Ecotoxicology (2017) 26:1293–1304
DOI 10.1007/s10646-017-1854-8

Competition and pesticide exposure affect development of invasive (*Rhinella marina*) and native (*Fejervarya vittigera*) rice paddy amphibian larvae

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Accepted: 12 September 2017

Abstract Increased pesticide use in rice agricultural ecosystems may alter competitive interactions between invasive and native amphibian species. We conducted an experiment with two rice paddy amphibians found in Luzon, Philippines, the invasive cane toad (*Rhinella marina*) and the endemic Luzon wart frog (*Fejervarya vittigera*), to determine whether exposure to a common herbicide, butachlor, drives competitive interactions in favor of the invasive amphibian. Our results revealed that competition had a strong effect on the development of both species, but in opposing directions; Luzon wart frog tadpoles were smaller and developed slower than when raised alone, whereas cane toad tadpoles were larger and developed faster. Contrary to our predictions, development and survival of endemic wart frog tadpoles was not affected by butachlor, whereas invasive cane toad tadpoles were affected across several endpoints including gene expression, body size, and survival. We also observed an interaction between pesticide exposure and competition for the cane toad, where survival declined but body size and expression of thyroid sensitive genes increased. Taken together, our findings indicate that the success of the cane toad larvae in rice fields may be best explained by increased rates of development and larger body sizes of tadpoles in response to competition with native Luzon wart frog tadpoles rather than lower sensitivity to a common pesticide. Our results for the cane toad also provide evidence that butachlor can disrupt thyroid hormone mediated development in amphibians, and further demonstrate that important species interactions such as competition can be affected by pesticide exposure in aquatic ecosystems.

Keywords Butachlor · Cane Toad · Endocrine Disruption · Luzon Wart Frog · Southeast Asia · Thyroid

Introduction

Amphibian populations are in critical decline worldwide (Wake and Vredenburg 2008). Knowledge of the status of Southeast Asian species is particularly limited, with approximately 36 percent currently classified as data deficient and 20 percent classified as threatened (Sodhi et al. 2004; Rowley et al. 2010). Rice agriculture comprises significant total landmass in Southeast Asia (GRiSP 2013) and provides native amphibians with important substitute wetland habitat (Bambaradeniya et al. 2004; Edirisinghe and Bambaradeniya 2006; Holzer et al. 2017). Amphibians utilize rice fields throughout larval, metamorphic, and adult life stages, and provide important ecosystem services. They consume arthropod pests (Attademo et al. 2005; Khatiwada et al. 2016), act as sentinels to reveal negative impacts of pesticide exposure, and are a staple food source for humans (Punhali 1995; Hocking and Babbitt 2014).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10646-017-1854-8) contains supplementary material, which is available to authorized users.

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Lowland irrigated rice fields in the Philippines harbor native amphibian species, including *Fejervarya vittigera*, *Occidozyga laevis*, *Polypedates leucomystax*, and *Kaloula picta* (McLeod et al. 2011; Ramirez 2014). However, non-native species including *Rhinella marina*, *Hoplobatrachus rugulosus*, and *Kalula pulchra* have spread rapidly throughout the country, and may be outcompeting natives (Diesmos et al. 2006; Diesmos 2008). The larvae, juveniles, and adults of the Luzon wart frog (*F. vittigera*) and the cane toad (*R. marina*), in particular, co-occur at high densities in rice fields throughout the rainy season. The Luzon wart frog is endemic to the Philippines, widespread throughout lowland Luzon (Diesmos et al. 2015), and commonly consumed and sold by locals (Howard 2015). The cane toad is an opportunistic invasive species that has an ability to disperse rapidly across new environments and negatively impact native species, particularly in Australia (Molloy and Henderson 2006; Shine 2010). The cane toad was introduced in the Philippines in the 1930s as a biological control agent in sugar cane crops, and now occupies all major islands except Palawan (Diesmos et al. 2006). Its extensive distribution and high abundance suggest it may pose a threat to native fauna in the country (Diesmos 2008); however, no reported studies have addressed this threat in rice fields.

We conducted an experiment to determine whether cane toad tadpoles have a competitive advantage over the Luzon wart frog, and furthermore, whether pesticide use, which has increased over the past two decades in rice (Horgan et al. 2016), could drive competitive interactions in favor of the invasive species. Pesticide exposure is a threat to biodiversity and human health, and many pesticide products are endocrine disrupting chemicals (EDCs) that impair vertebrate development and reproduction (McKinlay et al. 2008). Comparative studies of amphibians have revealed interspecific differences in sensitivity to pesticides (Bridges and Semlitsch 2000; García-Muñoz et al. 2010), which may be attributed to differences in exposure risk or genetics.

Genetic variability within populations can allow some species to adapt to novel stressors and persist in changing environments (Lande and Shannon 1996), and may play a key role in the success of invasive species (Prentis et al. 2008). In the case of the cane toad, traits such as heightened metabolic and immune system response have allowed toads to respond successfully to challenges encountered along the invasion front in Australia (Rollins et al. 2015). If invasive amphibians such as the cane toad have traits that decrease their susceptibility to common pesticides, this may provide them a competitive edge over native species in rice agroecosystems.

Interactions between pesticide and ecological stressors are poorly understood, but have the potential to produce cascading consequences at multiple levels of biological organization. For example, pesticide use in rice fields can alter community composition of beneficial insects (Cohen et al. 2014) and migratory waterbirds (Parsons et al. 2010). Sub-lethal pesticide exposure can also indirectly affect population size by altering predation rates of aquatic insects on amphibian larvae or changing competitive outcomes between species (Relyea and Hoverman 2008). At the organismal and tissue levels, pesticide and ecological stressors affect development, reproduction, and gene expression by acting directly upon endocrine targets, and indirectly via crosstalk between the stress and thyroid endocrine systems (Denver 1997, 2009; Kulkarni and Buchholz 2012).

The goals of our experiment were three-fold: (1) to determine the effects of competition on the larvae of the cane toad and Luzon wart frog, (2) to determine if species-specific differences exist in response to exposure to butachlor, a common herbicide used in rice, and (3) to test for an interaction between pesticide exposure and interspecific competition. To establish the level of sensitivity for each species to our competition, pesticide, and pesticide x competition treatments, we examined several endpoints at the organismal (morphology, activity, development, and survival) and tissue levels (gene expression).

We hypothesized that cane toad tadpoles would be less affected by butachlor exposure across all measured endpoints, compared with native Luzon wart frog tadpoles. Moreover, we predicted that this difference in sensitivity would affect competitive interactions with the native species, incurring an advantage to the cane toad in terms of growth and survival. At the tissue level, we expected both butachlor and competition would have a larger effect on the expression of thyroid sensitive genes controlling development in the endemic species, the Luzon wart frog.

Methods

Species collection and care

We collected wild Luzon wart frog and invasive cane toad tadpoles at Gosner stages 25–27 from experimental rice fields at the International Rice Research Institute (IRRI) in Los Baños, Philippines. To capture genetic diversity, three tadpole clusters from each species were collected from isolated paddies (SI Table 1). At the time of collection on June 23rd, 2016 paddies were flooded with rainwater, but were not cultivated, and the egg masses and tadpoles had not been exposed to pesticides directly from spraying. Following collection, we combined tadpoles of the same species, transferred them to 16 L tanks (36.8 × 33.8 × 23.6 cm plastic rice storage bins), and allowed them to acclimate for 1 day. We maintained each tank with 10 L of tap water aged for 24 h to allow any possible chlorine to

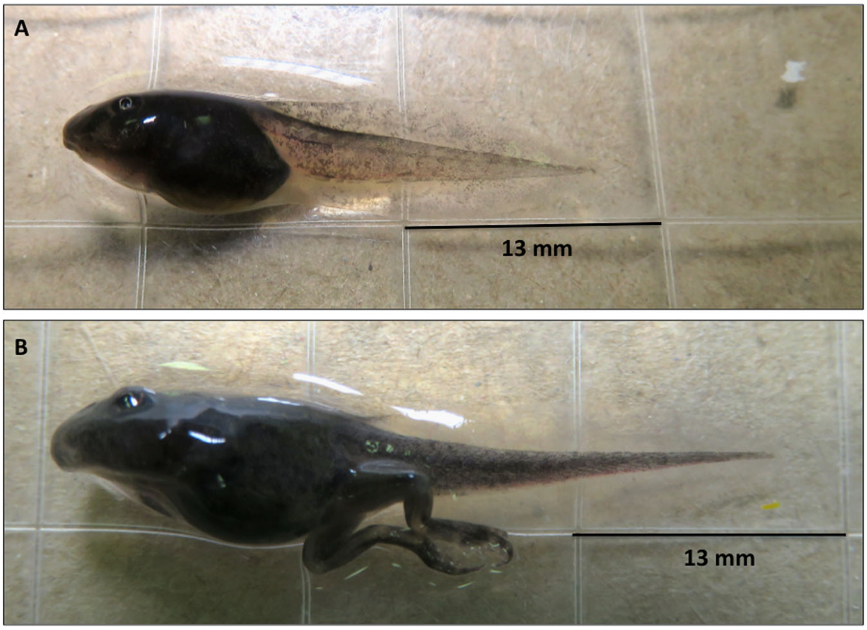

Fig. 1 Representative photographs of **a** Luzon wart frog (*Fejervarya vittigera*) tadpole at Gosner stage 29, and **b** cane toad (*Rhinella marina*) tadpole at Gosner stage 41 used to determine morphometric differences at day 14

evaporate. Throughout the experiment, tadpoles were fed *ad libitum* with a 1:1 mixture of crushed rabbit pellets and fish flakes, and deceased individuals were removed every morning.

Pesticide

We evaluated the effects of the acetanilide herbicide butachlor, one of the most extensively used herbicides in rice agriculture throughout the Philippines (Snelder et al. 2008) and Southeast Asia (Abigail et al. 2015), and a suspected endocrine disruptor to which paddy amphibians are exposed during development in the wild (Liu et al. 2011; Li et al. 2016). In particular, butachlor exposure has been observed to disrupt thyroid hormone mediated pathways in the African clawed frog, *Xenopus laevis* (Li et al. 2016), and zebrafish, *Danio rerio* (Chang et al. 2011). We used a commercial formulation, Machete EC (68% active ingredient, 600 mg/mL 2-chloro-2′,6′-diethyl-N-butoxymethyl-acetanilide, Sinochem Crop Protection Inc., Philippines), to which paddy amphibians are realistically exposed in the field (Snelder et al. 2008; Yap and Demayo 2015). We exposed tadpoles to 0.2 mg/L, a concentration we estimated to exist in fields 6 to 7 days after spraying based upon the recommended application rate of 4.8 mg/L (Geng et al. 2005), and the observed half life of 1.7–2.5 days in paddy water (Huarong et al. 2010). Amphibian breeding occurs when rice fields are irrigated, and corresponds with the typical application of pre-emergence herbicides such as Machete 1–3 days after transplanting. We estimated that 0.2 mg/L should be a realistic concentration to which recently hatched tadpoles of Gosner stage 25–27 could be exposed.

Experimental design

We conducted a full factorial laboratory experiment at IRRI with the following treatments: (1) tadpoles of only *R. marina*, (2) tadpoles of *R. marina* exposed to butachlor (3) tadpoles of only *F. vittigera*, (4) tadpoles of *F. vittigera* exposed to butachlor 3) tadpoles of both *R. marina* and *F. vittigera*, and (5) tadpoles of both *R. marina* and *F. vittigera* exposed to butachlor (Fig. 1). We replicated each treatment four times for a total of 24 experimental units (tanks), each containing 20 individual tadpoles. Water temperature and pH was kept at 26° C and between 8.2–8.6, respectively, within the temperature and pH range of paddy water (Kuwagata et al. 2008, Ramirez 2014). The facility was kept on a 13-hour light: 11-hour dark cycle to simulate field conditions in Luzon during June and July.

To generate a nominal concentration of 0.2 mg/L in each pesticide treated tank we performed a serial dilution. Trained pesticide applicators at IRRI created a solution stock 1) with a nominal concentration of 2 mg/mL (2000 mg/L), consisting of 2 mL of concentrated 600 mg/mL Machete added to 598 mL of water, and a second solution (stock 2) with a nominal concentration of 0.002 mg/mL (2 mg/L), consisting of 66 mL of stock 1 added to each of six bins containing 65.934 L of aged tap water (66 L total volume). To start the experiment, we added 1 L of stock solution 2 to each of our treatment tanks pre-filled 9 L to yield a nominal concentration of 0.0002 mg/mL (0.2 mg/L). We added the same amount of aged tap water to our non-pesticide tanks. In order to mimic degradation of butachlor over time in the field, we stored water from stock solution 2 for the duration of the experiment and added 1 L of this water to our tanks every water change. Although we were unable to verify the actual concentrations of butachlor to which our animals were exposed, appropriate conclusions can still be drawn from our experiment because our primary aim was to determine if an environmentally relevant pesticide exposure might affect competitive interactions. Furthermore, IRRI pesticide applicators perform the same 2 step dilution series when they prepare Machete for application, suggesting that our exposure is a conservative estimate of a concentration in rice fields.

We began our experiment on June 24th after placing 20 tadpoles at Gosner stages 25–27 in each tank, and adding Machete to the pesticide treatment groups. We took daily measurements of tadpole stage using the Gosner staging chart (Gosner 1960) and recorded survival and activity level by counting the number of live and moving tadpoles in the tank prior to feeding. We recorded temperature and pH every 5 days using a HI9829 multi-parameter meter (Hannah Instruments, Woonsocket, RI, USA). We conducted water changes every 5 days, re-filling each tank with a total of 10 L of aged tap water. In the pesticide-treated tanks, 1.43 L of the final 10 L volume was from stock solution 2. We terminated the experiment on day fourteen when the first tadpoles reached metamorphosis climax (Gosner stage 41), and euthanized all individuals in 0.02% MS222 over a subsequent 36-hour period. We took lateral photographs of individuals (Fig. 1) for morphometric analysis, and harvested tissue samples from the intestine and gonad. Samples were placed in RNAlater solution within a half hour after death (Thermofisher Scientific Inc., Nepean, ON, Canada), shipped on dry ice to Northern Arizona University, and stored at −80 °C.

Quantitative polymerase chain reaction (qPCR)

We conducted a qPCR assay to determine the effects of the competition, pesticide, and competition x pesticide treatments on thyroid sensitive genes. We examined intestinal tissue because the intestine undergoes a comprehensive physiological transformation during amphibian metamorphosis that is mediated by thyroid hormone (Shi et al. 2012). We extracted RNA from the intestine of both species using AllPrep DNA/RNA Mini Kits (Qiagen, Hilden, Germany), and determined concentrations using a Nanodrop ND-1000 (ThermoFisher Scientific Inc., Nepean, ON, Canada). We validated RNA quality using a 2100 Bioanlayzer (Agilent) with an RIN cut-off of 8, and then used Invitrogen SuperScript® VILO™ Master Mix (ThermoFisher Scientific Inc., Nepean, ON, Canada) to convert to cDNA using the manufacturer's instructions. All subsequent qPCR reactions were carried out using 19 uL iQ SYBR Green SuperMix (Bio-Rad Laboratories In., Hercules, CA, USA) containing 10 pmol of each primer and 1 ul sample cDNA on a Realplex 4 thermo-cycler (Eppendorf, Hamburg, Germany).

We tested nine genes (SI Table 2) for potential use in our wild species using a two-tiered approach for quality control (Veldhoen et al. 2014). We selected genes that had been previously established as thyroid hormone sensitive in *X. laevis* and *Rana catesbiana* (Veldhoen et al. 2014; Wolff et al. 2015). To confirm primer specificity, we first conducted qPCR using pooled intestinal cDNA across treatments for each species under the thermocycler conditions used by Veldhoen et al. (2014), and examined products on 1% agarose gels stained with ethidium-bromide. For primers that yielded products of the expected molecular weight, we then performed a 1:2 serial dilution with pooled cDNA to test for parallel amplification efficiency against the housekeeping gene, *rpl8* (Bustin et al. 2010). Transcript levels of *rpl8* were tested, and were not affected by experimental treatments.

For our final qPCR assay we used three primers that yielded the correct product and ran with parallel efficiency to our reference gene: thyroid hormone receptors alpha and beta (*thra* and *thrb*) and Krüppel-like factor 9 (*klf9*). *Thra* and *thrb* mediate the effects of thyroid hormone on amphibian metamorphosis (Shi et al. 2012). *Klf9* regulates expression of *thrb* in the model amphibian *X. laevis* (Kanamori and Brown 1992), and mediates actions of thyroid hormone on differentiation of nerve cells (Knoedler and Denver 2014). We used intestinal cDNA diluted 20-fold, and ran quadruplicate reactions using the following thermocycler sequence (1) enzyme activation at 95 °C for 3 min, (2) 38 cycles of DNA template denaturation at 95 °C for 15 s, primer annealing at 62 °C (*klf9, thra*) and 60 °C (*rpl8, thrb*) for 30 s, and elongation at 72 °C for 45 s, and (3) a melt curve. We used the comparative cycle threshold (Ct) method (ΔΔCt) to calculate fold change in transcript levels for *thra, thrb*, and *klf9* relative to our reference gene, *rpl8* (Schmittgen and Livak 2008).

Morphometric analyses

For morphometric analyses we enhanced and screened photos, discarding images in which tadpole features were not clearly visible. One individual performed analysis on all randomized photos, without knowledge of the associated treatments. We used ImageJ (NIH, Bethesda, Maryland) to obtain measures of tail width and lengths of total body, snout–vent, tail, and hind limb.

Statistical analysis

We used R for all statistical analyses. We examined data for normality using qqplots and Shapiro-Wilk tests, and tested for homogeneity of variance using Levene's tests. To visualize differences in survival over time, we computed nonparametric Kaplan-Meier survival curves. To determine increases/decreases in hazard ratios between our control and treatment groups, we then calculated Cox proportional-hazards regression coefficients for each species. Significant positive coefficients indicated a higher risk of tadpole death in a given treatment compared with the control. We used the R package ‘survival’ for both steps in our survival analysis (Therneau 2016).

Components of developmental stage and morphometric data were non-normally distributed or exhibited unequal variances. Activity and gene expression data met assumptions. Transformations did not improve the distribution of stage data, so we performed non-parametric Kruskal–Wallis tests followed by Dunn’s *post hoc* tests with Bonferroni correction for multiple comparisons (kruskal.test and dunn.test functions in R) to test for differences across treatments. Morphometric data met assumptions following log transformations. We used the nlme package in R to examine the effects of butachlor exposure crossed with competition on tadpole morphology, gene expression, and activity using mixed effects models that accounted for variance in our experimental unit by including tank as a random effect. For activity, we included day of measurement (1–10) as a fixed effect, to determine whether activity levels changed throughout the course of the experiment. We used the multcomp package in R to test for significant differences in treatment groups by setting our model reference (intercept) as control, butachlor, and competition to generate all possible comparisons.

Results

Survival analysis

We observed interspecific differences in survival across the butachlor and butachlor x competition treatments (Fig. 2). Across treatments, 93% of all Luzon wart frog tadpoles survived the two-week experimental period, and we observed no significant differences in survivorship of those in control water, exposed to butachlor, competition, or both. In contrast, 80% of cane toad tadpoles survived across all treatments, and those exposed to butachlor were 27% more likely (Cox Hazard Ratio = 2.74, 95% CI 6.61, 1.14, $P < 0.05$) to experience mortality than control tadpoles over the two-week experimental period. Competition alone did not affect survival of cane toad tadpoles, but cane toad larvae in the competition x butachlor treatment were 58% more likely (Cox Hazard Ratio = 5.78, 95% CI 14.06, 2.38, $P < 0.05$) to experience mortality than control animals, and 4.7% more likely (Cox Hazard Ratio = 0.45, 95% CI 0.94, 0.24, $P < 0.05$) to experience mortality than animals exposed to only butachlor (Table 1).

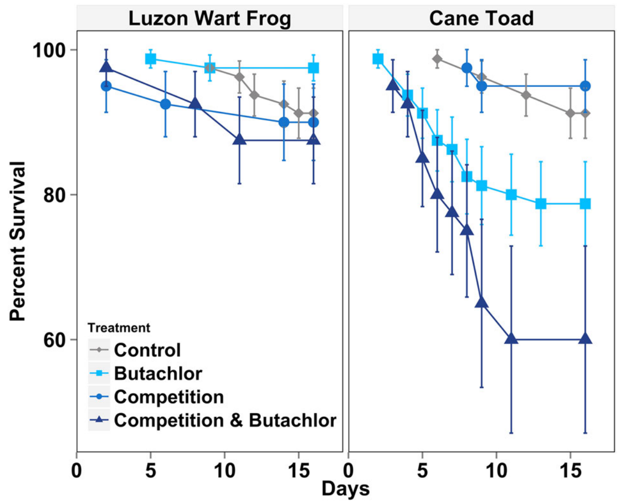

Fig. 2 Nonparametric Kaplan–Meier survival curves depicting percentages of Luzon wart frog (*Fejervarya vittigera*) and cane toad (*Rhinella marina*) tadpoles surviving over the course of the experiment in response to competition, butachlor, or competition × butachlor. Errors bars depict SE and stars represent treatments where survival was significantly reduced compared to control ($P < 0.05$)

Developmental stage

Cane toad tadpoles developed 1.4 times faster than Luzon wart frog tadpoles. All individuals began at Gosner stages 25–27, but after 14 days control cane toad tadpoles were approaching metamorphosis (Gosner stages 40–41) whereas control wart frog larvae were still growing limb buds (Gosner stages 26–32). Development of wart frog and cane toad tadpoles was significantly affected by treatment, but in opposing directions (Fig. 3; Kruskal–Wallis, $P < 0.001$ and $P < 0.001$, respectively). Cane toad tadpoles developed faster in response to competition, but were not affected by butachlor or the combined competition x butachlor treatment (Fig. 3 and Table 2). In contrast, Luzon wart frog tadpoles developed slower in the competition treatment compared with control animals. Butachlor alone had no effect on development, but in the competition x butachlor treatment, Luzon wart frog tadpoles were observed at a developmental stage intermediate to both control and competition treatments. In this treatment, decreased survivorship of the cane toad alleviated some, but not all, competitive pressure on the Luzon wart frog (Fig. 3 and Table 2).

Activity

The activity of Luzon wart frog tadpoles was not affected by exposure to butachlor, but was significantly reduced when in competition with the cane toad. We observed no

Table 1 Cox proportional-hazards regression coefficients and corresponding upper and lower CIs depict risk of tadpole death in response to competition, butachlor, or competition × butachlor

Species	Cox proportional hazards regression coefficients											
	Butachlor				Competition				Butachlor × Competition			
	Hazard ratio	CI upr	CI lwr	*P* value	Hazard Ratio	CI upr	CI lwr	*P* value	Hazard ratio	CI upr	CI lwr	*P* value
Cane toad (*Rhinella marina*)	2.74	6.61	1.14	**0.02**	0.57	2.75	0.12	0.49	5.78	14.06	2.38	**0.0001**
Luzon wart frog (*Fejervarya vittigera*)	3.53	1.37	0.06	0.12	1.19	4.08	0.35	0.78	1.50	4.74	0.67	0.49

Significant positive hazard ratios (indicated in bold) indicate a higher risk of tadpole death in a given treatment compared with the control

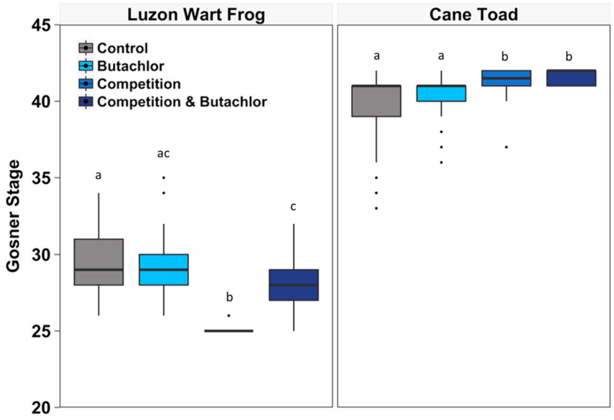

Fig. 3 Box plots depicting mean plus lower (25%) and upper (75%) quartiles of the effect of competition, butachlor, and competition × butachlor on developmental stage (Gosner stage) of Luzon wart frog (*Fejervarya vittigera*) and cane toad (*Rhinella marina*) tadpoles at day 14. Whiskers extend to lower and upper values excluding outliers, which are represented as points

interactions between competition and butachlor, and cane toad activity levels were not affected by any treatment (SI Fig. 2, SI Table 3). Activity levels for both species varied significantly over the course of the exposure period.

Morphology

We observed no differences in the measurements of Luzon wart frog tadpoles exposed to butachlor alone across morphological endpoints, with the exception of tail width, which was wider in exposed tadpoles (Table 3). Total body, tail, and hind limb lengths of Luzon wart frog tadpoles were affected by competition, and comparisons revealed tadpoles in the competition treatments were shorter across all morphological endpoints compared with controls (Fig. 4). We observed no interactions between competition x butachlor on morphology of the Luzon wart frog (Table 3).

Butachlor exposure affected the snout-vent length of cane toad tadpoles (Table 3), and comparisons revealed exposed tadpoles exhibited longer snout-vent lengths compared with control animals (Fig. 4). The same morphological endpoints that were affected by competition for the Luzon wart frog (total body, tail, and hind limb lengths) were also affected in the cane toad. However, cane toad tadpoles responded in the opposite direction, and exhibited longer total body lengths compared to control animals. For tail width, we observed a significant interaction in the competition x butachlor treatment (Table 3).

Gene Expression

We observed no differences in the relative expression of *klf9* and *thra* in Luzon wart frog tadpoles exposed to

Table 2 Dunn's test results examining differences in the final developmental stage of Luzon wart frog (*Fejervarya vittigera*) and cane toad (*Rhinella marina*) tadpoles across treatment groups at day 14

Luzon wart frog			
Treatment	Control	Butachlor	Competition
Butachlor	$Z = -1.99$, $P = 0.14$		
Competition	$Z = -9.69$, $\boldsymbol{P < 0.001}$[a]	$Z = 8.14$, $\boldsymbol{P < 0.001}$	
Competition × Butachlor	$Z = -2.83$, $\boldsymbol{P < 0.014}$	$Z = 1.25$, $P = 0.63$	$Z = -5.87$, $\boldsymbol{P} = \boldsymbol{< 0.001}$
Cane toad			
Treatment	Control	Butachlor	Competition
Butachlor	$Z = 1.08$, $P = 0.84$		
Competition	$Z = 3.5$, $\boldsymbol{P < 0.01}$	$Z = -2.50$, $\boldsymbol{P} = \mathbf{0.04}$	
Competition × Butachlor	$Z = 4.94$, $\boldsymbol{P < 0.001}$	$Z = 8.14$, $\boldsymbol{P < 0.001}$	$Z = -1.74$, $P = 0.24$

[a] Comparisons in bold indicate significant ($P < 0.05$) differences between treatment groups, where the probability of observing a random value from one group that is greater than a random value from a second group is ≠ ½ (differences are not due to random chance)

Table 3 Results of mixed effects models testing the effects of butachlor, competition, competition × butachlor on morphology of Luzon wart frog (*Fejervarya vittigera*) and cane toad (*Rhinella marina*) tadpoles

Luzon wart frog										
Treatment	Total Body		Snout—vent		Tail length		Tail width		Hind limb	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Butachlor	1.29	0.28	2.73	0.12	0.76	0.40	3.56	**0.04**	0.54	0.48
Competition	6.98	**0.02**[a]	2.14	0.17	8.95	**0.01**	5.36	0.08	6.84	**0.02**
Competition × Butachlor	0.38	0.55	0.31	0.59	0.25	0.63	0.05	0.83	1.56	0.24
Cane toad										
Treatment	Total Body		Snout—vent		Tail Length		Tail Width		Hind Limb	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Butachlor	3.66	0.08	4.99	0.05	2.87	0.12	1.58	0.24	3.69	0.08
Competition	6.95	**0.02**	3.73	0.08	7.46	**0.02**	0.43	0.53	6.45	**0.03**
Competition × Butachlor	0.92	0.36	3.12	0.10	0.24	0.86	10.36	**0.01**	0.33	0.58

We accounted for variance in our experimental unit by including tank as a random effect. Degrees of freedom were 1, 12

[a] Comparisons in bold indicate significant ($P < 0.05$) effects

butachlor alone, or in the competition x butachlor treatment. Competition significantly affected expression of both genes, but in opposite directions; expression of *klf9* increased and expression of *thra* decreased relative to control. For the cane toad, exposure to butachlor increased expression of *thrb* but did not affect expression of *klf9*. Competition alone did not affect expression of either gene, but we observed an interaction in our competition x butachlor treatment that resulted in increased expression of both *klf9* and *thrb* in cane toad tadpoles (Fig. 5).

Discussion

Contrary to our predictions, we demonstrated that invasive cane toad tadpoles were more sensitive to exposure to the common pesticide, butachlor, than endemic Luzon wart frog tadpoles. The population of cane toad larvae in our experiment also exhibited traits, including faster development and larger body size, which may lend them a competitive edge over the native Luzon wart frog in rice agroecosystems. Larger body sizes at metamorphosis improve chances of survival later in life (Johansson et al. 2010; Cabrera-Guzmán et al. 2013a), and faster development can limit the period of time larva are directly exposed to pesticide contaminants. Competition with the cane toad negatively affected growth and development of several native amphibian species in Queensland, and higher baseline activity levels and aggressive feeding behaviors have been suggested as a possible mechanism of success (Williamson 1999). However, exceptions exist. For instance, competition with one native Australian species has been found to negatively impact growth and development of cane toad larvae (Cabrera-Guzmán et al. 2013b), which suggests that

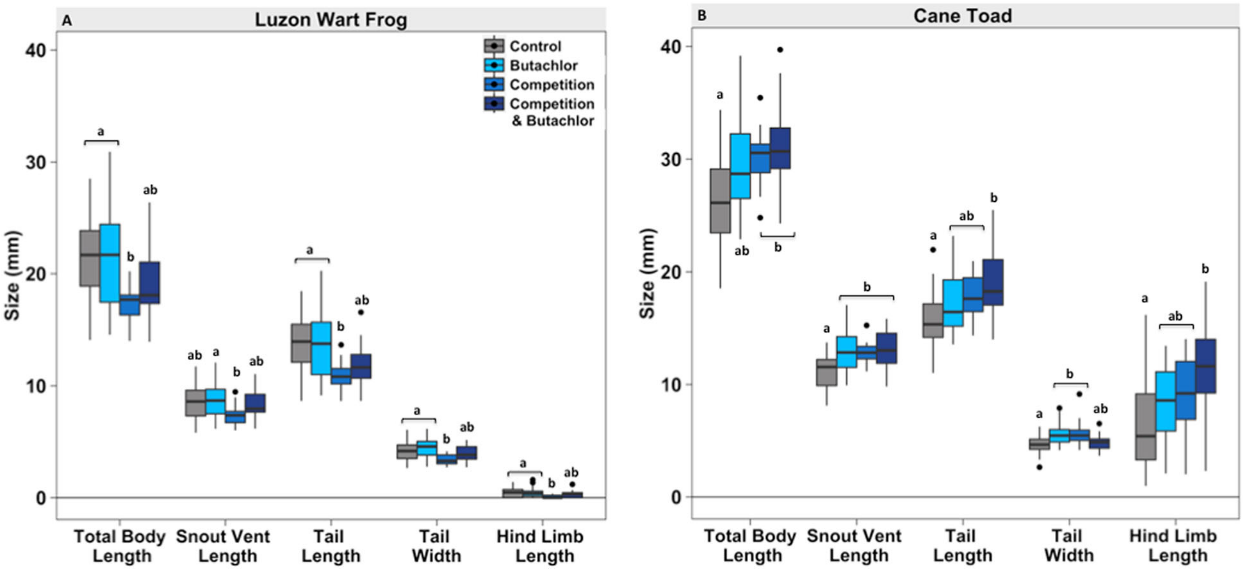

Fig. 4 Box plots depicting mean plus lower (25%) and upper (75%) quartiles of the effect of competition, butachlor, and competition × butachlor on morphometric measurements (body, snout-vent, and hind limb lengths and tail width) of **a**, Luzon wart frog (*Fejervarya vittigera*), and **b**, cane toad (*Rhinella marina*) tadpoles at day 14. Whiskers extend to lower and upper values excluding outliers, which are represented as points. Unique letters depict significant differences ($P < 0.05$) between treatments as determined using mixed effect model accounting for variance in our experimental unit, tank. Data were log transformed prior to statistical analysis

the effects of competition on development are both plastic and species-dependent.

Exposure to bufadienolide chemical cues may be partially responsible for the reduced activity levels and slowed growth and development of the Luzon wart frog in the competition treatments. Cane toads contain bufadienolide toxins that are present in eggs, in early larval developmental stages, and in adults (Hayes et al. 2009). Bufadienolides are cardioactive steroids known to inhibit Na^+/K^+-ATPase, but also have been observed to suppress immune system function (Cunha-Filho et al. 2010) and blood vessel growth (Lee et al. 1997). Future research should test the possibility that bufadienolides disturb developmental processes of native tadpoles; particularly given our results that competition with cane toad larvae slowed development and affected expression of two thyroid sensitive genes, *thra* and *klf9*, in the native species.

Intraspecific competition can also affect the development, growth, and morphology of amphibian larvae (Relyea 2002). In the wild, cane toad larvae are often found in aggregates consisting of thousands of tadpoles, and intense intraspecific competition including cannibalism of conspecific eggs and dead tadpoles has been well documented (Pizzatto and Shine 2008). Our findings that control cane toad tadpoles developed slower and were, on average, smaller compared with those in competition with the Luzon wart frog suggest that competition with conspecifics is more intense than interspecific competition at the same density.

The effects of both inter and intraspecific competition observed in the laboratory can be dampened in the field by interactions with predators and by abiotic factors (Loman 2001). For instance, differences in microhabitat use may dampen interspecific competition because wart frog larvae

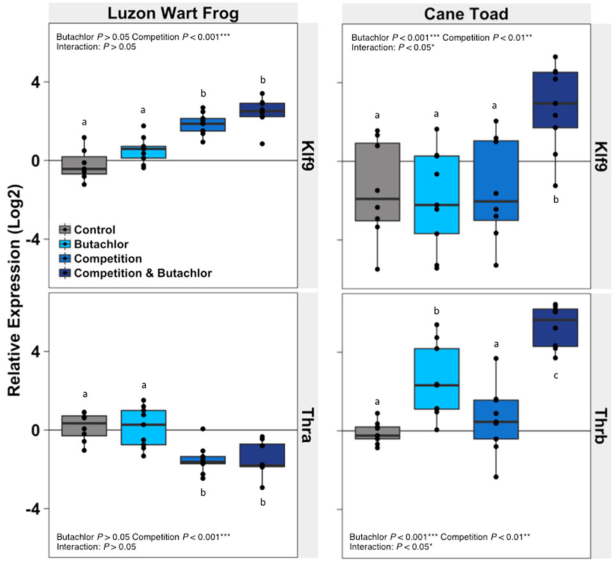

Fig. 5 Box plots depicting mean plus lower (25%) and upper (75%) quartiles of the effect of competition, butachlor, and competition × butachlor on relative expression of thyroid dependent genes (*thra, thrb*, and *klf*) in intestinal tissue of Luzon wart frog (*Fejervarya vittigera*) and cane toad (*Rhinella marina*) tadpoles. Points represent samples included in analysis, which consisted of $n = 9$ individual tadpoles per treatment, chosen at random from replicate tanks. Results of two-way ANOVAs are presented for each gene, and unique letters depict significant differences ($P < 0.05$) between treatments as determined using mixed effect models accounting for variance in our experimental unit, tank

appear to stay close to the bottom sediment, whereas cane toad larvae forage higher in the water column (M. Shuman-Goodier, personal observation). Differences in predation pressure could also plausibly affect the magnitude and direction of competitive interactions in rice fields. For example, aquatic insect predators have been observed to prefer cane toad tadpoles over native Australian species (Cabrera-Guzmán et al. 2012), perhaps because they are obvious, slow swimmers. Field studies are needed to determine whether the strong effects of interspecific competition we observed in the laboratory persist in rice agroecosystems.

Cane toad survival was reduced in the pesticide and pesticide x competition treatments, which alleviated some, but not all, competitive stress from the native species. The variation in sensitivity we observed may be attributed to differences in the rate each species bioaccumulates, biotransforms, and eliminates butachlor and its metabolites (Leney et al. 2006). Amphibian populations have been observed to exhibit tolerance to pesticides, particularly those that have been used for a long time period (Hua et al. 2015a, b), as is the case with the herbicide butachlor. It is also possible that the species introduction in the 1940's produced a founder effect, and that low genetic diversity of cane toad populations may have limited their ability to adapt to pesticide exposure compared with the Luzon wart frog. Alternatively, the Luzon wart frog may be vulnerable to butachlor exposure at later stages of development, which we did not test in our 15-day experiment. For example, survival of *Fejervarya limnocharis* tadpoles, a congener found in Taiwan, was negatively affected by butachlor exposure at the same concentration of 0.2 mg/L (Liu et al. 2011), but effects were only observed after 21 days of exposure. Realistically, however, exposure risk is likely to be highest at young developmental life stages for all amphibian species because butachlor is typically applied early in the rice-cropping season, as a pre-emergence herbicide, 1–3 days after transplanting.

A growing body of evidence indicates that butachlor exposure at environmentally relevant concentrations can disturb vertebrate development by disrupting the thyroid axis (Zhu et al. 2014; Li et al. 2016). The hypothalamic pituitary thyroid (HPT) axis plays a critical role in amphibian metamorphosis, and changes to levels of circulating thyroid hormones (THs), and expression of TH sensitive genes can disturb normal development (Opitz et al. 2006; Fort et al. 2007). Exposure to butachlor increased levels of circulating Ths, T3 and T4, in zebra fish (*Danio rerio*) at 50 and 100 ug/L (Chang et al. 2011), female rare minnows (*Gobiocypris rarus*) at 0.1, 1, and 10 ug/L (Zhu et al. 2014), and in *X. laevis* tadpoles at 100 ug/L. THs act at the level of the receptor (*thra* and *thrb*) to regulate gene expression. Increased expression of *thrb*, as we observed in our experiment for *R. marina* exposed to butachlor, can parallel increased levels of circulating THs, and result in a faster rate of metamorphosis (Fort et al. 2007). Our gene expression results for *R. marina* corroborate work done with *X. laevis*, which found that butachlor up-regulated *thrb*, increased total body and hind limb length, and promoted metamorphosis (Li et al. 2016). Moreover, corticosterone hormones typically act synergistically with THs to speed up metamorphosis (Denver 1997, 2009; Kulkarni and Buchholz 2012), and cross talk between the stress and HPT axes may explain the interaction between competition x butachlor we observed at the tissue level in *R. marina*.

Disruption of the HPT axis by endocrine disrupting chemicals can cause developmental and reproductive disorders in humans similar to those observed in wildlife (Choksi et al. 2003). The above studies observed interference with the HPT axis using butachlor concentrations within the range of those in the environment (Li et al. 2016), where concentrations ranging from 0.05 to 1.4 μg/L in surface water (Mamun et al. 2009; Toan et al. 2013) and 0.01 to 17.8 μg/L in paddy water (Li et al. 2016) have been detected. Butachlor exposure thus poses a realistic threat to both wildlife and human health, and the greatest risks are for organisms, such as amphibians, that utilize rice paddies during developmental and reproductive life stages.

Our experiment also demonstrates that exposure to a common pesticide can indirectly affect competitive interactions between native and invasive tadpole larvae, albeit in a direction contrary to our hypotheses. The sub-lethal effects of pesticides on species interactions, such as predation and competition, are understudied, but have the potential to shape the life history of a variety of taxa, including amphibians (Campero et al. 2007; Pestana et al. 2009; Janssens and Stoks 2013; Rasmussen et al. 2013). For example, competition decreased survival of bullfrog tadpoles exposed to the herbicide glyphosate at high densities (Jones et al. 2011), and exposure to the insecticide carbaryl promoted survival but increased competition between woodfrog tadpoles by killing off insect predators and reducing availability of food resources (Mills and Semlitsch 2004). As our own results demonstrate, the direction of these interactions is not always intuitive, and reflects a pressing need to incorporate ecological knowledge and complexity into ecotoxicological studies (Chapman 2002; Köhler and Triebskorn 2013).

Conclusion

We found that endemic Luzon wart frog larvae were negatively affected by competition with cane toad larvae, but contrary to our predictions, cane toad larvae were more susceptible to butachlor exposure. Our findings suggest that the success of the cane toad in rice fields may be best explained by increased rates of development and larger body sizes of tadpoles in response to competition with the native wart frog rather than species-specific sensitivity to butachlor. Our results for the cane toad also support evidence that butachlor can disrupt thyroid hormone mediated development in vertebrates, and further demonstrate that species interactions such as competition can be affected by pesticide exposure in aquatic ecosystems. To increase the environmental relevancy of the hypotheses we tested, we suggest that future work explore interactions between pesticide exposure and competition at additional juvenile and adult life stages, as well as use longer exposures to encompass species with slower developmental rates. There is also a need for studies that test for relationships between amphibian diversity and pesticide use in rice fields at the landscape level.

Acknowledgements We thank Frank von Hippel and Rachel Rubin for their editorial support. We are grateful to Linsey Benally, Alexander McCain, Renee Lorica, and Calsey Richardson for their assistance in field and laboratory data collection. We thank the anonymous reviewers who helped to improve the manuscript. Funding was provided by the Merriam Powell Center for Environmental Research's Integrative Graduate Education, Research, and Traineeship Program (IGERT) Fellowship, the Achievement Rewards for College Scientists (ARCS) Foundation, the Closing Rice Yield Gaps in Asia with a Reduced Environmental Footprint (CORIGAP) funded by the Swiss Agency for Development and Cooperation, the National Institute on Minority Health and Health Disparities of the NIH, Award Number T37MD008626 to CRP, and the National Cancer Institute of the NIH award for the Partnership of Native American Cancer Prevention U54CA143925 to Northern Arizona University.

Author contributions M.S.G., G.S., and C.R.P. conceived and designed the study. M.S.G. executed the study, analyzed the data, and wrote the manuscript. G.S. and C.R.P. contributed significant editorial guidance on the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests associated with this study, and that all applicable institutional and/or national guidelines for the care and use of animals were followed and approved by Northern Arizona's Institutional Animal Care and Use Committee (IACUC).

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