the tadpoles from each treatment to conspecific hatchlings. Conspecific hatchlings exhibit clear, dose-dependent responses to the cues produced by cane toad tadpoles [26–28]. These effects are associated with the induction of defence against cannibalism by older conspecifics, which is a major source of mortality for cane toad eggs and hatchlings. To defend against this threat, hatchlings accelerate their development upon detecting cues produced by conspecific tadpoles [27]. The magnitude of the subsequent effects depends upon the level of risk perceived, such that stronger cues lead to larger reductions in viability during the tadpole stage [28]. Although stronger cues may generally indicate greater risk (i.e. that the cannibals are more abundant or closer), a shift in the potency of the cues produced by a tadpole could also lead to changes in the magnitude of this response (e.g. if a single tadpole produces disproportionately strong cues, the level of risk perceived by the hatchling

may increase; J. L. DeVore 2020, personal communication). These effects are easily quantifiable in an experimental setting; indeed, the effects of exposure to conspecific tadpole cues are so pronounced that they were initially interpreted as allelopathic interference by older cannibal tadpoles [26]. Hatchlings, therefore, provide a useful indicator of the potency of the cues produced by conspecific tadpoles.

We assessed how our G1 and G2 treatments affected the impact of chemical cues from G2 tadpoles on conspecific hatchlings (figure 1, bottom right panel). To do so, we exposed hatchlings to 17-day-old G2 tadpoles from our DNA methylation manipulation experiments (details of experimental design are included in the electronic supplementary material). We separated hatchlings from older tadpoles by a fine mesh to prevent cannibalism. After 24 h, we removed the G2 tadpoles (see above, G2 methylation manipulation experiment) and left the hatchlings in the tubs containing G2 tadpole cues until they reached the free-swimming tadpole stage (stage 25; 48 h later). We then transferred them to new tubs, fed them ad libitum for the next 10 days (15 days old) and then euthanized them and assessed their weight and developmental stage, and calculated mortality for each tub.

(e) RRBS sequencing

We extracted DNA from whole G1 tadpoles ($n = 11$) for reduced representation bisulfite sequencing (RRBS) analyses and analysed these together with RRBS data from an additional 36 G1 tadpoles included in [16]. In total, we included the following G1 samples in our analyses (codes show region, generation and treatment group): R.G1.C ($n = 8$), R.G1.+A ($n = 8$), R.G1.+Z ($n = 8$), E.G1.C ($n = 8$), E.G1.+A ($n = 8$), E.G1.+Z ($n = 7$) (i.e. one individual per family per treatment). From G2, we extracted DNA from 64 individuals for RRBS analysis ($n = 8$ from each of the following groups; codes show parental G1 treatment/G2 treatment, see figure 1): G1.C/G2.C, G1.C/G2.+A, G1.+Z/G2.C, G1.+Z/G2.+A, G1.+A/G2.C, G1.+A/G2.+A, G1.+Z+A/

G2.C, G1.+Z+A/G2.+A). DNA was extracted using a Genra Puregene Kit (QIAGEN, USA) following the manufacturer's instructions. The Ramaciotti Centre for Genomics (UNSW) conducted RRBS library preparation and sequencing using 100 ng of DNA and the Ovation RRBS Methyl-seq System (NuGEN Technologies, San Carlos, CA). Sequencing (1 × 100 bp reads) was conducted on the NovaSeq 6000 platform (Illumina, San Diego, CA).

3. Statistical analyses

(a) Ecological data

We analysed data on tadpole mass and stage with linear mixed-effects models using JMP Pro 14.2.0 (SAS Institute Inc., Cary, NC, 1989–2019). We log-transformed data prior to analysis to meet the assumption of normality, as required. For experiments that assessed treatment effects on mortality at a single time period, we classified mortality of tadpoles as a binomial response (dead versus alive) and analysed these data with logistic regression based on the quasi-binomial distribution to account for over-dispersion of data [29], using the package *mass* [30] in R [31]. We analysed treatment effects on mortality of post-metamorphic (terrestrial-stage) toads aged 6–12 months using mixed-effects Cox regression (*coxme* package [32]). Details for fixed effects and random effects for each specific analysis are given in the electronic supplementary material. We estimated the main effects by sequentially removing non-significant higher-order interaction terms, starting with the highest-order terms followed by highest *p* values. Experimental treatment effects were exposure to zebularine (yes/no), exposure to alarm cue (yes/no) and geographic origin (range-core/range-edge).

(b) DNA methylation analyses

RRBS data quality control and filtering parameters are included in the electronic supplementary material. We used the EDMR package [33] in R to identify differentially methylated regions (DMRs) that contained a minimum of 10% mean DNA methylation difference and at least five CpGs (cytosines followed by guanines). We aligned DMRs to the cane toad genome [34] using the closest-features algorithm in BEDOPS [35]. We used DMRs for four sets of analyses investigating alarm cue exposure (figure 1, top right panel). First, for Analysis 1, we compared DMRs identified in G1 tadpoles exposed to alarm cues (+A) versus controls (C) with DMRs identified in G2 tadpoles exposed to alarm cues (G1.C/G2.+A) versus controls (G1.C/G2.C). Note that the RRBS data for half of the G1 individuals used here were previously investigated, where it was determined that strong geographic regional differences exist in DNA methylation data [16]. For this reason, we conducted these comparisons within-region. This analysis allowed us to characterize effects of direct alarm cue exposure on two generations from two populations (i.e. four independent groups of tadpoles). For Analysis 2, we compared DNA methylation of G2 tadpoles whose parents were exposed (G1.+A/G2.C) versus G2 tadpoles from parents who had no exposure to alarm cues (G1.C/G2.C). Note that none of these individuals had direct exposure to alarm cues. We compared these results with those from RRBS Analysis 1 to determine whether the experience of G1 tadpoles affected the DNA methylation of their offspring. For RRBS Analysis 3, we created 10 randomized datasets

containing all G2 individuals included in RRBS Analysis 2 so that each randomized treatment group contained 50% of the G2 tadpoles from G1 control family lines (G1.C/G2.C) and 50% of the G2 tadpoles from G1 families exposed to alarm cues (G1.+A/G2.C). This enabled us to discern whether RRBS Analysis 2 represented an actual effect or was just the result of random differences in DNA methylation between individuals. Finally, for RRBS Analysis 4, we compared DNA methylation of G2 tadpoles that were directly exposed to alarm cues and whose parents were exposed to alarm cues (G1.+A/G2.+A) versus control tadpoles that were directly exposed to alarm cues whose parents were controls (G1.C/G2.+A). We compared these results with those from RRBS Analysis 1, which also enabled us to determine whether the experience of G1 tadpoles affected G2 tadpoles and whether there was an additive effect of exposures in both generations on DNA methylation of G2 tadpoles. The individuals used in RRBS Analysis 2 were full-siblings of those used in RRBS Analysis 4. Finally, using the same methodology, we investigated methylation shifts in zebularine-exposed G1 tadpoles (G1.+Z versus G1.C, regions combined to be comparable with G2 individuals) and in their G2 unexposed offspring (G1.+Z/G2.C versus G1.C/G2.C).

4. Results

(a) Generation 1 DNA methylation manipulation experiment

At day 9, tadpoles that were exposed to zebularine exhibited greater mean body mass and developmental stage than controls (table 1*a,b*; electronic supplementary material, table S1*a,b* and figure S1*a,b*), but this effect was no longer evident at day 18 (electronic supplementary material, table S2*a,b*). There was no significant effect of geographic origin on body mass or developmental stage at day 9 (electronic supplementary material, table S1*a,b*). However, at day 18 there was an effect of geographic origin on tadpole body mass and development, with range-core tadpoles growing and developing faster than range-edge tadpoles (table 1*c,d*; electronic supplementary material, table S2*a,b* and figure S2*a,b*). At day 18, there was no significant effect of exposure to alarm cue on tadpole body mass or development (electronic supplementary material, table S2*a,b*). Tadpole density within the tanks also affected development (table 1*d*).

Neither geographic origin nor exposure to zebularine affected tadpole mortality at day 18 (electronic supplementary material, table S3). However, exposure to alarm cues resulted in a higher mortality (table 1*e*; electronic supplementary material, figure S3). There was no significant effect of geographic origin or exposure to zebularine on the probability of mortality for metamorphs (electronic supplementary material, table S3*a*), but exposure to alarm cues resulted in a marginally non-significant increase in the probability of metamorph mortality (table 1*f*). Unsurprisingly, there was an effect of both time and tank density on toad growth from 6 to 12 months (table 1*g*). There was also significant Time × Origin × Zebularine interaction for toad growth at 6–12 months (table 1*g*; electronic supplementary material, figure S4). The Time × Origin × Alarm Cue interaction for toad growth was marginally non-significant ($p = 0.053$, table 1*i*; electronic supplementary material, table S3*b*). There was no significant effect of origin,

Table 1. Summary of treatment effects for G1 (*a–h*) and G2 (*i–l*) (*p*-values <0.05 shown in bold). Full model results for all phenotypic analyses are listed in the electronic supplementary material. Zeb, zebularine treatment.

response variable	fixed effects	estimate	d.f.	t/F/z	<i>p</i>
Generation 1					
(a) day 9 tadpole growth	Zeb	−0.014	1, 476.5	6.529	0.011
(b) day 9 tadpole development	Zeb	−0.047	1, 479.2	4.158	0.042
(c) day 18 tadpole growth	Origin	5.259	1, 18.97	4.714	0.043
(d) day 18 tadpole development	Density	−0.463	1, 264.4	13.324	< 0.001
	Origin	0.608	1, 18.94	8.679	0.008
(e) day 18 tadpole mortality	Alarm Cue	0.487	250	2.599	0.010
(f) metamorph mortality	Alarm Cue	0.198	250	1.954	0.052
(g) adult growth 6–12 months	Time	0.006	1, 1221	2450.2	< 0.0001
	Density	−1.531	1, 1215	284.6	< 0.0001
	Time × Origin × Zeb	0.0002	1, 1212	4.369	0.037
	Time × Origin × Alarm Cue	0.01	1, 1212	3.766	0.053
(h) adult mortality 6–12 months	Density	−235.664	11.981	−19.670	< 0.0001
Generation 2					
(i) tadpole growth	Density	8.0968	1,64.85	19.6965	< 0.0001
(j) tadpole mortality	G1 Zeb × G2 Alarm Cue	−1.8875	68	−2.3750	0.0204
	G1 Alarm Cue × G2 Alarm Cue	−2.4516	68	−3.1543	0.0024
(k) potency of G2 tadpole cues—younger conspecific tadpole growth	G1 Alarm Cue	0.016	1,19.4	4.961	0.038
	G2 Alarm Cue	0.045	1, 143.3	89.323	< 0.0001
(l) potency of G2 tadpole cues—younger conspecific tadpole development	G2 Alarm Cue	0.379	1, 156.4	49.498	< 0.0001

zebularine or alarm cue on mortality of toads at 6–12 months, but density affected toad mortality (table 1*h*; electronic supplementary material, table S3c).

(b) Generation 2 DNA methylation manipulation experiment

There was no significant effect of G1 treatment on growth or development of G2 tadpoles at days 18–29, nor was there an effect of direct exposure of G2 tadpoles to alarm cues on growth or development (electronic supplementary material, table S4a,b). There was an effect of tank density on tadpole growth (table 1*i*). With respect to G2 tadpole mortality, there were interactions between G2 tadpole alarm cue treatment and their G1 parents' treatments (table 1*j*). G2 tadpoles from control parents that were directly exposed to alarm cues had a higher probability of mortality than those with no direct exposure to alarm cues (figure 2*a*, black points; electronic supplementary material, table S4, *p* = 0.009). However, G2 tadpoles from alarm cue exposed parents that were themselves directly exposed to alarm cues were no more likely to die than were G2 tadpoles from control parents that were not themselves directly exposed to alarm cues (i.e. tadpoles that experienced the same environment as their parents performed similarly, regardless of the nature of that environment; figure 2*a*, right red (grey in printed version) data point and left black data point; electronic supplementary material, table S4, *p* = 0.52). The same pattern was found with respect to parental exposure to zebularine (electronic supplementary material, figure S5 and table S4).

(c) Cue potency experiment

We observed significant alarm cue-induced plasticity in the potency of tadpole chemical cues; G2 tadpoles directly exposed to alarm cues affected the growth and developmental stage of younger conspecifics more strongly than did G2 tadpoles that were raised in control conditions (figure 2*b*; electronic supplementary material, table S5a,b). There was also a significant carry-over effect of G1 alarm cue treatment on the potency of G2 tadpole cues in terms of the growth of younger conspecifics: the offspring of G1 toads that were exposed to alarm cues as tadpoles produced slightly stronger cues than did the offspring of G1 toads that were reared in control conditions (table 1*k,l*; figure 2*b*). This generational carry-over effect of exposure to alarm cues did not modify the induced reaction norm during G2 (i.e. the previous exposure had an additive effect [36] (G1 Alarm Cue × G2 Alarm Cue interaction, *p* = 0.419; electronic supplementary material, table S5a)). There was no significant carry-over effect of G1 alarm cue treatment on the potency of G2 tadpole cues in terms of developmental responses of younger conspecifics (electronic supplementary material, table S5b).

(d) DNA methylation analyses

For G1 individuals in RRBS Analysis 1 (i.e. how direct alarm cue exposure affects DNA methylation, figure 1), we found 121 DMRs in range-edge samples and 100 DMRs in range-core samples (figure 3). Seventeen DMRs were identified in both regions, five of which were located in annotated genes (figure 3).

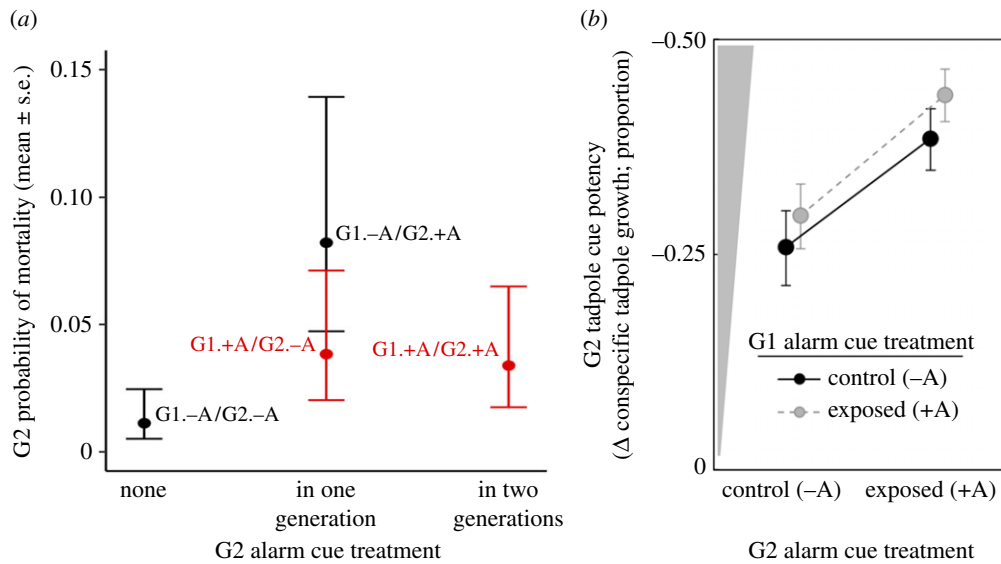


Figure 2. Phenotypic effects of methylation manipulations on second generation (G2) cane toad tadpoles. (a) Interaction of G1 alarm cue exposure \times G2 alarm cue exposure on the probability of mortality of G2 tadpoles. G2 tadpoles whose parents were not exposed to alarm cues and were not directly exposed to alarm cues themselves had the lowest rates of mortality (G1.-A/G2.-A, left data point) and significantly lower mortality rates than those whose parents had no exposure to alarm cues but were directly exposed to alarm cues themselves (G1.-A/G2.+A, upper-middle data point; electronic supplementary material, table S4, $p = 0.009$). G2 tadpoles whose parents were exposed to alarm cues (G1.+A/G2.-A, lower-middle data point; G1.+A/G2.+A, right data point) did not have significantly different mortality rates compared with tadpoles in any other treatment group. (b) Effect of G1 and G2 alarm cue exposure on tadpole cue potency (measured by the change in conspecific growth). Grey wedge represents the strength of cue. (Online version in colour.)

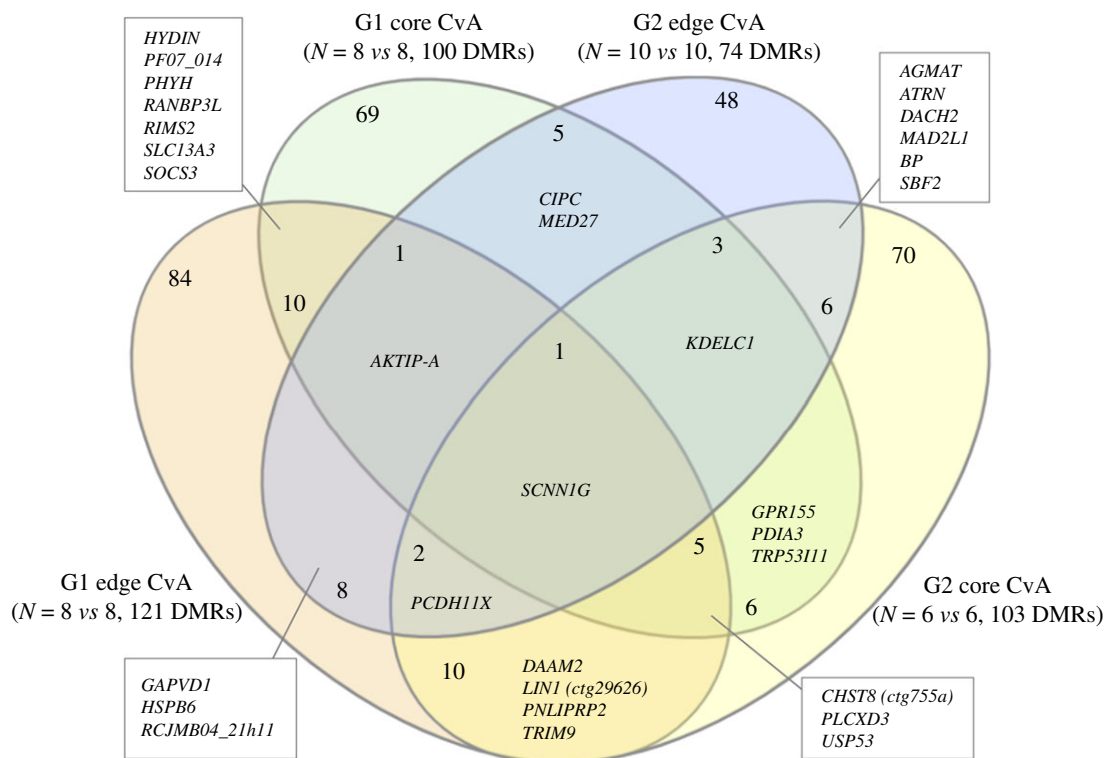


Figure 3. Venn diagram indicating the intersection of differentially methylated regions (DMRs) of the genome identified in first (G1) and second (G2) cane toad tadpoles in response to alarm cue exposure in range-core and range-edge samples (RRBS Analysis 1). Each comparison is alarm cue exposed (+A) versus control (C). Annotated genes associated with identified DMRs are shown.

In G2 individuals, we found 74 DMRs in range-edge samples and 103 DMRs in range-core samples (figure 3). Twelve DMRs were identified in both regions, eight of which were located in annotated genes (figure 3). Of the total of 328 distinct DMRs found in these four comparisons, 41 DMRs were found in both generations. Only one DMR was differentially methylated in all comparisons and this was located with the *SCNN1G* gene; six other DMRs were found in three out of four of these

comparisons, and an additional 24 genes were found to be differentially methylated in two comparisons (electronic supplementary material, table S6). Most of the DMRs listed in electronic supplementary material, table S6 were located within gene bodies; three DMRs were located upstream and one DMR overlapped with the transcription start site.

For RRBS Analysis 2 (i.e. how parental (G1) alarm cue exposure affects DNA methylation in G2 offspring, figure 1),

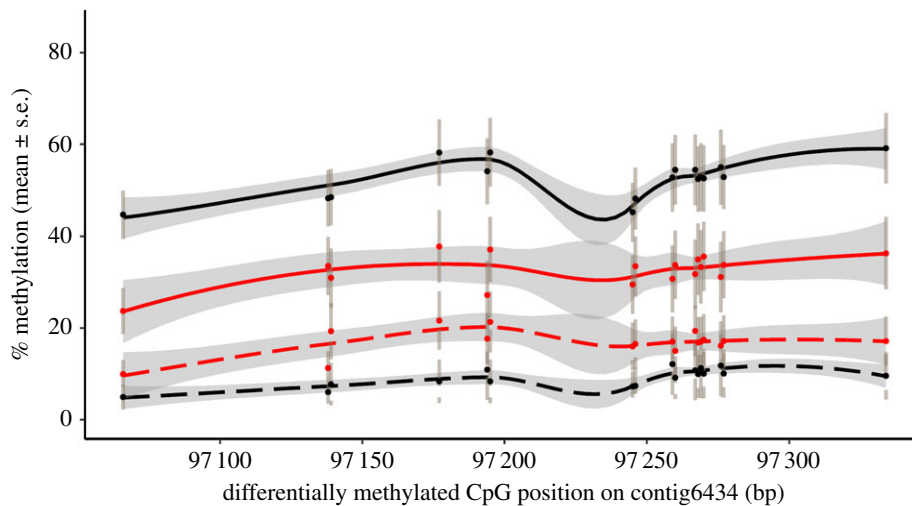


Figure 4. Mean percentage CpG methylation at the differentially methylated region contained in the *SCNN1G* gene of second generation (G2) cane toad tadpoles. Solid lines represent G2 individuals from first generation (G1) control families; dashed lines represent G1 alarm cue exposed families. Black lines represent unexposed G2 individuals; red lines represent alarm cue exposed G2s (grey in printed version). Mean methylation of differentially methylated CpGs is shown (dots; bars are standard errors). (Online version in colour.)

we found 332 DMRs between tadpoles from G1 control families and those from G1 families exposed to alarm cues (electronic supplementary material, figure S6 and table S7). Ninety-two of these DMRs were also found in RRBS Analysis 1, including the DMR in *SCNN1G*. For RRBS Analysis 3 (i.e. whether the result found in Analysis 2 is random, figure 1), we found fewer DMRs in random dataset comparisons (mean = 185, ranging from 140 to 253 DMRs) than in RRBS Analysis 2. We also found substantially fewer DMRs that overlapped with those identified in RRBS Analysis 1 (mean = 62, ranging from 50 to 75 overlapping DMRs). For RRBS Analysis 4 (i.e. whether there are additive effects of alarm cue exposure across generations, figure 1), we identified 678 DMRs, 110 of which overlapped with RRBS Analysis 1.

We examined DNA methylation of individual CpGs in the DMR found in *SCNN1G* (figure 4). This DMR contained 17 differentially methylated cytosines, all of which exhibited the highest levels of DNA methylation in G2s that were unexposed to alarm cues and were from G1 control families (figure 4, G1.C/G2.C). G2 tadpoles that experienced direct exposure to alarm cues but were from G1 control families (figure 4, G1.C/G2.+A) had less DNA methylation at this locus than did G1.C/G2.C individuals. G1 history had a larger impact on DNA methylation of this locus in G2 tadpoles than did the direct exposure to alarm cues on G2s (figure 4, top two lines represent G2 offspring from G1 control families, bottom two from G1 alarm cue exposed families).

We identified 44 DMRs in tadpoles exposed to zebularine in G1 (G1.+Z versus G1.C) that were also identified in the analysis of their unexposed offspring (G1.+Z/G2.C versus G1.C/G2.C) (electronic supplementary material, table S8). Twenty of these DMRs were also found in tadpoles exposed to alarm cues.

5. Discussion

In the current study, we investigated how methylation manipulation affects the phenotypic traits of cane toads from early developmental stages through to adulthood. We also investigated which genomic regions were affected by our

manipulations and asked whether associated changes in phenotype and DNA methylation were evident in the subsequent generation. Tadpole cue potency, growth, development and mortality were all affected by direct exposure to methylation manipulations. Interestingly, intergenerational effects were also identified: second-generation tadpoles of parents that had been exposed to alarm cues exhibited overall increases in cue potency and decreased mortality in environments where predation cues were present. Similarly, second-generation tadpoles of parents exposed to zebularine exhibited decreased mortality in environments where predation cues were present. DNA methylation changes were identified in all RRBS analyses, including in those that compared tadpoles of exposed parents that had not themselves been directly exposed to treatments. Many of these changes to DNA methylation were also seen in G2 tadpoles that had no direct exposure to treatments, demonstrating the stability of some of these shifts. Most DMRs identified were exclusive to a single comparison but one DMR within the *SCNN1G* gene was consistently differentially methylated; this gene plays an important role in the integrity of the epidermal barrier [37,38].

Exposure to both alarm cues and zebularine affected DNA methylation in cane toad tadpoles, albeit in different ways [16]. In the present study, we found no effect of alarm cue exposure on 18-day-old G1 tadpole growth or development, and only weak geographic region-specific support for impacts on growth in G1 juveniles aged between 6 and 12 months. However, we found differences in growth and development in 9-day-old G1 tadpoles exposed to zebularine. These zebularine-exposed tadpoles had accelerated development and they were *larger* than controls (results from both regions). Interestingly, juvenile (6–12 months old) toads from the range-core that were exposed to zebularine were *smaller* than controls but this effect was not seen in range-edge toads, suggesting the latter are less plastic in their response (as inferred by [39]).

We found evidence that direct exposure to alarm cues affects DNA methylation and, although dozens of DMRs were common between geographic regions and across generations, most DMRs were not found in multiple comparisons. We attribute this to three factors: (i) regional differences in population genetics within Australian toads [40] and the

association of genetic similarity with DNA methylation patterns [16]; (ii) DNA methylation is labile and affected by environment, so analyses that included comparisons across generations (i.e. at different times) will likely include subtle experimental differences that could affect methylation; and (iii) given the apparent intergenerational similarities between DNA methylation patterns we see here and the differences between range-core and range-edge environments (hotter and drier at the range-edge; [40]), lineages from different geographic regions were likely to have had different DNA methylation profiles prior to our manipulations that may have persisted throughout the experiment. Further, there are substantial differences in behavioural, morphological and physiological traits between range-core and range-edge toads [7] and some of these traits may affect predator interactions. For example, range-edge toads are more exploratory than those from the range-core [41], which may increase their likelihood of encountering predators. Parotoid glands are smaller in native-range toads than invasive Australian toads, presumably because the toxin is more effective against naive predators in introduced ranges [42]. Range-edge toads in Australia have relatively larger parotoid glands ([43], but see [42]), which may be a response to increased predation threat to these animals or a more unpredictable predator landscape in newly colonized areas. Fewer and potentially more predictable interactions with predators at the range-core could result in a lower cost of transmitting epigenetic marks to offspring in response to alarm cue exposure, potentially favouring intergenerational effects. While we did find regional differences in where DMRs were located in the genome, our sample sizes in the second generation required that we combine samples across regions, preventing analysis of the magnitude of these effects with respect to geographic region.

Despite the differences we found in methylation patterns across regions and generations, we did identify a single DMR found in range-core and range-edge comparisons in G1 and G2 individuals directly exposed to alarm cues (figure 1, RRBS Analysis 1) as well as in comparisons of G2 controls whose parents were exposed versus not exposed to alarm cues (figure 1, RRBS Analysis 2). This suggests that this DMR (within the *SCNN1G* gene) is located in a functionally important region of the genome with respect to tadpoles' response to alarm cues. The *SCNN1G* gene codes for the gamma subunit of the epithelial Na⁺ channel (ENaC), which regulates sodium absorption and secretion (reviewed in [44]), and is integral to the barrier function of skin [35,36]. The skin is the primary defence against physical, chemical or microbial exposure an individual might encounter. In amphibians, Na⁺ is absorbed into the skin (also urinary bladder and kidney, [45,46]) from the local environment or transcellular fluids [39]. ENaC also directs galvanotaxis in keratinocytes during the wound healing process [47]. Of the remaining six genes containing DMRs in three of four comparisons of direct exposure to alarm cues (RRBS Analysis 1), half were associated with gene ontology terms 'plasma membrane' or 'cell junction' (*AKTIP-A*, *PCDH11X*, *USP53*), as were an additional two genes found in two of four comparisons of direct exposure to alarm cues (*RIMS2* and *SLC13A3*; electronic supplementary material, table S6). Range-edge toads have significantly lower cutaneous resistance to water loss than do toads from the range-core [48], suggesting a possible role of ENaC in the adaptation of cane toads to their environmental conditions. It is plausible that the differential methylation we found at this gene was caused by the physiological response

that receiver tadpoles mounted when their skin contacted cues from crushed conspecifics. *SCNN1G*, *PCDH11X* and *SLC13A3* were all differentially methylated in the G2 offspring whose parents were exposed to alarm cues (G1.+A/G2.C versus G1.C/G2.C; electronic supplementary material, table S7). Given the lability of DNA methylation, it is remarkable that we were able to identify intergenerational DNA methylation changes in these genes. While we provide evidence here of shifts in DNA methylation in response to our treatments, future gene expression studies of these genes are needed to clarify the functional significance of these changes.

Our treatments affected tadpole growth, development and mortality, so it is unsurprising that we identified DMRs in a range of genes involved in growth and development. The *SOC53* gene inhibits leptin signalling [49], which regulates food consumption in tadpoles [50]. *DAAM2* regulates tissue and organ development [51] and is widely expressed across anuran embryogenesis [52]. *TRIM9* is highly expressed in the mouse brain during embryogenesis [53], and is key to neuronal development [54]. *ATRN* contains multiple epidermal growth factor domains and is involved in myelination, cell adhesion and homeostasis [55]. A number of other genes we identified as differentially methylated are also important to development (e.g. *DACH2*, *GPR155*, *HYDIN*, *MED27*), any of which could be responsible for the phenotypic effects we have identified.

We found evidence of inducible plasticity in the potency of tadpole chemical cues. G2 tadpoles that were directly exposed to alarm cues had stronger effects on conspecific hatchlings than did control tadpoles, causing greater reductions in their growth and development. Here, we also found intergenerational effects: G2 tadpoles whose parents (i.e. G1) were exposed to alarm cues had stronger effects on the growth of conspecific hatchlings than did G2 tadpoles from control families. However, this effect was weaker than the effect induced by directly exposing tadpoles to alarm cues, suggesting that this signal is attenuated when parentally transmitted. Regardless, our finding of intergenerational inducible defences demonstrates that experiences individuals have in the aquatic larval environment, long before reaching the terrestrial reproductive stage, can affect the larval defences of their offspring.

Although tadpoles whose parents had been exposed to alarm cues produced more potent chemical cues, perhaps the greatest benefit of a shared parent-offspring environment was evident in terms of the costs of producing the inducible defence. Although direct exposure to alarm cues increased mortality in tadpoles (in G1 tadpoles and in G2 tadpoles whose parents were unexposed), we also found intergenerational effects of both alarm cue and zebularine exposure with respect to G2 tadpole mortality. However, rather than having a negative effect, both treatments *increased* offspring survival when offspring were directly exposed to alarm cues themselves (i.e. offspring of G1 controls exhibited high survival rates when they were also raised in control conditions, but were more likely to die than other treatment groups if exposed to alarm cues). This result implies that the costs of producing the inducible defence were reduced for tadpoles whose parents had also been reared in the presence of predation cues. In environments where predators are frequently encountered, this intergenerational effect may reduce the costs of producing inducible defences. As the costs of producing inducible defences can significantly reduce fitness, processes through which these costs can be offset by parental experiences could strongly influence the

adaptive value of inducible defences. Interestingly, when we investigated DNA methylation responses in G2 tadpoles that were exposed to alarm cues and whose parents were or were not exposed to alarm cues (figure 1, RRBS Analysis 4), we found the highest number of DMRs of all comparisons ($n = 678$; compared with 332 in G2 tadpoles directly exposed, RRBS Analysis 2).

Our results suggest that experiences of G1 cane toads during early development affect the phenotype and DNA methylation of their offspring. Because primordial germ cells (PGCs) require totipotency, epigenetic marks are reprogrammed in many species during development [56,57]. Despite this, epigenetic inheritance has been demonstrated across a wide range of taxa (summarized in [2,58]). Nonetheless, it is not possible to determine from our experiments whether the methylation changes described here are transgenerational (i.e. heritable beyond a single generation). In amphibians, epigenetic reprogramming occurs in PGCs formed during Gosner stages 25–40 of their parents [57], which incorporates the window during which we exposed G1 tadpoles to alarm cues. Therefore, while epigenetic inheritance remains a possibility, it also is possible that G2 offspring were directly exposed as PGCs while their parents were developing.

Transgenerational plasticity can play an important role in enhancing offspring success and this may be especially true for invasive species. Because a multitude of environmental effects can shift rapidly during the invasion process (e.g. predator assemblages, conspecific densities, abiotic conditions), any process through which parents can use their experiences to facilitate the production of an adaptive phenotype in their offspring is likely to be favoured. Here, the intergenerational effects we detected appear to improve offspring fitness. These effects may, therefore, be under selection and have

evolutionarily important implications. However, the degree to which these effects persist across generations is unknown; if conditions shift rapidly, the relevance of the parental environment may be limited beyond the first generation. In contrast, if the environment is relatively stable, we could see ‘run-away’ effects of these traits: within predator-rich environments, multigenerational exposure to predation cues could produce stronger and stronger defences until physiological limits are reached—especially because the cost of mounting this response appears to be low. Alterations in DNA methylation appear to underlie these effects; both exposure to alarm cues and artificially induced shifts in DNA methylation (using zebularine) resulted in similar phenotypic effects in both exposed individuals and their offspring. This similarity implies that the effect of alarm cue exposure is mediated by DNA methylation changes, and supports the hypothesis that epigenetic responses may provide a multigenerational bridging strategy until traits can be canalized or until the environmental driver dissipates.

Data accessibility. CpG reports from RRBS data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.pk0p2ngmv> [59].

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