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Review

Amphibians and agricultural chemicals: Review of the risks in a complex environment

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The literature on the various mechanisms by which amphibians may be affected by agricultural chemicals is reviewed.

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ABSTRACT

Agricultural landscapes, although often highly altered in nature, provide habitat for many species of amphibian. However, the persistence and health of amphibian populations are likely to be compromised by the escalating use of pesticides and other agricultural chemicals. This review examines some of the issues relating to exposure of amphibian populations to these chemicals and places emphasis on mechanisms of toxicity. Several mechanisms are highlighted, including those that may disrupt thyroid activity, retinoid pathways, and sexual differentiation. Special emphasis is also placed on the various interactions that may occur between different agro-chemicals and between chemicals and other environmental factors. We also examine the indirect effects on amphibian populations that occur when their surrounding pond communities are altered by chemicals.

1. Introduction and scope of review

Since the early 1990's, countless journal articles have started out with the now familiar refrain "*Amphibians populations are declining in many parts of the world*", or words to that effect. Unfortunately, those words are as true today (Mackey and Boone, 2009) as they were in 1990 (Blaustein and Wake, 1990). This is not to say that our knowledge has languished in the intervening years; we have a far better understanding of various causal agents and the vulnerability of amphibians within a human landscape than we did 19 years ago. We also recognize that many factors must be at play, either in isolation or combination with each other (Collins and Storfer, 2003; Davidson and Knapp, 2007).

Globally, three broad categories of declining amphibian populations can be identified; (1) over-exploitation (Stuart et al., 2004) (2) those "enigmatic declines" that are associated predominantly with upland species and which are variously allied with chytridiomycosis, habitat modification, introduced species, climate change, as yet unknown causes, or combinations of the above (Hero and Morrison, 2004; Stuart et al., 2004) and, (3) those declines associated with lowland species and which are more often associated with habitat loss or modification (Hazell, 2003; Hero and Morrison, 2004).

Agriculture consumes a greater proportion of land than any other human activity (Devine and Furlong, 2007), and in the context of amphibian population decline, the "habitat loss" associated with agricultural expansion is likely the single most important human activity affecting lowland amphibian populations (Gallant et al., 2007). The fact that many species have been able to persist in agricultural landscapes is testimony to the one saving grace of agriculture, especially in Australia – the near permanent availability of water. Extraction of groundwater and the establishment of weirs, irrigation channels and dams has, in the case of some species, inadvertently provided breeding habitat where otherwise habitat has been destroyed. Amphibians have been able to exploit these water-bodies because they are able to colonize them by overland dispersal (Marsh et al., 2004; Vasconcelos and Calhoun, 2004) and in the absence of less mobile predators (i.e. fish) are able to persist as meta-populations across an altered landscape (Knutson et al., 2004; Mazerolle, 2005; Herzon and Helenius, 2008).

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However, agricultural practice changes continuously. In particular, chemicals in the form of pesticides and fertilizers are being applied in greater varieties, combinations, and to a greater extent than ever before, and represent a significant suite of pollutants. Data collated on the IUCN Red List of Endangered Species website for 2008 indicate that after habitat loss, pollution is the next major threatening process to amphibian populations (Fig. 1). The ability of amphibian populations to persist in a changing chemical environment forms the focus of this review.

Agricultural chemicals are receiving increasing attention as a potential cause of amphibian declines, acting singly or in combination with other stressors (Relyea and Mills, 2001). Surveys of natural populations have shown correlations between population declines and proximity to agricultural lands (Bishop et al., 1999; LeNoir et al., 1999; Davidson et al., 2002; Houlahan and Findlay, 2003; Davidson, 2004). Also, many malformed amphibians have been reported to occur in agricultural areas where pesticides and fertilizers are applied extensively (Ouellet et al., 1997; Taylor et al., 2005).

A large proportion of the amphibian life cycle occurs in ponds, streams, and temporary pools that are often associated with agricultural areas receiving pesticides. Therefore, some anuran species inhabiting such environments may be exposed to the toxic effects of pesticides. In addition, most temperate frog species are annual iteroparous breeders; breeding and larval development of amphibians occurs in spring and summer and coincides with the application of pesticides and fertilizers on agricultural lands. When considering these factors in addition to the large quantities of various herbicides, insecticides and fungicides presently used in agricultural production the resulting impacts on anurans have the potential to be significant. Although the links between amphibian population decline and increasing pesticide use has gained some credibility overseas (Davidson and Knapp, 2007), in Australia, relatively little attention has been given to this issue (Mann and Bidwell, 1999a; Story and Cox, 2001), with only anecdotal evidence available documenting the cessation of frog chorus following the application of pesticides (Tyler and Williams, 1996).

Despite the widespread and extensive use of pesticides, ecotoxicology studies examining the effects of these agricultural chemicals using amphibians as test organisms have been largely under represented for many decades compared to other aquatic organisms. The limited amphibian research undertaken until fairly recently has largely focused on the acute lethality of pesticides and has been the subject of a number of reviews (Power et al., 1989; Hall and Henry, 1992). However, increasing evidence is emerging indicating the adverse effects of agricultural pesticides on amphibian growth, development, reproduction and behaviour (Carey and Bryant, 1995). This review will examine the more recent studies on the sub-lethal effects of agricultural chemicals on anurans and evaluate their potential to affect field populations.

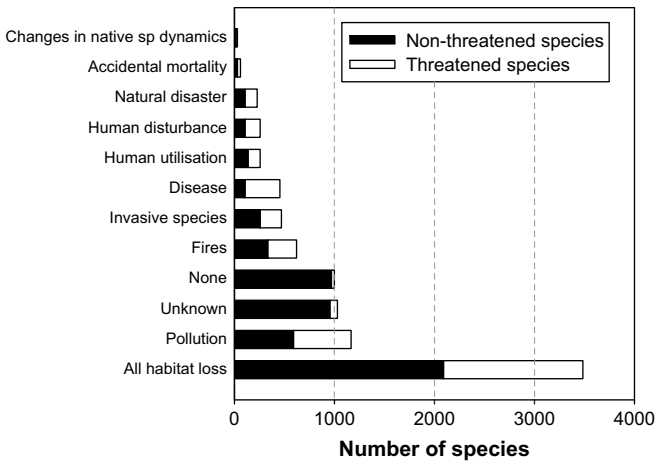

Fig. 1. Major threats to amphibians. Modified from http://www.iucnredlist.org/amphibians/major_threats.

2. Altered development following exposure to pesticides

While some pesticides have the potential to affect amphibian populations directly by causing mortality, the concentrations that occur in the environment are rarely so high as to directly cause mortality on a wide scale (e.g. Harris et al., 1998; Davidson, 2004; Fellers et al., 2004). As a consequence, studies that examine toxicity among amphibians generally observe and record numerous manifestations of sub-lethal toxicity. Some endpoints, such as teratogenesis and abnormal sexual development have been studied as specific responses to the widespread occurrence, or at least the perceived occurrence, of limb abnormalities and endocrine disruption among wild populations. Both these issues will be dealt with individually later. More routinely applied endpoints in sub-lethal toxicity tests include growth (as measured by mass and length, usually among tadpoles but also among newly emergent metamorphs) and time to metamorphosis. Because these are generalized manifestations of toxicity with numerous possible aetiologies, an exhaustive review of those studies that have examined such endpoints is unlikely to be instructive, especially since correlation with field observations is rarely possible. However, an examination of some of the potential underlying mechanisms may provide a basis for understanding how exposure to some agricultural chemicals is likely to influence larval growth and development.

2.1. Interactions with the thyroid axis

Normal metamorphosis is regulated by thyroid hormones (TH) (for review see Galton, 1992; Brown and Cai, 2007). Thyroxine (T_4) secreted by the thyroid gland is the main circulating TH. Thyroxine is converted in target tissues to the more active 3,5,3′-triiodothyronine (T_3), which acts at nuclear TH receptors in the cells of target tissues. In amphibians, circulating TH is low during the pre-metamorphic stages (up to and including hind-limb-bud stage), increases during prometamorphosis (encompassing limb development) and reaching a maximum at metamorphic climax (encompassing forelimb emergence and tail resorption).

The timing of metamorphosis can be manipulated by exposing tadpoles to exogenous chemicals with thyroid activity. Exposure to exogenous TH during pre-metamorphosis can result in precocious metamorphosis (Helbing et al., 1992). Conversely, inhibition of thyroid hormones can prevent metamorphosis completely (Gutleb et al., 2007). Various environmental contaminants are known to inhibit normal thyroid activity, including perchlorate (Hu et al., 2006; Theodorakis et al., 2006) nonylphenol (Christensen et al., 2005; Yang et al., 2005), the organochlorine pesticide, methoxychlor (Fort et al., 2004) and the DDT metabolite, DDE (Arukwe and Jenssen, 2005; Yang et al., 2005; Mortensen et al., 2006). On the other hand, some environmental contaminants appear to enhance thyroid activity. Acetochlor is a pre-emergent chloroacetanilide herbicide that is known to accelerate T_3-induced metamorphosis among *Rana pipiens* and *Xenopus laevis* when exposed to 10 nM (2.7 μg/L) acetochlor in combination with 1 nM T_3 (Cheek et al., 1999; Veldhoen and Helbing, 2001; Crump et al., 2002; Helbing et al., 2006). Although the exact mechanism by which the thyroid axis is affected remains unclear, it is likely associated with increased expression of TH receptors (Veldhoen and Helbing, 2001; Helbing et al., 2006). However, despite the clear interaction with

the thyroid axis, a recent mesocosm exposure to 10 μg/L acetochlor had no effect on metamorphic timing among *R. pipiens* and *Hyla versicolor* (Relyea, 2009).

Thyroid-mediated metamorphosis can also be induced by stress. Under natural conditions, stress associated with pond drying and/or crowding will accelerate metamorphosis as an adaptive stress response (Denver, 1997b). This response is mediated through a cascade of signalling events beginning with the hypothalamic secretion of corticotropin-releasing hormone (CRH) which in turn stimulates the pituitary to secrete both thyroid-stimulating hormone (THS) and adrenocorticotropic hormone (ACTH) which stimulate the thyroid gland to secrete TH and the interrenal gland to secrete corticosterone, respectively (Fig. 2, Denver, 1997a,b). Exogenously applied corticosteroids also accelerate metamorphosis in some species (Hayes, 1995, 1997; Hayes and Wu, 1995), although this may be stage-specific (Hayes et al., 1993). Therefore, although somewhat speculative, it seems likely that environmental contaminants can similarly affect metamorphosis by inducing a stress response. For example, some studies have demonstrated precocious metamorphosis among tadpoles exposed to pesticides that otherwise have no known thyroid activity (e.g. Boone et al., 2001; Boone and Semlitsch, 2002; Boone and Bridges, 2003; Greulich and Pflugmacher, 2003; Rohr et al., 2004; Cauble and Wagner, 2005; Forson and Storfer, 2006). It is surprising therefore that there are no studies (that we are aware of) that examine corticosteroid responses in tadpoles following exposure to a pesticide. Increases in corticosterone have been measured in adult *X. laevis* exposed to a cocktail of nine pesticides (each at 0.1 ppb) (Hayes et al., 2006a) and among tadpoles exposed to other environmental and chemical stresses (Belden et al., 2003; Peterson et al., 2009).

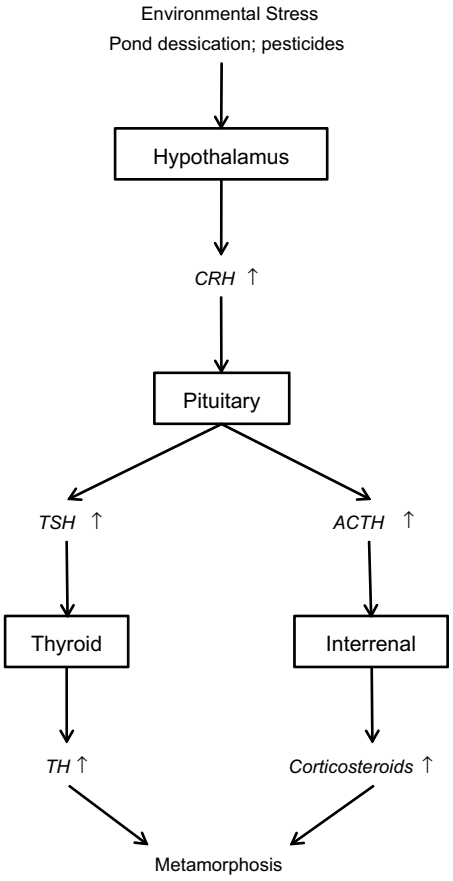

Fig. 2. Schematic diagram of cascade of events which may occur in tadpoles in response to environmental stress. Modified from Denver (1997a). Negative and positive feedback pathways are not shown. CRH – corticotropin-releasing hormone; TSH – thyroid-stimulating hormone; ACTH – adrenocorticotropic hormone; TH – thyroid hormone.

One of the consequences of accelerated metamorphosis is that the newly emerged juveniles are likely to be undersized (e.g. Cheek et al., 1999; Cauble and Wagner, 2005). General opinion would indicate that smaller animals are less ecologically fit (Semlitsch et al., 1988; Berven, 1990; Altwegg and Reyer, 2003; Chelgren et al., 2006), although Boone (2005) and Distel and Boone (in press) have presented data indicating that amphibians exposed to carbaryl were able to offset small size at metamorphosis with terrestrial growth.

It is also worth noting at this point, that an increase in corticosteroids has implications for normal immune function. Normal, spontaneous metamorphosis is associated with the destruction of about 40% of lymphocytes. However, during TH-induced precocious metamorphosis, 80% or more of lymphocytes are destroyed (for review see Rollins-Smith, 1998). The implication is that disruption of the thyroid and stress hormone axes prior to metamorphosis may also have implications for immune function in amphibians (see Section 4).

2.2. Other mechanisms for impaired growth and development

Far more frequently, researchers report delayed metamorphosis and/or developmental retardation in response to exposure to pesticides (e.g. Teplitsky et al., 2005), and as indicated above, the possible aetiologies are numerous. For example, organophosphorus and carbamate insecticides include many of the most commonly applied chemicals. They act through the inhibition of acetylcholine esterase (AChE) (Venturino and Pechen de D'Angelo, 2005), and have the effect of disrupting neurological systems. Exposure to these classes of chemicals is generally measured as a reduction in AChE activity both in laboratory studies (Shapira et al., 1998; Ozmen et al., 1999; Richards and Kendall, 2002; El-Merhibi et al., 2004; Colombo et al., 2005; Wacksman et al., 2006; Widder and Bidwell, 2006, 2008; Henson-Ramsey et al., 2008; for earlier studies see Mann and Bidwell, 1999a; Venturino and Pechen de D'Angelo, 2005) and among field collected frogs (Sparling et al., 2001; Lajmanovich et al., 2004; Attademo et al., 2007). Toxicity is manifested as impaired behavioural responses (Bridges, 1997, 1999a,b; Fordham et al., 2001; Punzo, 2005; Widder and Bidwell, 2008), impaired growth (Metts et al., 2005; Widder and Bidwell, 2008) and delayed metamorphosis (Metts et al., 2005; Boone, 2008); the latter possibly being as a consequence of inhibition of feeding behaviours (Bridges, 1999b) which likely impedes growth and development. Alternatively, growth may be inhibited because energy must be diverted to detoxification mechanisms (Rowe et al., 1998; DuRant et al., 2007). Numerous energy demanding detoxification pathways are initiated following exposure to pesticides (Greulich and Pflugmacher, 2004; Venturino and Pechen de D'Angelo, 2005).

Anti-AChE pesticides are among the more thoroughly investigated pesticides with regard to amphibians, and much of the literature describing developmental delays is likely to involve organophosphorus or carbamate pesticides. However, other studies have also reported impaired behavioural and growth responses following exposure to other pesticide classes; e.g. in response to pyrethroid pesticides (Materna et al., 1995; Greulich and Pflugmacher, 2003) and the fungicide, fenpropimorph (Teplitsky et al., 2005).

Another mechanism whereby pesticides are likely to affect growth and development is via non-specific narcosis. When taken up by an aquatic organism, lipophilic organic chemicals (which

encompasses the majority of pesticides), will induce non-specific narcosis as a consequence of disruption of cell membranes, and has the effect of reducing metabolism and behaviours necessary for successful growth and development (i.e. feeding, foraging, escape) (van Wezel and Opperhuizen, 1995). Subtle changes in behaviour that might be indicative of narcosis (in the absence of other toxic effects) are rarely described in the amphibian toxicology literature. One example is the narcotic effect seen among tadpoles exposed to non-ionic agricultural surfactants (Mann and Bidwell, 2001; Mann et al., 2003).

Any reduction in the growth rate following pesticide exposure extends the larval period thereby prolonging the period where they are within the size ranges for small aquatic predators such as salamanders and dragonfly nymphs (Caldwell et al., 1980) and therefore increases the risk of predation (Relyea and Mills, 2001; Broomhall, 2004). Furthermore, among amphibians that breed in ephemeral pools, there is increased risk that tadpoles will fail to reach metamorphosis before the pools dry (Roe et al., 2006; Relyea and Diecks, 2008). Therefore, even if pesticides do not have immediate impacts on anuran survival, sub-lethal effects could indirectly influence fitness and survival, and conceivably reduce population sizes over time when exposure occurs annually.

2.3. Deformities

Certainly one of the more dramatic manifestations of developmental anomalies in amphibians is the occurrence of gross external malformations, especially of the hind-limbs and digits. Although malformations of the digits, and to a lesser extent limbs, appears to be a normal occurrence among wild populations of frogs – estimates range between <1% (Gardiner and Hoppe, 1999) up to 5% of frogs (Read, 1997; Read and Tyler, 1994; Stocum, 2000; Johnson et al., 2001b; Schoff et al., 2003; Eaton et al., 2004; Piha et al., 2006) – increased prevalence of hind-limb and digit malformations has been reported among amphibians collected from agricultural regions (Linzey et al., 2003; McCallum and Trauth, 2003; Taylor et al., 2005; Gurushankara et al., 2007). The most prominent episode occurred in 1995 when a group of school children discovered a large number of deformed frogs in a Minnesota farm pond (Schmidt, 1997). The deformities took the form of missing or extra limbs and deformed eyes. The discovery preceded similar discoveries in other parts of Minnesota and in other states of the USA (Ouellet et al., 1997; McCallum, 1999; Meteyer et al., 2000; Vandenlangenberg et al., 2003). Although subsequent laboratory studies implicated a developmental contaminant (Burkhart et al., 1998; Fort et al., 1999a,b, 2001; Bridges et al., 2004) and identified numerous agricultural pollutants within water from sites where frog deformities occurred (Fort et al., 1999b), no single causative agent could be identified. To further complicate the issue, there was also some suggestion that mineral deficiencies in the water at sites where malformations occurred may be a contributing factor (Fort et al., 1999b; Tietge et al., 2000; Garber, 2002; Garber et al., 2004).

Numerous pesticides have been demonstrated to be teratogenic to developing amphibians (e.g. Fort et al., 1999b; Harris et al., 2000; Vismara et al., 2000, 2001; Kennedy and Sampath, 2001; Osano et al., 2002a,b; Bonfanti et al., 2004; Bridges et al., 2004; Bacchetta et al., 2008; Kang et al., 2008; Lenkowski et al., 2008; Sayim, 2008; Yoon et al., 2008; for studies prior to 1999 see Mann and Bidwell, 1999a). However, the majority of these studies report teratogenic effects at pesticide concentrations that exceed those reported in field samples. In addition, nearly all of these studies limit the assessment to the larval stages and limb malformations were not frequently reported. Exceptions include the study by Bridges et al. (2004), who reported limb deformities among *R. pipiens* exposed to a mixture of atrazine (5 μg/L) and carbaryl (5 μg/L), Fort et al. (1999b) who reported hind-limb deformities among *Xenopus laevis* exposed to Maneb ($EC50_{terata}$, 200 μg/L) and Rohr et al. (2003) who also reported significantly more limb asymmetries (deformed limbs or digits) among salamanders (*Ambystoma barbouri*) exposed to carbaryl during larval development.

The kinds of abnormalities observed among field collected frogs suggested that chemicals may be interfering with retinoid signalling pathways (Gardiner and Hoppe, 1999; Sessions et al., 1999; Degitz et al., 2000; Gardiner et al., 2003), and suspicion fell upon retinoid-like contaminants in the environment that might directly affect amphibian development by mimicking the action of retinoic acid (RA). Retinoic acid, an oxidized form of vitamin A (Fig. 3), has an endogenous role in embryological development, including limb development. However, imbalances in RA (both excesses and deficiencies) are also known to be teratogenic (for review see Loeffler et al., 2001; Lee et al., 2004). Following exposure to RA (250–1250 ng RA/ml), hind-limb malformations analogous to some of those observed in field collected animals could be induced among *X. laevis* and *Rana* sp., but only if tadpoles were exposed at the limb-bud stage (Degitz et al., 2000). Exposure to RA at the earlier mid-blastula stage, and at concentrations much lower than those required to induce hind-limb malformations, resulted in severe cranial–facial abnormalities that would ultimately result in mortality before metamorphosis (Degitz et al., 2000, 2003b). The implication from these studies (i.e. Degitz et al., 2000, 2003b) is that if retinoid analogues were present in a water body where amphibians breed, they would likely cause mortality among larvae prior to metamorphosis, and post-metamorphic juveniles or adults manifesting limb malformations, would not be found.

An early candidate as a source of retinoid activity was the insecticide Altosid®, which has been used since the mid 1970's for the control of mosquitoes (Henrick et al., 2002). The active constituent in Altosid® is *S*-methoprene (Fig. 3), a growth regulator that prevents pupation in mosquitoes (Henrick et al., 2002). *S*-Methoprene and some of its degradation products (e.g. methoprenic acid, Fig. 3) are known to have retinoid activity (Schoff and Ankley, 2004). However, in frog embryo teratogenesis assays (FETAX), exposure to degradation products of *S*-methoprene caused cranio-facial malformations among *Xenopus*, in much the same manner as RA (La Clair et al., 1998; Degitz et al., 2003a) and only at

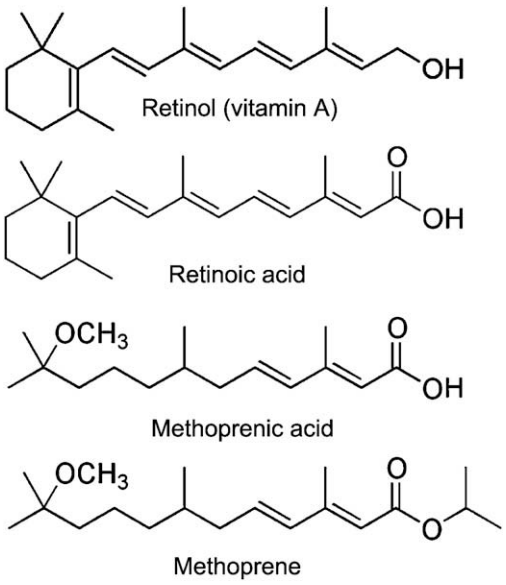

Fig. 3. Chemical structures of retinol, retinoic acid and the structurally similar compounds, methoprene and one of its degradation products, methoprenic acid.

very high concentrations that were unlikely to be found in waterways.

Supernumerary limbs, a dramatic but generally less common manifestation in field collected frogs (Meteyer et al., 2000; Loeffler et al., 2001), have not been observed among recent laboratory studies with RA or methoprene derivatives (La Clair et al., 1998; Degitz et al., 2000, 2003a,b; Kratke et al., 2000; Loeffler et al., 2001; Mahapatra et al., 2001; Alsop et al., 2004; but see Das and Mohanty-Hejmadi, 2000), nor among laboratory studies that examined the effects of natural waters collected from sites where malformed frogs were found (e.g. Fort et al., 1999a,b, 2001; Bridges et al., 2004). This is perhaps unexpected because limb duplications were demonstrated in earlier works that examined the role of RA in limb regeneration (for reviews see Niaze, 1996; Loeffler et al., 2001). Loeffler et al. (2001), attempting to disentangle the inconsistencies, suggested that damage to limb-buds through abrasions or predation (thereby triggering regeneration), may be prerequisite to limb malformation in the presence of retinoids. Furthermore, these authors (Loeffler et al., 2001) demonstrated that water from Ney Pond (the pond where malformed frogs were originally discovered by school children in Minnesota in 1995) could induce hind-limb malformations among *X. laevis*, including supernumerary limbs, but only if the limb-buds were surgically injured before exposure.

Despite the appeal of retinoids as the primary aetiology for frog malformations (Gardiner et al., 2003), several factors argue against it, including the widespread incidence of malformations across many different regions in the USA, and the failure to identify candidate chemicals with persistent retinoid activity in waterbodies associated with malformations (Loeffler et al., 2001; Henrick et al., 2002; Ankley et al., 2004). Therefore, the direct action of retinoids seems unlikely. However, interference with retinoid homeostasis as a consequence of pesticide exposure remains as a possible pathway for the induction of limb malformations. For example, subcutaneous administration of the organochlorine metabolite, DDE, is known to increase the concentration of retinol (vitamin A) and retinyl palmitate in the liver of *Rana temporaria* (Leiva-Presa and Jenssen, 2006; Leiva-Presa et al., 2006). More persuasive still, are reports showing reductions in plasma concentrations of retinol among bullfrogs (*Rana catesbeiana*) collected from rivers contaminated with multiple pesticides (Bérubé et al., 2005; Boily et al., 2005).

Despite compelling evidence that water contaminants are in some way responsible for a sizeable proportion of malformations in frogs (Gardiner et al., 2003; Taylor et al., 2005; Bacon et al., 2006; Fort et al., 2006), competing aetiologies have been postulated, including infection by the trematode *Ribeiroia ondatrae*, ultraviolet radiation (UV), and various combinations of all the above (for review see Ankley et al., 2004). The ability of trematode infection to induce limb malformations, including supernumerary limbs in frogs, and the relatively high infestation rate of trematode metacercariae in and around the developing limb-buds of frogs collected from some sites with high incidences of malformations, has raised the possibility that at least a proportion of limb malformations result as an indirect effect of agricultural practice on either the prevalence of the intermediate host snails, and/or the immune function of amphibians. Both these issues are discussed later in this review (Sections 4 and 8).

3. Effects on sexual differentiation

Just as various chemicals are known to affect the thyroid and stress hormone axes (Section 2.1), many environmental contaminants can also affect sexual differentiation and/or the levels of the circulating sex hormones, oestradiol and testosterone (Palmer et al., 1998; Noriega and Hayes, 2000; Sower et al., 2000; Bevan et al., 2003; Bögi et al., 2003; Cevasco et al., 2008). Altered sex ratios and gonadal abnormalities are examples of physical manifestations of hormone disruptions during the events leading up to metamorphosis. For example, Harris et al. (2000) found altered sex ratios in recently metamorphosed leopard frogs (*R. pipiens*) exposed to relatively high concentrations of either mancozeb (0.08 mg/L) or endosulfan (2.35 mg/L). One hundred percent females were found in both instances, although sample sizes were low.

At the centre of the recent attention concerning the potential of pesticides as endocrine disrupters in amphibians is atrazine, a triazine herbicide used extensively in agricultural production around the world. There is evidence suggesting that atrazine may adversely affect the reproductive capacity of anurans (Hayes et al., 2002, 2003; Tavera-Mendoza et al., 2002a,b; Oka et al., 2008) and as a consequence, potentially contribute to population declines.

3.1. Evidence for atrazine disrupting sexual differentiation of tadpoles

In amphibians the potential adverse effects of atrazine has focused on the proposal by Hayes et al. (2002, 2006b) and Tavera-Mendoza et al. (2002b) that atrazine-induced increases in aromatase (cytochrome P450-1 hydroxylase) activity (see also Holloway et al., 2008) was feminizing male frogs. Hayes et al. (2002, 2006b) reported gonadal abnormalities following exposure of *X. laevis* tadpoles throughout their larval development to atrazine at 25 μg/L, but also at the much lower concentration of 0.1 μg/L. Male tadpoles exposed to $\geq$ 1 μg/L also displayed lower concentrations of testosterone and an associated reduction in the sizes of larynges. In addition, Tavera-Mendoza et al. (2002b) reported that the exposure of *X. laevis* tadpoles to atrazine (21 μg/L) for periods as short as 48 h immediately prior to the gonadal differentiation stage (Nieuwkoop and Faber stage 56) resulted in a 57% reduction in testicular volume and a 70% reduction of the primordial germ cells.

Similar effects were obtained by Hayes et al. (2003) among tadpoles of the American leopard frog (*R. pipiens*), suggesting that the potential endocrine disrupting effects of atrazine may pose a risk for amphibians in general. The authors described varying degrees of testicular oogenesis among 29% of frogs exposed to 0.1 μg/L atrazine, with some males having gonads almost completely filled with oocytes. In the same study, tadpoles exposed to the much higher concentration of atrazine (25 μg/L) also displayed testicular oogenesis at metamorphosis, but at a lower incidence of 8%. The authors emphasized the apparently greater toxicity of low concentrations compared to higher concentrations (Hayes, 2005) and advocated an 'inverted U' response.

3.2. Evidence refuting the notion that atrazine is an endocrine disrupting chemical

Subsequent studies (for review see Solomon et al., 2008) have not corroborated the results of Hayes et al. (2002, 2003). Although Carr et al. (2003) also reported gonadal abnormalities in *X. laevis* at an atrazine concentration of 25 μg/L, they were unable demonstrate any such abnormalities at 1 and 10 μg/L, and the gonadal abnormalities were based on the physical appearance of the gonads that was not confirmed by histology. Moreover, the percentage of gonadal abnormalities (defined as intersex gonads) in *X. laevis* was less than 5% compared to the 16–20% reported by Hayes et al. (2002), and 20% reported for gonads of *R. pipiens* at the same concentration (Hayes et al., 2003).

More recent laboratory studies in *X. laevis*, *R. pipiens*, and *H. versicolor* exposed to atrazine concentrations between 0.1 and 200 μg/L have failed to demonstrate links between atrazine exposure and the development of abnormal gonads (at either the level of

gross morphology or histologically) at either the low or high concentrations (Coady et al., 2005; Orton et al., 2006; LaFiandra et al., 2008; Oka et al., 2008; Kloas et al., 2009). This is despite the fact that some of the studies included a treatment in which animals were exposed to positive control compounds (oestradiol) that did result in metamorphs displaying testicular oocytes (Coady et al., 2005; Oka et al., 2008; Kloas et al., 2009). Similarly, atrazine exposure in *X. laevis* has not been demonstrated to prejudice laryngeal development in males (Coady et al., 2005), affect germ cell development (Hecker et al., 2005a), induce aromatase activity (Coady et al., 2005; Hecker et al., 2005a,b; Oka et al., 2008), induce the production of vitellogenin (Oka et al., 2008), nor alter concentrations of steroidal hormones in male plasma (Hecker et al., 2005a), although Hecker et al. (2005b) did report a significant reduction in circulating testosterone in male *Xenopus* exposed to 250 μg atrazine/L. It is interesting to note however, that Oka et al. (2008) despite finding no physiological basis for emasculation, did demonstrate significantly higher numbers of phenotypically female metamorphs among *X. laevis* exposed to 10 and 100 μg/L of atrazine. Taken as a whole, these latter studies do not support the view that atrazine at environmentally relevant concentrations adversely affects amphibian gonadal development through oestrogenic action or via the inappropriate synthesis of oestrogen via aromatase induction.

3.3. Others factors affecting sexual development in amphibians

Several confounding factors must be taken into consideration when interpreting intersex data in amphibians. Firstly, carrier solvents, which are frequently used to solubilize compounds with potentially oestrogenic (anti-androgenic) properties, can themselves have oestrogenic properties (Hutchinson et al., 2006). When ethanol was used as a carrier solvent (50 μl/L) for atrazine and hormones in laboratory studies, it was shown to induce gonadal abnormalities in up to 6.5% of *X. laevis* tadpoles and testicular oocytes or intersex gonads in up to 20% of post-metamorphosis frogs (Coady et al., 2005). Ethanol (36–40 μl/L) was also used as a carrier in the studies by Hayes et al. (2002, 2003) although intersex gonads were not reported in ethanol controls. Tavera-Mendoza et al. (2000) also observed up to 80% incidence of sex-reversal among male testes of *X. laevis* tadpoles exposed to methanol (3.3 μl/L). Further testing is required to substantiate these solvent effects.

Temperature is known to affect sex ratios in amphibians. Specifically, several amphibian species are masculinized by high temperatures or feminized by low temperatures during larval life and manifested as distorted sex ratios obtained at or soon after metamorphosis (for review see Hayes, 1998; Wallace et al., 1999). Jooste et al. (2005) described a high incidence of testicular oocytes among Nieuwkoop and Faber stage 66 *X. laevis* metamorphs following exposure to atrazine (1–25 μg/L) and control animals during larval development. However, this was not consistent with previous laboratory studies that notably failed to find the same (see above), and may have been as a consequence of the low temperatures experienced during the early stages of the study (Hayes, 2005; Hayes et al., 2006b).

Developmental stage-specific differences are to be expected. In *X. laevis* the frequency of intersex individuals following larval exposure to oestradiol benzoate varies depending on the initial development stage of the tadpoles (Villalpando and Merchant-Larios, 1990). When exposure begins at Nieuwkoop and Faber stages 44–50, 50% of the tadpoles that develop have ovaries and 50% have ova-testes. Oestradiol exposure starting later, at stages 55–56, does not affect the gonadal sex ratio (Villalpando and Merchant-Larios, 1990).

Species differences must also be considered when considering the environmental risks posed by potentially oestrogenic (anti-androgenic) chemicals. For example, studies have shown a species-specific difference in sensitivity to 17α-ethinyloestradiol with no apparent effect of exposure on the green frog (*Rana clamitans*), whereas between 5 and 12.5% of male mink frogs (*Rana septentrionalis*) developed intersex gonads after exposure (Park and Kidd, 2005). The reason for pronounced species differences may lie in disparate patterns of gonadal development, which do not necessarily correspond with similar stages of somatic development. Some species that display retarded gonadal development (e.g. *Bufo* sp.) only reach gonadal maturity several weeks after metamorphosis, whereas other species (e.g. *Rana lessonae*, *Rana ridibunda*, *R. catesbeiana*, *R. pipiens*) display accelerated development; reaching gonadal maturity at metamorphosis or shortly after (Ogielska and Kotusz, 2004). Therefore, species with accelerated gonadal development are likely to be exposed to waterborne contaminants for a greater proportion of their sexual development and may be more susceptible to endocrine disruption (Storrs and Semlitsch, 2008). The majority of laboratory studies examining low concentration exposure to atrazine have used *X. laevis*; a species that exhibits basic (neither retarded nor accelerated) gonadal development. Therefore, *X. laevis* may not be representative of species that exhibit accelerated development. Much of the data pertaining to other species comes from observations of intersex among field collected animals. *R. pipiens* in particular is a species exhibiting accelerated development, and it was in this species that high incidences of gonadal abnormalities were described among animals collected from atrazine-contaminated field-sites (Hayes et al., 2003; McDaniel et al., 2008).

3.4. Hermaphroditic individuals in field populations

Concurrent with their laboratory studies with *R. pipiens*, Hayes et al. (2003) reported hermaphroditic frogs in localities associated with atrazine use and/or atrazine contamination. Male frogs examined at these sites were shown to exhibit gonadal abnormalities that were similar to those induced by atrazine in the laboratory. Varying incidences of testicular oocytes were observed among male leopard frogs at sites where atrazine was detected, with up to 92% of males examined at one site displaying this abnormality. Gonadal dysgenesis (characterized by poorly developed testicular lobules that lacked germ cells) was also observed in 28% of male frogs at one site where atrazine is widely used. The site with the highest incidence of testicular oocytes (92%) had relatively low concentrations of atrazine, and although Hayes (2005) has suggested that low atrazine concentrations present a greater risk because of an 'inverted U' response, a US-EPA data evaluation report (Steeger et al., 2003) on the Hayes et al. (2003) study observed that a clear dose relationship was not evident.

The field studies with *R. pipiens* (Hayes et al., 2003) are supported by a recent study by McDaniel et al. (2008) who found a high incidence (42%) of testicular oocytes among *R. pipiens* collected from regions of intensive row agriculture in southern Ontario where atrazine had been used extensively. Although the authors suggested a link with exposure to atrazine, they were unable to isolate atrazine as the causal agent among the numerous pesticides to which leopard frog populations had been exposed. An earlier field study by Reeder et al. (1998) also reported a weak correlation between the incidence of intersex gonads in cricket frogs (*Acris crepitans*) and atrazine contamination. However, the overall incidence of intersex gonads (ova-testes, mixed gonads and/or multiple testes) in that study was <3%. Furthermore, examination of cricket frogs collected prior to and after the introduction of atrazine in 1958, indicated a reduction in the prevalence of intersex

frogs over the last 60 years (Reeder et al., 2005), and the authors suggested that there was a better correlation between intersex phenotypes and the use, and subsequent reduction in use, of organochlorine compounds in the mid 1900s. Murphy et al. (2006) also found a variable incidence (0–14.3%) of testicular oocytes in male green frogs (*R. clamitans*), bullfrogs (*Rana catesbeiana*) and leopard frogs (*R. pipiens*) in both agricultural and non-agricultural areas – in contrast to the field observations reported by Hayes et al. (2003) – and suggested that their presence may be a natural part of the development of some frogs. Hermaphroditic individuals have been observed historically in healthy frog populations (Witschi, 1921, 1929). Finally, it is interesting to note that among *X. laevis* (South Africa), and *Litoria raniformis* and *Limnodynastes* sp (Australia) collected from natural populations living in either a corn growing region where atrazine was used, or a non-corn growing region, only a very low incidence of gonadal abnormality was observed (0–3%), with no indication that atrazine was a causal factor (Smith et al., 2005a; Hyne et al., 2009), contrasting not only with the field observations of Hayes et al. (2003), but also with relatively high background incidence of gonadal abnormalities in native American frogs (Murphy et al., 2006).

3.5. Atrazine effects on gonadal development – are they significant?

Recently the US-EPA evaluated the available literature on the potential effects of atrazine on amphibian gonadal development. The results of studies submitted by Syngenta, the principal registrant for atrazine, and two additional studies conducted in two independent laboratories (Kloas et al., 2009) were reviewed. These two additional studies addressed the design, methodology and quality limitations the US-EPA identified in the early studies (see US-EPA data evaluation reports http://www.epa.gov/scipoly/SAP/meetings/june/dataevaluationreports.htm). Based on an examination of all the studies and their results, the US-EPA concluded that atrazine does not adversely affect amphibian gonadal development when exposure falls within the range of 0.01–100 μg/L (US-EPA, 2007). However, the controversy over the endocrine-disruptive effects of atrazine is likely to continue, partly because of the perceived over-emphasis by the US-EPA on studies funded by Syngenta (Sass and Colangelo, 2006), and also because of the under representation of species other than *X. laevis*. It is arguable that the preoccupation with atrazine alone needs to be reduced and greater emphasis placed on pesticide mixtures, because there is mounting evidence that some amphibian species living in regions of intensive agriculture are being affected by contaminant-induced reproductive abnormalities (McCoy et al., 2008; McDaniel et al., 2008).

4. Increased susceptibility to disease from pesticide exposure

Exposure to pesticides can lead to suppression of the immune system, thereby preventing amphibians from developing a normal and adequate response against pathogens (Carey et al., 1999; Christin et al., 2004; Rollins-Smith et al., 2004; Fournier et al., 2005). Numerous biomarkers for immune-suppression have been developed, including simple counts of white blood cells and phagocytic cells (Kiesecker, 2002; Fink and Salibian, 2005; Houck and Sessions, 2006; Brodkin et al., 2007; Rohr et al., 2008b); assays of phagocytic and lytic capacity of polymorphonuclear cells (Christin et al., 2004; Fink and Salibian, 2005; Houck and Sessions, 2006; Brodkin et al., 2007); assays of antibody titres as a humoral response to an antigenic challenge including delayed-type hypersensitivity, a T cell-mediated inflammatory response, and oxidative burst as a measure of innate immunity (Gilbertson et al., 2003). Gilbertson et al. (2003) demonstrated significant suppression of the humoral response following intramuscular administration of DDT and dieldrin. Similarly, Albert et al. (2007) demonstrated the same kind of suppression of humoral response to an antigenic challenge following dietary exposure to low doses of dieldrin and DDT. Gilbertson et al. (2003) also demonstrated suppressed capacity to elicit this same humoral response among wild *R. pipiens* collected from a field location contaminated with DDT and dieldrin. In another series of studies, Christin et al. (2004) demonstrated that exposure to water contaminated with a mixture of pesticides (atrazine, metribuzine, endosulfan, lindane, aldicarb, and dieldrin) at environmentally relevant combinations and concentrations can alter aspects of the immune system (lymphocyte proliferation, spleen cellularity, and phagocytic activity of splenocytes) in *X. laevis* and *R. pipiens*. Importantly, increased susceptibility to infection following exposure to pesticides has also been demonstrated by several authors (Table 1), and in combination with concomitant biomarker responses, presents good evidence for pesticide-induced immuno-suppression.

Therefore, pesticide exposure might be expected to be an important cofactor facilitating the outbreaks of infectious diseases such as chytrid fungus (*Batrachochytrium dendrobatidis*) that have been associated with the declines of frog populations in six continents (http://www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm) (Alford and Richards, 1999; Hero and Morrison, 2004; Stuart et al., 2004). However, Davidson et al. (2007) found no interaction between the pesticide carbaryl and chytrid infection in yellow-legged frogs (*Rana boylii*). Species under threat from chytridiomycosis typically occur in relatively pristine upland habitats where cold temperatures play a role in the virulence of the infection (Berger et al., 1998; Hero and Morrison, 2004; Stuart et al., 2004). Chytrid fungus appears to be able to persist within lowland species inhabiting a relatively warm agricultural region without affecting populations (Hyne et al., 2009). It remains unclear if pesticide exposure and associated immuno-suppression may affect the virulence of chytrid fungus in colder environments.

A direct relationship between pesticide exposure and susceptibility to infection by trematodes and subsequent occurrence of limb deformities has recently been demonstrated (Table 2; Kiesecker, 2002; see also Fig. 5 in Section 8). Although infected amphibians may not necessarily die as a direct result of trematode infection, population viability may be affected through secondary effects associated with malformations, such as impaired mobility, decreased food intake, and an increased susceptibility to predators (Blaustein and Johnson, 2003). These studies suggest that agricultural chemicals through suppression of the immune system can also indirectly contribute to population declines by facilitating mortality or altering adult fitness resulting from infection. It is interesting to note however, that King et al. (2007) were unable to demonstrate an increased prevalence of parasites among leopard frogs collected from agricultural and urban environments. Also, there is some evidence that the virulence of trematode parasites themselves might be reduced by exposure to pesticides (Koprivnikar et al., 2006b).

Clearly more studies are required that correlate specific physiological biomarkers with increased rates of infectivity. Also, it needs to be established if there is indeed a link between increased prevalence of disease or parasitism among amphibians in habitats receiving inputs of pesticides/fertilizers.

5. Interacting chemical mixtures – pesticide formulations and cocktails

5.1. Pesticide formulations

In addition to the active ingredients, pesticides used in agricultural production often contain several additives, solvents,

Table 1
Evidence for pesticide-induced immune-suppression among amphibians.

Amphibian host	Pesticide treatment	Infective agent	Infectivity	Biomarker	Reference
Bufo woodhousii as adult male frogs	1.1 μg malathion/g toad or 11 μg malathion/g toad. Single topical application	*Aeromonas hydrophila*, (bacterium that causes 'red leg' disease) by intraperitoneal injection 30-day incubation	Statistically significant incidence of clinical symptoms of disease		(Taylor et al., 1999)
Rana pipiens as juvenile frogs	1.0× and 10× concentrated solutions of mixture of Atrazine (21 μg/L), Metribuzin (0.56 μg/L), Aldicarb (17 μg/L), Endosulfan (0.02 ng/L), Lindane (0.33 ng/L), Dieldrin (0.15 ng/L) 21-day exposure	*Rhabdias ranae* (nematode) 24-h exposure	100% prevalence of lung infection. Control treatments expressed 70/80% prevalence	Significant decrease in proliferation of T-lymphocytes in response to an infective agent	(Christin et al., 2003; Gendron et al., 2003)
Rana sylvatica as tadpoles	Atrazine (3 or 30 μg/L) or malathion (2000 μg/L) or esfenvalerate (180 or 1800 μg/L) 4-week exposure	Trematode cercariae of *Ribeiroia* sp. *Telorchis* sp. 4-h exposure	Increased proportion of cercariae successfully encysted	Decrease in number of white blood cells (eosinophils)	(Kiesecker, 2002)
Rana clamitans as tadpoles	Atrazine (201 μg/L) or glyphosate (3700 μg/L) or carbaryl (33.5 μg/L) or malathion (9.6 μg/L) 7-day pre-infection exposure + 7-day post-infection exposure	Trematode cercariae of *Echinostoma trivolis* 24-h exposure	Increased proportion of cercariae successfully encysted		(Rohr et al., 2008a)
Ambystoma macrodactylum as tadpoles	Atrazine (1.84–184 μg/L) 30-day exposure	*Ambystoma tigrinum* iridovirus 6-day exposure	Atrazine reduced infectivitity		(Forson and Storfer, 2006)
R. clamitans and *Rana palustris* as tadpoles	Atrazine (117 μg/L) 4-week mesocosm exposure	Co-occurrence with phlagiorchid trematode infected snails	Statistically significant increase in number of phlagiorchid trematode cysts	Decrease in number of eosinophils	(Rohr et al., 2008b)

carriers, excipients, stabilizers and wetters in the formulation (Mann et al., 2003). These additives are rarely specified, or simply listed as 'inerts' on product labels. In a comprehensive comparison of active ingredient toxicity versus formulation toxicity, Mayer and Ellersieck (1986) noted that formulation additives could increase toxicity by as much 2.5 orders of magnitude. The comparisons reported by Mayer and Ellersieck (1986) were criticized as being unreliable by Schmuck et al. (1994) because they were made between results from tests performed in separate laboratories under different test conditions. However, even in the comparisons reported by Schmuck et al. (1994), some pesticide formulations, particularly emulsifiable concentrates, displayed toxicities that were much higher than the active ingredients.

In regard to amphibians, few studies have compared formulation toxicity with that of active ingredients. Nebeker et al. (1998) showed that the commercially available formulation Guthion®, a widely used organophosphorus insecticide (azinphosmethyl), was significantly more toxic in terms of acute toxicity (96 h LC50, 1.47 mg/L) and growth inhibition (10-day LOEC, 0.17 mg/L) to tadpoles of the Pacific tree frog (*Pseudacris regilla*) than technical grade Guthion (96 h LC50, >3.60 mg/L; 10-day LOEC, 3.60 mg/L). Similarly, Linder et al. (1990) showed greater acute and chronic toxicity among leopard frogs (*R. pipiens*) exposed to a commercial formulation of paraquat (a widely used herbicide) than among those exposed to technical grade paraquat, and Boone (2008) described high rates of mortality among *Bufo americanus* tadpoles exposed to a commercial permethrin formulation which was not seen following exposure to a pure form of permethrin.

Surfactants are one of the most common 'inerts' used for pesticide formulation. They are a widely disparate group of compounds that are used as emulsifiers, dispersants, spreaders and wetting agents with pesticides because of their ability to reduce the interfacial tension between aqueous and non-aqueous materials. However, surfactants are recognized as toxic substances in their own right among aquatic fauna, including amphibians (Mann and Bidwell, 2000, 2001; Renner, 2005; Ying, 2006). The most thoroughly studied example of formulation-surfactant toxicity is that associated with glyphosate formulations.

5.2. Glyphosate formulations – a special case

Commercial formulations of glyphosate constitute one of the most ubiquitous groups of pesticides presently used around the world. Because of its anionic nature, glyphosate on its own does not penetrate the plant cuticle. Therefore, the herbicide's phytotoxicity is facilitated by the addition of a surfactant. The surfactant incorporated into most glyphosate-based products is a polyoxyethylene tallowamine (POEA). The toxicity of POEA among aquatic fauna (for review see Giesy et al., 2000) has challenged the reputation of glyphosate as a benign herbicide when used in frog habitat. Mann and Bidwell (1999b) examined the toxicity of a widely used commercial glyphosate formulation (Roundup® Herbicide) to four species of Australian anurans (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis* and *Litoria moorei*) and reported markedly lower 48 h LC50 values for these formulations (2.9–11.6 mg acid equivalent (ae)/L) compared to the active constituent, glyphosate isopropylamine (>340 mg ae/L). Subsequent studies in a variety of species have confirmed the relatively high toxicity of glyphosate-based products containing POEA when exposed to amphibian larvae (Smith, 2001; Lajmanovich et al., 2003; Chen et al., 2004; Edginton et al., 2004b; Relyea, 2004a, 2005a,b,c; Relyea and Jones, in press; Cauble and Wagner, 2005; Comstock et al., 2007) and similarly found POEA to be responsible for the toxicity (Perkins et al., 2000; Edginton et al., 2004a; Howe et al., 2004). In acute toxicity tests, the toxic effects of POEA on aquatic biota appears to be associated with interference with gill morphology (Cardellini and Ometto, 2001; Lajmanovich et al., 2003), with mortality likely being the result of either the loss of osmotic stability or asphyxiation. At sub-lethal concentrations, exposure to POEA or glyphosate/POEA formulations has been variously reported to result in delayed development (Howe et al., 2004), accelerated development (Cauble and Wagner, 2005), reduced size at metamorphosis (Howe et al.,

Table 2
Comparative nitrate toxicity data among amphibians. GS = Gosner stage, A = activity, G = growth measured as tadpole length, mass or developmental stage, D = deformity.

Nitrogen compound	Concentration range mg/L	Species and developmental stage	Test conditions	Lethal effect-concentration mg/L	Sub-lethal effect concentration mg/L	Reference
Nitrate as $NaNO_3$	0.44–22	*Nyctibatrachus major*, GS25	22 °C, 56 days	9.0 (96-h LC50) ≥4.4 (100% mortality)	≥4.4 (D)	(Krishnamurthy et al., 2008)
	0.44–22	*Fejervarya limnocharis*, GS25	22 °C, 35 days	33.7 (96-h LC50) ≥4.4 (100% mortality)	≥4.4 (D)	(Krishnamurthy et al., 2008)
	0.44–22	*Rana temporaria*, GS25, nitrate acclimatized	Formulated water, 17.4 °C, till metamorphosis	No effect on survival	No effect (G – size)	(Johansson et al., 2001)
	0.44–22	*R. temporaria*, GS25, nitrate naive	Formulated water, 17.4 °C, till metamorphosis	No effect on survival	5 (G – ↓size)	(Johansson et al., 2001)
	1.25–20	*Rana catesbeiana*, GS26	Aged tap water, 17–19 °C, 15 days	20 (>30% mortality)		(Smith et al., 2005b)
	1.25–20	*Rana sylvatica*, GS26	Aged tap water, 17–19 °C, 15-day test	20 (90% mortality)		(Smith et al., 2005b)
	10	*Rana pipiens*, GS25	Aged tap water, 21.8 °C, till metamorphosis	No effect on survival	No effect (G)	(Orton et al., 2006)
	10	*Hyla versicolor* tadpoles	Outdoor mesocosm, till metamorphosis	No effect on survival		(Boone and Bridges-Britton, 2006)
	5.5–89	*R. sylvatica*, GS26	17–19 °C, 96 h	No effect on survival		(Smith, 2007)
	5.5–89	*H. versicolor*, GS26	17–19 °C, 15 days	No effect on survival	No effect (G – mass, A)	(Vaala et al., 2004)
	40 & 100	*Bufo bufo*, GS25	Distilled water, 19–24 °C, 13 days	40 (85% mortality)	40 (G – ↓length)	(Baker and Waights, 1993)
	40 & 100	*Litoria caerulea*, GS25	Distilled water, 22.6–26 °C, 16 days	40 (54% mortality)	40 (G – ↓length)	(Baker and Waights, 1994)
	10–500	*R. temporaria*, GS25	Formulated water, 24.5 °C, 15 days	50 (40% mortality)	≥100 (G – ↓length)	(Oromí et al., 2009)
	100	*Rana sphenocephala*, GS25	Formulated water, 22 °C, 15 weeks	100 (100% mortality)	100 (G – stage of development)	(Ortiz-Santaliestra and Sparling, 2007)
	22–133	*Bufo terrestris*, GS25	Formulated water & Spring water, 19–22 °C, till metamorphosis	No effect on survival	No effect (G)	(Edwards et al., 2006)
	7–292	*Xenopus laevis*, GS25-26	Formulated water, 20–22 °C, till metamorphosis	No effect on survival	400 (G – ↑mass)	(Brown-Sullivan and Spence, 2003)
	133–13228	*P. regilla* early limb-bud stage	Natural well water, 22 °C, 10 days	7750 (4-day LC50), 1179 (10-day LC50)	133 (G – ↓mass)	(Schuytema and Nebeker, 1999b)
	291–13228	*X. laevis* early limb-bud stage	Natural well water, 22 °C, 10 days	7334 (4-day LC50), 5476 (10-day LC50)	560 (G – ↓mass)	(Schuytema and Nebeker, 1999b)
	10, 25 & 40	*R. sylvatica, Ambystoma jeffersonianum, Ambystoma maculatum, Bufo americanus* (all as embryos)	Formulated water, 10 °C	No effect on embryo survival	No effect (D)	(Laposata and Dunson, 1998)
	129–13545	*Rana aurora* GS11-12 embryos	Natural well water, 16 days	2818 (16-day LC50)	129 (G – ↓length), 1040 (G – ↓mass)	(Schuytema and Nebeker, 1999c)
	22–2200	*Ambystoma mexicanum, Hyla chrysoscelis, Rana clamitans* (all as embryos)	Formulated water, 16–25 °C, till hatching	No effect on embryo survival	No effect (G – length)	(Meredith and Whiteman, 2008)
	110–12 030	*P. regilla* embryos	Natural well water, 22 °C, 10 days	2848 (4-day LC50), 2560 (10-day LC50)		(Schuytema and Nebeker, 1999a)
	110–2082	*X. laevis* embryos	Natural well water, 22 °C, 5 days	1942 (5-day LC50)		(Schuytema and Nebeker, 1999a)
	110–2082	*X. laevis* embryos	FETAX solution, 24 °C, 4 days	3861 (4-day LC50)		(Schuytema and Nebeker, 1999a)

(*continued on next page*)

Table 2 (*continued*)

Nitrogen compound	Concentration range mg/L	Species and developmental stage	Test conditions	Lethal effect-concentration mg/L	Sub-lethal effect concentration mg/L	Reference
Nitrate as $Ca(NO_3)_2$	43–128	*R. temporaria* embryos	Outdoor mesocosm, till hatching	No effect on survival		(de Wijer et al., 2003)
Nitrate as KNO_3	22–111	*Ambystoma gracile*, GS25	Dechlorinated tap water, 15 °C, 15 days	104 (15-day LC50), 55 (>10% mortality), 111 (>50% mortality)		(Marco et al., 1999)
	22–111	*Rana pretiosa*, GS25	Dechlorinated tap water, 15 °C, 15 days	73 (15-day LC50), 55 (>35% mortality)		(Marco et al., 1999)
	22–111	*Bufo boreas*, GS25	Dechlorinated tap water, 15 °C, 15 days	No effect on survival		(Marco et al., 1999)
	22–111	*Hyla regilla*	Dechlorinated tap water, 15 °C, 15 days	No effect on survival		(Marco et al., 1999)
Nitrate as NH_4NO_3	5, 10 & 15	*Litoria aurea*, GS25	Formulated water, 22 °C	10–15 (20% mortality)		(Hamer et al., 2004)
	5, 10 & 15	*Crinia signifera*, GS28	Formulated water, 22 °C, 21 days	No effect on survival		(Hamer et al., 2004)
	5, 10 & 15	*Litoria peronii*, GS25	Formulated water, 22 °C, 21 days	No effect on survival		(Hamer et al., 2004)
	10 & 20	*R. clamitans*, GS25	Outdoor mesocosm, 95 days	No effect on survival	No effect (G)	(Boone et al., 2005)
	40 & 100	*Osteopilus septentrionais*, GS25	Aged tap water, 23 °C, 13 days, pH ≤7.22	40 (>84% mortality)	40 (G – ↓length)	(Punzo and Law, 2006)
	varied b/n 20 & 100	*R. temporaria*, *B. bufo*, GS22-28	Outdoor mesocosm – no substrate, till metamorphosis	Significant mortality	G – delayed metamorphosis	(de Wijer et al., 2003)
	varied b/n 120 & zero	*R. temporaria*, *B. bufo*, GS22-28	Outdoor mesocosm – with substrate, till metamorphosis	No effect on survival	No effect on survival or timing of metamorphosis	(de Wijer et al., 2003)
	38.8–388	*Triturus helvetica* larvae	Formulated water, 15 °C, pH 7.8–8.4, 100 days	Dose dependant mortality		(Watt and Jarvis, 1997)
	53–89 mg/L	*B. americanus*, GS25, nitrate naive	Dechlorinated tap water, 20 °C, 96 h	60 (96-h LC50)		(Hecnar, 1995)
	111–222	*B. americanus*, GS25, nitrate acclimatized	Dechlorinated tap water, 20 °C, 96 h	174 (96-h LC50)		(Hecnar, 1995)
	44–155	*R. pipiens*, GS25	Dechlorinated tap water, 20 °C. 96 h	100 (96-h LC50)		(Hecnar, 1995)
	111–200	*Pseudacris triseriata*, GS25	Dechlorinated tap water, 20 °C, 96 h	75 (96-h LC50)		(Hecnar, 1995)
	44–222	*R. clamitans*, GS25	Dechlorinated tap water, 20 °C, 96 h	144 (96-h LC50)		(Hecnar, 1995)
	50, 100 & 200	*R. sylvatica*, GS26	Aged tap water, 7 days, pH 8.5	50 (33% mortality) 200 (100% mortality)		(Burgett et al., 2007)
	13.3–1322	*P. regilla* early limb-bud stage	Natural well water, 22 °C, 10 days, pH 7.0–7.6	600 (4-day LC50), 246 (10-day LC50)	133 (G – ↓mass)	(Schuytema and Nebeker, 1999b)
	13.3–1322	*X. laevis* early limb-bud stage	Natural well water, 22 °C, 10 days, pH 6.7–7.6	446 (4-day LC50), 234 (10-day LC50)	560 (G – ↓mass)	(Schuytema and Nebeker, 1999b)
	50–10 000	*B. bufo*, GS32-35	Formulated water, 25–30 °C, 7 days, pH 7.37–7.41	2200 (4-day LC50), 2112 (7-day LC50)		(Xu and Oldham, 1997)
	50 & 100	*B. bufo*, GS32	Formulated water, 25–30 °C, till metamorphosis	100 (21% mortality)	100 (G – ↓time to metamorphosis)	(Xu and Oldham, 1997)
	400	*Discoglossus galganoi*, GS24	16–19 °C, pH 7.2–7.4, 11 days	No effect on survival	400 (G – ↓length)	(Ortiz-Santaliestra et al., 2006a)
	400	*D. galganoi*, GS21	16–19 °C, pH 7.2–7.4, 11 days	400 (>50% mortality)	400 (G – ↓length)	(Ortiz-Santaliestra et al., 2006a)
	400	*Pelobates cultripes*, GS24	16–19 °C, pH 7.2–7.4, 11 days	400 (>70% mortality)		(Ortiz-Santaliestra et al., 2006a)
	400	*P. cultripes*, GS21	16–19 °C, pH 7.2–7.4, 11 days	400 (>90% mortality)		(Ortiz-Santaliestra et al., 2006a)
	400	*P. cultripes*, GS19	16–19 °C, pH 7.2–7.4, 11 days	400 (>30% mortality)		(Ortiz-Santaliestra et al., 2006a)
	400	*P. cultripes*, GS13	16–19 °C, pH 7.2–7.4, 11 days	400 (>60% mortality)		(Ortiz-Santaliestra et al., 2006a)
	14–1386	*R. aurora*, GS11-12 embryos	Natural well water, 16 days	319 (16-day LC50)	59 (G – ↓length & mass)	(Schuytema and Nebeker, 1999c)
	14.6–452	*P. regilla* embryos	Natural well water, 22 °C, 10 days, pH 6.6–6.7	182 (4-day LC50), 111 (10-day LC50)		(Schuytema and Nebeker, 1999a)

14.6–452	*X. laevis* embryos	Natural well water, 22 °C, 5 days, pH 6.4–7.3	194 (5-day LC50)	149 (5-day EC50 (D))	(Schuytema and Nebeker, 1999a)
14.6–452	*X. laevis* embryos	FETAX solution, 24 °C, 4 days, pH 7.0–7.5	142 (4-day LC50)		(Schuytema and Nebeker, 1999a)
50, 100 & 200	*D. galganoi* embryos	16.3 °C, 15 days, pH 7.2–7.8	200 (>50% mortality)	200 (G – ↓length, D)	(Ortiz et al., 2004)
50, 100 & 200	*Hyla arborea* embryos	22.2 °C, 15 days, pH 7.2–7.8	50 (>75% mortality), 200 (95% mortality)	200 (G – ↓length)	(Ortiz et al., 2004)
50 & 200	*B. bufo* embryos	21.0 °C, 15 days, pH 7.2–7.8	200 (>35% mortality)	200 (G – ↓length, D)	(Ortiz et al., 2004)
50 & 200	*Pleurodeles waltl* embryos	18.7 °C, 15 days, pH 7.2–7.8	No effect on survival	200 (G – ↓length)	(Ortiz et al., 2004)
50 & 200	*P. cultripes* embryos	18.9 °C, 15 days, pH 7.2–7.8	No effect on survival	50 & 200 (G – ↓length)	(Ortiz et al., 2004)
50 & 200	*Bufo calamita* embryos	21.1 °C, 15 days, pH 7.2–7.8	No effect on survival	200 (G – ↓length, D)	(Ortiz et al., 2004)

2004; Cauble and Wagner, 2005), developmental malformations of the tail, mouth, eye and head (Lajmanovich et al., 2003; Howe et al., 2004), histological indications of intersex (Howe et al., 2004) and symptoms of oxidative stress (Costa et al., 2008). It is noteworthy that in selection trials, gray tree frogs avoided the pools contaminated with a glyphosate/POEA formulation and placed the majority of their eggs in the control pools (Takahashi, 2007).

All the studies mentioned above expressed the toxicity of glyphosate/POEA formulations in terms of glyphosate concentration, which can be deceptive considering the toxicity is allegedly as a consequence of exposure to the formulation surfactants. This situation arises because surfactants in general, including POEA, are typically a mixture of closely related oligomers (e.g. Mann and Boddy, 2000) and measuring surfactants as discrete compounds is difficult. However, if the quantities of surfactant used in a formulation varies, then the toxicity data will also vary because it is dependent on the ratio of glyphosate to surfactant. Furthermore, analysis of water samples for glyphosate as a proximate measurement of the concentrations of associated surfactants is likely to underestimate the risk because the environmental persistence of surfactants may be higher than the active ingredient. Glyphosate has an aquatic half-life ranging from 2 to 14 days, whereas that of the associated POEA surfactant (Monsanto's MON 0818) in the environment has been conservatively estimated at 21–41 days (Giesy et al., 2000).

Two points of view have developed regarding the environmental risk posed by the use of POEA and similar surfactants. One view is that when used in accordance with directions stipulated on product labels, the concentration of glyphosate (and by inference the concentration of POEA or associated surfactants) will be sufficiently diluted to avoid toxic concentrations in water-bodies likely to receive runoff or be contaminated by spray-drift (Giesy et al., 2000; Solomon and Thompson, 2003; Thompson et al., 2004, 2006; Wojtaszek et al., 2004; McComb et al., 2008; Struger et al., 2008). Solomon and Thomson (2003) modelled various scenarios for the application of glyphosate formulations to open water-bodies, including ponds, and concluded that the expected concentrations of either glyphosate or associated surfactants posed little risk to aquatic fauna. The opposing view is that amphibians may be particularly susceptible to the toxic effects of these pesticides because their preferred breeding habitats are often shallow, lentic or ephemeral pools that do not necessarily constitute formal water-bodies, and which can contain higher concentrations when compared to larger water-bodies (NRA, 1996; Mann et al., 2003; Howe et al., 2004; Relyea, 2005a,b,c, 2006).

Glyphosate formulations, including those incorporating POEA will continue to be widely used in the foreseeable future, and it seems likely that concern over their impact on amphibian populations is also likely to continue. The recent controversy over the impact on frog populations of the widespread use of glyphosate in Columbia's coca fields highlights concerns with the continued use of some glyphosate formulations (Lubick, 2007; Solomon et al., 2007).

5.3. Pesticide mixtures

Although it is now widely agreed that worldwide amphibian population declines are not likely to be ascribed to a single factor, only recently have researchers begun to examine experimentally the possible interactions among various co-occurring stressors affecting anuran populations, which can often interact synergistically.

This is particularly true for anuran populations inhabiting agricultural areas that are more than likely to be exposed to a mixture of agricultural chemicals and are potentially at risk from additive, synergistic, or antagonistic toxicological effects. A few studies have

examined the toxic effects of pesticide mixtures on survival, developmental or immunological parameters in amphibians (Howe et al., 1998; Christin et al., 2003, 2004; Relyea, 2004a, 2009; Hayes et al., 2006a), and as one might expect, a cocktail of pesticides is likely to affect some or all of these parameters. In most of these studies it is impossible to elucidate the contributing toxicity of individual pesticides, whether there is an interaction between the various pesticides, or the mode of interaction. For example, Hayes et al. (2006a) presented sub-lethal toxicity data for nine individual pesticides at low environment concentrations (0.1 ppb) in *R. pipiens*. They demonstrated no toxicity for the majority of them individually, but also demonstrated some indices of toxicity (i.e. delayed metamorphosis, and altered morphology at metamorphic climax) when presented as a mixture of nine pesticides (each at 0.1 ppb). While there is a place for this kind of exposure (especially if these pesticides are expected to be found together in the environment), it provides no mechanistic information. A more informative approach, albeit slower, is to examine binary or tertiary mixtures which are likely to provide information about the contribution of each pesticide. Relyea (2004a) exposed the larvae of several species of frog to binary mixtures of diazinon, carbaryl, malathion and glyphosate and examined survival and growth. Although the author (Relyea, 2004a) reported that the combined pesticides (i.e. 1 mg/L + 1 mg/L) caused greater effects on the measured endpoints than either pesticide alone, the effects were not greater compared to observed effects following exposure to twice the concentration (2 mg/L) of any single pesticide. Therefore the interaction was not greater than the additive effect of the two pesticides. Synergystic effects, where the combined effect of two or more compounds results in greater than additive toxicity has also been demonstrated. In some invertebrates, atrazine is known to act synergistically with certain organophosphorus pesticides (Wacksman et al., 2006), but synergism has also been demonstrated in amphibians. In *X. laevis*, atrazine alone had no effect at 5 mg/L, but 1 mg/L atrazine significantly increased the toxicity of the organophosphorus insecticide chlorpyrifos in a synergism; this same interaction was not observed in *R. clamitans* (Wacksman et al., 2006). Howe et al. (1998) also demonstrated that two herbicides, atrazine and alachlor, when applied as a 50:50 mixture had a synergistic toxic effect on *R. pipiens* and *B. americanus*. However, synergistic interactions between various pesticides or constituents are chemical-specific and not necessarily a general phenomenon applicable to all chemical contaminants.

Acute effects like those described above have only been demonstrated at relatively high concentrations, and as indicated by Hayes et al. (2006a), the potential endocrine- and immune-disruptive effects of pesticides and pesticide mixtures at very low environmental concentrations may present a more pervasive and insidious scenario. The alleged endocrine effects of atrazine at low environmental concentrations have been discussed above (Section 3), and although the immune-disruptive effect of some pesticide mixtures has been examined (Section 4), the potential effects of low concentration mixtures is yet to be addressed in any depth. Hayes et al. (2006a) noted that *R. pipiens* metamorphs exposed to a mixture of nine pesticides (each at 0.1 ppb) during development suffered from a bacterial infection. They subsequently described an increase in thymic plaques (as an index of immune disruption) among metamorphs following exposure to the mixture of nine pesticides, and among animals exposed to either atrazine or *S*-metolachlor singly or in combination as a preparation called Bicep II. However, it remained unclear if there was an interaction between pesticides suggestive of a more than additive effect.

Some studies have suggested that the widespread use of various pesticides together with nitrogen fertilizers may further exacerbate the effects on wild amphibian populations that inhabit agricultural landscapes (Howe et al., 1998; Orton et al., 2006). The combined effects of pesticide chemicals and nitrogen fertilizers are of high environmental relevance, particularly because fertilizer use often coincides both spatially and temporally not only with herbicide application but also the breeding season of amphibians (Allran and Karasov, 2000; Orton et al., 2006). Amphibian population declines observed in Poland, England, United States and Canada have been attributed to nitrogen fertilizers together with habitat loss and pesticides and will be discussed later (Section 6).

The limited studies conducted to date report few additive or synergistic effects on the growth and larval development of amphibians from combined exposures to pesticide and nitrate (Allran and Karasov, 2000; Boone, 2008; Boone and Bridges-Britton, 2006). Boone et al. (2005) described reduced survival and impaired growth and development among *R. clamitans* tadpoles exposed to 2.5 mg/L of carbaryl together with 20 mg/L of nitrate (as NH_4NO_3) which was not evident when the tadpoles were exposed to either carbaryl or nitrate alone. Also, Orton et al. (2006) described a synergistic effect on the sex ratios of amphibians exposed to nitrate and atrazine (Section 6.4).

6. Impact of fertilizers

Some of the more ubiquitous contaminants in agricultural landscapes are those associated with the use of fertilizers and in recent years a great deal of attention has been focused on these. Subsequent runoff after rainfall will result in elevated concentrations of inorganic nitrogenous compounds (i.e. NH_3/NH_4^+, NO_2^-, NO_3^-) in downstream waters. Rouse et al. (1999) cited nitrate concentrations of between 2 and 40 mg/L in streams traversing agricultural landscapes in North America. More recently, Egea-Serrano et al. (2008) cited some extraordinarily high concentrations for inorganic nitrogen species in agricultural waters sampled from the Segura River in Spain (154.6 mg NH_4^+/L, 74.4 mg NO_2^-/L 333 mg NO_3^-/L). In Australia, nutrient exports into waterways are generally lower than North America or Europe because agricultural land use is less intensive, particularly with respect to fertilizer application (for review see Vink et al., 2007).

Flooded rice agriculture within the Murray–Darling Basin in Australia provides examples of environments that are subject to intensive fertilizer treatment and that are also utilized by frogs (Wassens, 2005). Following application of urea at a rate of 80 kg N/ha into the floodwater of a young rice crop, nitrate was detected up to a maximum concentration of ~4.0 mg NO_3^-/L 2–9 days following application. Nitrite was detected up to a maximum of 5.2 mg NO_2^-/L on day 9 following application, and ammoniacal species (NH_3 + NH_4^+) fluctuated between 1.5 and 2.8 mg N/L (Simpson et al., 1984). To keep this in perspective, nitrogen exports from forested catchments (not receiving fertilizer) into receiving streams are comparable. During annual summer and autumn runoff events from a forested (primarily *Pinus radiata*) catchment, Vink et al. (2007) reported nitrate concentrations up to 100 mg NO_3^-/L with an average of 15 mg NO_3^-/L for years 2002–2004. During the same runoff events, nitrite was not detected and ammonium (NH_4^+) was recorded up to ~3 mg NH_4^+/L, with average concentrations of 0.2 mg NH_4^+/L.

6.1. Ammonia

Ammonia is toxic. Among fish and invertebrates 96 h LC50 values range between 0.13 and 0.80 mg NH_3/L (Camargo and Alonso, 2006). Relatively few studies have examined the toxic effects of NH_3 on amphibians. Diamond et al. (1993) generated NH_3 toxicity data for several species of fish, invertebrates and the

embryos/larvae of *R. pipiens* and *Hyla crucifer*. In cold water (12 °C), both frog species were more sensitive than other test-species (96 h LC50s, 0.42 and 0.46 mg NH_3/L, for *R. pipiens* and *H. crucifer*, respectively). Increasing the temperature to 20 °C increased the LC50 to 1.9 mg NH_3/L for *R. pipiens* (Diamond et al., 1993). This is consistent with data generated by Jofre and Karasov (1999) that indicated significant levels of mortality and deformities among *R. clamitans* and *R. pipiens* embryos exposed to NH_3 above 0.6 and 1.5 mg NH_3/L, respectively. In the same study, *B. americanus* embryos were unaffected up to 0.9 mg NH_3/L. Schuytema and Nebeker (1999a,b) also generated NH_3 toxicity data for *X. laevis* and *P. regilla* at 22–24 °C that ranged between 0.36 (10-day LC50, *P. regilla*) to 1.27 (96 h LC50, *X. laevis*) mg NH_3/L.

However, ammonia is not persistent in aqueous systems. Its presence and persistence is dictated by: (1) losses of NH_3 gas to the atmosphere; (2) a pH (and temperature) dependent equilibrium between un-ionized NH_3 and the relatively non-toxic ionized NH_4^+ (Thurston and Russo, 1981; Dejours et al., 1989); and (3) the sequential conversion of NH_4^+ to nitrite and nitrate by *Nitrosomonas* and *Nitrobacter* bacteria, respectively (Fig. 4).

6.2. Nitrite

Nitrite is also toxic. Nitrite has a high affinity for the Cl^- uptake mechanism, and having entered the haemolymph, induces the conversion of haemoglobin to methaemoglobin which is incapable of binding oxygen. Methaemoglobinemia following exposure to NO_2^- has been demonstrated in amphibians (Huey and Beitinger, 1980a; Punzo and Law, 2006) and is likely the root cause of mortality, sluggish growth and development, and behavioural traits observed among embryonic/larval amphibians exposed to up to ~20 mg NO_2^-/L (Huey and Beitinger, 1980b; Marco and Blaustein, 1999; Marco et al., 1999; Griffis-Kyle, 2005, 2007). It is notable that tolerance to high concentrations of nitrite (≥20 mg NO_2^-/L) has been documented among amphibian larvae (Smith, 2007; Smith et al., 2004; Shinn et al., 2008) and adult frogs (Egea-Serrano et al., 2008). In some cases tolerance may be a consequence of the ameliorative effect of Cl^- that effectively competes with NO_2^- at Cl^- transport sites (Huey and Beitinger, 1980a) or geographic adaptation to increased inorganic nitrogen contamination (Shinn et al., 2008).

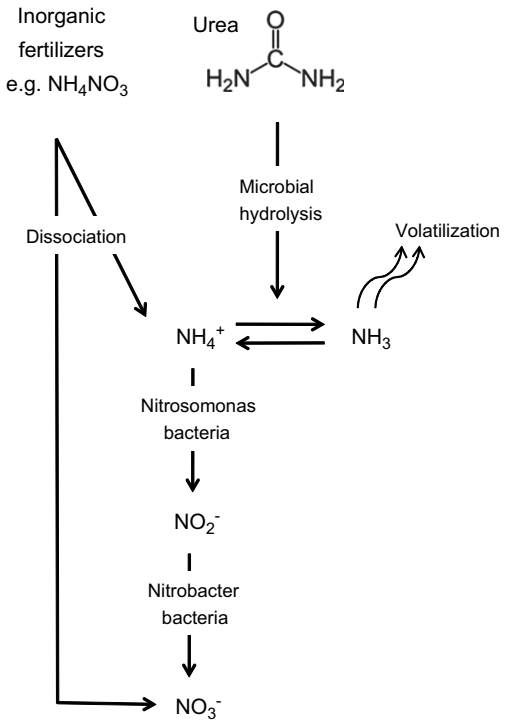

Fig. 4. Transformation pathway for nitrogenous fertilizers.

6.3. Nitrate

Because ammonia and nitrite are transient species within the environment, high environmental concentrations are usually only found at point source discharges or after heavy rainfall events. Concern about nitrogenous pollutants therefore tends to focus on nitrate. However, nitrate is not considered to be particularly toxic, or more to the point, a mechanism of toxicity has not been described. Indeed, following exposure to nitrite, crayfish have been shown to convert nitrite to nitrate as an endogenous mechanism of detoxification (Jensen, 1996). Unlike nitrite, nitrate is not actively transported, and following exposure to nitrate, branchial uptake (at the gills) in aquatic organisms is slow, entering the extracellular space by passive diffusion only (Camargo and Alonso, 2006).

Nevertheless, an extraordinarily wide range of effect-concentration data has been generated for amphibians exposed to nitrate (Table 2). Several studies reported high rates of mortality at concentrations ≤50 mg NO_3^-/L, while others have reported LC50 data only at NO_3^- concentrations well over 1000 mg/L. More typically, nitrate is reported to have no effect following exposure to concentrations up to ~100 mg NO_3^-/L. Unfortunately, consilience between studies is hindered because little consistency occurs with regard to test-species used, test-water, test durations and which nitrate salts were used for the tests.

Several authors have highlighted the confounding effects of ammonium in those tests which used NH_4NO_3 (Mann and Bidwell, 1999a; Johansson et al., 2001; de Wijer et al., 2003; Boone et al., 2005) because the ammonium ion (as a source of dissolved ammonia) is likely to have a pronounced toxic effect. Schuytema and Nebeker (1999a,b,c) made comparisons between different species and different ammonium and nitrate compounds. Their studies with *X. laevis* and *P. regilla* found sodium nitrate to be relatively non-toxic (96 h LC50s, 3860 and 2848 mg NO_3^-/L, respectively), and concluded that when nitrate is presented as ammonium nitrate, the ammonium ion (as a source of dissolved ammonia) was of greater concern (96 h LC50s, 142.2 and 182.0 mg NO_3^-/L for *X. laevis* and *P. regilla*, respectively) and may explain the relatively high toxicity described for newly hatched *Hyla arborea* (Ortiz et al., 2004), *Rana sylvatica* (Burgett et al., 2007), *Osteopilus septentrionalis* (Punzo and Law, 2006) and juvenile *Triturus helvetica* (Watt and Jarvis, 1997). In contrast, (Xu and Oldham, 1997) also used ammonium nitrate in their tests with *Bufo bufo* tadpoles, and reported 4- and 7-day LC50s of 1704 and 1637 mg NO_3^-/L, respectively. This discrepancy was not obviously related to differences in pH (a higher pH promotes the formation of toxic NH_3) and is not likely to be a consequence of species sensitivity differences, since such large species differences have not been described in the various studies where more than one species has been used (e.g. Hecnar, 1995; Schuytema and Nebeker, 1999a). Similarly perplexing, are the high indices of toxicity reported by several authors that exposed animals to $NaNO_3$ (Baker and Waights, 1993, 1994; Smith et al., 2005b; Krishnamurthy et al., 2008; Oromí et al., 2009), and points to the need to ascertain the mechanism of nitrate toxicity.

6.4. Is there a mechanism for nitrate toxicity?

Some mechanisms for nitrate toxicity have been proposed. Hecnar (1995) speculated that nitrate may be reduced to nitrite in the gut, thus resulting in nitrite toxicity. Nitrite-induced methaemoglobinemia as a secondary effect of exposure to nitrate may

provide an explanation for toxicity at relatively low levels of nitrate. Hecnar (1995) also speculated that nitrate may affect osmoregulation as had previously been suggested by Camargo and Ward (1992). Corroborating evidence for either of these mechanisms of toxicity is scarce and requires investigation.

A more intriguing mechanism is through the induction of hypothyroidism. Thyroid hormones play an important role during metamorphosis (Section 2.1). At metamorphic climax, which corresponds with the emergence of forelimbs, there is a corresponding peak in the primary circulating thyroid hormone, thyroxine. Thyroxine contains iodine, and data exist to indicate that nitrate can impair the formation of thyroid hormones through the inhibition of iodine uptake, transport and retention (Crow et al., 2001; Edwards et al., 2006). In an experiment designed to demonstrate this kind of endocrine disruption, Edwards et al. (2006) measured whole body thyroxine levels in recently metamorphosed *Bufo terrestris* that had been exposed to 22, 66 and 133 mg NO_3^-/L (as $NaNO_3$) during pre-metamorphic development. The trials were performed in both laboratory-formulated water and natural spring water. Although the authors described a significant relationship between nitrate exposure and thyroxine concentration, when the "flux" treatment (in which nitrate exposure was varied during the exposure) is removed from the statistical analysis, there is clearly no effect of nitrate on circulating thyroxine (Thea Edwards, personal communication). In contrast, it was clear that the type of water used for the experiment had a far greater impact on circulating thyroxine levels than did nitrate exposure.

Oestrogenic properties have also been ascribed to nitrate. Orton et al. (2006) suggest that combined effects of the herbicide atrazine and nitrate caused a significantly higher proportion of female *R. pipiens* tadpoles during larval development. These same authors reported increased size of ovarian follicles following exposure to 10 mg NO_3^-/L that they described as indicative of a local endocrine effect in the gonad because the hypothalamo-pituitary-gonadal axis becomes functional only shortly prior to metamorphic climax (Orton et al., 2006). Barbeau and Gullette (2007) also described altered follicular morphology and altered steroidogenesis among adult *X. laevis* exposed to 109.5–219.0 mg NO_3^-/L. Further study is required to establish whether nitrate acts as an endocrine disruptor.

The Orton et al. (2006) study also highlighted the importance of complex mixtures and their implications for amphibians in agricultural landscapes. The issue of combined effects of agricultural chemicals or their effect in combination with abiotic stressors is discussed in greater detail below (Section 7), including the combined effects of nitrogenous compounds and UV-B.

6.5. Are fertilizers toxic in the real world?

Under more natural conditions, nitrate toxicity is more difficult to demonstrate. Despite correlations between the occurrence of nitrates in agricultural practice and amphibian population declines (Berger, 1989; Bishop et al., 1999; Rouse et al., 1999; de Solla et al., 2002; Hamer et al., 2004), other studies provide a contrary view. As indicated above, only nitrate is likely to persist in appreciable quantities in natural ecosystems, but given suitable conditions, excess nitrate will be absorbed by and converted into organic biomass. Through this process, excess of nitrate can lead to eutrophication (Nijboer and Verdonschot, 2004). In extreme cases, this can lead to anoxic conditions (indeed this appears to have been the case in the study by Berger, 1989), and allow the persistence of toxic compounds like ammonia and nitrite. Alternatively, eutrophic conditions can lead to the detrimental proliferation of toxic algal blooms (Camargo and Alonso, 2006). However, under less than extreme conditions, the proliferation of algae and macrophytes that occurs in nitrate enriched systems appears to either benefit (by providing a source of food and habitat) or have no effect on amphibian communities (Beebee, 1987; de Wijer et al., 2003; Boone et al., 2007; Massal et al., 2007).

A unique exception to this appears to be the occurrence of limb malformations among North American amphibians where eutrophication may be the indirect cause of the phenomenon (Johnson and Chase, 2004; Johnson et al., 2007). Numerous studies by the same author have described a link between amphibian limb malformations and the occurrence of a parasitic trematode, *Ribeiroia* (Johnson et al., 1999, 2001a,b, 2002, 2003; Johnson and Sutherland, 2003) and more recently, Johnson et al. (2007) have demonstrated that even relatively small increases in nutrients (phosphate, nitrate), and the subsequent proliferations in periphyton and the intermediate trematode host, *Planorbella trivolvis* (a snail), resulted in the increased prevalence of trematode infection among resident amphibians (*R. clamitans*) (see also Fig. 5 in Section 8). In contrast, Koprivnikar et al. (2006a) were unable to find a correlation between trematode infection and nitrate in an agricultural area of southern Ontario. To our knowledge, this phenomenon has only been described for sites in North America.

6.6. Can amphibians detect and avoid nitrogenous pollution?

We are aware of five studies which have examined the direct toxic effects of fertilizers on adult amphibians (Oldham et al., 1997; Hatch et al., 2001; Marco et al., 2001; Ortiz-Santaliestra et al., 2006b; Egea-Serrano et al., 2008). The proposed pretext for these studies is that frogs are likely to come into close contact with fertilizer chemicals if they traverse terrain where fertilizer has recently been applied. Substrate selection trials indicte that some amphibians are able to detect and avoid paper impregnated with high concentrations of ammonium- and urea-based fertilizers (Table 3), but that this ability was not evident when trials were performed on natural soils (Hatch et al., 2001). Exposure to either paper or field-soil contaminated with NH_4NO_3 (between 11 and 87 kg N/ha) was also associated with respiratory stress (Oldham et al., 1997), although this effect was lost within an hour or so after application to soil. Prolonged exposure to urea-contaminated soils (100 kg N/ha) resulted in reduced survival and reduced food consumption among western toads and cascades frogs (Hatch et al., 2001), although long-toed salamanders and roughskin newts were not similarly affected. Marco et al. (2001) also described high levels of mortality within 48 h among red-backed salamanders and southern torrent salamanders exposed to urea-contaminated paper at either 225 or 450 kg N/ha but no mortality among roughskin newts exposed to the same concentrations. It seems likely that in all these trials amphibians were responding to direct contact with toxic ammonia; and as indicated earlier, ammonia will dissipate relatively quickly under field conditions, although it is clear that even short term exposure is likely to have deleterious effects.

7. Agricultural chemicals and abiotic/biotic stressors

Amphibian populations in the natural environment are subjected to a complex range of naturally occurring stressors in addition to those exerted by agricultural chemicals. Therefore, there is potential for interactions to occur between various stressors including those exerted by pesticides, which can have profound effects on anuran populations and contribute further to declines (Alford and Richards, 1999; Blaustein and Kiesecker, 2002). However, relatively few studies have considered the potential impacts of agricultural chemicals in the presence of naturally occurring stressors, which are likely to affect natural anuran

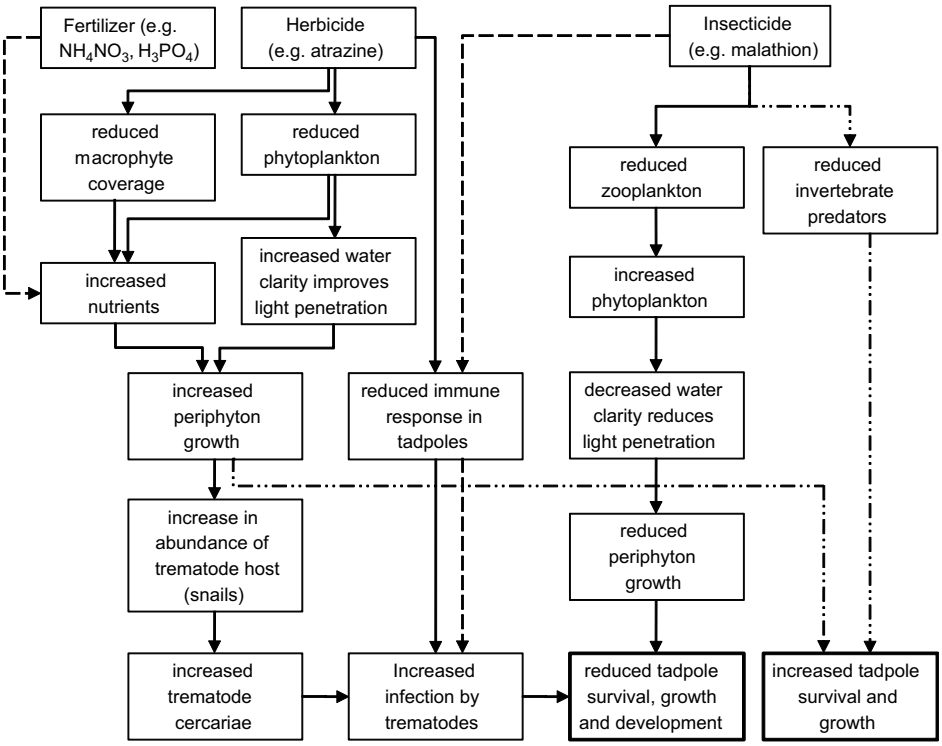

Fig. 5. Pathways for indirect community effects of agricultural chemicals on amphibian populations in agricultural landscapes. Scenarios are based primarily on data presented by Rohr et al. (2008a,b) and Relyea and Diecks (2008) (solid lines) but also on the data presented by Johnson et al. (2007) and Kiesecker (2002) (dashed lines) and various data cited above (dashed-dotted lines).

populations (Rohr et al., 2004; Sih et al., 2004). Recent data describing the combined effects of agricultural chemicals and external factors is presented in Table 4.

One natural stressor that has received concerted attention as a cause for amphibian population decline is UV radiation, and while the literature that examines the direct effects of UV is extensive (for review see Blaustein et al., 2003) only a handful of studies that we are aware of have examined the combined effects of UV and a pesticide. Zaga et al. (1998) investigated the interactions between carbaryl and UV radiation on African clawed frog (*X. laevis*) and gray tree frog (*H. versicolor*) embryos and larvae (Table 4). However, the Zaga et al. (1998) study is not strictly a demonstration of additive or synergistic effects of two independent stressors, because the increased toxicity of carbaryl observed in the presence of UV was most likely a result of photo-activation of carbaryl rather than a combined effect on the test animals. The authors were effectively testing the toxicity of carbaryl photo-degradation products rather than the combined effect of carbaryl and UV. In the case of studies that examined the combined effect of UV and nitrogenous compounds (Hatch and Blaustein, 2000, 2003; Macías et al., 2007) the interaction is less clear, possibly because a mechanism for nitrate toxicity on its own is not known.

The effect of acidity on amphibians has also received a great deal of attention in the past, primarily as a response to concerns about acid rain (for review see Rowe and Freda, 2000), and it can be expected that pH is likely to act as an additional stress among amphibians exposed to agricultural chemicals (Hatch and Blaustein, 2000). However, among the few existing studies we are aware of, moderate decreases in pH have been found to alter chemical speciation rather than have additive or synergistic effects on amphibian larvae. For example, triclopyr was more toxic at low pH (pH 5.5) than high pH (ph 7.5) because triclopyr degrades more quickly at a higher pH (Edginton et al., 2003; Chen et al., 2008). In contrast, POEA, the surfactant in most glyphosate formulations (Section 5.2), is more toxic at a high pH (Chen et al., 2004; Edginton et al., 2004b) because POEA becomes more non-ionic with increased pH.

Table 3
Evidence for avoidance of nitrogenous fertilizers among adult amphibians.

Species	Treatment	Concentration	Avoidance	Reference
Pelophylax perezi (Iberian water frog)	NH_4Cl solution in beakers	40 mg/L	No	(Egea-Serrano et al., 2008)
Pelophylax perezi	NH_4Cl Solution in beakers	40 mg/L	No	(Egea-Serrano et al., 2008)
Pelophylax perezi	$NaNO_3$ Solution in beakers	500 mg/L	No	(Egea-Serrano et al., 2008)
Pelophylax perezi	$NaNO_2$ Solution in beakers	10 or 100 mg/L	No	(Egea-Serrano et al., 2008)
Triturus boscai (Iberian newt)	NH_4NO_3 contaminated paper	25 kg N/ha.	Yes	(Ortiz-Santaliestra et al., 2006b)
Bufo boreas (Western toad); *Rana cascadae* (cascades frog)	Urea-contaminated paper	100 kg N/ha	Yes	(Hatch et al., 2001)
B. boreas (western toad); *R. cascadae* (cascades frog)	Urea-contaminated soil	50 or 100 kg N/ha	No	(Hatch et al., 2001)
Ambystoma macrodactylum (long-toed salamander); *Taricha granulose* (roughskin newt)	Urea-contaminated paper	100 kg N/ha	No	(Hatch et al., 2001)
A. macrodactylum; *T. granulose*	Urea-contaminated soil	50 or 100 kg N/ha	No	(Hatch et al., 2001)
Plethodon vehiculum (red-backed salamander); *T. granulose*; *Rhyacotriton variegates* (southern torrent salamander)	Urea-contaminated paper	225 kg N/ha	Yes	(Marco et al., 2001)

Table 4
Studies examining abiotic or biotic co-factors in the toxicity of agricultural chemicals. GS = Gosner stage.

Abiotic/biotic factor	Pesticide	Species	Effect	Mechanism	Comments	Reference
UV–B	Carbaryl (15 mg/L)	*Hyla versicolor* (tadpoles)	Reduced survival and reduced swimming ability	Photo-activation of carbaryl	4-day laboratory study with uniform exposure to UV	(Zaga et al., 1998)
UV–B	Carbaryl (0.86–1.76 mg/L)	*Xenopus laevis* (tadpoles)	Reduced survival and reduced swimming ability	Photo-activation of carbaryl	4-day laboratory study with uniform exposure to UV	(Zaga et al., 1998)
UV–B	Carbaryl (4.1 mg/L)	*Rana sphenocephala* (2 weeks old)	No effect on survival	–	Mesocosm study – tadpoles were able to avoid UV exposure	(Bridges and Boone, 2003)
UV–B	Nitrite (11.5 mg/L)	*Bufo bufo*, *Rana perezi*	Reduced survival when exposed to nitrite and UV-B	Unknown – likely due to additive stress from multiple stressors	15-day *in-situ* field exposures	(Macías et al., 2007)
UV–B	Nitrate (10–20 mg/L)	*Hyla regilla* *Ambystoma*	Lower mass and/or survival when exposed to among nitrate & UV-B	Unknown – likely due to additive stress from multiple stressors	3-week *in-situ* field exposures	(Hatch and Blaustein, 2003)
UV–B & pH (pH 5 vs pH 7)	Nitrate (5–20 mg/L)	*Rana cascadae* (GS23 – 24)	Reduced survival and activity when exposed to nitrate, UV-B and low pH	Unknown – likely due to additive stress from multiple stressors	3-week laboratory study	(Hatch and Blaustein, 2000)
pH (pH 5.5 vs pH 7.5)	Polyoxyethylene tallowamine (POEA) in glyphosate formulations	*Rana pipiens* (GS25)	Reduced survival at pH 7.5 compared to pH 5.5	Change in ionic state of POEA surfactant increases toxicity	10-day laboratory exposures	(Chen et al., 2004)
pH (pH 6 vs pH 7.5)	Polyoxyethylene tallowamine (POEA) in glyphosate formulations	*X. laevis*, *Bufo americanus*, *Rana clamitans*, *R. pipiens* (GS25)	Reduced survival at higher pH	Change in ionic state of POEA surfactant increases toxicity	96-h laboratory study	(Edginton et al., 2004b)
pH (pH 5.5 vs pH 7.5)	Triclopyr	*X. laevis*, *B. americanus*, *R. clamitans*, *R. pipiens* (GS25)	Reduced survival at lower pH	Hydrolysis of herbicide at higher pHs reduces toxicity	96-h laboratory study	(Edginton et al., 2003)
pH (pH 5.5 vs pH 7.5)	Triclopyr	*R. pipiens* (GS25)	Reduced survival at lower pH	Hydrolysis of herbicide at higher pHs reduces toxicity	10-day laboratory exposures	(Chen et al., 2008)
Temperature 17 °C–27 °C	Cabaryl (10.3–30 mg/L)	*R. clamitans* (GS25)	Reduced survival at higher temperatures	Increased uptake at higher temp	96-h laboratory study	(Boone and Bridges, 1999)
Variable temperatures (21 ± 7.5 °C) vs constant temp (20 ± 0.8 °C)	Endosulfan (0.8 μg/L)	*Litoria citropa* (GS25)	Reduced survival & susceptibility to predation increased with variable temperature	Unknown – likely due to additive stress from multiple stressors	96-h laboratory study	(Broomhall, 2002)
Temperatures during embryonic development 14 °C vs 20 °C	Endosulfan (0.3–1.3 μg/L)	*Limnodynastes peronii* (GS25)	Reduced growth	Possibly acclimation to lower temperatures improves metabolic efficiency		(Broomhall, 2004)
Temperatures during embryonic development 14 °C vs 20 °C	Endosulfan (0.3–1.3 μg/L)	*L. peronii* (GS25)	Increased susceptibility to predation if the tadpoles were reared at low embryonic temperature	Possibly lower developmental temperatures alters muscle phenotype		(Broomhall, 2004)
Density (20 tadpoles/kL vs 60 tadpoles/kL	Atrazine (200 μg/L)	*R. sphenocephala*	Reduction in survival when tadpoles are at high density compared to low density	Likely an indirect effect on food resources (Section 8)	No effect when exposed to carbaryl (3.5–7.0 mg/L)	(Boone and James, 2003)

	Carbaryl (3.5 mg/L) 1, 2 or 3 exposures	*R. clamitans*	Increase in developmental rate when animals are at high density compared to low density	Possible induction of thyroid/stress-mediated precocious metamorphosis (Section 2)	1000 L mesocosm study till metamorphosis	(Boone et al., 2001)
Hydroperiod (drying vs constant)	Carbaryl (5 mg/L)	*B. americanus*	In ponds that were drying there was significant increase in mass at metamorphosis	Unknown	1000 L mesocosm study till metamorphosis	(Boone and James, 2003)
Hydroperiod (drying vs constant)	Atrazine (200 μg/L)	*Ambystoma texanum*	In ponds that were drying there was significant decrease in mass at metamorphosis	Possible induction of thyroid/stress-mediated precocious metamorphosis (Larson et al., 1998) (Section 2)	1000 L mesocosm study till metamorphosis	(Boone and James, 2003)
Hydroperiod (drying vs constant)	Atrazine (200 μg/L)	*A. texanum*	Under condition of constant water volume, the pesticide abolishes the tendency for delayed development	Possible induction of thyroid/stress-mediated precocious metamorphosis	1000 L mesocosm study till metamorphosis	(Boone and James, 2003)
Hydroperiod (drying vs constant)	Atrazine (40–400 μg/L)	*Ambystoma barbouri*	Under condition of constant water volume, the pesticide abolishes the tendency for delayed development	Possible induction of thyroid/stress-mediated precocious metamorphosis	117-day laboratory study	(Rohr et al., 2004)
Hydroperiod (drying vs constant)	Atrazine (40–400 μg/L)	*A. barbouri*	The increased survival benefit of constant water volume is abolished with pesticide	Possible induction of thyroid/stress-mediated precocious metamorphosis	117-day laboratory study	(Rohr et al., 2004)
Food abundant vs food restricted	Atrazine (4.0–400 μg/L)	*A. barbouri*	No effect		117-day laboratory study	(Rohr et al., 2004)
Presence of caged predatory newt	Roundup® (0.1–1.0 mg/L)	*R. sylvatica* (GS25)	Predator cue increased toxicity of pesticide (i.e. reduced survival)	Synergistic consequences of stress and/or reduced foraging	16-day laboratory study. *R. pipiens*, *R. clamitans*, *R. catesbeiana*, *B. americanus*, and *H. versicolor* were not similarly affected	(Relyea, 2005c)
Presence of a caged predatory dragonfly larva	Atrazine (200 μg/L)	*H. versicolor* (GS25)	Predator cue increased toxicity of pesticide (i.e. reduced survival)	Synergistic consequences of stress and/or reduced foraging	Laboratory study till metamorphosis	(LaFiandra et al., 2008)
Presence of caged predatory salamander	Carbaryl (0.05–4.2 mg/L)	*H. versicolor* (GS25)	Predator cue increased toxicity of pesticide (i.e. reduced survival)	Synergistic consequences of stress and/or reduced foraging	10/16-day laboratory study	(Relyea and Mills, 2001)
Presence of caged predatory newt	Carbaryl (0.3–6.5 mg/L)	*R. pipiens*, *R. clamitans*, *R. catesbeiana*, *B. americanus*, *R. versicolor*	Predator cue increased toxicity of pesticide (i.e. reduced survival)	Synergistic consequences of stress and/or reduced foraging	16-day laboratory study. Survival of *R. sylvatica* was reduced by predator cue alone.	(Relyea, 2003)
Presence of caged predatory newt	Malathion (0.1–5.0 mg/L)	*H. versicolor*	Predator cue increased toxicity of pesticide (i.e. reduced survival)	Synergistic consequences of stress and/or reduced foraging	16-day laboratory study. *R. pipiens, R. clamitans, R. catesbeiana, B. americanus, and R. sylvatica* were not similarly affected	(Relyea, 2004b)

Another important environmental variable that is not often incorporated into toxicological studies is temperature. Typical amphibian habitat within agricultural landscapes such as farm dams, irrigation and drainage canals, because of their shallow nature are more likely to experience wide temperatures fluctuations that may subject amphibians to temperature stress, particularly during summer months which coincide with both pesticide application and sensitive development periods of anurans (Boone and Bridges, 1999). Although an increase in temperature may be expected to reduce the toxicity of some pesticides as a consequence of increased metabolic rate and subsequent increase in detoxification and excretion, it is not clear if a chemical is likely to be more or less toxic when exposures occur at higher temperatures. Boone and Bridges (1999) demonstrated that the toxicity of carbaryl was substantially enhanced with increasing temperature, and concluded that more rapid absorption at higher temperatures likely negated any increased capacity to detoxify the pesticide.

The temperature regime experienced by amphibians during development may also affect their capacity to cope with other natural or anthropogenic stressors later in life. Broomhall (2004) demonstrated that low embryonic developmental temperature had enduring implications for growth and ecological fitness in the face of predatory or chemical stressors. Cold-adapted tadpoles, if exposed to endosulfan concentrations as low as 0.03 μg/L were more susceptible to predation than warm-adapted tadpoles. Therefore, the entire life history of a larval amphibian can synergistically interact with a pesticide.

Other abiotic or biotic factors can act to increase the level of stress among tadpoles (Section 2), and an additional chemical stressor such as a pesticide may negatively affect survival or growth and development. Stress can take the form of extreme temperature regimes (Broomhall, 2002), changes in water level in ephemeral ponds (Boone and James, 2003; Rohr et al., 2004), high stocking densities (Boone and Semlitsch, 2001; Boone and James, 2003) and subsequent limitations in food availability (Rohr et al., 2004) and the presence of predators (Boone and Semlitsch, 2001; Relyea and Mills, 2001; Relyea, 2003, 2004b, 2005c). Tadpoles already at their tolerance limits or under physiological stress may be unable to deal with the additional stress imposed by chemical contamination. In all the studies listed above, the additional stress had the effect of exacerbating pesticide toxicity (Table 4).

8. Community effects

Natural ecosystems, even those that exist within highly altered agricultural landscapes are governed by ecological principles. At their simplest, these principles can be reduced to: competition for resources, and predator–prey or host–parasite relationships. Pesticides are designed to disrupt the community dynamics that exist within the context of ecosystems by eliminating one or more of the species within a community. It is no surprise therefore, that amphibians can be indirectly affected by the application of pesticides through the elimination of food resources, competitors or predators. For example, impairment of growth and development among *Ambystoma maculatum* and *R. pipiens* tadpoles exposed to atrazine (200 μg/L) was ascribed to the removal of algal food resources (Boone and James, 2003), as were the deleterious effects on *Rana sylvatica* tadpoles exposed to atrazine (25 μg/L) (Rohr and Crumrine, 2005). In both those studies the methods used to evaluate algal resources differ. In the Boone and James (2003) study, water samples were used to determine chlorophyll *a* concentrations, providing an estimate of phytoplankton but not of periphyton. In contrast, Rohr and Crumrine (2005) measured chlorophyll *a* concentrations from periphyton specifically, which may be a better estimate of food resources for rasping tadpoles, although it remains unclear which food resources are likely to be more important for individual species (Altig et al., 2007).

A more recent study has demonstrated a more complex interaction following the application of atrazine (117 μg/L) to mesocosms containing several species of amphibians and the snails which were host to trematode parasites known to parasitize developing tadpoles (Rohr et al., 2008b). Firstly, atrazine was demonstrated to directly compromise the immune function of *R. clamitans* and *Rana palustris* tadpoles (Section 4). At the same time, atrazine also reduced the phytoplankton resources, and as an indirect consequence of increased water clarity, promoted the growth of periphyton, which in turn promoted the subsequent growth of trematode-bearing snails, thereby increasing the prevalence of trematode cercariae, and the likelihood of trematode infection among immuno-suppressed tadpoles (Fig. 5).

Conversely, several studies have also demonstrated that amphibians can benefit from the application of an insecticide, by eliminating competitors and thereby increasing the food resources available for tadpoles (Boone and Semlitsch, 2001, 2002; Bridges and Boone, 2003; Boone et al., 2004; Rohr and Crumrine, 2005; Boone and Bridges-Britton, 2006) or by eliminating predators (Boone and Semlitsch, 2003; Mills and Semlitsch, 2004; Relyea et al., 2005) that were more sensitive to the pesticides (Fig. 5). However, Relyea and Diecks (2008) recently demonstrated that a cascade of events can still counteract the beneficial consequences removing competitors/predators. Following the addition of malathion (10–250 μg/L) to 1000 L mesocosms, heterospecific competitors (zooplankton) were quickly eliminated, which promoted the proliferation of phytoplankton (as measured by chlorophyll *a* concentrations). However, 3–4 weeks after the initial application of malathion, the phytoplankton bloom also reduced light penetration, which subsequently reduced periphyton – the main source of food for foraging tadpoles. As a consequence, growth and development among leopard frog (*R. pipiens*) tadpoles was impaired (reduced survival, lower mass at metamorphosis, delayed metamorphosis) several weeks after the pesticide had dissipated (Fig. 5).

9. Conclusion

The issue of amphibian population decline, has lead to an increasing public awareness of the importance of remnant amphibian populations wherever they might occur. The presence of amphibians within highly altered agricultural environments is remarkable, but the subsequent disappearance of some species raises concern about the risk of application of chemicals associated with agriculture to both larval and adult populations. However, demonstrating a link between population declines and the toxic effects of agricultural chemicals is difficult. In one case where there is strong evidence that pesticide exposure has directly impacted on amphibian populations (Davidson and Knapp, 2007), the actual mechanism of toxicity remains unknown, and the links between declines and pesticide usage are drawn from multivariate statistical treatments of data about the presence or absence of *Rana muscosa* in California's Sierra Nevada. The authors still needed to speculate as to the mechanisms by which pesticides affect individual frogs.

Ecotoxicology as a discipline is only slowly developing the tools required to elucidate the role of agricultural chemicals in amphibian population decline. If the last 20 years have taught us anything, it is that traditional laboratory toxicity assays examining a single stressor or chemical will not provide the answers we seek. Despite this assertion, there has been a wealth of studies over the last decade investigating the sub-lethal effects of agricultural chemicals and fertilizers to anurans. Some important steps have been made in understanding the risks associated with chemical use in these environments including the identification of potentially

hazardous chemicals and the mechanisms by which these interfere with development, growth, reproduction and survival of amphibians. Important findings linking effects with particular modes of action or pathways, such as via the endocrine system (both hypothalamus-pituitary–thyroid and hypothalamus–pituitary–gonad axes), retinoid homeostasis and immune responses to name a few, have advanced the knowledge base. However, these approaches are still in their infancy, and will need further development. Also, the majority of these studies have involved single chemical exposures, and although they provide important first steps in understanding responses, they do little to draw conclusions regarding the health of populations in complex natural environments.

The uncertainties lie with the fact that the agricultural landscape is constantly in a state of flux. Seasonal cropping; crop rotation and land use changes; differences in chemical use, formulations and application rates; regional variation in pond community structure and environmental chemistry; and both broad-scale and local changes in climatic conditions. All these factors create difficulties in defining and attributing cause and effect.

A tiered approach is essential. At one end of the spectrum of ecotoxicology studies, statistical treatments of presence/absence data will provide insights about which factors are likely involved in population declines. At the other end of the spectrum, single species, single chemical studies are still required to understand mechanisms of toxicity. It is arguable that these kinds of studies should be performed with a reference species. *X. laevis* is already well entrenched as the standard laboratory model (Mann, 2005), but it should also be used as a template against which species variability can be gauged. Test methodology should also be standardized. The larval (tadpole) stages encompass a large suite of transient morphological and physiological changes which occur between fertilization and metamorphosis. Therefore, chemical exposures, even if they are relatively short, or targeted towards a specific developmental stage, should be applied within the context of the full developmental cycle, because developmental effects might not be apparent until much later.

Both these approaches can be performed independently. However neither will provide the links between population decline and chemical toxicity. Between the two extreme tiers in ecotoxicology described above, there has been a growing trend to investigate mixtures of different pesticides and fertilizers, and in the presence of various biotic and abiotic factors, in an attempt to inject some environmental realism in experimental exposures. Mesocosm studies in particular play an important part in bridging the divide between laboratory and field by providing a more relevant and realistic approach to an examination of risk in an agricultural landscape (Mann, 2005). Clearly, the resource intensive nature of these kinds of studies and the countless combinations of chemicals and environmental factors that are likely to interact make it impossible to examine all possible scenarios. However, they provide a means to establish a range of paradigms that are likely to affect amphibian health and survival when agricultural chemicals are present within a complex environment. For example, evidence from mesocosm studies has highlighted the intricate nature by which ecological and chemical principles interplay and produce results such as trophic cascades.

With increased coordination among these various experimental approaches, the risks that exposure to agricultural chemicals poses towards amphibian populations may become more apparent.

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