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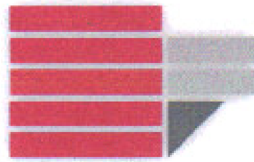

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Effects of long-term exposure to two fungicides, pyrimethanil and tebuconazole, on Italian tree frog (*Hyla intermedia*)

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Introduction

A consequence of human population growth is the increase of global agricultural production and therefore the increased use, abuse, or misuse of pesticides all over the world (Köhler and Triebskorn, 2013). Aquatic biota inhabiting agricultural areas are often exposed to a wide variety of pesticides that reach freshwater ecosystems through un-intended direct application, spray drift and runoff, thus posing a potential risk for non-target species (Wagner *et al*., 2014).

Amphibians are currently the most globally threatened group of vertebrates and have experienced a drastic population decline. Among other established causes for this decline (e.g. habitat loss, increased disease susceptibility, climate changes), pesticide contamination is considered a primary factor (Bernabò *et al*., 2011a; Brunelli *et al*., 2009; Smalling *et al*., 2013, 2015). Amphibians are particularly sensitive to pollution due to their complex life cycle and permeable skin; in fact, in amphibians skin permeability is higher than in any other order of vertebrates and therefore the percutaneous absorption of xenobiotics is greater (Quaranta *et al*., 2009). Aquatic habitats, in which amphibians live, breed and develop, are easily contaminated by a range of pollutants, and in agricultural landscapes the likely exposure scenario is represented by pesticides (Aldrich *et al*., 2016; Brühl *et al*., 2011, 2013; Fryday and Thompson, 2012; Mann *et al*., 2009). Many of these substances have been shown to exert their adverse effects through modulation and/or disruption of endocrine functions, and are known as endocrine disrupting chemicals (EDCs) (Hayes *et al*., 2006; McKinlay, 2008; Orton and Tayler 2015, Orton *et al*., 2011,). Given the crucial role of the endocrine system in the

maintenance of numerous biological, physiological and behavioural functions, damage in any part of this complex system can lead to serious disease or death.

A number of laboratory-based studies demonstrated that in amphibians, a perturbation of the endocrine system has the potential to dramatically affect all biological processes, including growth, development, gonadal differentiation, hormone levels and liver function (Bernabò *et al*., 2011b; Hayes *et al*., 2006, 2010; Higley *et al*., 2013; Kloas, 2002; Navarro-Martín *et al*., 2014; Orton and Tayler, 2015). There is also good evidence that amphibian populations, living in agricultural areas, have been affected by many endocrine-related disorders that can be linked to endocrine disrupting potential of pesticides (e.g. skewed sex ratio, greater incidences of gonadal anomalies, male and female reproductive dysgenesis, altered secondary sex characteristics, sex steroid and thyroid hormone disruption) (McCoy *et al*., 2008; McDaniel *et al*., 2008; Orton and Routledge, 2011; Papoulias *et al*., 2013).

In recent years pesticide use is changing and fungicides have become the most important component of pest and disease management programs in modern agriculture (Knäbel *et al*., 2014; Reilly *et al*., 2012). Unlike most other types of agrochemicals, fungicides are mainly applied several times throughout the growing season for prophylactic purposes (Knäbel *et al*., 2014). As a result of repeated applications, chronic exposure scenario becomes most likely, and the concentrations often exceed the chronic toxicity values of concern (Belden *et al*., 2010; Deb *et al*., 2010; Reilly *et al*., 2012). Furthermore, most fungicides do not have specific modes of action and they may be toxic to a wide range of organisms (Maltby *et al*., 2009). Despite the widespread occurrence of fungicides in aquatic

environments, ecotoxicological data are surprisingly sparse, compared with other types of pesticides (Reilly *et al*., 2012; Smalling *et al*., 2013; Wightwick *et al*., 2012). Fungicides are generally or entirely overlooked by amphibian conservation biologists (Ghose *et al*., 2014).

Available data indicate that fungicides, at environmentally relevant concentrations, can induce several harmful effects on amphibians, such as increased mortality and deformity (Brühl *et al*., 2013; Méndez *et al*., 2016; Yu *et al*., 2013a), decreased and/or increased growth rate and development (Brande-Lavridsen *et al*., 2010; Hartman *et al*., 2014; Higley *et al*., 2013; Yu *et al*., 2013b), alteration of behaviour (Teplitsky *et al*., 2005), immunosuppression and lipid peroxidation (McMahon *et al*., 2011; Strong *et al*., 2016a). Most if not all of those harmful effects could be related to endocrine disturbance and actually a variety of fungicides are known or suspected to act as EDCs (Matthiessen and Weltje, 2015; McKinlay, 2008; Orton *et al*., 2011; Poulsen *et al*., 2015).

In this context, we simulated a chronic contamination using two different common-used compounds, tebuconazole and pyrimethanil. These two fungicides are extensively applied on cereals, vineyards, fruits, vegetables and ornamentals with protective, curative, and eradicative purposes (EFSA, 2006, 2014) and are among the most frequently detected and relatively persistent pesticides in both surface waters and sediments (De Gerónimo *et al*., 2014; Herrero-Hernández *et al*., 2013; Robles-Molina *et al*., 2014; Smalling *et al*., 2012, 2015; Thomatou *et al*., 2012; Wightwick *et al*., 2012).

Information on pyrimethanil acute and chronic toxicity is scarce especially on vertebrates. Seeland and colleagues (2012) reported that pyrimethanil LC_{50}/EC_{50} values (concentration causing 50% mortality/effect of exposed population) for

aquatic organism range from 1.18 to 46.1 mg/L. Available study on fish reported that lethal concentration for the rainbow trout (*Oncorhynchus mykiss*) ranges between 14 and 35 mg/L (van Leeuwen and Vonk, 2008), whereas Araújo and collaborators (2014a) found that pyrimethanil, even at non-lethal concentrations, could be environmentally disruptive by triggering spatial avoidance in juveniles of *Danio rerio* exposed to a pyrimethanil gradient. The same authors demonstrated in two amphibian species exposed to pyrimethanil-contaminated water that spatial distribution was influenced by the presence of fungicide (Araújo *et al.*, 2014b).

Tebuconazole is moderately toxic to freshwater fishes and $LC_{50\text{-}96h}$ values range from 2.37 to 19.6 mg/L (EFSA, 2014; Kreutz *et al.*, 2008; Sancho *et al.*, 2010; Toni *et al.*, 2011a). The exposure to sublethal concentrations of tebuconazole may affect fish growth and survival, and induce oxidative stress, physiological impairment, changes in metabolism, chronic inhibition of the stress response and severe hepatic cell injuries (Ferreira *et al.*, 2010; Koakoski *et al.*, 2014; Sancho *et al.*, 2010; Toni *et al.*, 2011a,b). Moreover, it has been shown that this fungicide has bioaccumulation potential in fish (Andreu-Sánchez *et al.*, 2012; Konwick *et al.*, 2006). Concerning amphibians, previous studies have reported the occurrence of tebuconazole in frog tissue (Hansen *et al.*, 2014; Poulsen *et al.*, 2015; Smalling *et al.*, 2013).

In spite of their widespread occurrence in environmental compartments, no previous study has examined sublethal effects of tebuconazole and pyrimethanil on amphibians. On this basis, we performed a long-term exposure to environmentally relevant concentrations (5 and 50 µg/L) of tebuconazole and pyrimethanil, on a native species to Italy *Hyla intermedia.* This species was chosen

because typically lives in inland water bodies and it is frequently found in agricultural areas.

In order to have a more comprehensive overview of the fungicides induced effects we evaluated survival, growth, developmental traits and incidence of deformities, and we also explored indicators of post-metamorphic fitness such as mass at and time to metamorphosis. This analysis is very informative to determine the factors that may affect amphibian population in nature.

In evaluating the effects of a toxicant in amphibians it is important to consider also lagged effects that may not become evident until metamorphosis (Bernabò *et al*., 2011b; Hayes *et al*., 2006; Tamschick, 2016b). On this basis, here we focused on the effects induced by an exposure to pyrimethanil during the whole developmental period, in *H. intermedia* juveniles.

Several studies in mammalian and fish models, both *in vitro* and *in vivo*, suggested that pyrimethanil may influence the biosynthesis of sexual hormones and/or interact with sexual hormone receptor thus acting as EDC (Ankley *et al*., 2005; Medjakovic *et al*., 2014; Orton *et al*., 2011; 2014; Prutner *et al*., 2013;). Given the fact that EDCs are responsible of many adverse reproductive outcomes in developing amphibians we first evaluated gonads histology, in order to identify putative effects on sex ratio and gonadal differentiation. EDCs may also act through broader mechanisms/pathways than firstly recognized, exerting different effects in a tissue specific manner (Bernabò *et al*., 2014; Haselman *et al*., 2016), therefore we also analysed morphological alterations in two organs highly susceptible to xenobiotic toxicity.

The kidney is an important site of injury after chemical exposure, due to their involvement in a number of interrelated functions (i.e. maintenance of internal

water, ion, and acid-base balance, selective reabsorption and secretion of ions and organic molecules, and excretion of nitrogenous and other waste products of metabolism) (Cakici, 2015; Fenoglio *et al*., 2011; Strong *et al*., 2016b). The liver has long been considered the major target organ for most chemicals, including EDCs, in consequence of its essential functional features (i.e. maintaining of the metabolic homeostasis of the body including protein synthesis, storage metabolites, detoxification and inactivation of harmful substances) (Bernabò *et al*., 2014; de Oliveira *et al*., 2016; Melvin *et al*., 2013).

To our knowledge no previous studies have investigated the effects of pyrimethanil on the selected organs in amphibians.

Chapter 1

Endocrine Disruptor Pesticides

Endocrine disrupting chemicals (EDCs) are compounds that are able to modify the normal functioning of the endocrine system of both wildlife and humans. A large number of chemicals have been identified as endocrine disruptors, among them several pesticides (Mnif *et al.*, 2011).

In industrialized countries, with the Green Revolution of the 1960s the agricultural productivity greatly increased and numerous pesticides have been developed and used extensively worldwide, in order to eradicate the threatening pathogens, with few guidelines or restrictions (Mellanby, 1992; Briggs, 2009). This fight, therefore, requires the massive use of pesticides, which are hazardous chemicals designed to repel or kill rodents, fungi, insects, and weeds that undermine intensive farming. Pesticides represent a great benefit for increased food production (Cooper and Dobson, 2007). However, many pesticides for agricultural use, or agrochemicals, are harmful to the environment, in fact, they can persist in soils and aquatic sediments, bioconcentrate in the tissues of invertebrates and vertebrates, move up trophic chains, and affect top predators including humans (Mnif *et al.*, 2011).

Worldwide consumption of agrochemicals is steadily rising and consequently humans and wildlife are now continuously exposed to various of agrochemicals though the

environment (surface water, groundwater, soil), food and drinking water (Kolpin *et al.*, 2000)

The World Health Organization has reported that roughly three million pesticide poisonings occur annually, resulting in 220,000 deaths worldwide (WHO, 1992, 2007)

In some cases, it has been suggested that diseases such as cancer, allergies, neurological and reproductive disorders may be connected to pesticide exposure.

1.1. Effects of Endocrine Disruptor Pesticides

More than 100 substances of the several identified EDCs are pesticides (Andersen *et al.*, 2002; Kojima *et al.*,2004; Lemaire *et al.*, 2006a,b; Vinggaard *et al.*, 2000). Of these, 46% are insecticides, 21% herbicides and 31% fungicides (Mnif, *et al.*, 2011). In general, EDCs interfere with the hormonal homeostasis binding to estrogen or androgen receptors. More in detail, EDCs can act as receptor agonist miming the natural hormone's action, thus binding to and activating various hormone receptors, including androgen, estrogen and aryl- hydrocarbon receptors. EDCs can also act as an antagonist by binding to these receptors without activating them and consequently inhibiting their action. Finally, EDCs can interfere through different feedback mechanisms with the synthesis, transport, metabolism and elimination of hormones, thus decreasing the concentration of natural hormones (Tabb and Blumberg, 2006).

At the environmental level, wildlife is particularly vulnerable to the endocrine disrupting effects of pesticides (Mnif *et al.*, 2011). Endocrine-related effects have been amply observed in invertebrates (Ellis and Pattisina, 1990; Gooding *et al.*, 2003; Heidrich *et al.*, 2001), reptiles (Bishop *et al.*, 1991,1995; Guillette *et al.*, 1995, 1996,

1999), fish (Munkittrick *et al*.,1991; Purdom *et al*., 19994), birds(Crisp *et al*., 1998; Fry, 1981; Fry *et al*., 1987; Tyler *et al*., 1998) and mammals (Facemire *et al*., 1995; Oskam *et al*., 2003; Reijnders, 1986) as reviewed by Mnif and colleagues (2007*)*. Most of this effects are related to exposure to organochlorine pesticides (OC) and disturb the reproductive function. For example, endosulfan sulphate affects embryonic development and juvenile hormone activity of *Daphnia magna* (Palma *et al*., 2009a,b). Another OC, linuron, influences the production of reproductive hormones in rats after an in utero exposure; testosterone levels was strongly reduced, whereas progesterone production was not affected (Wilson *et al*., 2009).

Endocrine disrupting pesticides are also able to interfere with the reproductive and sexual development in humans, and many of these detrimental effects occur during gametogenesis and the early development of the fetus (Anderson, 2000; Hardell *et al*., 2006; Sharpe, 2006; Skakkebaek, 2001; Sultan *et al*., 2001; Waliszewski *et al*., 2000). Nevertheless, the effects may not become apparent until adulthood. Also, fetuses and newborns receive larger doses of EDCs because of the mobilization of maternal fat stores during pregnancy and breastfeeding (Anderson, 2000; Hardell *et al*., 2006; Przyrembel *et al*., 2000; Skakkebaek, 2001; Waliszewski *et al*., 2000). Infants are tremendously susceptible to pre and postnatal exposure to these compounds, resulting in a large variety of negative health effects, including the likely long-term impact on intellectual function (Eskenazi *et al*, 2006 ;. Jacobson and Jacobson, 1996) and the lagged effects on the central nervous system functioning (Beard, 2006; Ribas-Fito *et al*., 2003).

1.2. Fungicides

Nowadays many fungicides are known or suspected to act as EDCs (Orton *et al*., 2011; Poulsen *et al*., 2015; Matthiessen and Weltje, 2015; McKinlay, 2008). Fungicides are chemical or biological agents that specifically inhibit or kill fungi underlying diseases important to man. Fungal infections may cause severe damage in agriculture resulting in critical loss of yield, quality and profit (Oruc, 2010; Rouabhi, 2010).

1.2.1. Use

Fungicides are used both in agriculture and in the struggle against fungal infections in humans and animals. In agriculture, these pesticides are applied to protect the fruits, tubers and vegetables during storage or are applied directly to grapes, ornamental plants, trees, field crops, cereals and turf grasses (Gupta and Aggarwal, 2007).

In veterinary medicine, fungicides are commonly used in the treatment of foot rot disease but are also utilized to repel and kill slugs and snails (Ortolani *et al*., 2004). Another example of dual use of fungicides is the treatment of intestinal parasites in both human and veterinary medicine with the widely used agricultural fungicide thiabendazole (Lorgue *et al*., 1996).

In addition, many fungicides are used to protect industrial products during shipment, remove molds from painted surfaces, wood preserving, controlling fungal growth in paper production and protect the carpets integrity (Osweiler *et al*., 1985).

1.2.2. Classification

The classification systems proposed by different authors for fungicides are numerous. Most of these are based on the chemical structure but this kind of classification somewhat lead to confusion rather than providing a streamlined list. In addition to classification for chemical structural group, fungicides can be classified according to their utilization (agricultural, clinical).

According to their origin, fungicides can be classed into two main groups (Rouabhi, 2010):

- ***Biologically based fungicides*** (biofungicides) that contain living microorganisms, bacteria or fungi, that are antagonistic to the pathogens that cause the disease.
- ***Chemically based fungicides*** that are synthesized from organic and inorganic chemicals. most of the fungicides that are sold throughout the world are chemically-based.

A further classification of fungicides can be made according to the topical activity, we distinguish four groups (Rouabhi, 2010):

- ***Contact fungicides***. They are able to act only on the surface of the plants without being absorbed from leaves, stems or roots, so they cannot inhibit the fungal growth from the inside of the plants (dithiocarbamates, nitriles, aromatic hydrocarbons, peroxides, phenylpyrolles, cyanoimidazoles).
- ***Localized penetrants.*** These fungicides inhibit fungi on treated plant surfaces and inside treated leaves. They are absorbed only by the treated leaves and

cannot move from one leaf to another. They are not absorbed by the roots (dicarboximides, most of the strobilurins).

- ***Acropetal penetrants.*** These fungicides can penetrate inside the plants through roots, shoots and leaves. They inhibit fungi on and in treated plant surfaces and inside plant parts that lie above the treated surface. (benzimidazoles, triazoles, pyrimidines, carboximides, acylalanines, plus the strobilurins azoxystrobin and fluoxastrobin).
- **Systemic fungicides.** They are the only fungicides able to be absorbed into xylem and phloem and moves up and down in plants. These fungicides inhibit fungi on and in treated plant surfaces and inside plant parts that lie above or below the treated surfaces (phosphonates).

Fungicides can be also divided into two groups based on mode of action in fungal cells (Rouabhi, 2010):

- ***Site-specific inhibitors,*** that target individual sites within the fungal cell.
- ***Multi-site inhibitors,*** that target many different sites in each fungal cell.

1.2.3. Environmental risk and toxicology

Given the crucial role in the agricultural sector, in recent years the use of fungicides has increased significantly (Battaglin *et al.,* 2010). In fact, most of the diseases caused by fungi is difficult to eradicate and requires a massive and repeated use of fungicides. Furthermore, modern farming practices also include the adoption of preventive strategies to control the spread of fungal diseases and this involves a regular

application of these substances for the whole plant growing season, even when infection is not present (Wightwick *et al.,* 2012).

As a result, the frequent use of fungicides may pose a risk to the environment, particularly if the residues persist in soil or migrate out of the application site, with negative consequences for the health of both terrestrial and aquatic ecosystems (Komarek *et al.,* 2010; Wightwick and Allinson, 2007; Wightwick *et al.,* 2010). A limited number of studies have taken into account the presence of these compounds in surface waters and sediments. The few available data reveals that residues of fungicides are the most frequent and can reach high percentages in the samples analyzed (Gregoire *et al.* 2010; Rabiet *et al.* 2010; Smalling *et al*., 2012; Wightwick *et al*. 2010; 2012). These studies also indicate that the risk of contamination for aquatic ecosystems is more likely ascribable to long-term rather than short-term exposure (Wightwick *et al*., 2012). Aquatic organisms, therefore, may be exposed to fungicides through: atmospheric transport, runoff, leaching and runoff, the direct application of spray, the spray drift and the movement of animals through the fields during application (Junges *et al*., 2012; Belden *et al*., 2010). Moreover, for the majority of fungicides on the market there are no guide values on the concentrations which may cause adverse effects on non-target organisms (e.g. LC_{50}, EC_{50}) (Frampton *et al.,* 2006; Maltby *et al.,* 2009; Wightwick *et al.* 2010; 2012).

The understanding of the specific mechanisms of action of fungicide and their toxicity is important because even humans, cattle and pets meet these substances through a wide range of applications. In fact, every year, livestock are poisoned accidentally by these pesticides. The available toxicological data examined the detrimental effects of

fungicides on model laboratory animals (rats, mice, rabbits) offering limited data on livestock, wild animals and pets. (Gupta and Aggarwal, 2007). In general, fungicides have been defined as low/moderate toxic for mammalian, although they are believed to have a higher overall incidence than other pesticides to cause developmental toxicology and oncogenesis (Costa, 1997).

It was estimated that over 80 per cent of oncogenic risk related to pesticides use, comes from a few fungicides (NAS, 1987).

Theoretically, since morphology and physiology of fungi greatly differ from those of other forms of life, fungi can be efficaciously fought by low toxicity compounds to other organisms, particularly mammals. (Edwards *et al*., 1991). However, since the mechanism of injury to pathogenic fungi may be different from that affecting mammals, it is possible that the two properties may coexist in a fungicide molecule

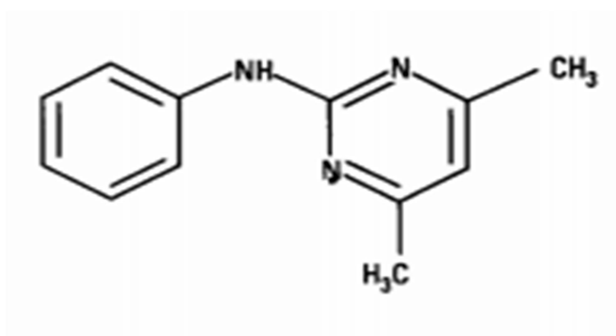
(Marrs and Ballantyne, 2004). Since the mechanisms of action and metabolic clearance differ among the various existing fungicides, the specific effects (reproductive, teratogenic, mutagenic, carcinogenic) that may occur will be very different according to the poison ingested.(Hayes and Laws, 1990; USEPA, 1999). In addition, some animals may be more vulnerable to fungicide contamination than others due to their physiology and/or behaviour. For example, some fungicides (e.g., copper sulphate, thiram, chlorothalonil and captan) have especially toxic effects on fish (Pimentel, 1971; Lorgue *et al*., 1996; Tomlin, 2000), and bees (Hartley and Kidd, 1983). The kinds of fungicides used in both agricultural and industrial practices range from those of relatively low toxicity to those that may be lethal to animals.

Fungicides that are frequently used around the home pose a serious risk to pets and livestock consequently to lack of attention and misapplication (Osweiler *et al*., 1985; Gupta and Aggarwal, 2007; Oruc *et al*., 2009). For example, fungicides have caused systemic poisoning in animals such as sheep (Ortolani *et al*., 2004; Oruc *et al*., 2009), poultry (Guitart *et al*., 1999), and humans (Israeli *et al*., 1983; Kintz *et al*., 1997; Chodorowski, 2003; Kayacan *et al*., 2007; Calvert *et al*., 2008; Mortazavi and Jafari-Javid, 2009). incorrect application while using fungicides are probably responsible for a disproportionately large number of injuries irritating to skin and mucous membranes, as well as skin sensitization. Fungicides are often utilized in combination with other pesticides and adjuvants or solvents, which, together, may be more toxic (Osweiler *et al*., 1985). In France, Lorgue *et al*. (1996) reported that pesticides are the most common cause of animal poisoning (45.5%), with fungicides accounting for 6.1% of all pesticides. The two most commonly involved species are dogs and cattle. In 2003, 992 cases involving dogs and cats were confirmed as poisoning in France, and fungicides caused 2.8% of all poisonings (Barbier, 2005). Acute fungicide poisonings was 4.4% in 129 poisoning cases in Greece (Berny *et al*., 2009). In Italy, poisoning related with fungicides account was 8.1% of pesticides in pet poisonings (Caloni *et al*., 2004).

Chapter 2

Pyrimethanil and Tebuconazole

2.1. Pyrimethanil



The fungicide pyrimethanil [N-(4, 6-dimethylpyrimidin-2-yl)-aniline; CAS number 53112-28-0] is an anilinopyrimidine fungicide that inhibits the secretion of fungal enzymes produced in the infection process (FAO/WHO, 2007; EFSA, 2011). It was developed to act on resistant fungi strains, mainly to control *Botrytis cinerea* in grapes, *Venturia inaequalis* in apples and *Botrytis* spp. in protein peas (EFSA, 2006), consequently its use has increased greatly in the lasted years (Smilanick *et al.*, 2006; Sugar and Basile, 2008). Pyrimethanil rapidly penetrates the cuticle and inhibits the secretion of fungal enzymes required for the infection process, blocking the ability of fungi to degrade and digest the plant tissues, thus stopping the disease (Araújo *et al*., 2015; EFSA, 2006).

The commercial products that contain pyrimethanil as active ingredient are Clarinet®, Mythos®, Rubin®, Scala®, Siganex®, Vision®, and Walabi®, which are

currently used both pre- and post-harvest to protect various crops such as apple, banana, carrot, citrus, grape, melon, onion, potato, strawberry, and tomato (Smilanick *et al.*, 2006; EFSA, 2011; Sirtori *et al.*, 2012)

According to the EFSA report (2006), pyrimethanil does not bioaccumulate, is quickly eliminate once orally absorbed, is not teratogenic and has a low acute toxicity. However, several authors have demonstrated that pyrimethanil has the potential to induce negative effects on non-target organism after both acute and chronic exposure (Verdisson *et al.*, 2012; Shinn *et al.*, 2015; Seeland *et al.*, 2012, 2013; Araújo *et al.*, 2014a,b). Unluckily, despite the intensive agricultural use of this fungicide, there is a lack of information regarding the effects on adjacent aquatic ecosystems.

This is perhaps in relation to the assumption that pyrimethanil has a short half-life and, consequently, the possible toxic effects may occur in the short term, but are reduced to a minimum at long-term (EFSA, 2006; PPDB, 2009). Chemical and (eco)toxicological characteristics of pyrimethanil, published by EFSA, are summarized in Table 2.1 (Araújo *et al*., 2015; EFSA, 2006).

The detected concentrations in surface water and sediments for pyrimethanil range between 0.06–90 μg/L for pyrimethanil (EFSA, 2006; Gregoire *et al*., 2010; Herrero Hernández *et al*., 2013; Kreuger *et al*., 2010; Seeland *et al*., 2013; Thomatou *et al*., 2012; Wightwick *et al*., 2012).

Chemical name (IUPAC)		*N*-(4, 6-dimethylpyrimidin-2-yl) aniline
Chemical name (CA)		4, 6-dimethyl-*N*-phenyl-2-pyrimidinamine
Molecular formula		$C_{12}H_{13}N_3$
Molecular mass		199.28 g mol^{-1}
Temperature of decomposition		189.54 to 344.74 °C
Flammability		Not flammable
Explosive properties		Not explosive
Skin irritation		Not irritating
Eye irritation		Not irritating
Genotoxicity		No evidence
Degradation time in water and sediment	DT50 water	8.9 to 24 days
	DT90 water	70 to 99 days
	DT50 whole system	40 to 121 days
	DT90 whole system	Not stated and 134 days
Toxicity for aquatic organisms	Rainbow trout	LC_{50} (96 h):10.56 mg/L
	Daphnia sp.	EC_{50} (96 h): 2.9 mg/L
	Green alga	EbC_{50}/ ErC_{50} (96 h): 1.2/5.84 mg/L
	Daphnia magna	NOEC (reproduction, 21 d):0.94 mg/L
	Chironomus riparius	NOEC (emergence, 28 d) 4.0 mg/L
Ecotoxicological data		Harmful

CA: Chemical Abstract; DT50 and DT90: period required for 50% and 90% dissipation; EC_{50}: median effective concentration;EbC_{50}: the concentration at which 50% reduction of biomass is observed, ErC_{50}: the concentration at which 50% reduction of growth rate is observed, IUPAC: International Union of Pure and Applied Chemistry; LC_{50}: median lethal concentration; NOEC: no observed effect concentration.

Table 2.1. Chemical and (eco)toxicological characteristics of pyrimethanil (from Araújo *et al.*, 2015).

2.1.1. Toxicity of pyrimethanil

The toxic effects induced by pyrimethanil exposure on different aquatic animals have been investigated in the last years. For *Daphnia magna* the 96 h LC_{50} (lethal concentration to 50% of exposed organisms) of pure pyrimethanil ranged from 1.2 to 2.9 mg/L , while the NOEC (no observed effect concentration) on reproduction after 21 days of exposure range from 0.5 to 0.9 mg/L (EFSA, 2006; Seeland *et al.*, 2012). In addition, *D. magna* exposed to 1.0 mg/L pyrimethanil did not produce a F1-generation (Seeland *et al.*, 2012). The EC_{50} (Half maximal effective concentration) for the reproduction of the *D. pulex* was 0.69 mg/L and the NOEC was 0.015 mg/L (Scherer *et al.*, 2013). The 96 h LC_{50} for a rainbow trout population (*Oncorhynchus mykiss*) was 14 mg/L- pyrimethanil, whereas the NOEC for the parameter dry weight was 0.07 mg/L pyrimethanil (van Leeuwen *and* Vonk ,2008).

In a recent study conducted by Mosleh and collegous (2014) the aquatic worm *Tubifex tubifex* has been used as model species to assess the toxicity of pyrimethanil. In particular, the endpoints of the study were survival rate and oxidative stress index. Despite the LC_{50} values after 7days was 39 mg/L, and after 1 day was 49 mg/L, the authors observed after exposure to a sub-lethal concentration (25 mg/L) an increased activity of catalase and a decreased activity of glutathione-*S*-transferase (Mosleh *et al*., 2014).

A novel approach to evaluate the toxicity of pyrimethanil in a probable global change scenario has been developed by a group of German researchers (Müller *et al*., 2012; Seeland *et al*., 2012; Scherer *et al*., 2013). Scientists have based these studies on the assumption that the warm and humid climate expected in the coming years, will

probably lead to the appropriate conditions for the fungus growth, and consequently an increase in the use of fungicides (Müller *et al*., 2012). Thus, they assessed if the harmful effects of pyrimethanil on the studied species (*C. riparius, D. magna, D. pulex, P. acuta*) change with increasing temperature. Lethal pyrimethanil toxicity to *C. riparius* increased when combined with increasing temperature (Seeland *et al*., 2012). They observed that when exposed to 2 mg/L of pyrimethanil the genetic diversity in *C. riparius* cohorts decreased for multiple generations in dependence of thermal variation; genetic diversity was reduced by about 20% under thermal simulation of a typical cold or hot year and by 42% in a temperature regime for a warm hypothetical year (Müller *et al*., 2012). Even the thermophilic snail *P. acuta* showed higher susceptibility to toxic effects of pyrimethanil at warmer temperatures (Seeland *et al*., 2013).

Recently, in an in vitro study it was demonstrated that pyrimethanil is able to modulate the pathway of estrogen (ERα) and androgen (AR) receptors and the activity of the aryl-hydrocarbon receptor (AhR). It has been observed that an AhR-agonist effect may involve alteration in several physiological pathways such as: cellular differentiation and division, hormonal and growth factors metabolism. Moreover, the activation of the AhR may also have antiestrogenic effects (Medjakovic *et al.,* 2013).

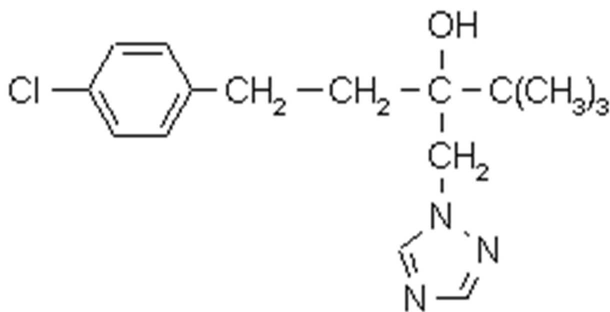
As regards mammals, short term toxicity of pyrimethanil was studied in dietary 90-days studies in rats and mice, and in 90- days and 1-year studies in dogs. In the rat study the main detrimental observed effect were increase of weight and hypertrophy of the liver and follicular epithelial hypertrophy and colloid depletion of thyroid. In

mice, the relevant findings concerned the liver were changes in clinical parameters and increased organ weight; in the thyroid necrosis of follicular epithelial cells were frequently detected along with tubular dilatation of the kidneys and urinary bladder hyperplasia. In dogs relevant findings comprised clinical signs, retardation of body weight gain and some minor effects in hematological and biochemical parameters. In addition, in both dog studies, a dose-related marked decrease in water intake was observed, which was considered an adverse effect. (EFSA, 2006).

For assessing long term toxicity of pyrimethanil a combined chronic toxicity/carcinogenicity 2-year study was conducted in rats and an 18-months carcinogenicity study in mice (EFSA, 2006). In rats liver and thyroid have been identified as the target organs. Liver pathology comprised changes in biochemical parameters, increased organ weight and histological alterations at 5000 ppm. In the thyroid, microscopic examination revealed higher incidences of colloid depletion, hypertrophy of the follicular epithelium, deposition of intra-cytoplasmic brown pigment and focal hyperplasia of the follicular epithelium also at 5000 ppm. In addition, increased incidences of benign follicular cell tumors of the thyroid gland were evident in males and females at this high dose level. However, statistical significance was not reached. In mice, there were no treatment-related increases in the incidence of tumors following long-term treatment with pyrimethanil up to 1600 ppm suggestive of a carcinogenic effect. Additionally, there were no treatment-related differences in mortality, clinical signs, body weight or hematological parameters at any dose level. There was an increased incidence in morbidity and mortality in males of all groups, particularly during the first 52 weeks of the study, which was associated

with lesions in the urogenital tract. These findings (with no evidence of a clear dose-response relationship) were mostly considered to be caused by male aggression. However, the slightly increased incidence of urinary bladder distension evident in decedent males at 1600 ppm was suggested to be a possible effect of treatment (EFSA, 2006).

2.2. Tebuconazole



Tebuconazole (TBZ) (*α-[2 etile (4-chlorophenyl)] -1 dimethylethyl di a (1,)-1H-1,2, 4 triazole1-ethanol*) is a broad-spectrum azole fungicide that inhibits the biosynthesis of ergosterol, a component of yeast and fungal cell membranes (EFSA, 2014). Tebuconazole is used in agriculture and viticulture to control a range of fungal diseases. Tebuconazole is currently registered for use on peanuts, and recently on turf (golf courses and sod farms), ornamentals (residential and commercial uses), almonds, asparagus, barley, beans, corn (foliar and seed treatment), cotton, cucurbits, hops, lychee, okra, pecan, pistachio, pome fruit, soybean, stone fruit (except cherries), sunflower, turnip, and wheat (USEPA, 2007).

The chemical structure of TBZ is of synthetic nature and some of its chemical and toxicological characteristics are summarized in the Table2.2.

Field monitoring studies reported for tebuconazole a large variation in surface water concentrations depending on the pesticide application calendar and entry routes (e.g. direct overspray, runoff and rainfall events). The detected concentrations range between 0.02–200 μg/L (Berenzen *et al*., 2005; Deb *et al*., 2010; Elsaesser *and* Schulz, 2008; Herrero-Hernández *et al*., 2013; Knäbel *et al*., 2014; Rabiet *et al*., 2010; Robles-Molina *et al*., 2014; Wightwick *et al*., 2012)

Citing the assessment report of the Standing Committee on Biocidal Products: "Tebuconazole is not readily biodegradable and the biodegradation half-life in surface water is estimated to about 198 days. However, tebuconazole will be adsorbed to the sediment and therefore a dissipation half-life in surface water is estimated to be 43 days based on a water/sediment study. Tebuconazole is not metabolised rapidly in soil in laboratory experiments, the half-life for primary degradation is greater than one year. In field studies the dissipation half lives are 77 days. An accumulation of Tebuconazole in soil is not anticipated when tebuconazole is used as a wood preservative" (SCBP, 2013).

The action mechanism of tebuconazole is similar to that of the other azole fungicides. Tebuconazole is able to penetrate into the tissues of plants, showing an excellent antifungal activity (Zarn *et al.,* 2003).

The molecular mechanism behind the antifungal activity is due to the inhibition of the ergosterol synthesis. Ergosterol is a sterol precursor of vitamin D_2, an essential component of the cell membrane of fungi and yeasts, which performs the same

function in fungi that cholesterol does in animal cells. More in detail, the main effect is to inhibit 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway (Vanden Bossche *et al*., 1995). In consequence of the ergosterol depletion, the normal permeability and fluidity of the fungal membrane is altered, with secondary consequences for membrane-bound enzymes, such as those involved in cell wall synthesis (Marichal *et al*., 1985). The principal molecular target of azole antifungals is a cytochrome P450–Erg11p or Cyp51p, which catalyses the oxidative removal of the 14α-methyl group of lanosterol and/or eburicol in fungi by a typical P450 mono-oxygenase activity (Frank *et al*.,2003).

Furthermore, tebuconazole belongs to the group of triazole fungicides which are suspected to have endocrine disrupting properties (EFSA, 2008).

Chemical name (IUPAC)	(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4- triazol-1-ylmethyl)- pentan-3-ol
Chemical name (CA)	(±)-α-[2-(4-chlorophenyl)ethyl]- α -(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol
Molecular formula	$C_{16}H_{22}ClN_3O$
Molecular mass	307.8 g/mol
Temperature of decomposition	DTA-measurement: Exothermal reaction above 350 °C. TGA-measurement: A weight loss was observed above 165 °C. (99.5%)
Flammability	Not highly flammable (purity 98.1%)
Explosive properties	No explosive properties (purity 97.6%)
Skin irritation	Not irritating
Eye irritation	Not irritating
Genotoxicity	No evidence for genotoxic potential
Absorption, distribution, excretion and metabolism (toxicokinetics)	
Rate and extent of oral absorption	> 98% (based on urinary (7.4%) and biliary (90.9%) excretion within 48 hours
Distribution	Widely distributed, highest concentrations in kidney and liver
Potential for accumulation	No potential
Rate and extent of excretion	Rapid and extensively. 65-80% via faeces and 16-35% via urine
Metabolism in animals	Extensively metabolised by phase-1 oxidation and phase-2 conjugation
Toxicologically relevant compounds (animals and plants)	Tebuconazole and triazole metabolites
Toxicologically relevant compounds (environment)	Tebuconazole and triazole metabolites

Table 2.2. Chemical and (eco)toxicological characteristics of pyrimethanil (from EFSA., 2014).

2.2.1. Toxicity of tebuconazole

Single-dose toxicity testing with tebuconazole indicates that it is low in toxicity to mammals, bees, and worms but moderately toxic to birds, fish and other aquatic organisms In detail, the LD_{50} corresponded to: 3,252 mg/kg for mammalian, 1,988 mg/kg for avian species, > 83 ug/bee for honey bee or other insect, 1,381 mg/kg for anellida (USEPA, 2011; IUPAC, 2012); the LC_{50} calculated for fish and crustacean were respectively 4.4 mg/L and 2.8 mg/L, whereas no data is available for amphibians and mollusk (IUPAC, 2012). Unrefined risk assessments show that the level of concern is exceeded for freshwater fish, marine fish, and other aquatic organisms from runoff following three applications to turf at the rate of 1.4 pounds per acre (USEPA, 2011). Potential long-term exposures to birds eating contaminated vegetation and insects from large scale applications or exposures to eggs may cause toxicity (USEPA, 2011). The $LC_{50\text{-}96h}$ for tebuconazole on zebrafish was found to be 26.8 mg/L (Andreu-Sánchez, *et al*., 2012), while for Rainbow trout was 4.4 mg/L and for Bluegill sunfish 5.7 mg/L (Tomlin, 200).

In a recent study, adult males of *Danio rerio*, were exposed to a sublethal tebuconazole concentration of 230 mg/L for 7 or 14 days and allowed to recover for 7 or 14 more days, respectively. After the first hours of contact with the toxic substance, all the fish showed clear signs of poisoning, with pale skin, a reduction in swimming performance followed by periods of inactivity. These clinical signs tend to disappear slowly during the recovery period in pesticide-free water. In addition, the same authors showed that tebuconazole induces increased production of vitellogenin (Vtg) during treatment and during the 14 days following treatment. Even the levels of glucose, cholesterol,

triglycerides and lactate increased after 7 days of treatment and 14 days after exposure (Sancho *et al.,* 2010). Further studies on zebrafish have highlighted the many negative effects of such fungicide on homeostasis of thyroid hormones. In fact, after treatment with tebuconazole T4 levels are very low compared to control animals, while T3 levels are increased, indicating that the tissue of the thyroid gland may suffer significant damage (Yu *et al*., 2013a).

Sub-lethal concentration of tebuconazole are also able to induce changes in oxidative stress parameters as well as hepatic cell injuries in Silver catfish (Ferreira *et al*., 2010)

Studies conducted on adult male *Xenopus laevis* showed that the accumulation of tebuconazole is tissue-specific. High levels of this fungicide were found in adipose tissue, kidney, liver and brain; the presence of tebuconazole in the brain, indicates that it is able to pass through the blood-brain barrier and to determine neurological and neuroendocrine disorders (Poulsen *et al.,* 2015).

To assess the toxicity of tebuconazole on mammalian species the EFSA committed some short- term and long-term studies (EFSA, 2008).

Two separated studies are conducted on rats: a 90-day oral and a 21-day inhalation study. With dogs a 90-day and two 1-year oral studies were performed. With rabbits a 21-day dermal study was presented. The NOAEL in the rat 90-day oral study was set at 9 mg/kg bw/d based on liver enzyme induction, growth retardation and histopathology in the adrenals. In the inhalation study with rats a NOAEL of 0.0106 mg/L was obtained based on observations of induction of liver enzymes and slight clinical symptoms.

From the 90-day dog study a NOAEL of 8.3 mg/kg bw/d was derived based on body weight effects and clinical changes at the next higher dose while an overall NOAEL of 3 mg/kg bw/d was derived from the two 1-year studies based on findings of hypertrophy in zona fasciculata cells of the adrenals. No adverse effects were seen in the dermal study in rabbits up to the highest dose of 1000 mg/kg bw/d (EFSA, 2008).
Long term toxicity test were carried out on rats and mouse: a 2-year rat study and two 21-month mouse studies. In the chronic rat study a systemic NOAEL of 55.0 mg/kg bw/d was derived based on liver effects (pigment deposits in Kupffer cells). No tumours were observed up to the top dose.
From the two mouse carcinogenicity studies (employing the same strain) an overall systemic NOAEL of 5.9 mg/kg bw/d was derived from liver effects (changes in clinical chemistry and vacuolisation).
The experts concluded that the liver tumours occurring in the second study should be considered as not relevant for human risk assessment since the strain used was highly susceptible and the tumours occurred only at a dose exceeding the maximum tolerated dose (i.e. at the highest dose of 280 mg/kg bw/d). (EFSA, 2008).

Chapter 3

Studied species *(Hyla intermedia)*

Phylum	*Chordata*
Subphylum	*Vertebrata*
Class	*Amphibia*
Order	*Anura*
Family	*Hylidae*
Genus	*Hyla*
Species	*Hyla intermedia*

3.1. Taxonomy

The Italian tree frog (*Hyla intermedia*) is an endemic species of the Italian peninsula; based on genetic studies, it was distinct from the common tree frog (*Hyla arborea*), nevertheless, the two tree frogs share some morphological, ethological and ecological characteristics (Dubois, 1995; Nascetti *et al.,* 1995; Sindaco *et al.,* 2006)

3.2. General distribution

This species is largely restricted to mainland Italy and the island of Sicily (Italy); smaller populations are present on the edge of its range in southern Switzerland and western Slovenia (a single site on the Italian border) (Andreone *et al*., 2009; Lanza *et al.,* 2006; Sindaco *et al*., 2006).

The Italian tree frog appears common and relatively well distributed across much of the Italian territory, except for Sardinia, the Tuscan Archipelago, the Alpine regions and the Apennine ridge, where is rare and usually limited to the valley floors. In Valle d'Aosta may be extinct as the last known observation dates back to 1983. In addition, it is uncommon in Liguria, where it is replaced by *Hyla meridionalis* (Sindaco *et al.,* 2006). It appears also scarce in the southern regions of the peninsula, with the exception of Calabria where it is fairly widespread (Tripepi *et al.,* 1999) (Fig. 3.1). The species has an altitudinal range ranging from sea level to at least 1,855m asl (Andreone *et al*., 2009). However, the Italian tree frog appears to be linked mainly to lowland areas and hill (with a marked preference for altitudes of less than 400 m), although it may exceed 1000 m s.l.m. in some regions and reach 1800 m of altitude in Nebrodi Mountains, in the province of Messina (Lanza *et al.,* 2006; Sindaco *et al.,* 2006).

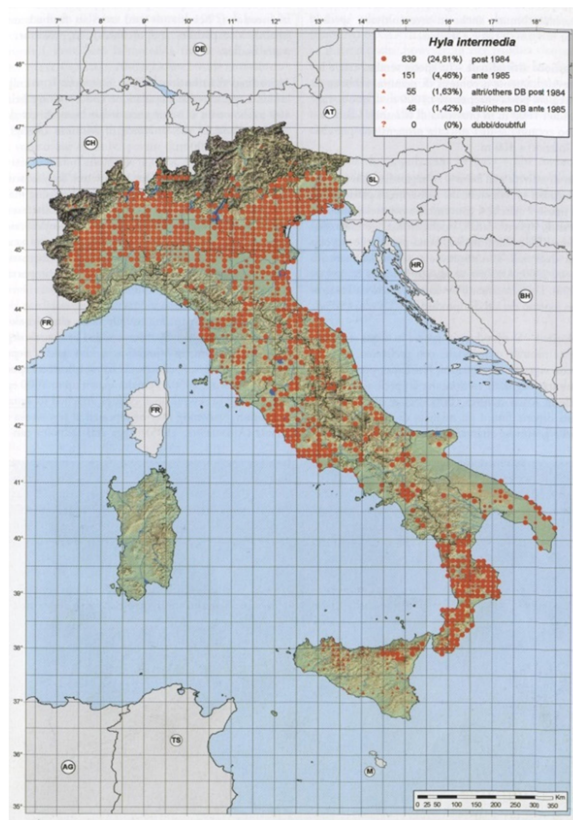


Fig. 3.1. Distribution map of *H. intermedia* (from Sindaco *et al*., 2006)

3.3. Morphology

The Italian tree frog is a small arboreal anuran that rarely exceeds 5 cm in length from the apex of the snout to the cloaca. The eyes, on the sides, have a horizontal pupil; the golden iris is more or less colored with brown lines. The eardrum has a diameter at most equal to half of that of the eye. The long and slender legs are characterized by the presence of adhesive disc-shaped expansion at the ends of the fingers; the disks of the fingers are as big as the eardrum. The fingers of the forelimbs have a webbing barely visible, while the rear toes are webbed by half to 2/3 of their length (Lanza *et al.,* 2006).

The skin is perfectly smooth dorsally but grainy on the abdomen, on the lower face of the thighs and, in females, even under the throat. In the dorsal area, the Italian tree frog shows a typical bright green color rather uniform, but the color variations are not uncommon; the color variations range from very dark shades to very clear in function of the fluctuations of some environmental parameters such as brightness, temperature and substrate. The throat and the ventral area are usually whitish, while the fingers have a pink or yellow pigmentation. Both males and females have a gray or black marginal strip (rimmed of white, cream or pale yellow) which runs from the nostrils, through the eye and the eardrum, along the sides of the body to the groin (Tripepi *et al.,* 1999; Lanza *et al*., 2006).

As regards the sexual dimorphism, the males are slightly smaller than the females and are provided with a great vocal sac of spherical shape with a yellowish-brownish coloration; when inflated this sac is spherical and larger than the head, at rest does

not form longitudinal folds under the throat. In the breeding period the males develop nuptial pads under the first finger of the hand (Lanza *et al.,* 2006).

3.4. Habitat and ecology

The Italian tree frog, as all species of the genus *Hyla*, has mainly arboreal habits but can also live in a wide range of environmental conditions. It inhabits open and well sunny environments, characterized by the presence of shrub or arboreal vegetation. It is a skilled swimmer and jumpers, it also has great qualities of climber (thanks to the presence of adhesive disks at the apex of the fingers that enable it to move easily even on smooth and vertical surfaces), therefore, it prefers the vegetation at a certain height from the ground, such as trees, shrubs and reed beds. On the contrary, juveniles stay close to the land becoming more distinctly arboreal over time (Lanza *et al.,* 2006; Sindaco *et al.,* 2006; Gentilli *and* Scali, 2007).

It is often observed in clearings, heathlands, scrub areas and is quite common even in cultivated areas, especially on the edge rice fields and orchards.

The Italian tree frog has mainly crepuscular and nocturnal habits. It is a eurythermal species and both hibernation and aestivation (that in particular conditions may not occur) occur not far from the breeding sites and usually take place in the ground or in burrows previously occupied by other animals, under rocks, in crevices between rocks, under or inside rotting logs, and in the hollows of tree roots (Lanza, 1983). This species feeds mainly on insects and other small invertebrates, which also captures in flight. Its predators are mammals, water birds, water snakes, carnivorous fish; when leading arboreal life it can be preyed upon by various diurnal and nocturnal raptors.

The main means of defense of the species is mimicry. It spends much of its life on land and its presence in water is limited to the breeding season; at the end of March, chorusing males concentrate near well-vegetated reproductive sites (Sindaco *et al*., 2006).

3.5. Reproduction and development

H. intermedia has annual cyclic reproductive activity regulated by endogenous hormonal factors, the seasonal cycles as well as various environmental factors such as environmental and water temperature. The breeding season begins between March (in more temperate locations and at low altitude) and May, and can last up to July-August; under optimal conditions they may also have autumn depositions. The breeding sites of the Italian tree frog are represented by standing water bodies (of natural or artificial origin), often only temporary, characterized by shrub or arboreal vegetation; its presence in these environments, such as pools, ponds, lakes, marshes, rice fields, marshes, reservoirs, channels and troughs, is limited to the reproductive period (Tripepi *et al.,* 1999; Bologna *et al.,* 2000; Ebisuno *and* Gentilli, 2002).

During night and evening hours the males in spawning emit their songs call audible even at great distances. The embrace is of type axillary and occurs mostly at dusk and during the night. For each breeding season, each female lays up to 1000 eggs with a diameter between 1.5 and 2 mm; the eggs are divided into small spherical gelatinous masses of 3-4 cm in diameter and attached to aquatic vegetation (Lanza *et al*., 2006). Hatching occurs after about 15 days of laying. The tadpoles show an olive-brown or yellowish-brown colour with golden hueshe in the dorsal part; in the sides are present

golden spots, while the belly is whitish with golden or nacreous spots. The larvae of tree frogs are distinguished from those from the other Anurans for the great distance between the two eyes, which are located in lateral position, and for the high and convex caudal ridges with the dorsal portion that extends forward up to eye level. The tail is relatively large and with more or less sharp apex. Under normal temperature conditions, the development of the larvae to the metamorphosis takes 2-3 months. The newly metamorphosed, already very similar to the adults, are approximately long 1.5 cm. They remain tied to the land and shrub vegetation for some time, then take arboreal habits; sexual maturity is probably reached at the second to third year of age (Lanza *et al.,* 2006; Bologna *et al.,* 2000; Andreone, 1995; Gentilli *and* Scali, 2007).

3.6. Population and conservation actions

The status and distribution of *H. intermedia* have not been studied in detail (Sindaco *et al*., 2006). Overall, by an analysis at regional and provincial levels of the distribution and populational consistency of the Italian tree frog, some authors state that this widespread species is subject to a low risk of extinction, thanks to its wide ecological value and its ability to colonize also altered environments (Andreone, 1995; Tripepi *et al*., 1999; Andreone *and* Luiselli, 2000). This opinion is reinforced by the inclusion in the category "least concern - LC" of the IUCN Red List (International Union for the Conservation of Nature), given to species with a stable trend of populations because widespread and abundant (Andreone *et al*., 2009; Temple and Cox, 2009). However, although the Italian tree frog is not, at the time, one of the most threatened species, the authors seem to agree in identifying numerous factors of human pressure

affecting the species, which can locally determine a regression (Giacoma and Balletto, 1993; Scoccianti, 2001; Lanza *et al*., 2006; Sindaco *et al.,* 2006). The researchers identified as risk factors at the local level the disappearance and alteration of wetland breeding grounds (also intended as a simplification of the agricultural landscape and the changing modes of cultivation techniques management that can cause the modification of breeding sites or ecotone environments often used by this species), e.g. intensive agricultural exploitation of the plain areas, overuse of pesticides, removing marginal / riparian vegetation belts, remediation and draining, filling of ditches, canals, ponds, lakes and marshes. Furthermore, the placing of allochthonous fish in the aquatic environment is a serious problem that affects in particular eggs and larvae, but which can also be harmful to adults individuals (Scoccianti, 2004). Similar problems may arise from the placing of non-native freshwater crayfish, such as *Procambarus clarkii* (Cruz *and* Rebelo, 2005).

and is protected by national legislation in Italy and Switzerland. It is present in a number of protected areas. With regard to conservation measures, the species is protected by numerous international, national and regional laws: it is listed on Appendix III of the Bern Convention and and Annex IV of the EU Habitats Directive 92/43/CEE. Therefore, detention, capture, or sale of the Italian tree frogs are prohibited as it is forbidden to make any modification, likely to cause excessive disturbance, destruction or deterioration of the environment in which it lives and reproduces (Gentilli *and* Scali, 2007).

Chapters 4

Target organs

4.1. Urogenital System

The excretory and reproductive system are strictly associated in amphibians, as they are in all vertebrate, although these structures originate from different embryonic tissue (Duellmann and Trueb, 1986). Francis (1934) furnished a description of the urogenital system of salamanders, while Bhaduri (1932) and Bhaduri and Basu (1957) provided a detailed comparative explanation of this system in anurans. The morphology of the urogenital system in caecilians was described by Wake (1968, 1970a, 1970b,1972).

4.1.1. Kidneys

The kidneys in adult amphibians are bilateral structures lying on either side of the dorsal aorta. They develop from larval nephrostomes. In caecilians, anterior and posterior nephrostomes persist in the formation of the kidney; they have an opisthonephric kidney in which evident segmentation are visible in adult. Adult anurans and salamanders have a mesonephric kidney, in fact the larval pronephros is lost and the middle and posterior nephrostomes were they maintained.

By observing the gross morphology of the kidney in the three order of amphibians, great differences could be detected. In caecilians the kidneys are slender and long and extend from the heart's region to the cloaca. In some salamanders the kidneys are long

with no differences from males and females, while in other salamanders, the kidneys are shorter and sexual dimorphism in shape is evident. The kidneys of most anurans species vary from long and slender three to four times as long as broad. (Duellman and Trueb, 1986).

The kidneys are highly vascularized organs; they are supplied with blood via both renal arteries and hepatic portal veins. The renal arteries are branches from the dorsal aorta, while the hepatic portal veins carry blood from the posterior part of the animal. The renal arteries enter the kidneys medially and branch into arterioles which branch further into capillary tufts known as glomeruli. Therefore, there is an afferent arteriole going to the glomerulus and an efferent arteriole carrying blood from the glomerulus, which then connect to peritubular capillaries. The peritubular capillaries also contain branches of the renal portal veins which enter the kidney from a dorsolateral direction. These anastomoses between venous and arterial supplies means the peritubular capillary carry both arterial and venous blood to the interstitium of the kidney. This peritubular capillary blood drains via efferent renal veins to the postcava where it enters the sinus venous.

The functional unit of the kidney is the nephron. The kidney is comprised of thousands of nephrons. Each nephron consist of a glomerulus, Bowman's capsule surrounding the glomerulus, a slender ciliated neck region of the proximal convoluted tubule, a large-diameter proximal convoluted tubule, a thin ciliated intermediate segment, a slender distal convoluted tubule and finally a collecting tubule which drains into the collecting duct. The combination of a glomerulus and its surrounding Bowman's capsule is known as the renal corpuscle. Bowman's capsule is drained by

the ciliated neck segment. The hydrostatic pressure difference moves urine from Bowman's space capsule to the proximal convoluted tubule. The cilia of the neck region contribute in maintaining this pressure difference. The distal end of the proximal convoluted tubule is connected to a second ciliated tubular segment, the intermediate segment, which is also partially responsible for contributing to the pressure difference that move fluid from the proximal to the distal tubule. The collecting ducts from all nephrons drain into the ureter. (Hillman *et al*., 2009)

4.1.2. Gonads

In anurans, the gonads of male and female larvae are paired structures, located near the kidneys, that develop from sexually undifferentiated primordial gonads. Gonadal differentiation starting at different time in a specie specific manner: in *Xenopus* at Nieuwkoop/Faber stage 49, in *R. ridibunda* at Gosner 26 (Ogielska and Wagner, 1990) and in *Rana nigromaculata* at Gosner stage 29 (Tanimura and Iwasawa, 1988) The differentiation is completed in young frog.

The primordial gonads appear as longitudinal bilateral thickenings covering the ventromedial region of the opisthonephros and are attached by their central region, the mesogonium, to the inner body wall. The central region leads to the gonad, while the anterior region, progonium, will develop in the fat bodies.

The primordial gonad is made up by medullary and cortical regions covered with a basal lamina, and its differentiation start from anterior to posterior region (Iwasawa and Yamaguchi, 1984; Lopez, 1989; Merchant-Larios and Villalpando 1981; Ogielska and Wagner, 1990; Tanimura and Iwasawa, 1986, 1988, 1989, 1991).

Medullary and cortical regions of primordial gonads have no ultra-structural differences indicating a cortical origin of the medullary tissue. In later larval stages, the central region is flooded by blood vessels, nerves and opisthonephric interstitial tissue. A primitive germinal epithelium, constituted by the primordial germ cells (PGCs) together with surrounding prefollicular cells, is located in the cortical region of the undifferentiated gonad. The primordial germ cells, in connotation with proliferation and digestion of yolk platelets, usually become smaller after they enter the gonadal ridge (Lopez, 1989; Ogielska andWagner, 1990; Züst and Dixon, 1975, 1977).

Gonadal sex differentiation differs temporally among species but usually coincides with metamorphic climax (McDiarmid and Altig, 1999). Female and males gonads are distinguishable by the number and size of germ cells and the amount of medullary tissue. (Ijiri and Egami; 1975; Tanimura and Iwasawa, 1987; Zaccanti *et al.,* 1977).

Male gonads can develop in a differentiated, semidifferentiated, or undifferentiated mode. The semidifferentiated and undifferentiated types often expressed spontaneous early female sexuality (Tanimura and Iwasawa, 1989; Takahashi, 1971; Zaccanti *et al.,* 1977). In most anuran species, differentiation of testes starts prior to metamorphosis (Iwasawa and Yamaguchi, 1984; Lopez,1989; Ogielska and Wagner, 1990; Tanimura and Iwasawa,1989,1991). The primitive germinal epithelium, due to disintegrative modification, becomes a simple surface epithelium. The male gonial cells or spermatogonia, together with their enveloping follicular cells, are scattered throughout the compact medullary tissue. In the center of the medulla, a network of tubules becomes increasingly distinct and finally differentiates into seminiferous

tubules. Medullary cords, near the hilus of the primordium testis, form the rete testis. Spermatogenesis is practically identical among species (Deuchar, 1975; Kalt, 1973; Kerr and Dixon, 1974). In premetamorphic larvae, are detectable two types of spermatogonia: primary and secondary spermatogonia. Primary single spermatgonia are characterized by a lobed nucleus with diffuse chromatin, while secondary spermatogonia, which derive from primary spermatogonia, occur in cluster of cells of similar size (Fawcett *et al.,* 1959; Rastogi *et al.,* 1983). Meiosis typically begins in late metamorphosis when secondary spermatogonia will transform into primary spermatocytes.

Larval ovaries are bigger than testes and have a lightly uneven outline. Ovaries typically comprise both medullary and cortical tissue in which are present group of proliferating oogonia and oocytes (Iwasawa and Yamaguchi, 1984; Lopez, 1989; Ogielska and Wagner, 1990; Tanimura and Iwasawa, 1986, 1988, 1989, 1991). The cortex is distinctly separated from medulla by an acellular collagenous layer, and the medullary tissue has degenerated and lost its sex cord pattern. In this way small lumens are formed, and with the proceeding of development, they finally fuse to the ovarian cavity. The medullary connective tissue, at this point, becomes a simple epithelial layer covering the ovarian cavity. Oogonia, similar to the primordial germ cells despite devoid of pigmentation granules, undergo periodic proliferation to replenish oogonial nests (Coggins, 1973; Lopez, 1979; Redshaw, 1972). The passage from primary to secondary oogonia (primary oocyte) is associated with change in darkening cytoplasm (Eddy and Ito, 1971) and modification of nuclei from a lobed to a roundish shape.

Prior to metamorphosis, the primary oocytes enter into prophase (Coggins, 1973; Lopez, 1989; Ogielska and Wagner, 1990). In the larval ovaries, at metamorphic climax, coexist oogonia and leptotene, zygotene, pachytene and diplotene oocyte (McDiarmid and Altig, 1999). When an oocyte of a cell nest isolates from the others, it is enclosed by follicular cells which differentiate into steroid-producing follicular epithelium (Redshaw and Nicholls, 1971; Saidapur and Nadkarni, 1974). During folliculogenesis oocyte begin to develop asynchronously and the cytoplasm and the nucleus of diplotene oocytes greatly increase (Coggins, 1973).

4.1.3. Urogenital ducts

The Wolffian and Müllerian ducts of anurans transport gametes and, especially in males, nitrogenous wastes and water. Therefore, the anatomical structure of the genital ducts reflects their functions (Wake, 1979). The Wolffian ducts originate from the anterior region of each kidneys and continue to the cloaca; they are present in both sexes and will develop into primary nephric ducts.

In female they retain their excretory functions, while in males they also serve as urogenital ducts. In fact, in male they present several modification especially near the cloaca where a seminal vesicle develops in some species (Amer, 1972; Bhaduri and Basu, 1957). In young female anuran the Müllerian ducts or oviducts arise at metamorphosis (Hillman *et al*., 2009). The oviducts lie parallel and lateral to the kidneys and connect to the cloaca. In larvae the Müllerian ducts are thin walled tubules, in adult are greatly convoluted especially during the breeding season. Each oviduct comprises a short ostium and a long, coiled pars convoluta. The terminal

portion of the oviduct is enlarged to form the ovisacs (Amer, 1972; Bhaduri and Basu, 1957). In males, Müllerian ducts either degenerate or persist with no functions.

4.1.4 Fat bodies

The fat body, derived from the progonium, consists of proliferating somatic cells that form fingerlike projections aggregated at the anterior end of the gonads. No data are available on the functions of fat body in larvae (McDiarmid and Altig, 1999). In adults, fat bodies are a source of nutrients for the gonads, in fact, they are largest just before hibernation an smallest after breeding (Duellmann and Trueb, 1986).

4.2. Liver

In anurans, the liver occupies the cranioventral quadrant of the coelom. The parietal face lies directly under the ventral abdominal wall. The liver consists of two completely separated lobes and each of which may be subdivided into smaller lobes. A third, median lobe is present in many species. A large gallbladder lies on the midline in the interlobar connective tissue. The lungs lie dorsal to each lobe of the liver. The left lobe may also make contact with the stomach, whereas the caudal edge of the right lobe borders the intestine as it crosses the coelom and turns caudally. In mature females, the ova extend cranially and cover the parietal surface. The ventral abdominal vein runs forward inside the ventral coelomic wall, travels cranially between the lobes of the liver, and divides with a branch entering each lobe. As it approaches the liver, the vein is joined by the hepatic portal vein and vessels from the gallbladder (Crawshaw, 2000).

The gross appearance of the amphibian liver is more variable than in mammals; in fact, the color ranges from orange to deep brown, and from pale gray to black in a specie-specific manner. The liver of all amphibian species contains pigmented macrophages, the melanomacrophages, which gives the typical spots of black coloration. These cells, which are distinct from the melanocytes that give the skin normal pigmentation, are found either diffusely or in clusters (Crawshaw, 2000).

The histological structure of the liver is similar to that in other vertebrates, but the lobular pattern is less distinct than in mammals (Crawshaw, 2000).

However, the traditional concept of structural and functional unit of the liver is the acinus, containing both the hepatic lobule and portal triad. The hepatic lobule is formed by hepatocytes and sinusoids. The sinusoids are capillary networks and are localized in the space between hepatic plates (clusters and cords) in which hepatocytes are arranged (Akiyoshi and Inoue, 2012; Rappaport, 1967)

The portal triads are located in the portal spaces between the hepatic lobules and contain branches of the portal vein and hepatic artery, bile duct and lymph vessels which are surrounded by connective tissue (Akiyoshi and Inoue, 2012).

The amphibian liver performs the same physiological functions as in other taxa, including energy and protein metabolism, synthesis of urea, excretion of bile salts, biotransformation, and detoxification. Amphibians that live in temperate environments, in the autumn, accumulate large source of energy in the liver consisting in glycogen granules and fat droplets; these reserves are used during hibernation. In females, the liver is responsible for the production of vitellogenin, the protein/lipid compound that constitutes the yolk of the amphibian egg.

In the embryonic stages the liver also plays an important role in hematopoiesis. Histiocytic and erythroblastic cell types predominate, but lymphocytic and granulocytic precursors are also present. After metamorphosis, other organs such as the bone marrow take over this role, but residual activity may be seen in adult amphibians, particularly beneath the liver capsule. The liver also has a significant immune function. In detail, the large population of melanomacrophages together with the Kupffer cells, that are located within the sinusoids, act both as phagocytes and in antigen recognition. They also scavenge embryonic erythrocytes in the transition phase from embryo to larva (Corsaro *et al.,* 1995; Sichel *et al.,* 1997).

Hepatic melanomacrophages appear to increase in number with antigenic stimulation, with the age of the animal, and with other factors causing tissue breakdown, but seasonal variations are also seen. Among many metabolic pathways, the liver is the site for the ornithine and urea cycle, an enzyme system that converts ammonia into urea. Ammonia, produced from protein catabolism, is toxic to higher animals and must be excreted or detoxified. Aquatic amphibians and larvae excrete ammonia via the gills, but most adult terrestrial amphibians are ureotelic. The induction of the ornithine cycle at metamorphosis enables the conversion of ammonia into urea (Crawshaw, 2000).

Chapter 5

Materials and methods

5.1. Collection and animal husbandry

Newly laid (up to 3 d) egg masses were collected with handheld dip nets from natural and unpolluted ponds (free from pluvial runoff from agricultural areas) in a location close to Cosenza in Calabria (Southern Italy; 39°21'36''N 16°9'3''E; elevation 387 m a.s.l.) during the spring of 2013.

We collected 6 clutches to increase genetic variability in our samples and, in the laboratory, eggs were randomly assigned to 40 L glass aquaria (60 cm × 35 cm × 30 cm) filled with aerated and aged tap water. Tadpoles were staged according to the appropriate developmental table (Gosner, 1960). After hatching, tadpoles were sorted into groups of 100 animals per aquarium with continuous gentle aeration until they reached developmental Gosner stage 25 (GS 25, feeding and free-swimming tadpoles). During acclimation in the laboratory, tadpoles were held at 22 ± 1°C and median pH 7.3, on a natural light:dark photoperiod, and fed boiled organic spinach *ad libitum* every other day along with the renewal of water.

5.2. Experimental design and exposure conditions

Experimental treatments started once larvae reached the developmental GS 25 (Fig.1). Exposure period (a total of 78 days) lasted from GS 25 until GS 46 (end of metamorphosis and complete tail resorption). For each experimental unit, twenty

tadpoles of comparable body dimension were randomly chosen and assigned to 15 L constantly aerated glass tanks (40 cm × 25 cm × 20 cm) containing the appropriate treatment solution. Each treatment was conducted in quadruplicate.

Test solutions were prepared by dissolving tebuconazole (purity 99.5%, Cas No: 107534-96-3, Sigma-Aldrich Chemie, Steinheim, Germany) and pyrimethanil (purity 99.9%, Cas No: 53112-28-0, Sigma-Aldrich Chemie, Steinheim, Germany) in 100 μL of acetone and then diluted in dechlorinated tap water to obtain the respective nominal fungicide concentrations; the control group was maintained in dechlorinated tap water added with the adequate amount of vehicle. Therefore, acetone concentration was the same in all treatment tanks, including control, and was equal to 9.3×10^{-5} mL. According to Marquis and colleagues (2006) and OECD Guidelines (2015) no acetone controls were used.

We used two nominal concentrations: 5 μg/L and 50 μg/L of tebuconazole or pyrimethanil, for simplicity, we will refer to 5 μg/L as the "low" and 50 μg/L as the "high" concentration. A complete renewal of the water volume was performed every seven days.

Water samples were collected for chemical analysis before each renewal and after renewal of the test solutions; the actual concentrations of both fungicides were verified via high resolution gas chromatography with a pulsed splitless injection mode and simultaneous detection by ECD (electron capture detector) and NPD (nitrogen-phosphorus detection) coupled in parallel (the procedures followed were according to the method described in APAT CNR IRSA 5060 Man 29/2003). Water fungicides concentrations remained fairly constant over the 7-day exposure period ($p>0.05$); this

is in agreement with literature data (Andreu-Sánchez *et al*., 2012; Seeland *et al*., 2013) indicating that both fungicides are stable in water for at least a week. Therefore, nominal concentrations are referred in this article.

Water quality parameters for all holding aquaria were recorded before and after renewal of the test solutions and measured with a handheld multi-parameter PCE-PHD 1 (PCE Instruments UK Ltd). The water temperature was maintained at 22 ± 1°C and median pH 7.3, conductivity 300 µS/cm, dissolved oxygen 8±1 mg/L, and hardness 180 mg. The experiment was conducted in natural light:dark photoperiod. Throughout the exposure period, tadpoles were fed boiled organic spinach *ad libitum* every three days until feeding ceased at the beginning of metamorphic climax (GS 41); at this time, mouth and digestive organs are remodeled for juvenile life and the animals were not fed as tail resorption and fat reserves provide adequate energy during this time period (Hourdry *et al*., 1996). Food waste and debris were removed daily using a fine mesh net.

Tadpoles were maintained under the conditions described above for the duration of their pre-metamorphic and pro-metamorphic larval development. Day 50 of the experiment is the time point corresponding to the longest exposure window before metamorphic climax took place in any one of the experimental groups; then as animals began to metamorphose (i.e., emergence of at least one forelimb – GS 42), we moved individuals into semi-aquatic tanks containing a thin layer of respective treatment water and dry areas (stones and soil) to complete metamorphosis. Animals were not fed throughout this period, since anuran larvae typically do not feed for several days during metamorphosis (due to remodelling of mouth and digestive

organs) (Hourdry *et al*., 1996). Mortality and/or completion of tail resorption (GS 46), in each experimental unit, were monitored daily. Immediately after metamorphosis, the froglets were transferred in plastic terrariums with a moist substrate, water in shallow Petri dishes, and fed *ad libitum* with *Drosophila melanogaster*. At this point the individual recognition was performed and each animal was given a unique identification number; time to initiate and complete metamorphosis, length, and weight for each frog were recorded (Bernabò *et al* 2016).

All experimental procedures were undertaken in compliance with approved Animal Care and Use Committee (Ministerial permit PNM-2011-0002086). All animals not used in experiments were released in the place of collection.

5.3. Endpoints

To avoid any confusion in the measurements and to make statistical comparisons among treatments, two time periods were defined and distinctly studied: from GS 25 to 41 (pre-metamorphic and pro-metamorphic larval development), and from GS 42 to 46 (metamorphic climax).

During pre-metamorphic and pro-metamorphic development, tadpoles in each treatment tank were counted and carefully inspected daily for survival (deceased were promptly removed) and presence of morphological abnormalities. Developmental stage (GS), measurements of snout vent length (SVL) and wet body mass were recorded at the beginning of the experiment and then weekly as an index of growth. All measurements were taken on a subsample of 10 tadpoles per replicate tank of each treatment. Developmental stage was determined using a

stereomicroscope (Leica MZ APO, Leica Microsystems, Wetzlar, Germany); tadpole was quickly towel-dried and weighed to the nearest milligram (Mettler Bas Bal 300), then put on a sheet of waxed millimeter graph paper to measure SVL.

After the beginning of metamorphic climax, semi-aquatic tanks were monitored daily for metamorphic animals; we assessed survival to GS 42, success at metamorphosis, mass and body length at metamorphosis, time to metamorphosis and presence of deformities. Time to metamorphosis was the number of days for tadpoles to complete metamorphosis, with day zero as the day when pre-metamorphic (GS 25) tadpoles were placed into experimental tanks. For calculations of time to metamorphosis and statistics, tadpoles that had not completed metamorphosis by the study's end date were considered not metamorphosed.

5.4. Statistical analyses

All analyses were performed using Graph Pad Prism 5.00 (GraphPad Software Inc., San Diego, CA, USA) at significance level of 0.05. For all experiments, replicates were statistically compared for all endpoints using Mann-Whitney test. Because no significant differences were found among four replicates ($p>0.05$), no tank effect was identified and data were pooled for all subsequent analyses. Assumptions of normality and homoscedasticity were tested with D'Agostino and Pearson omnibus and Bartlett's tests, respectively. Nonparametric tests were performed if data could not be transformed to meet the assumptions for analysis of variance (ANOVA).

Survival analysis, using a Log-rank (Mantel-Cox) Test, was carried out to compare the survival of tadpoles in the five experimental conditions through the entire exposure

period. A Kaplan-Meier survival graph was used to illustrate cumulative survival. In addition, to statistically compare fungicide exposure groups to control groups with respect to mortality for each time-point, we used Fisher's exact probability test (two-way). Fisher's exact probability test was also used to compare fungicide exposure groups to control groups with respect to deformity incidence. To test the effects of the different fungicides exposure on body mass, SVL and developmental stage, Kruskal-Wallis test was performed for all testing periods (every 7 days) separately, followed by Dunn's Multiple Comparison post test to compare fungicide exposure groups with the control. Time (days) to complete metamorphosis and body size (body length and mass) at metamorphosis were compared using one-way ANOVAs followed by Dunnett's Multiple Comparison Test.

5.5. Morphofunctional analysis

One week after completion of metamorphosis, froglets were deeply anesthetized by immersion in 0.1% tricaine methanesulfonate (MS-222, Sigma-Aldrich Chemicals Co., St. Louis, MO). A subset of the surviving juveniles from control and pyrimethanil exposed groups was utilized for histological analysis (Control n = 30, Pyr-L, 5 μg /L n=28, and Pyr-H, 50 μg/L n=22). Gross morphology evaluation and phenotypic sex determination were performed under a stereomicroscope (Leica MZ APO, Leica Microsystems, Wetzlar, Germany equipped with Canon camera) using a drop of Bouins' solution to enhance coloration.

Liver and gonad-mesonephros complex were quickly excised. Each organ was weighed in an analytical precision scale (0.001 g) (MettlerBas Bal 300) for calculation

of liver somatic index (LSI) and gonad-mesonephros complex index as follows: LSI = (liver weight/body weight) x 100; GMCI = (gonad-mesonephros complex weight/body weight) x 100 (Melvin *et al*. 2013).

Samples were placed in Bouins' liquid for 24 h at 4°C and then dehydrated in an increasing series of ethanol, cleared in xylene and embedded in paraffin wax. Sections (7 μm) were serially cut and mounted on positive charged slides. For liver, step sections were taken at 50 μm intervals until the maximum diameter of the samples was attained. For gonad-mesonephros complex, serial sections from the dorsal, middle, and ventral region were taken.

Before evaluation, slides were randomized and coded, and all histological sections were blindly screened to validate phenotypic sex, identify morphological abnormalities, evaluate the progression of gonadal maturation and immunoresponse.

5.5.1. Light microscopy analysis

Sections for morphological analysis were stained with hematoxylin and eosin (Panreac, Barcelona, Spain), and photographed by LM equipped with a digital camera (Leica DME, ICC50 HD, Leica Microsystems, Wetzlar, Germany). Analyses of ovaries and testes, and staging of gonadal development were performed according to Ogielska and Kotusz (2004) and Haczkiewicz and Ogielska (2013). Froglets were defined as females based on the presence of an ovarian cavity and oogonial cells and/or nests of oocytes (early meiotic and/or diplotene); animals were defined as male based on the presence of a large medullary region and of a distinct testicular structure, with spermatogonia into early seminiferous tubules.

In order to determine *H. intermedia* gonad differentiation pattern (i.e. differentiated, semi-differentiated or undifferentiated), individuals raised apart were also analysed at selected developmental Gosner stages (27, 30, 37, and 42).

5.5.2. Immunofluorescence

Dewaxed sections were rinsed with distilled water and phosphate buffer (PBS) and then incubated for 30 min in a moist chamber with 20% normal serum to block non-specific sites. Unwashed sections were incubated overnight at 4°C with a rabbit polyclonal antibody to caspase-3 (diluted 1:100 - Sigma–Aldrich Chemical Co., St. Louis, MO, USA). After several washes in PBS, sections with fluorescein isothiocyanate-conjugated γ-globulins sheep anti-rabbit (diluted 1:100 - Sigma–Aldrich Chemical Co., St. Louis, MO, USA) for 30 min at room temperature. Finally, slides were counterstained with propidium iodide (1:200 - Sigma–Aldrich Chemical Co., St. Louis, MO, USA), which binds to ribonucleic acid and labels cell nuclei.
To examine non-specific binding (negative control) primary antibody was substituted with non-immune normal serum in control sections. The sections were observed under a Leica TCS SP2 confocal laser scanning microscope (LSM).

5.5.3. **Sex ratio, LSI, GMCI.**

Analyses of data were performed using Graph Pad Prism 5.00 (GraphPad Software Inc., San Diego, CA, USA), at significance level of 0.05. No tank effect was detected and data from replicates per treatment groups were pooled into one data set. Assumptions of normality and homoscedasticity were tested with D'Agostino and Pearson omnibus

and Bartlett's tests, respectively. Nonparametric tests were used if data could not be transformed to meet the assumptions for analysis of variance (ANOVA).

To test whether sex ratio in each group deviated from the expected 50:50 (female:male), χ^2 test was performed. For evaluations of frequency of ovarian developmental stages, we first used χ^2 test; if differences (exact $p \leq 0.05$) were found, Fisher's exact tests (2-sided) were applied for pairwise comparisons between control and exposure groups with respect to frequency of underdeveloped versus developed ovary.

To test the effect of pyrimethanil exposure on LSI, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test was performed. Significant differences in GMCI between fungicide exposed and control animals were tested using a One-Way ANOVA followed by Bonferroni's Multiple Comparison Test.

Chapter 6

Results

6.1. Effects of long-term exposure to pyrimethanil and tebuconazole, on survival and life history traits

The whole exposure period lasted 78 days. This period corresponded to the longest exposure window during which the major part of individuals reached GS 46; a few individuals (n=4) in pyrimethanil exposed groups remaining as tadpoles by the end of the experiment and were excluded from statistical analysis of metamorphic success.

6.1.1. Survival

Overall survival to day 78 was significantly influenced by fungicides exposure during the whole experimental period, compared to the control group (Log-rank Mantel-Cox: χ^2= 57.38, 4° of freedom (*df*), p<0.0001) (Fig. 6.1a). Until day 60, survivorship was 100% in the control group, whereas in exposed groups the cumulative survival was affected by fungicides in a manner inversely proportional to their concentration (Fig. 6.1a). In detail, pyrimethanil produced significantly higher mortality in the low concentration group starting from day 6 of exposure (Fisher's exact test, p<0.01), and a further significant decrease in survival was observed from day 14 to the end of the experiment (Fisher's exact test, all p<0.001) compared with the control (Fig. 6.1a). At the high concentration of pyrimethanil, a significant reduction of survival occurred

from day 33 of exposure (Fisher's exact test, $p<0.05$) compared with the control group (Fig. 6.1a). Tadpoles exposed to tebuconazole had significantly greater mortality, compared to control, starting from day 6 of exposure in both low and high concentration groups (Fisher's exact test, $p<0.001$ and $p<0.01$, respectively) and this trend was maintained until the end of the experiment (Fisher's exact test, all $p<0.001$). Before of day 60, survival was greater in the high concentration group than in the low concentration group for both fungicides whereas the opposite trend was observed starting from this time point until the end of experiment (Fig. 6.1a).

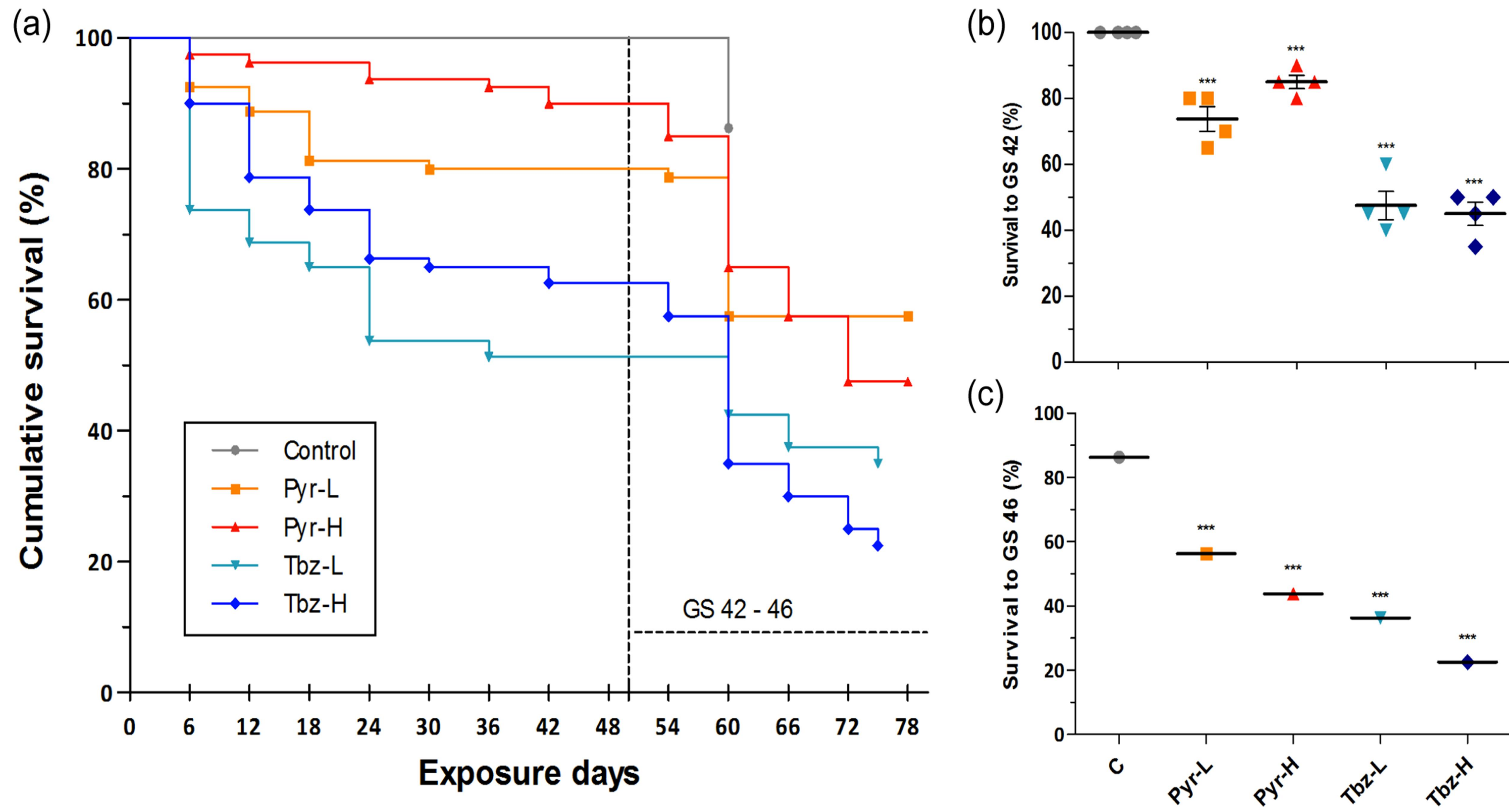
A highly significant reduction (Fisher's exact test, all $p<0.001$) in the percentage of individuals that reached GS 42 (onset of metamorphic climax) has been observed in fungicides exposed groups compared with control (Figs. 6.1a and b; Table 1). The percentage of tadpoles that reached GS 42 was much lower in the tebuconazole exposed groups than in pyrimethanil exposed groups (Fig. 6.1b, Table 1). A great reduction in survival occurred from GS 42 to GS 46 when mortality reached high point in both exposed and control groups (Fig. 6.1a).

The fungicides exposure caused a highly significant effect on success at metamorphosis compared to the control group (Fisher's exact test, all $p<0.001$) (Fig. 6.1c; Table 1). In the control group 86.25% of individuals successfully completed metamorphosis; conversely, by the end of the experiment, in tebuconazole exposed groups only 22.5% (high concentration) and 36.25% (low concentration) of individuals completed metamorphosis and in pyrimethanil exposed groups the percentage of survived to GS 46 was 43.75% (high concentration) and 56.25% (low concentration) (Fig. 2c; Table 1).

Table 1. Summary table of evaluated endpoints. Percentage of survival to GS 42 and to GS 46 are calculated as the number of tadpoles surviving each developmental stage divided by the initial sample size (n = 80). The data presented are the means ± S.E. Asterisks indicate the treated groups that differ from the control using: the two-tailed Fisher's exact probability test, and one-way ANOVA followed individual comparisons by Dunnett's *post hoc* test (*p<0.05, **p<0.01 and ***p<0.001).

Treatment	Survival to GS 42 (%)	Cumulative deformity (%)	Survival to GS 46 (%)	Deformity at GS 46	Time to complete metamorphosis (days)	Body length at GS 46 (mm)	Mass at GS 46 (g)
Control	100	0	86.25	0	58.5 ± 0.2	15.1 ± 0.2	0.31 ± 0.01
5 μg/L Pyr-L	73.75 ± 3.75***	22.5 ± 2.5***	56.25***	2.5	62.3 ± 0.9***	15 ± 0.2	0.36 ± 0.01**
50 μg/L Pyr-H	85 ± 2***	8.8 ± 1.2*	43.75***	1.25	63.2 ± 1***	14.6 ± 0.2	0.35 ± 0.01*
5 μg/L Tbz-L	47.5 ± 4.3***	6.25 ± 1.2	36.25***	2.5	61.9 ± 1**	15.1 ± 0.2	0.44 ± 0.01***
50 μg/L Tbz-H	45 ± 3.5***	5 ± 2.5	22.5***	1.5	60.9 ± 1	14.7 ± 0.2	0.38 ± 0.01**

Figure 6.1. (**a**) Kaplan-Meier cumulative survival curves of *H. intermedia* tadpoles exposed to 50 and 5 μg/L of pyrimethanil and tebuconazole during the entire larval developmental period (from GS 25 to 46). p<0.0001 from tests between survival trajectories by Log-rank Mantel-Cox Test. Treatment types are represented by different symbols. The vertical dashed line (at day 50) indicates the time point corresponding to the longest exposure window before metamorphic climax for all experimental groups. (**b**) Survival (%) of tadpoles reaching GS 42. Each symbol represents a replicate tank and the black lines indicate mean ± S.E. (**c**) Survival (%) to GS 46. Each symbol represents a semi-aquatic tank per treatment. ***p<0.001 when compared to the control group using the two-tailed Fisher's exact probability test.



6.1.2. Morphological abnormalities

During development, several malformations were noticed in individuals from exposed groups. In both concentration groups of pyrimethanil (Fig. 6.2a,b,d, h-j) and tebuconazole (Fig. 6.2e-g), deformities mainly observed were irregular profile of the tail fin, axial and tail malformations (lateral/dorsal flexure, and wavy tail), abnormal mouth, and limb deformities. Tadpoles displayed malformations that increased in degree of severity as development proceed. None of the tadpoles belonging to the control group showed morphological abnormalities (Fig. 6.2c).

The incidence of total malformations in tadpoles exposed to pyrimethanil was significantly higher compared to control group; the cumulative incidence of deformity was equal to 22.5% in tadpoles exposed to the lowest concentration (Fisher's exact test, $p < 0.001$) and to 8.8% (Fisher's exact test, $p < 0.05$) in those exposed to the high concentration group (Table 1). Axial and tail malformations were observed starting from day 7 in tadpoles treated with the low concentration and from day 14 in the high dose reared ones (Fig. 6.2a,b,d, h).

As regards tebuconazole, the cumulative incidence of deformity was 6.25% and 5% in tadpoles exposed to the low and high concentration groups, respectively (Table 1). Skeletal malformations, consisting of lateral flexure of the spine originating at the midpoint or near the tail base, were observed starting from day 14 in tadpoles treated with the low concentration and from day 21 in the high dose reared ones (Fig. 6.2e-g). In the low concentration group of tebuconazole we found an individual (GS 41) affected by hyperextension of the hind limbs (Fig. 6.2g). In the high concentration

group of pyrimethanil we found a specimen without the right limb (ectromelia) and with curved tail; this animal has reached the GS 42 but was not able to successfully complete metamorphosis (Fig. 6.2h). At the end of the experiment, from a quantitative point of view, the percentage of malformed froglets has decreased (Table 1); this datum needs to be interpreted in the light of the high mortality that occurred during the metamorphic climax. Moreover, the tail resorption has implied the disappearance of deformities previously detected in this organ. In both fungicides exposed groups, the greater part of malformed individuals surviving to GS 46 showed the presence of edema or swelling (Fig. 6.2i and j).

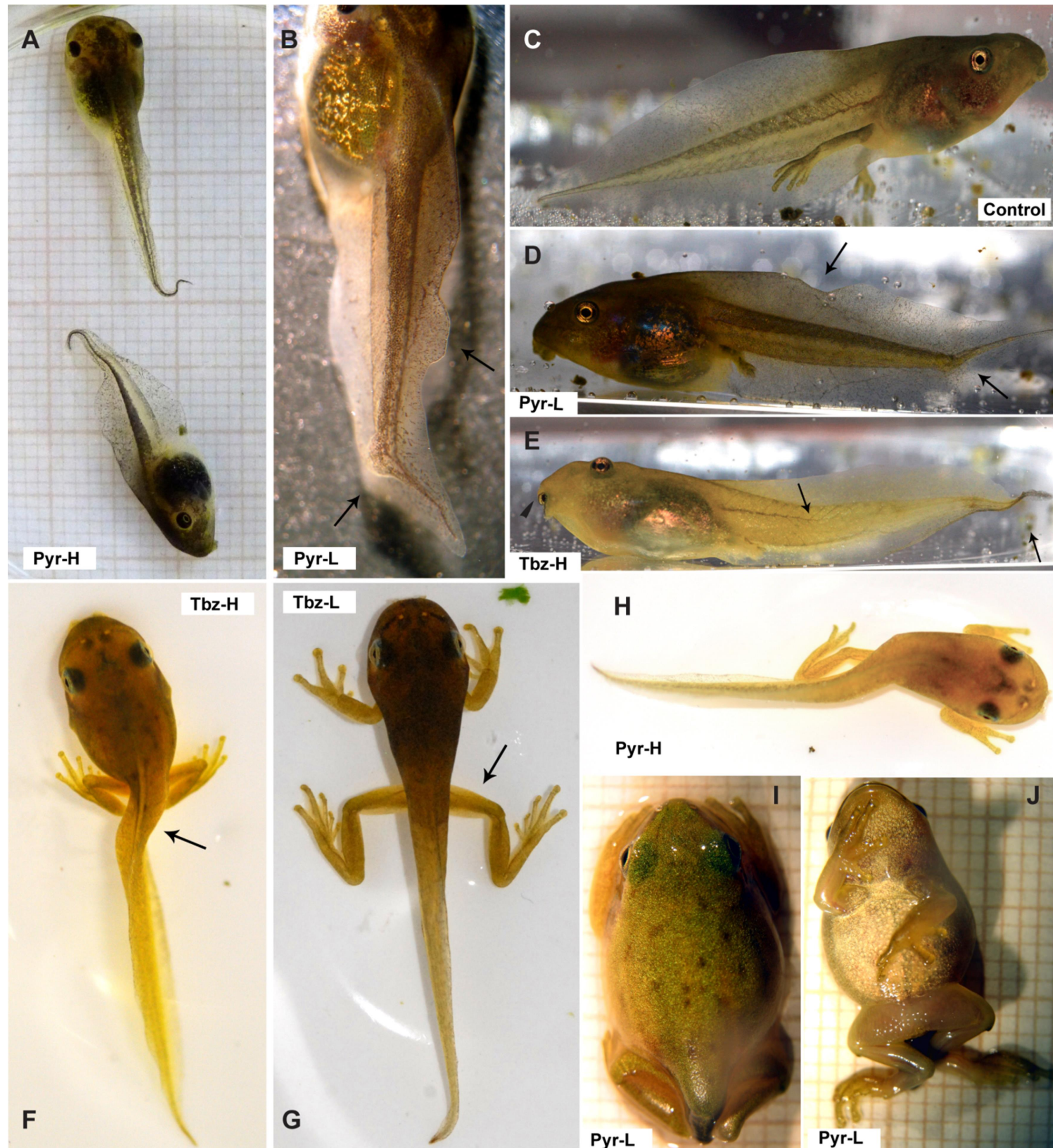


Figure 6.2. *H. intermedia* individual from the control group (**c**) and representative types of morphological alterations found at different developmental stages in tadpoles exposed to tebuconazole (Tbz-L = 5 μg/l and Tbz-H = 50 μg/l) and pyrimethanil (Pyr-L = 5 μg/l and Pyr-H = 50 μg/l) from GS 25 to GS 46.Note axial and tail malformations, such as lateral or dorsal flexure of the spine, with varying degrees of severity (**a, b, d, e, f, h**), irregular profile of the tail fin (**d-e**), hyperextension of the hind limbs (**g**) and ectromelia (**h**), abnormal mouth (**e**), and the presence of edema or swelling in froglets (**i-j)**. Arrows or arrowhead indicate the abnormalities.

6.1.3. GS 25-42: growth and general development

Mean GS of development was not significantly affected by exposure to both fungicides prior to day 49 (Fig. 6.3a). Conversely, at this time point, immediately before the onset of metamorphic climax, development was significantly delayed in both low and high concentration groups of pyrimethanil (Kruskal-Wallis test = 92.98 and 103.8, respectively, *df* = 4, $p<0.001$) and in both low (Kruskal-Wallis test = 56.60, *df* = 4, $p<0.001$) and high concentration groups of tebuconazole (Kruskal-Wallis test = 44.85, *df* = 4, $p<0.01$) compared with the control (Fig. 6.3a).

Exposure to fungicides did not caused significant differences in SVL throughout the exposure period until day 49, compared to control group (Kruskal-Wallis test, all $p>0.05$) (Fig. 6.3b).

Mean body mass of the individuals in the exposed groups was not significantly different compared to that of tadpoles in the control group until day 42; at this time point Kruskal-Wallis tests revealed that animals in the low tebuconazole concentration group were significantly heavier than controls (Kruskal-Wallis test = 21.05, *df* = 4, $p<0.05$) (Fig. 6.3c). At day 49 significant differences were also detected in body mass in both the low (Kruskal-Wallis test = 19.42-21,74, *df* = 4, $p<0.01$) and high tebuconazole (Kruskal-Wallis test = 21.74, *df* = 4, $p<0.01$) concentration groups compared with the control (Fig. 6.3c).

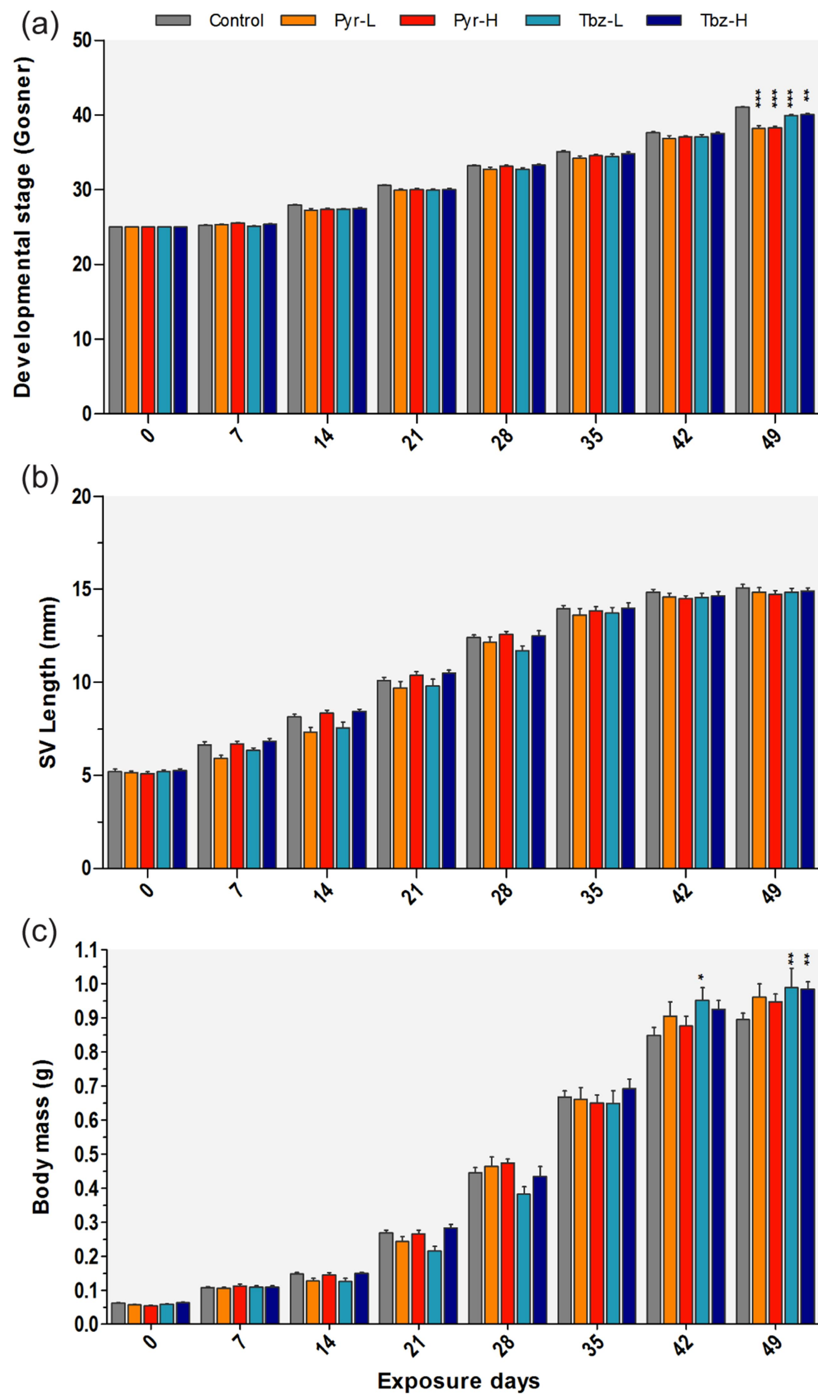


Figure 6.3. Developmental Gosner stages (**a**), SVL (**b**) and body mass (**c**) until day 49 in *H. intermedia* tadpoles exposed to 50 and 5 μg/l of pyrimethanil and tebuconazole. The bars show mean ± S.D. Asterisks indicate the treated groups that differ from the control, **p*<0.05, ***p*<0.01 and ****p*<0.001 (Kruskal-Wallis test followed by Dunn's multiple comparison test).

6.1.4. GS 46: length, mass and time to metamorphosis

Exposure to fungicides did not significantly affect the final body length at metamorphosis in any concentrations groups (One way ANOVA, $F_{4,191} = 1.072$, $p=0.371$) (Fig. 6.4a; Table 1).

In contrast, the final body mass at metamorphosis was strongly influenced by exposure to both fungicides (One way ANOVA, $F_{4,191} = 16.02$, $p<0.0001$) (Fig. 6.4b; Table 1); individual comparisons showed that metamorphs in both concentrations of pyrimethanil (Dunnett's test; low $p<0.01$ and high $p<0.05$) and tebuconazole (Dunnett's test; low $p<0.001$ and high $p<0.01$) were significantly heavier than those in the control group (Fig. 6.4b; Table 1).

The mean time to complete metamorphosis was significantly delayed by exposure to fungicides (One way ANOVA, $F_{4,19} = 7.858$, $p<0.0001$). In detail, tadpoles exposed to both the low and high concentration of pyrimethanil took a significantly longer time to complete metamorphosis than those in the control group (Dunnett's test, $p<0.001$) or may not have completed metamorphosis at all (n=4) (Fig. 6.4c; Table 1). Time to metamorphosis was significantly prolonged in tadpoles reared in the low concentration group of tebuconazole when compared with control group (Dunnett's test; $p<0.01$), while no difference was detected in the high concentration group (Dunnett's test; $p>0.05$) (Fig. 6.4c; Table 1).

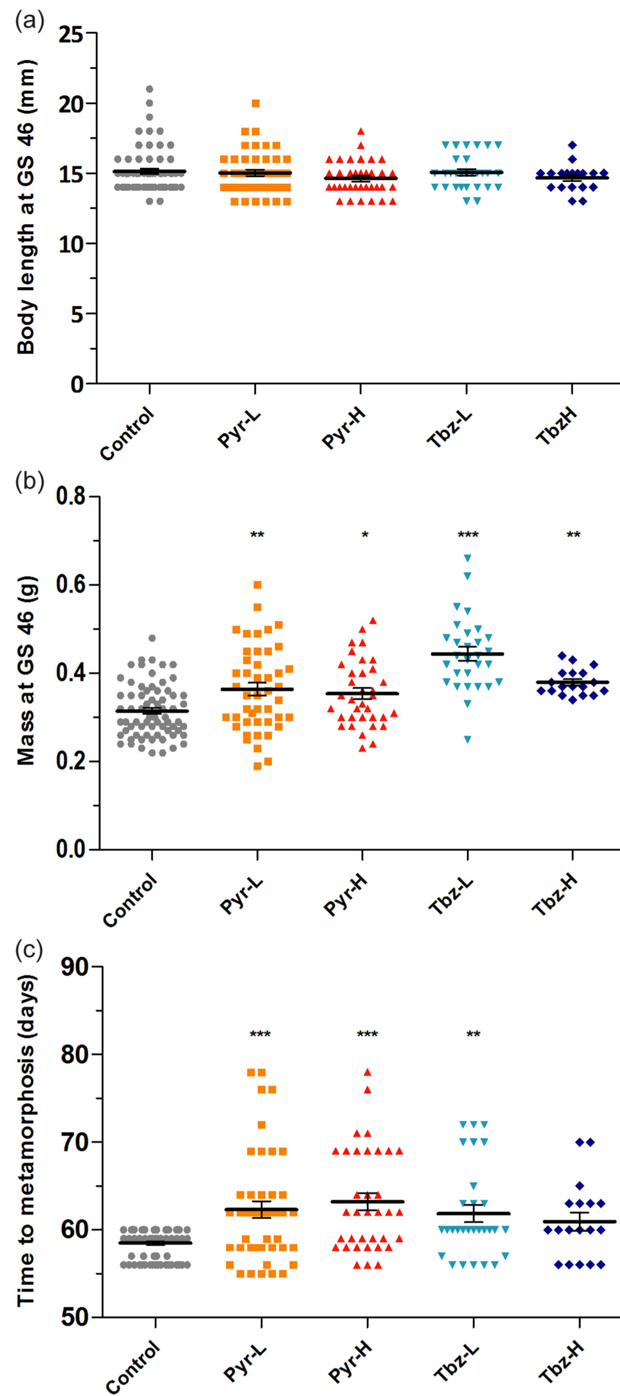


Figure 6.4. Scatter plot of body length (**a**), mass at metamorphosis (**b**), and time to complete metamorphosis (**c**) by experimental groups. Each symbol represents a metamorph. The black lines indicate the mean ± S.E. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with controls (One way ANOVA followed by Dunnett's *post hoc* test).

6.2. Effects of long-term exposure to pyrimethanil on gonads, liver and kidney.

6.2.1. Control group

6.2.1.1. Gonadal differentiation and histology

We first described the pattern of both ovarian and testicular differentiation for *H. intermedia* under basal condition (data not shown). Histological analysis revealed that by stages 27-30 (n=5) ovary differentiation has begun. The first event was the reduction of the medulla and the appearance of a central lumen lined by newly formed epithelial cells. In the cortical region it was possible to distinguish proliferating primordial germ cells and primary oogonia. By stage 30 (n=5), in developing testes undifferentiated somatic cell populated the medullary region and primary spermatogonia were visible; a central lumen was not observed.

In *H. intermedia* one week after metamorphosis (age: 11-12 weeks), gonads were sexually differentiated and definitive testes or ovaries were clearly distinguishable. Observed under the stereomicroscope, the differentiated ovaries appeared as paired large, long sacs with an evident external lobulation; the left gonad was typically slightly bigger than the right one (Plate 1a). From histological point of view two stages of ovarian maturation were distinguishable in females: the majority (56%) of ovaries, categorized as ovary development stages VIII *sensu* Ogielska and Kotusz, 2004, was characterized by large discontinuous external areas of proliferation containing oogonia and/or nests of leptotene-pachytene meiocytes situated in the external part of the cortex, whereas numerous diplotene oocytes in primary growth (previtellogenesis) occupied ovarian lumen (Plate 1b; Table 2). With further

enlargement diplotene oocytes appeared globular in shape with highly basophilic cytoplasm, large, spherical and central nuclei with non-condensed chromatin, and many peripheral nucleoli of different sizes; a monolayer of follicular cells surrounded diplotene cells (Plate 1c). In 38% of samples, the cortex was mostly occupied by diplotene oocytes increasing in number and size whereas small patches of proliferation were restricted in the periphery of the ovary (ovary development stages IX) (Plate 1d; Table 2).

Testes were bilateral compact organs and, by stereomicroscope, it was possible to note the granular appearance and the absence of lobulations; left testis was longer and larger than right one (Plate 1e).

Based on histological observations, in the males examined (stage VIII *sensu* Haczkiewicz and Ogielska, 2013), testes displayed the formation of the typical arrangement in seminiferous tubules (Plate 1f-h). Each developing seminiferous tubule had a germ tissue with primary spermatogonia and few secondary spermatogonia (Plate 1g,h). Primary spermatogonia were large cells with irregular, aspect, voluminous eosinophilic cytoplasm and a highly polymorphic nucleus; these germ appeared singly located adjacent to the basal lamina of the seminiferous tubules (Plate 1g,h). Early secondary spermatogonia were recognizable within developing membranous cyst; they were smaller, with a more pronounced colour and less lobulated nuclei than primary spermatogonia (Plate 1g,h). Darkly stained and flattened somatic cells (Sertoli cells) were located in the central part of testis where they will develop in so-called *rete testis* (Plate 1f-g).

Sex ratio. Based on both gross morphology and histology, the observed phenotypic sex ratio of the control juveniles was 53% females and 47% males, which did not significantly differ from the 50:50 (female:male) sex ratio expected for amphibians (Table 3).

6.2.1.2. Kidney morphology

The gross morphology of the kidneys, in animals from control group, resembled the definitive adult mesonephros. The kidneys were paired structures highly vascularized in close contact with the gonads; each kidney showed an elongated oval shape with narrow apical portions and a wider median part (Plate 1a,e). By light microscope, it was possible to distinguish renal corpuscles and distal tubules distributed in the ventromedial zone of renal parenchyma whereas proximal tubules and collecting tubules were located in the dorsal portion of the kidney (Plate 2a). As previously described for other anuran species, renal corpuscles showed complete Bowman's capsule and well-organized central glomeruli composed of capillary loops (Plate 2a-c). The renal tubule was divided into the neck segment, the proximal tubule, the intermediate segment and the distal tubule, which opens into the collecting tubule (Plate 2a). The proximal tubule was made by cuboidal, granulated epithelial cells with acidophilic cytoplasm and was characterized by a dense brush border at luminal surface (Plate 2b,d). The distal tubule was composed of simple cuboidal epithelium (Plate 2a,b).

6.2.1.3. Liver morphology

H. intermedia liver showed an orange-brown pigmented coloration with black spots due to the melanomacrophages (Plate 2e). The hepatic parenchyma had a compact appearance and was formed by hepatocytes arranged in clusters and cords; sinusoids of different size were interspersed within hepatocytes (Plate 2f-h). A wide population of large melanin-containing cells (i.e. melanomacrophages) was distributed close or within sinusoids (Plate 2f-h). Macrophages (or Kupffer cells) could also be recognized (Plate 2h). In the hepatic portal area it was possible to note branches of the portal vein and hepatic artery along with bile duct composed by simple cuboidal epithelium (Plate 2g). Hepatocytes were polyhedric in shape with round nuclei and a clear, granulated cytoplasm (Plate 2h).

6.2.2. Pyrimethanil exposed groups

6.2.2.1. Sex ratio and gonadal morphology

Based on gross morphology and successive histological validation, phenotypic sex ratio was not significantly different from an equal sex ratio (χ^2 = 3.019, *df* = 2, p = 0.221) (Table 3) in all experimental groups. Histological analysis also revealed that none of the exposed animals showed sex reversal or abnormal gonadal intersex. Furthermore, exposure to fungicide did not cause significant differences in gonad-mesonephros complex index (GMCI) (One-Way ANOVA, $F_{2,79}$ = 0.7185, p = 0.4906) compared to control group (Table 3).

During dissection under stereomicroscope, it was possible to note that gross

morphology of females from pyrimethanil exposed groups (both high and low concentrations) was maintained and all individuals showed well-developed ovaries with typical external lobulation (Plate 3a,d). On the contrary, the light microscope analysis revealed severe abnormalities in both oogenesis progression and histological features (Table 2; Plate 3b,c,e-m). In fact, despite typical ovarian structure with presence of the central cavity, underdevelopment of the meiotic oocytes nests was detected. The ovarian tissue was mostly filled by oogonia and nests of early meiotic prophase oocytes and none or few newly formed diplotene oocytes were observed (Plate 3b,c,e-g,i).

Statistical analysis of gonad differentiation, revealed that this delay was significant for both pyrimethanil exposed groups ($\chi^2 = 56.75$, df = 4, $p < 0.0001$). In detail underdevelopment was recognized in the 50% of ovaries from the low concentration group and in the 21.4% from the high one (Fisher's exact test, $^{***}p < 0.001$ and $^{**}p < 0.01$, respectively) (Table 2).

Moreover, ovarian tissue was affected by several types of alterations, such as conspicuous degeneration phenomena involving all germ cell types (Plate 3b,c,h-m), large presence of macrophages and apoptotic bodies (Plate 3g,l); enlargement of intercellular spaces and detachment of diplotene oocytes from the enveloping follicular cells (Plate 3b,c,j,l,m), and diffuse mononuclear cell infiltration (Plate 3b,k).

Males from pyrimethanil exposed groups showed no significant differences in testes gross morphology compared to control (Plate 4a). However, in low concentration group we found two individuals with only one testis (the right one was absent) (Plate

4d), unfortunately we did not obtain the histology and therefore these were exclude from successive analysis; these animals exhibited edema at metamorphosis (see below). In samples of both concentrations groups, most of individuals displayed a well preserved histological organisation and the testes appeared well organized in seminiferous tubules (Plate 4b,c,) with big primary spermatogonia and several cysts of round shape secondary spermatogonia (Plate 4e,f). However in some samples from both experimental groups, alterations of testicular tissue have been observed: seminiferous tubules tended to be less well organized with an enhancement of the intercellular spaces, primary spermatogonia/germ cells showed signs of degeneration; mononuclear cell infiltrates were frequently detected (Plate 4g,h).

6.2.2.2. Kidney histopathology

Histological analysis revealed many morphological changes in renal parenchyma of animals exposed to both fungicide concentrations (Plate 5), despite gross morphology was preserved (Plate 4a). Intensity of impairment varied depending on individual response, but the regular arrangement of kidney was always impaired by a general disorganisation of tissue and consistent histologic features of necrosis. In all samples, we observed some pathological effects in both renal tubules and corpuscles. One of the most frequent alteration were the inflammatory response, associated with infiltration of both macrophages and mononuclear cell (Plate 5a,e-g,j), the presence of haemorrhage and apoptotic bodies(Plate 5a,c,e,g,h,j). Renal tubules displayed severe

damages such as a pervasive tubular dilation (Plate 5a,d,h,j), hydropic swelling, with or without cytoplasmic vacuolization, and a general loss of cytoplasmic detail (pale-staining and poor cytoplasm of necrotic cells) (Plate 5). The presence of proteinaceous fluid within the renal tubules (characterized by homogenous dark pink material) was frequently observed (Plate 5a,b,g-i). Moreover, at higher magnification was evident the destruction of brush border in the proximal renal tubule (Plate 5b,f). A marked widening of Bowman spaces and shrinkage of the capillaries were observed (Plate 5d,e,h-j).

6.2.2.3. Liver histopathology

Exposure to fungicide did not cause significant differences in liver somatic index (LSI) (Kruskal-Wallis test, $p = 0.3584$) compared to control group.

Histological examinations revealed, in liver of animals exposed to pyrimethanil, considerable morphological changes (Plate 6). In samples from the low concentration group, the liver appeared highly vascularized and the numerous blood vessels appeared often occluded (Plate 6a). The large amount of blood resulted in sinusoidal congestion and dilation giving to the liver a loose appearance (Plate 6a). In addition, the liver parenchyma dyschromia, due to degenerative phenomena, was evident at higher magnification (Plate 6b). In some areas, hepatocytes showed a clear, foamy cytoplasm whereas in other cases the cytoplasm appeared highly hypereosinophilic (Plate 6c). In the portal area, also cuboidal cells forming interlobular bile ducts displayed signs of degeneration (Plate 6d). Signs of inflammation (e.g. infiltration of

mononuclear cells) in sinusoids and among hepatocytes were frequently detected (Plate 6e). In addition, a large amount of apoptotic bodies could be seen (Plate 6f).

In samples from the high concentration group, the intensity of degenerative phenomena greatly increased (Plate 6g-m). The hepatic architecture was completely lost and diffuse degeneration along with hepatic dyschromia were observed in all samples (Plate 6g). Both hypereosinophilic and clear degenerating hepatocytes could be detected (Plate 6h). In large areas, massive or sub-massive necrosis were observed (Plate 6i) and it was possible to note haemorrhage and cellular debris (Plate 6j). Mononuclear cell infiltration (Plate 6k) and vessels congestion (Plate 6l) were conspicuous. Bile ducts disorganization was observed in portal area (Plate 6m).

6.2.3. Immunofluorescence

Caspase-3 immunodetection revealed no labeling or an extremely weak one in all tissues from control group (Plate7 a-d). After exposure to both pyrimethanil concentrations, it was possible to note a marked increase in Caspase-3 immunoreactivity (Plate7 e-l).

The increase of staining is tissue-specific and both ovaries (Plate7 e,i) and liver (Plate7 h,l) exhibited the most intense caspase positivity.

We detected a dose-related enhancement of signal in kidney (Plate7 g,k) and testes (Plate7 f,j), whereas in liver (Plate7h) and ovaries (Plate7e) the peak of expression has been observed in samples from low concentration group.

In both ovaries and testes, the signal was particularly evident at the periphery of the organs but all gonadal tissue was stained (Plate7 e,f,i,j). Similarly, in the liver the signal was mainly detected at peripheral region of the organ (Plate7 h,l). On the contrary, in kidney the signal was uniform through the section (Plate7 g,k).

Group	Ovary stage (%)		
	Underdeveloped[a]	Stage VIII[b]	Stage IX[c]
Control (n = 16)	6	56	38
5 μg/L (n = 14)	50 ***	35.7	14.3
50 μg/L (n = 14)	21.4 **	57.2	21.4

Table 2. Frequency of stages of ovarian development in *H. intermedia* one week after metamorphosis

[a] Underdeveloped ovary were determined by the presence of large areas of proliferation (numerous oogonia and nests of leptotene-pachytene oocytes) and none and/or few early diplotene oocytes.

[b,c] Categorization of ovarian developmental stages following the criteria described by Ogielska and Kotusz (2004).

Sample size and frequency are reported. Asterisks indicate statistically significant differences between treatments when compared to the control using the two-tailed Fisher's exact test (***$p < 0.001$ and **$p < 0.01$).

Group	n[a]	Metamorphic success (n)[b]	DTM[c]	n[d]	% Females[e]	% Males[f]	Sex ratio (♀:♂)	GMCI[g]	LSI[h]
Control	80	69	58.5	30	53	47	1:0.9	3.29 (0.13)	3.37 (0.14)
5 μg/L	80	45	62.3	26	54	46	1:0.9	3.12 (0.15)	3.46 (0.16)
50 μg/L	80	35	63.2 (1)	22	64	36	1:0.6	3.34 (0.12)	3.69 (0.20)

Table 3. Summary table of evaluated endpoints

[a] Initial sample size;

[b] Number of tadpoles completing metamorphosis;

[c] DTM = number of days to complete metamorphosis;

[d] Number of one week recent metamorphs of *H. intermedia* used for histological observations;

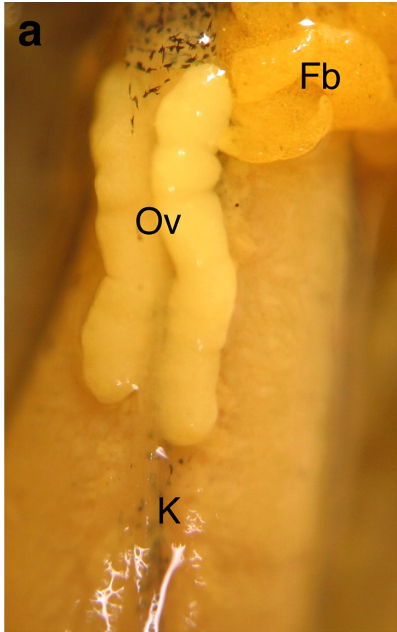
Percentage of phenotypic [e]female and [f]male based on both gonadal gross morphology and histological examination;

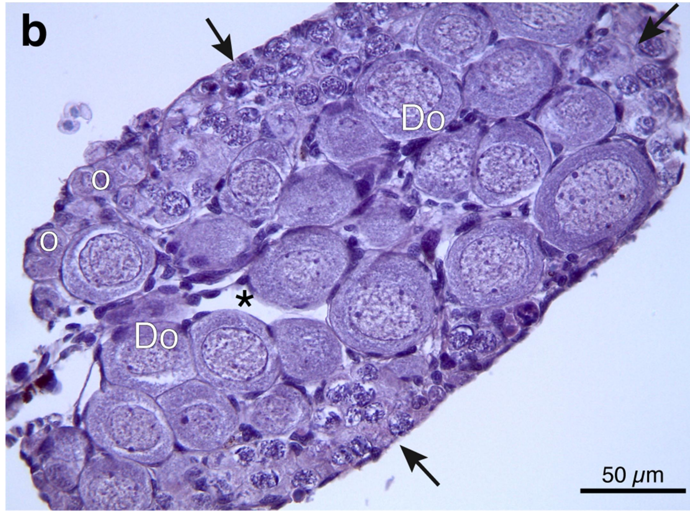
[g] GMCI = gonad-mesonephros complex index;

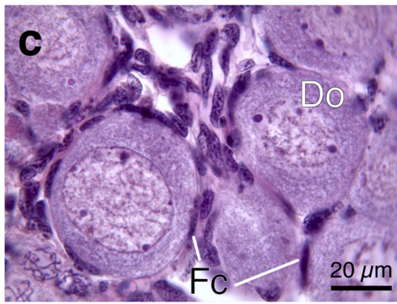
[h] LSI = liver somatic index;

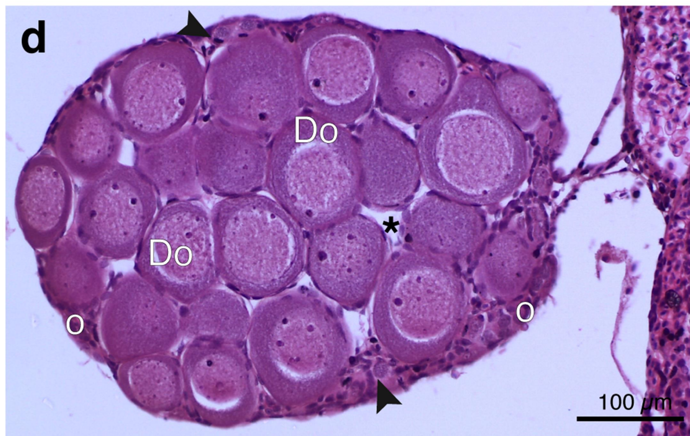
Values are reported as means (± standard error).

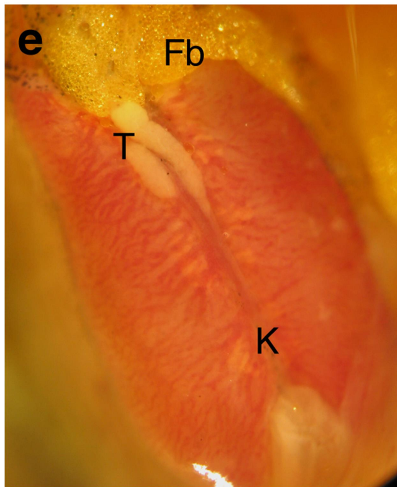
Plate 1

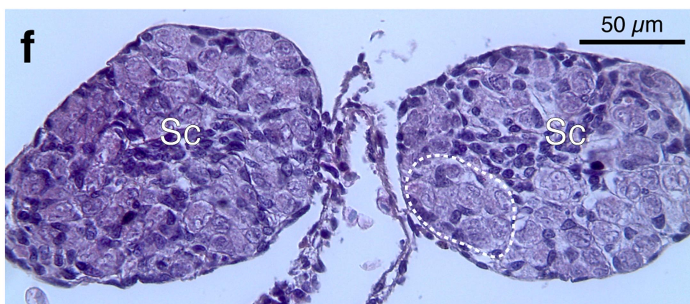


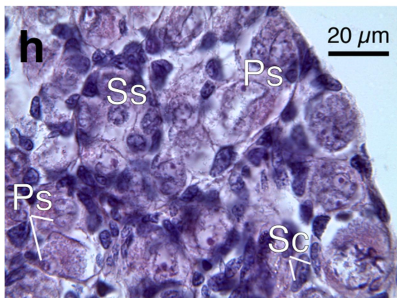


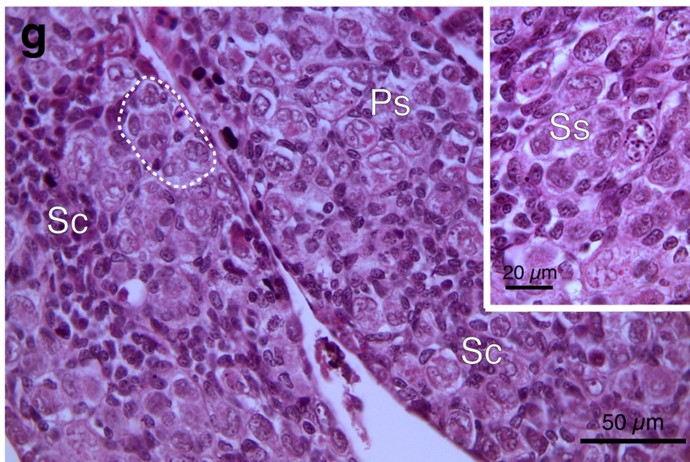












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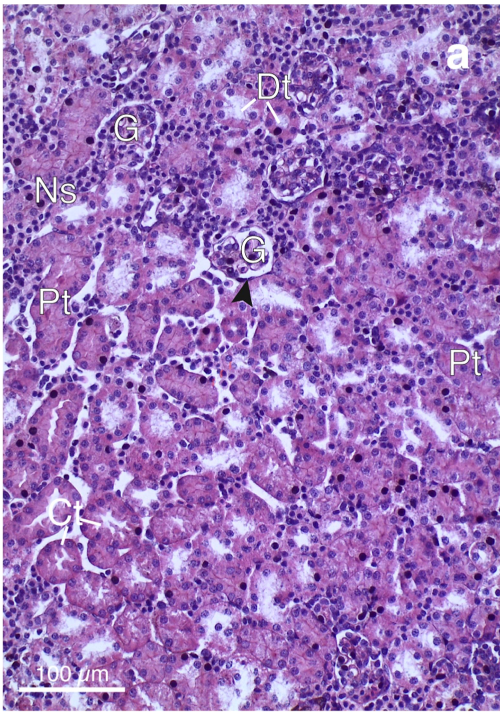

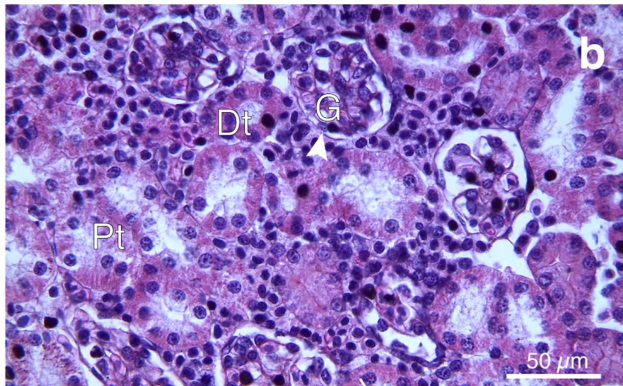

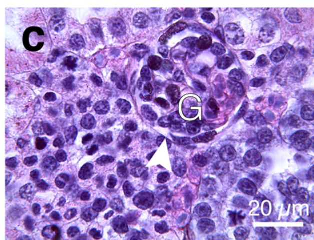

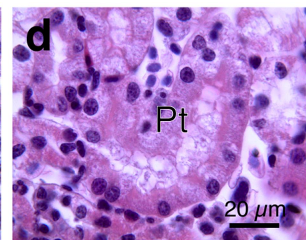

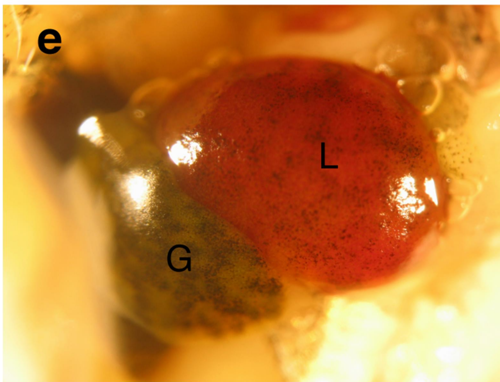

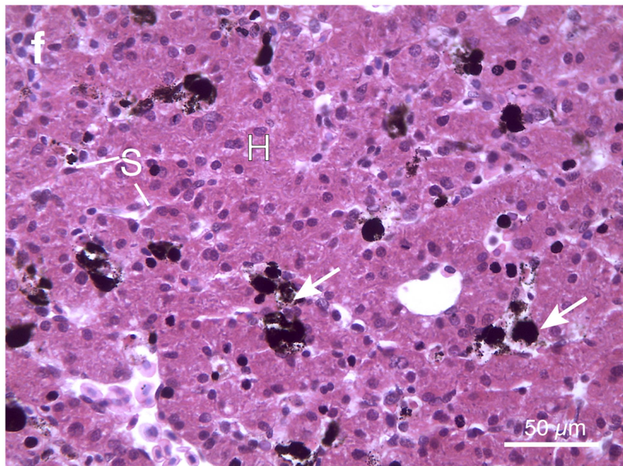

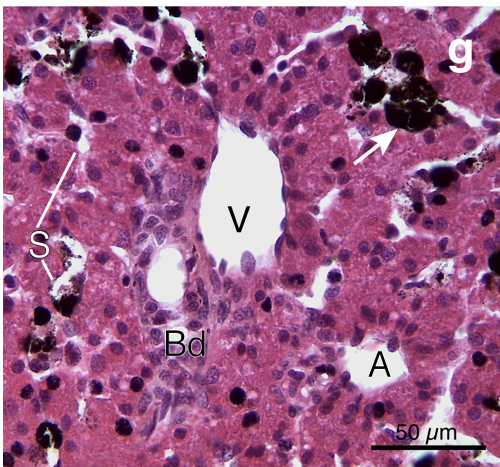

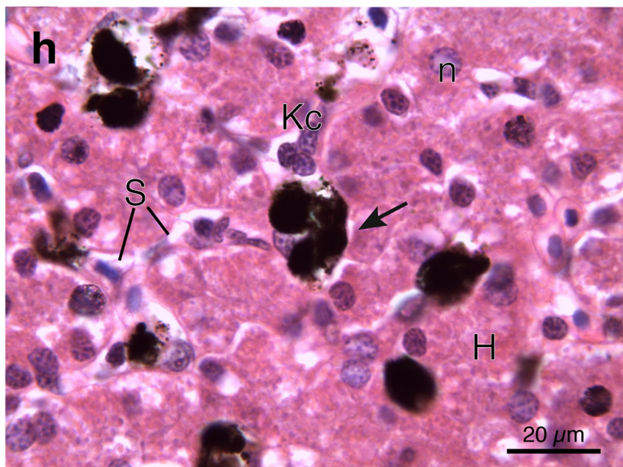

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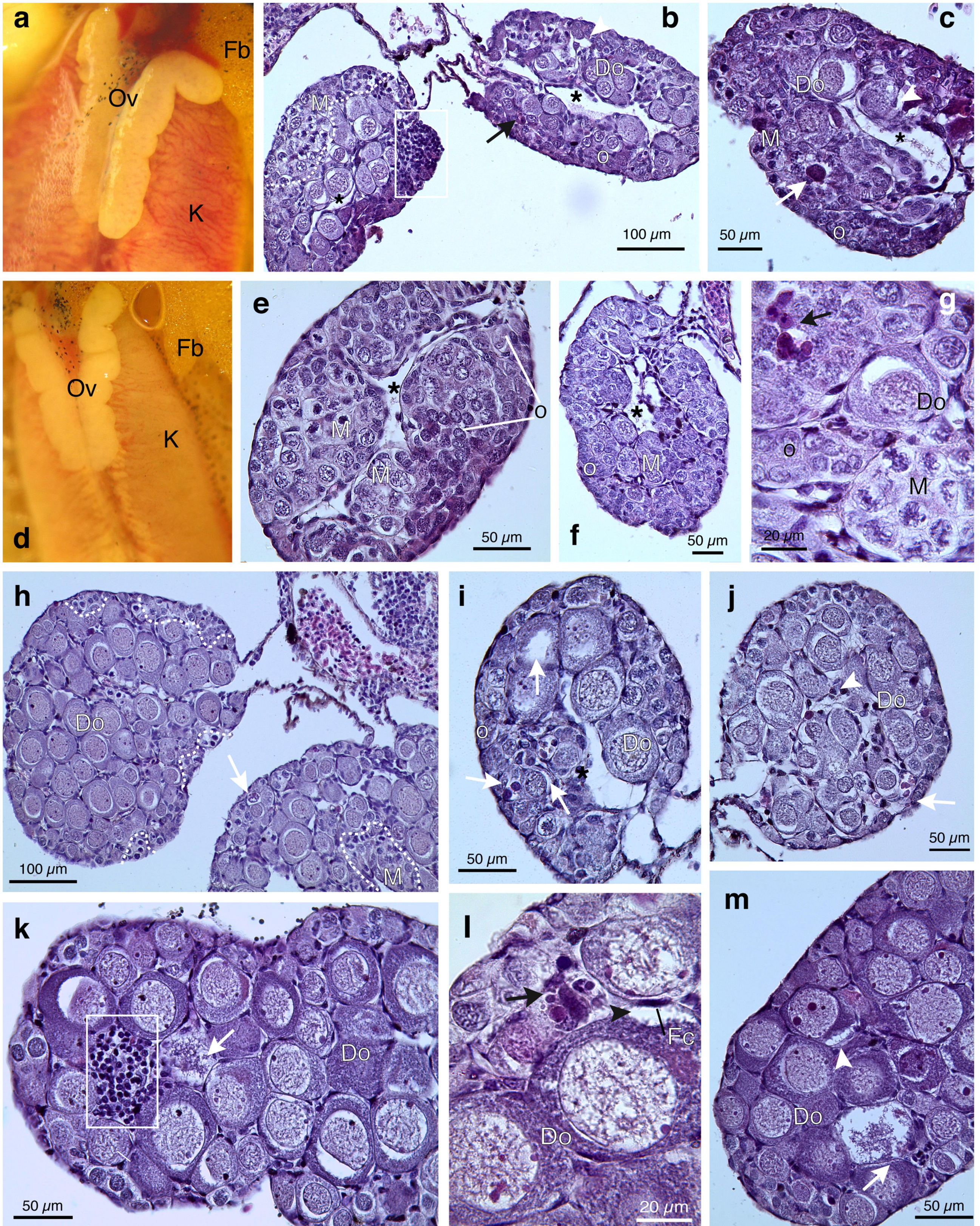

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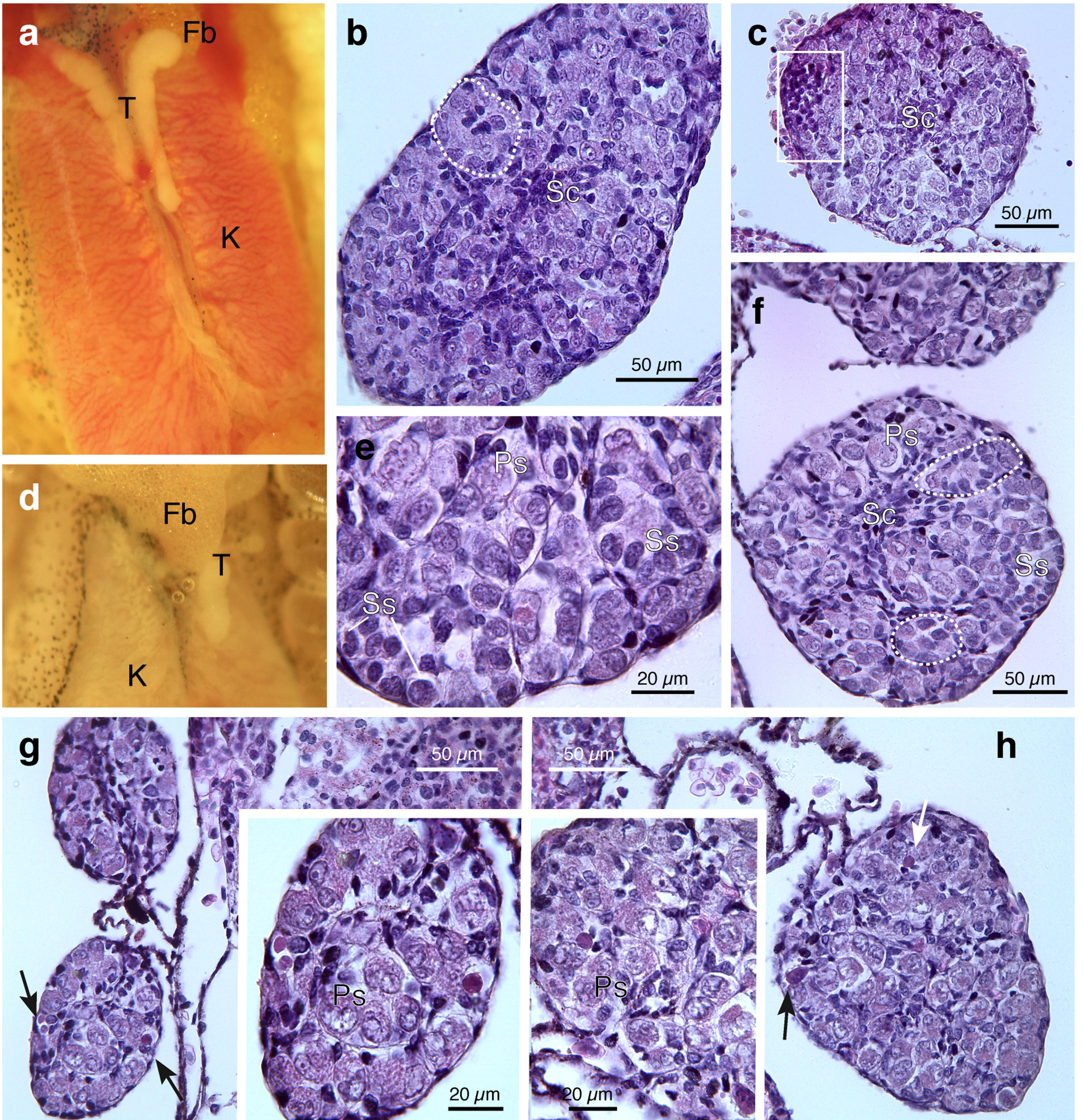

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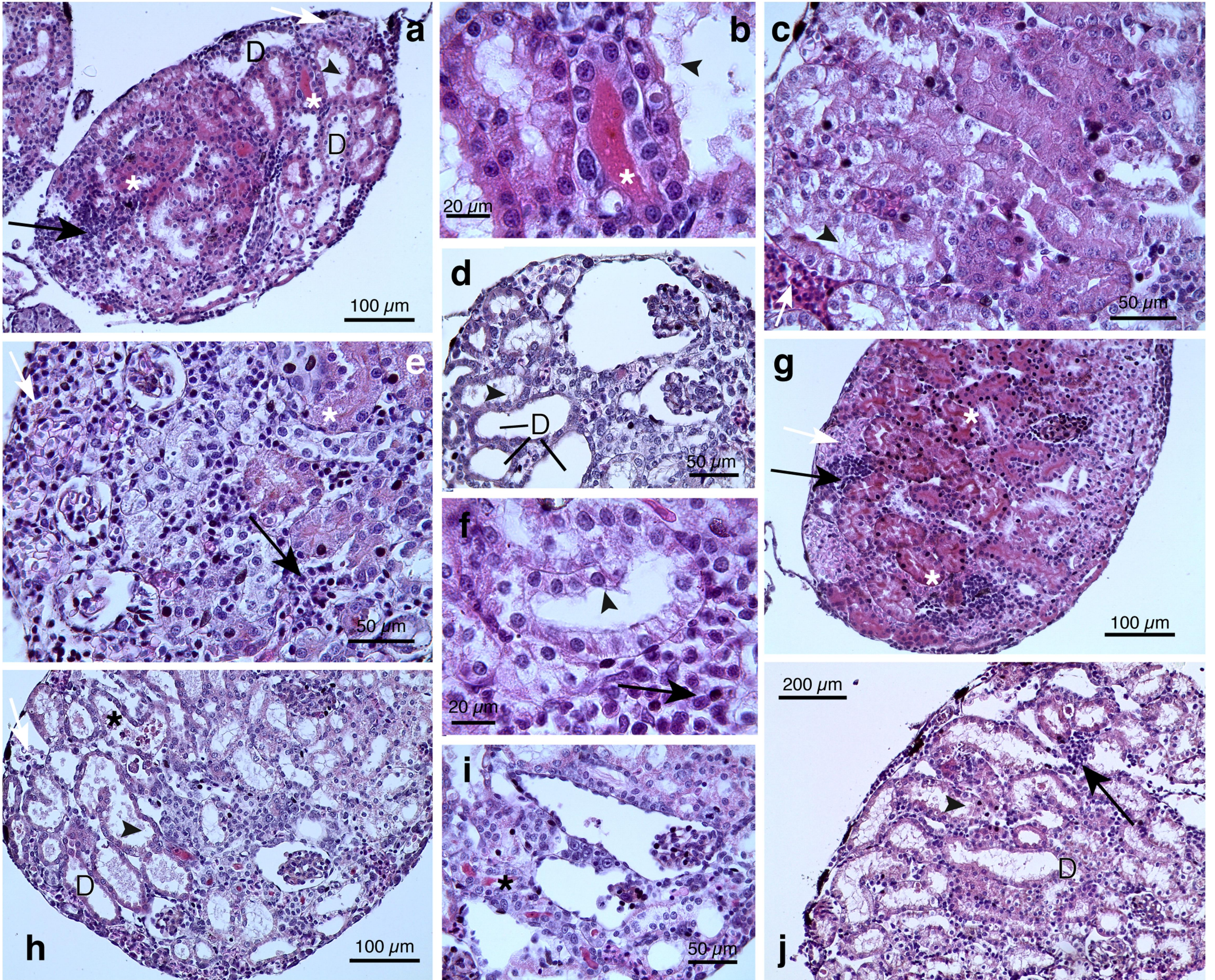

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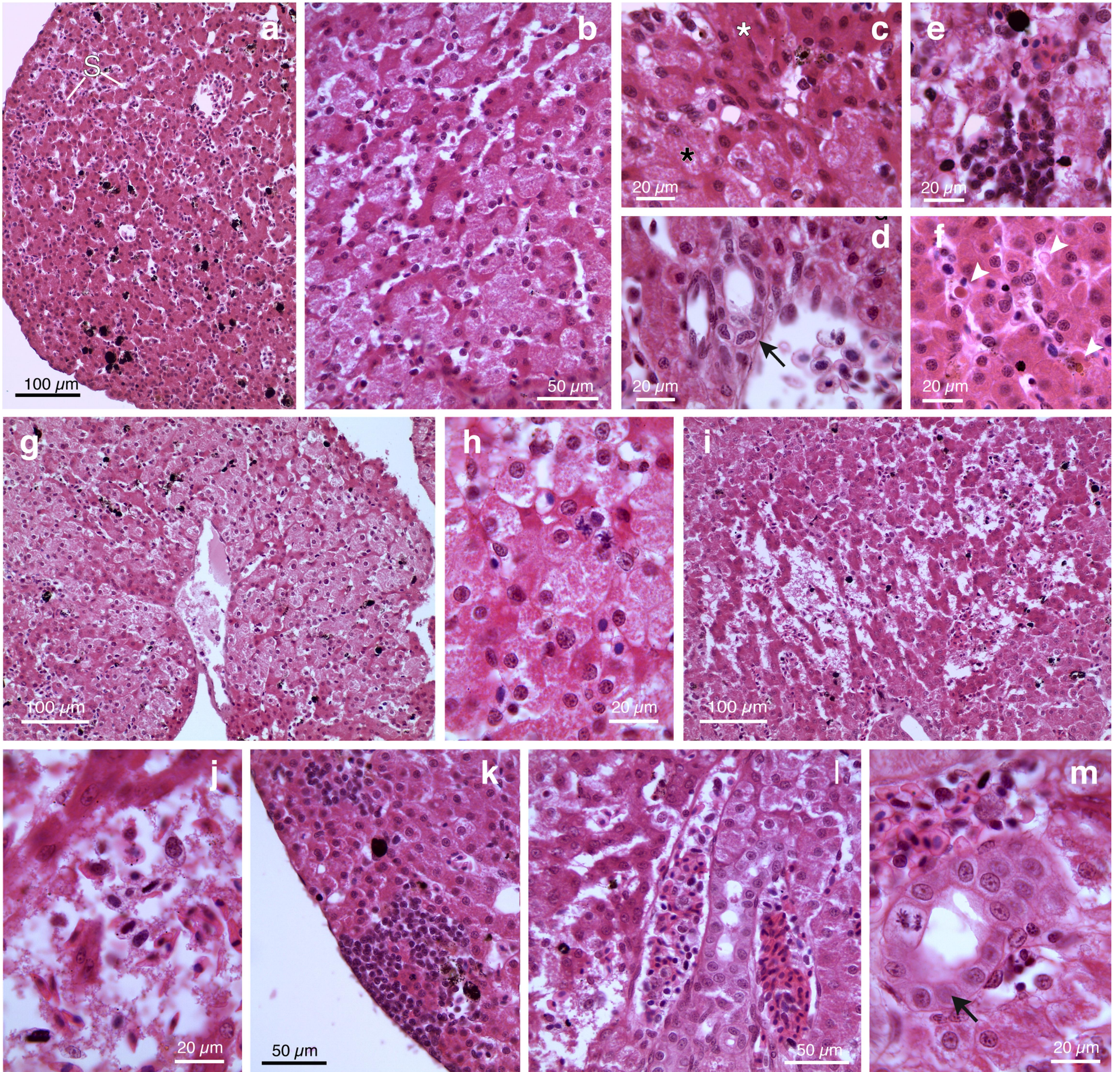

Plate 7

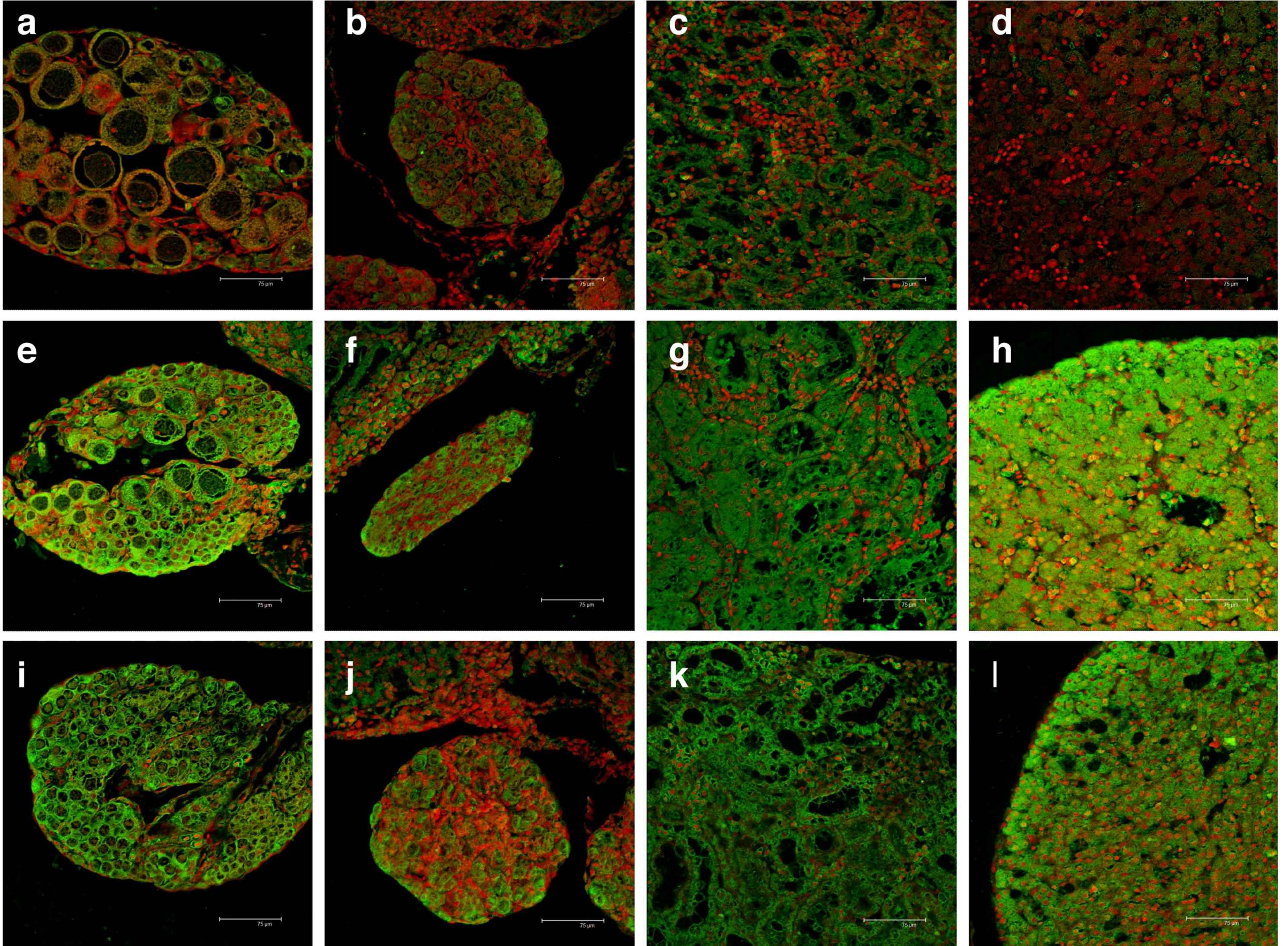

Plate legends

Plate 1. Ovaries and testes from control *Hyla intermedia* juveniles. (**a**) Ovaries and (**e**) testes in macroscopic ventral view attached to kidney; the fat bodies are present at the left side. (**b, c**) Cross-sections showing typical ovarian tissue of an ovary at stage VIII: the ovarian cavity is filled with growing diplotene oocytes; note in the cortex proliferating areas of oogonia and nests of leptotene-pachytene meiocytes (arrows). (**c**) Particular of diplotene oocytes surrounded by follicular cells. (**d**) Cross-section of an ovary at stage IX packed with numerous and large growing diplotene oocytes; note proliferating germ cells restricted in peripheral spots (arrowheads). (**f**) Cross section of testes depicting proliferating spermatogonia into seminiferous tubules in formation (marked by dotted lines); somatic cell are flattened and darkly stained. (**g**) At higher magnification is possible to distinguish primary and secondary spermatogonia. (**h**) Longitudinal sections of testicular tissue showing developing seminiferous tubules. (Ov = ovaries; T = testes; K = kidney, Fb = fat body; Do = diplotene oocytes; Fc = follicular cells; o = oogonia; * = ovarian cavity; Sc = somatic cells; Ps = primary spermatogonia; Ss = secondary spermatogonia). All sections are HandE.

Plate 2. Kidney and liver from control *Hyla intermedia* juveniles. (**a**, **b**) Renal parenchyma showing typical architecture with filtration unit, the capsule of Bowman surrounding the glomerulus, and renal tubules. (**c**) Particular of renal corpuscles formed by a globular mass of specialized capillaries, enclosed into the Bowman's capsule. (**d**) Note the luminal brush borders of the proximal tubules. (G = glomerulus; Dt = distal tubule; Pt = proximal tubule; Ns = neck segment; Ct = collecting tubule; arrowheads = Bowman's capsule). (**e**) External view of liver showing an orange-brown color and pigmented cells. A large dark-green gallbladder lies on the liver. (**f**) Histological section depicting the homogenous organization of hepatic tissue. (**g**) Hepatic portal area constituted by bile duct, branches of both portal vein and hepatic artery. (**h**) Particular at higher magnification showing large hepatocytes with rounded nuclei and granulated cytoplasm; Kupffer cells are anchored to sinusoid endothelium. (L = liver; G = gallbladder; H = hepatocytes; S = sinusoid; V = branch of the portal vein; A = branch of the hepatic artery; B = bile duct; Kc = Kupffer cells; n = nucleus; arrows = melanomacrophages complex). All cross-sections are HandE.

Plate 3. Ovaries from animals exposed to 5 μg/L (**a - c, f - h**) and 50 μg/L (**d**, **i - m**) of pyrimethanil. (**a, d**) Typical ovarian gross morphology in exposed animals with no sign of alterations compared to control. (**b**, **c**, **e - g**, **i**) Females, at histological level, that have a poorly developed ovarian structure with respect to control; even though ovarian cavity lined by somatic cells is well visible (*) note that the cortex is filled with oogonia and leptotene-pachytene oocytes and almost all of the germ cells are oogonia or oocytes at early stage of meiotic division; a smaller number of diplotene oocytes are present. (**h, j - m**) Ovaries at stage VIII or IX: the progression of oocyte development is manifested by an ovary tissue fully composed by growing previtellogenic diplotene oocytes; note external patches of proliferation containing dividing oogonia and/or nests of leptotene-pachytene oocytes (marked by dotted lines). (**b, c, h - m**) Diffuse degeneration phenomena affecting all germ cell types (arrows). (**b**, **c**, **j**, **l**, **m**) Note the enlargement of intercellular spaces and the detachment of diplotene oocytes from the follicular cell (arrowheads) and (**b, k**) mononuclear cells infiltration (marked by rectangle). (**g, l**) At higher magnification the presence of numerous apoptotic bodies, visible as dark pink spots, frequently engulfed by macrophages is clearly detected (arrows). (Ov = ovaries; K = kidney, Fb = fat body; Do = diplotene oocytes; Fc = follicular cells; o = oogonia; M = meiocytes at leptotene-pachytene stages). All cross-sections are HandE.

Plate 4. Testes from animals exposed to 5 μg/L (**a - d**) and 50 μg/L (**e - h**) of pyrimethanil. (**a**) Testicular normal gross appearance in exposed animal with no significant differences compared to control. (**b**, **e, f**) Cross sections showing well-preserved testicular tissue with primary and secondary spermatogonia within seminiferous tubules (marked by dotted lines). (**c**) Note the presence of mononuclear cells infiltration (marked by rectangle). (**d**) Macroscopic ventral view of a specimen with only one left testis with reduced dimension. (**g**, **h**) In some treated animals seminiferous tubules tend to be less well organized and alterations of testicular tissue are detected: including enhancement of the intercellular spaces, primary spermatogonia/germ cells showed hydropic tumefaction or signs of focal necrosis (arrows) The cells of interest are magnified in the inset. *(*T = testes; K = kidney, Fb = fat body; Sc = somatic cells; Ps = primary spermatogonia; Ss = secondary spermatogonia). All sections are HandE.

Plate 5. Representative light micrographs showing marked kidney impairments in *H. intermedia* after exposure to 5 μg/L (**a - f**) and 50 μg/L (**h - j**) of pyrimethanil. Extensive signs of tubular degeneration are detected including: (**a**, **d**, **h**, **j**) renal tubular dilation (D); (**b**, **f**) destruction of brush border (arrowheads) clearly appreciable at higher magnification; (**b**, **c**, **e**, **f**, **h-j**) diffuse loss of the integrity of the cells, hydropic swelling, vacuolization and apoptotic cells. (**a**, **b**, **g - i**) The renal tubules contain intraluminar proteinaceous deposits (*) that appear as dark pink eosinophilic material. **(d**, **e**, **h - j**) Considerable glomerular shrinkage and enlargement of Bowman's capsula can be observed. (**a**, **c**, **e - h**, **j**) Inflammatory phenomena are frequently detected: infiltration of inflammatory cells, mainly mononuclear cells (black arrows); haemorrhage (white arrows). All cross sections are HandE.

Plate 6. (**a - f**) Representative light micrographs depicting alterations observed in *H. intermedia* liver after exposure to 5 μg/L of pyrimethanil. (**a**) Congestion and dilatation of sinusoids (S). (**b**) Liver parenchyma dyschromia. (**c**) Hepatocytes with weakly (white asterisk) and strongly (black asterisk) eosinophilic cytoplasm. (**d**) Degenerating bile ducts (arrow). (**e**) Foci of mononuclear cell infiltration. (**f**) Apoptotic cells (arrowheads). (**g - m**) Representative light micrographs depicting alterations observed in *H. intermedia* liver after exposure to 50 μg/L of pyrimethanil. (**g**) Note the diffuse and extensive degenerative phenomena in liver parenchyma. (**h**) Particular at higher magnification of hepatocytes in degeneration. (**i**, **j**) Hepatic parenchyma completely disorganized in which is possible to note extended necrotic and hemorrhagic zones. (**k**) Mononuclear cell infiltration. (**l**) Blood vessels congestion. (**m**) Note the disorganization in bile ducts (arrow). All cross sections are HandE.

Plate 7. Confocal micrograph of *H. intermedia* ovaries (**a**,**e**,**i**), testes(**b**,**f**,**j**), kidney (**c**,**g**,**k**) and liver (**d**,**h**,**l**) sections labeled with rabbit polyclonal antibody against caspase-3 (green – FITC labeled); nuclei are labeled with propidium iodide (red). (**a-d**) In control specimen no or extremely weak immunoreaction could be revealed. (**e-l**) After exposure to both pyrimethanil concentrations, it was possible to note a marked increase in caspase-3 immunoreactivity in samples from both low (**e-h**) and high (**i-l**) concentration groups.

Chapter 7

Discussion

7.1. Effects of long-term exposure to pyrimethanil and tebuconazole, on survival and life history traits

In this study we showed that a chronic exposure to environmentally realistic concentrations of tebuconazole and pyrimethanil affects *H. intermedia* survival, development and metamorphic traits, causing also severe malformations. Our results are consistent with previous literature data examining the potential toxicity of tebuconazole and pyrimethanil on other aquatic organisms (Andreu-Sánchez *et al*., 2012; Ferreira *et al*., 2010; Konwick *et al*., 2006; Sancho *et al*., 2010; Toni *et al*., 2011a,b; Seeland *et al*., 2012, 2013; Araújo *et al*., 2014a,b).

7.1.1. Survival

We demonstrated that exposure to tebuconazole has a greater effect on *H. intermedia* tadpoles and froglets than does exposure to pyrimethanil.

Before the onset of metamorphic climax, we demonstrated for both fungicides that the low concentrations (5 μg/L) induced significantly greater effects than the higher ones (50 μg/L) on survival and deformity incidence. Such nonlinear relationship between concentration and effect has been previously reported in other anuran species after long-term exposure to the fungicide chlorothalonil (McMahon *et al*., 2011), and to the herbicide atrazine (Brodeur *et al*., 2013; Storrs and Kiesecker, 2004). Low dose effects and non-monotonic dose responses

represent a widely discussed topic with particular referral to endocrine disrupting chemicals (EDCs) (Beausoleil *et al*., 2013; Lagarde *et al*., 2015; Vandenberg *et al*., 2012). Several *in vitro* and *in vivo* studies, in mammalian and fish models, revealed that tebuconazole and pyrimethanil have the potential to induce endocrine disruption by inhibition of key enzymes required for androgen and estrogen synthesis (CYP family) (Medjakovic *et al*., 2014; Orton *et al*., 2014; Prutner *et al*., 2013; Taxvig *et al*., 2007; Yu *et al*., 2013a). In amphibians, it has been recently demonstrated that tebuconazole concentrations ranging from 0.1 to 500 µg/L are able to disrupt steroidogenesis (Poulsen *et al*., 2015). The endocrine disruption potential of these fungicides may represent a plausible explanation for the low dose effects observed here before metamorphic climax.

During the metamorphic climax, a complete reversal of the survival trend takes place, for both fungicides, leading to a greater metamorphic success in the low concentration groups. Our results strongly indicate that to determine whether the trend of an endpoint is either linear or nonlinear it is necessary to consider different developmental stages, since fungicides may show unexpected and modifying effects. To explore and validate a putative non-monotonic dose-response curves for two tested fungicides further studies, testing more than two concentrations and/or other sensitive endpoints, are needed.

7.1.2. Morphological abnormalities

It is well known that agrochemicals are able to induce morphological abnormalities in amphibians (Agostini *et al.*, 2013; Taylor *et al.*, 2005; Bernabò *et al.*, 2011a; Brunelli *et al.*, 2009), but very few data are available regarding fungicides (Yu *et al.*, 2013b). Within this context, another important finding from our study is the strong correlation emerged between fungicides exposure and the incidence of morphological abnormalities (i.e. tail malformations, scoliosis, edema, mouth and limb deformities). The decrease of deformity incidence at metamorphosis is an artifact due to two factors: the resorption of the tail, that accounted for a good portion of deformities, and the high mortality of deformed individuals during metamorphic climax. For instance, malformed individuals under controlled laboratory conditions have been able to grow and develop but the consequences on performance and fitness could be dramatic in nature.

7.1.3. Metamorphic traits

During metamorphic climax we recorded a mortality increase in both control and exposed groups. Metamorphosis represents the most critical event in amphibian life during which larvae undergo morphological, biochemical and physiological changes and they experience a higher probability of mortality; this explains the slight increase observed also in control group.

7.1.3.1. Success at metamorphosis

We observed a drastic decrease in the success at metamorphosis in all exposed groups, compared to control. This finding is consistent with previous studies that

have shown a similar effect after exposure to different pesticides (Bridges, 2000; Brunelli *et al*., 2009; Lavorato *et al*., 2013; Teplitsky *et al*., 2005). As suggested for other lipophilic compounds, it might be valid for pyrimethanil and tebuconazole that once accumulated and stored in body tissue (i.e. fat of the tail) they might suddenly be released and mobilized during tail resorption thus causing the increase in mortality or other detrimental effects (Bridges, 2000; Fioramonti *et al*.,1997; Hartman *et al*., 2014; Rosenshield *et al*., 1999). We showed that tebuconazole was able to induce a higher mortality than pyrimethanil and this is in agreement with its higher lipophilicity (log K_{ow} 3.7 for tebuconazole and 2.84 for pyrimethanil). Moreover, recent evidences demonstrated bioaccumulation ability of tebuconazole revealing high levels of residues in whole-body extracts from wild Pacific chorus frogs (Smalling *et al*., 2013) and in adipose tissue, kidney, liver and brain in African clawed frogs (Hansen *et al*., 2014; Poulsen *et al*., 2015).

7.1.3.2. Mass and time to metamorphosis

It is well documented that pollutants may affect amphibian metamorphosis, and that age and size at metamorphosis are the most sensitive indicators of developmental toxicity (Brande-Lavridsen *et al*., 2010; Brodeur *et al*., 2013; Brunelli *et al*., 2009; Hayes *et al*., 2006, 2010; Lavorato *et al*., 2013). Amphibian larvae exhibit phenotypic flexibility in growth and development rate in response to different environmental factors and to the presence of stressors (i.e. osmotic or thermal stress, food level, habitat desiccation, larval density, competitors or predators and presence of pollutants) (Cabrera-Guzmán *et al*., 2013; Denver, 2009; and references therein). The presence of environmental perturbations may induce

an energy re-allocation for activation and maintenance of detoxification mechanisms thus limiting resources for growth and development (Bernabò *et al.*, 2013).

We showed that metamorphs from exposed groups weighed more than controls; in this regard it must be considered the appearance of edema that accounted for a great amount of body mass. This response has also been reported in leopard frog after exposure to polychlorinated biphenyl 126 and the authors suggested that body masses of metamorphs could be skewed by the presence of edema (Rosenshield *et al.*, 1999). The same authors suggest that the appearance of edema may be related to the mobilization of lipophilic compounds, stored in larval tissue, during metamorphic climax.

Another important finding of our study was the significantly longer time to complete metamorphosis in the fungicides exposed groups. This resulted from the reduced developmental rate just prior to the onset of metamorphic climax; in the high tebuconazole concentration group the absence of a significant difference is an artifact that may be attributed to the high mortality rate that reduced sample size by the end of experiment.

A greater both body mass and a longer time to metamorphosis have been reported after exposure to the fungicides prochloraz and triphenyltin (Brande-Lavridsen *et al.*, 2010; Fioramonti *et al.*, 1997); only a longer time to metamorphosis has also been observed after exposure to mancozeb, triphenyltin and propiconizole (Hayes *et al.*, 2006; Shenoy *et al.*, 2009; Teplitsky *et al.*, 2005).

One possible explanation for the observed metamorphosis delay could be the disruption of thyroid hormones playing the most important role but also the

detrimental effects on one or several target tissues; regardless of the underlying mechanism further studies are needed.

Many amphibian species, such as *Hyla intermedia*, often breed in unpredictable and ephemeral freshwater habitats and this implies that tadpoles initiating and completing metamorphosis late are subjected to an increased risk of habitat drying and predation. Our results strongly support the hypothesis that the stressful environments experienced by organisms during early development shape the timing of developmental processes and growth trajectories, leading to different probabilities of survival in the developmental habitat, and often limiting the potential for adaptive plastic responses later in life (Brande-Lavridsen *et al*., 2010; Denver, 2009).

Conclusion

This study represents the first evidence of sublethal effects exerted by tebuconazole and pyrimethanil on amphibians and highlights the hazardous properties of these two common-used fungicides for non-target species. Our results clearly indicate that, through their effects on survival, development and metamorphic traits, these fungicides may have detrimental long-term individual and population consequences. In our opinion, a more research effort, in both laboratory and field conditions, must be conducted to better elucidate the impacts of fungicides to amphibians, in order to determine amphibian conservation strategies.

7.2. Effects of long-term exposure to pyrimethanil on gonads, liver and kidney

Our results were successful in demonstrating that the anilinopyrimidine fungicide pyrimethanil has the potential to induce histological alterations in tissues of the Italian tree frog *Hyla intermedia*. We revealed that a long-term exposure to environmentally realistic concentrations of pyrimethanil caused severe histopathologic damages in gonads, liver and kidney. To the best of our knowledge, this is the first report describing the effects of pyrimethanil on the morphology of these organs in amphibian. The findings of this study are consistent with our previous results showing the reduced survival rate, the alteration in development and metamorphic traits and the incidence of severe malformations induced by this fungicide in the same species. Moreover, we described for the first time the histological features of *H. intermedia* gonads, liver and kidney, under basal condition.

7.2.1. Gonads

Histological observations on *H. intermedia* tadpoles and juveniles under basal conditions, revealed that both ovaries and testes exhibit a general arrangement similar to that described for *H. arborea* and for other species belonging to Hylidae family (Goldberg, 2015; Takahashi, 1958; Ogielska and Kotusz, 2004; Haczkiewicz and Ogielska, 2013; Gramapurohit *et al.*, 2000); in addition we demonstrated that in *H. intermedia*, during pre-metamorphic larval stages, developing gonads directly differentiated into ovaries or testis and the gonadal differentiation is completed by the end of metamorphosis.

Anuran gonads are most susceptible to malformation during the period of sexual differentiation and the time in which this takes place has been demonstrated to differ between species (Ogielska and Bartmanska, 1999; Ogielska and Kotusz, 2004; Storrs and Semlitsch, 2008). After exposure to pyrimethanil, we detected an unmodified phenotypic sex ratio in both experimental groups; also morphological analysis revealed no sex reversal or abnormal gonadal intersex at metamorphosis thus supporting the hypothesis that this fungicide is not able to induce feminization or masculinization in *H. intermedia*. Numerous studies in both fishes and amphibians indicate the incidence of intersex as the main gonadal abnormality induced by exposure to EDCs (Abdel-moneim *et al*., 2015; Orton and Tyler, 2015). According with previous findings on the effects induced by several EDCs on amphibians sexual development, the susceptibility and the incidence of sex reversals is a species-specific response (Mali and Gramapurohit, 2016 and references therein). Our results emphasize the importance of studies on non-model species when evaluating the effects of EDCs and other pollutants since an extrapolation of single-species data to other may be misleading (Tamschick *et al*. 2016a).

We clearly showed that a chronic exposure to pyrimethanil causes severe abnormalities in both oogenesis progression and histological features of gonads, despite the well maintained gross morphology, and the absence of intersex and/or sex reversal. The presence of unpaired gonad observed in two males from the low concentration group has been previously reported by Goldberg (2015) in both males and females of *Scinax fuscovarius*, another species belonging to Hylidae, in natural condition. Aside, the unique gonad in male from our experiments,

displayed a normal appearance thus suggesting that no correlation exists between the incidence of this anomaly and the exposure to xenobiotic.

The role of histological evaluation is largely recognized as the key tool in phenotypic sex determination (Bernabò *et al*., 2011b; Tompsett *et al*. 2012, Wolf *et al*., 2010). Our results strongly support the concept that histological analysis is able to disclose a host of alterations, beyond intersex incidence, that may underpin reproductive pathologies, also when anatomical organization is largely unaffected. One of the most important findings of our study is the highly significant underdevelopment of the meiotic oocytes as it clearly indicates that female fertility in *H. intermedia* may be compromised by pyrimethanil, even at very low concentrations. A similar effect, although much more pronounced, has been reported in the South American toad, *Rhinella arenarum* after exposure to Maxim® XL, a broad spectrum fungicide (Svartz *et al*., 2016). As pyrimethanil interferes with normal sexual differentiation, causing a delay in gonads development, their endocrine disruption potential is evident and largely supported by this study.

There is surprisingly few data on the effects of fungicides on reproductive system, despite they are known or presumed to act as EDCs. Reproductive function in Amphibians may be impaired through multiple mechanisms acting individually and/or synergically. As for other pesticides, aryl hydrocarbon receptor (AhR)-mediated effects have been hypothesized for pyrimethanil in studies *in vitro* (Medjakovic *et al*., 2014). The AhR is a transcriptional factor belonging to the basic helix–loop–helix (bHLH)-Per-ARNT-Sim (PAS) family that mediates a wide range of biological and toxicological responses in several different species and tissues.

It has been suggested that AhR-mediated cellular responses may have a pivotal role in female reproductive process (Pocar *et al.*, 2005). A growing body of evidence established the interactions between the AhR and different intracellular pathways as a result of exposure to a variety of synthetic and naturally occurring chemicals (Denison *et al.*, 1988). Whether the exact molecular events is determined or not, xenobiotic compound that are able to modulate endogenous endocrine messengers inevitably impair reproduction and developmental events (Pocar *et al.*,2005).

In addition to the alterations of gonads maturation pattern, we also detected a number of histological abnormalities in both testes and ovaries after exposure to pyrimethanil. Both apoptosis and degeneration was considerably increased suggesting the coexistence of different pathological pathway. Programmed cell death in the reproductive systems intervenes removing damaged cells (Chen *et al.*, 2015) thus explaining in part the reduction of gonadal maturation induced by pyrimethanil.

We revealed an intense labeling for caspase-3 in gonads of both male and females exposed to pyrimethanil and in particular the most intense immunoreactivity has been observed in females from low concentration group. In mammals oocyte, apoptosis is regulated by concentrations of circulating sex steroids with estrogens preventing atresia and androgens increasing this process (Billing *et al.*, 2003). Mackenzie and colleagues (2003) suggested that the testosterone increase, due to the treatment with an aromatase inhibitor, may be the cause of the ovarian atresia observed in two species of Ranidae. The role of steroids in amphibians gonad differentiation has not definitely been clarified and available information on the

steroid hormonal control of gonad differentiation are scarce and conflicting (Mali and Gramapurohit, 2016; Nakamura, 2010). However, one may speculate that aromatase inhibition would be one of the mechanisms involved in gonadal impairment after exposure to pyrimidine fungicide (Sanderson, 2006; Saxena *et al*., 2015).

The AhR-mediated action does not exclude other alternative physiological responses and pyrimethanil also induce inflammation as demonstrate by the presence of both macrophage and mononuclear cellular infiltrate.

7.2.2. Liver

Our observations on healthy *H. intermedia* liver confirm the general organization previously described for other amphibian species, and other vertebrates (Akiyoshi and Asuka, 2012; Bruni and Porter, 1965; Ells, 1954; Goldblatt*et al*., 1987; Spornitz, 1975). The hepatic parenchyma is composed of hepatocytes, arranged in clusters and cords, surrounding a network of sinusoids of different size. We showed the presence of a well-defined portal triad, made by bile duct branches of the portal vein and hepatic artery), thus contributing to the discussion on whether a portal triad would be a common structural feature of Vertebrate liver or not (Akiyoshi and Asuka, 2012; Odokuma and Omokaro, 2015).

For their crucial role in detoxification of most xenobiotic compounds the liver is considered as the main target organ for the assessment of histopathological changes. In the present study we clearly demonstrated that a chronic exposure to pyrimethanil low concentrations resulted in modifications of liver morphology in a dose related manner.

The dilation and congestion of both blood vessels and sinusoid were one of the most evident result of pyrimethanil application. Similar findings were reported in several amphibian species after exposure to both pesticides and heavy metals (Bandara *et al*., 2012; Çakıcı *et al*., 2015; Ikechukwu and Ajeh, 2011; Păunescu *et al*., 2010, 2012). Some authors suggested that endothelial cells alterations may affect the blood flow from the hepatic artery and veins to the central vein thus resulting in extravasations of red blood cell and sinusoids dilation (Bandara *et al*., 2012; Olurin *et al*. 2006).

We also showed a remarkable liver tissue dischromia after exposure to pyrimethanil highlighting the establishment of different pathologic response in the same tissue. Numerous interrelated biochemical and molecular mechanism are involved in cellular outcomes to chemical stressors; proliferative status, repair enzyme capacity, and the ability to induce proteins promoting/inhibiting cell death determine the cellular fate. It is now becoming increasingly clear that both necrosis and apoptosis can occur in response to the same type of insult (Elmore *et al*., 2016; Venturino and de D'Angelo, 2005).

After exposure to pyrimethanil, a common alterations observed in *H. intermedia* hepatocytes were the appearance of numerous apoptotic bodies. As confirmed by immunodetection of caspase-3, a key mediator of apoptosis, this process peaked in the hepatocyte of low concentration groups. Programmed cell death is an important mechanism for the removal of senescent, or damaged cells and apoptosis have been demonstrated to be induced by several EDCs *in vitro* studies (Aoki *et al*., 2004; Yao *et al*., 2006, 2007). Moreover it has been reported that in

fish liver other xenobiotics exert their toxicity via apoptosis (Kaptaner and Ünal, 2010; Piechotta *et al*., 1999).

In our experiment the apoptosis occurred in conjunction with the necrosis thus demonstrating the attempt of liver tissue to restore homeostasis, and the otherwise strong cytotoxic effect of pyrimethanil. The higher fungicide concentration probably favours the onset of the degenerative phenomena.

The necrotic cells in were easily recognizable *H. intermedia* liver by their pale cytoplasm; these are characterized by cell and nuclear swelling that led to the accumulation of cellular debris into the cytosol and interstitial space and subsequent recruitment of inflammatory cells. Vacuolization and necrosis in hepatic cells and hemorrhage have been reported in *B.variabilis* after exposure to the insecticide carbaryl (Cakıcı *et al*., 2015) and in both fish and amphibians after exposure to copper (Figueiredo-Fernandes *et al*., 2007; Gürkan and Hayretdağ, 2009; Osman *et al*., 2009).

7.2.3. Kidneys

The gross morphology and histology of kidney of *H. intermedia* under basal conditions correspond to that previously described for other amphibian species (Holz and Raidal 2006; Møbjerg *et al*., 2000, 2004; Pessier, 2009), besides this topic appears overlooked by researchers. As in other vertebrates amphibians kidney, like the liver, plays a major role in maintaining a stable internal milieu and it is well acknowledged as an important target for xenobiotic. We showed that, despite a well preserved gross morphology, pyrimethanil is able to affects morphological organisation of the kidney in *H. intermedia* and all exposed samples displayed

some pathological alterations, involving both renal tubules and corpuscles. The most remarkable effects were observed in samples from the high concentration group, although the intensity of injury varied, to some extent, depending on the individual response.

Histological alterations most frequently detected were a considerable infiltration of inflammatory cells, a pervasive tubular dilation, a widening of Bowman spaces and a shrinkage of the capillaries. Both the inflammatory cell infiltration and the dilation of renal parenchyma can be induced by different types of toxic injury to the tubule epithelium (Loumbourdis, 2003). In addition, the destruction of brush border observed in the proximal renal tubule of *H. intermedia* have also been observed in amphibians after exposure to heavy metals and the authors suggested that this may explain the well-known alteration of both reabsorptive and secretory functions of proximal tubules induced by metals (Herak-Kramberger and Sabolic, 2001; Loumbourdis, 2003).

Other histopathological changes frequently detected were hydropic swelling, the presence of proteinaceous fluid within the renal tubules. Moreover both necrotic cells with a pale cytoplasm and apoptotic cells were scattered through the kidney tissue.

Limited studies are available for comparison with our results to evaluate the consequences of pesticides on the amphibian kidney (Cakıcı *et al*., 2015) but our findings are consistent with the few previous reports on this organ. Similar changes were reported in adult of *B. variabilis* after carbaryl oral administration (Cakıcı *et al*., 2015) and in adult of *Pelophylax ridibundus* after intraperitoneal injections of both the copper-containing fungicide Champion 50WP (Păunescu and

Ponepal, 2011) and the pyrethroid insecticide Talstar 10EC (Păunescu *et al.*, 2012). Comparable pathological effects have also been reported in frogs inhabiting contaminated area (Fenoglio *et al.*, 2011) and after exposure to several heavy metals (Loumbourdis, 2003; Medina *et al.*, 2016).

Independently from the administration route, it seems that the most common pathological effects on kidney are not exclusive to a specific contaminant. Nevertheless, the high sensitivity of the kidney to xenobiotic indicate histopathological alterations of this organ as a good indicators for risk assessment studies.

Conclusion

This is the first evidence of the effects induced by pyrimethanil on gonads, liver and kidney histology and point out the hazardous properties of this commonly used fungicide for non-target species. On the basis of our data, it is conceivable that the noxious effect of pyrimethanil can result from several pathological process affecting different key compartments of amphibian. In our opinion, when evaluating the harmful potential of a xenobiotic it is important to conduct a broad and in-depth study through a multi-organ evaluation in order to disclose all the putative and subtle pathological effects. Moreover our results strongly support the essential role of cross-check data from both laboratory and field studies, when assessing a site-specific risk. In fact, according with our earlier results, when such severe multi-organ alterations are detected in amphibian from an apparently uncontaminated site, a previous contamination event cannot be excluded.

References

Abdel-moneim, A., Coulter, D.P., Mahapatra, C.T., Sepúlveda, M.S., 2015. Intersex in fishes and amphibians, population implications, prevalence, mechanisms and molecular biomarkers. J. Appl. Toxicol., 3511, 1228-1240.

Agostini, M.G., Kacoliris, F., Demetrio, P., Natale, G.S., Bonetto, C., Ronco, A.E., 2013. Abnormalities in amphibian populations inhabiting agroecosystems in Northeastern Buenos Aires Province, Argentina. Dis. Aquat. Organ. 104, 163-171, http,//dx.doi.org/10.3354/dao02592.

Akiyoshi, H., Inoue, A.M., 2012. Comparative histological study of hepatic architecture in the three orders amphibian livers. Comparative hepatology, 111, 2.

Aldrich, A J unghans, M., Aeberli, C., Brühl, C. A., Streissl, F., Schmidt, B. R., 2016. Amphibians and plant-protection products, what research and action is needed? Environ. Sci. Eur. 28, 17, doi, 10.1186/s12302-016-0085-6

Amer, F.I., 1972. Observations on the urogenital ducts of the Egyptians toad, *Bufo regularis* Reuss. Zool. Sc. Egypt Bull. 24, 15-19.

Andersen, H.R., Cook, S.J., Waldbillig, D., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol. Appl. Pharmacol. 179, 1-12.

Anderson, H., Wolff, M.S., 2000. Environmental contaminants in human milk. J. Expo. Anal. Environ. Epidemiol. 10, 755-760.

Andreone, F., 1995. Valutazione e categorizzazione dello status della batracofauna in Piemonte e Valle d'Aosta. Quaderni della Civica Stazione Idrobiologica, 19, 27-40.

Andreone, F., Luiselli, L., 2000. The Italian batrachofauna and its conservation status, a statistical assessment. Biological Conservation, 96, 197-208.

Andreone, F., Schmidt, B.,Vogrin, M., Corti, C., Sindaco, R., Romano A., 2009*. Hyla intermedia.* The IUCN Red List of Threatened Species 2009, http,//dx.doi.org/10.2305/IUCN.UK.2009.RLTS.T55517A11323168.en

Andreu-Sánchez, O., Paraíba, L.C., Jonsson, C.M., Carrasco, J.M., 2012. Acute toxicity and bioconcentration of fungicide tebuconazole in zebrafish (*Danio rerio)*. Environ. Toxicol. 27, 109-16, http,//dx.doi.org/10.1002/tox.20618.

Ankley, G.T. Jensen, K.M., Durhan, E.J., Makynen, E.A., Butterworth, B.C., Kahl, M.D., Wilson, V.S., 2005. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow *Pimephales promelas*. Toxicol. Sci. 86, 300.

Aoki, M., Kurasaki, M., Saito, T., Seki, S., Hosokawa, T., Takahashi, Y., Fujita, H., Iwakuma, T., 2004. Nonylphenol enhances apotosis induced by serum deprivation in PC12 cells. Life Sci. 74,2301–2312.

APAT IRSA-CNR, 2003. Metodi analitici per le acque. Manuali e Linee guida [Water analytical methods. Manuals and guidelines] 29/2003.

Araújo, C.V., Shinn, C., Müller, R., Moreira-Santos, M., Espíndola, E.L., Ribeiro, R., 2015. The Ecotoxicity of Pyrimethanil for Aquatic Biota. In: Toxicity and Hazard of Agrochemicals. Eds. Larramendy, M.L., Soloneski, S. ISBN 978-953-51-2145-9, INTECH, pp.127-141.

Araújo, C.V.M., Shinn, C., Mendes, L.B., Delello-Schneider, D., Sanchez, A.L., Espíndola, E.L.G. 2014a. Avoidance response of *Danio rerio* to a fungicide in a linear contamination gradient. Sci. Total Environ. 484, 36-42, http,//dx.doi.org/10.1016/j.scitotenv.2014.03.037.

Araújo, C.V.M., Shinn, C., Vasconcelos, A.M., Ribeiro, R., Espíndola, E.L.G., 2014b. Preference and avoidance responses by tadpoles, the fungicide pyrimethanil as a habitat disturber. Ecotoxicology, 23, 851-860, http,//dx.doi.org/10.1007/s10646-014-1225-7.

Bandara, M.G.D.K., Wijesinghe, M.R., Ratnasooriya, W.D., Priyani, A.A.H., 2012. Chlorpyrifos-induced histopathological alterations in *Duttaphrynus melanostictus (*Schneider 1799) tadpoles, evidence from empirical trials. Journal of Tropical Forestry and Environment, 2.2, 27-36.

Barbier, N., 2005. Bilan d'activité du Centre National d'Informations Toxicologiques Vétérinaires pour l'année 2003. Thèse de doctorat vétérinaire, pp.220, Lyon.

Battaglin, W., Mark, W., Sandstrom, K.M., Kolpin, D.W., Meyer, M.T., 2010. Occurrence of Azoxystrobin, Propiconazole, and Selected Other Fungicides in US Streams, 2005–2006. Water Air Soil Pollut. 218, 307-322.

Beard, J., 2006. Australian rural health research collaboration. DDT and human health. Sci. Total Environ. 355, 78-89.

Beausoleil, C., Ormsby, J.N., Gies, A., Hass, U., Heindel, J.J., Holmer, M.L., Nielsen, P.J., Munn, S., Schoenfelder, G., 2013. Low dose effects and non-monotonic dose responses for endocrine active chemicals, science to practice workshop, workshop summary. Chemosphere, 93, 847-856, http,//dx.doi.org/10.1016/j.chemosphere.

Belden, J., McMurry, S., Smith, L., Reilley, P., 2010. Acute toxicity of fungicide formulations to amphibians at environmentally relevant concentrations. Environ. Toxicol. Chem. 29, 2477-2480, http,//dx.doi.org/10.1002/etc.297.

Berenzen, N., Lentzen-Godding, A., Probst, M., Schulz, H., Schulz, R., Liess, M., 2005. A comparison of predicted and measured levels of runoff-related pesticide concentrations in small lowland streams on a landscape level. Chemosphere, 58, 683-691, http,//dx.doi.org/10.1016/j.chemosphere.2004.05.009.

Bernabò, I., Biasone, P., Macirella, R., Tripepi, S., Brunelli, E., 2014. Liver histology and ultrastructure of the Italian newt *Lissotriton italicus*, Normal structure and modifications after acute exposure to nonylphenol ethoxylates. Exp. Toxicol. Pathol. 66, 455-468.

Bernabò, I., Bonacci, A., Coscarelli, F., Tripepi, M., Brunelli, E., 2013. Effects of salinity stress on *Bufo balearicus* and *Bufo bufo* tadpoles, Tolerance, morphological gill alterations and Na^+/K^+-ATPase localization. Aquat. Toxicol. 132-133, 119-133, http,//dx.doi.org/10.1016/j.aquatox.2013.01.019.

Bernabò, I., Brunelli, E., Sperone, E., Tripepi, S., 2011a. Toxicity of chlorpyrifos to larval *Rana dalmatina*, acute and chronic effects on survival, development, growth and gill apparatus. Arch. Environ. Contam. Toxicol. 61, 704-718, http,//dx.doi.org/10.1007/s00244-011-9655-1.

Bernabò, I., Gallo, L., Sperone, E., Tripepi, S., Brunelli, E., 2011b. Survival, development, and gonadal differentiation in *Rana dalmatina* chronically exposed to chlorpyrifos. J. Exp. Zool. Part A 315, 314-327.

Berny, P., Caloni, F., Croubels, S., Sachana, M., Vandenbroucke, V., Davanzo, F., Guitart,R., 2009. Animal poisoning in Europe. Part 2, Companion animals. The Veterinary Journal, 183, 255-259.

Bhaduri, J.L., Basu, S.L., 1957. A study of the urinogenital system of Salientia. Part I, Ranidae and Hyperoliidae of Africa. Musée Royal du Congo Belge.

Bhaduri, J.L., 1932. Observations on the urino-genital system of the tree-frogs of the genus *Rhacophorus* Kuhl, with remarks on their breeding habits. Anat. Anz., 74,336–343.

Billig, H., Furuta, I., Hsueh, A.J., 1993. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology, 133,2204–2212.

Bishop, C.A., Brooks, R.J., Carey, J.H., Norstrom, P.N.R.J., Lean, D.R.S., 1991. The case for a cause-effect linkage between environmental contamination and development in egg of the common snapping turtle *Chelydra serpentina* from Ontario. Canada. J. Toxicol. Environ. Health, 33, 521-547.

Bishop, C.A., Lean, D.R.S., Brooks, R.J., Carey, J.H., Norstrom, P.N.R.J., 1995. Chlorinated hydrocarbons in early life stages of the common snappling turtle chelydra serpentina serpentina from a coastal wetland on lake Ontario. Canada. Environ. Toxicol. Chem., 14, 421-426.

Bologna, M.A., Capula, M., Carpeneto, G.M., 2000. Anfibi e rettili del Lazio. Fratelli Palombi Editori, Roma, 160.

Bossche, H.V., Koymans, L., Moereels, H., 1995. P450 inhibitors of use in medical treatment, focus on mechanisms of action. Pharmacol. Ther. 67, 79–100.

Brande-Lavridsen, N., Christensen-Dalsgaard, J., Korsgaard, B., 2010. Effects of Ethinylestradiol and the fungicide Prochloraz on metamorphosis and thyroid gland morphology in *Rana temporaria*. The Open Zoology Journal, 3, 7-16, http,//dx.doi.org/10.2174/1874336601003020007.

Bridges, C.M., 2000. Long-term effects of pesticide exposure at various life stages of the southern leopard frog *Rana sphenocephala*. Arch. Environ. Contam. Toxicol. 39, 91-96, http,//dx.doi.org/10.1007/s002440010084.

Briggs, J., 2009. Green revolution. Int. Encycl. Hum. Geogr. 634-638.

Brodeur, J.C., Sassone, A., Hermida, G.N., Codugnello, N., 2013. Environmentally-relevant concentrations of atrazine induce non-monotonic acceleration of developmental rate and increased size at metamorphosis in *Rhinella arenarum* tadpoles. Ecotoxicol. Environ. Saf. 92, 10-17, http,//dx.doi.org/10.1016/j.ecoenv.2013.01.019.

Brühl, C.A., Pieper, S., Weber, B., 2001 Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. Environ. Toxicol. Chem. 30, 2465-2472.

Brühl, C.A., Schmidt, T., Pieper, S., Alscher, A., 2013. Terrestrial pesticide exposure of amphibians, an underestimated cause of global decline? Sci. Rep. 3, 1135, http,//dx.doi.org/10.1038/srep01135.

Brunelli, E., Bernabò, I., Berg, C., Lundstedt-Enkel, K., Bonacci, A., Tripepi, S., 2009. Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behavior in *Bufo bufo* tadpoles. Aquat. Toxicol. 91, 135-142, http,//dx.doi.org/10.1016/j.aquatox.2008.09.006.

Bruni, C., Porter, K.R., 1965. The fine structure of the parenchymal cell of the normal rat liverI. General observations. Am. J. Pathol.1965, 46,691–755.

Cabrera-Guzmán, E., Crossland, MR., Brown, GP., Shine, R., 2013. Larger Body Size at Metamorphosis Enhances Survival, Growth and Performance of Young Cane Toads *Rhinella marina*. PLoS ONE 8, e70121. http,//dx.doi.org/10.1371/journal.pone.0070121.

Çakıcı, Ö., 2015. Histopathologic changes in liver and kidney tissues induced by carbaryl in *Bufotes variabilis* Anura, Bufonidae. Exp. Toxicol. Pathol. 67, 237-243.

Caloni, F., Scarpa, P., Pompa, G., Davanzo, F., 2004. Epidemiologia degli avvelenamenti degli animali domestici in Italia anni 2000-2002, Casistica del centro antiveleni di Milano. Archivio Veterinario Italiano, 55, 1-6, 0004-0479.

Calvert, G.M., Karnik, J., Mehler, L., Beckman, J., Morrissey, B., Sievert, J., Barret, R.,Lackovic, M., Mabee, L., Schwartz, A., Mitchell, Y., Moraga-McHaley, S., 2008. Acute pesticide poisoning among agricultural workers in the United States, 1998–2005. Am. J. Und. Med., 51, 883–898.

Chen, X., Wang, J., Zhu, H., Ding, J., Peng, Y., 2015. Proteomics analysis of *Xenopus laevis* gonad tissue following chronic exposure to atrazine. Environ. Toxicol.and Chem. 348, 1770-1777.

Chodorowski, M.D., 2003. Acute oral suicidal intoxication with captan-a case report. J Toxicol-Clin. Toxic., 41, 5, 603, 0731-3810.

Coggins, L.W., 1973. An ultrastructural and radioautographic study of early oogenesis in the toad *Xenopus laevis*. J. Cell Sci. 121, 71-93.

Cooper, J., Dobson H., 2007. The benefits of pesticides to mankind and the environment. Crop Prot., 26, 1337-1348.

Corsaro, C. Scalia, M., Blanco, A.R., Aiello, I., Sichel, G., 1995. Melanins in physiological conditions protect against lipoperoxidation, A study on albino and pigmented Xenopus. Pigment Cell Res. 8, 279-282.

Costa, L.G., 1997. Basic toxicology of pesticides. Occup. Med. State of the Art Rev. 12, 251–268.

Crawshaw, G.J., Weinkle, T.K., 2000. Clinical and pathological aspects of the amphibian liver. In Seminars in Avian and Exotic Pet Medicine Vol. 9, No. 3, pp. 165-173. WB Saunders.

Crisp, T.M., Clegg, E.D., Cooper, R.L., Wood, W.P., Anderson, D.G., Baetcke, K.P., Hoffmann, J.L., Morrow, M.S., Rodier, D.J., Schaeffer, J.E., Touart, L.W., Zeeman, M.G. Patel, Y.M., 1998. Environmental endocrine disruption, An effects assessment and analysis [Review]. Environ. Health Perspect., 106, 11-56.

Cruz, M.J., Rebelo, R., 2005. Vulnerability of Southwest Iberian amphibians to an introduced crayfish, *Procambarus clarkii*. Amphibia-Reptilia, 26, 293-303.

De Gerónimo, E., Aparicio, V.C., Barbaro, S., Portocarrero, R., Jaime, S., Costa, J.L., 2014. Presence of pesticides in surface water from four sub-basins in Argentina. Chemosphere, 107, 423-431.

de Oliveira, C. R Fraceto, L.F., Rizzi, G.M., Salla, R.F., Abdalla, F.C., Costa, M.J., Silva-Zacarin, E.C.M., 2016. Hepatic effects of the clomazone herbicide in both its free form and associated with chitosan-alginate nanoparticles in bullfrog tadpoles. Chemosphere, 149, 304-313

Deb, D., Engel, B.A., Harbor, J., Hahn, L., Lim, K.J., Zhai, T., 2010. Investigation potential water quality impacts of fungicides used to combat soybean rust in Indiana. Water Air Soil Pollut. 207, 273-288, http,//dx.doi.org/10.1007/s11270-009-0135-4.

Denison, M.S., Fisher, J.M. Whitlock, 1988. The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. J. Biol. Chem. 263, 17221–17224.

Denver, R.J., 2009. Stress hormones mediate environment-genotype interactions during amphibian development. Gen. Comp. Endocrinol. 164, 20-31, http,//dx.doi.org/10.1016/j.ygcen.2009.04.016.

Deuchar, E.M., 1975. Xenopus, the South African clawed frog. New York, Wiley.

Dubois, A., 1995. The valid scientific name of the Italian tree frog with comments on the status of some early scientific names of Amphibia Anura and on some articles of the Code concerning secondary homonyms. Dumerilia, 2, 55-71.

Duellmann, W.E., Trueb, L., 1986. Biology of Amphibians. McGraw–Hill Book Company, New York.

Ebisuno, M., Gentilli, A., 2002. Reproductive site selection and characteristic of sources and sinks in an Italian tree frog metapopulation *Hyla intermedia* Boulenger, 1882. Terre Vie, 57, 263-278.

Eddy, E.M., Ito, S., 1971. Fine structural and radioautographic observations on dense perinuclear cytoplasmic material in tadpole oocytes. The Journal of cell biology, 491, 90.

Edwards, I.R., Ferry, D.G., Temple, W.A., 1991. Fungicides related compounds, In,Handbook of Pesticide Toxicology. Hayes, W.J. Laws, E.R., Eds. Academic Press, New York, NY,.Vol., 3, pp, 1409–1470.

EFSA – European Food Safety Authority, 2006. Conclusion regarding the peer review of the pesticide risk assessment of the active substance pyrimethanil. EFSA Sci. Rep. 61, 1-70.

EFSA – European Food Safety Authority, 2011. Authority. Review of the maximum residue levels MRLs for pyrimethanil according to Article 12 of Regulation EC No 396/2005. EFSA Journal,11 2454.

EFSA – European Food Safety Authority, 2014. Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole. EFSA Journal 2014, 121,3485, 98 pp. http,//dx.doi.org/10.2903/j.efsa.2014.3485.

Ellis, D.V., Pattisina, L.A,. 1990. Widerspread neogastropod imposex, a biological indicator of TBT contamination. Mar. Pollut. Bull., 21, 248-253.

Ells, H.A., 1954. The gross and microscopic anatomy of the liver and gall bladder of the lizard, *Sceloporus occidentalis biseriatus* Hallowell. Anat. Rec. 119,213–29.

Elmore, Susan A., Dixon, D., Hailey, J. R., Harada, T., Herbert, R. A., Maronpot, R. R., Satoh, H., 2016. Recommendations from the INHAND Apoptosis/Necrosis Working Group. Toxicol. Pathol., 44.2, 173-188.

Elsaesser, D., Schulz, R., 2008. Mitigation of fungicide pollution in vegetated agricultural surface waters, GIS modeling and monitoring in the field. Conference proceeding from the SETAC Europe 18th Annual Meeting. SETAC, Warsaw, pp.406-407.

Eskenazi, B., Marks, A.R., Bradman, A., Fenster, L., Johnson, C., Barr, D.B., 2006. In utero exposure to dichlorodiphenyltrichloroethane DDT and dichlorodiphenyldichloroethylene DDE and neurodevelopment among young Mexican American children. Pediatrics, 118, 233-241.

Facemire, C.F., Gross, T.S., Guillette, L.J., 1995. Reproductive impairment in the Florida panther, Nature or nuture? Environ. Health Perspect. 103, 79-86.

FAO/WHO. Pesticide residues in food. Part II — toxicological. Joint FAO/WHO meeting on pesticide residues, Switzerland, 529 pp, 2007.

Fawcett, D.W., Ito, S., Slautterback, D., 1959. The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. The Journal of biophysical and biochemical cytology, 53, 453-460.

Fenoglio, C., Albicini, F., Milanesi, G., Barni, S., 2011. Response of renal parenchyma and interstitium of *Rana* snk. *esculenta* to environmental pollution. Ecotox. Environ. Safe, 745, 1381-1390.

Ferreira, D., da Motta, A.C., Kreutz, L.C., Toni, C., Loro, V.L., Barcellos, L.J., 2010. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. Chemosphere 79, 914-921, http,//dx.doi.org/10.1016/j.chemosphere.2010.03.024.

Figueredo-Fernandes, A., Ferreira-Cardoso, J.V., Garcia-Santos, S., Monteiro, S.M, Carola, J., Matos, P., Fontainhas-Fernandes, A., 2007. Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. Pesquisa Veterinária Brasileira, 27, 103-109.

Fioramonti, E., Semlitsch, R.D., Reyer, H.U., Fent, K., 1997. Effects of triphenyltin and pH on the growth and development of *Rana lessonae* and *Rana esculenta* tadpoles. Environ. Toxicol. Chem. 16, 1940-1947, http,//dx.doi.org/10.1002/etc.5620160925.

Frampton, G.K., Jänsch, S., Scott-Fordsmand, J.J., Römbke, J., Van den Brink, P.J., 2006. Effects of pesticides on soil invertebrates in laboratory studies, a review and analysis using species sensitivity distributions. Environm. Toxicol. Chem. 25, 2480-2489.

Francis, E.T.B., 1934. The Anatomy of Salamander. London, Oxford Univ Press.

Fry, D.M., Toone, C.K., 1981. DDT-induced feminization of gull embryos. Science, 213, 922-924.

Fry, D.M., Toone, C.K., Speich, S.M., Peard, R.J., 1987. Sex ratio skew and breeding patterns of gulls, demographic and toxicological considerations. Stud. Avian Biol. 10, 26-43.

Fryday, S., Thompson, H., 2012. Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural

environments. EFSA Supporting Publications, EN-343 pp. 348. http,//www.efsa.europa.eu/en/supporting/pub/343e.htm

Gentilli, A., Scali, S., 2007. Biologia e conservazione della raganella italiana Hyla intermedia Boulenger, 1882 Quad. Staz. Ecol. civ. Mus. St. nat. Ferrara, 17, pp. 77-82.

Ghose, S.L., Donnelly, M.A., Kerby, J., Whitfield, S.M., 2014. Acute toxicity tests and meta-analysis identify gaps in tropical ecotoxicology for amphibians. Environ. Toxicol. Chem. 33, 2114-2119, http,//dx.doi.org/10.1002/etc.2665.

Giacoma, C, Balletto, E, 1993. Una valutazione delle probabilità di estinzione delle popolazioni europee di *Hyla arborea.* In, Ferri V. ed., Atti del Primo Convegno italiano sulla Salvaguardia degli Anfibi, Quad. Civ. Staz. Idrobiol. Milano, 20, 21-28.

Goldberg, J., 2015. Gonadal differentiation and development in the snouted treefrog, *Scinax fuscovarius* Amphibia, Anura, Hylidae. Journal of Herpetology, 493, 468-478.

Goldberg, J., 2015. Gonadal differentiation and development in the snouted treefrog, *Scinax fuscovarius* Amphibia, Anura, Hylidae. Journal of Herpetology, 493, 468-478.

Goldblatt PJ, Hampton JA, Didio LN, Skeel KA, Klaunig JE., 1987. Morphologic and his-tochemical analysis of the Newt *Notophthalmus viridescens* liver. Anat Rec. 217,328–38.

Gooding, M.P., Wilson, V.S., Folmar, L.C., Marcovich, D.T., LeBlanc, G.A., 2003. The biocide tributyltin reduces the accumulation of testosterone as fatty acid esters in the mud snail *Ilyanassa obsoleta*. Environ. Health Perspect. 111, 426-230.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 12, 249-259.

Gramapurohit, N.P., Shanbhag, B.A., Saidapur, S.K., 2000. Pattern of gonadal sex differentiation, development, and onset of steroidogenesis in the frog, *Rana curtipes*. Gen. Compar. Endocrin. 1193, 256-264.

Gramapurohit, N.P., Shanbhag, B.A., Saidapur, S.K., 2000. Pattern of gonadal sex differentiation, development, and onset of steroidogenesis in the frog, *Rana curtipes*. General and comparative endocrinology, 1193, 256-264.

Gregoire, C., Payraudeau, S., Domange, N., 2010. Use and fate of 17 pesticides applied on a vineyard catchment. Int. J. Environ. Anal. Chem. 90, 406-420, http,//dx.doi.org/10.1080/03067310903131230.

Guillette, L.J., Brock, J.W., Rooney, A.A., Woodward, A.R., 1999. Serum concentration of various environmental contaminants and their relationship to sex steroid concentration and phallus size in juvenile American alligators. Arch. Environ. Contam. Toxicol. 36, 447-455.

Guillette, L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodward, A.R., 1995. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 1995, 102, 680-688.

Guillette, L.J., Pickford, D.B., Crain, D.A., Rooney, A.A., Percival, H.F. 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environnement. Gen. Comp. Endocrinol. 101, 32-42.

Guitart, R., Manosa, S., Guerrero, X. Mateo, R., 1999. Animal poisonings, the 10 year experience of a veterinary analytical toxicology laboratory. Veterinary and Human Toxicology, 41, 5, 331-335, 0145-6296.

Gupta, P.K. Aggarwal, M., 2007. Toxicity of fungicides. In Veterinary Toxicology, Gupta,R. C. Ed., 1st ed., 587-601, Elsevier, 978-0-12-370467-2, New York.

Gürkan M, Hayretdağ S., 2009. Morphological and histological effects of copper sulfate on the larval development of green toad *Bufo viridis*. Turk. J. Zool. 36, 231–40.

Haczkiewicz, K., Ogielska, M., 2013. Gonadal sex differentiation in frogs, How testes become shorter than ovaries. Zoolog. Sci. 30, 125–134.

Hansen, M., Poulsen, R., Luong, X., Sedlak, D.L., Hayes, T., 2014. Liquid chromatography tandem mass spectrometry method using solid-phase extraction and bead-beating-assisted matrix solid-phase dispersion to quantify the fungicide tebuconazole in controlled frog exposure study, analysis of water and animal tissue. Anal. Bioanal. Chem. 406, 7677-7685, http,//dx.doi.org/10.1007/s00216-014-8207-8.

Hardell, L., Bavel, B., Lindström, G., Eriksson, M., Carlberg, M., 2006. In utero exposure to persistent organic pollutants in relation to testicular cancer risk. Int. J. Androl. 29, 228-234.

Hartley, D., Kidd, H. Eds., 1983. The Agrochemicals Handbook. Royal Society of Chemistry, 0851864066, Nottingham.

Hartman, E.A., Belden, J.B., Smith, L.M., McMurry, S.T., 2014. Chronic effects of strobilurin fungicides on development, growth, and mortality of larval Great Plains toads *Bufo cognatus*. Ecotoxicology, 23, 396-403 http,//dx.doi.org/10.1007/s10646-014-1203-0.

Haselman, J.T., Sakurai, M., Watanabe, N., Goto, Y., Onishi, Y., Ito, Y., Iguchi, T., 2016. Development of the Larval Amphibian Growth and Development Assay, Effects of benzophenone-2 exposure in *Xenopus laevis* from embryo to juvenile. J. Appl. Toxicol. 36,1651-1661.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J., Tsui, M., 2006. Pesticide mixtures, endocrine disruption, and amphibian declines, are we underestimating the impact? Environ. Health. Perspect. 114, 40-50, http,//dx.doi.org/10.1289/ehp.8051.

Hayes, T.B., Falso, P., Gallipeau, S., Stice, M., 2010. The cause of global amphibian declines, a developmental endocrinologist's perspective. J. Exp. Biol. 213, 921-933.

Hayes, W.J., Laws, E.R., 1990. Handbook of Pesticide Toxicology, Vol. 3, Classes of Pesticides.Academic Press, 0123341604, New York.

Heidrich, D.D., Steckelbroeck, S., Klingmuller, D., 2001. Inhibition of human cytochrome P450 aromatase activity by butyltins. Steroids, 66, 763-769.

Herak-Kramberger, C.M., Sabolic, I., 2001. The integrity of renal cortical brush-border and basolateral membrane vesicles is damaged in vitro by nephrotoxic heavy metals. Toxicology, 156,139–147.

Herrero-Hernández, E., Andrades, M.S., Álvarez-Martín, A., Pose-Juan, E., Rodríguez-Cruz, M.S., Sánchez-Martín, M.J., 2013. Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region. J. Hydrol., 486, 234-245, http,//dx.doi.org/10.1016/j.jhydrol.2013.01.025.

Higley, E., Tompsett, A.R., Giesy, J.P., Hecker, M., Wiseman, S., 2013. Effects of triphenyltin on growth and development of the wood frog *Lithobates sylvaticus*. Aquat. Toxicol. 144-145, 155-161, http,//dx.doi.org/10.1016/j.aquatox.2013.09.029.

Hillman, S.S., Withers, P.C., Drewea. R.C., Hillyard, S.D., 2009. Ecological and environmental physiology of amphibians. Oxford University Press.

Holz, P.H., Raidal, S.R., 2006. Comparative renal anatomy of exotic species. Vet. Clin. North Am. Exotic Anim. Pract. 9,1-11.

Hourdry, J., Hermite, A., Ferrand, R., 1996. Changes in the digestive tract and feeding behavior of anuran amphibians during metamorphosis. Physiol. Zool. 69, 219-251.

Ijiri, K.I., Egami, N., 1975. Mitotic activity of germ cells during normal development of *Xenopus laevis* tadpoles. Development, 343, 687-694.

Ikechukwu, E.L., Ajeh, E.A., 2011. Histopathological alterations in the liver and lungs of *Hoplobatrachus occipitalis* exposed to sublethal concentrations of cadmium. Aust. J.Basic. Appl. Sci. 5, 1062–1068.

Israeli, R., Sculsky, M. Tiberin, P., 1983. Acute intoxication due to exposure to maneb and zineb. Scand. J. Work Env. Hea. 9, 47-51.

IUPAC, 2012. International Union of Pure Applied Chemistry. Pesticide Properties Database. Tebuconazole Ref, HWG 1608. Accessed 2/22/2012.

Iwasawa, H., Yamaguchi, K., 1984. Ultrastructural study of gonadal development in *Xenopus laevis*. Zool. Sci. 1,591–600.

Jacobson, J.L., Jacobson, S.W., 1996. Intellectual impairment in children exposed to polychlorinated biphenyls in utero. N. Engl. J. Med. 335, 783-789.

Junges, C.M., Peltzer, P.M., Lajmanovich, R.C., Attademo, A.M., Cabagna Zenklusen, M.C., Basso, A., 2012. Toxicity of the fungicide trifloxystrobin on tadpoles and its effect on fish-tadpole interaction. Chemosphere, 87, 1348-1354, http,//dx.doi.org/10.1016/j.chemosphere.2012.02.026.

Kalt, M.R., 1973. Ultrastructural observations on the germ line of *Xenopus laevis*. Cell and Tissue Research, 1381, 41-62.

Kaptaner, B., Ünal, G., 2011. Effects of 17α-ethynylestradiol and nonylphenol on liver and gonadal apoptosis and histopathology in *Chalcalburnus tarichi*. Environ. Toxicol. 26(6), 610-622.

Kayacan, S.M., Vatansever, S., Akkaya, V., Erk, O., Tukek, T., Kılıcaslan, I., Yildiz, A. Guler, K. 2007. A case of copper sulfate intoxication that is presented with prolonged hemolysis and acute renal failure. Journal of Istanbul Faculty of Medicine,70, 47-50.

Kerr, J.B., Dixon, K.E., 1974. An ultrastructural study of germ plasm in spermatogenesis of *Xenopus laevis*. Development, 323, 573-592.

Kintz, P., Jamey, C., Doray, S. Ludes, B., 1997. Acute fatal poisoning with dichlorophen. International Journal of Legal Medicine, 110, 95-96, 0937-9827.

Kloas, W., 2002. Amphibians as a model to study endocrine disruptors. Int. Rev. Cytol. 216, 1-57.

Knäbel, A., Meyer, K., Rapp, J., Schulz, R., 2014. Fungicide field concentrations exceed FOCUS surface water predictions, urgent need of model improvement. Environ. Sci. Technol. 48, 455.463, http,//dx.doi.org/10.1016/j.chemosphere.2012.04.023.

Koakoski, G., Quevedo, R.M., Ferreira, D., Oliveira, T.A., da Rosa, J.G., de Abreu, M.S., Gusso, D., Marqueze, A., Kreutz, L.C., Giacomini, A.C., Fagundes, M., Barcellos, L.J., 2014. Agrichemicals chronically inhibit the cortisol response to stress in fish. Chemosphere 112, 85-91, http,//dx.doi.org/10.1016/j.chemosphere.2014.02.083.

Köhler, H-R., Triebskorn R., 2013. Wildlife Ecotoxicology of Pesticides, Can We Track Effects to the Population Level and Beyond? Science 341, 759, http,//dx.doi.org/10.1126/science.1237591.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., Kobayashi, K., 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environ. Health Perspect. 112, 524-531.

Kolpin, D.W., Thurman, E.M., Linhart, S.M., 2000. Finding minimal herbicide concentrations in ground water? Try looking for their degradates. Sci. Total Environ. 248, 115-122.

Komarek, M., Cadkova, E., Chrastny, V., Bordas, F., Bollinger, J.C., 2010. Contamination of vineyard soils with fungicides, A review of environmental and toxicological aspects. Environment International. 36, 138-151.

Konwick, B.J., Garrison, A.W., Avants, J.K., Fisk, A.T., 2006. Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout *Oncorhynchus mykiss*. Aquat. Toxicol. 80, 372-381, http,//dx.doi.org/10.1016/j.aquatox.2006.10.003.

Kreuger, J., Graaf, S., Patring, J., Adielsson, S., 2010. Pesticides in surface water in areas with open ground and greenhouse horticultural crops in Sweden 2008. Ekohydrologi 117, 1-45.

Kreutz, L.C., Barcellos, L.J.G., Silva, T.O., Anziliero, D., Martins, D., Lorenson, M., Marteninghe, A., Silva, L.B., 2008. Acute toxicity testing of agricultural pesticides on silver catfishes *Rhamdia quelen*, fingerlings. Ciência Rural 38, 1050-1055.

Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M., Roussell, C., 2015. Non-monotonic dose–response relationships and endocrine disruptors, a qualitative method of assessment. Environ. Health 14, 13, http,//dx.doi.org/10.1186/1476-069X-14-13.

Lanza, B., Nistri, A., Vanni, S., 2006. Iconografia degli Anfibi D'Italia/ Iconography of Italian Amphibians [Ministero dell'Ambiente e delle Tutela del Territorio e del Mare Direzione per la Protezione della Natura]. Grandi, Grandi Editori, Savignano sul Panaro Mo.

Lanza. B., 1983. Anfibi e rettili Amphibia, Reptilia. Guida per il riconoscimento delle specie animali dell'acque interne italiane, 27. Collana del progetto finalizzato "Promozione della qualità dell'ambiente" C.N.R., Roma.

Lavorato, M., Bernabò, I., Crescente, A., Denoël, M., Tripepi, S., Brunelli, E., 2013. Endosulfan effects on *Rana dalmatina* tadpoles, quantitative developmental and behavioural analysis. Arch Environ. Contam. Toxicol. 64, 253-262, http,//dx.doi.org/10.1007/s00244-012-9819-7.

Lemaire, G., Mnif, W., Mauvais, P., Balaguer, P., Rahmani, R., 2006a. Activation of alpha- and beta- estrogen receptors by persistent pesticides in reporter cell lines. Life Sci. 79, 1160-1169.

Lemaire, G., Mnif, W., Pascussi, J.M., Pillon, A., Rabenoelina, F., Fenet, H., Gomez, E., Casellas, C., Nicolas, J.C., Cavailles, V., Duchesne, M. J., 2006b. Identification of new human PXR ligands among pesticides using a stable reporter cell system. Toxicol. Sci. 91, 501-509.

Lopez, K., 1989. Sex differentiation and early gonadal development in *Bombina orientalis* Anura, Discoglossidae. J Morphol, 199, 299–311.

Lorgue, G., Lechenet, J. Rivière, A., 1996. Clinical Veterinary Toxicology, 5-194, Blackwell Science, 0-632-03269-3, London.

Loumbourdis, N.S., 2003. Nephrotoxic effects of lead nitrate in Rana ridibunda. Archives of toxicology, 779, 527-532.

Mackenzie, C.A., Berrill, M., Metcalfe, C. Pauli, B.D., 2003. Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. Environ. Toxicol. Chem. 22, 2466-2475.

Mali, P.V., Gramapurohit, N.P., 2016. Are sex steroids essential for gonadal differentiation of the ornate frog, Microhyla ornata?. General and comparative endocrinology, 233, 63-72.

Maltby, L., Brock, T.C.M., Brink, P.J., 2009. Fungicide risk assessment for aquatic ecosystems, importance of interspecific variation, toxic mode of action, and exposure regime. Environ. Sci. Technol. 43, 7556-7563, http,//dx.doi.org/10.1021/es901461c.

Mann, R.M., Hyne, R.V., Choung, C.B., Wilson, S.P., 2009. Amphibians and agricultural chemicals, review of the risks in a complex environment. Environ. Pollut. 157, 2903-2927.

Marichal, P., Gorrens, J., Bossche, H.V., 1985. The action of itraconazole and ketoconazole on growth and sterol synthesis in *Aspergillus fumigatus* and *Aspergillus niger*. Sabouraudia, 22, 13–2.

Marquis, O., Millery, A., Guittonneau, S., Miaud, C., 2006. Solvent toxicity to amphibian embryos and larvae. Chemosphere 63, 889-892, http,//dx.doi.org/10.1016/j.chemosphere.2005.07.063.

Marrs, C.T., Ballantyne, B., 2004. Pesticides toxicology and international regulation. John Wiley and Sons, Ltd. 554 pages.

Matthiessen, P., Weltje, L., 2015. A review of the effects of azole compounds in fish and their possible involvement in masculinization of wild fish populations Crit. Rev. Toxicol. 45, 453-467.

McCoy, K.A. Bortnick, L.J., Campbell, C.M., Hamlin, H.J., Guillette Jr, L.J., St Mary, C.M., 2008. Agriculture alters gonadal form and function in the toad *Bufo marinus*. Environ. Health Persp. 116, 1526-1532.

McDaniel, T.V. Martin, P.A., Struger, J., Sherry, J., Marvin, C.H., McMaster, M. E., Tetreault, G., 2008. Potential endocrine disruption of sexual development in free ranging male northern leopard frogs *Rana pipiens* and green frogs *Rana clamitans* from areas of intensive row crop agriculture. Aquat. Toxicol. 88, 230-242.

McDiarmid, R.W, Altig, R. 1999. Tadpoles, the biology of anuran larvae. University of Chicago Press.

McKinlay, R., Plant, J.A., Bell, J.N.B., Voulvoulis, N., 2008. Endocrine disrupting pesticides, implications for risk assessment. Environ. Int. 34,168-183.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W., Boughton, R.K., Martin, L.B., Rohr, J.R., 2011. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. Environ. Health Perspect. 119, 1098-1103, http,//dx.doi.org/10.1289/ehp.1002956.

Medina, M.F., González, M.E., Klyver, S.M.R., Odstrcil, I.M.A., 2016. Histopathological and biochemical changes in the liver, kidney, and bloodof amphibians intoxicated with cadmium. Turk. J. Biol., 401, 229-238.

Medjakovic, S., Zoechling, A., Gerster, P., Ivanova, M.M., Teng, Y., Klinge, C.M., Schildberger, B., Gartner, M., Jungbauer, A., 2014. Effect of non persistent pesticides on estrogen receptor, androgen receptor, and aryl hydrocarbon receptor. Environ. Toxicol. 29, 1201-1216, http,//dx.doi.org/10.1002/tox.21852.

Mellanby, K., 1992. The DDT Story, British Crop Protection Council, Hampshire, UK.

Melvin, S.D., Lanctôt, C.M., Craig, P.M., Moon, T.W., Peru, K.M., Headley, J.V., Trudeau, V.,L. 2013. Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles. Environ. Pollut. 177, 22-27.

Méndez, M. Obando, P., Pinnock-Branford, M., Ruepert, C., Castillo, L.E., Mena, F., Alvarado, G., 2016. Acute, chronic and biochemical effects of chlorothalonil on Agalychnis callidryas, *Isthmohyla pseudopuma* and *Smilisca baudinii* tadpoles. Environ. Sci. Pollut. Res., 1-11.

Merchant-Larios, H, Villalpando, I., 1981. Ultrastructural events during early gonadal development in *Rana pipiens* and *Xenopus laevis*. Anat. Rec 199, 349–360.

Mnif, W., Hassine, A. I. H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B., 2011. Effect of endocrine disruptor pesticides, a review. Int. J. Environ. Res. Public Health, *86*, 2265-2303.

Mnif, W., Pillon, A., Balaguer, P., Bartegi, A., 2007. Endocrine xenoestrogenics disrupters, molecular mechanisms and detection methods. Therapie, 62, 369-386.

Møbjerg, N., Jespersen, Å., Wilkinson, M., 2004. Morphology of the kidney in the west African caecilian, *Geotrypetes seraphini*, Amphibia, Gymnophiona, Caecilidae. J Morphol, 262, 583-607, 2004.

Møbjerg, N., Larsen, E.H., Jespersen, Å., 2000. Morphology of the kidney in larvae of *Bufo viridis* (Amphibia, Anura, Bufonidae). J. Morphol. 245, 177-195.

Mortazavi, F. Jafari-Javid, A. 2009. Acute renal failure due to copper sulphate poisoning, a case report. Iranian Journal of Pediatrics. 19, 1, 75-78.

Mosleh, Y.Y., Mofeed, J., Afifi, M., Almaghrabi, O.A., 2014. Biological effects of pyrimethinal on aquatic worms *Tubifex tubifex* under laboratory conditions. Bull. Environ. Contam. Toxicol, 92, 85–89.

Müller, R., Seeland, A., Jagodzinski, L.S., Diogo, J.B., Nowak, C., Oehlmann, J., 2012. Simulated climate change conditions unveil the toxic potential of the fungicide pyrimethanil on the midge *Chironomus riparius*, a multigeneration experiment. Ecol. Evol. 2, 196–210.

Munkittrick, K.R., Port, C.B., Van Der Kraak, G.J., Smith, I.R., Rokosh, D.A., 1991. Impact of bleach kraft mill effluent on population characteristics, liver MFO activity, and serum steroid levels of a Lake Superior white sucker *Catostomus commersoni*. Can. J. Fish Aquat. Sci., 48, 1371-1380.

Nakamura, M., 2010. The mechanism of sex determination in vertebrates—are sex steroids the key-factor? J. Exp. Zool. 313A, 381–398.

NAS, 1987. Regulating Pesticides in Food. The Delaney Paradox. Report of Committee on Scientific and Regulatory Issues. Unlikely Pesticide Use Patterns. National Academy of Sciences, National Academy Press, Washington, DC

Nascetti, G., Lanza, B., Bullini, L., 1995. Genetic data support the specific status of the Italian treefrog (Amphibia, Anura, Hylidae). Amphibia-Reptilia. 16, 215–227.

Navarro-Martín, L., Lanctôt, C., Jackman, P., Park, B.J., Doe, K., Pauli, B.D., Trudeau, V.L., 2014. Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frogs *Lithobates sylvaticus* tadpoles. I, Chronic laboratory exposures to VisionMax®. Aquat. Toxicol. 154, 278-290 2014.

Odokuma, E. I., Omokaro, E.I., 2015. Comparative histologic anatomy of vertebrate liver. Annals of Bioanthropology, 31, 1.

OECD - Organisation for Economic Co-operation and Development – 2015. Test No. 241, The Larval Amphibian Growth and Development Assay LAGDA, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris. DOI, http,//dx.doi.org/10.1787/9789264242340-en.

Ogielska, M., Bartmańska, J., 1999. Development of testes and differentiation of germ cells in water frogs of the *Rana esculenta*-complex (Amphibia, Anura). Amphibia-Reptilia 20, 251–263.

Ogielska, M., Kotusz, A., 2004. Pattern and rate of ovary differentiation with reference to somatic development in anuran amphibians. J. Morphol. 259, 41-54.

Ogielska, M., Wagner, E., 1990. Oogenesis and development of the ovary in European green frog, *Rana ridibunda* Pallas. I, Tadpole stages until metamorphosis. Zoologische Jahrbücher. Abteilung für Anatomie und Ontogenie der Tiere, 1203, 211-221.

Olurin, K.B., Olojo, E.A.A., Mbaka, G.O., and Akindele, A.T., 2006. Histopathological responses of the gill and liver tissues of *Clarias gariepinus* fingerlings to herbicide, glyphosate. African Journal of Biotechnology, 5,24, 2480.

Ortolani, E.L., Machado, C.H., Sucupira, M.C.A., 2003. Assessment of some clinical and laboratory variables for early diagnosis of cumulative copper poisoning in sheep. Vet. and Hum.Toxicol. 45(6), 289-293.

Orton, F., Ermler, S., Kugathas, S., Rosivatz, E., Scholze, M., Kortenkamp, A., 2014. Mixture effects at very low doses with combinations of anti-androgenic pesticides, antioxidants, industrial pollutant and chemicals used in personal care products. Toxicol. Appl. Pharmacol. 278, 201-208, http,//dx.doi.org/10.1016/j.taap.2013.09.008.

Orton, F., Rosivatz, E., Scholze, M., Kortenkamp, A., 2011. Widely used pesticides with previously unknown endocrine activity revealed as in vitro anti-androgens. Environ. Health Persp. 119, 794-800.

Orton, F., Routledge, E., 2011. Agricultural intensity in ovo affects growth, metamorphic development and sexual differentiation in the Common toad *Bufo bufo*. Ecotoxicology, 20, 901-911.

Orton, F., Tyler, C.R., 2015. Do hormone-modulating chemicals impact on reproduction and development of wild amphibians? Biol. Rev. 90, 1100-1117.

Oruc, H.H., 2010. Fungicides and Their Effects on Animals, Fungicides, Odile Carisse Ed., ISBN, 978-953-307-266-1, InTech, Available from, http,//www.intechopen.com/books/fungicides/fungicides-and-theireffects-on-animals

Oskam, I.C., Ropstad, E., Dahl, E., Lie, E., Derocher, A.E., Wiig, O., Larsen, S., Wiger, R., Skaare, J.U., 2003. Organochlorines affect the major androgenic hormone, testosterone, in male polar bears Ursus maritimus at Svalbard. J. Toxicol. Environ. Health A, 66, 2119-2139.

Osman, M.M., El-Fiky, S.A., Soheir, Y.M., Abur, A.I., 2009. Impact of Water Pollution on Histopathological and Electrophoretic Characters of *Oreochromis niloticus* Fish. Research Journal of Environmental Toxicology, 31, 9-23.

Osweiler, G.D., Carlson, T.L., Buck, W.B. Von Gelder, G.A., 1985. Organic SyntheticFungicides. In: Clinical and Diagnostic Veterinary Toxicology, 3rd ed., 231-242,Kendal/Hunt, 0-8403-3332-3, Dubuque.

Palma, P., Palma, V.L., Matos, C., Fernandes, R.M., Bohn, A., Soares, A.M.V.M., 2009a. Barbosa, I.R., Effects of atrazine and endosulfan sulphate on the ecdysteroid system of *Daphnia magna*. Chemosphere, 74, 676-681.

Palma, P., Palma, V.L., Matos, C., Fernandes, R.M., Bohn, A., Soares, A.M.V.M., Barbosa, I.R., 2009b. Assessment of the pesticides atrazine, endosulfan sulphate and chlorpyrifos for juvenoid-related endocrine activity using Daphnia magna. Chemosphere, 76, 335-340.

Papoulias, D.M., Schwarz, M.S., Mena, L., 2013. Gonadal abnormalities in frogs Lithobates spp. collected from managed wetlands in an agricultural region of Nebraska, USA. Environ. Pollut. 172, 1-8 .

Păunescu, A., Ponepal, C.M., Grigorean, V.T., Popescu, M., 2012. Histopathological changes inthe liver and kidney tissues of Marsh Frog *Pelophylax ridibundus* induced bythe action of Talstar 10EC insecticide. An. U O Fasc. Biol. 19, 5–10.

Păunescu, A., Ponepal, M.C., Draghici, O., 2010. Liver histopathologic alterations in the frogRana *Pelophylax ridibunda* induce by the action of Reldan 40 EC insecticide. A.U O Fasc. Biol. 17, 166–169.

Păunescu, A., Ponepal, M.C., 2011. Nephrotoxic effects of Champion 50WP fungicides in the marsh frog, *Pelophylax ridibundus*. Oltenia. Studii şi comunicări. Ştiinţele naturii, 27.1, 119-122.

Pessier, A.P., 2009. Edematous frogs, urinary tract disease, and disorders of fluid balance in amphibians. Journal of Exotic Pet Medicine, 181, 4-13.

Piechotta, G, Lacorn, M., Lang, T., Kammann, U., Simat, T., Jenke, H.S., Steinhart, H., 1999. Apoptosis in dab *Limanda limanda* as possible new biomarker for anthropogenic stress. Ecotoxicol. Environ. Saf. 42, 50–56.

Pimentel, D.,1971. Ecological effects of pesticides on nontarget species. Executive Office of the President's Office of Science and Technology. U.S. Government Printing Office,Washington, DC.

Pocar, P., Fischer, B., Klonisch, T., Hombach-Klonisch, 2005. Molecular interactions of the aryl hydrocarbon receptor and its biological and toxicological relevance for reproduction. Reproduction, 129, 379-389.

Poulsen, R., Luong, X., Hansen, M., Styrishave, B., Hayes, T., 2015. Tebuconazole disrupts steroidogenesis in *Xenopus laevis*. Aquat. Toxicol. 168, 28-37, http,//dx.doi.org/10.1016/j.aquatox.2015.09.008.

PPDB., 2009. The Pesticide Properties Database PPDB Developed by the Agriculture Environment Research Unit AERU, University of Hertfordshire, Funded by UK National Sources and the EU-funded FOOTPRINT Project FP6-SSP-022704.

Prutner, W., Nicken, P., Haunhorst, E., Hamscher, G., Steinberg, P., 2013. Effects of single pesticides and binary pesticide mixtures on estrone production in H295R cells. Arch. Toxicol. 87, 2201-2214, http,//dx.doi.org/10.1007/s00204-013-1081-x.

Przyrembel, H., Heinrich-Hirsch, B., Vieth, B., 2000. Exposition to and health effects of residues in human milk. Adv. Exp. Med. Biol. 478, 307-325.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8, 275-285.

Quaranta, .A, Bellantuono, V., Cassano, G., Lippe, C., 2009. Why amphibians are more sensitive than mammals to xenobiotics. PLoS ONE 4, e7699, http,//dx.doi.org/10.1371/journal.pone.0007699.

Rabiet, M., Margoum, C., Gouy, V., Carluer, N., Coquery, M., 2010. Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment - Effect of sampling frequency. Environ. Pollut. 158, 737-748, http,//dx.doi.org/10.1016/j.envpol.2009.10.014.

Rappaport, A.M., 1967. Diseases of the Liver. In Anatomic considerations. Second Editionth edition. Edited by Schiff L, J. B. Lippincott Company. Philadelphia, Asian Edition Hakko Company Limited,1–46.

Rastogi, R.K., Rastogi, R.K., Izzo-Vitiello, I., Meglio, M.D., Matteo, L., Franzese, R., Costanzo, M.G., Minucci, S., Iela, L., Chieffi, G., 1983. Ovarian activity and reproduction in the frog, *Rana esculenta*. J. Zool. 2002, 233-247.

Redshaw, M.R., Nicholls, T.J., 1971. Oestrogen biosynthesis by ovarian tissue of the South African clawed toad, *Xenopus laevis* Daudin. General and comparative endocrinology, 161, 85-96.

Redshaw, M.R., 1972. The hormonal control of the amphibian ovary. American Zoologist, 122, 289-306.

Reijnders, P.J., 1986. Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature, 324, 456-457.

Reilly, T.J., Smalling, K.L., Orlando, J.L., Kuivila, K.M., 2012. Occurrence of boscalid and other selected fungicides in surface water and groundwater in three targeted use areas in the United States. Chemosphere, 89, 228-234, http,//dx.doi.org/10.1016/j.chemosphere.2012.04.023.

Ribas-Fito, N., Cardo, E., Sala, M., Eulalia de Muga, M., Mazon, C., Verdu, A., Kogevinas, M., Grimalt, J.O., Sunyer, J., 2003. Breastfeeding exposure to organochlorine compounds and neurodevelopment in infants. Pediatrics. 111, 580-585.

Robles-Molina, J., Gilbert-Lopez, B., García-Reyes, J., Molina-Díaz, A., 2014. Monitoring of selected priority and emerging contaminants in the Guadalquivir River and other related surface waters in the province of Jaén, South East Spain. Sci. Total Environ. 479-480, 247-257, http,//dx.doi.org/10.1016/j.scitotenv.2014.01.121.

Rosenshield, M.L., Jofré, M.B., Karasov, W.H., 1999. Effects of polychlorinated biphenyl 126 on green frog *Rana clamitans* and leopard frog *Rana pipiens* hatching success, development, and metamorphosis. Environ. Toxicol. Chem. 18, 2478-2486.

Rouabhi, R., 2010. Introduction and Toxicology of Fungicides, Fungicides, Odile Carisse Ed., ISBN,978-953-307-266-1, InTech, Available from, http,//www.intechopen.com/books/fungicides/introduction-andtoxicology-of-fungicides.

Saidapur, S.K., Nadkarni, V.B., 1974. Steroid-synthesizing cellular sites in amphibian ovary. A histochemical study. Gen. Comp. Endocr. 224, 459-462.

Sancho, E., Villarroel, M.J., Fernández, C., Andreu, E., Ferrando, M.D., 2010. Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish *Danio rerio*. Ecotoxicol. Environ. Saf. 73, 370-376, http,//dx.doi.org/10.1016/j.ecoenv.2009.09.020.

Sanderson, J.T., 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicological sciences, 941, 3-21.

Saxena, A.K., Devillers, J., Bhunia, S.S., Bro, E., 2015. Modelling inhibition of avian aromatase by azole pesticides." SAR and QSAR in Environmental Research, 26.7-9, 757-782.

SCBP, 2013. Regulation EU n°528/2012 concerning the making available on the market and use of biocidal products. Evaluation of active substances,. Tebuconazole PT 7 Assessment report Finalised in the Standing Committee on Biocidal Products. Denmark, 27 September 2013.

Scherer, C., Seeland, A., Oehlmann, J., Müller, R., 2013. Interactive effects of xenobiotic, abiotic and biotic stressors on *Daphnia pulex* – results from a multiple stressor experiment with a fractional multifactorial design. Aquat. Toxicol. 138–139 105–115.

Schmidt, B., Vogrin, M., Corti, C., Sindaco, R., Romano, A., 2009. *Hyla intermedia*. The IUCN Red List of Threatened Species 2009, http,//dx.doi.org/10.2305/IUCN.UK.2009.RLTS.T55517A11323168.en

Scoccianti, C., 2001. Amphibia, aspetti di ecologia della conservazione. Editore Guido Persichino Grafica, Firenze.

Scoccianti, C., 2004. Amphibians, threats and conservation. Ital. J. Zool., 71, Suppl. 1, 9-15.

Seeland, A., Albrand, J., Oehlmann, J., Müller, R., 2013. Life stage-specific effects of the fungicide pyrimethanil and temperature on the snail *Physella acuta* Draparnaud, 1805 disclose the pitfalls for the aquatic risk assessment under global climate change. Environ. Pollut. 174, 1-9, http,//dx.doi.org/10.1016/j.envpol.2012.10.020.

Seeland, A., Oehlmann, J., Müller, R., 2012. Aquatic toxicity of the fungicide pyrimethanil, effect profile under optimal and thermal stress conditions. Environ. Pollut. 168, 161-169, http,//dx.doi.org/10.1016/j.envpol.2012.04.020.

Sharpe, R.R.M., 2006. Pathways of endocrine disruption during male sexual differentiation and masculinisation. Best. Pract. Res. Clin. Endocrinol. Metab., 20, 91-110.

Shenoy, K., Cunningham, B.T., Renfroe, J.W., Crowley, P.H., 2009. Growth and survival of northern leopard frog *Rana pipiens* tadpoles exposed to two common pesticides. Environ. Toxicol. Chem. 28, 1469-1474, http,//dx.doi.org/10.1897/08-306.1.

Shinn, C., Delello-Schneider, D., Mendes, L.B., Sanchez, A.L., Müller, R., Espíndola, E.L., Araújo, C.V., 2015. Immediate and mid-term effects of pyrimethanil toxicity on microalgae by simulating an episodic contamination. Chemosphere, 120, 407-413, http,//dx.doi.org/10.1016/j.chemosphere.2014.08.023.

Sichel, G., Scalia, M., Mondio, F., 1997. The amphibian Kupffer cells build and demolish melanosomes. An ultrastructural point of view. Pigment Cell Res. 105, 271-287.

Sindaco, R., Doria, G., Razzetti, E., Bernini, F., 2006. Atlas of Italian Amphibians and Reptiles. Societas Herpetologica Italica. Firenze, Edizioni Polistampa.

Sirtori, C., Zapata, A., Malato, S., Agüera, A., 2012. Formation of chlorinated by-products during photo-Fenton degradation of pyrimethanil under saline conditions. Influence on toxicity and biodegradability. J. Hazard. Mater. 217–218,217–223

Skakkebaek, N.N., 2002. Endocrine disrupters and testicular dysgenesis syndrome. Horm. Res., 57, 43.

Smalling, K.L., Fellers, G.M., Kleeman, P.M., Kuivila, K.M., 2013. Accumulation of pesticides in pacific chorus frogs *Pseudacris regilla* from California's Sierra Nevada Mountains, USA. Environ. Toxicol. Chem. 32, 2026-2034, http,//dx.doi.org/10.1002/etc.2308.

Smalling, K.L., Orlando, J.L., Calhoun, Daniel, Battaglin, W.A., Kuivila, K.M., 2012. Occurrence of pesticides in water and sediment collected from amphibian habitats located throughout the United States, 2009-10, U.S. Geological Survey Data Series 707, 36 p.

Smalling, K.L., Reeves, R. Muths, E., Vandever, M., Battaglin, W.A., Hladik, M.L., Pierce, C.L., 2015. Pesticide concentrations in frog tissue and wetland habitats in a landscape dominated by agriculture. Sci. Total Environ. 502, 80-90, http,//dx.doi.org/10.1016/j.scitotenv.2014.08.114.

Smilanick, J.L., Mansour, M.F., Mlikota Gabler, F., Goodwine, W.R., 2006. The effectiveness of pyrimethanil to inhibit germination of *Penicillium digitatum* and to control citrus green mold after harvest. Post. Biol. Techn. 2006, 42, 75–85.

Spornitz, U.M., 1975. Studies on the liver of **Xenopus laevis**. I. The ultrastructure of theparenchymal cell. Anat. Embryol. 146, 245–64.

Storrs, S.I., Semlitsch, R.D., 2008. Variation in somatic and ovarian development, predicting susceptibility of amphibians to estrogenic contaminants. Gen. Comp. Endocrin. 1563, 524-530.

Storrs, S.L., Kiesecker, J.M., 2004. Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. Environ, Health Perspect. 112, 1054-1057, http,//dx.doi.org/10.1289/ehp.6821.

Strong, R.J., Halsall, C.J., Jones, K.C., Shore, R.F., Martin, F.L. 2016a. Infrared spectroscopy detects changes in an amphibian cell line induced by fungicides. Comparison of single and mixture effects. Aquat. Toxicol. 178, 8-18.

Strong, R.J. Halsall, C.J., Ferenčík, M., Jones, K.C., Shore, R F., Martin, F.L., 2016b. Biospectroscopy reveals the effect of varying water quality on tadpole tissues of the common frog *Rana temporaria*. Environ. Pollut. 213, 322-337.

Sugar, D., Basile, S,R., 2008. Timing and sequence of postharvest fungicide and biocontrol agent applications for control of pear decay. Post. Biol. Techn., 49, 107–112.

Sultan, C., Balaguer, P., Terouanne, B., Georget, V., Paris, F., Jeandel, C., Lumbroso, S., Nicolas, J., 2001. Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. Mol. Cell. Endocrinol. 178, 99-105.

Svartz, G., Meijide, F., Coll, C. P. 2016. Effects of a fungicide formulation on embryo-larval development, metamorphosis, and gonadogenesis of the South American toad Rhinella arenarum. Environmental toxicology and pharmacology, 45, 1-7.

Tabb, M.M., Blumberg, B., 2006. New modes of action for endocrine-disrupting chemicals. Mol. Endocrinol. 20, 475-482.

Takahashi, H., 1958. Gonadal reaction in the tree frog larvae *Hyla arborea japonica* Guenther to the androgen. Jour. Fac. Sci. Hokkaido Univ., Ser. VI, Zool., 14, 92-99.

Takahashi, H., 1971. Sex differentiation and development of the gonad in *Rana rugosa*. Zool. Mag., 80, 15-21.

Tamschick, S., Rozenblut-Kościsty, B., Ogielska, M., Lehmann, A., Lymberakis, P., Hoffmann, F., Lutz, I., Schneider, R.J., Kloas, W., Stöck, M., 2016a. Impaired gonadal and somatic development corroborate vulnerability differences to the synthetic estrogen ethinylestradiol among deeply diverged anuran lineages. Aquat. Toxicol. 177, 503-514.

Tamschick, S., Rozenblut-Kościsty, B., Ogielska, M., Lehmann, A., Lymberakis, P., Hoffmann, F., Lutz, I., Kloas, W., Stöck, M., 2016b. Sex reversal assessments reveal different vulnerability to endocrine disruption between deeply diverged anuran lineages. Sci. Rep. 6, 23825, doi, 10.1038/srep23825.

Tanimura, A., Iwasawa, H., 1986. Development of gonad and Bidder's organ in *Bufo japonicus formosus*. Histological observation. Sci Rep Niigata Univ Ser D Biol 23, 11–21.

Tanimura, A., Iwasawa, H., 1987. Germ cell kinetics in gonadal development in the toad *Bufo japonicus formosus*. Zool Sci., 4, 657–664.

Tanimura, A., Iwasawa, H., 1988. Ultrastructural observations on the origin and differentiation of somatic cells during gonada development in the frog *Rana nigromaculata*. Dev. Growth Differ.,30, 681–691.

Tanimura, A., Iwasawa, H., 1989. Origin of somatic cells and histogenesis in the primordial gonad of the Japanese tree frog *Rhacophorus arboreus*. Anat. Embryol., 180, 165–173.

Tanimura, A., Iwasawa, H., 1991. Proliferative activity of somatic cells during gonadal development in the Japanese pond frog, *Rana nigromaculata*. J. Exp. Zool. A, 2593, 365-370.

Taxvig, C., Vinggaard, A.M., Hass, U., Axelstad, M., Metzdorff, S., Nellemann, C., 2008. Endocrine-disrupting properties in vivo of widely used azole fungicides. Int. J. Androl. 31, 170-177.

Taylor, B., Skelly, D., Demarchis, L.K., Slade, M.D., Galusha, D., Rabinowitz, P.M., 2005. Proximity to pollution sources and risk of amphibian limb malformation. Environ. Health Perspect. 113, 1497-1501, http,//dx.doi.org/10.1289/ehp.7585.

Temple, H.J., Cox, N.A., 2009. European Red List of Amphibians. Office for Official Publications of the European Communities, Luxembourg.

Teplitsky, C., Piha, H., Laurila, A., Merilä, J., 2005. Common pesticide increases costs of antipredator defenses in *Rana temporaria* tadpoles. Environ. Sci. Technol. 39, 6079-6085, http,//dx.doi.org/10.1021/es050127u.

Thomatou, A.A., Zacharias, I., Hela, D., Konstantinou, I., 2012. Determination and risk assessment of pesticide residues in lake Amvrakia W. Greece after agricultural land use changes in the lake's drainage basin. Intern. J. Environ. Anal. Chem. 93, 780-799, http,//dx.doi.org/10.1080/03067319.2012.656099

Tomlin, C.D.S., 2000. The Pesticide Manual, 12th edition. Farnham,UK, The British Crop Protection Council. 9p.

Tompsett, A. R., Wiseman, S., Higley, E., Pryce, S., Chang, H., Giesy, J. P., Hecker, M., 2012. Effects of 17α-ethynylestradiol on sexual differentiation and development of the African clawed frog *Xenopus laevis.* Comp. Biochem. Phys. C, 1563, 202-210.

Toni, C., Ferreira, D., Kreutz, L.C., Loro, V.L., Barcellos, L.J., 2011a. Assessment of oxidative stress and metabolic changes in common carp *Cyprinus carpio* acutely exposed to different concentrations of the fungicide tebuconazole. Chemosphere 83, 579-584, http,//dx.doi.org/10.1016/j.chemosphere.2010.12.022.

Toni, C., Loro, V.L., Santi, A., de Menezes, C.C., Cattaneo, R., Clasen, B.E., Zanella, R., 2011b. Exposure to tebuconazol in rice field and laboratory conditions induces oxidative stress in carp *Cyprinus carpio*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 128-132, http,//dx.doi.org/10.1016/j.cbpc.2010.09.008.

Tripepi, S., Serroni, M., Brunelli, E., 1999. Guida Atlante degli Anfibi della provincia di Cosenza. Pellegrini Editore, Cosenza, 119.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife, a critical review of the evidence. Crit. Rev. Toxicol. 28, 319-361.

USEPA, 1999. U.S. Environmental Protectin Agency. Reregistration Eligibility Decision RED, Captan, EPA-738-R-99-015, Office of Pesticide Programs, U.S. GovernmentPrinting Office, Washington, DC.

USEPA, 2007. U.S. Environmental Protection Agency. Environmental Fate and Effects Division Risk Assessment for the Section 3 New Use Registration of Tebuconazole. Washington, D.C. 20460

USEPA, 2011. **U.S. Environmental Protection Agency**.Office of Chemical Safety and Pollution Prevention. MEMORANDUM Subject, TEBUCONAZOLE, Human Health Risk Assessment to Harmonize Tolerances of Tebuconazole in/on Oats and Wheat with Canada. June 14, 2011.

van Leeuwen, L., Vonk, J.W., 2008. Environmental risk limits for pyrimethanil. RIVM National Institute for Public Health and the Environment, Letter report 601716010/2008, The Netherlands, 20p.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.H., Shioda, T., Soto, A.M., vomSaal, F.S., Welshons, W.V., Zoeller, R.T., ZoellerMyers, J.P., 2012. Hormones and endocrine-disrupting chemicals, low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378-455, http,//dx.doi.org/10.1210/er.2011-1050.

Venturino, A., de D'Angelo, A.M.P., 2005. Biochemical targets of xenobiotics, biomarkers in amphibian ecotoxicology. Applied Herpetology, 23, 335-353.

Verdisson, S., Couderchet, M., Vernet, G., 2012. Effects of procymidone, fludioxonil and pyrimethanil on to non-target aquatic plants. Chemosphere, 44, 467–474.

Vinggaard, A.M., Hnida, C., Breinholt, V., 2000 Larsen, J.C. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol. In Vitro, 14, 227-234.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambré, C., Tarazona, J.V., Brandt, I., Vethaak, A.D., 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European sitation. Crit. Rev. Toxicol. 30, 71-133.

Wagner, N.D., Rödder, C.A., Brühl, M., Veith, P.P., Lenhardt, Lötters, S., 2014. Evaluating the risk of pesticide exposure for amphibian species listed in Annex II of the European Union Habitats Directive. Biol. Conserv. 176, 64-70, http,//dx.doi.org/10.1016/j.biocon.2014.05.014.

Wake, M. H., 1968. Evolutionary morphology of the caecilian urogenital system. Part I. The gonads and the fat bodies. J. Mrphol. 1263, 291-331.

Wake, M.H., 1970a. Evolutionary morphology of the caecilian urogenital system. Part II. The Kidneys and urogenital ducts. Acta Aat. 753, 321-358.

Wake, M.H., 1970b. Evolutionary morphology of the caecilian urogenital system. Part III. The bladder. Herpetologica, 120-128.

Wake, M.H., 1972. Evolutionary morphology of the caecilian urogenital system. Part IV. The cloaca. J. Morphol.,136, 353-366.

Waliszewski, S., Aguirre, A.A., Infanzón, R.M., Siliceo, J., 2000. Carry-over of persistent organochlorine pesticides through placenta to fetus. Salud. Publica Mex., 42, 384-390.

WHO, 1992. Our Planet, Our Health, Report of the WHO Commission on Health and Environment, WHO, Geneva, Switzerland.

Wightwick, A., Walters, R., Allison, G., Reichman, S., Mensien, N., 2010. Environmental Risks of Fungicides Used in Horticoltural Production Systems Environmental risks of fungicides used in horticultural production systems. In: Carisse O ed Fungicides. InTech, Reijka, 273–304, doi: 10.5772/13032.

Wightwick, A.M., Allinson, G., 2007. Pesticide residues in Victorian waterways, a review. Australian Journal of Ecotoxicology, 13, 91–112.

Wightwick, A. W., Duyen Bui, A., Zhang, P., Rose, G., Allinson, M., Myers, J. H., Reichman, S. M., Menzies, N. W., Pettigrove, V., Allinson, G., 2012. Environmental fate of fungicides in surface waters of a horticultural-production catchment in southeastern Australia. Arch. Environ. Contam. Toxicol. 62, 380-390. http,//dx.doi.org/0.1007/s00244-011-9710-y.

Wilson, V.S., Lambright, C.R., Furr, J.R., Howdeshell, K.L., Gray, L.E., 2009. The herbicide linuron reduces testosterone production from the fetal rat testis during both in utero and in vitro exposures. Toxicol. Lett. 186, 73-77.

Wolf, J. C., Lutz, I., Kloas, W., Springer, T. A., Holden, L. R., Krueger, H. O., Hosmer, A. J. 2010. Effects of 17 β-estradiol exposure on *Xenopus laevis* gonadal histopathology. Environmental Toxicology and Chemistry, 295, 1091-1105.

Yao, G., Ling, L., Luan, J., Ye, D., Zhu, P., 2007. Nonylphenol induces apoptosis of Jurkat cells by a caspase-8 dependent mechanism. Int. Immunopharmacol. 7, 444–453.

Yao, G., Yang, L., Hu, Y., Liang, J., Liang, J., Hou, Y., 2006. Nonylphenol- induced thymocyte apoptosis involved caspase-3 activation and mitochondrial depolarization. Mol. Immunol. 43,915–926.

Yu, S., Wages, M. R., Cobb, G. P., Maul, J. D., 2013b. Effects of chlorothalonil on development and growth of amphibian embryos and larvae. Environ. Pollut. 181, 329-334, http,//dx.doi,10.1016/j.envpol.2013.06.017.

Yu. L., Chen, M., Liu, Y., Gui, W., Zhu, G., 2013a. Thyroid endocrine disruption in zebrafish larvae following exposure to hexaconazole and tebuconazole. Aquat. Toxicol. 138-139, 35-42.

Zaccanti, F., Tognato, G., Tommasini, S., 1977. Proposta di un metodo di valutazione del grado di differenziamento sessuale dei corpi genitali in corso di sviluppo di Anfibi Anuri. Arch. Ital. anal. Embriol., 82, 233-252.

Zarn, J.A., Brüschweiler, B.J., Schlatter, J. R. ,2003. Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14 alpha-demethylase and aromatase. Environ. Health Persp. 1113, 255.

Züst, B., Dixon, K.E., 1977. Events in the germ cell lineage after entry of the primordial germ cells into the genital ridges in normal and uv-irradiated *Xenopus laevis*. Development, 411, 33-46.

Züst, B., Dixon, K.E., 1975. The effect of uv irradiation of the vegetal pole of *Xenopus laevis* eggs on the presumptive primordial germ cells. Development, 341, 209-220.