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EFFECTS OF MICROPLASTIC EXPOSURE ON ENDANGERED ANURAN AMPHIBIANS

WITH AN UPDATE ON THE USE OF VIDEO-TRACKING TECHNIQUES FOR BEHAVIOURAL STUDIES

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INTRODUCTION

1.1 The worldwide decline of amphibians

In 1989, during the First World Congress of Herpetology, the problem of the global decline of amphibian populations and species arose. The declining trend was already documented since the 1970s, with thousands of reports from across the planet. In the 1990s, the increasing number of studies and data convinced most herpetologists of the non-randomness of the worldwide amphibian decline. The Global Amphibian Assessment (GAA) initiative, led by the International Union for Conservation of Nature (IUCN), helped to evaluate the extent and severity of the problem collecting data on distribution, abundance, population trends, habitats, and threats for the 5743 amphibian species described at the time, determining their conservation status according to the IUCN Red List criteria. The results showed a global threat status, with 1856 species (32%) belonging to the IUCN Red List categories Vulnerable (VU), Endangered (EN), or Critically Endangered (CR), a percentage higher than that for birds (12%, 1211 species) and mammals (23%, 1130 species). In addition, 2468 amphibian species (43.2%) were declining, 1552 (27.2%) were stable, and only 28 (0.5%) were increasing, while the trend of the remaining species (29.1%) was unknown. Moreover, it should be noted that the threat level for amphibians was underestimated, as 1294 species (22.5%) fell into the Data Deficient (DD) category.

The number of CR amphibian species has approximately doubled since 1980 (231 species, 4.0%), with 34 extinct species since 1500 (9 of them after 1980), not including the 122 "Possibly Extinct" species (which cannot be declared extinct for the lack of exhaustive surveys), of which 113 have disappeared since 1980. 435 species that have been moved to IUCN categories of higher threat since 1980; these species are defined as "rapidly declining" and are divided into three groups according to the cause of the decline: overexploitation (50 species), habitat loss (183 species), and enigmatic decline (unknown reasons, possibly including infective disease and climate change, 207 species), with some species falling into more than one category (Stuart *et al*., 2004). Recently, environmental contaminants, UV-B radiation, emerging diseases, introduction of non-native species, and climate change have been added to the main causes of amphibian decline (Beebee & Griffiths, 2005), with recent studies focusing on their interactions rather than their individual effects (Blaustein *et al*., 2011).

1.1.1 Habitat loss and fragmentation

Habitat loss and fragmentation are among the greatest threats to amphibian populations, directly impacting on most of the risk factors for this taxon. These include their relatively low movement capacity, which leads to a high risk of dying crossing roads or inhospitable terrain and lowers population growth rates; low habitat tolerance, which exacerbates the effects of habitat loss; and finally, high vulnerability to pathogens, invasive species, climate change, increased exposure to UV-B radiation, and environmental pollution (Cushman, 2006).

Examples of factors causing habitat destruction and fragmentation include intensive agriculture and road infrastructure, which can have impacts on migration to mating sites that can remain undetected for decades. Moreover, habitat alteration can have long-term consequences, with effects that may still be underestimated despite the growing number of studies on this subject (Beebee & Griffiths, 2005). A negative correlation between amphibian species richness and decreased landscape connectivity has been observed, in which isolation of suitable sites for the species, density of road infrastructure, and urbanization represented the most significant variables (Lehtinen *et al*., 1999).

Extensive conversion of natural habitats for human use causes a sudden change in the ecological and evolutionary scenario and can therefore cause the decline of native species. A comprehensive phylogenetic analysis found that species favoured in a habitat conversion context tend to belong to the same clades within the amphibian phylogenetic tree. Thus, habitat use conversion results in non-random decline of more sensitive taxa and loss of biodiversity, which results in a large-scale phylogenetic homogenization (Nowakowski *et al*., 2018).

Many amphibian species are threatened by the loss and fragmentation of terrestrial habitats not strictly related to reproductive events. Some research have highlighted the effects of habitat fragmentation on the dispersal ability of juveniles as one of the major issues in the conservation of pond-breeding amphibians. Population connectivity is critical for regional survival and occurs primarily through juvenile dispersion, which is often lower than required for maintaining the population in fragmented landscapes. Although species with high dispersal capability are more vulnerable to the short-term effects of habitat loss and fragmentation, species with limited dispersal capacity are equally endangered in the long-term maintaining of populations. In summary, habitat loss and fragmentation impact accessibility to breeding and non-breeding sites, dispersal, survival rates, and population dynamics in many amphibian species (Cushman, 2006).

1.1.2 Environmental pollution

Chemical contaminants originating by the extensive use of fertilizers and pesticides in agriculture represent a major threat for amphibian populations. A meta-analytic review (Egea-Serrano *et al*., 2012) has shown a general negative impact of environmental chemical pollution on amphibians, highlighting its role into their overall decline. The main studies focused on the effects of different chemical pollutants (phosphorus and nitrogen compounds, pesticides, heavy metals, and other water contaminants) on survival, body mass, frequency of malformations, hatching time, and metamorphosis, highlighting various degree of correlation between these factors and exposure to chemical contaminants. Moreover, correlations were generally independent of amphibian phylogeny, therefore representing a general threat for the whole taxon.

Among the wide range of pollutants that can negatively impact amphibian populations, heavy metals and their effects on this taxon have been widely investigated. Essential and toxic metals naturally occur in ecosystems, but anthropogenic activities may alter their concentrations (Tchounwou *et al*., 2012). A frequent impact caused by exposure to metal ions (e.g., cadmium, copper, and lead) is growth inhibition (Pérez-Coll & Herkovits, 1990; Herkovits *et al*., 1997; Mouchet *et al*., 2006a, b; García-Muñoz *et al*., 2008; Ranatunge *et al*., 2012), while accelerated metamorphosis is uncommon, but still observed in tadpoles of *Fejervarya limnocharis* (Gravenhorst, 1829) exposed to cadmium (Patar *et al*., 2016). Cadmium exposure during early development generates severe malformations such as microcephaly, axial curvature, and incomplete gill development in *Rhinella arenarum* (Hensel, 1867) (Pérez-Coll *et al*., 1986, 1988). Exposition to cadmium in smooth xenopus (*Xenopus laevis* Daudin, 1802) during early life stages, causes major morphological disruptions such as axial curvature, reduction in size, growth and pigmentation, caudal fin and eye malformations, and microcephaly (Herkovits *et al*., 1997).

Effects of metal exposure on amphibian activity are commonly a consequence of malformations. This was observed in tadpoles of *R. arenarum* exposed to cadmium, which showed swimming impairment due to severe axial curvature and tail malformations (Pérez-Coll *et al*., 1986). In the same species, lead exposure led to neurological disorders such as tremor and swimming and balance alterations (Pérez-Coll & Herkovits, 1990). A decrease in swimming activity was also observed in tadpoles of *Duttaphrynus melanostictus* (Schneider, 1799) exposed to cadmium (Ranatunge *et al*., 2012). Other studies showed that even in the absence of morphological and growth alterations, changes in tadpole behaviour may still be observed. Bullfrog tadpoles (*Lithobates catesbeianus* Shaw, 1802) exposed to lead showed increased learning latency associated with reduced avoidance behaviour (Strickler-Shaw & Taylor, 1991). Natterjack toad tadpoles (*Epidalea calamita* Laurenti, 1768) exposed to copper

exhibited decreased reactivity, with abnormal and reduced movements (García-Muñoz *et al*., 2008). Exposure to metals can also modify secondary sexual traits and sexual behaviour, as reported in the frog *Pelophylax nigromaculatus* (Hallowell, 1861) exposed to cadmium. The observed effects included abnormalities in larynx structure in adult individuals, delayed development, reduced cross-sectional area, and alterations in muscle fibre size (Duan & Huang, 2016), but also severely altered calling and delayed responses to female calling stimuli (Huang *et al*., 2015).

Amphibians are also non-target species of pesticides used in extensive agriculture or for disease vector control (Slaby *et al*., 2019). Crop protection products can have toxic effects on amphibians (Aldrich *et al*., 2016), causing mortality and sublethal effects i.e., reduced growth and increased susceptibility to disease, with species-specific variations. A meta-analysis (Baker *et al*., 2013) investigated the role of different chemicals on amphibian survival and growth: inorganic fertilizers, organophosphates, chloropyridinyl, phosphoglycines, carbamates, and triazines highly impacted survival, while growth was negatively impacted by organophosphates and phosphoglycines. Pesticide exposure can also induce morphological alterations (Slaby *et al*., 2019). American toad (*Anaxyrus americanus* Holbrook, 1836), leopard frog (*Lithobates pipiens* Schreber, 1782), and wood frog (*Lithobates sylvaticus* LeConte, 1825) tadpoles exposed to atrazine, showed dose-dependent malformations such as facial edema, blistering, axial shortening, and latero-dorsal tail flexion (Allran & Karasov, 2001). Atrazine can also lead to weight loss in individuals of *X. laevis* and gray treefrog (*Dryophytes versicolor* LeConte, 1825) (Diana *et al*., 2000; Sullivan & Spence, 2003).

Insecticides are also reported to affect amphibians' general activity levels. Exposure to Carbaryl in *Lithobates blairi* (Mecham *et al*., 1973) caused a reduction in activity time associated with reduced swimming speed and distance (Bridges, 1997), but also lowered activity in *D. versicolor* (Relyea & Mills, 2001). Exposure to another carbamate contained in Marshal® (Carbosulfan) has been shown to be responsible for abnormal swimming activity and morphological alterations in tadpoles of *D. melanosticus* (Samarakoon & Pathiratne, 2017). In *Phyllodytes luteolus* (Wied-Neuwied, 1824), hypoactivity was recorded after exposure to a commercial malathion formulation (Formitek®) (Egea-Serrano & Solé, 2017). Malathion (20 ppm) and parathion (2 ppm) both induced a lowered activity in *R. arenarum* (Anguiano *et al*., 2001). Loss of equilibrium has been reported in tadpoles of *L. catesbeianus* (Fordham *et al*., 2001) following chronic exposure to Malathion. Activity level alteration caused by insecticides (Abate®, Depe®, and Introban®) were observed in *R. arenarum*, *Physalaemus albonotatus* (Steindachner, 1864), and *Rhinella fernandezae* (Gallardo, 1957) (Junges *et al*., 2017).

Herbicides can also affect tadpole activity and behaviour. *X. laevis* exposed to atrazine showed abnormalities in swimming (Carr *et al*., 2003). Exposure to Roundup® Original induced increased activity levels, associated with tachycardia in *L. catesbeianus* (Costa *et al*., 2008). Exposure to different concentrations of glyphosate-based herbicides in tadpoles of agile frog (*Rana dalmatina* Fitzinger, 1839) caused significant effects: after exposure to high concentrations, tadpoles were less active and hid more than controls (Mikó *et al*., 2017). Exposure to Roundup® Power 2.0 for 96 hours caused a reduction in length and caudal membrane in tadpoles of marsh frog (*Pelophylax ridibundus* Pallas, 1771) exposed to 7.6 mg/L and an increase in body length at lower concentrations (0.7 and 3.1 mg/L); moreover, tadpoles exposed to the highest concentration showed reduced lateralization intensity, whereas all concentrations were correlated with reduced activity level (Bolis *et al*, 2020). Finally, the organotin compound Triphenyltin chloride, used as a fungicide and antifouling, can induce a decrease in swimming activity and an increase in time spent feeding (Semlitsch *et al*., 1995).

Another effect of chemical contaminants is sex reversal, which has been well documented in amphibians. In *X. laevis*, exposure to atrazine has been observed to induce primary sex organ defects in tadpoles i.e., intersex, multiple, or altered gonads (Hayes *et al*., 2002; Tavera-Mendoza *et al*., 2002; Carr *et al*., 2003), reduce testosterone levels in adult males, and alter secondary sexual characters (e.g., reduction in larynx size; Hayes *et al*., 2002). Atrazine promotes feminization by enhancing the conversion of testosterone into estrogen and, like other environmental endocrine disruptors, may play an important role in the overall decline of amphibians (Hayes *et al*., 2002). Feminization after exposure to DDT or derivatives has been recorded in *Hyperolius argus* (Peters, 1854), with female-skewed sex ratios (Noriega & Hayes, 2000). Finally, exposure to the fungicide Vinclozolin has been observed to alter the song of *X. laevis* males (Hoffmann & Kloas, 2010).

Emerging organic contaminants are often classified as Endocrine Disrupting Chemicals (EDCs), mainly hormones and hormone-like substances (Slaby *et al*., 2019). Exposure to EDCs during early life can lead to profound and long-term effects such as gonadal sex reversal, reduced gametogenesis, and impaired fertility in adult frogs. An experiment performed on western clawed frog (*Xenopus tropicalis* Gray, 1864) showed that larval phase exposure to the progestin Levonorgestrel (LNG) causes arrested oocyte development, oviduct agenesis, and infertility (Kvarnryd *et al*., 2011). This contraceptive drug is common in the environment due to its high presence in civil wastewater and is sometimes dispersed in cultivated fields via sludge spreading (Tang *et al*., 2012).

Although direct mortality and physiological alterations caused by chemical pollutants are one of the major causes of the amphibian decline, sublethal concentrations of contaminants can also present a threat. Prevention efforts and strategies are usually focused on preventing the release of lethal doses

into the environment, but low levels of pollutants are ubiquitous and often go unnoticed. A recent study (Polo-Cavia *et al*, 2016) shows that sublethal concentrations of two common water contaminants, namely Humic acid and Ammonium nitrate, can impair the ability of Iberian spadefoot toad tadpoles (*Pelobates cultripes* Cuvier, 1829) to recognize predator chemical signals (*Anax imperator* Leach, 1815), therefore increasing the risk of predation. In the absence of contaminants, tadpoles reduced swimming activity in response to predator's chemical signals, whereas, when one or both contaminants were added to the water, even in low concentrations, the anti-predatory response was absent. Moreover, exposition to both contaminants did not alter tadpoles' basal activity levels, therefore indicating the absence of toxic effects.

1.1.3 Alien species

Non-native species, otherwise known as allochthonous, exotic, or alien species, can pose an ecological threat to native species in a variety of ways: directly, through predation, competition, and transmission of pests and diseases, or indirectly, through habitat alteration. According to the IUCN, more than a thousand (16%) of Red listed amphibian species are threatened by invasive alien species. Evidence on the impact of non-native species on amphibian populations are based on the negative correlations between the presence of alien predators and population declines, and on experiments evaluating how alien species may affect amphibians.

In the case of introduced predators, native prey may not have evolved the ability to recognize and respond to the new threat. Amphibian larvae provide an excellent system for examining anti-predatory responses: tadpoles can respond to predator scent and chemical alarm signals released by injured conspecifics, modifying their behaviour, life history traits, and morphology. A global meta-analysis of the impacts of alien species on native amphibians (Nunes *et al*., 2019) showed that amphibian performance was consistently lower in the presence of alien species, with strong effects on all measures of fitness. Moreover, exposure to alien species caused a significant decrease in amphibian behavioural activity when compared with controls, with a stronger response towards controls of native impacting species.

Local declines and extinctions have been widely ascribed to introduced fish, other amphibians such as *L. catesbeianus* and the cane toad (*Rhinella marina* Linnaeus, 1758), and freshwater crayfishes (Kats & Ferrer, 2003). Competition and predation can cause reductions in growth and survival, while altering behaviour and habitat use. Eggs and larvae are usually the most vulnerable stages (Vonesh & De la Cruz, 2002).

The red swamp crayfish (*Procambarus clarkii*, Girard, 1852) is a highly invasive (Gherardi, 2006; Aquiloni *et al*., 2008) species native to Mexico and the USA, that preys on a wide range of freshwater species, including anuran larvae (Gherardi *et al*., 2001; Cruz *et al*., 2006; Ficetola *et al*., 2011). This voracious predator can have dramatic impacts on freshwater fauna and alter aquatic ecosystems through trophic cascades (Souty-Grosset *et al*., 2016). Its introduction has been related to the local decline or extinction of several amphibian species (Cruz *et al*., 2008), including the Italian agile frog (*Rana latastei*), an endemic threatened species of northern Italy (Ficetola *et al*., 2012).

Another danger associated with species introductions concerns the pathogens they may carry. *R. catesbeiana* is an effective vector of chytridiomycosis (Daszak *et al*., 2004) and this frog has become established in many areas outside of its original distribution range.
Introduction may also cause hybridization with local species. In Switzerland and southern England, the introduced Italian crested newt (*Triturus carnifex* Laurenti, 1768) hybridized with the native northern crested newt (*Triturus cristatus* Laurenti, 1768), impacting local populations (Arntzen & Thorpe, 1999; Brede *et al*., 2000). Pool frog (*Pelophylax lessonae* Camerano, 1882) has been completely replaced by *P. ridibundus* following its introduction and hybridization in many areas of western and central Europe (Vorburger & Reyer, 2003).

1.1.4 Emerging infectious diseases

Batrachochytrium dendrobatidis (Bd) is a pathogenic fungus that causes chytridiomycosis in amphibians (Daszak *et al*., 1999, 2000; Fisher *et al*., 2009). This disease can cause population declines and local extinctions, contributing to species extinctions (Stuart *et al*., 2004; Skerratt *et al*., 2007; Olson *et al*., 2013). Another fungal pathogen that causes chytridiomycosis is *Batrachochytrium salamandrivorans* (Bsal), a recently discovered pathogen that mainly infects salamanders (Martel *et al*., 2013). In addition, iridoviruses of the genus *Ranavirus* (Rv) are also implicated in declines and mass mortality events in amphibians (Green *et al*., 2002; Chinchar *et al*., 2009; Teacher *et al*., 2010; Kik *et al*., 2011; Miaud *et al*., 2016).

Bd has multiple hosts (Fisher *et al*., 2009; Olson *et al*., 2013) and has been associated with numerous population declines and some extinctions (Berger *et al*., 1998; Lips, 1998; McCallum, 2005). Recent evidence suggests that Bd originated from the Korean Peninsula and became established from the early 20th century with the expansion of the global amphibian trade (O'Hanlon *et al*., 2018). This pathogen has a complex life cycle which includes an aquatic free-living infectious stage (zoospore) and a non-mobile stage (zoosporangium). Zoospores are chemically attracted by the keratin in

amphibian hosts (Berger *et al*., 2005; Greenspan *et al*., 2012) and infection can cause hyperkeratosis and hyperplasia of the dermal layer, erosions and ulcerations of the skin, and disruption of the epidermal cell cycle (Berger *et al*., 1998; Nichols *et al*., 2001; Berger *et al*., 2005; Voyles *et al*., 2009; Greenspan *et al*., 2012). However, not all infected animals are symptomatic. After infection, zoosporangia mature and develop into pathogenic zoospores, which are then released from the host into the aquatic environment.

The recent isolation and characterization of the fungal pathogen Bsal may explain some declines in amphibian populations. The dramatic decline of the spotted salamander (*Salamandra salamander* Linnaeus, 1758) in the Netherlands, Germany, and Belgium, has been linked to Bsal (Spitzen-van der Sluijs *et al*., 2014, 2016; Sabino-Pinto *et al*., 2015). The proposed origin of Bsal is East Asia (Martel *et al*., 2014), where it would have coevolved with salamanders for millions of years; the introduction of Bsal in Europe has probably been mediated by the lack of biosecurity in international pet trade. As with Bd, Bsal infection results in lethal skin lesions, but Bsal pathogenesis is still to be elucidated (Blaustein *et al*., 2018).

Ranaviruses are a group of dna-based viruses belonging to the family Iridoviridae and use fishes, reptiles, and amphibians as hosts (Chinchar, 2002). The first Rv was isolated in the frog *L. pipiens* in 1965 (Granoff *et al*., 1965). The Global Ranavirus Reporting System (https://mantle.io/grrs/map), shows that Rvs are somewhat widespread in Canada and the USA west of the Rocky Mountains. The Rv genus consists of 6 identified viral species, three of which can infect amphibians: *Ambystoma tigrinum* virus (ATV), Bohle iridovirus (BIV), and Frog Virus 3 (FV3) (Chinchar, 2002). Laboratory experiments have shown that introduced Rvs can be significantly more virulent than endemic Rvs (Storfer *et al*., 2007). Amphibians can get infected with Rv via direct physical contact, dermal exposure to contaminated water, or direct ingestion (Harp & Petranka, 2006; Brunner *et al*., 2007). Infection can cause cell apoptosis and tissue necrosis within hours (Chinchar, 2002; Williams *et al*., 2005). Fatal cases may be associated with internal organ injury and haemorrhage (Cunningham *et al*., 1996; Docherty *et al*., 2003; Miller and Gray, 2010). However, the precise mechanisms of Rv infection are still uncertain, especially during the earliest stages of infection. A recent study demonstrated that FV3 can alter the blood-brain barrier in tadpoles of *X. laevis*, allowing the virus to spread into the central nervous system (Andino *et al*., 2016). Pearman *et al*. (2004) tested the effects of FV3 on tadpoles of Italian agile frog (*Rana latastei* Boulenger, 1879); exposure to increasing concentrations of the virus produced dose-dependent survival rates, and cannibalism of infected carcasses increased mortality from FV3. In addition, it has been shown that *R. latastei* populations

with low genetic variability are more susceptible to emerging pathogens, particularly FV3 (Pearman & Garner, 2005).

Amphibians' susceptibility to pathogens varies according to different factors such as species, host age, life stage, biotic (e.g., presence of competitors or predators) and abiotic (e.g., temperature, presence of contaminants) conditions, but also strain and dose of the pathogen to which they are exposed (Blaustein *et al*., 2018).

1.1.5 Climate change

Climate change results in a general warming in the climate system, with many of the observed changes unprecedented over the past millennia: warming of the atmosphere and the ocean, melting of glaciers and sea ice, sea level rise, and increasing concentrations of greenhouse gases (Intergovernmental Panel on Climate Change, 2013).

This phenomenon is causing profound modifications in the phenology of organisms, and amphibians present particularly strong phenological responses to increasing temperatures. Assessing the consequences of climate change on individual performance and population dynamics can be extremely difficult (Merilä & Hendry, 2014), therefore, these aspects present a wider knowledge gap than phenology and distribution (Dunn & Moller, 2014). However, population dynamic represents a key aspect in assessing the impact of climate change (Ficetola & Maiorano, 2016).

Climate change is expected to have a particularly strong impact on ectothermic vertebrates (Buckley *et al*., 2012), as temperatures directly influence their activity patterns and metabolism, which in turn determine individual fitness (Kearney & Porter, 2009). Mating anticipation has been observed to occur in amphibians in response to climate warming (Beebee, 1995), and different studies suggested that this taxon is showing strong phenological alterations in response to global warming, with an average anticipation of reproduction of 6.1 days per decade (While & Uller, 2014), compared to the average 2.8 measured in other taxa (Parmesan, 2007).

Few attentions have been paid to the consequences of changes in precipitation and water availability. Many amphibian species depend on wet environments for their survival and reproduction and are therefore susceptible to change in water availability. In tropical areas, many amphibian declines and extinctions have been attributed to climate change (Pounds *et al*., 1997, 1999; Laurance, 2008; Menéndez-Guerrero & Graham, 2013). Even more drastic declines are expected in the future,

especially under extreme climate change scenarios, due to the narrow ecological niche and limited dispersal ability of these organisms (Araujo *et al*. 2006; Courtois *et al*. 2016).

Earlier breeding season in response to global warming is probably the best-documented consequence of climate change on amphibian populations (Beebee, 1995; While & Uller, 2014). A meta-analysis (Ficetola & Maiorano, 2016) performed on more than 50 species worldwide investigated the role of temperature and precipitation trends on amphibian populations. Both factors strongly influence amphibians, but temperature is the major driver of phenological alterations, while precipitation plays a major role in population dynamics, with dryer years causing reductions in population densities. Dry periods have been associated with lower adult survival in *Hemisus marmoratus* (Peters, 1854) and lower reproductive success in *E. calamita* (Banks *et al*. 1994; Grafe *et al*. 2004), while warm periods have been correlated with reduced body condition index in the common toad (*Bufo bufo* Linnaeus, 1758) and reduced survival in the Northern crested newt (*Triturus cristatus* Laurenti, 1768) (Reading, 2007; Griffiths *et al*. 2010). The above-mentioned meta-analysis (Ficetola & Maiorano, 2016) confirmed that temperature is the primary driver of phenological anticipation. Amphibians rely on multiple signals to initiate reproductive activity, and for many species, the onset of reproduction occurs during rainy periods and/or when temperature exceeds a specific threshold (Timm *et al*., 2007). Although some species show early reproduction in rainy years, the relationship between precipitation and phenology was usually weak (Ficetola & Maiorano, 2016).
On the other hand, precipitation plays a major role in determining population size. Population declines are usually associated with dry periods. Water availability is a key determinant for amphibian fitness, increasing larval survival and reproductive success in species that require water bodies for reproduction (Banks *et al*. 1994). In addition, most species have a limited tolerance to desiccation; therefore, high humidity and wet periods are necessary for adult activity (Zug *et al*. 2001; Ficetola *et al*. 2012a, b).

Although global warming results in a trend of earlier reproduction, it is difficult to predict how this will impact population dynamics. On the one hand, earlier reproduction may cause earlier maturity, which could have positive effects (Alvarez & Nicieza, 2002; Altwegg & Reyer, 2003), although some evidence does not support this thesis (Schmidt *et al*., 2012; Earl & Semlitsch, 2013). On the other hand, changes in phenology may differ among related species within communities, with potential demographic effects, i.e., mismatch between predator and prey phenologies (Both *et al*., 2006; Moller *et al*., 2008). Moreover, knowledge on the potential effects of phenological discrepancies between amphibian reproduction and trophic resource is still limited.

Analyses of phenology show that the response of amphibians to climate change is spatially heterogeneous. With respect to temperature, climate change is strongest at high latitudes (Intergovernmental Panel on Climate Change, 2013), and populations in those areas experience higher impacts, also exhibiting greater phenological alteration in response to warming (Mazaris *et al*., 2013; While & Uller, 2014). On the other hand, populations in wet tropical climates appear to be less tolerant to dry periods. Many tropical species that do not need large water bodies to reproduce (and can even use very small ones, e.g., phytotelmas, aquatic microecosystems formed by water retained by some terrestrial plants), can reproduce out of water (Gomez-Mestre *et al*., 2012) and have longer periods of activity. Therefore, these organisms require higher levels of moisture to avoid desiccation. Climate change scenarios suggest that reduction in precipitation could be severe in some tropical areas (e.g., northern Australia, Mesoamerica, Amazon Basin, and Madagascar) (Intergovernmental Panel on Climate Change 2013), which currently host most part of amphibian diversity.

1.2 The problem of microplastics

Tons of plastic waste have been released into the environment over the past few decades (Hoellein *et al*., 2014; Geyer *et al*., 2017) as a result of the increasing demand for these polymers. Increased production, along with improved chemical and mechanical resistance of plastics, has made these contaminants more persistent and potentially more dangerous to the environment (Lithner *et al*., 2011).

Microplastic particles (MP) are classified according to origin, size, shape, and polymer composition. In terms of origin, primary MPs are plastic pellets used in the plastic industry as raw material to make plastic products, or as an ingredient in personal care products (e.g., exfoliants and shower gels) (Wagner *et al*., 2014; Nizzetto *et al*., 2016). Secondary MPs originate from larger plastic objects which can degradate into MP less than 5 mm in diameter directly in the environment. Sources of secondary MP include household and manufacturing products, old tyres, paint flaking, etc. MPs with sizes > 25 mm, 5-25 mm, 1-5 mm, 20 μm-1 mm, and 1-1000 nm are classified as macro, meso, large micro, small micro, and nano-plastics, respectively (Gigault *et al*., 2018). According to shape MPs can be classified as, fragments, pellets, fibres, and granules. In terms of composition, MPs of high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) are among the most common in the environment (European Commission, 2013). The fate of MPs depends on their size, shape, surface area, porosity, roughness, morphology, solubility, and surface chemistry, while their biodegradation depends on

their susceptibility to degradation by microbial communities present in the environment (Anbumani & Kakkar, 2018).

1.2.1 Microplastic pollution

Recently, MP contamination has been one of the most widely investigated forms of environmental pollution (Andrady, 2017; Chae & An, 2018; Li *et al*. 2018). The presence of MP in lakes, rivers, and estuaries has been widely documented (Williams & Simmons, 1996; Moore *et al*., 2011; Faure *et al*., 2012; Imhof *et al*., 2013; Morritt *et al*., 2014; Sadri & Thompson, 2014; Zbyszewski *et al*., 2014; Klein *et al*., 2015). MP concentration tends to be higher in proximity to high-populated areas in proximity to freshwater ecosystems. Moreover, a significant correlation was observed between high-populated areas and MPs concentration in 18 coastal sites across all continents, suggesting that the number of MPs dispersed into the environment will likely increase following the growth of the human population (Browne *et al*., 2011). MPs can enter freshwater environments through degradation of plastic material in the water column, leaching of from land or watersheds, and through direct discharge from urban and industrial effluents (Campbell *et al*., 2017). MPs are released into the environment by human activities or improper waste disposal (Barnes *et al*., 2009) primarily through manufacturing, wastewater treatment, and sewage sludge.

The most common sources of primary MPs include personal hygiene products, cosmetics, abrasive microspheres, and resin powders and pellets (Gregory, 1977; Shiber, 1979; Zitko & Hanlon, 1991; Gregory, 1996; Redford *et al*, 1997; Moore *et al*., 2001; Derraik, 2002; Thompson *et al*., 2004; Reddy *et al*., 2006; Betts, 2008; Moore, 2008), but also synthetic textile fibres, which can enter the environment from urban drains. Indeed, the release of MPs in the form of fibres resulting from textile washing is well documented (Browne *et al*., 2011; Dris *et al*., 2015; Essel *et al*., 2015; GESAMP, 2015; Napper & Thompson, 2016; Wentworth & Stafford, 2016).

Abundance, persistence, ubiquity, and small size (< 5 mm; Hartmann *et al*., 2019) put MPs among the most dangerous contaminants for many plant and animal organisms, causing a wide range of toxic effects, such as alteration of feeding, reproductive performance, metabolism, physiological changes in the liver, synergistic action with other hydrophobic organic pollutants, etc., at all trophic levels (Anbumani & Kakkar, 2018). Thanks to their small size, which facilitates their uptake by biota, MPs are prone to trigger bioaccumulation in the trophic chain; moreover, they can adsorb other chemical contaminants, increasing the exposure of organisms to a wide range of toxic substances (Rillig, 2012; Wagner *et al*., 2014; Kärrman *et al*., 2016).

Harmful effects can also be ascribed to the release of constituent compounds of plastics (e.g., monomers and additives), which have been shown to be carcinogenic and endocrine disruptors (Oehlmann *et al*., 2009; Talsness *et al*., 2009). Particles can be ingested by various freshwater organisms, including plankton, fish, birds, and mammals (Batel *et al*., 2016). The ecological impacts of MPs on marine ecosystems are well studied in invertebrates (Thompson *et al*., 2004; Ward & Shumway, 2004; Browne *et al*., 2008; Graham & Thompson, 2009; Murray & Cowie, 2011; Cole *et al*., 2013; Goldstein & Goodwin, 2013) and vertebrates (Eriksson & Burton, 2003; Ryan *et al*, 2009; Thompson *et al*., 2009; Boerger *et al*., 2010; Davison & Asch, 2011; Fossi *et al*., 2012, 2014), but there is little information regarding the effects of MPs on freshwater biota (Wagner *et al*., 2014; Eerkes-Medrano *et al*., 2015).

1.2.2 Effects of microplastics on anuran amphibians

The ecotoxicological effects of MPs on anuran amphibians, one of the most threatened taxa globally (Stuart *et al*., 2004; Becker *et al*., 2007), are still lagging behind, with only a few recent studies highlighting their effects on tadpoles' physiology and behaviour (De Felice *et al*., 2018; Boyero *et al*., 2020; Da Costa Araújo *et al*., 2020 a, b, c). Furthermore, studies on the impact of MPs on species of marked conservation interest are still scarce or completely absent.

Anuran tadpoles are suspensivore/grazer primary consumers and are therefore extremely likely to ingest MPs while feeding (Altig *et al*., 2007; Boyero *et al*., 2020). Ingestion and accumulation of MPs has been proven in tadpoles both under laboratory and natural conditions (De Felice *et al*., 2018; Hu *et al*., 2016; Hu *et al*., 2018; Kolenda *et al*., 2020; Karaoğlu & Gül, 2020). Although some studies have shown that tadpoles are capable of tolerating and expel MPs relatively fast (Hu *et al*., 2016; De Felice *et al*., 2018), others showed significant physiological alterations and high mortality levels in tadpoles of *Xenopus laevis* and *Alytes obstetricans* exposed to MPs (Tussellino *et al*., 2015; Boyero *et al*., 2020). Exposure to polyethylene (PE) MPs has been proven to cause histopathological damage in *Physalaemus cuvieri* tadpoles (Da Costa Araújo *et al*., 2020a): after seven days of exposure to MPs (60 mg/L), tadpoles showed liver bioaccumulation of MPs, associated with vessel dilation, infiltration, congestion, hydropic degeneration, hypertrophy, and hyperplasia. Moreover, mutagenic, and cytotoxic effects associated with a significant increased number of abnormalities in nuclear erythrocytes were observed (Da Costa Araújo *et al*., 2020b). Finally, behavioural alterations such as locomotion issues and defective anti-predatory defensive response have been observed in tadpoles exposed to PE MPs (Da Costa Araújo *et al*., 2020c).

Along with the global decline of amphibian species and populations, there are two main reasons why the effects of MPs on amphibian should be carefully addressed. Firstly, tadpoles are primary consumers in many freshwater ecosystems, and their feeding activity may influence key processes such as primary production or nutrient cycling (Seale, 1980; Whiles *et al*., 2012). Therefore, if MPs ingestion results in alteration of tadpoles' trophic role, it may also lead to significant ecological alterations. Secondly, predator species such as fishes, crayfishes, snakes, and mammals may be subject to bioaccumulation of MPs if they prey on contaminated amphibians. Therefore, amphibian larvae and adults may represent an important transfer path for these contaminants through higher trophic levels and between freshwater and terrestrial ecosystems (Larsen *et al*., 2016; Da Costa Araújo *et al*., 2021).

1.3 Amphibian behaviour in conservation studies

A change in behaviour is the promptest reaction that animals can adopt to respond to external change, potentially enhancing individual fitness and, therefore, the probability of species' long-term survival (Wong & Candolin, 2015). Habitat alterations, chemical pollutants, parasites, and introduced predators are among the main drivers of change in amphibian behaviour (Sievers *et al*., 2019; Bower *et al*., 2019; Nunes *et al*., 2019). Amphibian larvae are easy to collect and cheap to rear in captivity, while their complex life cycle offers the chance to investigate many different behavioural traits (Watt *et al*., 1997; Crane & Ferrari, 2017; Gazzola *et al*., 2018). On these grounds, they constitute an ideal candidate to investigate the effects of anthropic pressure on animal behaviour.

Tadpoles have been largely used as model system for investigating predator-prey relationships (Van Buskirk, 2001; Moore *et al*., 2004; Gazzola *et al*., 2017). Invasive predators (which have no common evolutionary history with prey species) are the major cause of the decline of rare or endemic prey species (Dick & Platvoet, 2000; Fukasawa *et al*., 2013), therefore, studies on the behavioural response to non-native predators are paramount for amphibian conservation (Hobbs & Huenneke,1992; Chytrý *et al*., 2012). Moreover, behavioural alterations (e.g., reduced activity, defective anti-predatory responses) induced by emerging contaminants (such as MPs) may play an important and synergistic role in the decline of anuran populations, especially in those already threatened by other anthropogenic alterations, such as habitat loss and fragmentation, infective disease, and alien species.

1.4 Aims of the PhD thesis

The present thesis aims to evaluate the effects of microplastic contamination on the behaviour and survival of two endangered anuran species typical of northern Italy, namely the Italian agile frog (*R. latastei*) and the Balearic green toad (*B. balearicus*). Although the effects of MPs have been investigated on a wide variety of taxa, the impact of these widespread pollutants is virtually unknown in anuran amphibians. In this work, we used an integrated ecotoxicological-behavioural approach supported by digital video-tracking techniques in order to develop a low-cost, standardized protocol for testing MPs on amphibian larvae. The main objectives can be resumed and will be treated as follows:

I. Evaluate and quantify the anti-predatory response of tadpoles to native and alien predators under laboratory conditions (Chapters 2, 3, and 4)

II. Evaluate the suitability of video-tracking techniques in collecting behavioural data on tadpoles (Chapters 2, 3, 4, and 6)

III. Test the effect of an MP mixture on the growth, survival, activity, and anti-predatory behaviour of tadpoles (Chapters 5 and 6)

1.5 Thesis outline

Chapter 1 provided a general overview of the problems affecting the global decline of amphibians and a list of our main objectives. Following this brief consultation guide, this chapter will be concluded with a book of abstracts, providing the reader a quick and accessible synthesis of our main findings. The main corpus of the work will outline as a collection of the scientific papers published over the three years of my PhD project.

Chapters 2, 3, and 4 present the basic knowledge on which we built the experiments described in Chapters 5 and 6. As well as representing the foundation of our work, these chapters add interesting bits of knowledge to different aspects of anuran behaviour. In Chapter 2 we evaluated the behavioural defensive responses of *R. latastei* tadpoles induced by a native predator's odour, using digital video-tracking techniques to investigate the role of intra- and inter-specific alarm cues on the intensity of tadpole response. Chapter 3 investigates *R. latastei* differences in the response to native and alien predators, paying particular attention to lateralization and sinuosity using a C++ code we developed for the analysis of digitally recorded tadpole tracks. Chapter 4 evaluates the effect of group living and conspecific density on the individual performance of *B. balearicus* tadpoles exposed to native predator cues.

In Chapter 5 we used classical ecotoxicological techniques to investigate the effects of microplastics on the activity, growth, development, and survival of *R. latastei* and *B. balearicus*, which led to the identification of important interspecific differences in the response to MP treatments. Finally, in Chapter 6, we investigated the effects of exposure to MP on the defensive response of *R. latastei* tadpoles using a combination of the techniques presented in the previous chapters.

1.6 Book of abstracts

Ethology, Volume 126, Issue 9, https://doi.org/10.1111/eth.13072

STRONG BEHAVIOURAL DEFENSIVE RESPONSES OF ENDEMIC *RANA LATASTEI* TADPOLES INDUCED BY A NATIVE PREDATOR'S ODOUR

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Abstract

Prey species must constantly acquire information on predator identity, abundance, and dangerousness from the environment. In aquatic habitats, this information is mainly propagated by water-borne chemical signals, either predator-specific odours or prey alarm cues. Anuran larvae innately respond to conspecific alarm cues and are able to associate them to predator cues during their lifetime. In this study, we investigated the anti-predatory responses of endemic Italian agile frog (*Rana latastei*) tadpoles exposed to either conspecific or heterospecific alarm cues and a native predator's (*Anax imperator* larvae) odour. Pre-and post-stimulus behaviours of each tadpole were recorded by a digital camera and analysed by a source executable software for image-based tracking. We found that Italian agile frog tadpoles responded to fasted dragonfly odour by strongly reducing their activity, both in terms of the amount of time they spent active and path length covered in comparison to control groups. Contrary to previous studies, predators' diet had a negligible effect on tadpole response and our experiment did not bring any evidence of the phylogenetic-relatedness hypothesis. The innate or early-in-development recognition of dragonfly larvae is clearly adaptive and may increase tadpole survival with relatively low costs, but, at the same time, may increase the risk of ignoring novel potential threats.

Journal of Experimental Biology, Volume 224, Issue 9, https://doi.org/10.1242/jeb.240465

CONTEXTUAL BEHAVIOURAL PLASTICITY IN ITALIAN AGILE FROG (*RANA LATASTEI*) TADPOLES EXPOSED TO NATIVE AND ALIEN PREDATOR CUES

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Abstract

Predation is a strong driver for the evolution of prey behaviour. To properly assess the actual risk of predation, anuran tadpoles mostly rely on water-borne chemical cues, and their ability to evaluate environmental information is even more crucial when potential predators consist of unknown alien species. Behavioural plasticity – that is, the capacity to express changes in behaviour in response to different environmental stimuli – is crucial to cope with predation risk. We explored the defensive behaviour of Italian agile frog (*Rana latastei*) tadpoles when exposed to the chemical cues of two predator species, one native (dragonfly larvae) and one alien (red swamp crayfish). Firstly, we observed whether a plastic life history trait (i.e., hatching time) might be affected by native predatory cues. Secondly, we recorded a suite of behavioural responses (activity level, lateralization, and sinuosity) to each cue. For assessing lateralization and sinuosity, we developed a C++ code for the automatic analysis of digitally recorded tadpole tracks. Hatching time seemed not to be affected by the potential risk of predation, while both predator species and diet affected tadpoles' defensive behaviour. Tadpoles responded to a predator threat by two main defensive strategies: freezing and 'zig-zagging'. While the first behaviour had previously been reported, the analysis of individual trajectories indicated that tadpoles can also increase path complexity, probably to prevent predators from anticipating their location. We also recorded a decrease in lateralization intensity, which suggests that under predation risk, tadpoles tend to scrutinize the surrounding environment equally on both sides.

Animal Behaviour, Volume 180, https://doi.org/10.1016/j.anbehav.2021.08.009

EFFECTS OF A GROUP-LIVING EXPERIENCE ON THE ANTIPREDATOR RESPONSES OF INDIVIDUAL TADPOLES

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Abstract

The tendency to aggregate during the larval stage is widespread and highly variable among anuran species. Several studies have highlighted the link between tadpole group density and their activity level, confirming that, usually, living in groups brings several antipredator benefits. However, nearly all studies have focused on the average behavioural responses of tadpoles tested in groups. In this study, we explored the effects of living in groups of three different sizes (1, 5 and 25 individuals per group) on the antipredator behaviour of individual green toad, *Bufotes balearicus*, tadpoles. We first assessed their basal activity and then examined changes in mobility rate and total distance after exposure to the chemical cues of predatory dragonfly, *Aeshna cyanea*, larvae. For both the pre- and post-stimulus activity levels, we also tested the effects of the presence of conspecifics' chemical cues in the experimental tub. Our results showed that (1) a previous brief (8 days) experience of group living is sufficient to affect the basal level of activity of individual tadpoles, which increased with group size; (2) tadpoles that were reared alone did not lower their activity further when exposed to predators' odour; (3) the antipredator response of high-density-reared tadpoles decreased in the presence of conspecifics' cues, supporting the so-called dilution effect, which, anyway, may need a minimum group size to be apparent. We conclude that both previous group-rearing experience and current perception of the surrounding environment may affect antipredator behaviour in individual tadpoles.

Environmental Pollution, Volume 303, https://doi.org/10.1016/j.envpol.2022.119137

DIFFERENTIAL EFFECTS OF MICROPLASTIC EXPOSURE ON ANURAN TADPOLES: A STILL UNDERRATED THREAT TO AMPHIBIAN CONSERVATION?

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Abstract

Microplastics (MPs) have been reported to threaten a wide variety of terrestrial, marine, and freshwater organisms. However, knowledge about the effects of MPs on anuran amphibians, one of the most threatened taxa worldwide, is still limited. To assess the effects of MPs on the growth and survival of the Italian agile frog (*Rana latastei*) and green toad (*Bufotes balearicus*), we exposed tadpoles to three different concentrations (1, 7, and 50 mg L^{-1}) of an environmental relevant mixture of microplastics (HPDE, PVC, PS and PES), recording data on their activity level, weight, and mortality rates. While the effects of MPs on green toad tadpoles were negligible, Italian agile frog tadpoles were severely affected both in terms of growth and activity level, with high mortality rates even at the lowest MP density (1 mg L^{-1}). Our results suggest that MP contamination of freshwater habitats may contribute to the ongoing decline of anuran amphibians.

Environmental Science and Pollution Research, https://doi.org/10.1007/s11356-022-23131-4

ANTI-PREDATOR BEHAVIORAL RESPONSES OF ITALIAN AGILE FROG TADPOLES (*RANA LATASTEI*) EXPOSED TO MICROPLASTICS

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Abstract

Microplastics (MPs) are nowadays abundant, persistent, and ubiquitous in the environment, representing a new threat for terrestrial, marine, and freshwater ecosystems. Although anuran populations and species are globally declining, the effect of MP exposure on this taxon has been poorly investigated. With the aim of assessing the effects of microplastic exposure on the defensive responses of Italian agile frog (*Rana latastei*) tadpoles, we exposed them to three different concentrations (1, 7, and 50 mg L^{-1}) of a mixture of plastic polymers (HPDE, PVC, PS, and PES) for 2 weeks. Then, we measured the total distance covered by individual tadpoles before and after exposure to tadpole-fed dragonfly larvae (*Aeshna cyanea*) cues. As expected, predation risk sharply lowered the total distance travelled by tadpoles; however, MP concentration did not affect their defensive performances. We also collected data on tadpole development, activity, and mortality. In contrast with previous experiments, neither tadpole growth nor mortality varied with MP concentration. Our results indicate that the intensity of MP effects on growth and development may depend on tadpole size, with large tadpoles being less susceptible to the negative effects of MP exposure.

2.

STRONG BEHAVIOURAL DEFENSIVE RESPONSES OF ENDEMIC *RANA LATASTEI* TADPOLES INDUCED BY A NATIVE PREDATOR'S ODOUR

2.1 Introduction

In predator–prey interactions, selection is often asymmetric, being stronger on prey than predators. According to the life-dinner principle (Dawkins & Krebs, 1979), whenever an attack fails, generalist predators may still be able to switch on alternative prey, while the consequences for a prey missing the prompt detection of a predator (injuries or death) are evidently more severe for its fitness. As a consequence, prey evolve defensive responses more rapidly than predators generate compensatory abilities (Brodie & Brodie, 1999).

Amphibian larvae have been largely used as model system for investigating predator–prey relationships (e.g., Moore, Van Buskirk, 2001; Griffiths *et al*., 2004; Gazzola *et al*., 2017). In aquatic habitats, information on predator identity, abundance and dangerousness is mainly propagated by water-borne chemical signals, either predator-specific cues or prey alarm cues (chemicals released by injured individuals and digestion-released cues; Van Buskirk & Arioli, 2002; Hettyey *et al*., 2015). Anuran tadpoles may innately respond to conspecific alarm cues and are able to associate them to predator cues during their lifetime (Brown, 2003; Ferrari *et al*., 2010). While prey-borne cues can induce defensive responses towards a broad range of threats, including novel predators (Sih *et al*., 2010), the calibration of innate strong predator-specific behavioural responses implies the exposure of prey species to a predator archetype over evolutionary time (Cox & Lima, 2006).

Several studies agree that tadpoles' strongest responses are triggered by the synergistic effects of both predator-borne cues and prey alarm cues. This is the case of *Rana* species, such as leopard frog *R. pipiens* (Schoeppner & Relyea, 2009), common frog *R. temporaria* (Marquis *et al*., 2004; Hettyey *et al*., 2015) and agile frog *R. dalmatina* (Gazzola *et al*., 2018), which show full defensive responses towards native predators fed with conspecific tadpoles. The leopard frog occurs from Hudson Bay to New Mexico in North America (Conant & Collins, 1998), while common and agile frogs are both widespread throughout most of Europe (Sindaco *et al*., 2006). Through such wide distribution ranges, these species can be expected to face a large variety of predators and environmental conditions. Broad-spectrum chemical signals, such as conspecific-borne alarm cues, may offer selective advantages by inducing defensive responses towards any tadpole-feeding predator and allowing

individuals to recognize the odour of potential predators through learning (i.e., the association between alarm cues and predator-borne cues).

Few studies have investigated how endemicity may influence species interactions; endemic plant species are functionally different from non-endemic congeners and invest more in anti-predator defence (Gorman *et al*., 2014). Several studies have investigated the response of native anuran larvae to alien predators, showing a general lack of behavioural response to predation-threat (e.g., Nunes *et al*., 2013; Gazzola *et al*., 2018), while, to our knowledge, there is a lack of studies focusing on the defensive behaviour of endemic species towards native predators. Available information suggests that species confined to a relatively small area might show strong innate responses to local predators and rely to a lesser extent on conspecific-released alarm cues (e.g., *Rana pirica*, Kishida & Nishimura, 2005; *Pelodytes ibericus*, Nunes *et al*., 2013). Accordingly, Griffiths, *et al*. (1998) recorded a strong inherited response of endemic midwife toad of Mallorca, *Alytes muletensis* towards the chemical cues of a native predator, Mallorcan *Natrix maura*.

The Italian agile frog *R. latastei* is a monotypical, endemic species which occurs in residual lowland hygrophilous woods of the Po-Venetian plain (northern Italy) and, locally, in some neighbouring countries (Canton Ticino, Istria, Slovenia and Croatia; Barbieri & Mazzotti, 2006). This species is threatened by the destruction of residual lowland woods due to increasing urbanization and intensive agriculture. Predation by non-native fish and crayfish is a further cause for concern and may have caused the extinction of the Italian agile frog from part of its range (Ficetola *et al*., 2012). The species is included in Annexes II and IV of the Habitat Directive (EC 43/1992) and filed as Vulnerable in the IUCN Red List. The action plan for the conservation of the Italian agile frog in Europe asks, among others, for scientific research investigating its ecology (Edgar & Bird, 2006).

Although the role of interference competition with syntopic *R. dalmatina* has been extensively investigated (Hettyey & Pearman, 2003; Ficetola & De Bernardi, 2005; Sacchi *et al*., 2015), predator–prey relationships have been poorly studied. Egg clutches and larvae of R. latastei are more likely to occur in ponds with high predation risk (Indermaur *et al*., 2010), and Italian agile frogs are associated with wetlands invaded by red swamp crayfish (*Procambarus clarkii*) (Ficetola *et al*., 2011).

In this study, we investigated behavioural, anti-predatory responses of endemic Italian agile frog tadpoles with the aim of comparing those triggered by exposure to both a native predator's odour (late instar larvae of emperor dragonfly *A. imperator*) and alarm cues (produced by either conspecific or heterospecific tadpoles) to the responses triggered only by the predator's odour. As reported for other Rana species, we expected Italian agile frog tadpoles to exhibit the weakest response towards fasted

dragonfly larvae and to record a conspecific-to-heterospecific gradient in the strength of behavioural responses. These were recorded using a software (ToxTrac) for image-based tracking, which allows the simultaneous and accurate recording of several locomotor parameters.

2.2 Materials & Methods

2.2.1 Animal collection and husbandry

Female Italian agile frogs lay their eggs in single clutches (one per year), which are usually deposited within 50 cm from the water surface of lentic waters in wooded areas (Barbieri & Mazzotti, 2006). This species is considered an explosive breeder, with most ovipositions occurring within 2–15 days between February and April. Eggs hatch 10–15 days after deposition, and tadpoles complete their metamorphosis in about 3 months (Sindaco *et al*., 2006).

In March 2019, we collected five freshly laid Italian agile frog clutches from each of three distinct populations: Sorgenti della Muzzetta (MZ: 45°27′N, 9°22′E), Bosco Castagnolo (BC: 45°15′N, 8°58′E) and Bosco Negri (BN: 45°10′N, 9°8′E), all included in Lombardy region (N Italy). MZ pond is located a few kilometres east of Milan, in a small protected natural area surrounded by agricultural land. Water depth was <1 m, with high turbidity and aquatic vegetation cover. BC humid area is located in the riparian forest of the river Ticino, included in a large, protected area. It is an elongated elliptical pond fed by groundwater, with shallow water (80–100 cm) and low aquatic vegetation cover (<10%). BN pond is located in a natural protected area close to the city of Pavia, consisting of a residual wetland forest intermingled with crops and rural and suburban areas. It is a small circular pond with water depth >1 m and rich in submerged branches and leaves.

Egg masses were immediately brought to the laboratory and individually kept in 11 L tanks filled with well water. Upon hatching, tadpoles were transferred into 50-L tanks (one per clutch), kept in an unheated room, under natural light conditions, and fed ad libitum with rabbit chow. Mean water temperatures ranged between 15°C and 18°C throughout the study period and water was partially (50% ca.) changed every 2 days.

Late instar *A. imperator* larvae were collected from an artificial pond in the Botanical Garden of Pavia (45°11′N, 9°10′E), by using dip nets. Predators were individually kept in plastic tubs filled with 0.5 L of aged tap water. A small leaf was provided as perching site for each larva. When tadpoles reached Gosner's developmental stage 27–29 (Gosner, 1960), we performed the experiment and recorded tadpole behavioural responses to a suite of predatory cues.

Using dip nets, we collected agile frog (*R. dalmatina*) and common toad (*Bufo bufo*) tadpoles from two different locations in the province of Varese (45°46′N, 8°38′E and 45°45′N, 8°39′E, respectively). They were sorted according to size, transferred into 50-L tanks and kept in the same conditions as Italian agile frog tadpoles.

2.2.2 Preparation of odour cues

The experiment included five odour treatments: (a) aged tap water (as control); (b) fasted dragonfly kairomones; (c) Italian agile frog tadpole-fed dragonfly; (d) agile frog tadpole-fed dragonfly; (f) common toad tadpole-fed dragonfly.

To obtain the odour stimuli, five predators were assigned randomly to each of four diet treatments (overall 20 dragonfly larvae). Three groups were fed for two consecutive days, at 9:00 a.m., with, respectively, Italian agile frog tadpoles, common toad tadpoles or agile frog tadpoles; the fourth group received no food (fasted predators). Each predator was provided, as far as possible, with a similar prey weight (usually two tadpoles). Average tadpole weights per treatment (day 1–day 2) were as follows: Italian agile frog = 130–140 mg; common toad = 134–138 mg; agile frog = 136–140 mg.

Prey was always consumed within a few minutes after provision and, 45 min after feeding, an aliquot of water (50 ml) was collected from each of the 20 predator tubs. Aliquots were sorted by treatment and stored in separated plastic containers. For each experimental arena, 2 ml of the resulting mixture was used as odour stimulus during behavioural experiments. Every time, predator tubs were refilled to keep the water volume constant. Water used for all treatments was collected from two 150 L tanks filled with 80 L each of aged tap water. The same water was used for experimental arenas.

2.2.3 Experimental procedure

To assess the level of activity before and after the infusion of predatory cues, tadpoles were individually put into opaque plastic tubs (15 × 10.5 cm) filled with 250 ml of aged tap water and left to acclimatize for 15 min. The trials consisted of a 5 min pre-stimulus (before infusion), ca 1 min required for the injection and a 5 min post-stimulus (after infusion) recording periods (overall, each session lasted ca. 26 min) (Figure 2.1). The odour stimulus was slowly injected with a 5-ml disposable syringe, in order to minimize disturbance. The concentration of the odorous stimulus during the trials (1:125) was consistent with previous studies (e.g., Gomez-Mestre & Diaz-Paniagua, 2011; Gazzola *et al*., 2018). Tadpoles were exposed to cues 1–4 hr after their preparation, while cues have been

observed to still trigger strong behavioural responses after 36–48 hr of ageing in well water (Peacor, 2006; Van Buskirk *et al*., 2014); thus, we were confident in their effectiveness. All trials were performed indoor, and video recorded by a digital camera (Canon Legria) hanged up 1 m above the arena, which was shielded by opaque panels and uniformly lightened by spotlights.

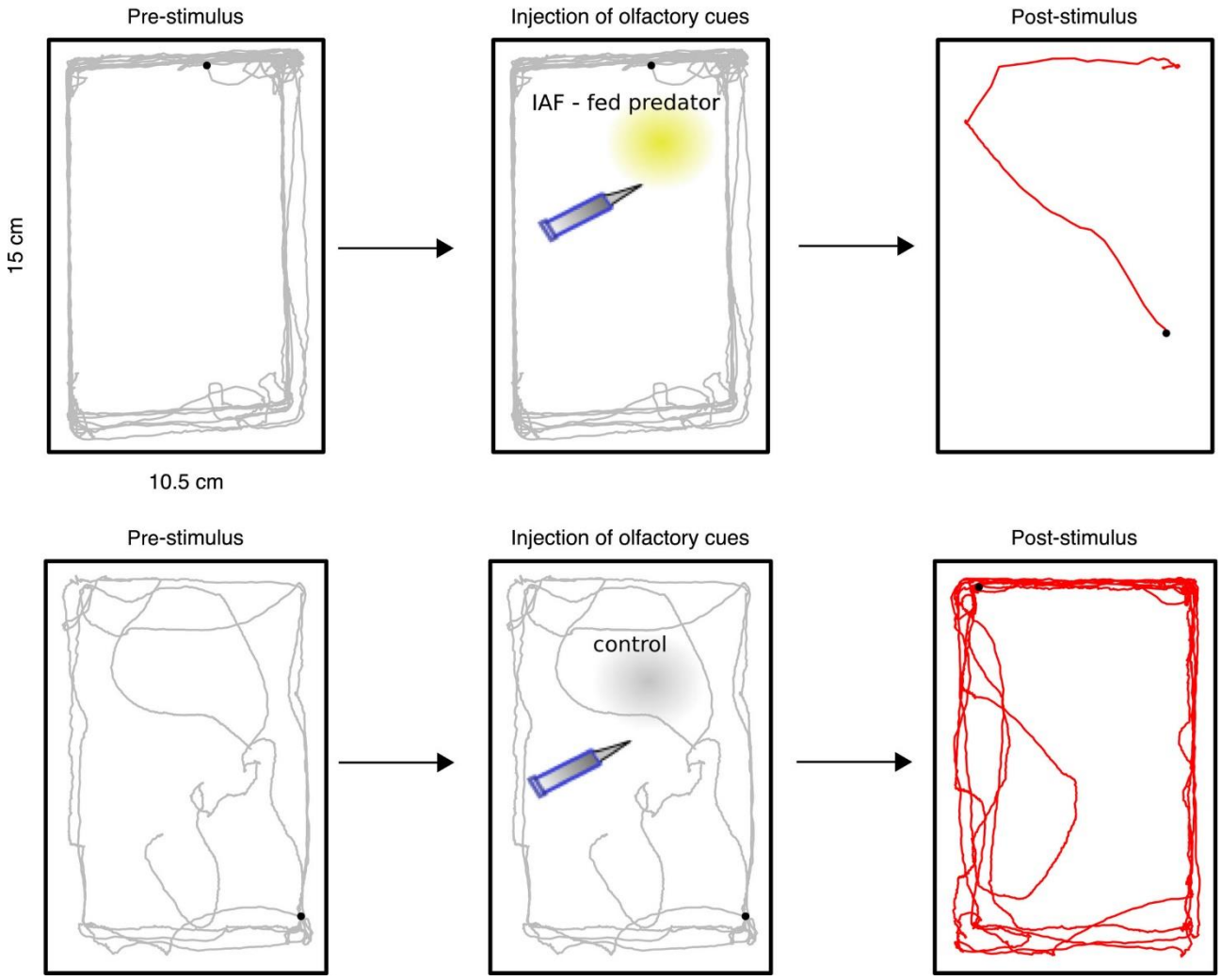

Figure 2.1. Trajectory of a single tadpole from control (below, grey patch) and Italian agile frog fed predator (IAF, yellow patch above) groups. Plots represent the schedule used in the experiment: 5 min of pre-stimulus video recording, injection of olfactory cues and 5 min post-stimulus video recording. Trajectories were obtained using R package trajr (McLean & Volponi, 2018).

We tested 70 tadpoles for each population, 14 for each of the five signals within the same population, for a total of 210 tested individuals. Each tadpole was tested only once. All trials were randomly (with respect to treatment and population) performed in the span of 2 days, between 10 a.m. and 4 p.m. At

the end of the experiment, all video clips were analysed by a source executable software for image-based tracking (ToxTrac; Rodriguez *et al*., 2018), which provides locomotor information (by recording the x and y coordinates of the central point of each tadpole every 0.04 s).

2.2.4 Statistical analysis

In order to assess the behavioural responses of tadpoles exposed to predatory cues, we tested two locomotor variables, namely the mobility rate, defined as the rate of instant speed above a threshold of 1 mm/s, and the total distance, that is the total swimming distance (in mm) covered by each tadpole during the trial (pre- and post-stimulus intervals).

To test for variation in tadpole level of activity in relation to odour type and population of origin, we applied linear mixed models (LMMs), including either mobility rate or total distance as response variables. First, we explored inter-population variation in the pre-stimulus mobility rate, including population as fixed factor and clutch within population as random effect. A second model was run to investigate the effect of predatory cues on tadpole defensive behaviour; the model included post-stimulus mobility rate as response variable, pre-stimulus mobility rate as covariate (to control for individual variation in the activity level), the interaction cue × population ×pre-stimulus mobility rate as main factor and clutch within population as random effect; all two-levels interactions were also included. The same models were run using total distance as response variable.

Planned comparisons between either controls or fasted dragonfly-exposed tadpoles and all other treatments were obtained from the model using emmeans R package (Lenth, 2019). To check the appropriateness of experimental procedures, the pre- and post-stimulus levels of activity of control groups, as expressed by each of the two variables, were compared by Wilcoxon rank sum test with continuity correction.

Finally, to compare variances in behavioural responses, that is the consistency among tadpole responses, we used Fligner–Killeen test of homogeneity of variances, which is not sensitive to departures from normality; the test was conducted for both behavioural variables by comparing the responses of each population before and after cue injection for all treatments.

2.3 Results

Mobility rate and total distance before the injection of cues did not vary among populations ($F = 0.284$, $df = 2$, $p = .76$ and $F = 0.546$, $df = 2$, $p = .59$, respectively; Figure 2.2).

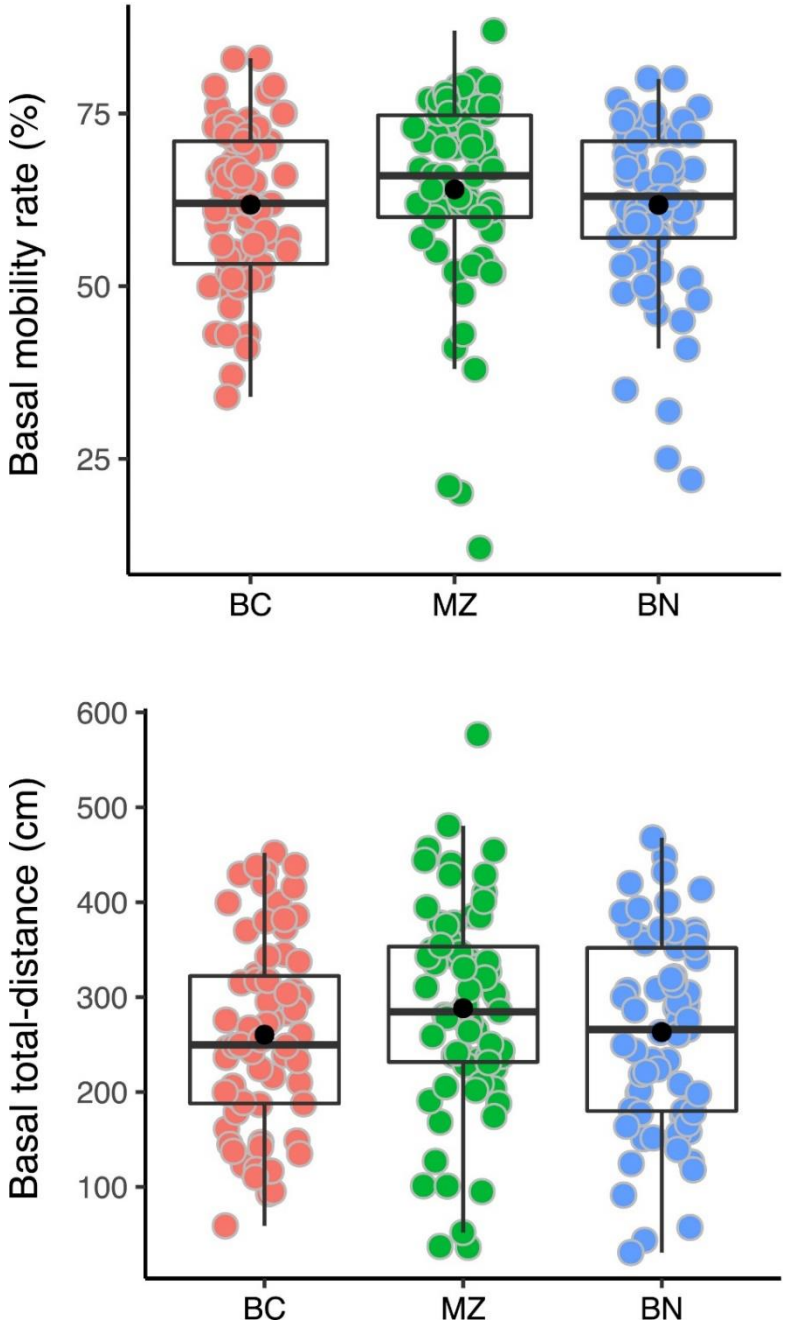

Figure 2.2. Tadpole mobility rate and total distance before (pre-) chemical cue injection for all populations (n = 70 for BC and MZ; n = 69 for BN). Values reported are means (filled black dots) and medians (horizontal black lines inside boxplots; n = 209)

Tadpoles from control groups did not modify their level of activity after water injection, whether it was assessed using mobility rate (W = 1,049, p = .136) or total distance (W = 959, p = .493; n = 42). For both behavioural responses, LMMs showed a main significant effect of treatment, the respective pre-stimulus behavioural responses and their interaction (all p < .001: see Table 2.1 and Figures 2.3, 2.4). No significant three-way interaction was recorded (Table 2.1). The analysis of total distance showed a significant interaction of population × treatment (Table 2.1; Figure 2.4).

Variable	***Mobility rate***		***Total distance***		
	F	*P*	*F*	*P*	*d.f.*
Treatment	74.01	**<0.001**	90.73	**<0.001**	4
Population	0.09	0.90	0.83	0.46	2
Pre-response	24.40	**<0.001**	58.31	**<0.001**	1
Population × Treatment	1.62	0.12	2.76	**0.006**	8
Pre-response × Population	1.59	0.20	0.44	0.64	2
Pre-response × Treatment	8.42	**<0.001**	24.28	**<0.001**	4
Pre-response × Population × Treatment	1.07	0.38	1.31	0.24	8

Table 2.1. Linear mixed models of mobility rate and total distance in relation to experimental treatment, population, and pre-injection behavioural response (pre-response in the table, both for mobility rate and total distance recorded before the infusion of chemical cues). Numbers in bold indicate significant results (p value < .05).

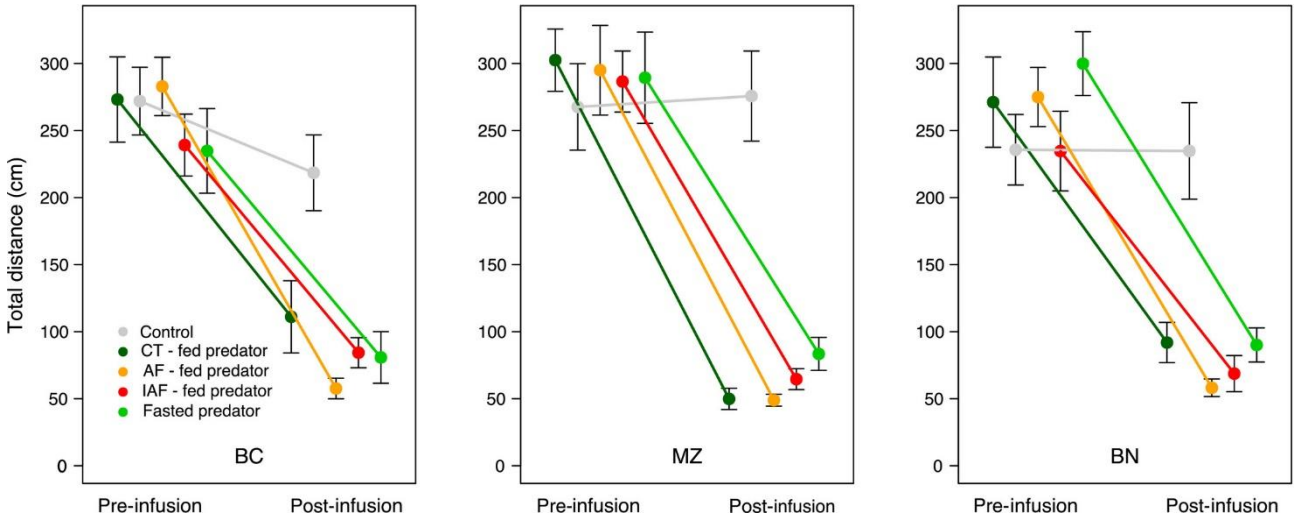

Figure 2.3. Tadpole mobility rate before (pre) and after (post) chemical cue injection for all treatments and all populations. Values reported are means and standard errors (n = 209; CT, common toad; AF, agile frog; IAF, Italian agile frog).

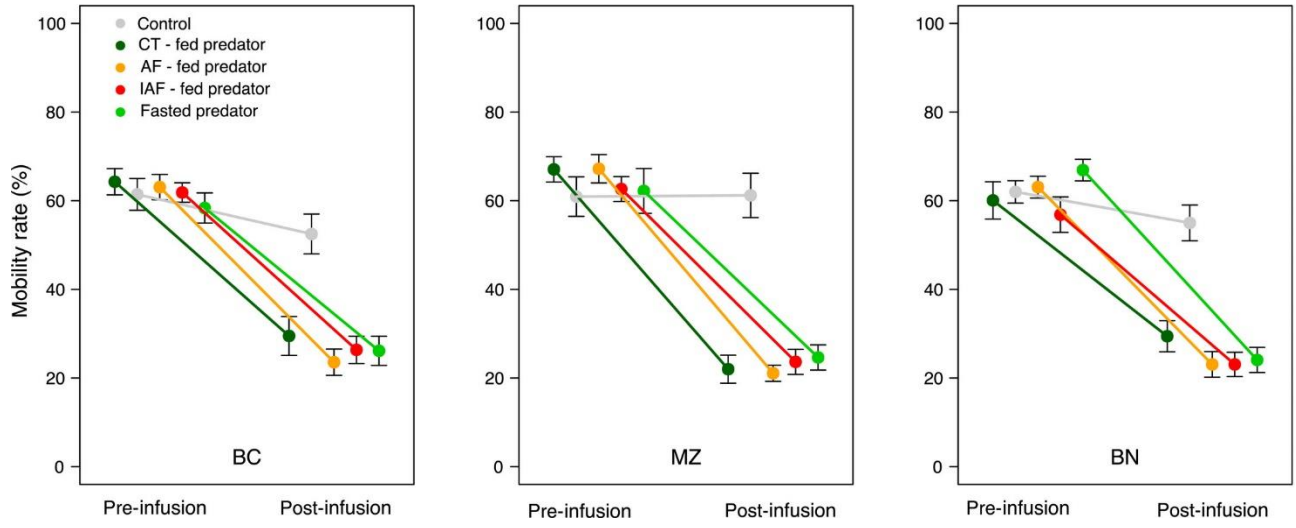

Figure 2.4. Tadpole total distance covered before (pre) and after (post) chemical cue injection for all treatments and all populations. Values reported are means and standard errors (n = 209; CT, common toad; AF, agile frog; IAF, Italian agile frog).

Respect to controls, all treated tadpoles responded by strongly decreasing both mobility rate and total distance, with a seemingly negligible effect of predator diet on the intensity of the behavioural responses (Figures 2.3, 2.4; Tables 2.2, 2.3). Behavioural responses of tadpoles exposed to fasted dragonfly larvae did not significantly differ from those of tadpoles exposed to fed predators (Tables 2.2, 2.3).

Contrasts (difference)	**BC**			**MZ**			**BN**		
	Est.	***t.ratio***	***P***	*Est.*	***t.ratio***	***P***	*Est.*	***t.ratio***	***P***
CT - control	-24.25	-5.84	**<0.001**	-33.28	-6.96	**<0.001**	-22.86	-4.79	**<0.001**
AF - control	-29.63	-6.64	**<0.001**	-34.17	-7.13	**<0.001**	-29.82	-6.16	**<0.001**
IAF - control	-26.43	-5.94	**<0.001**	-29.41	-6.18	**<0.001**	-28.87	-6.00	**<0.001**
Fasted p - control	-25.86	-5.78	**<0.001**	-28.08	-5.90	**<0.001**	-29.66	-6.17	**<0.001**
Fasted p - CT	3.06	0.71	0.47	2.51	0.58	0.560	-3.98	-0.90	0.37
Fasted p - AF	2.90	0.68	0.49	5.29	1.23	0.218	5.29	1.23	0.22
Fasted p - IAF	3.46	0.72	0.47	1.28	0.31	0.755	1.28	0.31	0.75

Table 2.2. Comparison of mobility rate between control and fasted dragonfly cues and all other treatments, respectively, for each population. Estimates have been extracted from LMM using emmeans package (see methods; CT, common toad; AF, agile frog; IAF, Italian agile frog; Fasted p, fasted predator). Numbers in bold indicate significant results (p value < .05).

Contrasts (difference)	BC			MZ			BN		
	Est.	***t.ratio***	***P***	*Est.*	***t.ratio***	***P***	*Est.*	***t.ratio***	***P***
CT - control	-107.6	-5.38	**<0.001**	-225.32	-10.88	**<0.001**	-184.00	-8.90	**<0.001**
AF - control	-159.7	-7.93	**<0.001**	-229.95	-11.38	**<0.001**	-217.60	-10.33	**<0.001**
IAF - control	-128.7	-6.20	**<0.001**	-215.73	-10.69	**<0.001**	-204.08	-9.63	**<0.001**
Fasted p - control	-128.6	-6.25	**<0.001**	-198.65	-9.87	**<0.001**	-182.94	-8.56	**<0.001**
Fasted p - CT	-21.00	-1.02	0.31	26.67	1.27	0.20	1.06	0.05	0.96
Fasted p - AF	31.12	1.51	0.13	31.30	1.54	0.12	34.66	1.65	0.10
Fasted p - IAF	0.06	0.00	0.99	17.08	0.84	0.40	21.14	0.99	0.32

Table 2.3. Comparison of total distance between control and fasted dragonfly cues and all other treatments, respectively, for each population. Estimates have been extracted from LMM using emmeans package (see methods; CT, common toad; AF, agile frog; IAF, Italian agile frog; Fasted p, fasted predator). Numbers in bold indicate significant results (p value < .05).

For mobility rate, variances before and after cue injection differed neither in control groups nor treatments ($p > .08$ for all Fligner–Killeen tests). For total distance, variances were very similar for control groups, while decreased significantly for most predatory cues (Table 2.4).

	BC		**MZ**		**BN**	
Treatment	χ^2	**P**	χ^2	**P**	χ^2	**P**
Control	0.24	0.62	0.09	0.76	1.20	0.27
IAF - fed predator	8.96	**0.002**	3.92	**0.047**	4.32	**0.037**
AF - fed predator	2.86	0.09	15.16	**<0.001**	5.97	**0.014**
CT - fed predator	1.23	0.26	9.23	**0.002**	8.10	**0.004**
Fasted predator	4.32	**0.037**	2.50	0.11	9.98	**0.001**

Table 2.4. Fligner–Killeen test of homogeneity of variances for the variable total distance, comparing the responses of each population before and after cue injection (CT, common toad; AF, agile frog; IAF, Italian agile frog). Numbers in bold indicate significant results (p value < .05).

2.4 Discussion

Tadpoles responded to dragonfly cues by strongly reducing their activity, both in terms of the amount of time they spent active and path length covered in comparison to control groups. The response was consistent among populations, suggesting that Italian agile frog tadpoles may be highly sensitive to dragonfly cues.

Contrary to previous studies (e.g., Laurila *et al*., 1997; Nunes *et al*., 2013), predators' diet had a negligible effect on tadpole response and our experiment did not bring any evidence of the phylogenetic-relatedness hypothesis, which predicts stronger responses when closely related species are preyed upon (Chivers & Mirza, 2001; Schoeppner & Relyea, 2005). As we did not test alarm cues separately, we cannot infer any conclusion on tadpole sensitivity to prey-borne signals, which means that the predator's odour may have completely overcome all the other stimuli. Nonetheless, this eventuality would not undermine the effectiveness of dragonfly larvae's odour as an indicator of predation risk in this Rana species.

Although fasted predators have been reported to induce defensive responses in prey other than anurans, for example freshwater snails (McCarthy & Fisher, 2000; Dalesman *et al*., 2006), mussels (Shin *et al*., 2009) and crayfish (Hazlett & Schoolmaster, 1998), most species generally show no or weak responses with respect to those elicited by conspecific-fed predators (reviewed by Scherer & Smee, 2016). The general lack of strong responses by tadpoles to starved predators has led to hypothesize that predators emit detectable infochemicals only while feeding (Schoeppner & Relyea, 2009).

Notwithstanding, larval ringed salamanders (*Ambystoma annulatum*) show innate defensive responses (Mathis *et al*., 2003) and, more recently, fasted-predator odours have been reported to trigger defensive responses, although weaker than those induced by conspecific-fed predators, in predator-naïve tadpoles of *Hyla meridionalis*, B. bufo and *Pelodytes ibericus* (Nunes *et al*., 2013), suggesting that, at least in some anuran species, native predator odours may be sufficient to induce behavioural responses.

The ability of recognizing predator-specific cues and trigger appropriate defences is clearly adaptive. In the case of a very common tadpole predator such as A. imperator, its prompt identification, whatever prey it previously consumed, can be expected to be favoured by natural selection in environments with high predation risk. The sharp response shown by Italian agile frog tadpoles may thus depend on a long coevolutionary history in a relatively restricted range where emperor (or *Anax spp.*) dragonfly larvae were one of their main predators. Alternatively, we cannot exclude that

tadpoles learnt to recognize the predator prior to hatching (see Ferrari & Chivers, 2009 about *R. sylvatica* as prey and *A. tigrinum* as predator), although embryos of sympatric *R. dalmatina* do not seem to respond to dragonfly odour by changing their time of hatching (Gazzola *et al*., 2018).

The innate or early-in-development recognition of dragonfly larvae may increase tadpole survival with relatively low costs if they could easily escape attacks (Sih *et al*., 2010). Although this hypothesis needs to be tested, the counter-intuitive selection for breeding sites with high predation risk recorded by Indermaur *et al*. (2010) may suggest that Italian agile frogs have evolved efficient mechanisms to face predation risk.

Supporting the effectiveness of dragonfly odour, the total distance travelled by each tadpole in the pre-injection period showed a greater variance than that covered after exposure to the predator's odour. This result suggests that environments perceived as “safe” or “inscrutable” (DeWitt & Scheiner, 2004) allow the exhibition of a whole set of behaviours ranging from the meticulous exploration of the water volume to short swims in search of shelter, while predator threat, constraining the range of behaviours which are adaptive (i.e., enhance survival in a risky environment), may result in the smoothing of individual behavioural differences. Conversely, tadpole mobility rate did not show as much variation in the pre- and post-stimulus variances, pointing out that individual differences were mainly related to swimming speed and the choice of movement variables to measure is pivotal to answering questions such as the extent to which behaviour can change as a function of external stimuli.

Video-tracking techniques, although used in a variety of behavioural studies, have seldom been applied to amphibians and mainly to assess the effects of chemicals on tadpole behaviour (Denoël *et al*., 2010, 2013; Junges *et al*., 2017). In our study, ToxTrac allowed to automatically record a plethora of quantitative data for each individual and calculate movement variables with a level of precision that traditional methods cannot allow. Moreover, by recording individually exposed tadpoles, we could remove confounding effects, such as the so-called “domino” effect (the “burst” of an individual which causes a chain of sudden movements in the other group members; Cresswell *et al*., 2000), and “dilution” effect (group size-dependent reduction in the predation risk perceived by each individual; Lehtonen & Jaatinen, 2016), thus achieving a greater accuracy in the assessment of tadpole responses.

2.5 Conclusion

To our knowledge, these are the first behavioural data recorded for *R. latastei* larvae. By avoiding unnecessary responses, anuran tadpoles which rely on specific cues lower the costs of defensive behaviour but may increase the risk of not recognizing novel potential threats (Sih *et al*., 2010). As alien species are a major cause of amphibian decline worldwide (Kats & Ferrer, 2003), we argue that further behavioural studies are urgently needed to assess the risk posed to this endemic species by the spread in northern Italy of invasive predators such as the red swamp crayfish and spiny-cheek crayfish (*Orconectes limosus*).

3.

CONTEXTUAL BEHAVIOURAL PLASTICITY IN ITALIAN AGILE FROG (*RANA LATASTEI*) TADPOLES EXPOSED TO NATIVE AND ALIEN PREDATOR CUES

3.1 Introduction

Behavioural plasticity – the ability to detect and respond to environmental signals – is a necessary requirement for survival and reproduction in all organisms, and in the last three decades it has come to include virtually all behavioural traits that may show some kind of variation in response to environmental conditions (West-Eberhard, 1989; DeWitt & Scheiner, 2004). 'Contextual plasticity', as distinct from 'developmental plasticity', indicates an individual's response to a stimulus to which it has been exposed immediately before (Stamps, 2016).

A change in behaviour is the promptest reaction that animals can adopt to respond to external changes and can potentially improve individual fitness and enhance a species' long-term survival by preventing drastic population declines (Wong & Candolin, 2015). However, irrespective of its contribution to fitness, plasticity stands as an important benchmark to explore how organisms respond to environmental changes at the individual, population, and community level (Stearns, 1989; Ghalambor *et al*., 2007).

Currently, human activity is the main agent of environmental change. Human-induced rapid environmental changes (HIREC; Sih *et al*., 2011) are considered the greatest threat to biodiversity (Tilman *et al*., 1994; Pimm & Raven, 2000), forcing species to face conditions never encountered previously (Wong & Candolin, 2015; Sih *et al*., 2016). Behavioural plasticity can play a major role in allowing species to cope with anthropogenic environmental changes: variation in each species' ability to express adaptive behaviours may explain why some species survive or even benefit from the new conditions while others decline, sometimes irreversibly (Sih *et al*., 2011; Tuomainen & Candolin, 2011; Van Buskirk, 2012).

The invasion of non-native species is a major contribution to HIREC, and can strongly affect the distribution, abundance, use of resources and habitats, reproduction, interspecific interactions, and evolution of many native species (Strauss *et al*., 2006). Currently, freshwater ecosystems are amongst the most invaded ecosystems and are particularly vulnerable to introduced predators, which are considered to be one of the most important causes of biodiversity loss around the globe (Vitousek *et al*., 1997; Cox & Lima, 2006). Introduced predators can drive amphibian populations to extinction

(Bradford *et al*., 1994; Gamradt & Kats, 1996; Matthews *et al*., 2001), amphibian eggs and larvae being particularly vulnerable to alien aquatic predators (Kats & Ferrer, 2003). Invasive predators are the major cause of the decline of rare or endemic prey species (Dick & Platvoet, 2000; Fukasawa *et al*., 2013), and predicting the impact of non-native predators is essential for their conservation, especially in human-altered environments (Hobbs & Huenneke, 1992; Chytrý *et al*., 2012).

In the intensively cultivated and urbanized lowlands of northern Italy, the red swamp crayfish (*Procambarus clarkii*) is by far the most widespread of four alien crayfish species (Morpurgo *et al*., 2010). Since the early 1990s, it has probably been introduced several times in ponds and streams (Gherardi, 2006), from which it has rapidly spread out over the extensive network of canals that crosses the whole lower catchment of the River Po (Gherardi *et al*., 1999; Fea *et al*., 2006).

This highly invasive (Gherardi, 2006; Aquiloni *et al*., 2008), and voracious predator preys on a wide range of freshwater species, including anuran larvae (Gherardi *et al*., 2001; Cruz *et al*., 2006; Ficetola *et al*., 2011), and can have dramatic impacts on freshwater fauna and alter aquatic ecosystems through trophic cascades (Souty-Grosset *et al*., 2016). Its introduction has been related to the local decline or extinction of several amphibian species (Cruz *et al*., 2008), including the Italian agile frog (*Rana latastei*), an endemic threatened species of northern Italy (Ficetola *et al*., 2012).

The Italian agile frog is a monotypical, small brown frog which occurs in the floodplains of northern Italy and small areas of the adjacent Swiss canton Ticino (Grossenbacher, 1997), western Slovenia and north-western Croatia (Burlin & Dolce, 1986; Barbieri & Mazzotti, 2006). Rana latastei is threatened by multiple factors, including loss of habitat due to agricultural intensification, increased isolation of populations and loss of genetic diversity (Ficetola & De Bernardi, 2004; Pearman & Garner, 2005; Ficetola *et al*., 2007; Canova & Balestrieri, 2018). It is considered globally vulnerable by the IUCN (http://www.iucn.it/scheda.php?id=-1527036578), and the Action Plan for its conservation in Europe includes the eradication of alien crayfish as an urgent priority action (Edgar & Bird, 2006).

In the current study, we tested the defensive behaviour of Italian agile frog tadpoles. Firstly, we investigated whether the presence of water-borne kairomones of a native predator (dragonfly larvae) alters the timing of hatching. The ability of anurans to modify the timing of hatching may represent an effective way to cope with the upcoming risk of falling prey both before and after hatching (Warkentin, 2011). For example, the presence of predators preying on hatchlings may lengthen the permanence time inside the protective jelly. Alternatively, the occurrence of predators that prefer feeding on eggs may induce an early exit from the egg mass (Warkentin, 2011). External conditions

set the stage for a potential trade-off; that is, tadpoles need to make the most advantageous choice, relying on available information in the surrounding environment (Warkentin, 1995; Ireland *et al*., 2007).

A few days after tadpole hatching, we performed a second experiment to investigate how tadpoles alter their behaviour when briefly exposed to water-borne cues coming from either a native or alien predator, and how these changes are affected by predator's diet, i.e., the chemical cues actively or passively released by injured or preyed conspecifics. With this aim, we explored tadpole activity level (time spent active, time frozen, total distance covered) when exposed to different types of predatory cues (contextual behavioural plasticity). We included a further behavioural analysis by investigating lateralization (tadpole preference for a rotational direction) and sinuosity (the tortuosity of an animal's path; Benhamou, 2004).

By assessing behavioural and life history responses from different breeding sites, we explored the effect of genotype by environment interactions on the expression of defensive behaviour in the presence of different sources of information (i.e., predator species and diet). Consistently with current knowledge, we expected the strongest defensive responses from tadpoles exposed to both conspecific alarm cues and native predator odour, and the weakest towards fasted, alien predator cues.

3.2 Materials & Methods

3.2.1 Sample collection

During March 2019, we collected 18 freshly laid Italian agile frog (*Rana latastei* Boulenger 1879) clutches from three different breeding sites (six clutches from each site) located in the Lombardy region (northern Italy). Permits were obtained from the Italian Ministry of Environment, Land and Sea (0006075–23/03/2018–PNM).

The first site, known as Sorgenti della Muzzetta (MZ: 45°27′N, 9°22′E), is a large pond located a few kilometres east of Milan, in a small protected natural area surrounded by agricultural land; maximum water depth was >1 m, with high turbidity and aquatic vegetation cover. Bosco Castagnolo (BC: 45°15′N, 8°58′E) is a humid area, consisting of several small ponds connected by narrow canals, included in the riparian forest of the protected valley of the river Ticino. The main waterbody is an elongated elliptical pond fed by groundwater, with shallow water (80–100 cm) and low aquatic vegetation cover (<10%). Bosco Negri (BN: 45°10′N, 9°8′E) is a natural protected area close to the city of Pavia, consisting of a residual wetland forest intermingled with crops and rural and suburban

areas. It includes some small ponds with a water depth <1 m and rich in submerged branches and leaves. In each site, egg clutches were collected along the banks of breeding ponds. During sampling, several juveniles, and adults of *P. clarkii* were recorded in BN, both in the sampling pond and in nearby canals, while only a few individuals were found in MZ, crayfish being more widespread in adjacent agricultural canals than in the pond where frogs reproduce. Finally, in BC, we never observed any crayfish (see also Gazzola *et al*., 2018), although *P. clarkii* has been reported to occur in the area since the start of the 21st century (Gherardi *et al*., 1999; Fea *et al*., 2006).

Egg masses were immediately transported into the laboratory and individually kept in 11 litre tanks filled with well water. A subsample of 10 eggs from each clutch was used to determine the Gosner level of development (mean ± s.d.: MZ 9.7±0.3, BN 7.4±0.5, BC 9.7±0.3; Gosner, 1960). After hatching (one BC clutch did not hatch), tadpoles were kept in 50 l tanks in an unheated room, under natural light conditions, and fed ad libitum with rabbit chow. Throughout the study period, mean water temperature ranged between 15 and 17°C. Water was partially changed (ca. 50%) every 2 days.

Using dip-nets, 10 adult red swamp crayfish and 10 late instar dragonfly larvae (*Anax imperator*) were collected from a small canal near Pavia and an artificial pond located inside the botanic garden of the city, respectively. All predators were transferred to the laboratory and kept individually in plastic tubs containing, respectively, 0.5 and 2.0 l of aged tap water.

3.2.2 Preparation of odour cues

To assess predation risk, amphibian larvae generally rely on water-borne chemical cues (Kats & Dill, 1998). The chemicals to which prey respond may be predator-specific odours, cues released by conspecifics or, more frequently, a combination of the two (Chivers & Smith, 1998; Fraker, 2009; Schoeppner & Relyea, 2009; Hettyey *et al*., 2010). Several studies have shown that fed predators commonly elicit stronger antipredator defences than starved predators (Stirling, 1995; Ślusarczyk, 1999; Petranka & Hayes, 1998; Van Buskirk & Arioli, 2002; Schoeppner & Relyea, 2005, 2009). As a predator may become chemically ‘labelled’ by its diet via learning processes, recognition of a novel predator can be facilitated by its association with conspecific cues (reviewed in Ferrari *et al*., 2010).

To obtain the odour stimuli of fed predators, dragonfly larvae and crayfish were fed every day at 13:30 h with Italian agile frog tadpoles (total mass 100–150 mg) from an early-laid clutch. Prey was always consumed within an hour and 100 ml of water were collected from each predator tub at 14:30 h. Aliquots from the same treatment were poured into the same container and 50 ml of the resulting

mixture was used as an odour stimulus. Each time, predator tubs were refilled to keep the water volume constant. The same procedure was used to obtain chemical cues from fasted predators.

For both experiments, the final concentration of the odour stimulus was consistent with previous studies (e.g., Gomez-Mestre & Díaz-Paniagua, 2011; Gazzola *et al*., 2015, 2018). Cues were collected the same day as the trials. As predator cues have been observed to still trigger strong behavioural responses after 36–48 h of ageing (Peacor, 2006; Van Buskirk *et al*., 2014), we were confident in their effectiveness in stimulating behavioural responses.

3.2.3 Effect of predator cues on hatching time

The first experiment was performed to assess the influence of the risk of predation by dragonfly larvae on the hatching time of frog embryos. From the day of collection, two subsamples (50 eggs each) were taken from each clutch and placed into separated plastic containers (30×20×20 cm), filled with 6 l of aged tap water (n=36, 18 for each treatment). Embryos were then randomly exposed to two different odour treatments, one for each egg clutch subsample: (i) 50 ml of well water (control group), (ii) 50 ml of tadpole-fed dragonfly cue. Treatments were provided daily until the first egg hatched. Time of hatching for each experimental container was defined as the Julian date when 50% of tadpoles were detached from the jelly and 5 cm away from the egg mass (Ireland *et al*., 2007; Gazzola *et al*., 2015), and was recorded, as precisely as possible, by checking all containers 3 times per day (07:00 h, 13:00 h, 21:00 h). Throughout the experiment, water temperature ranged between 10 and 15°C, and random daily measures of temperature from different containers differed ≤0.7°C.

3.2.4 Tadpole behavioural responses to predator cues

After hatching, when tadpoles had reached Gosner developmental stage 26–28, we recorded tadpole behavioural responses to a suite of different signals: fasted dragonfly cue, tadpole-fed dragonfly cue, fasted crayfish cue, tadpole-fed crayfish cue, control (tap water). Five predator specimens were used to obtain each type of predator chemical cue (for a total of 20 predators). To assess the activity of the larvae before and after cue infusion, 10 min individual trials were conducted, testing tadpoles that had not previously been exposed to predator cues (at least after clutch collection). Tadpoles were put into white, opaque, circular cups (12 cm in diameter) filled with 200 ml of aged tap water, and left to acclimate for 15 min. The trials consisted of a 5 min pre-stimulus recording period (i.e., before cue

infusion), and a 5 min post-stimulus recording period (after cue infusion). To minimize disturbance, the odour stimulus (2 ml) was injected slowly (ca. 30 s) by a 5 ml syringe on one side of the cup.

Tadpoles were video recorded over the whole trial by a Canon Legria digital video camera. Each tadpole was tested once (375 tadpoles in total, 125 per site, 25 for each cue–site combination). All video clips were analysed using a source executable software for image-based tracking (ToxTrac; Rodriguez *et al*., 2018), which tracks the position of the centre of the animal's detected shape.

3.2.5 Statistical analyses

Hatching time was explored by linear mixed models (LMMs), with the total time embryos took to hatch as the response variable. Models included embryonic treatment (presence or absence of predator odour) and site as fixed factors, and developmental stage at collection as a covariate; clutch identity, nested within the site of origin, was included as a random effect (intercepts varying among sites and among clutches within sites). The final model, obtained after AIC exploration, included treatment × stage at collection and treatment × site interactions.

Behavioural responses were based on three variables provided by the tracking software: mobility rate, time frozen and total distance. Mobility rate was calculated as the rate of instant speed (v) above a certain threshold (v>1 mm s−1). Total time frozen was calculated as the total time the animal remained still during the recording period, while total distance was measured as the total length of the trajectory covered by the animal during the trial.

As tadpole activity was observed both before (i.e., basal mobility rate) and after exposure to cues, we could assess individual behavioural reaction norms (Dingemanse *et al*., 2010; Stein & Bell, 2019) expressed by each tadpole group in response to each chemical stimulus. In addition, by analysing the trajectory of each individual, we assessed both sinuosity and lateralization in the pre- and post-stimulus phases. To this purpose, we developed a C++ code based on ROOT (Brun & Rademakers, 1997) for the automatic analysis of tadpole tracks recorded by the digital video camera (Fig. 3.1).

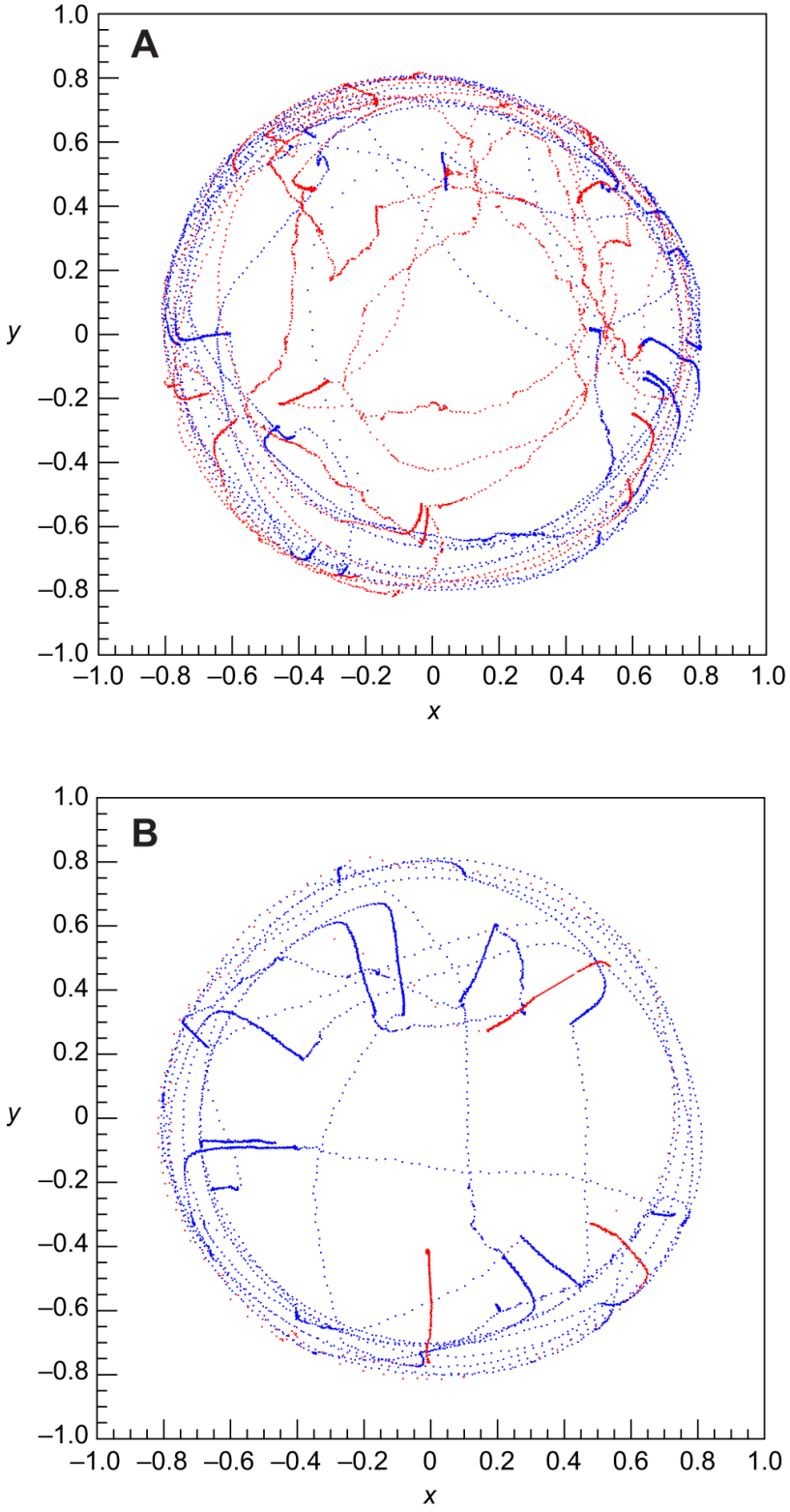

Figure 3.1. *Rana latastei* tadpole responses to control and fed odonate cues. (A) Response to control cue, showing similar pre-stimulus (blue) and post-stimulus (red) mobility. (B) Response to fed odonate cue, showing lower mobility in the post-stimulus phase.

As testing cups were placed in different positions with respect to the video camera, raw coordinates were first normalized. To this purpose, the centre (Xc, Yc) and maximum and minimum values of both X and Y were recorded for each cup, and coordinates were normalized to the interval [−1,1] by means of the following transformations:

(1) $x = \frac{X - X_c}{R}$

(2) $y = \frac{Y - Y_c}{R}$

where R is the cup radius (6 cm). Both x and y are dimensionless. Velocity and acceleration vectors were evaluated numerically, both step by step and as an average for each 1 s frame, for which 25 tadpole locations were recorded.

Sinuosity was defined as the ratio between the curvilinear length (actual tadpole trajectory) and Euclidean distance (straight line) between the end points of the curve: this dimensionless quantity ranges from 1 to ∞. Sinuosity was calculated for each time frame (1 s) and averaged over the total observation period (300 s pre-stimulus and 300 s post-stimulus). An index of sinuosity was calculated as: (sinuosity post-stimulus−sinuosity pre-stimulus)/sinuosity pre-stimulus.

The time spent by each tadpole moving clockwise and anti-clockwise was assessed for each time frame based on the angular momentum, i.e., (i) by identifying the frame mid-point and (ii) by measuring tadpole position (r) and the velocity (v) in the mid-point: as the motion is planar, the z-component of the angular momentum points up or down depending on the rotation direction and can be used to distinguish between anti-clockwise and clockwise motion (i.e. the sign of L modulus is, respectively, positive or negative; Fig. 3.2). Two lateralization indices were then calculated following Lucon-Xiccato *et al*. (2017): LR = [(clockwise swimming time−anticlockwise swimming time) / (clockwise swimming time + anticlockwise swimming time)] ×100; and LA = |LR|, which assesses the intensity of lateralization. These procedures were applied to all 375 tadpoles, each recorded for 300 s before and after the stimulus for a total of about 15,000 locations for each tadpole. For both responses, we imposed v>0.1 mm s−1 to exclude all frames when tadpoles remained still. Subsequently, after inspecting the velocity distribution (Fig. 3.3), all tadpoles moving less than a minimum threshold length (377 mm, i.e., the perimeter of the testing cup) over the sampling time (300 s) were removed from the analysis to prevent bias.

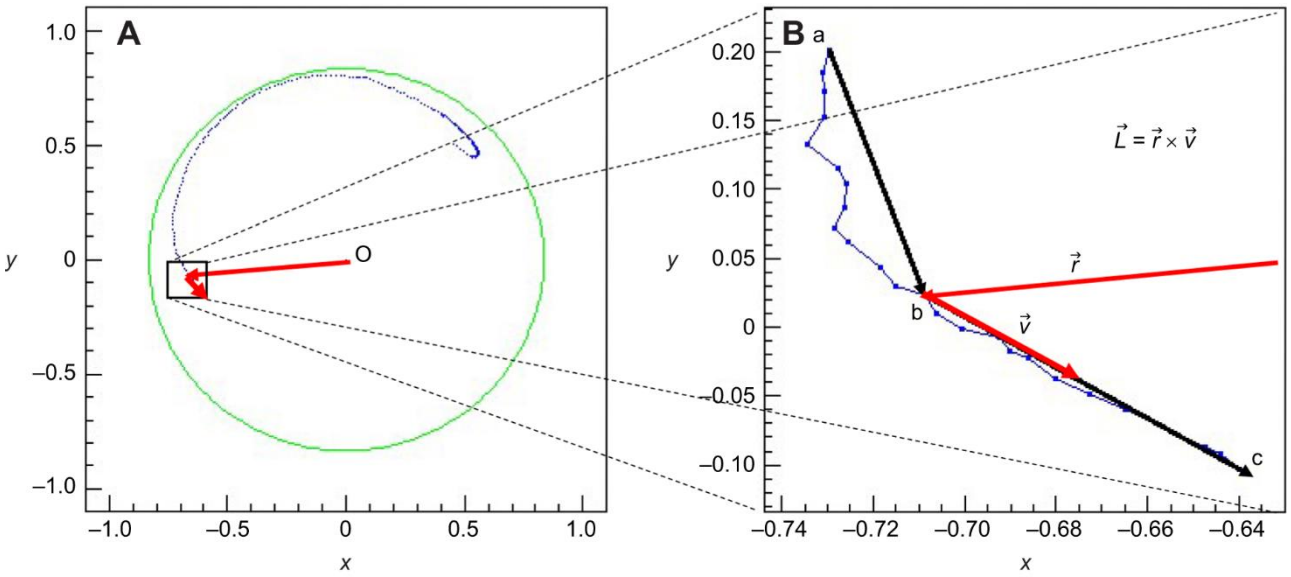

Figure 3.2. Assessment of tadpole lateralization using the angular momentum of their motion. (A) Snapshot of the track reconstruction code (green, cup; blue, tadpole track). Red vectors represent tadpole position and velocity. (B) Expanded view of the boxed area in A, representing a 1 s time frame with 25 sampled tadpole positions on the path between a and c. The mid-point b is taken as a reference to evaluate tadpole position r and velocity v and calculate the angular momentum L as a vectorial quantity: being the motion planar, L points upwards or downwards depending on the motion direction and defines whether the motion is anti-clockwise or clockwise.

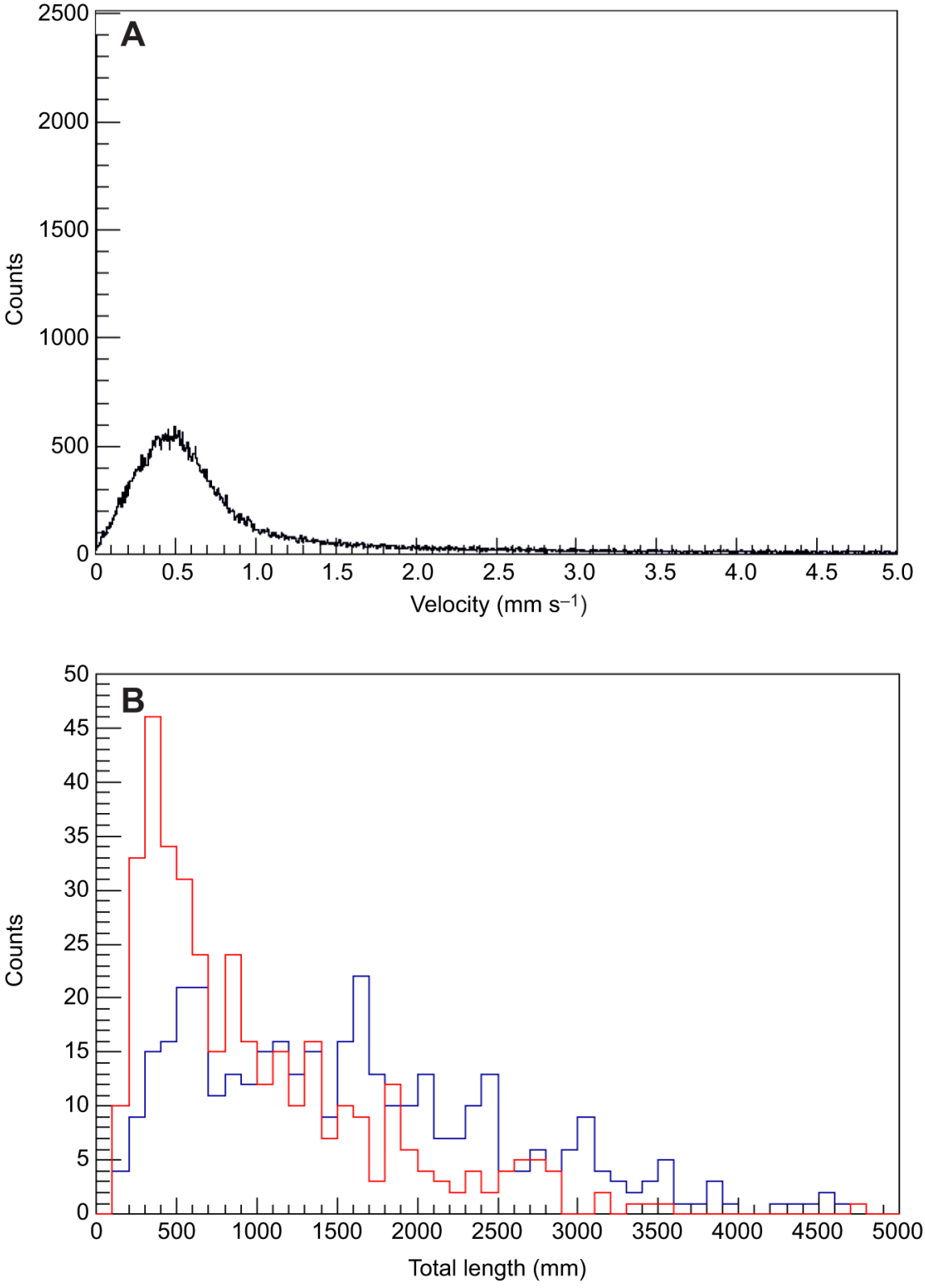

Figure 3.3. Velocity and track length distribution. (A) Post-stimulus velocity distribution for one individual. (B) Track length distribution for all tadpoles (blue, pre-stimulus; red, post-stimulus).

Mobility rate, time frozen and total distance were explored by LMMs. We investigated behavioural plasticity by including the effects of chemical predation cue (i.e., environmental effect), site of origin (genetic background) and their interaction (see also Carter *et al*., 2015) as main predictors in the models for each behavioural response (i.e., mobility rate, total distance, frozen time) after odour infusion. The corresponding behavioural response before the infusion of the odour was included as a covariate and clutch nested within site as a random effect (intercepts varying among sites and among clutches within sites). Basal level of activity, considered to be the mobility rate expressed before cue infusion, was explored with a LMM with site as the main factor (pairwise comparison between sites were tested using Tukey adjustment); paired Wilcoxon signed rank test (V) was used to compare behavioural variables after and before water infusion (controls) between sites. All planned comparisons with the control treatment were obtained from LMMs by the emmeans package in R (https://github.com/rvlenth/emmeans).

As sinuosity and lateralization violated the assumptions of LMMs, these variables were tested by a non-parametric Kruskal–Wallis test, using Mann–Whitney test for post hoc comparisons. All statistical analyses were conducted in R version 3.6.0 (http://www.R-project.org/).

3.3 Results

The chemical treatment provided during embryonic development did not significantly affect the time of tadpole emergence from the jelly in any breeding site (Table 3.1), despite all groups showing a weak effect of hatching time when predator cues were injected. Developmental stage at collection was highly significant (P=0.002), with hatching time inversely related to developmental stage, but no significant effect of either treatment × site or treatment × stage interactions was detected.

Tadpole basal mobility rate differed among breeding sites (F=38.06, d.f.=2, P<0.0001), with BC and MZ showing the lowest and highest level of activity, respectively (BC: 31.6±1.4; MZ: 50.3±1.6; BN: 41.5±1.6; pairwise comparisons, BN–BC: t-ratio=4.60, P<0.0001; BN–MZ: t-ratio=−4.12, P=0.0001; BC–MZ: t-ratio=−8.72, P<0.0001; Fig. 3.4).

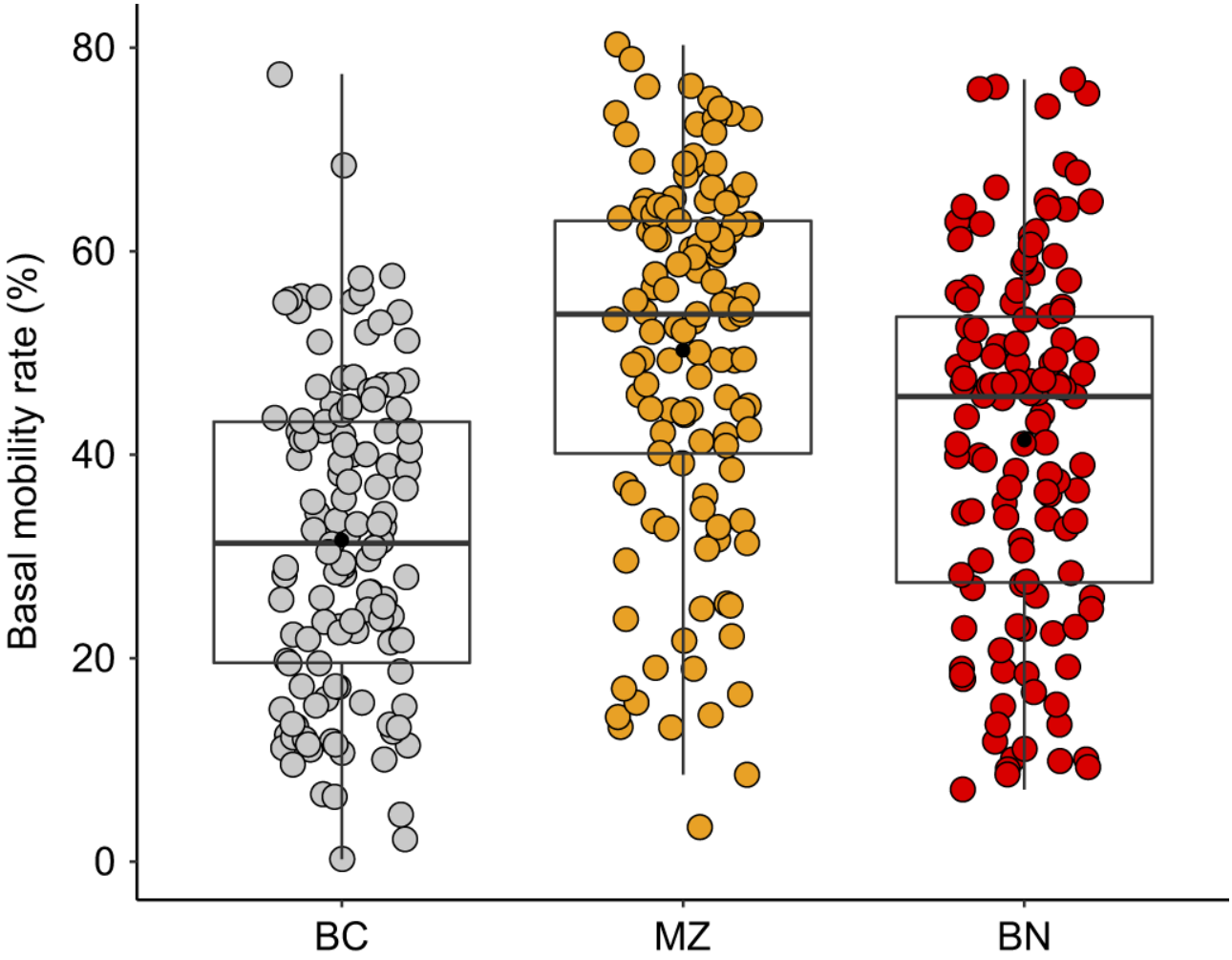

Figure 3.4. Mean (±s.e.m.) basal mobility rate error for each breeding site. BC, Bosco Castagnolo; MZ, Sorgenti della Muzzetta; BN, Bosco Negri. Box plots indicate median values (horizontal line), upper and lower quartiles (box) and 1.5× interquartile range (whiskers).

After infusion of the control cue (water), tadpoles did not significantly modify their level of activity for either BC or BN sites (V=137, P=0.507 and V=119, P=0.252, respectively), while MZ tadpoles showed a significant decrease (V=77, P=0.02). Total distance was not affected by water infusion in all the sites examined, while time frozen revealed a significant increase for MZ (V=257, P=0.01).

For all variables, after infusion of the cues, the degree of behavioural activity showed significant effects of treatment, site, and basal mobility rate (i.e., pre-infusion activity). No significant interaction was detected for treatment×site or basal mobility rate×site (Table 3.1).

	Mobility rate		***Total distance***		***Time frozen***		
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*d.f.*
Treatment	25.9	**<0.001**	12.2	**<0.001**	30.1	**<0.001**	4
Site	6.11	0.013	5.22	**0.021**	4.23	**0.037**	2
Basal mobility rate	53.6	**<0.001**	144.7	**<0.001**	72.9	**<0.001**	1
Treatment x site	1.13	0.344	1.48	0.161	1.74	0.087	8
Pre-mobility rate x site	2.53	0.08	0.21	0.816	1.59	0.205	2

Table 3.1. fixed effects of linear mixed models for tadpoles (n=375) behavioural responses after cue infusion. Bold indicates significance.

The comparison with control treatments indicated a marked decrease in mobility rate after infusion of the tadpole-fed odonate cue in all groups (Table 3.2). The fasted odonate cue and tadpole-fed crayfish cue clearly lowered the activity of both BN and MZ tadpoles but had no significant effect on BC tadpoles (Table 3.2, Fig. 3.5). The fasted crayfish cue had a weak effect (P=0.04) only on BN tadpoles (Table 3.2).

	BC			MZ			BN		
Contrasts	*Est.*	*t*	*P*	*Est.*	*t*	*P*	*Est.*	*t*	*P*
Fasted crayfish – control	6.88	0.59	0.90	-5.8	-0.50	0.93	-30.5	-2.55	**0.04**
Fed crayfish – control	-14.6	-1.24	0.53	-35.3	-3.11	**0.007**	-53.3	-4.60	**<0.001**
Fasted odonate – control	-25.8	-2.20	0.10	-47.2	-4.16	**<0.001**	-66.3	-5.74	**<0.001**
Fed odonate - control	-38.3	-3.24	**0.005**	-56.4	-4.97	**<0.001**	-75.0	-6.46	**<0.001**

Table 3.2. Comparison among different chemical cues and control treatment (water) for mobility rate post-cue infusion as a response variable. BC, Bosco Castagnolo; MZ, Sorgenti della Muzzetta; BN, Bosco Negri. P-value adjustment with Dunnett method for four tests for each population. Bold indicates significance.

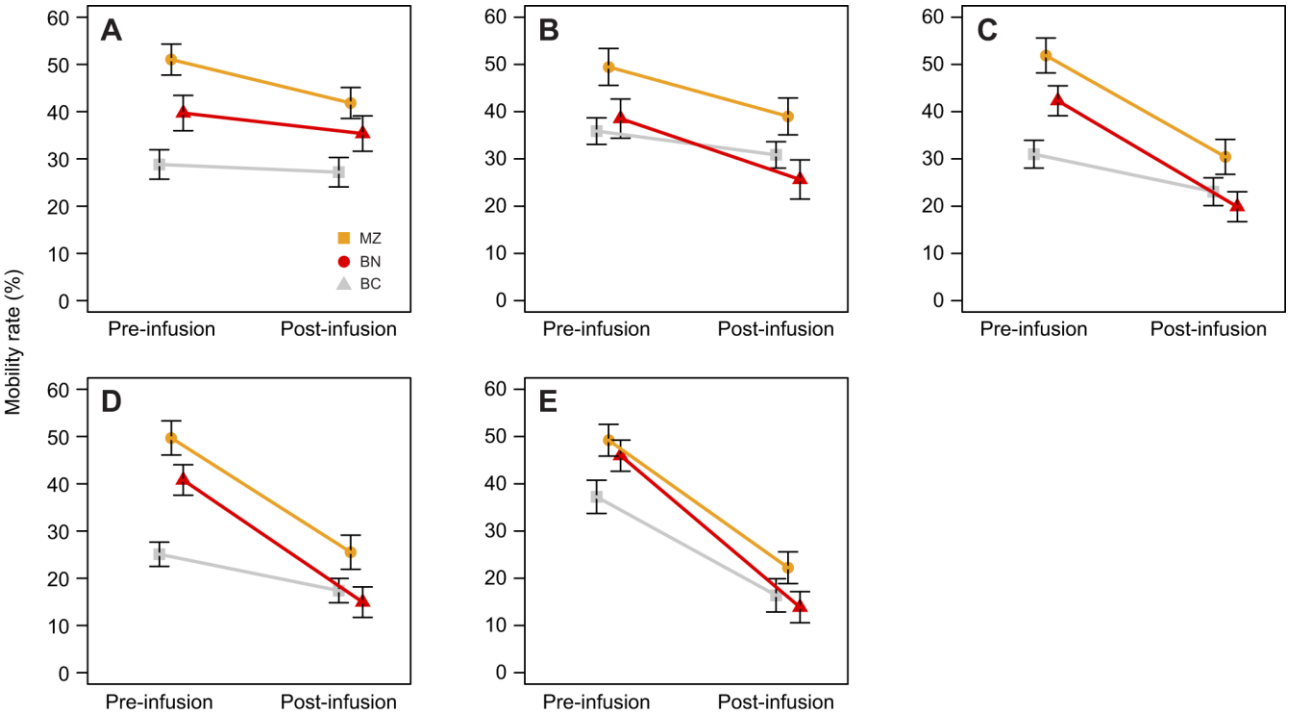

Figure 3.5. Mean (±s.e.m.) mobility rate for each breeding site. (A) Control cue, (B) fasted crayfish cue, (C) fed crayfish cue, (D) fasted odonate cue and (E) fed odonate cue. Each plot shows the effect of predation risk (pre- and post-infusion of predatory cues) and genetic background (site) on tadpole mobility rate (n=75 for each plot).

The examination of behavioural reaction norms showed that more than 80% (range: 84–100% in BC and BN) of tadpoles lowered their mobility rate when exposed to the fed odonate cue. The same pattern was recorded for tadpoles exposed to either fed crayfish or fasted odonate cue, except for BC tadpoles, which were less consistent in their responses (64% and 72%, respectively; Fig. 3.6).

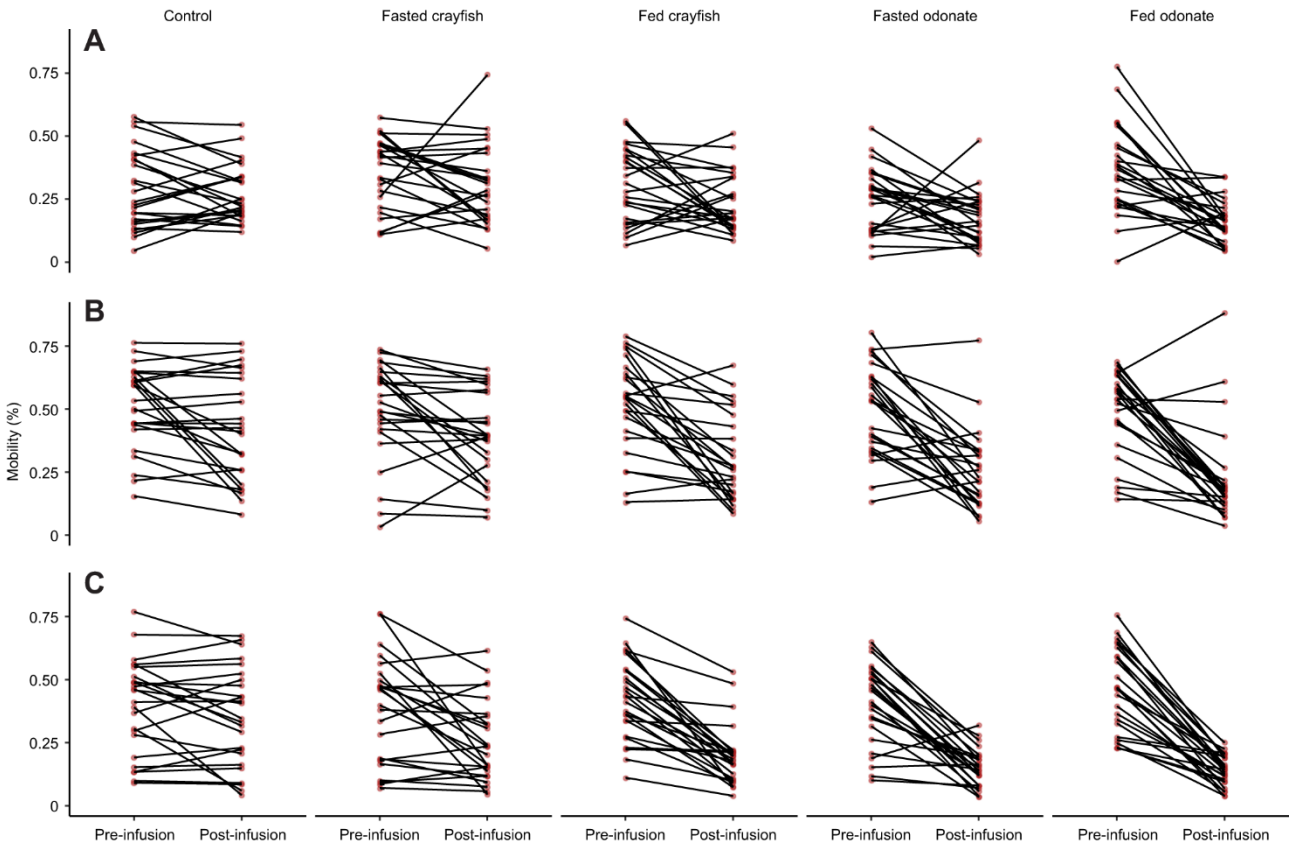

Figure 3.6. Individual reaction norms showing mobility rate before and after the infusion of chemical cues. Mobility rate is shown at each site (A, BC; B, MZ; and C, BN) pre- and post-infusion of the five different cues, as indicated (n=375; n=25 for each subplot).

Total distance varied in all sites but BC. The highest reduction in path length was obtained using the tadpole-fed odonate cue for both BN and MZ. The fasted odonate cue did not induce any significant reduction in the overall path length of MZ tadpoles, while BN tadpoles strongly decreased the distance covered. The tadpole-fed crayfish cue induced a strong distance reduction for both MZ and BN tadpoles, while exposure to the fasted crayfish cue only weakly affected the response of BN tadpoles. Behavioural reaction norms were highly consistent (>80%) for both MZ and BN tadpoles exposed to fed predators.

Time spent frozen provided a pattern similar to that obtained for mobility rate. Comparisons with controls showed that the tadpole-fed odonate cue induced the strongest response in all sites, with BC showing the weakest difference. Both fasted odonate and tadpole-fed crayfish cues significantly increased time spent frozen in both BN and MZ tadpoles, while only the former treatment affected the behaviour of BC tadpoles. Individual tadpoles from all sites showed consistent responses to both fed and fasted odonate cues, as well as to the fed crayfish cue, except for BC.

After cue infusion, the mean length of tadpole paths did not differ among treatments ($\chi2$=9.2, d.f.=4, P=0.06), ranging between 1048 mm (tadpole-fed odonate cue) and 1444 mm (control), while their sinuosity varied among treatments ($\chi2$=9.23, d.f.=4, P=0.05). Overall, sinuosity increased for tadpoles exposed to the cues of both fasted and fed odonates with respect to controls ($\chi2$=31.5, d.f.=4, P<0.001; Fig. 3.7). Post hoc comparisons showed the same trend for each breeding site (P<0.03 for all comparisons), except for MZ, which responded only to the fasted odonate cue. For BN, a nearly significant response (P=0.06) was also recorded for tadpoles exposed to the tadpole-fed crayfish cue.

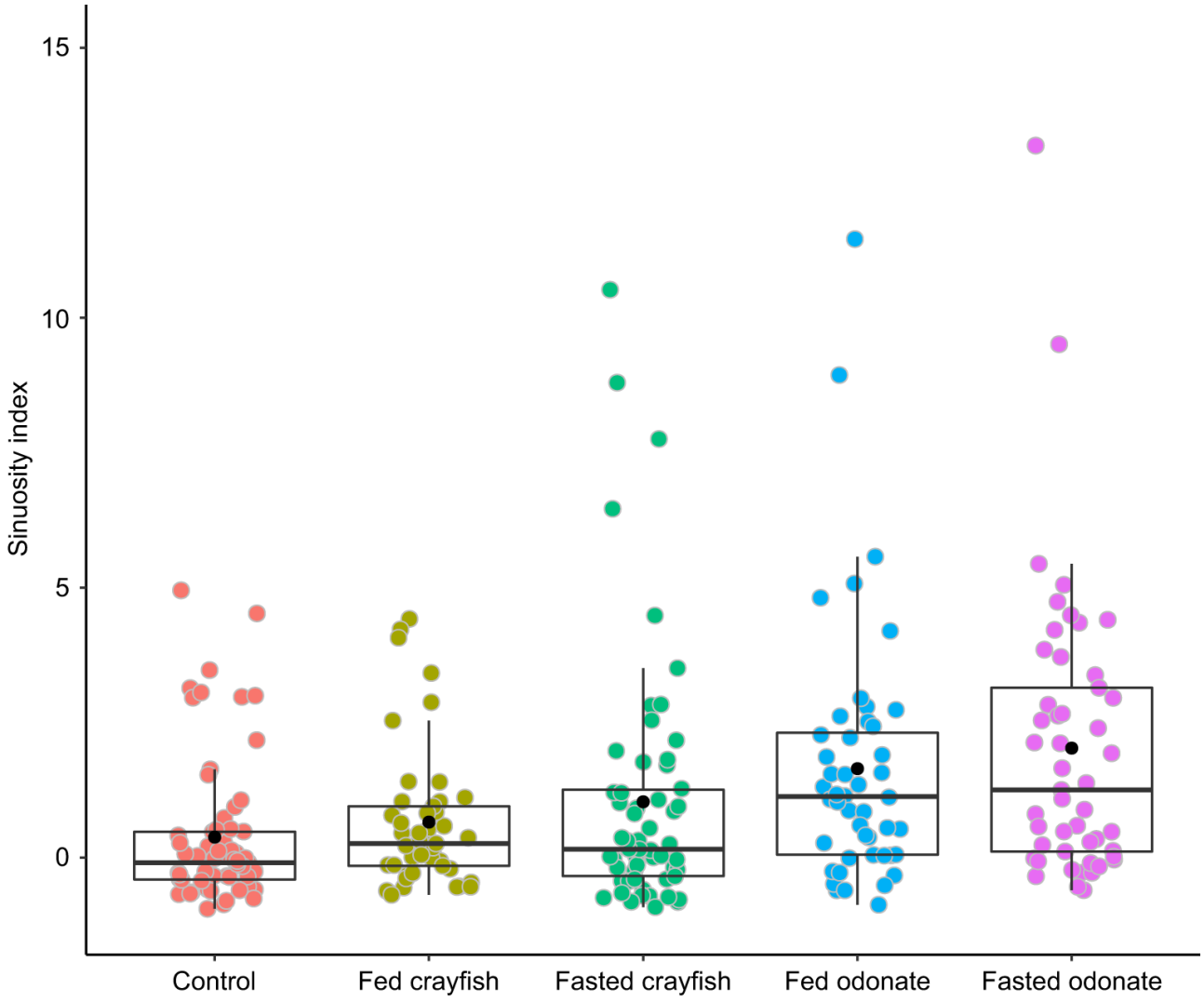

Figure 3.7. Boxplot of the sinuosity index for each chemical cue. Black circles represent means.

Before cue infusion, neither the directionality ($\chi 2$=5.5, d.f.=2, P=0.07) nor the intensity ($\chi 2$=4.5, d.f.=2, P=0.1) of lateralization differed among sites, while both fasted and fed odonate cues lowered the intensity of lateralization ($\chi 2$=18.1, d.f.=4, P<0.001; Fig. 3.8). At the site level, BC and BN responded to the tadpole-fed dragonfly larvae cue (P=0.012 and 0.002, respectively), while MZ showed no statistically significant response.

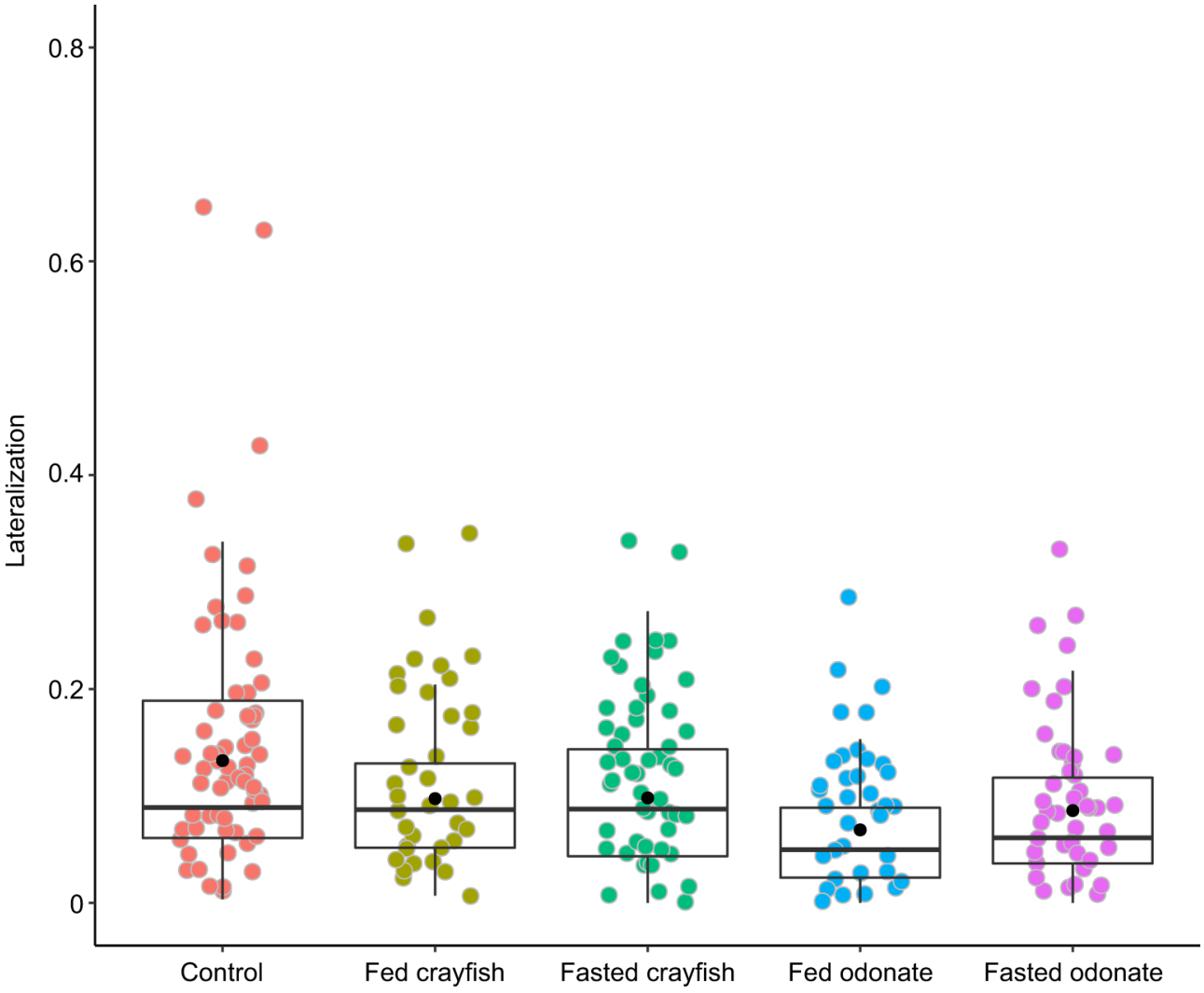

Figure 3.8. Boxplot of the lateralization index for each chemical cue. Black circles represent means.

3.4 Discussion

Several studies have recently reported a certain degree of behavioural plasticity in the defensive responses of a wide range of anuran species when exposed to water-borne chemical stimuli, usually highlighting the synergistic role played by conspecific alarm cues in enhancing the response to predator kairomones (Schoeppner & Relyea, 2009; Sih *et al*., 2010; Gazzola *et al*., 2018). Differently from most Rana species, the Italian agile frog has been shown to display strong behavioural responses towards the cues of native dragonfly larvae in the absence of conspecific alarm cues (Scribano *et al*., 2020). Although this ability may allow Italian agile frogs to efficiently escape predation from native predators, weak sensitivity towards conspecific signals may increase the risk of misleading novel threats, such as introduced potential predators (Sih *et al*., 2010). To test for this hypothesis, we exposed tadpoles to the cues of either native dragonfly larvae or alien crayfish and assessed their efficacy when infused both alone and with prey alarm cues. The use of ToxTrac allowed the precise recording of individual paths, from which we could assess several behavioural variables with greater accuracy than with traditional methods (Scribano *et al*., 2020).

Overall, when exposed to odonate cues, tadpoles showed a strong defensive response, particularly consistent among breeding sites, towards tadpole-fed dragonflies, suggesting that, although kairomones are sufficient to elicit a prompt reduction in tadpole activity, the synergistic effect of predator and prey cues may trigger a more shared and intense response (Schoeppner & Relyea, 2009; Hettyey *et al*., 2015).

Reduction of activity, to lower the probability of encountering or being detected by predators, is a common behavioural response shown by threatened tadpoles, usually measured as either the proportion of active individuals in a group or duration of activity over a standard period (Van Buskirk, 2001; Steiner, 2007; Gazzola *et al*., 2017). By measuring the variables ‘time frozen’ and ‘sinuosity’, we highlighted that tadpoles respond to a predator threat by two different, not mutually exclusive defensive strategies: freezing and zigzagging. While the first behaviour had previously been roughly described by counting the number of tadpoles actively moving, individual recordings and analysis allowed the more precise assessment of the proportion of time that each tadpole spent still before and after being exposed to predator cues. The analysis of individual trajectories indicated that under predation threat, tadpoles incorporate protean elements into their movement, increasing path complexity. Protean behaviour (Humphries & Driver, 1970) prevents predators from anticipating the future position of their prey, lowering their targeting accuracy (Jones *et al*., 2011; Richardson *et al*., 2018), especially in small arenas, where the distance between opponents is short (Furuichi, 2002). Both strategies, and their combination, are expected to be effective in the face of attacks by dragonfly

larvae, which detect their prey at a distance by sight or vibration (freezing) and then move towards it until the prey is within range of their labium (zigzagging) (Rowe, 1994).

As, in principle, predators may reach their prey and attack on any side, lateralization, which is an asymmetrical perceptual system, is expected to be detrimental to prey, which would be more vulnerable on their deficient side (Corballis, 1998). Nonetheless, lateral bias seems to be ubiquitous in animals and it has been hypothesized that it may arise from the need to coordinate behaviours in asymmetrically organized groups (Vallortigara & Rogers, 2005). The decrease in lateralization intensity in tadpoles exposed to odonate cues may suggest that under predation risk, tadpoles tend to scrutinize the surrounding environment equally on both sides. This behaviour may be enhanced by the chemical nature of the signal, which rapidly disperses throughout the test arena, while an increase in lateralization intensity may be expected in tadpoles exposed to caged predators, a hypothesis that needs further testing.

Because of the absence of a common evolutionary history (Gamradt & Kats, 1996; Freeman & Byers, 2006; Banks & Dickman, 2007; Smith *et al*., 2008) and, consequently, the lack of defensive adaptations, native preys are likely to exhibit weak or inappropriate antipredator responses when facing novel predation threats. The degree of naivety, and thus the impact of the alien predator, may depend on its phylogenetic relatedness to native predators (Cox & Lima, 2006; Sih *et al*., 2010). As expected, tadpole responses towards alien crayfish were less sharp and, in general, were mostly elicited by tadpole-fed crayfish. The role played by conspecific cues in eliciting a defensive response in tadpoles exposed to alien predators has been shown for several species (Nunes *et al*., 2013), and our results confirm that alarm cues from damaged conspecifics are also able to elicit behavioural responses in Italian agile frog larvae, at least in laboratory conditions. Interestingly, we recorded inter-site variability in tadpole responses to crayfish, as BC tadpoles did not react significantly to either fed or fasted alien predators, while BN tadpoles also showed a weak but significant response to fasted crayfish. This inter-site gradient (BN>MZ>BC) in the defensive response of tadpoles was consistent with the relative abundance of *P. clarkii* visually recorded at the sites during the sampling of egg clutches. These results suggest that coexistence may enhance behavioural adaptations to a novel predatory threat – that is, native species are able to learn to recognize cues from novel invasive predators (Strauss *et al*., 2006) – probably by associating conspecific alarm cues with predator kairomones (Ferrari *et al*., 2010). This association may occur during egg development (before collection) or be genetically based, given that enough time has been allowed for evolution (Strauss *et al*., 2006). The latter hypothesis seems improbable, as crayfish were introduced to northern Italy only recently (<30 years).

Despite all tadpoles being kept in standard conditions after hatching, basal mobility rates differed among populations, in agreement with previous studies (Nunes *et al*., 2013). Different environmental conditions and pressures are known to affect activity and motor behaviours (Richardson, 2001), and studies on the relationship between predator cues and hatching time have reported discordant results (Ireland *et al*., 2007; Gazzola *et al*., 2015, 2018). Consistent with the potential effect of environmental conditions (Vences *et al*., 2002), our results agree with those reported for syntopic *Rana dalmatina*, for which Gazzola *et al*. (2018) did not observe any effect of odonate cues on hatching time.

3.5 Conclusion

Anuran larvae are a well-studied system to test for predator–prey interactions, behavioural responses usually being used for analysing contextual plasticity (Relyea, 2003; Ferrari *et al*., 2010). By measuring sinuosity and lateralization in tadpoles individually exposed to predation threat, we could identify details about tadpole escape strategies that are usually missed when employing activity-based behavioural variables. To gather this kind of information is pivotal for endemic, threatened species as, although prey may evolve the ability to recognize and respond to alien predators, time may be insufficient to prevent the extinction of several fragmented populations. Finally, we suggest that video-tracking techniques, which have seldom been used for assessing tadpole behaviour (Scribano *et al*., 2020), offer several opportunities to further investigate predator–prey relationships in aquatic habitats.

4.

EFFECTS OF A GROUP-LIVING EXPERIENCE ON THE ANTIPREDATOR RESPONSES OF INDIVIDUAL TADPOLES

4.1 Introduction

Aggregation, considered in all its forms and variations, is widespread in many taxa, affecting individuals' behaviour on a large scale. Living in a group has been reported to bring several benefits, in terms of increased cooperation, foraging efficiency, reproduction and antipredator vigilance (Alcock, 1989; Krause & Ruxton, 2002). Regarding the latter, the mechanisms that can lead to benefits from grouping, such as the 'dilution effect' (Turner & Pitcher, 1986; Lehtonen & Jaatinen, 2016), 'many eyes theory' (Lima & Dill, 1990; Lima, 1995; Roberts, 1996) and 'confusion effect' (Landeau & Terborgh, 1986), have been well studied. These studies have focused on the effects of group living on predation rate in many taxa (e.g., *Oncorhynchus kisutch*, Grand & Dill, 1999; *Nasua narica*, Hass & Valenzuela-Galván, 2002; *Lampropholis delicata*, Downes & Höfer, 2004; protozoan, rotifer, and crustacean species, Tollrian *et al*., 2015), suggesting that prey may use information about the density of both conspecifics and predators to determine their investment in inducible defences.

Prey can gather information on predation risk in multiple ways: direct cues include the visual, auditory, or chemical detection of predators (kairomones), while indirect cues consist of any signal from injured or attacked conspecifics (or heterospecifics), as well as exudates produced by predators after consuming their prey (see Chivers & Smith, 1998; Kats & Dill, 1998). In aquatic environments, direct or indirect predation cues are mainly chemosensory (Van Buskirk & Arioli, 2002; Hettyey *et al*., 2015). Using anuran tadpoles as model organisms, it has been demonstrated that the detection of water-borne cues may elicit significant responses in prey species, in terms of changes in morphological characteristics, behaviour and life history traits (Marquis *et al*., 2004).

Behavioural responses, such as 'freezing' and reduction of activity rates, can be promptly elicited as predation risk is perceived, and have been recorded in many anuran larvae species (e.g. *Rana perezi*, Gómez-Mestre & Díaz-Paniagua, 2011; *Rana dalmatina*, Gazzola, Russo *et al*., 2018; *Rana latastei*, Scribano *et al*., 2020), including toads (*Bufotes viridis*, Stav *et al*., 2007; *Bufo bufo*, Maag *et al*., 2012; *Bufo nebulifer*, Preston & Forstner, 2014).

Anuran tadpoles show different degrees of social aggregation, with bufonids usually forming large social groups (Waldman, 1991; Blaustein & Waldman, 1992), especially under predation risk (Stav *et al*., 2007). Consistently with the 'dilution effect', highly dense tadpole groups are subject to a higher number of attacks, but per capita predation rates are lower than in small groups (Watt *et al*., 1997; Spieler, 2005). Accordingly, lone tadpoles show lower activity levels and more cautious behaviours than tadpoles in pairs or groups (McCoy, 2007; McClure *et al*., 2009), even in the absence of predators' cues (Golden *et al*., 2001; Awan & Smith, 2007).

The relationship between conspecific density and predation risk assessment was investigated by Peacor (2003), who claimed that a single prey individual cannot accurately assess predation risk without gathering information on conspecific density. If this information is not included, the estimation of actual predation risk will be under- (at low prey density) or overestimated (at high prey density). Although, in tests of *Rana temporaria* tadpoles, the predictions of Peacor's risk assessment model have gained some support (Van Buskirk *et al*., 2011), the model has been poorly tested and little evidence of risk assessment based on conspecific density is available (Guariento *et al*., 2015). 'Thinning', i.e., density decline as an effect of predation, does not seem to decrease *R. dalmatina* tadpoles' activity levels, as the model would imply (Gazzola *et al*., 2018). Notwithstanding, Peacor's model provides a useful starting point to investigate the effects of conspecific density on the assessment of predation risk.

In this study, we explored the effects of group living on the defensive behaviour of Balearic green toad, *Bufotes balearicus*, tadpoles. As previous experiments mainly tested the collective behavioural responses of tadpoles in groups, here we specifically aimed at investigating the individual responses of tadpoles reared at three different densities (1, 5 or 25 individuals/container). Following Peacor (2003), we first hypothesized rearing conditions would affect tadpole antipredator behaviour (i.e., after exposure to a predatory cue), with tadpoles reared at the highest densities showing the lowest post-stimulus reduction in activity levels. Second, since anuran larvae largely rely on chemosensory cues to assess the density of both conspecifics and predators, we expected responses to be more strongly elicited in tadpoles also exposed to conspecific chemical cues.

4.2 Materials & Methods

4.2.1 Animal Collection

The Balearic green toad is native to Italy and Corsica and was introduced in the Balearic Islands in prehistoric times (Sindaco *et al*., 2009). It occurs in plain and hilly areas characterized by sandy and freshwater habitats, regularly used as sites for breeding and laying clusters (‘strings’) of eggs (Lanza *et al*., 2007; Gasparri *et al*., 2013; Fiacchini & Cavalieri, 2015; Canestrelli *et al*., 2017). During May 2020, six freshly laid green toad egg strings were collected from a network of canals flowing in an intensively cultivated area south of Milan (45°26′N, 9°20′E, Lombardy region, northern Italy). Egg clutches were immediately transported to the laboratory and prepared for the experiments. After hatching, tadpoles were transferred to 10 different containers (15 litres) and reared until the onset of the experiment, coinciding with Gosner's developmental stage 26–28, when they became appreciably active and social.

Twenty late-instar dragonfly, *Aeshna cyanea*, larvae, native predators of anuran tadpoles, were collected from an artificial pond located inside the protected natural area ‘Bosco del Vignolo’ (45°13′N, 8°56′E), using dip-nets. In the laboratory, they were kept individually in 0.8-litre plastic tubs.

4.2.2 Experimental Design

The experiment was planned as a 3 × 2 × 2 full factorial design, which combined three different population density levels with two types of test environments (water with conspecifics' chemical cues versus aged tap water) and predation risk (predators’ chemical cues versus water; Fig. 4.1). In the first, conditioning, phase of the experiment we prepared water containers (1.5 litres) with three levels of population density. On the first day, we prepared 35 containers, 25 at the lowest density (1 tadpole/container), five at the intermediate density (5 tadpoles/container) and five at the highest density (25 tadpoles/container). An equal number of containers for each density were prepared on each of the following 3 days, for a total of 140 rearing tubs. Each set was kept for 8 days before entering the testing phase. Throughout the conditioning period, tadpoles were fed rabbit food (dried grass pellets) ad libitum, to exclude the potential behavioural effects of food availability (Relyea, 2002), and water was changed every other day. Overall, 300 tadpoles were tested (25 individuals × 3 density treatments × 4 cues). On each of the 4 test days (the ninth since the start of the conditioning period of each set of tubs), we recorded the behavioural responses of 25 individual tadpoles from

each density treatment. For the highest density treatment, five tadpoles from each container were tested; the remaining individuals were not included in the experiment. Tadpoles from each density treatment were selected, as far as practicable, to have a similar size and were randomly assigned to different stimuli (chemical cues).

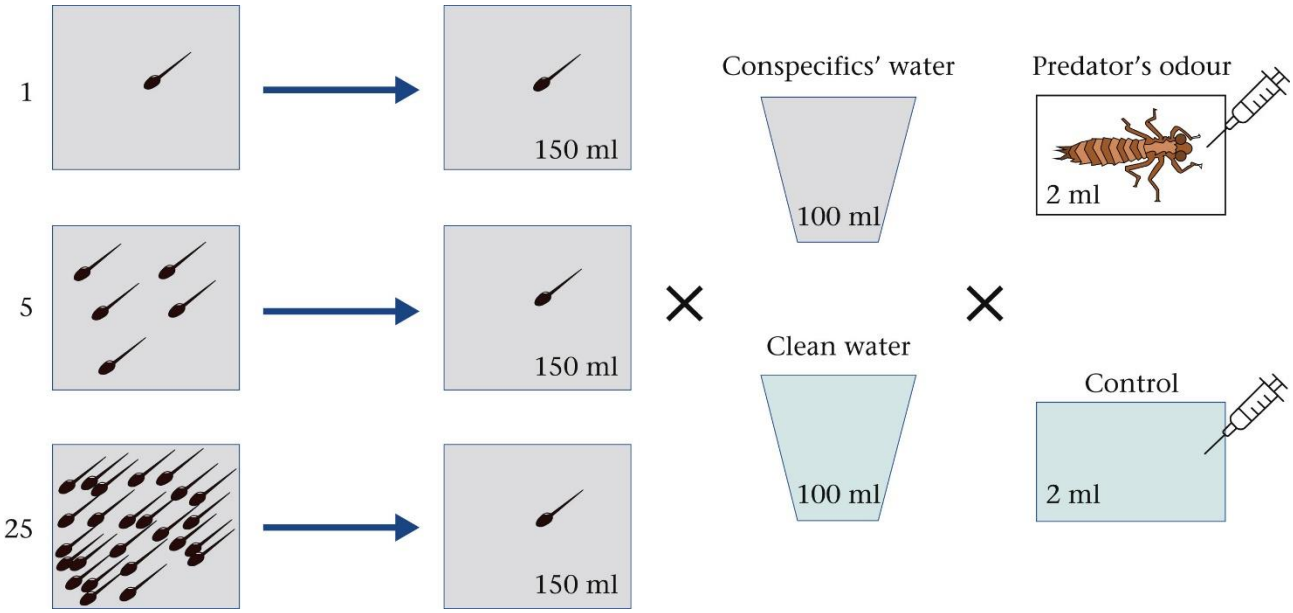

Figure 4.1. Experimental design. Tadpoles were placed in one of three density treatments (1, 5, 25 tadpoles) for 8 days. Individual tadpoles were then placed into the test tubs (N = 100 for each density treatment) in two types of test environment (presence or absence of conspecifics' cues × presence or absence of predators' cues).

4.2.3 Preparation of Odour Cues

To obtain the predators' cues, the day before the start of each test session, five dragonfly larvae were randomly selected and fed (at 2000 hours) green toad tadpoles. At the beginning of the experiment, we collected 50 ml aliquots of water from each predator's tub and mixed them in a separate container (Scribano *et al*., 2020). Aged tap water collected from a large tank (150 litres) was used as a control cue. Predators' cues have been observed to trigger strong behavioural responses after 36–48 h (even up to 72 h) of ageing in well water (Van Buskirk *et al*., 2014); thus, we were confident in their effectiveness.

4.2.4 Behavioural Trials

On each test day, tadpoles were randomly collected from the rearing containers and transferred individually in tubs (15 × 10.5 cm), filled with either 250 ml of aged tap water or 150 ml of aged water and 100 ml of water collected from the same container into which each individual tadpole had been conditioned, which, for the medium- and high-density treatments, was assumed to contain the chemical cues of conspecifics. Tadpoles were left to acclimatize for 20 min before the beginning of the test.

All tests were performed indoors and video-recorded with a digital camera (Canon Legria) hung 1.2 m above an arena, shielded with opaque panels and uniformly lit by spotlights. Overall, each trial lasted 20 min, 10 min before and 10 min after the injection of the stimulus, which consisted of 2 ml of either predator mixture or water (control) carefully injected with a 5 ml disposable syringe to minimize disturbance. The concentration of the predators' cues in the test tub (1:125) was consistent with previous studies (e.g., Gómez-Mestre & Díaz-Paniagua, 2011; Gazzola *et al*., 2021), and was expected to trigger a clear defensive response.

Each tadpole was tested only once. On each test day, all trials were performed in random order between 1000 and 1500 hours. Videos were recorded at 25 frames/s and analysed using a source executable software for image-based tracking (ToxTrac; Rodriguez *et al*., 2018), which provides locomotor information by recording the x and y coordinates of the central point of each tadpole every 0.04 s.

4.2.5 Statistical Analysis

We tested two locomotor variables, namely 'mobility rate', i.e., the rate of instant speed above a threshold of 1 mm/s, and 'total distance', defined as the total distance (mm) covered by each tadpole in the test arena. Behavioural responses were recorded for both the pre- and post-stimulus intervals. To test for variation in the tadpoles' level of activity with respect to the predators' cues, density treatment and presence of conspecifics' cues, we applied linear mixed models (LMMs), including, as response variable, the proportional change with respect to the pre-stimulus baseline in either mobility rate or total distance.

First, we explored the variation in the basal level of activity among treatments, i.e., pre-stimulus mobility rate and total distance, including density treatment (three levels), conspecific cues (two

levels) and their interaction as fixed effects. The day of the experiment and behavioural trial within each day were inserted as random effects. To assess the proportional change in activity level after cue injection (for both mobility rate and total distance), we used a three-way interaction including predation risk (water or tadpole-fed dragonfly cue), test environment (presence or absence of chemical cues from conspecifics) and density treatment (1, 5, 25 tadpoles/container); the day of the experiment and behavioural trial within each day were included as random effects (all two-way interactions were also included in the model). LMMs were fitted through restricted maximum likelihood in the R package nlme (Pinheiro *et al*., 2019). Planned comparisons of interest were obtained using the R package emmeans (Lenth, 2018). We visually inspected the residuals to assess model fit and obtained confidence intervals for the estimates of fixed effects using the R package effects (Fox & Weisberg, 2018).

4.3 Results

Pre-stimulus mobility rate was significantly affected by rearing conditions, increasing with density (density treatment: $\chi^2 = 43.98$, $P < 0.001$; N = 50 for each density level), but not by the presence of conspecifics' chemical cues ($\chi^2 = 0.06$, $P = 0.80$) or the density treatment x conspecifics' cues interaction ($\chi^2 = 0.67$, $P = 0.71$; Fig. 4.2). Pairwise comparisons showed a strong difference between the lowest and the highest density treatments, both in the presence ($t = -3.839$, $P = 0.002$) and in the absence ($t = -4.759$, $P < 0.001$) of conspecifics' cues. Similarly, the intermediate density treatment differed significantly from the highest one, both in the presence ($t = -2.91$, $P = 0.01$) and in the absence ($t = -3.18$, $P = 0.004$) of conspecifics' cues.

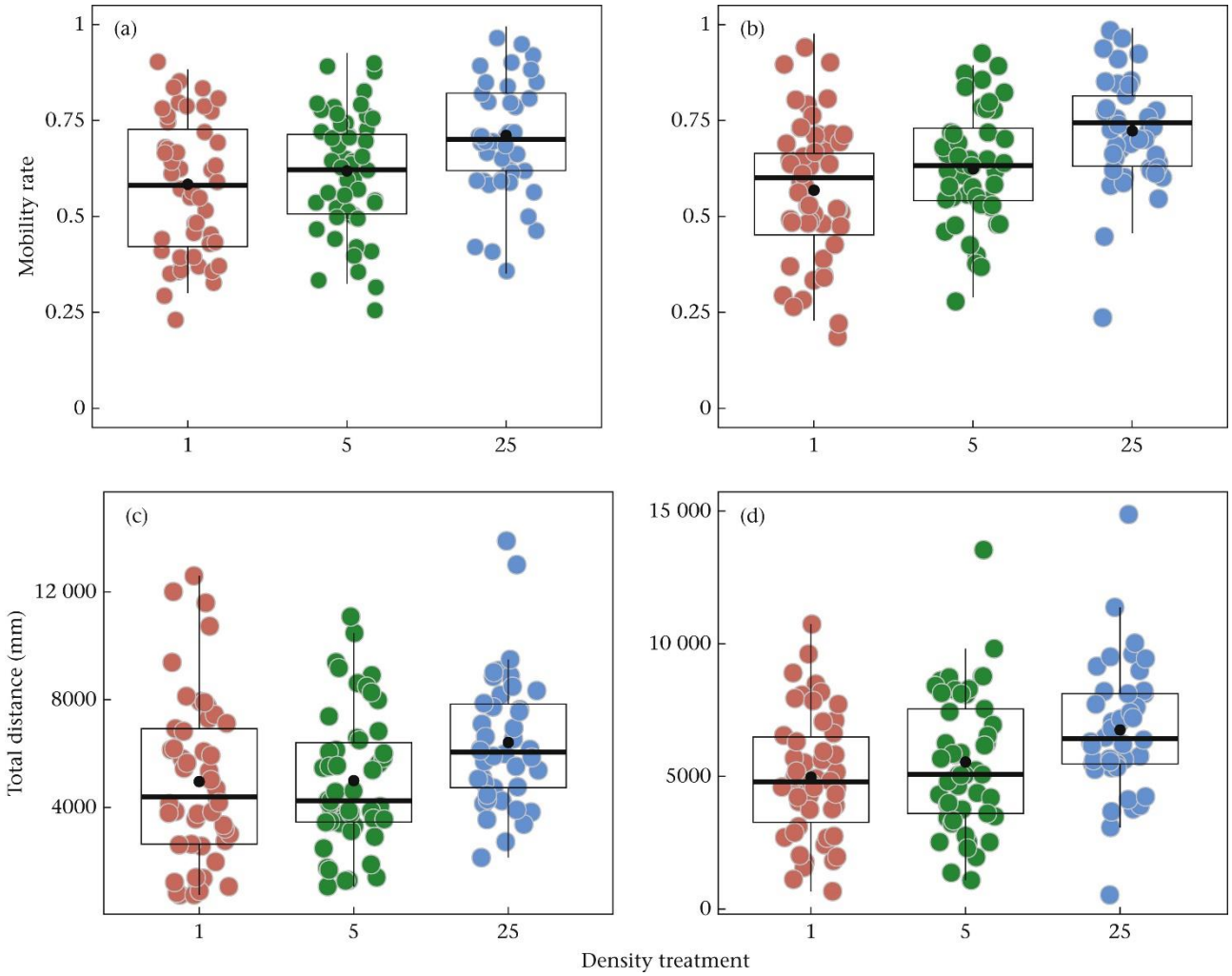

Figure 4.2. Prestimulus mobility rate and total distance moved for each of the three density treatments (1, 5, 25 tadpoles) (a, c) in the presence or (b, d) in the absence of conspecifics' cues. Coloured dots represent individuals' data; black dots represent means for each treatment (N = 50 for each combination of density and water-borne cues); box plots show medians (lines in the boxes), 25% and 75% quartiles (boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers) and outliers (circles).

Pre-stimulus total distance increased with density ($\chi^2 = 20.46$, $P < 0.001$, $N = 50$ for each density level) and was not affected by the presence of conspecifics' chemical cues ($\chi^2 = 0.76$, $P = 0.38$) or by the density treatment x conspecifics' cues interaction ($\chi^2 = 0.79$, $P = 0.67$; Fig. 4.2). Pairwise comparisons showed a strong difference between the lowest and highest density treatment, both in the presence ($t = -2.70$, $P = 0.02$) and in the absence ($t = -3.38$, $P = 0.002$) of conspecifics' cues. The intermediate density treatment differed significantly from the highest density treatment when in the presence of conspecifics' cues ($t = -2.64$, $P = 0.02$) but not in their absence ($t = -2.0$, $P = 0.09$). For both locomotor variables, no significant difference was detected between the lowest and intermediate density treatments ($P > 0.60$ for all).

We found a significant interaction between predators' cues and density treatment ($\chi^2 = 8.67$, $P = 0.01$) in the proportional change in mobility rate, while none of the other two-way interactions was significant (density treatment x conspecifics' cues: $\chi^2 = 1.88$, $P = 0.38$; predators' cues x conspecifics' cues: $\chi^2 = 0.01$, $P = 0.91$; Fig. 4.3). The interaction among the three factors was marginally nonsignificant ($\chi^2 = 5.92$, $P = 0.051$). Lone-reared tadpoles did not respond differently from controls, either in the presence (estimated difference = 0.07 ± 0.07, $t268 = 0.96$, $P = 0.33$) or in the absence (estimated difference = -0.05 ± 0.07, $t268 = -0.73$, $P = 0.46$) of conspecifics' cues. Predators' cue-exposed tadpoles from the intermediate density group showed a significant reduction in mobility rate with respect to controls, in both environments (conspecifics' cues: estimated difference = 0.25 ± 0.07, $t268 = 3.60$, $P < 0.001$; clean water: estimated difference = 0.17 ± 0.07, $t268 = 2.45$, $P = 0.01$). Tadpoles from the highest density treatment showed a strong reduction in mobility rate when exposed to the predators' cues only in clean water (estimated difference = 0.23 ± 0.07, $t268 = 3.00$, $P = 0.003$).

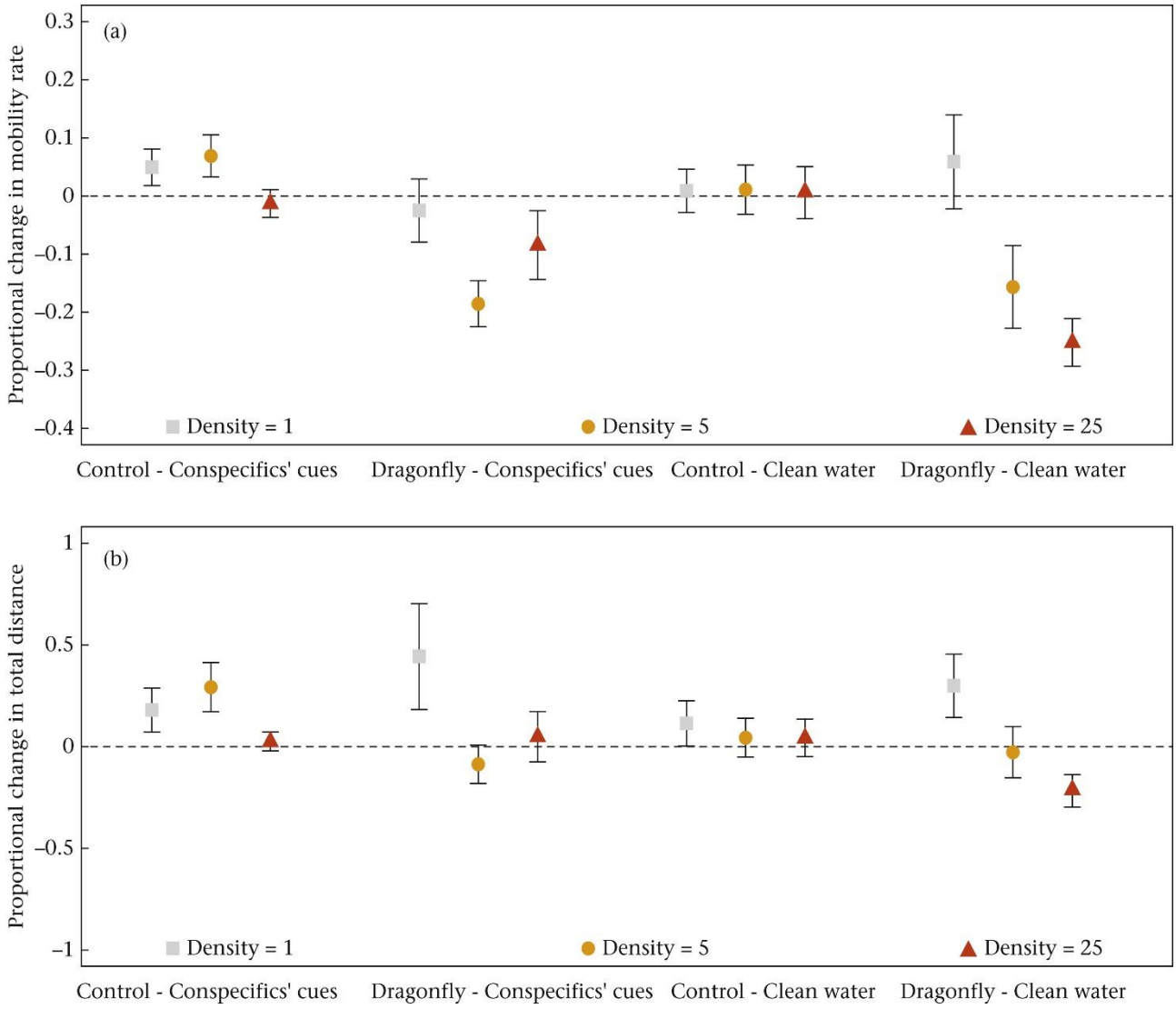

Figure 4.3. Mean (± SE) proportional change in (a) mobility rate and (b) total distance moved for tadpoles exposed to predatory cues (tadpole-fed dragonfly or control) either in the presence or in the absence of conspecifics' cues. Different colours and shapes indicate density treatments (1, 5 or 25 tadpoles: N = 25 for each combination).

With proportional change in total distance as a response variable, we detected a significant interaction between predators' cues and density treatment (χ^2 = 6.23, P = 0.04); no other interaction was significant (P > 0.25 for all). Only the intermediate density treatment showed significant variation, total distance decreasing when both predators' and conspecifics' cues were present relative to the control treatment (estimated difference = 0.37 ± 0.18, t268 = 2.03, P = 0.04; Fig. 4.3).

4.4 Discussion

Many studies have explored the importance of group density for antipredator behaviour in a wide variety of species, demonstrating that group living is often intertwined with predation risk assessment, providing antipredator benefits while implying inevitable costs due to increased intraspecific competition and increased attack rate on large groups (Krause & Ruxton, 2002). In this respect, anuran larvae have been poorly investigated, with studies using different approaches and experimental conditions, mainly with the aim of estimating the behavioural responses of tadpoles exposed to predation risk (e.g., Nicieza, 1999; Spieler, 2003; McCoy & Bolker, 2008; Gazzola *et al*., 2018). Nearly all experiments have recorded the behavioural responses of tadpoles tested in groups of different size, hence acquiring data on activity levels by assessing the proportion of active individuals in each group. To investigate the effects of group-living experience on antipredator responses, we focused on individual *B. balearicus* tadpoles, showing that a brief conditioning period (8 days) was sufficient to later affect both their basal level of activity and defensive responses.

Increased basal activity in tadpoles raised at growing densities may be explained by each individual's perception of conspecifics as a potential threat for future (as food was not limiting during the conditioning period) resource availability (i.e., the risk of future food shortage; Tollrian *et al*., 2015). Larger rearing containers might have lowered perceived competition, but at the risk of reducing proximity among tadpoles and erasing the effects of density conditioning and thus undermining the aim of our experiment.

The behaviour of green toad tadpoles after cue injection differed between density treatments. Although single tadpoles usually respond to predators' cues (*Hyla femoralis*, McCoy, 2007; *Hyla squirella*, McCoy & Bolker, 2008; *Rana sylvatica*, McClure *et al*., 2009; *R. perezi*, Gómez-Mestre & Díaz-Paniagua, 2011; *R. latastei*, Scribano *et al*., 2020), green toad tadpoles reared alone in our experiment showed the lowest activity level and did not significantly reduce their activity level when exposed to predators' cues.

Toad tadpoles rarely grow alone, and usually tend to aggregate with up to thousands of siblings and congenerics as a predator avoidance behaviour (Watt *et al*., 1997). In the absence of conspecifics, competition is no longer crucial and, whenever food is not a limiting factor, activity levels can be sharply reduced (Van Buskirk & McCollum, 2000; Gazzola *et al*., 2018). In the lowest density treatment, activity levels were probably too low to decline further in the presence of predation risk (Nunes *et al*., 2014). Accordingly, Peacor (2003) stated that prey response to predation risk is a function of the per capita amount of predators' cues. This implies that individuals cannot accurately assess predation risk without gathering information on conspecific density. Since predation risk may

exist even in the absence of cues (Lima & Dill, 1990), low conspecific density systems (in which cues propagate more slowly and irregularly) should hypothetically always induce 'higher-risk phenotypes', that is, more cautious and risk-avoiding behaviours.

On the other hand, individuals living in high-density groups should be better able to evaluate predation risk, and thus invest less energy and resources in inducible defences to the benefit of a more efficient competitive behaviour. Consistently, tadpoles reared within the largest groups strongly decreased their mobility rate when exposed to predators' cues in clean water, but their response was substantially weakened when conspecifics' cues were present; the latter probably provided information on group size, mimicking the dilution effect. The behaviour of tadpoles from the intermediate density treatment, which responded to predation risk in both test environments, may suggest that there needs to be a minimum group size, or concentration of conspecifics' cues, for individuals to experience the antipredator benefits of group living. Indeed, we may argue that water from a five times higher group density might have conveyed more reliable information on the number of conspecifics and, consequently, might also have induced a different perception of the per capita amount of cues present in the environment.

Total distance moved gave less straightforward results when tadpoles were exposed to predators' cues. Recently Gazzola *et al*. (2021) have shown that under predation threat tadpoles incorporate protean elements into their movement, increasing path complexity. Such changes in movement patterns may explain why the total distance covered by high-density-reared tadpoles did not vary compared to controls, despite the decrease in their mobility rate.

4.5 Conclusion

By applying video-tracking techniques to individual tadpoles, we have provided evidence of the effects of group living on the defensive responses of green toad tadpoles. Peacor's model predictions were only partially confirmed, and our results suggested that the contextual occurrence of conspecifics' cues plays a bigger role than previously experienced group size in shaping tadpole responses to predators' cues.

Our results cannot rule out the possibility that the short-term (30 min) aggregation experienced by tadpoles before the trials may have overridden the previous conditioning period (8 days) altering their activity levels. Further research would be needed to evaluate this possibility and to underline the differences that may arise with different density treatments' exposure times.

Anuran larvae are considered to rely mainly on chemical stimuli to assess both predation risk and conspecifics' density. To assess both predation risk and conspecifics' density, anuran larvae are considered to rely on chemical rather than visual stimuli. As neither dragonfly larvae nor conspecifics were physically present in the test tubs, visual information was not anyway available to the potential prey. The reinforcement of tadpole behavioural responses by visual stimuli, however, is worth exploring further in future studies.

5.

DIFFERENTIAL EFFECTS OF MICROPLASTIC EXPOSURE ON ANURAN TADPOLES: A STILL UNDERRATED THREAT TO AMPHIBIAN CONSERVATION?

5.1 Introduction

Amphibian populations are globally declining (Hayes *et al*., 2010; Converse & Grant, 2019), with >30% of amphibian species categorized as at risk of extinction by the IUCN (Stuart *et al*., 2004). Several anthropogenic factors are contributing to this trend, including habitat loss (Houlahan & Findlay, 2003; Cushman, 2006), pollution (Bridges & Semlitsch, 2000; Slaby *et al*., 2019), disease epidemics and climate change (Kiesecker *et al*., 2001; Ficetola & Maiorano, 2016). Among the several pollutants that can contaminate freshwater habitats, microplastics (MPs), defined as plastic particles smaller than 5 mm in diameter (Thompson *et al*., 2004), have recently gained the attention of the scientific community (Eerkes-Medrano *et al*., 2015), raising concerns about their toxicity for aquatic species and potential bioaccumulation through food webs (Imhof *et al*., 2017; Windsor *et al*., 2019; Araújo *et al*., 2021).

Major input pathways for MPs into freshwaters are run offs from urban and agricultural areas, treatment plant effluents (Anderson *et al*., 2016) and atmospheric deposition (Dris *et al*., 2016). "Primary" MPs are manufactured for specific purposes, such as the microspheres contained in several cleaning products, while "secondary" MPs result from the biological and mechanical degradation of large plastic materials (Castro-Castellon *et al*., 2021). Out of the over 5300 synthetic polymers in commerce (Wagner & Lambert, 2018), polyethylene (PE), polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS) are the most widespread in the environment. PE and PS can sorb high concentrations of priority pollutants, mainly polychlorinated biphenyls, while PVC and PS contain mutagenic vinyl chloride and vinyl benzene, respectively (Rochman *et al*., 2017). In addition, polyester fibres (PES) are dominant in waterbodies (Hu *et al*., 2018).

Microplastics can enter aquatic food webs at different trophic levels through direct ingestion, filter feeding, or predation on prey previously exposed to plastic debris (Wagner *et al*., 2014; Mattsson *et al*., 2015; Anderson *et al*., 2016). They adhere to periphyton, increasing the probability of ingestion by grazers (Boyero *et al*., 2019).

Most tadpoles have opportunistic feeding habits, filtering phytoplankton from the water column, scraping algae off the substrate, and ingesting a wide range of potentially edible particles, which they break into suitable sizes by their jaw sheaths and labial teeth (Altig *et al*., 2007; Wells, 2007). Their

feeding habits make tadpoles prone to the ingestion of MPs, which has been demonstrated under field conditions. Although these pollutants have been reported to affect tadpole growth and development (Hu *et al*., 2018), available information is still scarce. Microplastics may damage tadpoles through two major pathways: 1) exposing them to plasticizers or associated pollutants (e.g., persistent organic pollutants; Anderson *et al*., 2016); and 2) blocking their digestive tracts or inducing a false sense of satiation, thus impairing their feeding success (Watts *et al*., 2015; Welden & Cowie, 2016).

Exposure to polyethylene MPs has been reported to cause histopathological changes (Araújo *et al*., 2020a), abnormalities in nuclear erythrocytes (Araújo *et al*., 2020b) and affect both growth and behavior in Cuvier's foam froglet (*Physalaemus cuvieri*) tadpoles (Araújo & Malafaia, 2020; Araújo *et al*., 2020b). In contrast, polystyrene MPs neither affected the growth nor the activity of African clawed frog (*Xenopus laevis*) larvae (De Felice *et al*., 2018).

Although some studies have shown that tadpoles are capable of egesting MPs fast (Hu *et al*., 2016; De Felice *et al*., 2018), recently it has been demonstrated that exposure to polystyrene microspheres can impair both feeding and growth of common midwife toad (*Alytes obstetricans*) tadpoles, with lethal effects at high MP concentration (1800 part. mL^{-1}; Boyero *et al*., 2019).

Furthermore, despite widespread concern for amphibian conservation, studies on the impact of MPs on threatened species are lacking. To assess the effects of MP exposure on both the growth and behaviour of anuran tadpoles, we tested two species, differing in both habitat requirements and conservation status: the Italian agile frog (*Rana latastei*, Boulenger 1879) and Balearic green toad (*Bufotes balearicus*, Boettger 1880).

The Italian agile frog is an endemic species which occurs in residual lowland hygrophilous woods of northern Italy, Canton Ticino, Istria, Slovenia, and Croatia (Barbieri & Mazzoti, 2006). Loss of suitable habitats caused by urbanization and intensive agriculture and non-native fish and crayfish are considered the main threats to this species and have been imputed as causes of some local extinction events (Edgar & Bird, 2005; Ficetola *et al*., 2012). Ongoing decline in both habitat quality and population size led the IUCN to list the Italian agile frog as “Vulnerable”; it is also included in Annexes II and IV of the Habitats Directive (EC 43/1992). The native range of the Balearic green toad includes the Italian peninsula and north-eastern Sicily, Sardinia, Corsica, and the Balearic Islands. This species is widespread along sandy coasts and lowland floodplains, also occurring in urbanized and agricultural areas. Breeding sites usually consist of small, temporary, and brackish water bodies (Sindaco *et al*., 2006; Gasparri *et al*., 2013). Despite being less vulnerable to anthropogenic environmental changes than other anurans, the Balearic green toad is included in Annex IV of the Habitats Directive.

Freshwater habitats where both species deposit their egg-clutches can be widely contaminated by MPs (Cera *et al*., 2020), especially in the proximity of urban areas (McCormick *et al*., 2016).

To mimic field conditions, both Italian agile frog and Balearic green toad tadpoles were exposed to three different environmental concentrations of a MP mix composed of four of the most common polymers (PVC, PS, High-Density PE, and PES). We expected a reduction in activity, and therefore, foraging and growth of tadpoles exposed to increasing MP concentrations. We also expected B. balearicus to perform better than R. latastei under stressful conditions, due to its higher tolerance towards anthropic pressures.

5.2 Materials & Methods

5.2.1 Animal collection

In February 2021, we collected 20 fragments of Italian agile frog egg clutches from three ponds located inside a natural protected area (Bosco del Vignolo, 45°13′N, 8°56′E; Lombardy, N Italy). Maximum water depth was 1 m, with moderate turbidity and low (<10%) aquatic vegetation cover. In April 2021, 20 fragments of green toad egg strings were collected from three artificial fountains located inside the Botanical Garden of Pavia (45°11′N, 9°10′E; Lombardy, N Italy); water depth was 20 cm with high turbidity and no vegetation cover. Animal collection, husbandry and experiments were authorized by ISPRA (Prot. 1790 of January 18, 2021).

Both egg clutches and string fragments were immediately brought to the laboratory and kept until hatching in ten 21 L rearing tanks (2 clutches or strings per tank), filled with dechlorinated tap water.

5.2.2 Production of MPs

We generated an environmentally relevant MP mixture using common plastic objects made of four different synthetic polymers: expanded polystyrene (PS) from black foam food tray, high-density polyethylene (HDPE) from red bottle caps, polyester fibres (PES) from blue-colored synthetic fabrics, and polyvinyl chloride (PVC) from common orange pipes for building. PS, HDPE, PES, and PVC had the following densities: 0.028–0.048, 0.94, 1.38, and 1.3–1.45 g cm−3, respectively. HDPE, PVC, and PS were fragmented into smaller pieces using a metal pincer, plunged into a bath of liquid nitrogen and subsequently cryomilled using a Fritsch Pulverisette 11 mill, at a speed of 10 000 rpm for 30 s. PES fibres from synthetic fabrics were generated by manually cutting a blue polyester t-shirt

and collecting fibres from the exhaust filter of a dryer machine after the drying process of a mixture of synthetic fabrics.

We filtered the obtained materials on cellulose membranes and assessed the size of MPs (maximum diameter or length for powders and fibres, respectively), using a Leica EZ 4D stereomicroscope equipped with a digital camera and ImageJ. Mean size was 0.75 mm (SD 0.36) for HDPE fragments (n = 59), 0.28 mm (SD 0.24) for PVC (n = 105) and 0.59 mm (SD 0.40 for PS (n = 85). Mean length of PES fibres (n = 60) was 1.01 mm (SD 0.73).

We then pooled powders and fibres to obtain a mixture of MPs in three concentrations: 1, 7, and 50 mg L−1; the lowest was consistent with mean concentrations at the outlet of wastewater treatment plants (6400 MP m−3, Schmidt *et al*., 2020; assuming spherical MPs with a mean diameter of 700 μm and a density of 1 g cm−3, 6400 MP m−3 correspond to 1.15 mg L−1), while the other two concentrations (7 and 50 mg L−1) followed a geometrical increase and were tested as worst-case scenarios based on the highest concentration (450 000 MP m−3) reported by Schmidt *et al*. (2020). The highest concentration was also consistent with previous toxicity studies (Besseling *et al*., 2014; Araújo *et al*., 2020a). To prevent low-density polymers from being overrepresented in terms of particle numbers, for each concentration we weighed the MPs using a high-precision scale and metal cutlery as to obtain the following constant weight-ratio: 3 PVC: 3 HDPE: 3 PES: 1 PS.

5.2.3 Experimental procedure

To test for the effects of MP exposure on tadpole growth, behaviour, and survival, we performed two experimental trials, one using Italian agile frog tadpoles and one using Balearic green toad tadpoles. Each trial consisted of 24 tanks (38 × 28 × 20 cm), filled with aged well water, positioned in groups of four on six tables (blocks) in the same room, with natural light and air temperature conditions. Following a randomized and balanced design, we assigned MP treatments to tanks so that each block included one replicate for each of the three densities to test (1, 7, and 50 mg L−1) and one control (0 mg L−1), with 6 replicates per treatment. During trials, we recorded water temperature twice every day, at 10am and 4pm, using a digital thermometer, and provided a fixed quantity of rabbit chow (60 mg, i.e., ca.15% of the wet mass of tadpoles at the start of the trials) to each tank every day.

We recorded mortality rates by carefully inspecting all tanks twice a day. To assess the number and type of ingested MPs, dead tadpoles were collected separately for each tank and frozen until analysis. We assessed the lethal concentration expected to cause 50% mortality in the tested agile frog

population (LC50) at 240 h by plotting mortality vs. MP density and Log-probit regression. MP concentrations causing 10% and 90% mortality were also reported.

To assess the activity levels of tadpoles belonging to different treatments, tadpoles were recorded for ten consecutive minutes using a digital camera (Olympus Tough TG-5) hung up 1 m above each block. We assessed the proportion of active (swimming or foraging) tadpoles every second within a 10-s interval each minute (10 s × 10 min = 100 observations per tank). Activity was recorded five times per trial.

5.2.4 Trial I

One week after hatching, 480 *R. latastei* tadpoles were moved into the 24 experimental tanks (20 tadpoles per replicate). A further sample of 30 tadpoles was used to assess mean ± SD wet-weight (20 mg, SD 1) and stage (27.1, SD 0.65; min-max: 26–28, following Gosner, 1960) at the start of the trial. Mean water temperature was 16.8 °C (SD 0.7, min-max: 16.0–18.0) throughout the trial period (from 17 to March 24, 2021).

At the end of the trial, we wet-weighed (precision of 0.01 mg) all survived tadpoles from both the medium and high-density treatments and 60 randomly chosen tadpoles from low density and control treatments (10 tadpoles per tank). To assess their capacity to recover, we moved all survived specimens from the highest MP exposure and as many randomly selected individuals from the other treatments into four 21 L tanks filled with 8 L of aged well water (one tank per treatment). During the two successive weeks we fed 74icrophes with rabbit chow and recorded mortality rates.

5.2.5 Trial II

One week after hatching, 480 *B. balearicus* tadpoles were moved into experimental tanks (20 tadpoles per replicate). We assessed mean Gosner's stage (27, SD 0.75; min-max: 26–28) and weight (21 mg, SD 2.5) were assessed following the same protocol used for trial I. Tadpoles were exposed to MP treatments for one week, from 13 to May 20, 2021. During the trial, mean water temperature was 20.7 °C (SD 0.3, min-max: 20–21.4). At the end of the trial each tadpole was moved from its tank to a numbered (from 1 to 20), white plastic cup. Then, we used a Random Integer Set Generator to select and weight half of the tadpoles (for a total of 240 tadpoles).

5.2.6 MP extraction from dead tadpoles

To remove the organic content and extract the MPs, dead *R. latastei* tadpoles from trial I were chemically digested using hydrogen peroxide (30% H2O2). In total, we collected 53 daily samples, out of which 30 (from different days, blocks, and treatments) were processed for MP analysis. Each sample included between 1 and 10 tadpoles, depending on daily mortality rates. Samples were placed in beakers, filled with 20 mL of H2O2, for 1 h at 50 °C and overnight at room temperature. Using a glass filtration apparatus, we then filtered the solutions on a cellulose membrane (pore size: 0.45 μm; filtering area: 1193.985 mm2). Both the beaker and reservoir flask of the filter were rinsed with filtered Milli-Q. Filter membranes were placed in a glass Petri dish with a closed lid and left to dry in a desiccator for 48 h. To assess the number and size range of ingested MPs, for each sample we took ten images at 20× magnification (19 228 mm2, 1,6% of filtering area) and one at 8× magnification (119 983 mm2, 10% of filtering area). We used the latter for PVC and HDPE MPs, while PS and PES items were analyzed using 20x images. The total number of MPs was then extrapolated for the whole filtering area, except for 1 mg L−1 samples, for which, expected numbers of PVC and HDPE particles being low, whole membrane areas were inspected. Finally, for each polymer, we calculated the number of ingested MPs per tadpole by dividing the total number of MPs per the number of dead tadpoles in each daily sample.

5.2.7 Statistical analysis

We used linear mixed models (LMMs) to explore variation in both weight and activity levels. In each model, we included 'Treatment', 'species' and their interaction as fixed factors, and 'tank within block' as a random factor. We used Akaike's Information Criterion (AIC) to select the best model. For each experimental trial, comparisons among MP treatments were extracted from the models using "emmeans" package in R (75icrop *et al*., 2018). Degrees-of-freedom were calculated using Kenward and Roger's method (1997), while Tukey's test was used for post-hoc comparisons. Mortality rates were compared by Kruskal-Wallis' test, using Mann-Whitney tests for post-hoc comparisons. Residual plots were inspected to check model assumptions. To assess treatment-related variation in the number of ingested MPs per dead tadpole of Rana latastei, the normality of variables (size distribution of each polymer) was tested using Shapiro-Wilk's normality test. We applied one-way ANOVA (followed by Tukey's post hoc test for multiple comparisons) to test for normally distributed MPs (PVC and HDPE), otherwise (PES and PS) data were tested by Kruskal-Wallis' test (followed by Dunn's pairwise test). We calculated Bonferroni's confidence intervals for the proportion of use (White & Garrot, 1990) to compare the frequency of use of each size class with its availability in the

experimental tanks. Statistical analyses were conducted using R (version 3.2.1; R Core Development R Development Core Team, 2013) and lme4 package (Bates, 2010).

5.3 Results

Italian agile frog tadpoles were highly affected by exposure to MPs, showing significant negative effects in terms of growth, activity, and survival even at low concentrations, while Balearic green toad tadpoles did not.

The models (LMMs) showed that final weights were significantly affected by both treatment and species (treatment x species: $F_{3, 34} = 10.44$, $p < 0.001$; Table 5.1). The weight of frog tadpoles decreased with MP density (Fig. 5.1), while none of the treatments differed significantly from controls for toad tadpoles. However, we recorded a slight increase in final weight for both low and medium MP concentrations (Table 5.1, Fig. 5.1).

	Rana latastei				***Bufotes balearicus***			
Treatment	Emmean (N)	df	Lower CL	Upper CL	Emmean (N)	df	Lower CL	Upper CL
0	69.3 (60)	35	60.2	78.4	84.3 (120)	35	75.2	93.5
1	58.4 (60)	35	49.3	67.5	91.0 (120)	35	81.9	100.1
7	32.5(49)	43.4	22.8	22.8	90.4 (120)	35	81.3	99.5
50	20.9 (47)	45.9	11.2	30.7	74.2 (120)	35	65.1	83.3
Contrast	estimate	df	t.ratio	P-value	estimate	df	t.ratio	P-value
0-1	10.95	32.6	1.824	0.28	-6.65	32.6	-1.108	0.68
0-7	36.82	36.9	5.889	<0.0001	-6.05	32.6	-1.008	0.74
0-50	48.37	38.1	7.694	<0.0001	10.12	32.6	1.685	0.35
1-7	25.87	36.9	4.138	0.001	0.60	32.6	0.10	0.99
1-50	37.42	38.1	5.952	<0.0001	16.77	32.6	2.793	0.04
7-50	11.55	42.2	1.772	0.3	16.17	32.6	2.693	0.052

Table 5.1. Estimated means from LMMs for tadpole weight after exposure to four MP treatments and comparisons for all treatment pairs (N = number of weighted tadpoles.

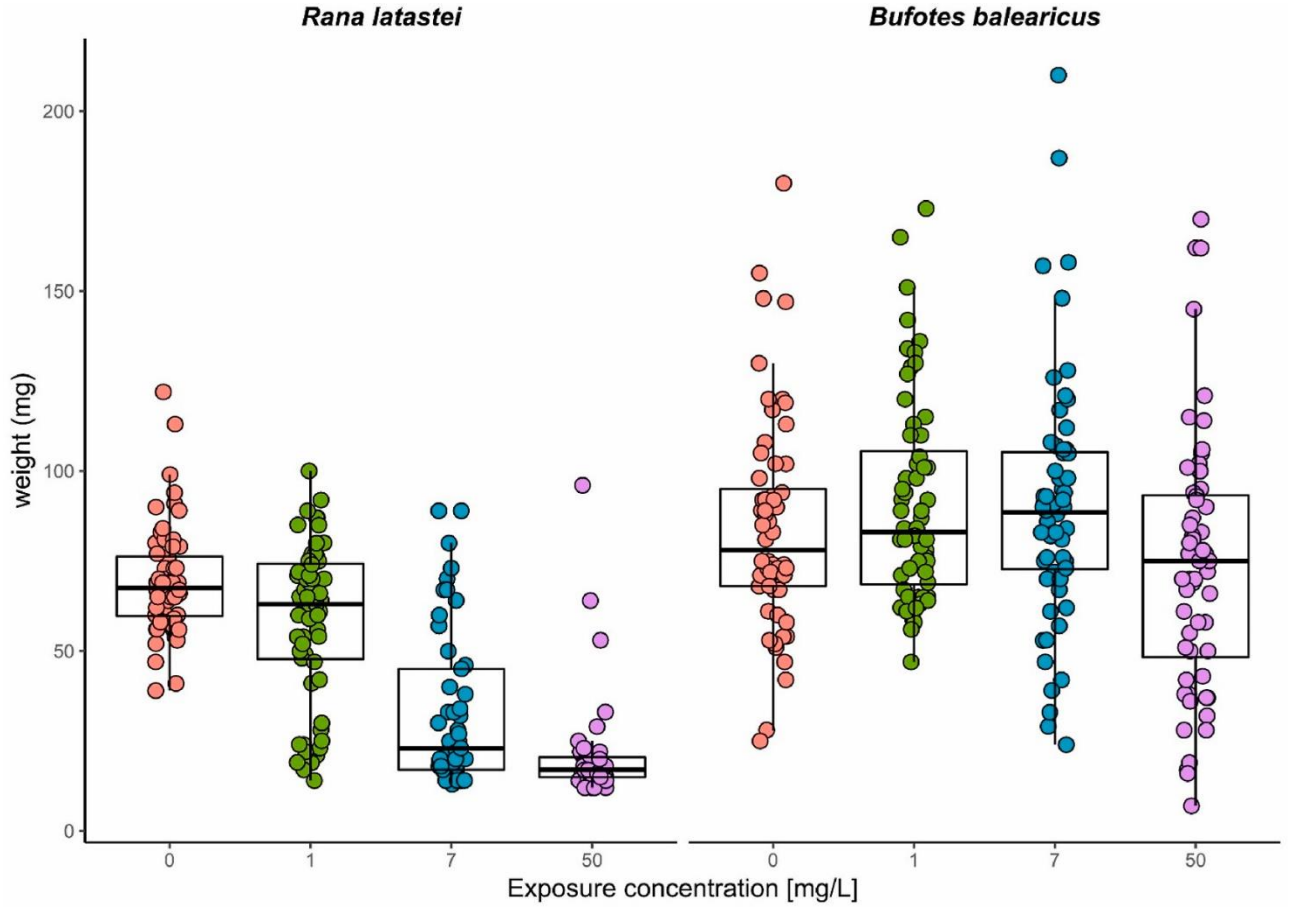

Figure 5.1. Final tadpole weights for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}).

Activity levels varied among treatments and species (treatment x species: $F_{3, 30} = 3.9$, $p = 0.018$; Table 5.2). Activity levels of frog tadpoles exposed to any MP density (treatment) were significantly lower than controls (Fig. 5.2), while in toad tadpoles a reduction in activity level was recorded only for those exposed to the highest MP density (Table 5.2, Fig. 5.2).

Treatment	*Rana latastei*				*Bufotes balearicus*			
	emmean	df	Lower CL	Upper CL	emmean	df	Lower CL	Upper CL
0	0.22	39.2	0.17	0.28	0.70	43.3	0.65	0.76
1	0.11	39.2	0.05	0.17	0.66	39.2	0.61	0.72
7	0.05	39.2	-0.004	0.1	0.66	39.2	0.60	0.71
50	0.03	39.2	-0.02	0.09	0.48	39.2	0.42	0.53
Contrast	estimate	df	t.ratio	P-value	estimate	df	t.ratio	P-value
0-1	0.11	30.2	3.07	0.022	0.036	31.9	0.98	0.76
0-7	0.17	30.2	4.65	0.0003	0.045	31.9	1.23	0.61
0-50	0.19	30.2	5.08	0.0001	0.222	31.9	6.07	0.0001
1-7	0.06	30.2	1.58	0.4	0.009	30.2	0.24	0.99
1-50	0.07	30.2	2.02	0.2	0.186	30.2	5.02	0.0001
7-50	0.02	30.2	0.43	0.97	0.177	30.2	4.78	0.0002

Table 5.2. Estimated means from LMMs for tadpole activity level after exposure to four MP treatments and comparisons for all treatment pairs.

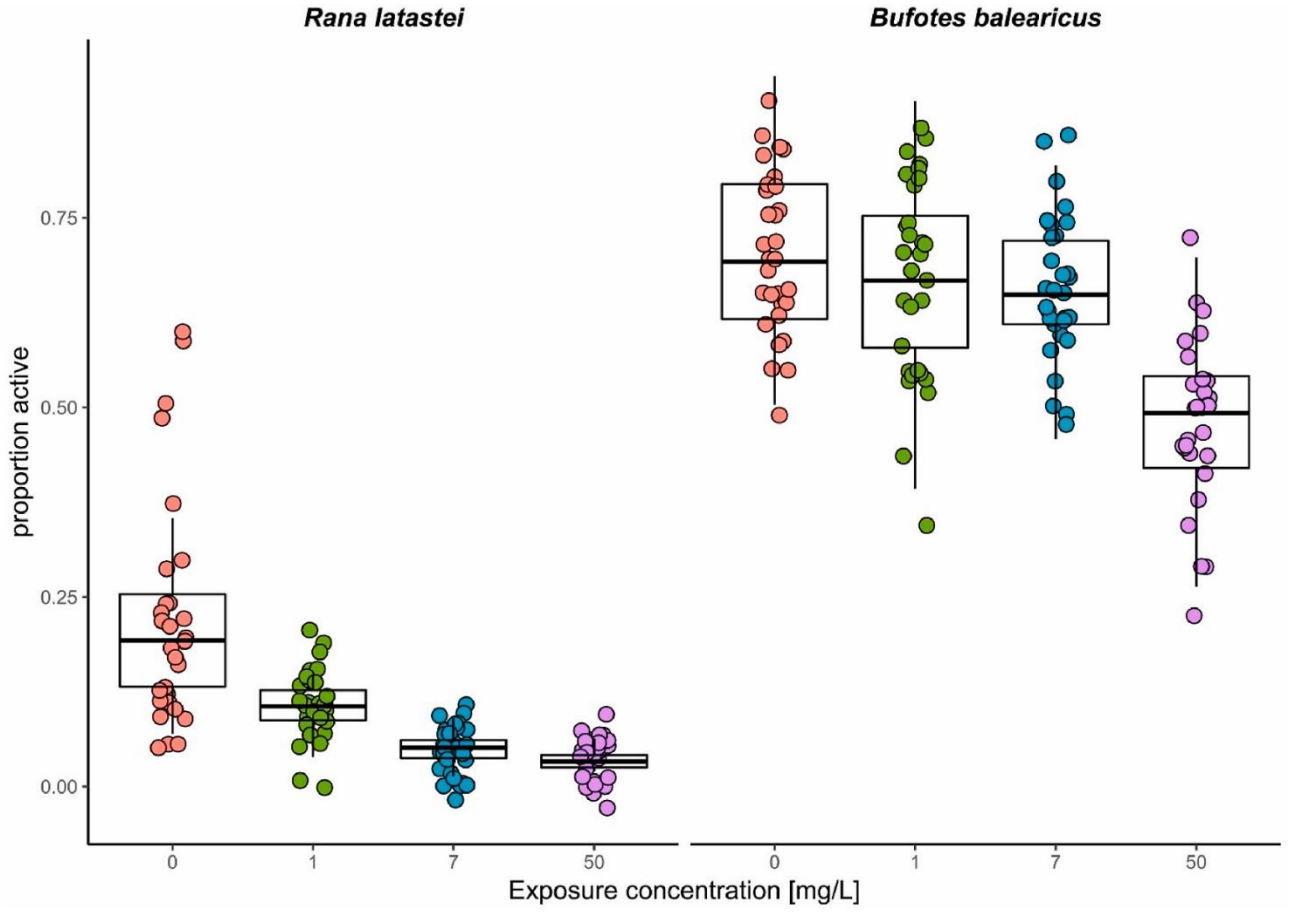

Figure 5.2. Tadpole activity levels for each of the four MP concentrations (0, 1, 7, 50 mg L−1).

Mortality occurred from day 3. Mean mortality rates of frog tadpoles at the end of the experimental period increased with MP concentration (control: 1.67%, SD 1.0; 1 mg L−1: 20.8%, SD 5.5; 7 mg L−1: 63.3%, SD 7.8; 50 mg L−1: 69.2%, SD 4.2), differing significantly between any treatment and controls (Kruskal-Wallis' $\chi 2 = 18.74$, 3 df, $p < 0.001$; $p < 0.01$ for all paired tests). After the recovery period, mortality rate was null in the control tank, while reached 1.94%, 61.11%, and 97.22% in tadpoles from the 1 mg L−1, 7 mg L−1, and 50 mg L−1 treatments, respectively. LC50 at 10 days was assessed as 2.5 mg L−1 ($p < 0.001$; LC10 – LC90 = 0.5–12.6 mg L−1).

In toad tadpoles, mortality rate was null in control and low-density tanks, while it reached 2.5% and 6.7% in the 7 and 50 mg L−1 treatment groups, respectively.

The number of ingested MPs per dead Italian agile frog tadpole was recorded for different treatments, replicates and dates. The smallest, lethal mean intake was 253 MPs (1 mg L−1) while the highest was 760 MPs (50 mg L−1), MPs mostly consisting of PES fibres (76% and 88%, respectively). The treatment did not affect the number of ingested HDPE and PS, while the number of ingested PVC particles increased with MP concentration ($F = 19.7$, 2 df, $p < 0.001$; Tukey's post hoc tests: 1–7 mg L−1: $p = 0.047$; 1–50 mg L−1: $p < 0.001$; 7–50 mg L−1: $p = 0.006$), and the number of ingested PES particles differed significantly between the lowest and highest concentrations ($\chi 2 = 12.6$, 2 df, $p = 0.002$; Dunn's multiple comparison test: $Z = 3.54$, $p = 0.001$; Fig. 5.3).

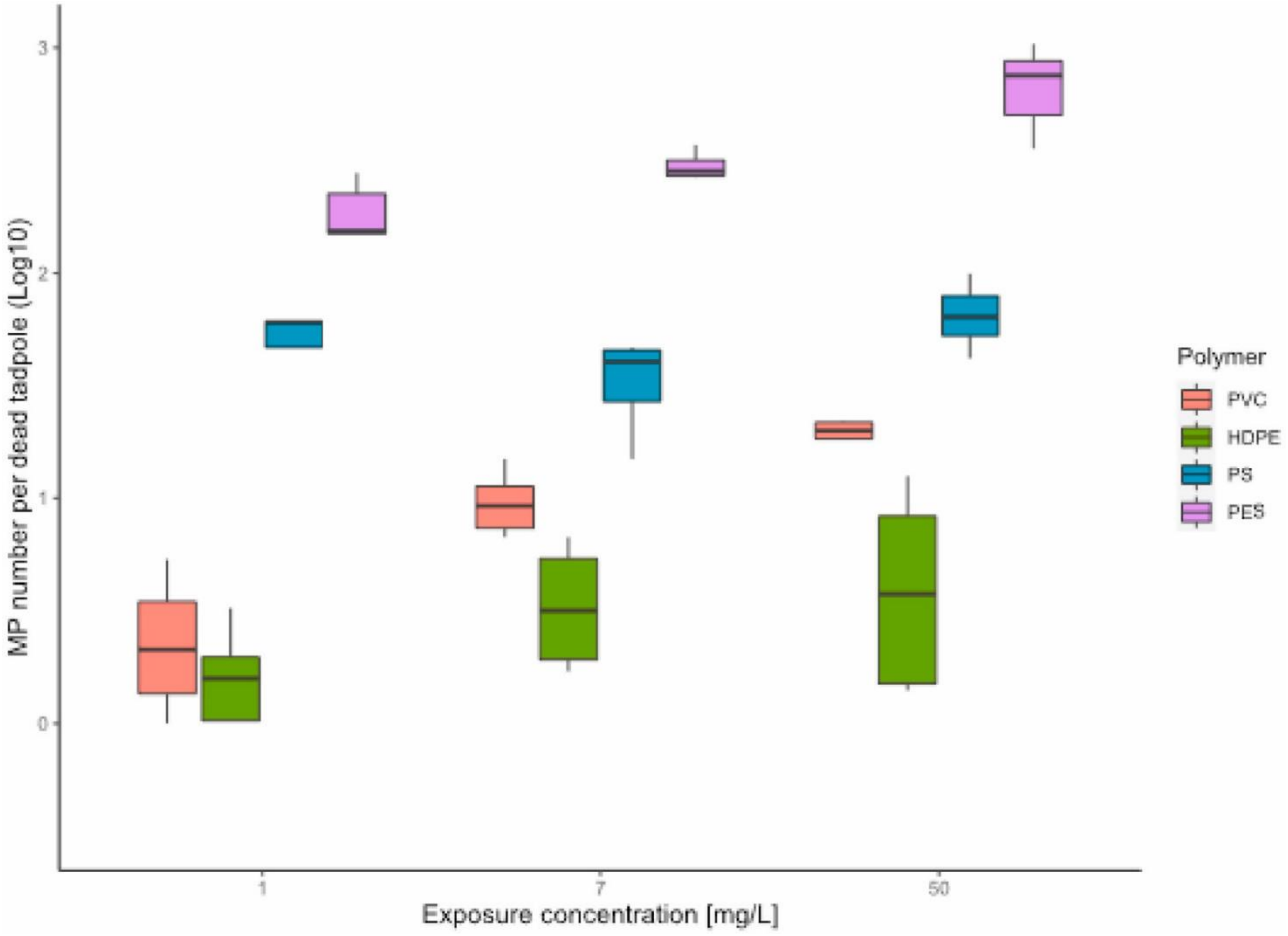

Figure 5.3. Ingested MPs per dead Italian agile frog tadpole for the three exposure concentrations (1, 7, 50 mg L−1). MP numbers are Log10 transformed for visual purposes.

Excluding PES fibres, 50% of ingested MPs were in the size range of 80–245 μm. Mean fragment size was 0.097 mm (SD 0.06) for PS (n = 120), 0.27 mm (SD 0.12) for HDPE (n = 36), 0.24 mm (SD 0.11) for PVC (n = 121); mean length of ingested PES fibres (n = 121) was 0.51 mm (SD 0.36). The smallest ingested MP particles were less than 0.2 mm, while the largest fragment was 0.853 mm; the longest ingested fiber was 1.65 mm. Intake frequency tended to decrease with increasing MP size (Fig. 5.4); in general, tadpoles selected particles in the range 0.2–0.5 mm, while fibres shorter than 0.2 mm and between 0.5 and 0.7 mm were ingested more than expected based on their availability (Fig. 5.4).

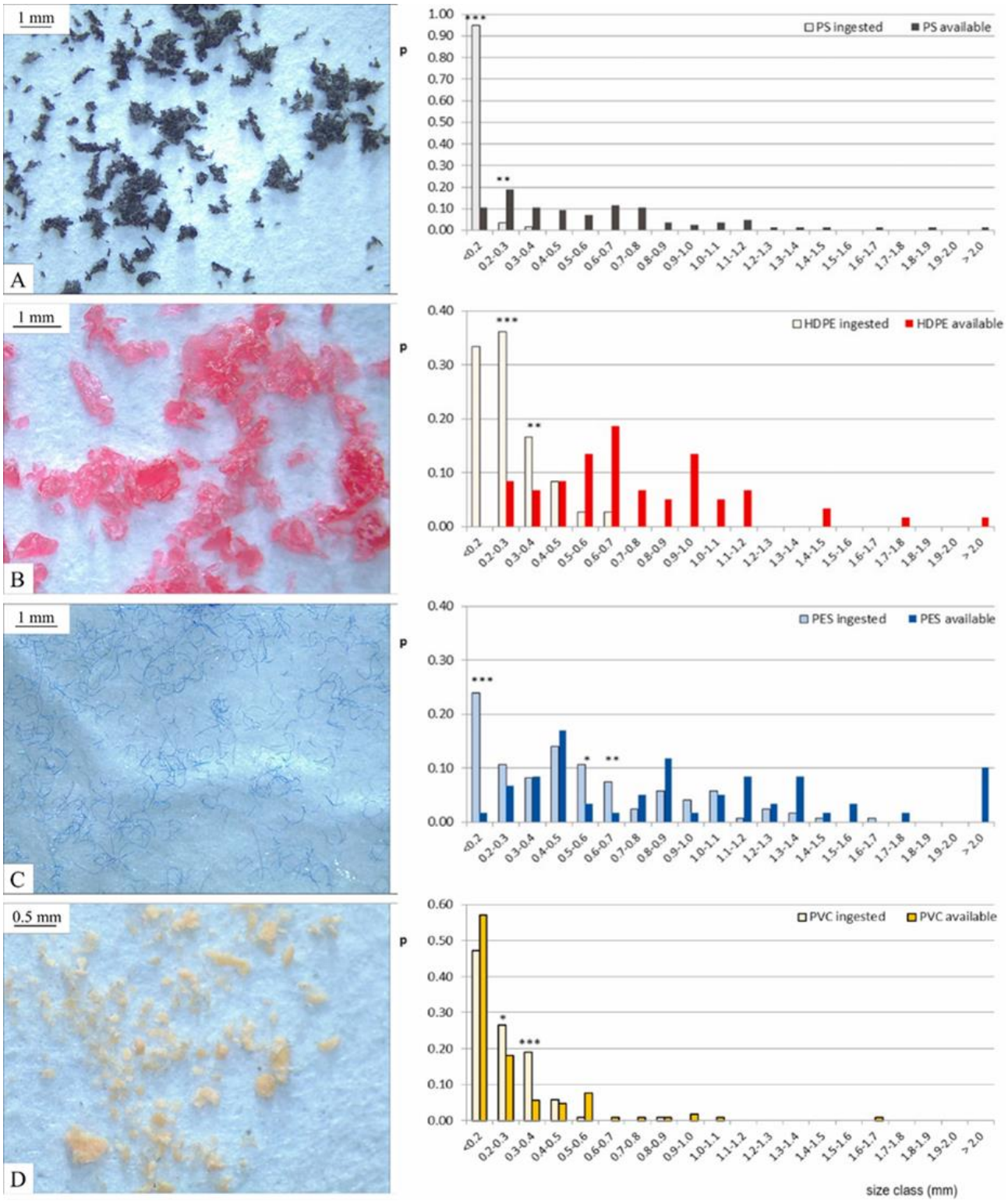

Figure 5.4. Percentage size distribution of MPs ingested by Rana latastei (use) vs. MP availability for all tested polymers (Bonferroni's confidence intervals for the proportion of use; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

5.4 Discussion

Anuran tadpoles usually eat almost continuously, needing a constant food intake, which is pumped with water through the oral cavity and then sorted by size to be channelled into the oesophagus either directly or through the pharynx (Wells, 2007). Food intake is based on random searching, which exposes tadpoles to the ingestion of a wide variety of non-food particles (Kinne *et al*., 2004; Savage, 2009). Moreover, MPs are in the same size range as plankton, increasing the risk for unintentional ingestion (Browne *et al*., 2007).

Despite being the same size and stage and raised using the same protocol, Italian agile frog and Balearic green toad tadpoles behaved and were consequently affected by MP exposure in sharply different ways. As recorded for common goby (*Pomatoschistus microps*) juveniles (de Sà *et al*., 2015), frog tadpoles actively fed on MPs, whilst toad tadpoles gathered around edible food, apparently ignoring MPs. Hence, interspecific variation in the effects of MP exposure seemed to depend on differential attractiveness and uptake rather than efficacy in the egestion of MPs.

The size of mouth opening may be excluded as a cause of variation in feeding behaviour, as toad tadpoles are 82icrophagous, while Rana species usually ingest smaller particles (Savage, 1950). Knowledge is still insufficient to understand whether variation in other mouth features, such as the pattern of marginal papillae, which should have tactile and chemosensory functions and thus may control the flow of food particles towards the mouth (Altig & McDiarmid, 1999), may involve more efficient discrimination of edible particles by bufonid larvae (Bonacci *et al*., 2008). The two patterns may also depend on interspecific differences in behavioural plasticity (Luniak, 2004), with such a typical forest-dwelling species as the Italian agile frog being less able than habitat-generalist green toads to cope with polluted habitats and avoid anthropogenic materials, a hypothesis which is worthy of further testing.

Intake by Italian agile frog tadpoles increased with MP concentration. Mortality rates were by far higher than expected, increasing exponentially since the third day of exposure and with the lowest number of survivors for the highest treatment. Although available data for several taxa show that in freshwater habitats the number of MPs per individual is usually lower (ranging between 0.2 and 24; Hu *et al*., 2018) than that suspected to be lethal for anuran larvae (>200), our data demonstrate that short-term exposure to low, environmentally relevant MP concentrations can affect tadpole growth and survival.

Impaired growth indicated that MPs altered the feeding efficiency of tadpoles, either causing direct damage (Araújo *et al*., 2020a; 2020b), or inducing satiety, as suggested by the lowering of activity

levels with MP density. The post-treatment phase allowed us to observe that the effects of MP entanglement are little reversible, suggesting that even short-term exposure to high MP concentrations may severely affect the reproductive success of agile frogs.

The highest MP numbers in dead tadpoles were recorded for the smallest PS (<0.2 mm) fragments and PES fibres up to 1.7 mm long, suggesting that particle size, morphology or colour may affect MP intake. Fibres are the most spread MPs in freshwaters (Hu *et al*., 2018), where more than 100 fibres per litre of effluent were recorded (Browne *et al*., 2011). Elongated PES fibres may be mistaken with aquatic plants and their morphology may enhance the ingestion of particles longer than the average recorded for the other tested polymers.

The long intestine of anuran tadpoles (>10 times their body length) forms a tight double spiral (Pretty *et al*., 1995), the *ansae* of which may favour the entanglement of fibres. PES fibres have been demonstrated to adhere to the intestinal epithelium of *X. laevis* tadpoles (Bacchetta *et al*., 2021), forming "balls" which are likely to affect gut functions. As a benchmark, the highest number of fibres recorded in a dead Italian agile frog tadpole corresponded to a total length (ca. 340 mm) which was assessed to be more than twice the length of its intestine.

The pattern of mortality in Balearic green toad tadpoles was by far less dramatic and consistent with the previous study by Boyero *et al*. (2019), who reported high (7 out of 8 tested individuals) mortality in common midwife toad tadpoles only when exposed to high MP density (1800 MP mL^{-1}). Dead Balearic toad tadpoles were not analysed, but visual inspection using a stereomicroscope confirmed the ingestion of blue fibres, suggesting that PES played a major role as a cause of mortality also for this species.

5.5 Conclusion

With the aim of addressing the potential adverse effects of water-borne MP on freshwater species, we tested two amphibian species under realistic contamination conditions. We showed that adverse effects of MP depended on the species and that MP intake varied depending on MP characteristics such as density, size, shape, and colour. Our results suggest that MP pollution may contribute to the decline of vulnerable anuran species, highlighting the need for further studies on the effects of these pollutants on the reproductive success of amphibian populations.

To point out the actual threat posed by these pollutants, we stress the need to test a variety of polymers, as to mimic environmental conditions and assess shapes and sizes that are more prone to be mistaken with food by anuran larvae. Concentrations being equal, MP availability or attractiveness to tadpoles may differ between laboratory and natural conditions. Mesocosms may be used to bridge this gap between the laboratory and real ecosystems.

Amphibian populations face many threats, which, acting synergistically, have been imputed of their worldwide decline. We note that the role that most factors play in affecting amphibian abundance and diversity in relatively undisturbed areas such as those usually exploited by Italian agile frogs is still poorly understood. Therefore, we suggest that future studies should not focus only on highly polluted habitats.

6.

ANTI-PREDATOR BEHAVIOURAL RESPONSES OF ITALIAN AGILE FROG TADPOLES (*RANA LATASTEI*) EXPOSED TO MICROPLASTICS

6.1 Introduction

In the last decades, thousands of metric tons of plastic waste have ended up in the environment (Hoellein *et al*. 2014; Geyer *et al*. 2017) because of the constantly increasing human demand for these polymers. Production increase, coupled with the improvement of plastics' chemical and mechanical resistance, has made these contaminants more persistent and potentially more hazardous to the environment (Lithner *et al*. 2011). Microplastic particles (MPs, plastic particles <5 mm in size) are commonly used as raw materials in plastic industries and are contained in several everyday consumer products (e.g., cleaning supplies, toothpaste, synthetic clothes); therefore, these contaminants may be spread in the environment through industrial and domestic wastewaters (Ross *et al*. 2021). Moreover, MPs can originate from the mechanical and biological fragmentation of plastic waste. Recently, MP contamination has become one of the most addressed forms of environmental pollution (Andrady 2017; Chae & An 2018; Li *et al*. 2018). MP abundance, persistence, ubiquity, and small size (Hartmann *et al*. 2019) put these contaminants among the most threatening for a wide range of plant and animal organisms (Anbumani & Kakkar 2018). However, the ecotoxicological effects of MPs on anuran amphibians, one of the most threatened taxa globally (Stuart *et al*. 2004; Becker *et al*. 2007), are still lagging behind, with only a few recent studies highlighting their effects on tadpole physiology and behaviour (De Felice *et al*. 2018; Boyero *et al*. 2020; da Costa Araújo *et al*. 2020a, b; Balestrieri *et al*. 2022). Furthermore, studies on the impact of MPs on anuran species of major conservation interest are still lacking.

Anuran tadpoles are suspensivore/grazer primary consumers and are therefore extremely likely to ingest MPs while feeding (Altig *et al*. 2007; Boyero *et al*. 2020). Ingestion and accumulation of MPs have been proven in tadpoles both under laboratory and natural conditions (Hu *et al*. 2016; De Felice *et al*. 2018; Hu *et al*. 2018; Karaoğlu & Gül 2020; Kolenda *et al*. 2020; Balestrieri *et al*. 2022). Although some studies have shown that tadpoles are capable of tolerating and expelling MPs relatively fast (Hu *et al*. 2016; De Felice *et al*. 2018), significant physiological alterations and high mortality levels have been recorded in tadpoles of *Xenopus laevis* and *Alytes obstetricans* (Tussellino *et al*. 2015; Boyero *et al*. 2020). Exposure to polyethylene (PE) MPs has been proven to cause histopathological damage in Cuvier's foam froglet (*Physalaemus cuvieri*) tadpoles (da Costa Araújo *et al*. 2020a), as well as mutagenic and cytotoxic effects (da Costa Araújo *et al*. 2020b). Finally,

behavioural alterations such as locomotion issues and defective anti-predator defensive response have been observed in tadpoles exposed to PE MPs (da Costa Araújo & Malafaia 2020).

Along with the global decline of amphibian species, there are two main reasons why the effects of MPs on amphibians should be carefully addressed. First, tadpoles are primary consumers in many freshwater ecosystems, and their feeding activity may influence key processes such as primary production or nutrient cycling (Seale 1980; Whiles *et al*. 2013). Secondly, amphibian larvae and adults may represent an important transfer path for these contaminants through higher trophic levels and between freshwater and terrestrial ecosystems (Larsen *et al*. 2016; da Costa Araújo & Malafaia 2021).

The Italian agile frog (*Rana latastei*, Boulenger 1879) is an endangered endemic species occurring in northern Italy, Canton Ticino, Istria, Slovenia, and Croatia (Barbieri *et al*. 2006) in highly fragmented populations. The main threat to this species is the loss of natural habitat caused by urbanization and intensive agriculture, while non-native predator fish and crayfish may have caused the extinction of subpopulations. For these reasons, the Italian agile frog is included in the Annexes II and IV of the Habitats Directive (EC 43/1992) and filed as "Vulnerable" in the IUCN Red List.

Recently, lowered activity levels and development and high mortality rates have been observed in Italian agile frog tadpoles exposed to MPs during early developmental stages (Balestrieri *et al*. 2022).

Although exposure to anthropogenic pollutants has been shown to affect tadpole behaviour in several ways (Rohr & Crumrine 2005; Lavorato *et al*. 2013; Polo-Cavia *et al*. 2016; Sievers *et al*. 2018; Bolis *et al*. 2020), the effects of MPs on defensive responses have been poorly investigated. Behavioural alterations (e.g., reduced activity, defective anti-predator responses) induced by MPs may play an important role in the decline of anuran population, especially those already threatened by other anthropogenic alterations, such as habitat loss and fragmentation, and alien species.

With the aim of assessing the effects of MPs on the anti-predator responses of Italian agile frog larvae, we exposed 480 tadpoles to three concentrations of a MP mix composed of polyester (PES), polystyrene (PS), polyethylene (HDPE), and polyvinyl chloride (PVC). We predicted the impact of MP exposure to be proportional to MP concentration. Considering the little available information regarding tadpole anti-predator behaviour (da Costa Araújo & Malafaia 2020), after MP exposure, we did not expect any unidirectional effect on the intensity of the defensive response. Secondarily, we recorded survival and weight at the end of the experiment, and activity levels during the conditioning period. In this case, we expected a MP concentration-dependent reduction in all variables with respect to controls (Balestrieri *et al*. 2022).

6.2 Materials & Methods

6.2.1 Animal collection and husbandry

In February 2021, we collected 20 fragments of Italian agile frog egg clutches from three ponds located in a natural protected area (Bosco del Vignolo, 45° 13' N, 8° 56' E; Lombardy, N Italy), characterized by several springs, canals, and high forest cover. Water depth was less than 1 m with moderate turbidity and low (<10%) aquatic vegetation cover. Animal collection, husbandry, and testing were authorized by the Ministry of Environment (ISPRA Prot. 1790, 18/01/2021).

Egg clutch fragments were immediately brought to the laboratory and kept in ten, 21-L rearing tanks (2 clutch fragments per tank) filled with dechlorinated tap water until hatching. All tanks were placed in an unheated room under natural light conditions. Mean water temperature ± SD was 20.4 ± 0.7°C throughout the study period. Ten late instar dragonfly larvae (*Aeshna cyanea*) were collected using dip nets from ponds located into the Botanical Garden of Pavia. Predators were individually kept in 0.8-L tubs filled with 0.5 L of dechlorinated tap water. A small piece of mesh was provided as perching site in each tub.

6.2.2 Production of MPs

Following the procedure described in Balestrieri *et al*. (2022), we prepared a MP mix consisting of polyvinyl chloride (PVC) from orange pipes, high-density polyethylene (HDPE) from red bottle caps, polyester fibres (PES) from blue-coloured synthetic fabrics and expanded polystyrene (PS) from black foam food trays. Polymers were then mixed in three concentrations: 1, 7, and 50 mg L^{-1}. The lowest tested concentration was consistent with mean concentrations at the outlet of wastewater treatment plants (6400 MP m^{-3}, Schmidt *et al*. 2020; assuming spherical MPs with a mean diameter of 700 μm and a density of 1 g cm^{-3}, 6400 MP m^{-3} correspond to 1.15 mg L^{-1}). The other two concentrations (7 and 50 mg L^{-1}) followed a geometrical increase and represented the worst-case scenarios based on the highest concentration (450 000 MP m−3) reported by Schmidt *et al*. (2020). To prevent low-density polymer particles from being quantitatively overrepresented, MPs were weighed using a high-precision scale to obtain a constant weight-ratio of 3 PVC : 3 HDPE : 3 PES : 1 PS. All tested concentrations were environmentally relevant (Schmidt *et al*. 2020) and were lower than those previously used for assessing the effects of MP exposure on anuran tadpoles (da Costa Araújo *et al*. 2020a).

6.2.3 Experimental procedure

Two weeks after hatching, a subsample of tadpoles (N = 20, which were excluded from trials) was staged following Gosner (1960) and wet-weighted (mean stage ± SE= 28 ± 0.15; mean weight ± SE = 57 ± 2.6 mg). A total of 480 tadpoles was then selected for the experiment (120 tadpoles per treatment and 120 as controls). Tadpoles were distributed into 24 tanks filled with 8 L of dechlorinated tap water (31.5 × 22.5 × 25 cm; 20 tadpoles per tank, two from each of the 10 rearing tanks), which were grouped into 6 blocks, each including all MP treatment levels (1, 7, 50 mg L−1) and a control tank (0 mg L-1). Within each block, treatments were randomly assigned to tanks. All tanks were checked for dead tadpoles twice a day (at 9 a.m. and 6 p.m.), and a standardized quantity of rabbit chow (170 mg, i.e., ca. 15% of the wet mass of 20 tadpoles at the start of the trial) was provided daily. At the end of the experimental period (when tadpoles were 30 days old), ten randomly chosen tadpoles from each tank (240 in total) were wet weighted with a high precision scale (± 0.01 mg).

6.2.4 Activity

To assess the activity level of tadpoles belonging to different treatments, we recorded the percentage of active tadpoles (i.e., swimming or foraging) during five 10-min sessions at day 3, 5, and 7 of exposure, twice from 9 to 10 am and three times from 3 to 4 pm. All sessions were video recorded using a digital camera (Olympus Tough TG-5), hung up 1 m above the testing tanks. Tanks belonging to the same block were recorded simultaneously. The number of active tadpoles was assessed in a 10-s interval within each minute, comparing consecutive 1-s frames and counting the number of individuals which changed their position inside the tank at each 1-s interval. A total of 2000 observation were made for each tank (20 tadpoles × 10 1-s intervals × 10 min) and the activity level was assessed as (total N of movements / 2000) × 100. Frame to frame movements shorter than tadpole body depth and rotations were excluded from the analysis. The observer was blind with respect to the treatment assigned to each experimental tank.

6.2.5 Defensive response

To obtain olfactory cues for anti-predator tests, dragonfly larvae were fed with Italian agile frog tadpoles. Each predator was provided with the same prey weight (usually two tadpoles, ≈ 130 mg). Before the beginning of every trial (1 h after feeding), we collected 10 ml of water from 5 randomly selected predator tubs. Aliquots were mixed in the same container and 2 ml of the resulting mixture was then used as odour cues for anti-predatory trials. Every day, predator tubs were carefully washed and refilled to keep the water volume constant and prevent signal contamination.

To test for tadpole anti-predator responses after 2 weeks of exposure to MPs, 36 tadpoles per treatment (6 tadpoles per tank) were individually moved into white plastic arenas (15 × 10.5 cm) filled with 250 ml of dechlorinated tap water and left to acclimatize for 15 min. Arenas were shielded by opaque panels and uniformly lightened by spotlights. All trials included a 15-min pre-stimulus (before cue infusion) and a 15-min post-stimulus (after cue infusion) video recording periods, which were recorded using a digital camera (Canon Legria) hung up 1.2 m above the arenas. Each trial included 12 arenas, in which tadpoles from different treatment levels were randomly distributed. To minimize disturbance, the stimuli, either 2 ml of predator cues or water (control), were gently injected with a 10-ml disposable syringe. A total of 144 tadpoles were tested (18 tadpoles × 2 cues × 4 MP treatments) in 2 days (6 trials per day, between 9 a.m. and 14 p.m.). The concentration of predator cues used for behavioural trials (1:125) was consistent with previous studies (e.g., Gazzola *et al*. 2018, 2021; Scribano *et al*. 2020).

All video clips were analysed using ToxTrac (Rodriguez *et al*. 2018), which provides locomotor information by recording the x and y coordinates of the central point of each tadpole every 0.04 s. We used the locomotor variable "total distance," namely the total distance (mm) covered by each tadpole during the trial (pre- and post-stimulus), as an index of tadpole activity level.

6.2.6 Statistical analysis

To analyse the effects of MP exposure on tadpole behaviour (i.e., activity levels recorded within the experimental containers), we ran a linear mixed model (LMM) with the proportion of active tadpoles as response variable. MP treatment, recording session (factor with five levels), and their interaction were included as fixed effects, block as random effect.

Tadpole weights at the end of the experiment were explored using a LMM, with the mean tadpole mass recorded for each tank (experimental unit) as response variable. Treatment and block were included as fixed and random effects, respectively.

Tadpole behaviour during anti-predatory tests was also explored by a LMM, using the proportional change in total distance [pctd = (post-stimulus – pre-stimulus) / pre-stimulus)] as response variable. Tank within block was included as random factor, and predator cue (factor with two levels), MP treatment and their interaction as fixed factors. We used the varIdent function (implemented in R package nlme) to account for unequal variances between predator treatments. The same method was used to explore tadpole total distance before stimulus injection (i.e., in absence of predatory cues), with MP treatment as fixed effect.

LMMs were run using the R package lme4 (Bates *et al*. 2015) and nlme (Pinheiro *et al*. 2022). The estimated means and planned comparisons among MP treatments and predator cues were obtained using the emmeans package (Lenth 2022). The "Anova" function of R package car was used for the analysis of deviance tables (Fox & Weisberg 2019), reporting Wald chi-squared tests. All model assumptions were explored by checking residual distribution against fitted values (Tukey–Anscombe plot) and residual normality against the theoretical normal distribution (quantile-quantile plot).

6.3 Results

Tadpole activity was not affected by treatment ($\chi2 = 6.71$, df = 3, P = 0.08), while was highly influenced by the recording session ($\chi2 = 439.15$, df = 4, P < 0.0001). The interaction between these factors was not significant ($\chi2 = 7.83$, df = 12, P = 0.80). Moreover, during all recording sessions, no significant difference was detected between each treatment and the respective control (lower P = 0.31; Fig. 6.1).

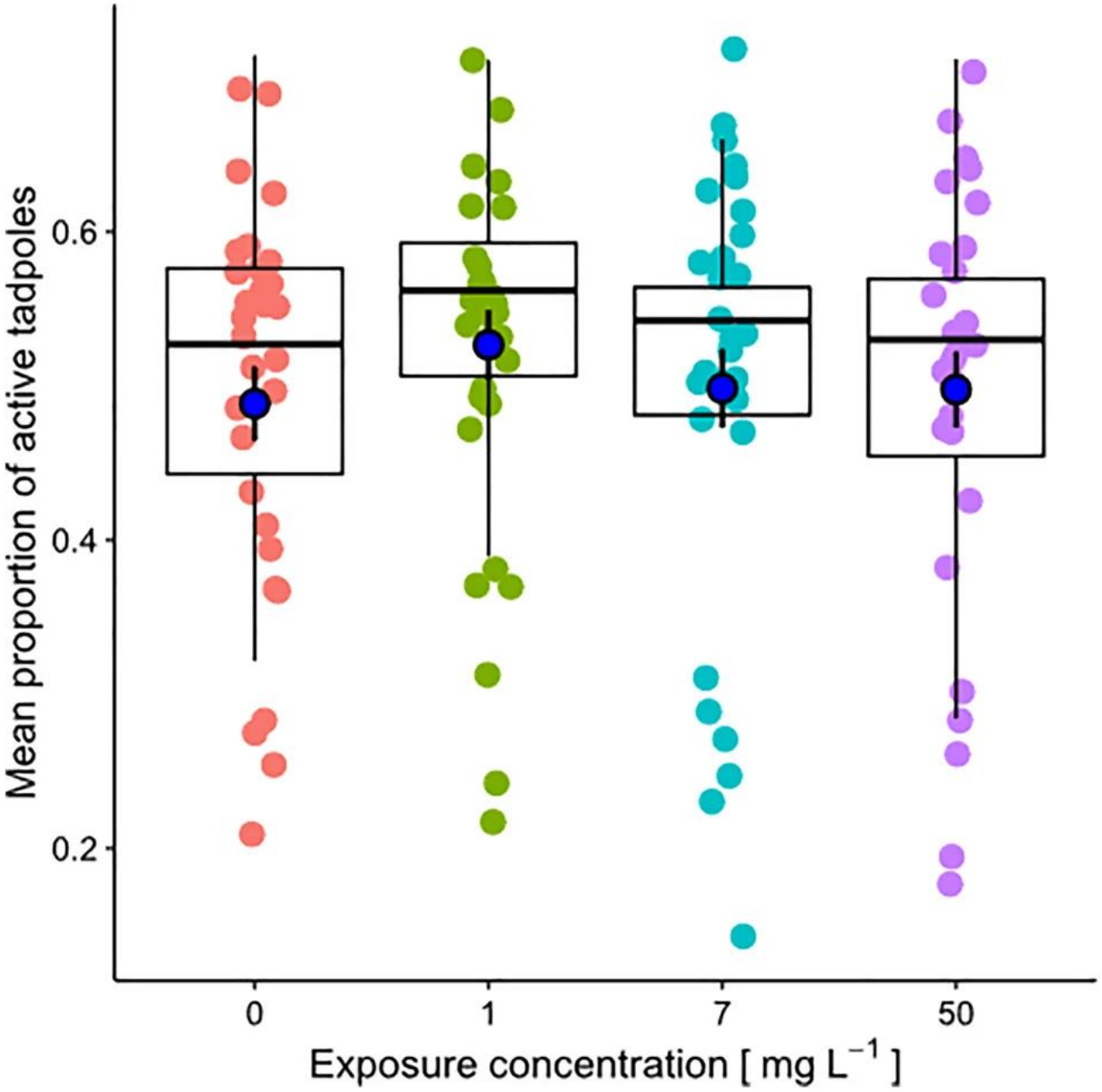

Figure 6.1. Mean proportion of active tadpoles (blue dots represents grand means ± SE) recorded during the entire conditioning period of the experiment for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}). Each coloured dot represents the mean proportion of active tadpoles per tank, during the five periods of recording (N = 30 for each MP concentration); box plots show medians (lines in the boxes), 25% and 75% quartiles (boxes).

During the anti-predator experiment, the total distance covered by tadpoles before stimulus injection was not affected by MP treatment ($\chi2 = 5.86$, df = 3, P = 0.12, Fig. 6.2), although a nearly significant increase was observed when comparing the highest MP concentration and controls (estimated difference: 0–50 = −845± 476, df = 141, t.ratio = −1.77, P = 0.07). Tadpoles belonging to all treatments responded to predator cues by strongly decreasing their total travelled distance respect to controls (water injection; Fig. 6.3). Proportional change in total distance (pctd) was significantly affected by predator cue ($\chi2 = 810.11$, df = 1, P < 0.0001), but neither by MP treatment ($\chi2 = 2.89$, df = 3, P = 0.40) nor the interaction between cue and treatment ($\chi2 = 2.99$, df = 3, P = 0.39). Planned contrasts showed that pctd of predation cue-exposed tadpoles was significantly lower than the respective control (water) for all microplastic treatments (lowest estimated difference = 0.66 ± 0.05, df = 116, t-ratio = 12.79, P < 0.0001; Fig. 6.3). However, pctd of tadpoles exposed to predator cues did not differ between any MP treatment and the control (0 mg L^{-1}) (highest estimated difference = 0.03, df = 131, P = 0.52), that is all five treatments showed a similar defensive behaviour.

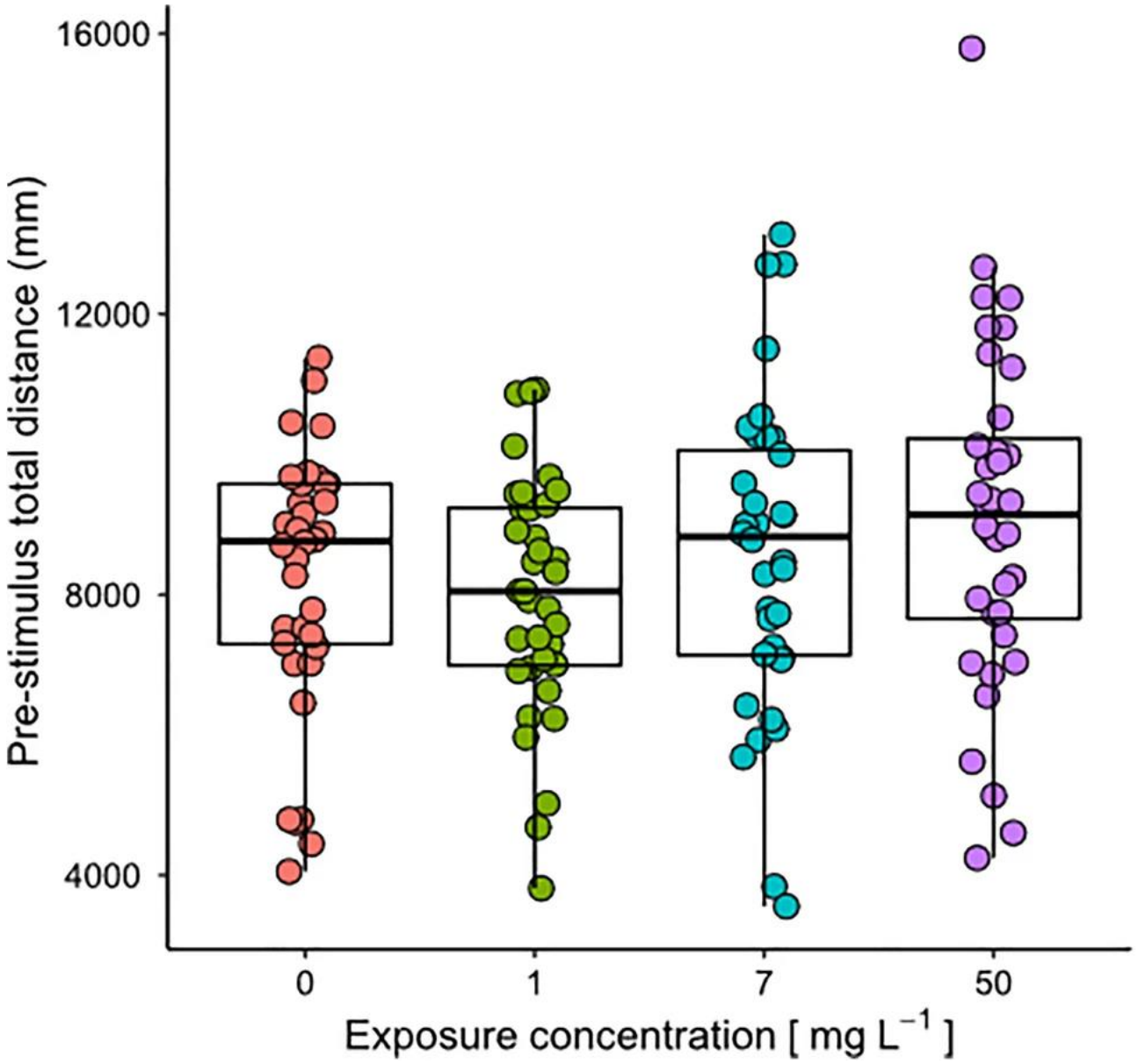

Figure 6.2. Total distance covered by tadpoles before the stimulus injection during the anti-predatory experiment for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}). Coloured dots represent individual data (N = 36 for each MP concentration); box plots show medians (lines in the boxes), 25% and 75% quartiles (boxes).

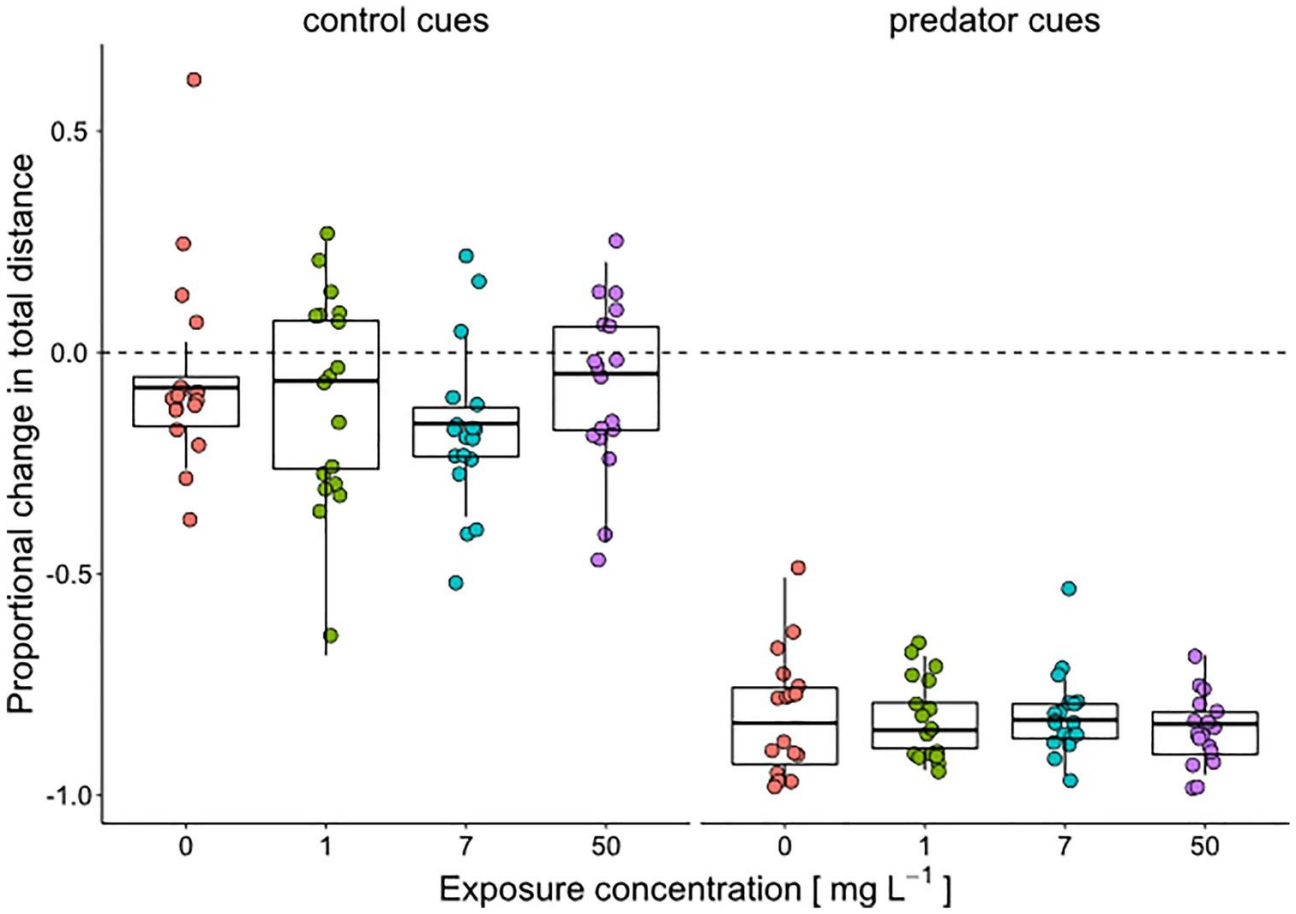

Figure 6.3. Variation in total distance covered by tadpoles after the injection of the stimulus (water as control or predator cue) respect to the pre-stimulus baseline for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}). Coloured dots represent individual data (N = 18, for each MP concentration); box plots show medians (lines in the boxes), 25% and 75% quartiles (boxes). The dashed line indicates a reference point for equal pre- and post-stimulus distance.

Mortality rate at the end of the experimental period was null for all treatments. Tadpole mass (mean ± SE final weights: control = 227.3 ± 10.8 mg; 1 mg L^{-1}= 225.2 ± 8.7 mg; 7 mg L^{-1} =234.2 ± 10.4 mg; 50 mg L^{-1} = 222.6 ± 9 mg) was not affected by long term exposure to microplastic treatments ($\chi 2$ = 2.20, df = 3, P = 0.53), and no difference was observed for any treatment respect to controls (lower P = 0.12; Fig. 6.4).

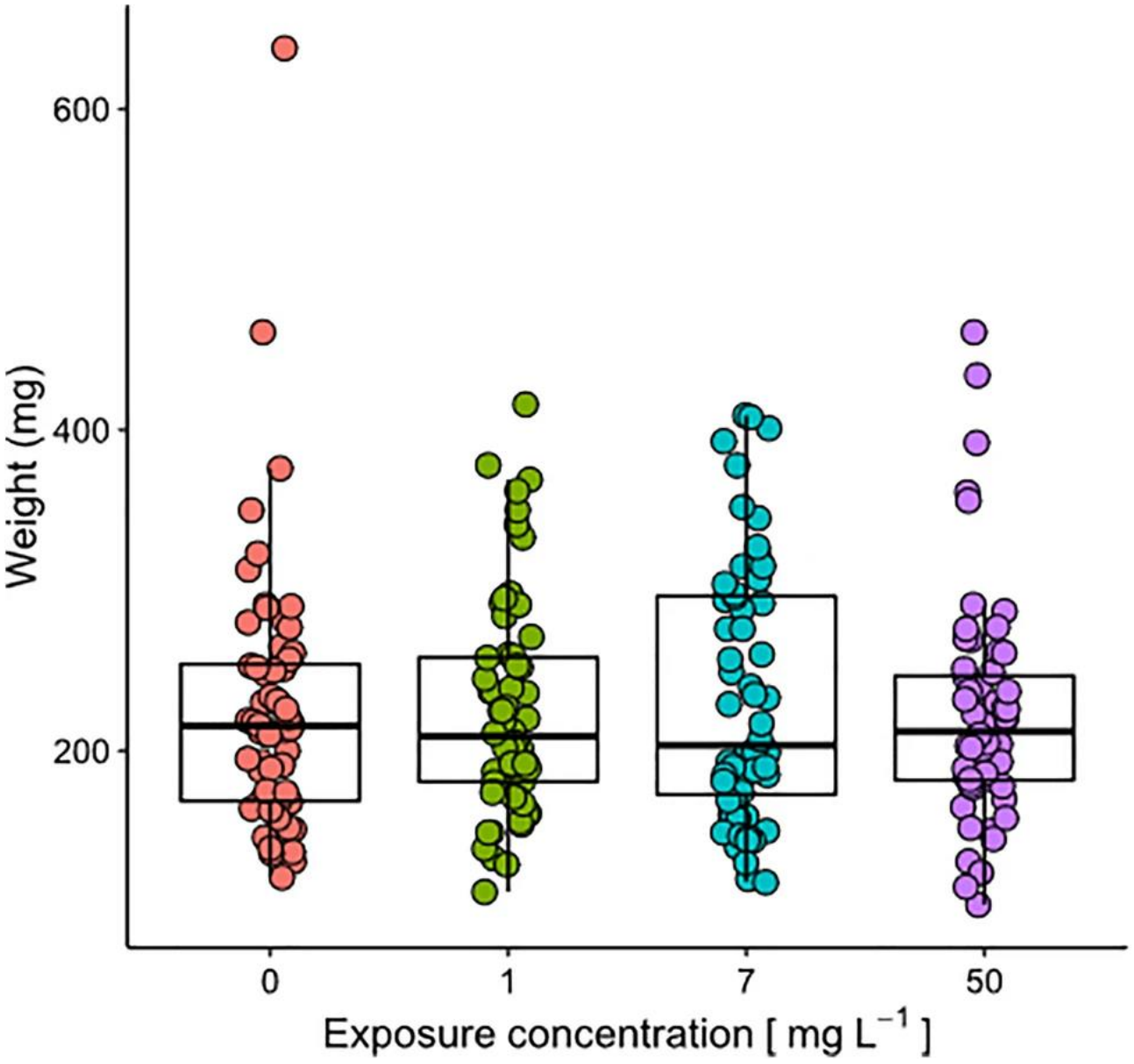

Figure 6.4. Tadpole weights at the end of the experiment for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}). Coloured dots represent individual data (N = 60 for each MP concentration); box plots show medians (lines in the boxes), 25% and 75% quartiles (boxes).

6.4 Discussion

Unexpectedly, MP exposure affected neither tadpole defensive behaviour, nor activity levels and growth. The chemical cues of tadpole-fed dragonfly larvae sharply lowered the total distance travelled by tadpoles. A reduction in activity levels is a widespread anti-predator response of prey species threatened by conspecific-fed, native predators, with which they can be assumed to share a long history of co-evolution (Schoeppner & Relyea 2009; Hettyey *et al*. 2015; Gazzola *et al*. 2018).

Contrary to the tadpoles of Cuvier's foam froglet (da Costa Araújo & Malafaia 2020), exposure to MPs had no effect on the performance of Italian agile frog tadpoles during anti-predatory trials. As the developmental stage of tadpoles was similar for both studies, conflicting results may depend on MP concentration, which was higher in the previous study (60 mg L−1), or tadpole size, Cuvier's froglet larvae being smaller than Italian agile frog's (139 ± 47 mg vs. > 222 mg, respectively, at the end of the experiments), and thus possibly less efficient in egesting MPs. Moreover, the defensive response of froglet tadpoles was assessed using an index of social aggregation, with the aim of testing the anxiogenic effect of MPs, which is why we cannot exclude contradictory results to have been provided by differing experimental protocols (i.e., testing of single individuals vs. groups of 8 tadpoles) or targeted stressor mechanisms (i.e., the choice of which type of response to measure). Another possible explanation might be intrinsic to experimental methods; for example, the concentration of predator odour might have been too high to reveal reliable behavioural differences among MP treatments. Moreover, the behavioural variable we collected might have been not ideal for revealing the potential effects of MP exposure, or again, potential differences were not observable by merely exploring behavioural (Gazzola *et al*. 2015) or life history traits.

The lack of changes in the behavioural defensive responses agrees with the recording of no effects of MP exposure on tadpole growth and activity levels and suggests that either agile frog tadpoles could avoid MP ingestion or efficiently egested them. Based on our previous knowledge on MP exposed tadpoles of the same species, these results were unexpected, as, when tested in a slightly earlier stage of development, tadpoles showed low activity levels, arrested development, and concentration-dependent, high mortality rates (Balestrieri *et al*. 2022).

Two hypotheses can be made to explain these differences. First, as captivity conditions can affect development (Matson *et al*. 2010; Mendelson III & Altig 2016), tadpole size may be a more effective parameter to compare the two experiments. At the beginning of our experiment tadpoles were by far larger (57 ± 1.2 mg vs. 20 ± 1 mg) than those tested by Balestrieri *et al*. (2022), suggesting that, as hypothesized for Cuvier's foam froglet (da Costa Araújo & Malafaia 2020), tadpole size may have

played a major role in driving the effects of MP exposure (see also De Felice *et al*. 2018 about *Xenopus laevis* and *X. tropicalis*).

Secondly, different from the first experiment, which was carried out on 1-week-old larvae naïve to both rabbit chow and MPs, we fed tadpoles for ten days before the beginning of the trials. Since the same food was provided during trials, it is possible that habituation to food enhanced the avoidance of MPs. Since *Rana* tadpoles are opportunistic feeders which usually ingest a wide variety of edible and non-edible particles (Pozzi 1980; Altig *et al*. 2007; Lanza *et al*. 2007), further studies are needed to understand if they can select specific food resources.

The recorded lack of MP effects of growth and activity is consistent with the findings of De Felice *et al*. (2018), who tested *Xenopus laevis* embryos at MP concentrations ranging between 0.125 and 12.5 mg L−1, suggesting that also species-specific differences may account for discrepancies in the results.

In response to the high level of threat affecting amphibians (Stuart *et al*. 2004; Beebee & Griffiths 2005), translocations have sometimes been carried out to enhance the recolonization of suitable areas (Denton *et al*. 1997; Fisher 1999; Sarrazin & Legendre 2000; Thompson *et al*. 2022). Nonetheless, considering the low success rate of reintroduction attempts (e.g., for *R. latastei*: Scali *et al*. 2001; Bernini & Razzetti 2002; Pellitteri-Rosa *et al*. 2008), several authors have questioned the effectiveness of these practices (Burke 1991; Dodd & Seigel 1991; Moritz 1999).

The quality of released individuals is a major factor to consider in any amphibian reintroduction or relocation program (Mendelson III & Altig 2016). Usually, the introduction of egg masses or early life stages is preferred, both for testing the suitability of the ponds for larval development and reducing the impact on donor populations (Buckley & Foster 2005). While translocation success has been reported to be independent from life stage (Germano & Bishop 2009), our results suggest that the size of released tadpoles may affect their probability of survival in waters contaminated by MPs.

6.5 Conclusion

Despite the growing number of studies investigating the effects of microplastics on a wide variety of organisms, their ecotoxicological effects on anuran amphibians have been poorly addressed, as so as those on behavioural responses. Moreover, anuran larvae may act as an entry for MPs in trophic webs and may prime bioaccumulation in higher trophic levels. Accordingly, da Costa Araújo & Malafaia (2021) recorded the transfer of MPs through an experimental food chain including *Physalemus cuvieri* tadpoles, fish, and Swiss mice; in a short time, MPs were transferred along the food chain affecting the activity and anti-predator responses of the highest trophic level (mice). Up to now, the effects of MPs on behaviour have been poorly investigated, leading to contrasting results, possibly depending on laboratory protocols and interspecific variation in susceptibility. In the case of anuran larvae, although the effects of MPs are far to be elucidated, our results indicate that tadpole size, either depending on intra- or inter-specific differences, and/or feeding habituation may reduce the negative effects of MPs in polluted environments. Whether size or the length of the larval stage may also shape interspecific variation in bioaccumulation levels is worth of further investigations.

7.

FINAL REMARKS AND FUTURE PERSPECTIVES

Our preliminary investigations, presented in Chapters 2, 3, and 4, laid the foundation of our main experiment on the effects of microplastics on anuran survival and behaviour. Moreover, every preliminary experiment individually shed some light on different aspects of anuran behaviour.

The experiment described in Chapter 2 provided the first behavioural data recorded for *R. latastei* larvae and stresses the need for further behavioural studies to assess the risk posed by the spread of invasive predators. Tadpoles exposed to fasted dragonfly odour strongly reduced their activity, both in terms of the amount of time spent active and path length covered in comparison to control groups. Predators' diet had a negligible effect on tadpole response, bringing no evidence of the phylogenetic relatedness hypothesis (a conspecific-to-heterospecific gradient in the strength of behavioural responses was expected). The innate or early-in-development recognition of dragonfly larvae is adaptive in *R. latastei* and may increase tadpole survival with relatively low costs but may simultaneously increase the risk of ignoring novel potential threats.

Relying on specific cues, anuran tadpoles lower the costs of defensive behaviour, but may increase the risk of not recognizing novel potential threats such as newly introduced alien species. Therefore, we stress out the need for behavioural studies designed to assess the risk posed to endemic species by the spread of invasive predators such as the red swamp crayfish and spiny-cheek crayfish.

In Chapter 3, we used sinuosity and lateralization in tadpoles exposed to predation threat, both from alien and native predators. Using these variables, we gathered fine details on tadpole escape strategies that usually go unnoticed when only activity-based variables are considered. Once again, tadpoles showed a strong response to native predator, while alien predators, either fed or starved, elicited extremely low or null responses. However, an inter-site gradient in the defensive response of tadpoles, consistent with the relative abundance of *P. clarkii* in tadpole origin sites, was observed. Since crayfish were introduced to northern Italy only recently (<30 years), a genetic-based response to this predator seems unlikely. Therefore, these results suggest that coexistence may enhance behavioural adaptations to a novel predatory threat by associating conspecific alarm cues with predator kairomones.

The information gathered in this Chapter are pivotal in the conservation of endemic threatened species. Although prey may evolve the ability to perform anti-predatory responses to alien predators, the time needed for adaptation may be long enough to cause the extinction of small, fragmented

populations. Therefore, further study on adaptation and evolution in the presence of alien predators are fundamental in the development of new conservation measures. Finally, we suggest that video-tracking techniques, which have seldom been used for assessing tadpole behaviour, offer several opportunities to further investigate predator–prey relationships in aquatic habitats.

In Chapter 4, we used video-tracking techniques provide evidence of the effects of group living on the defensive responses of green toad tadpoles. Our results suggested that the contextual occurrence of conspecifics' cues plays a more important role than previously experienced group size in shaping tadpole responses to predators' cues. Tadpoles reared in large groups strongly decreased their mobility rate when exposed to predators' cues in clean water, but their response was substantially weakened when conspecifics' cues were present; the latter probably provided information on group size, mimicking the dilution effect. The behaviour of tadpoles from the intermediate density treatment, which responded to predation risk in both test environments, may suggest that a minimum concentration of conspecifics' cues, may be necessary for individuals to experience the antipredator benefits of group living.

Our results cannot rule out the possibility that the short-term (30 min) aggregation experienced by tadpoles before the trials may have overridden the previous conditioning period (8 days) altering their activity levels. Further research would be needed to evaluate the differences that may arise with different density treatments' exposure times. To assess both predation risk and conspecifics' density, anuran larvae usually rely on chemical rather than on visual stimuli. As neither predators nor conspecifics were physically present in the test tubs, visual information was not available to tested individuals. The reinforcement of tadpole behavioural responses by visual stimuli, however, is worth further exploring.

In Chapter 5 we addressed the problem of microplastic exposure on our target species using a classic ecotoxicological approach. Exposition to three different concentrations (1, 7, and 50 mg L^{-1}) of an environmental relevant mixture of microplastics (HPDE, PVC, PS and PES) caused negligible effects on green toad tadpoles, while Italian agile frog tadpoles were severely affected both in terms of growth and activity, presenting high mortality rates even at the lowest MP concentration. We showed that the magnitude of MPs' adverse effects may depend on species; however, MP intake varied depending on MP characteristics such as density, size, shape, and colour.

Therefore, we stress the need to test a wider variety of polymers, paying particular attention to their colour, shape, and size, to identify those that are more prone to be mistaken with food by anuran larvae. Another problem that may go unnoticed is that MP availability or attractiveness to tadpoles may also differ between laboratory and natural conditions. We suggest that mesocosms may be used

to bridge this gap between laboratory and natural conditions. Finally, we note that the role played by most factors affecting amphibian abundance and diversity in relatively undisturbed areas is still poorly understood. Therefore, we suggest that future studies should not focus only on highly polluted habitats.

Chapter 6 integrated the methodology used in Chapter 5 with the investigation of the effects of MP exposure on the anti-predatory response of Italian agile frog tadpoles. As in previous experiments, predation risk sharply lowered the total distance travelled by tadpoles; however, MP concentration did not affect tadpole defensive performances. Moreover, neither tadpole growth nor mortality varied with MP concentration. These results were in contrast with our previous experiment and indicate that the intensity of MP effects on growth and development may depend on tadpole size, with large tadpoles being less susceptible to the negative effects of MP exposure.

The effects of MPs on animal behaviour have been poorly investigated. The wide range of contrasting results observed, likely depends on differences in experimental design and interspecific variation in target species susceptibility. In our case, although the effects of MPs are far to be elucidated, our results indicate that tadpole size, either depending on intra- or inter-specific differences, and/or feeding habituation may reduce the negative effects of MPs in polluted environments. For these reasons, whether size or the length of the larval stage may also shape interspecific variation in bioaccumulation levels is worth of further investigations.

Additionally, anuran larvae may act as an entry for MPs in trophic webs and may prime bioaccumulation in higher trophic levels, being simultaneously a transfer vector for MPs from the aquatic to terrestrial environment. Bioaccumulation of MPs has been largely demonstrated in freshwater macroinvertebrates and fishes, although studies on other taxonomic groups are scarce or completely absent. MPs were found in the diet of the Italian crested newtand and tadpoles of different species, while no reptiles have been studied except for sea turtles. The presence of MPs in freshwater birds such as ducks, geese, loons, and cormorants has been confirmed by few studies, while the otter is to date the only freshwater mammal to have been studied. Most of the abovementioned organisms are capable of preying on anurans and can in turn became prey to other predators, including humans. Therefore, bioaccumulation of MPs and associated pollutants in tadpoles should be more carefully investigated considering both the direct implications for anuran conservation and the possible impacts on different organisms and ecosystems.

In conclusion, the present work contributed to the evaluation of the anti-predatory response of tadpoles to native and alien predators (Aim I), highlighting the vulnerability of endemic species to introduced predators. The use of digital video-tracking techniques allowed us to analyse a huge amount of data in a faster and more reliable way compared to classical methods, thus confirming their suitability for application in behavioural studies (Aim II). Finally, the combination of behavioural trials, video-tracking techniques, and ecotoxicology allowed us to identify the effects of microplastics on the growth, survival, activity, and anti-predatory behaviour of anuran tadpoles (Aim III). In light of the need of every drop of knowledge to fight the tsunami of human destructiveness, we hope that this work may represent a useful background and inspiration for future studies on the effects of human induced change on endangered species.

8.

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