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INDIVIDUAL AND MIXTURE TOXICITY OF PHARMACEUTICALS NAPROXEN, CARBAMAZEPINE, AND SULFAMETHOXAZOLE TO AUSTRALIAN STRIPED MARSH FROG TADPOLES (*Limnodynastes peronii*)

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Nonsteroidal human pharmaceuticals are prevalent in domestic wastewater and may find their way into the environment at low concentrations. Since most pharmaceuticals are designed to be biologically active at low concentrations, there is a risk that these compounds may affect aquatic wildlife. Of particular concern is the occurrence of pharmaceutical mixtures, which may lead to increased adverse effects compared to individual compounds. Interactive effects were previously demonstrated for amphibians exposed to pesticide mixtures, but no such studies investigating responses of amphibians to pharmaceutical mixtures are apparently available. Results demonstrated increased toxicity (loss of tactile response) of striped marsh frog (*Limnodynastes peronii*) tadpoles exposed to a mixture of naproxen, carbamazepine, and sulfamethoxazole, compared to exposures to the individual compounds. Significant time × treatment interactions were observed for tadpole development following chronic exposures to 10 or 100 μg/L of each compound and the mixture; however, responses were weak and main treatment effects were not significant. Despite minor effects at low exposure concentrations, results demonstrated a potential for mixtures of nonsteroidal pharmaceuticals commonly occurring in wastewater to influence amphibian development. With the vast numbers of pharmaceuticals that exist and are found in the environment, this work highlights a need for further research into mixtures of pharmaceutically active wastewater contaminants. Further, since pharmaceuticals exert extremely varied biological actions, it is suggested that future investigations would benefit from inclusion of endpoints that are indicative of physiological or metabolic performance, as well as assessment of sensitive behavioral responses.

Nonsteroidal human pharmaceuticals represent important but relatively understudied groups of wastewater contaminants (Boxall et al., 2012). High concentrations of diverse groups of pharmaceuticals are commonly observed in aquatic environments in close proximity to highly populated areas (Metcalfe, 2013; Spongberg and Witter, 2008), with lower concentrations in areas farther removed from industrial and social centers (MacLeod and Wong, 2010; Wang et al., 2010). In the face of an ever-growing population and thus an expanding market for development and distribution of pharmaceutically active compounds, it is foreseeable that environmental concentrations may continue to rise. In many cases, the biological properties that make these compounds beneficial for human use may pose risks for nontarget wildlife, such that there is need to investigate and understand the potential toxicological consequences of pharmaceuticals and pharmaceutical mixtures on sensitive aquatic organisms (Corcoran et al., 2010; Mennigen et al., 2011).

Larval amphibians are highly sensitive to stressors in the aquatic environment because

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they (1) grow and develop in water, (2) have permeable skin and gills, and (3) undergo the complex energy-intensive process of metamorphosis (Shi, 2000). This sensitivity, in conjunction with widespread introductions of anthropogenic contaminants into aquatic ecosystems (Ferguson et al., 2013; Kolpin et al., 2002; Yoon et al., 2010), may be a contributing factor leading to global amphibian population declines (Houlahan et al., 2000; Stuart, 2004). Despite this, there have been few studies investigating responses of amphibian larvae to pharmaceuticals (Richards and Cole, 2006) or pharmaceutical mixtures (Fraker and Smith, 2004; Smith and Burgett, 2005). The few published examples that exist indicate a potential for adverse outcomes in amphibian tadpoles exposed to representative nonsteroidal pharmaceuticals.

Naproxen (NPX), carbamazepine (CBZ), and sulfamethoxazole (SMX) represent three commonly identified pharmaceuticals in domestic wastewater (Ferguson et al., 2013; Gagné et al., 2006; Hernando et al., 2006; Yoon et al., 2010). Each of these compounds has been detected in domestic wastewater samples in the μg/L range. The nonsteroidal anti-inflammatory drug (NSAID) NPX is used to treat pain and inflammation with conditions such as arthritis, tendinitis, and menstrual cramps, and was detected at concentrations as high as 38.8 μg/L (Li et al., 2013). The anticonvulsant drug CBZ is used to treat seizures and nerve injury and was identified at 4.9 μg/L (Hijosa-Valsero et al., 2010), and the slow-absorption sulfonamide antibiotic SMX was identified at 4.1 μg/L (Conkle et al., 2008). Each of these compounds has received attention for possible adverse effects on aquatic organisms (Crane et al., 2006), but few if any studies examined interactive effects, and none to our knowledge assessed the impacts of such a mixture on amphibian larval development. The aim of this study was thus to investigate the potential for NPX, CBZ, and SMX to influence tactile responsiveness, growth, and development of Australian striped marsh frog (*Limnodynastes peronii*) tadpoles with exposure to individual compounds or a complete mixture.

MATERIALS AND METHODS

Pharmaceutical Test Compounds

Technical-grade standards of NPX, CBZ, and SMX were purchased from Sigma-Aldrich (Australia). Physicochemical characteristics and structural information for each compound is provided in Table 1. Concentrated stock solutions (500 m*M*) were prepared by dissolving each compound in 1 ml ethanol (EtOH; 96%). Working stocks were prepared such that solvent volumes for all experiments were less than 0.2% of the holding water with the exception of acute tests with SMX, where as much as 3.15% EtOH was required to reach the highest target concentrations. Verification of the test concentrations was not possible and thus calculations are based on nominal exposure concentrations.

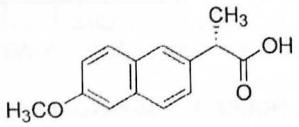
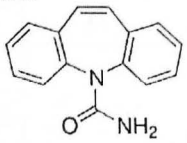
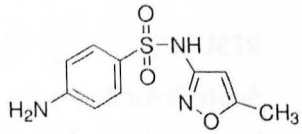
Tadpole Collection and Husbandry

Foam nests of striped marsh frog (*L. peronii*) were collected from a wetland near Tannum Sands, Queensland, Australia, following heavy rainfall events in late May 2013 (acute tests and chronic test 1), and again in late July 2013 (chronic test 2). Nests were maintained in 60-L aquaria filled with aerated carbon-filtered rainwater until the majority of tadpoles hatched. Hatched larvae were fed Sera micron fry food until commencement of the tests, when the majority had reached Gosner stage (Gs) 26 (Gosner, 1960).

Acute Toxicity

Acute tests were carried out to identify half-maximal effective concentrations (EC_{50}) for each of the compounds, alone and in a complete mixture resulting in a loss of tactile responsiveness to prodding. Tests were performed in acid-washed 150-ml glass beakers filled with 100 ml carbon-filtered rainwater. Three replicates were used for each test

TABLE 1. Physical–Chemical Properties and Chemical Structures of All Compounds Tested

	Naproxen	Carbamazepine	Sulfamethoxazole
Therapeutic class	NSAID	Anticonvulsant	Antibiotic
CAS number	22204-53-1	298-46-4	723-46-6
Molecular weight (g/mol)	230.26	236.27	253.28
Formula	$CH_3OC_{10}H_6CH(CH_3)CO_2H$	$C_{15}H_{12}N_2O$	$C_{10}H_{11}N_3O_3S$
log K_{ow}[a]	3.18	2.45	0.89
Chemical structure			

[a]Source: http://toxnet.nlm.nih.gov

TABLE 2. Exposure Concentrations of the Pharmaceutical Compounds in the Acute Tests, Individually and in the Full Mixture

Individual compounds			Mixture*		
NPX	CBZ	SMX*	NPX	CBZ	SMX
25	50	0	0	0	0
30	60	500	6.25	6.25	25
35	70	600	12.5	12.5	50
40	80	700	25	25	100
45	90	800	50	50	200

Note. Concentrations were chosen based on results from preliminary range-finding tests. Asterisk indicates solvent control used because EtOH concentration was >0.2%.

concentration including solvent control, and each replicate contained four Gs26 tadpoles. Initial range-finding tests were used to determine the more focused test concentrations employed for calculations of EC_{50} values (Table 2).

Chronic Toxicity

Two chronic exposures were carried out to evaluate the potential for sublethal effects on larval growth and development at concentrations similar to those observed in domestic wastewater. Chronic tests were carried out in 15-L glass aquaria filled with 10 L rainwater. The first chronic test included 4 replicates each of solvent control, NPX, CBZ, SMX, and a complete mixture (MIX) of all compounds all at 10 μg/L. Ten tadpoles each were exposed from Gs26 until commencement of front limb emergence. The second chronic test employed the same experimental setup, but with 8 animals per replicate and concentrations of 100 μg/L. Stocking densities were kept to a maximum of 1 tadpole/L to ensure density-dependent effects did not confound any possible treatment effects (Melvin and Houlahan, 2012, 2013).

Tadpoles were fed ad libitum twice daily with Sera micron powdered fry food supplemented with algal pellets throughout the experiment. Full water changes were performed twice weekly to renew the treatments. At this time, tadpoles were developmentally Gosner staged and photographed on a 10 mm × 10 mm grid for measurement using ImageJ image analysis software (National Institutes of Health, USA). Following exposures, tadpoles were euthanized in 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich) dissolved in water, measured for weight and length, and livers were dissected and weighed.

Statistical Analysis

Data were analyzed using IBM SPSS version 20 for Mac OS X. Probit analysis was used to determine EC_{50} concentrations following the acute tests. All other data were tested for normality and homogeneity of variances using Levene's test and visual inspection of normal probability plots. Data were analyzed for interactive and main effects of the pharmaceutical treatments over time on tadpole length and developmental stage, using repeated-measures analysis of variance (ANOVA). Additional assumption of sphericity

was therefore tested with Mauchley's test, and if this was violated a Greenhouse–Geisser correction factor was applied. Differences in tadpole condition and liver weight were analyzed using analysis of covariance (ANCOVA). The criterion for significance was set at $p < .05$.

RESULTS

Acute Toxicity

No mortality or loss of tactile responses was observed in solvent controls throughout the duration of the acute experiments. The 24-, 48-, 72-, and 96-h EC_{50} values corresponding to a loss of tactile response of tadpoles exposed to NPX, CBZ, and SMX alone and in a mixture are provided in Table 3. Results of the acute tests revealed EC_{50} values that were markedly lower when tadpoles were exposed to a mixture of all three pharmaceuticals, compared to exposures with the individual compounds (Figure 1).

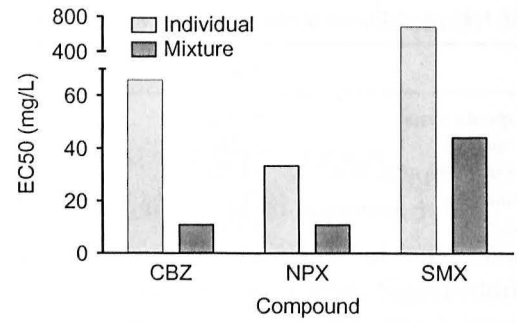


FIGURE 1. Half-maximal effective concentrations (EC_{50}) for loss of tactile responsiveness in *Limnodynastes peronii* tadpoles exposed to naproxen (NPX), carbamazepine (CBZ), and sulfamethoxazole (SMX), individually and in a mixture, for 96 h.

Chronic Toxicity

A significant time × treatment interaction was observed for tadpole development (Gosner stage) in the 10-μg/L chronic test, but the response was minor and the main effect of treatment nonsignificant (Figure 2a). There was also a significant interaction for snout–vent length (SVL; Figure 2b), but no main effect of treatment was observed. The pharmaceuticals exerted no marked effects on weights or liver weights of tadpoles exposed to 10 μg/L (data not shown).

In the 100-μg/L chronic test, a significant time × treatment interaction was also noted for tadpole development, but the main effect of treatment was nonsignificant (Figure 2c). There was no interactive or main effect of any of the pharmaceuticals of the mixture on SVL in tadpoles exposed to 100 μg/L (Figure 2d). None of the compounds tested exerted any marked effect on tadpole weight or liver weight with exposure to 100 μg/L (data not shown).

DISCUSSION

Increased toxicity resulting in a loss of tactile responsiveness was observed with acute exposure of *L. peronii* tadpoles to a mixture of NPX, CBZ, and SMX compared to exposures to the individual compounds. While loss of responsiveness was the endpoint of interest for the acute tests, this was almost always indicative of mortality. Estimates of EC_{50} values for all compounds were relatively high compared to concentrations occurring in natural waterways (i.e., maximal concentrations in the nanograms per liter to micrograms per liter range), indicating that acutely toxic effects are unlikely to result from mixtures of these pharmaceuticals in the aquatic environment.

TABLE 3. Half-Maximal Effective Concentrations (EC_{50}) for Loss of Tactile Responsiveness in Striped Marsh Frog Tadpoles (*Limnodynastes peronii*) Exposed to NPX, CBZ, and SMX Alone and in a Mixture After 24, 48, 72, and 96 h. All units are mg/L.

	24 h		48 h		72 h		96 h	
	Alone	Mixture	Alone	Mixture	Alone	Mixture	Alone	Mixture
NPX	37.52	15.13	37.52	12.47	33.40	11.70	33.40	10.99
CBZ	65.70	15.13	65.70	12.47	65.70	11.70	65.70	10.99
SMX	1257.09	60.91	688.30	49.78	672.22	46.73	672.22	44.04

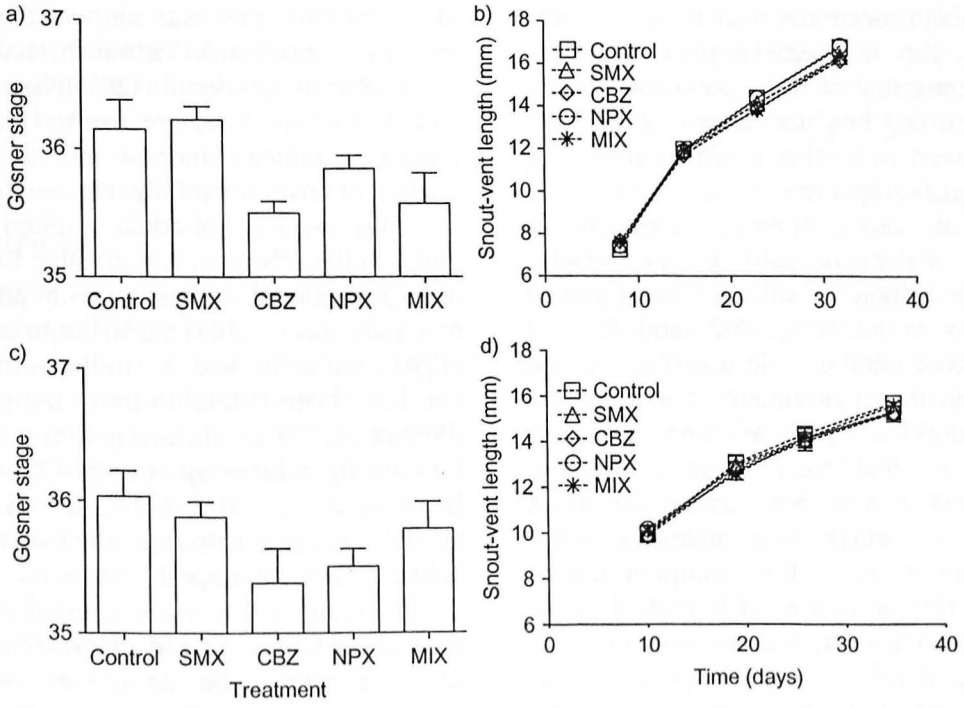


FIGURE 2. (a) Snout–vent length (SVL) and (b) developmental stage at d 21 of *Limnodynastes peronii* tadpoles exposed to 10 μg/L naproxen (NPX), carbamazepine (CBZ), and sulfamethoxazole (SMX), individually and in a mixture (MIX). (c) SVL and (d) development at d 21 for exposure to the same compounds at 100 μg/L. Bars represent 1SE.

This conclusion is consistent with studies investigating the acute toxicological effects of similar pharmaceutical mixtures on aquatic invertebrates and algae (Cleuvers, 2004; Borgmann et al., 2007), but differs from others where significant concerns regarding the potential for toxicological effects at low concentrations have been suggested (Flaherty and Dodson, 2005; Gust et al., 2013). Data demonstrate the differential sensitivities that may exist among compounds and organisms, and indicate the inherent complexity involved with understanding toxicological effects of pharmaceutical mixtures (Corcoran et al., 2010).

There is a clear need to investigate sublethal effects of pharmaceutical mixtures on aquatic organisms at realistic exposure concentrations (Pomati et al., 2008), but studies of this nature have generally been quite limited. Two recent comprehensive reviews summarized aquatic risks associated with chronic pharmaceutical exposures, and both of these revealed a scarcity of information relating to amphibians, in particular (Crane et al., 2006; Santos et al., 2010). This is surprising since amphibian metamorphosis has long been suggested as a useful tool for assessing mixture toxicity (Dawson and Wilke, 1991) and since mixtures of other common contaminants, for example, pesticides, have been well documented for their impacts on amphibian growth and development (Bernal et al, 2009; Relyea, 2009; Boone, 2008). In the present study, the pharmaceuticals tested had no discernable main effects on the growth and development of *L. peronii* tadpoles, individually or in a mixture, but interactive effects on development were observed at both exposure concentrations. These results support the general conclusion that low levels of pharmaceuticals can adversely impact amphibian tadpoles (Fraker and Smith, 2004; Smith and Burgett, 2005; Richard and Cole, 2006). However, effect sizes were quite negligible and we conclude that the specific compounds and concentrations tested have limited overall effects directly related to amphibian growth and development.

At the high concentrations used in the acute exposures, the nonsteroidal pharmaceuticals tested demonstrated the potential to act together causing heightened toxicity, and this has been seen with other similar studies using various aquatic organisms (Galus et al., 2013; Quinn et al., 2009). Therefore, although we observed only minor evidence of negative impacts on tadpole growth and development with a mixture of NPX, CBZ, and SMX at sublethal concentrations close to those found occurring in the environment, it is important to fully consider the broad range of possible responses that might occur with exposures to these compounds. Assessing mixtures toxicity is a particularly complex issue for pharmaceuticals, since these compounds possess many diverse modes of biological activities. The present study focused only on gross morphological effects of the exposed generation, but future studies need to consider more subtle endpoints. For example, metabolic disruption was observed in tadpoles exposed to low exposure concentrations of polychlorinated biphenyls (PCB) and petrogenic contaminants (Gillardin et al., 2009; Melvin et al., 2013), but these types of effects have not been investigated in amphibians exposed to pharmaceuticals. Changes to discrete animal behaviors may also be realized with exposure to low concentrations of pharmaceuticals and other contaminants (De Lange et al., 2006; Melvin and Wilson, 2013), and there are noted sensitive responses in amphibians exposed to pesticides (Denoël et al., 2012, 2013). Moreover, behavioral responses may be indicative of higher level effects, as observed following exposures of invertebrates and fish to pharmaceuticals (Guler and Ford, 2010; Nassef et al., 2010). These areas have received little attention relating to amphibians and need to be considered in future assessments of pharmaceutical toxicity.

While this is apparently the first study to investigate the effects of low concentrations of nonsteroidal pharmaceuticals on amphibian larval growth and development in a mixture, similar studies using fish as a vertebrate model were reported. In one example, a mixture of pharmaceuticals was shown to decrease embryo production in zebrafish (Galus et al., 2013). Parrott and Bennie (2009) found a comparable mixture exposure exerted little or no impact on fathead minnow growth or reproduction at environmentally relevant concentrations, but exposure of adults resulted in deformities in the offspring. It is possible that similar multigenerational exposures with amphibians may yield comparable results, but to our knowledge reproductive toxicity studies with amphibians have been limited to those using *Xenopus* (Berg et al., 2009). However, with advances in hormonally induced spawning of captive frogs (Trudeau et al., 2010, 2013), it may soon be possible to investigate reproductive endpoints in locally relevant amphibian species.

In summary, the environmental impacts of mixtures of human pharmaceuticals on aquatic wildlife remain to be determined, and doing so represents an arduous task. It is therefore important to emphasize that the three compounds tested represent an extremely small subset of the vast numbers and types of pharmaceuticals that exist (Daughton and Ternes, 1999). As such, it is difficult to postulate broad conclusions regarding the toxicology of pharmaceutical mixtures on amphibian larvae from this study alone. Our results suggest limited overall effects of SMX, CBZ, and NPX individually and in a mixture on amphibian growth and development at environmental concentrations; however, there are countless other pharmaceuticals and possible combinations that need to be considered. Finally, with the inherent biological activity of pharmaceutical compounds and the observed potential for subtle effects at low exposure concentrations, assessment of physiological and metabolic endpoints or of effects on sensitive behavioral parameters may be important to consider for future studies.

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