

Università degli Studi di Pavia
Dipartimento di Scienze della Terra e dell'Ambiente

SCUOLA DI ALTA FORMAZIONE DOTTORALE
MACRO-AREA SCIENZE E TECNOLOGIE

DOTTORATO DI RICERCA IN SCIENZE DELLA
TERRA E DELL'AMBIENTE

Andrea Gazzola

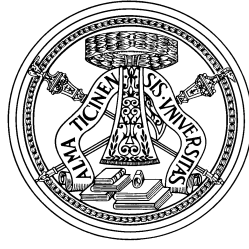
**Predator-induced phenotypic plasticity in
anuran larvae and embryos**

Anno Accademico 2015-2016

Ciclo XXIX

Coordinatore
Prof. Annamaria Picco

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"Evolution is the control of development by ecology"

Leigh Van Valen

"It is very difficult to find a black cat in a dark room," warns an old proverb.

"Especially when there is no cat".

Stuart Firestein

INTRODUCTION

Phenotypic plasticity

The century long quest to understand the relationship between genotype and phenotype has often relegated environmental factors to a minor and passive role (Pigliucci 2001). This perspective has arisen from the common practice of wording evolutionary explanations almost completely in terms of genes while we should focus on the phenotype, which is produced by both the environment and genotype (West-Eberhard 2003) and represents the target of natural selection.

When you consider the influence, during development, of environmental conditions on the phenotype, you are in the world of phenotypic plasticity. A common definition of phenotypic plasticity is the “environmentally sensitive production of alternative phenotypes by given genotypes” (DeWitt and Scheiner 2004). Such a definition is very flexible and lets great freedom in deciding what kinds of traits are plastic. For this reason some scientists prefer to restrict the concept of phenotypic plasticity to developmental processes rather than other ephemeral means of expressing phenotypes, such as physiological or behavioural changes. A different perspective considers plasticity as a general form of environment-dependent gene expression, which can include gene regulatory processes that may have no evident phenotypic effects. As a consequence of this too broad definition, as the environment may influence, to a certain extent, all biological processes, the range of phenomena that may fall in the field of plasticity is very large. DeWitt and Scheiner (2004) do not see this as a problem, as long as the main object of the study, under the name of “phenotypic plasticity”, is focused on the genotype-environment interaction.

Common examples of phenotypic plasticity include the formation of pigment in the skin of humans as a response to ultraviolet radiation (i.e. tanning), and the ability of plants to grow longer stems in shady places to capture more sunlight. Phenotypic plasticity is found in almost every trait, from the level of gene-expression to behaviour and morphology (reviewed in Whitman and Ananthakrishnan 2009). Literature about plasticity is full of dichotomies attempting to describe different aspects of phenotypic variation under environmental influences. As an example phenotypic plastic traits can be regarded as adaptive or non-adaptive, reversible or irreversible (Stearns 1989), active or passive (William 1992), continuously variable or discontinuously variable (Smith-Gill 1983, West-Eberhard 2003). This kind of dichotomy gave birth to a plethora of names when referring to the same underlying phenomenon of plasticity. However, because a given trait can change from one developmental or evolutionary time to another, to avoid confusions West-Eberhard (2003) suggested the use of the single term “plasticity” (as inclusive for all kind of responses) to avoid confusions, as the framework of the phenomena remains the same.

A fundamental tool, used to model and visualize phenotypic plasticity, is the reaction norm, which plots values of a phenotypic trait across two or more environments or treatments (Schlichting and Pigliucci 1998, Sarkar 2004). Strictly speaking, a reaction norm is a function, of whatever shape, and is a property of the genotype; if the function is linear the plasticity of a trait is represented by the slope of the line (a slope different from zero means that the trait under study is plastic for the environments considered; see Fig. 1). This kind of representation is helpful as the magnitude of variation for a given trait is immediately captured, and the idea of plasticity is easy to understand. When the study includes a number of levels of a factor (i.e. experimental treatments) that cannot be ordered as for a continuous variable, the reaction norm can still be useful to visualize the comparison between treatments and the control group and each factor level. Plasticity can be studied by experimental designs with some kind of genetic

structure such as half-siblings families, multiple populations, or a more simply, several individuals of a species randomly assigned to different treatments. This can give a good estimate of plasticity (Whitman and Agrawal 2009).

Plasticity may allow an organism's phenotype to improve its own fitness in a variable environment: when the optimal phenotype is a function of environmental conditions, the evolution of phenotypic plasticity is expected (Houston and McNamara 1992). However, it is worth to clarify that, within behavioural ecology, the interest in plasticity, within behavioural ecology, is not restricted to cases in which it is adaptive. Plasticity can also be maladaptive; this happens when a canalized phenotype (reaction norm is a flat line) is the best phenotype in a given environment (Eshel and Matessi 1998, West-Eberhard 2003, Ghalambor et al. 2007, 2015). This may occur when members of a population are exposed to new environmental conditions (e.g. as a consequence of introductions), or when environment abruptly changes. Besides, plasticity can be neither adaptive nor maladaptive, for example when physiological constraints are imposed by environmental conditions that restrict or modify the expression of a trait with no fitness effect (Gotthard and Nylin 1995, West-Eberhard 2003). These cases need to be considered in the study of plasticity, if we aim to properly understand the evolutionary and ecological basis of phenotypic variation across environments (Pigliucci 2005, Postma and van Noordwijk 2005).

Among the traits commonly studied under the name of plasticity, behaviour deserves a special mention. Contrary to morphological traits behavioural traits are capable of changing rapidly and are often reversible, which is why many behavioural ecologists do not consider appropriate to comprehend them inside the field of phenotypic plasticity (Piersma and Drent 2003, Sih 2004). However, as argued by Sih (2004), such a point of view oversimplifies the two kinds of plasticity (i.e. developmental and behavioural). There are examples of ecologically relevant behaviours that are hardly reversible and need time to be expressed (e.g. species that form

long-mating bonds, dietary specializations, use of refuges by prey species). On the other hand, developmental plastic traits are not always fixed or irreversible. Life history decisions can be reversible and repeated many times during ontogeny (Stearns 1992). Some striking examples can be found in larval anurans, where a diet- or predator-induced morphology can be fully reversed by changing the diet or removing predators (Pfenning 1992, Kishida and Nishimura 2006). The acquisition that behaviour can be legitimately studied under the framework of phenotypic plasticity has been recognized to have useful applications. As reaction norm shows how environmental conditions shape behavioural variation, many of the fundamental questions of behavioural ecology can be properly tested, such as those attempting to evaluate how behavioural variation within populations is related to adaptation, and how selection forces drive the evolution of behavioural differences between species and populations. A reaction norm based approach can easily combine information about the environmental conditions experienced by individuals, the way behavioural responses change as a function of the environment, and the differences among genotypes within the population under study. Moreover, many important topics of behavioural ecology concern traits that clearly manifest plasticity, like learning, optimal foraging, habitat selection, mating strategies, predator avoidance (to cite just a few examples).

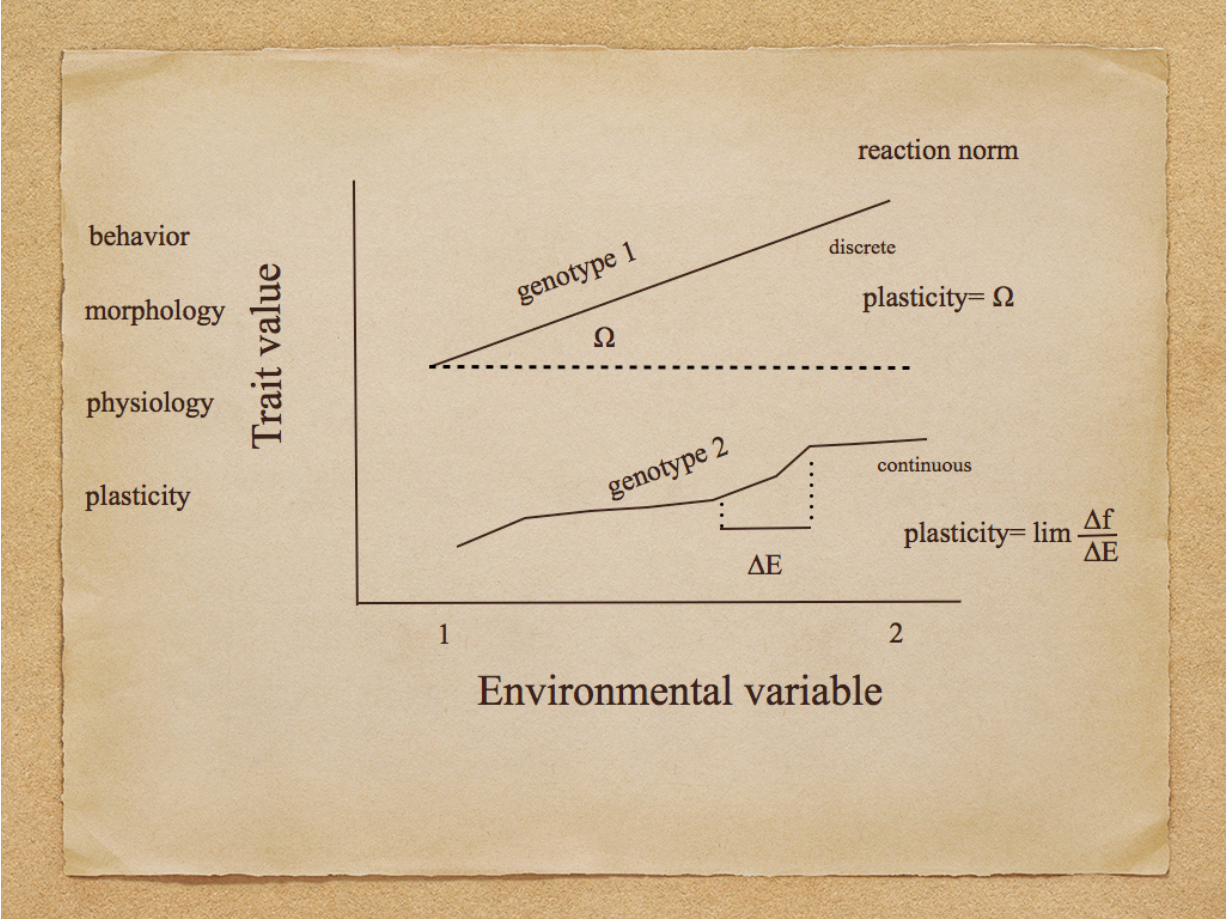


Fig. 1. Graphical representation of the reaction norm

How the risk of predation shapes phenotypes

Predation is a major environmental factor affecting life-history and population dynamics of prey (Lima and Dill 1990, Roff 1992, Agrawal 2003). In order to survive in the presence of predators, prey have evolved many defensive strategies. The pervasive condition of predation acts on prey individuals through the process of natural selection and the lifetime-induction to shape prey phenotypes (developmental plasticity). Some prey defenses can be constantly expressed (constitutive defenses) while others are produced as a consequence of predation risk perception (inducible defenses). Constitutive defenses may evolve when the risk of predation is constantly present in the environment (Edgell et al. 2009), while inducible defenses require a spatial and temporal variation of predation risk to evolve (Ghalambor et al. 2007). Further necessary conditions for the evolution of inducible defensive traits are the presence of reliable predation cues (that allow prey to accurately assess the risk of predation), and the underpinning cost in term of fitness (Ghalambor et al. 2007). For example *Daphnia* with predator-induced spines, useful to deflect predatory attacks, may have reduced fecundity compared to those lacking spines (Black and Dodson 1990). Consequently, in the absence of predators it would be better to not produce spines (Tollrian and Harwell 1998). This example provides evidence of how defenses, that are costly to produce, might favor the evolution of plasticity. Inducible defenses are, in fact, a common form of phenotypic plasticity and have been reported to occur in many organisms (Whitman and Agrawal 2009). Well studied examples are represented by the chemical defenses produced by plants (Berenbaum and Zangerl 1998), the formation of defensive structure in cladocerans (Boersma et al. 1998), the modifications of shell shape in mollusks (Hoverman and Relyea 2007), and changes in body shape and behaviour of fish and anurans (Brönmark and Petterson 1994, McCollum and Van Buskirk 1996). Many studies show that predator-induced plasticity is widespread in embryonic and larval anurans (Kats and

Dill 1998, Bernard 2004, Warkentin 2011). Life-history transitions can be strongly modified by predation risk, an effect that can be better observed in animals with complex life cycles. Amphibians can alter the timing of hatching in order to reduce the risk of predation when the environment of eggs/embryos is not safe (Warkentin 1995, 2000, 2011). The timing of metamorphosis is plastic as well; in many prey species the presence of predators may increase the rate of development to lower mortality risk in pre-metamorphic stages (Relyea 2007).

Size at metamorphosis can also experience plastic changes in response to predation pressure (Relyea 2007, Tejedo et al. 2010); this trait is a critical life-history attribute that greatly affects the fitness of early metamorphosed individuals (Smith 1987, Semlitsch et al. 1988). When potential predators are present, amphibians can metamorphose earlier and increase their chances of survival to the current danger of predation (Moore et al. 2004, Higginson and Ruxton 2009) by reducing the amount of time spent with predators (i.e. exposure to predation risk); alternatively prey can reduce their feeding rate and forage when the risk is lower. Both these strategies may incur the cost of having a smaller size after metamorphosis. Larval size at metamorphosis can also be increased as an effective response to predation, since a larger size can help to avoid mortal attack and gain protection from a large number of predatory insects (Urban 2007). Large body sizes at metamorphosis are proportionally related to large sizes at sexual maturity, and then to an improved reproductive success (Smith 1987, Semlitsch et al. 1988, Berven 1990).

More recently also plasticity of internal organs has started to receive attention. Gonda et al. (2010) performed an experimental study, and found that predation risk has a negative effect on brain growth in common frog tadpoles (*Rana temporaria*); in fact, tadpoles developed relatively smaller brains in presence of caged predators. Interestingly, they found an enlargement of the optic tectum. Although olfaction plays the main role in predator detection, this finding both reinforces the importance of vision for tadpoles to assess predation risk

(Hettyey et al. 2012) and discloses the importance of structural changes, usually hidden to our observation.

Anuran larvae exhibit a wide range of phenotypic changes in response to predation risk (Miner et al. 2005); in particular, morphological responses have been recorded for many species and for different types of predators (Skelly and Werner 1990, Laurila et al. 2001, Lardner 2000, Van Buskirk and McCollum 2000, Relyea and Werner 2000, Relyea 2001). In general, under prolonged exposure to predation risk tadpoles show increased tail depth, decreased body size and changes in tail colouration. Even though the fitness value of these changes is not completely clear, it seems that, when exposed to free hunting predators, tadpoles with predator-induced phenotypes survive longer than not-induced tadpoles (Van Buskirk and McCollum 2000). Deeper tails may enable tadpoles to perform faster accelerations and a bright color may direct predator attacks away from more vulnerable parts of the body (Van Buskirk et al. 2003). However, behaviour is the first line of defense for tadpoles coping with a risky environment. A common defensive response by larval anurans is aggregation, which is likely to both reduce the chance of being selected and attacked by a predator (dilution effect) and increase the probability to detect predators (many-eyes effect). Tadpoles can also move to safer places increasing their own distance from a detected predation cues (spatial avoidance). The most common behavioural defense adopted by tadpoles is the reduction in activity level, and it has been proved to be an effective way to decrease the rate of predation (Van Buskirk and McCollum 2000). In fact, many aquatic predators rely on movement to detect prey and their foraging efficiency can be strongly reduced if prey do not move (Skelly 1994). Activity is closely related to foraging, and, as a consequence, there is a conflicting trade-off between the need to forage and risk of predation. For this reason prey need to carefully choose when to move or when to be static, and their ability to accurately assess the actual level of risk has substantial fitness consequences.

Model system

The great diversity of plastic responses observed in amphibians makes them suitable for investigating not only behavioural and morphological traits, but also the molecular, genetic and physiological basis of phenotypic plasticity (Mori et al. 2005, Fraker et al. 2009, Barry and Syal 2013). In this study I used *Rana dalmatina* (agile frog) tadpoles and embryos, and to a smaller extent *Rana temporaria* (common frog) and *Rana esculenta* tadpoles, as model organisms to study phenotypic plastic traits such as behaviour, morphology and neuronal activity. The choice of agile frog is based on the well-known capacity of this species to produce plastic responses to chemical cue of predation (Lardner 2000, Teplitsky et al. 2003, 2004, 2005a, 2005b, Hettyey et al. 2010, 2011) and also on its availability in northern Italy (Bernini et al. 2004). The agile frog is widely distributed in Europe (Grossenbacher 1997) and in northern Italy (Bernini et al. 2004). It occurs in many habitat types, particularly in mixed deciduous forests up to 800 m above sea level (Grossenbacher 1997). This species spawns in small ponds and ephemeral pools in early spring. Females leave ponds soon after laying up to 500-1000 eggs in a single egg mass, and only 18% of broods results in multiple paternity (Lodé and Lesbarreres 2004). Tadpoles reach metamorphosis after two or three months (Bühler et al. 2007). Embryos are vulnerable to predation by birds, fish, newts and leeches, while larvae are subjected to a wider range of predators: dragonfly larvae, water beetles and bugs, crayfish, fish, snakes and birds.

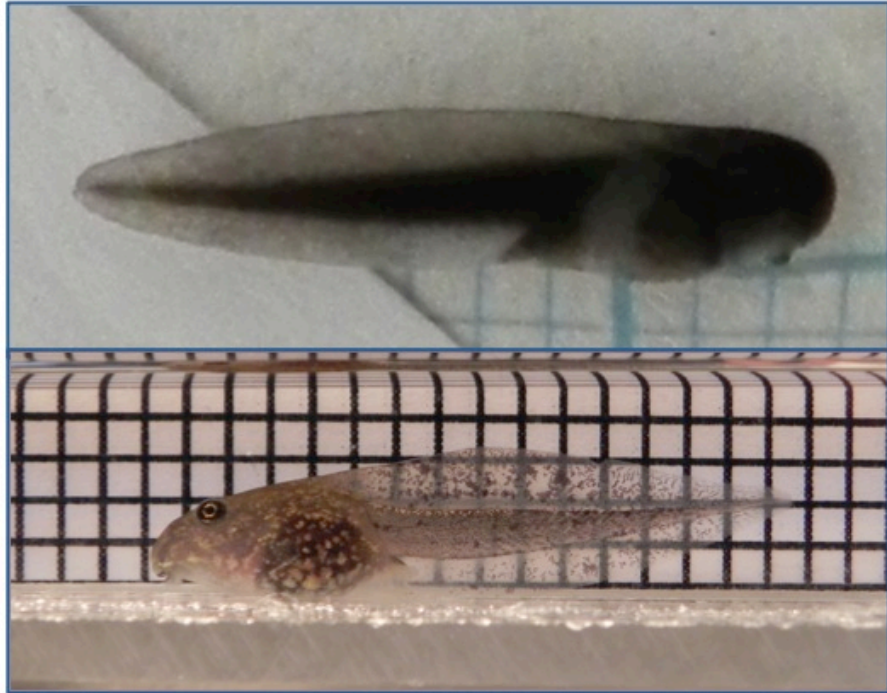


Fig. 2. Photographs showing agile frog embryo (up) and tadpole (down).

Thesis outline

The general aim of this study was to explore predator-induced phenotypic plasticity in frog embryos and tadpoles. Several experimental studies have shown that predator-induced phenotypic plasticity is widespread in embryonic and larval amphibians (Warkentin 1995, Laurila et al. 2001, Kats and Dill 1998, Benard 2004). As response to high predation pressure, many amphibian larvae alter their life-history, morphology, and behaviour. While plasticity in behaviour, morphology and life-history traits in response to predator cues has been thoroughly explored in this group, few works have been devoted at studying the of physiological traits of plasticity (Steiner and Van Buskirk 2008, Barry and Syal 2013) or have attempted to explore the physiological basis underpinning phenotypic plasticity (Maher et al. 2013). Moreover, only few studies, focused on a single species (*Rana pirica*), aimed to investigate the genetic basis of predator-induced morphological defenses (Mori et al. 2005, 2015). As tadpoles assess predation risk mainly through chemical cues dissolved in water, the starting idea of this research was to combine the study of behaviour and morphology to that of activity of tadpoles' olfactory neurons, in order to obtain a further insight into the mechanics of phenotypic plasticity in this system.

In the second part of this study I used behavioural plasticity triggered by predation risk as a tool to explore some open issues of tadpoles behavioural ecology (i.e. prey specific responses to different predator types, risk assessment hypothesis, starvation-predation risk trade-off). Although behavioural plasticity has been widely explored in anuran larvae, the growing literature is providing somehow new and unexpected results. As long as new species are included in behavioural studies, and the experimental designs change over time new data and unpredicted outcomes will help to face this endeavor and to refine our current behavioural models.

Chapter1

A first insight into the proximate causes of predator induced plasticity in anuran embryos

While the ultimate causes of phenotypic plasticity have been widely explored in behavioural studies, there is a lack of information about the mechanics of plasticity. Anuran larvae have been largely used as a model to explore morphological and behavioural plastic traits, but the physiological bases of environmental predator-induced changes in phenotype are poorly known (Fraker et al. 2008, Ferrari et al. 2010). The aim of this study was to explore, along with behavioural and morphological plasticity, the electrophysiological activity of tadpoles' olfactory neurons. As water borne chemical cues bring the main signal that triggers plastic responses in embryos and anuran larvae (Ferrari et al. 2010), olfaction comes to be the primary sensory system for assessing the risk of predation in this group. To explore these issues I exposed *Rana dalmatina* embryos to predator scent (kairomones) produced by *Anax imperator* larvae fed with gammarids. After hatching, at two different developmental stages, I recorded both behavioural and morphological traits of tadpoles together with the activity of olfactory neurons (mitral cells). Our current limitation about the perception of predation risk (i.e. the information available to prey and the way this information is used to evaluate predation risk) is that “an animal's actual perception of predation risk has *rarely* been measured *per se*” (modified from Lima and Steury 2005). To make the process clearer, Blumstein and Bouskila (1996) reviewed the framework of risk perception and came up with three basic steps:

- 1) Information acquisition
- 2) Assessment of the level of risk through information processing
- 3) Combination of the information about risk with information about animal's state (energetic or reproductive) and related environmental information (resources availability).

So spliced, the process of risk perception resembles the general framework, suggested by DeWitt and Scheiner (2004), used to make explicit the general mechanics of phenotypic plasticity. They argued “Plasticity can be seen as a chain of steps going from a particular environmental condition to a particular phenotype. The chain starts with a cue indicating the environment. This cue may lead to another, secondary cue. The cue can be detected by a receptor that generates a signal, which in turn may lead to a second or even third signal. This signal may be transported and stored, after which it has to be read and translated into a process in which the phenotype is formed” (DeWitt and Scheiner 2004). This representation of plasticity comes to be very useful to find out where the “unknown” lies. In anuran embryos-tadpoles system, what we currently know are the end points of the chain: the sources of the cue (kairomones from predators and alarm cue from prey) and the phenotypic product induced by these cues (in term of behaviour and morphology). The real gap lies in the middle rings of the chain, where many unsolved questions deserve to be explored in this prey model. To date, we do not know which types of molecular receptors are involved in risk assessment, and little is known about the chemical structure of water-borne cues triggering plastic changes in larval anurans (Van Buskirk et al. 2014). We also do not have any information on how larval anuran brain computes information coming from water borne cues deriving from predation events (Fraker 2009, Ferrari et al. 2010).

Chapter 2

Developmental and behavioural plasticity in a narrow window of time

Inducible plastic traits of tadpoles can be analyzed by conditioning animals with predatory cues over long periods, as to easily evaluate developmental or morphological measurements (Steiner

2007, Van Buskirk 2001). In this experiment I measured tadpoles' behaviour (hiding and level of activity) and consequent changes in life history traits (development rate and mass) induced by predation risk of common frog tadpoles (*Rana temporaria*). I performed the experiment exposing tadpoles, for a short period of time (two weeks) to the non lethal presence of two syntopic predators: backswimmers (*Notonecta glauca*) and dragonfly larvae (*Anax imperator*). These predators' species show different feeding behaviours and hunting efficiency (backswimmers are visual predators of the open water, while dragonflies hide inside aquatic vegetation and wait for prey), which I assumed to correspond to different degrees of dangerousness during development as perceived by tadpoles (Van Buskirk 2001). A previous study (Van Buskirk 2001), performed standardizing each predator's diet (feeding all predators with the same amount of tadpoles mass), showed that young common frog tadpoles (14-16 days old) seemed to be more sensitive to backswimmers (*Notonecta glauca*), while older tadpoles (41-46 days old) showed a stronger behavioural response to dragonfly (*Aeshna* sp. and *Anax* sp.). These results suggest that tadpoles, during development, are able to differentiate their behavioural responses in relation to predator's identity and tune their behaviour to refine defenses. On this basis I tested if, during a short time span and in a transitional larval developmental stage, different predator types could be perceived as posing different levels of risk. Moreover, I evaluated if life-history traits such as mass and rate of development can be modified in such a short period of time.

Chapter 3

The contribution of thinning to the risk assessment hypothesis in anuran tadpoles

The risk assessment hypothesis (R-AH) (Peacor 2003) states that prey must consider both conspecific density and cue concentration to evaluate the actual level of predation risk. The main prediction of this model is that prey risk-averse behaviour should vary with the intensity of indirect cue (coming from predators or from events of predation) and the density of prey, but should not change when cue:density ratio is constant (i.e. the per capita amount of cue). Another variable, slightly different from density, that may play a role in risk assessment is thinning, i.e. the progressive reduction in conspecific numbers (i.e. density reduction) due to predation. Very few studies have considered prey conspecific density reduction as a mechanism affecting the assessment of predation risk (Van Buskirk and Yurewicz 1998, Relyea 2002). In order to better understand how thinning could modify prey's behaviour and its potential interaction with R-AH, I used agile frog tadpoles, as a prey model, and adopted the following approach: (i) I held cue:density ratio at a fixed value, (ii) I applied a constant removal rate of individuals every other day, and (iii) evaluated the combined effect of both variables on tadpoles' activity. Density was controlled manually by removing tadpoles from assigned treatments, while the chemical cue (obtained from dragonfly larvae fed with tadpoles) was consequently adjusted. Being constant the cue:density ratio, I could assess of the effects of density reduction on tadpoles' defensive behavioural responses.

Chapter 4

Prey energetic state and predation risk can interact to affect behavioural plasticity

The activity level of a prey reflects a trade-off between the need to gain resources and predation risk (Lima, 1998). A reduction in activity lowers the probability to encounter predators but also the amount of time devoted to food intake (Werner and Anholt 1993, Matassa et al. 2016). Theoretical models have greatly improved our comprehension of how prey balance foraging decisions and proposed a context-dependent effect of predation risk (Houston et al 1993, Matassa et al. 2016). The main prediction is that prey energetic state will severely influence when, where and how much prey will forage in the presence of predation risk. Generally a low energetic state can induce prey to increase foraging efforts and become more exposed to predators (Werner and Anholt 1993, Schmitz et al. 2004). To test this prediction I investigated how food deprivation (i.e. energetic state) affects *R. esculenta* tadpoles' capacity to respond to predation risk. I combined a food regime (fed vs. fasted tadpoles) with a cue treatment (absence or presence of attack-released cue), and assessed the effect of their interaction on tadpoles' level of activity.

Chapter 5

Phenotypic responses to alien and native predators by agile frog embryos and tadpoles

The red swamp crayfish *Procambarus clarkii* is an invasive predator widespread in Europe and since 1990, it has been found in numerous ponds and streams of northern and central Italy. *P. clarkii* is an aggressive and resistant species and it seems able to prey upon both eggs and larval anurans. I conducted a laboratory experiment, to explore the effect of predator scent on

life history traits (i.e. hatching time, mass, developmental stage, body measurements) of *Rana dalmatina* embryos, exposing eggs (from two different *R. dalmatina* populations) to kairomones produced by either the invasive predator *P. clarkii* or native *Anax imperator* larvae.

By a second experiment I aimed to explore behavioural antipredator defences (activity level, hiding behaviour and sudden acceleration) of agile frog tadpoles (from two different populations) in response to *P. clarkii* chemical cues and compared them to their defences towards *A. imperator*. To investigate whether chemical cues produced by conspecifics are more effective in shaping the phenotypic responses with respect to those borne from other prey species, tadpoles were exposed to either tadpole-fed or gammarid-fed predators. At the end of the conditioning period, to explore predator-induced morphological changes, I recorded some standard tadpoles' body measures (tail depth, tail length, body depth, body length, tail muscle depth).

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CHAPTER 1

Fear is the mother of invention: anuran embryos exposed to predator cues alter life-history traits, post-hatching behaviour and neuronal activity patterns

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Fear is the mother of invention: anuran embryos exposed to predator cues alter life-history traits, post-hatching behaviour, and neuronal activity patterns

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ABSTRACT

Neurophysiological modifications associated to phenotypic plasticity in response to predators are largely unexplored, and there is a gap of knowledge on how the information encoded in predator cues is processed by prey sensory systems. To explore these issues, we exposed *Rana dalmatina* embryos to dragonfly chemical cues (kairomones) up to hatching. At different times after hatching (up to 40 days), we recorded morphology and antipredator behaviour of control and embryonic-treated tadpoles as well as their neural olfactory responses, by recording the activity of their mitral neurons before and after perfusion of a kairomone solution. Embryonic-treated embryos hatched later and originated smaller hatchlings than control siblings. In addition, embryonic-treated tadpoles showed a stronger antipredator response than controls at

10 (but not at 30) days post-hatching, though the intensity of the contextual response to the kairomone stimulus did not differ between the two groups. Baseline neuronal activity at 30 days post-hatching, as assessed by the frequency of spontaneous excitatory postsynaptic events and by the firing rate of mitral cells, was higher among embryonic-treated tadpoles compared to controls. At the same time, neuronal activity showed a stronger increase among embryonic-treated tadpoles than among controls after a local kairomone perfusion. Hence, a different contextual plasticity between treatments at the neuronal level was not mirrored by the antipredator behavioural response. In conclusion, our experiments demonstrate ontogenetic plasticity in tadpole neuronal activity after embryonic exposure to predator cues, corroborating the evidence that early-life experience can contribute to shaping the phenotype at later life stages.

KEY WORDS: Behavioural plasticity, Defensive behaviour, Kairomone, Life-history traits, Mitral neurons, Neuronal plasticity, Olfactory sensory system, Phenotypic plasticity

INTRODUCTION

Phenotypic plasticity is the ability of a single genotype to modify its phenotype (physiology, morphology, and behaviour) in order to track environmental changes (West-Eberhard, 1989). Thus, phenotypic plasticity can improve fitness, despite its inherent tradeoffs (Scheiner, 1993; DeWitt, 1998; Auld et al, 2010). The concept was first applied to morphological traits (Woltereck, 1909), but virtually any trait can show plasticity in response to environmental variation (Agrawal, 2007). However, though all types of plasticity represent (or result from) altered physiology, the proximate mechanisms involved are still poorly known (Whitman and

Agrawal, 2009, Ferrari et al. 2010, Forsman, 2014). Phenotypic plasticity can be described as a chain process where sensory systems are the first step by which environmental information is passed on to the organisms, and the phenotypic modification is the final product (DeWitt and Scheiner, 2004). To help clarifying how plasticity has evolved, we need to know both the mechanisms producing the plastic phenotype and the selective pressures driving the evolution of such a phenotype.

Predator-prey interactions have proven very useful in understanding the ecology and evolution of phenotypically plastic traits (Benard, 2004; Ferrari et al., 2010 ; Warkentin, 2011). In fact, predator-prey interactions can be viewed as an arm race of sensory systems where both actors are each rewarded when they gain an information advantage over the other. Early detection is the key to achieving such an advantage, which can translate into fitness benefits (Ferrari et al., 2010; Lima and Dill, 1990). In aquatic ecosystems, where information between conspecifics and other species is mostly shared via chemical cues, olfaction is the dominant sensory system and all major groups of aquatic organisms, from protists to amphibians, display defensive behaviours upon detection of predator odour (kairomone) or alarm cues released by damaged and consumed prey (Laurila et al., 1997; Wisenden, 2003; Lass et al., 2005). These chemicals often invoke immediate behavioural responses (and, if prolonged in time, morphological and life-history trait modifications) functioning to reduce the chances of predation (Ferrari et al., 2010).

The literature on kairomone and alarm cue effects is extensive and focused on fishes, crustaceans (e.g. *Daphnia* sp., Tollrian and Harvell, 1999), molluscs (e.g. Lukowiak et al., 2008) and particularly amphibian larvae (Ferrari et al., 2010). Indeed, tadpoles show extreme sensitivity to many different environmental stimuli during their development, and phenotypic effects induced by predator cues can be conspicuous. It is well known that developing embryos and tadpoles modify timing of hatching/metamorphosis as well as morphology and behaviour

in response to the perception of chemical cues of predation (Van Buskirk, 2001; Laurila et al., 2002; Orizaola and Braña, 2004; Ireland et al., 2007; Ferrari and Chivers 2009; Ferrari et al., 2010) and this plasticity may result in increased survival (Mathis et al., 2008).

On the other hand, the neurophysiological changes underlying phenotypic behavioural plasticity induced by predator cues remain largely unexplored (Orr et al., 2007). Life-history, morphological and behavioural changes in tadpoles may be elicited through the olfactory neural system by modulation of e.g. corticosteroid production (Denver, 2009; Maher et al., 2013). However, whether and how kairomones alter the olfactory bulb's (OB) neuronal activity remains unknown. The olfactory bulb (OB) is a phylogenetically conserved cortical structure with a multi-layered cellular architecture (Ramon y Cajal, 1894); in tadpoles it includes two main neuron classes: the mitral cells (MCs) and the local interneurons (granule cells, GCs; Manzini et al. 2003, Nezhlin, 2000), which can be distinguished on the basis of their distance from the surface of the bulb and their electrophysiological properties (resting potential, input resistance, and firing pattern). Morphology, projections, and synaptic interactions of MCs have been extensively described in rodents (Shipley and Ennis, 1996), but little is known about how MCs in amphibians and their larvae integrate electrical inputs evoked by predators' chemical signals.

To bridge these gaps, we ran a series of experiments using clutches of a frog species, the agile frog (*Rana dalmatina*). We exposed embryos to the kairomone of larval predators (dragonfly larvae, *Anax imperator*) for nine days and analysed its effects on key life-history traits (time of hatching, developmental stage, hatchling size) and post-hatching anti-predator behaviour before (ontogenetic behavioural plasticity) and after (contextual behavioural plasticity) a postnatal kairomone exposure (see Stamps 2015 for plasticity terminology). Thirty days after hatching we analysed the neurophysiological responses by *in vivo* whole cell recording of mitral cells (MCs) activity from control and tadpoles exposed to the kairomone at the embryo

stage (embryonic-treated tadpoles hereafter) before (ontogenetic neuronal plasticity) and after (contextual neuronal plasticity) a postnatal kairomone exposure.

We predicted that embryonic exposure to the kairomone of a larval predator would induce an adaptive ontogenetic plastic response in hatchlings (delayed hatching and morphological changes, Moore et al., 1996), ultimately functioning to enhance tadpole survival. Since larval anurans typically reduce their activity level when exposed to predator cues ('freezing behaviour', Skelly, 1994; Mathis et al., 2003; Ferrari and Chivers 2009), we predicted that embryonic-treated tadpoles would show a lower baseline post-hatching activity than controls. In addition, we expected a differential contextual behavioural response according to embryonic experience, i.e. a stronger decrease of activity among embryonic-treated tadpoles compared to controls when postnatally exposed to the same kairomone. Finally, we expected that the neuronal activity of MCs (the main output neurons of the OB, Czesnik et al., 2003; Davison and Katz, 2007) would be modified by embryonic kairomone exposure, since MCs are directly connected to different nuclei involved in defensive responses (Herrick, 1921). Similarly to the behavioural response, we expected a differential contextual neuronal response according to embryonic treatment.

Materials and Methods

Model species, animal collection and housing

The agile frog spawns in small ponds and ephemeral pools in early spring. Females leave ponds soon after laying up to 2000 eggs in a single egg mass, and only 18% of broods show multiple paternity (Lodé and Lesbarrères, 2004). Eggs are vulnerable to predation by birds, fish, newts, and leeches, while larvae are subjected to a wider range of predators: dragonfly larvae, water beetles and bugs, crayfish, fish, snakes and birds; tadpoles of this species are

known to show specific phenotypic responses (either morphological or behavioural) to different aquatic predators (Teplitsky et al., 2005). *Anax imperator* larvae are considered sit-and-wait predators, which require movements, detected either visually or via mechanosensory hairs, in order to elicit a predatory response, since immobile or dead tadpoles do not trigger predatory strikes (Skelly, 1994); dragonflies are important predators of amphibian larvae, but they do not consume amphibian eggs (PG, AG, pers. obs.).

On 1 March 2013, we collected 10 freshly-laid frog clutches from a natural, ephemeral pool near Pavia (Po Plain, Northern Italy). At the same time, twenty late-instars dragonfly larvae were collected in a different stable pond within the University Campus (Pavia, Northern Italy). We can reasonably exclude predator effects on embryos before clutch collection, due both to the ephemeral nature of the original pool, limiting dragonfly colonization, and to water temperature at collection time (+4° C), reducing dragonfly metabolism, and hence kairomone spreading.

We kept all animals in an unheated room with open windows under natural light conditions and mean water temperatures between 8° (March) and 25° C (May). *Rana dalmatina* clutches were individually held in opaque plastic tanks (60 × 60 × 80 cm) containing 150 l of aged tap water and equipped with aerators. After hatching, we fed tadpoles *ad libitum* with rabbit chow and changed 50% of water every other day. *Anax* larvae were held individually in 250-ml plastic cups (complete water change every other day) and were fed with living freshwater amphipod shrimps *Gammarus* sp. every other day. At the end of the experiments (mid May), all survivors (95% tadpoles, 100% dragonflies) were returned to their original sites.

Effects of embryonic kairomone exposure on tadpole life-history traits

The same day of clutch collection, we gently removed ca. 100 eggs from each clutch ball and split them into two samples of ca. 50 eggs (each consisting of a single mass with the egg jelly

intact), which were placed in matched plastic tanks (30 × 20 × 20 cm) containing 8 l of aged well water and equipped with aerators for a total of 20 tanks and 1000 eggs. Embryos were at Gosner developmental stages 12–18 (Teplitsky et al., 2005), and treatment began immediately. In our split-brood design, 10 half-clutches served as a control (infusion of 50 ml of well water with no predator cues), while the other 10 half-clutches were subjected every day to infusion by a syringe of 50 ml of water containing chemical stimuli (kairomone) from 3 different *Anax* larvae fed with *Gammarus* shrimps since the time of collection, but kept fasting for 48 hr before infusion. Both treatments (well water and kairomone) were stopped in all tanks when the first egg in whichever kairomone tank hatched, which occurred nine days after the onset of the experiment. During the treatment period, water temperature ranged from 5 to 10°C, and random temperature checks revealed only a 0.3°C mean daily difference among the 20 tanks. Hatching time of each half-clutch was defined when 50% of the embryos were completely detached from the yolk sac and remained immobile on the substrate surface, and calculated as hours since the start of the treatments. Hatchling developmental stage was determined according to Gosner (1960). Immediately after hatching (stage Gosner 23-24) and 40 days later (stage Gosner 34, range 30-37), 5 tadpoles/tank were collected and preserved in 10% formalin to measure weight and morphological traits.

Tadpoles were weighed three times (Sartorius R200D balance, accuracy 0.01 mg) and photographed (three times) in lateral view within a small glass chamber under standardized condition (light, exposure and distance of the subject set constant for all pictures) by a Panasonic Lumix DMC FZ28 digital camera (10.1 Mp sensor resolution, 3.648 × 2.736 pixels output images). Pictures were processed with ImageJ 1.48 software to determine developmental stage and measure total length, body and tail length, maximum body thickness, maximum tail fin thickness, maximum tail muscle thickness, eye size at hatching and close to metamorphosis. Measurements were taken by the same observer (AG) blind of embryonic

treatment. Repeatability of these measurements calculated on a subsample of 20 tadpoles was very high (r_i or intra-class correlation coefficient varying from 0.94 to 0.99, with F -values ranging from 48.9 to 4439, all P -values < 0.0001 ; Measey *et al.* 2003). Ratios between tail length and body length, between tail thickness and tail length, between body thickness and body length and between tail muscle thickness and tail fin thickness were also calculated.

Effects of embryonic and postnatal kairomone exposure on tadpole anti-predator behaviour

Ten days after hatching (stage 25-26 Gosner), we conducted 10-min trials in experimental tubs ($15 \times 10 \times 10$ cm) using matched pairs of embryonic-treated and control tadpoles from each strain, to assess the activity of the larvae before and after kairomone infusion (20 pairs, 40 individuals in total). Pairs of embryonic-treated and control tadpoles from each strain were inserted in two transparent, adjacent tubs, filled by 250 ml of well water, and left to acclimatize for 15 min. The tubs were visually isolated from each other by a cardboard barrier. The trials consisted of 5 min pre-stimulus recording period (before infusion), a 30 s infusion period (water with kairomone) and 5 min post-stimulus recording period (after infusion). Behaviour (activity) of the tadpoles was recorded, and a decrease in activity was considered a fright response (Petranka and Hayes 1998). To measure activity, we drew on the outer bottom of the tub two perpendicular lines across the centre and counted the number of line crosses during the two observation periods. We considered that a tadpole crossed a line when its entire body was on the other side of the line. During the infusion period, 5 ml of water containing kairomones from 3 different *Anax* larvae kept fasting for 48 h before trials was emptied slowly by a syringe on the side of the cup to minimize disturbance. We could rule out that any contextual effect of kairomone infusion on motility was simply due to mechanic disturbance of syringe infusion. Indeed, the movements of a sample of control tadpoles did not differ before and after a well

water infusion (i.e. without kairomone) at 10 days of age (paired samples t-test on \log_{10} -transformed activity data: $t_{39} = 0.50$, $P = 0.62$). The trials were performed indoor and tadpoles were video-recorded over the whole trial (JVC GZ-MG140E digital videocamera). Videos for 3 kairomone trials at 10 days of age failed and were discarded, thus reducing the sample size to 34 tadpoles. Each tadpole was tested once and then discarded from the next behavioural trials. Thirty days after hatching, we replicated the previous test using pairs of embryonic-treated and control tadpoles (mean stage 29 Gosner, range 28-31, 30 pairs, 60 individuals in total) from each strain, in order to test long-term behavioural differences between embryonic-treated tadpoles and their control siblings. All trials were video-recorded for 5 minutes both before and after the infusion period.

Effects of embryonic and postnatal kairomone exposure on MCs activity of tadpoles

Thirty days after hatching (an age that permits neurophysiological tests on tadpoles to be done), concomitantly to the second behavioural test, we measured, by patch-clamp recordings, the neurophysiological response in MCs of both control and embryonic-treated tadpoles. We first recorded, for both groups, the baseline neuronal activity through perfusion of a bath solution (see below for details). Then, we puffed in front of the OP a solution composed of bath solution mixed with dragonfly kairomone (concentrated 10 mM) to detect the effect of predator chemical cues on MCs activity. We used the minimum sample sizes to obtain statistically meaningful data, while keeping the number of sacrificed animals to a minimum. In total, we used 13 control and 16 embryonic-treated tadpoles (mean stage 29 Gosner, range 27-32) coming from different strains, where samples' size varied according to each experiment performed (see Results), since some neurons exit the range of accepted stability parameter (series resistance) during the recording (see below for further details)

***In vivo* preparation for patch-clamp recordings**

Tadpoles were anesthetized in a mixture of ice and water for dissection. A custom built harp was placed over the tadpole and the preparation was fixed in place in the recording chamber with 3% low-melting point agarose, slice anchor (Warner instruments) and insect pins (Sigma). The skin of the head was cut and the brain was opened along the midline of the recording. The ending part of the tail was also cut to reduce animal movements during experiments. For recording, the tadpole was held submerged in a custom-shaped Sylgard chamber with slice anchor and insect pins and constantly perfused with a fresh external bath solution (see below); all experiments were performed at the room temperature. Heart and breath rhythm could be observed in healthy animals and were monitored through the experiments. The brain was then viewed using Nomarski optics (OLIMPUS BX51WI, Japan).

After allowing the preparation to stabilize for 15 minutes, mitral cell *in-vivo* whole cell recordings were obtained from the OB using the blind patch-clamp method (Blanton et al. 1989). Since it is impossible to visually identify the mitral cells (patched neurons) by this method, we measured some electrophysiological parameters of cells such as the resting potential, the input resistance and firing pattern, which allow to distinguish the MCs from the granule cells (GCs).

Patch electrodes with a tip diameter of 1-2 μm and approximately 7-10 $\text{M}\Omega$ resistance were fabricated from borosilicate glass with a 1.8 mm outer diameter (Hilgenberg, Malsfeld, Germany) using a two-stage electrode puller (Narishige, Tokyo, Japan) and fire-polished. The patch pipette was filled with an intracellular solution (see below). Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in voltage-clamp mode at an holding potential of -70 mV, while cells firing at resting potential were recorded in current-clamp mode. A 200B amplifier (Axon Instruments) was interfaced to pClamp command/record software through a Digidata 1440A analog/digital converter (Molecular Devices; low-pass filter = 10 kHz,

sampling rate = 100 kHz). Voltage pulses were delivered from a microcontroller to a D/A converter and then to the patch clamp amplifier to assess the impedance in the whole-cell configuration. Series resistance was monitored by measuring passive current transients induced by -10 mV hyperpolarizing voltage steps from a holding potential of -70 mV as previously described (Brandalise et al., 2012; D'Angelo et al. 1993). Accepted deviations for this parameter in transient currents was less than 15%. The data were digitalized off-line using an 8-pole Bessel filter, an A/D converter and a PC. Experimental data were analysed using PClamp (Molecular Devices) and Origin (Microcal Software, Northhampton, MA) software.

Solutions used in neurophysiological trials

The composition of the bath solution was (mM): 135 NaCl, 2 KCl, 3 CaCl₂, 1.5 MgCl₂, 10 glucose, 10 HEPES, PH 7.3, osm: 255-260 The pipette solution used for whole-cell recording contained (mM): 5 NaCl, 47KCl, 1.5 MgCl₂, 120 potassium gluconate, 20 HEPES, 1 EGTA, 2 Na₂-ATP, 0.3 Na₂-GTP..

The kairomones (collected with the same procedure used for postnatal behavioural trials) were first dissolved in bath solution (10 mM stock) and then puffed locally in front of the OP. A fast perfusion system was located close to the puffer pipette in order to have a constant, anterior-to-posterior flux of bath solution which rapidly washes the puffed kairomones. In this way the applied kairomones can exert a local action on the olfactory receptor neurons, but thanks to the fast removing from the extracellular space, we avoided a possible significant spill-over in the proximity of the recorded mitral cells.

Short pressure pulses (2.7 kPa; 1.5 s) were delivered by a Picospritzer (PDES-2L npi Electronics, Germany) and were made it in order to eject a small and local amount of kairmones solutions. Patch pipette of 3-4 MΩ were loaded with solution containing kairmones. Kairomone solution was puffed 3 to 4 times per experiment with intervals of at least 3 sec

between one puff and the other. Recordings for both the spontaneous activity and the firing frequency were made from 5 minutes before kairomone applications until 3 to 4 minutes after the last puff application to record the neuronal activity also when kairomone solution was washed out.

Statistical analysis

We first ran linear mixed models (LMMs) (Zuur et al., 2009) to investigate differences in hatching time between embryonic-treated and control clutches, using embryonic kairomone treatment as a fixed factor, and stage at collection of each clutch as a covariate. Clutch of origin (strain hereafter) was included as a random intercept effect.

The effects of embryonic kairomone exposure on embryo development (Gosner stage) and morphology at hatching were analysed by means of LMMs, with embryonic treatment as a fixed factor and strain as a random intercept effect. We dismissed developmental stage at collection as covariate since the factor 'strain' fully captured the variance due to clutch age. We explored the existence of genotype by environment interactions by testing, for each trait, whether treatment effects significantly varied among strains. This was achieved by including a by-strain random slope for the kairomone treatment effect in linear mixed models of morphological traits. Significance of random slope effects and of fixed effects were tested by means of likelihood ratio tests, by computing the difference in $-2 \log$ -likelihood of the model including and the one excluding only the model term of interest, which is χ^2 -distributed. Similar analyses were ran to investigate the effects of embryonic kairomone treatment on tadpole development (Gosner stage) and morphology 40 days after hatching.

LMMs were used to compare activity (\log_{10} -transformed values to improve normality) of embryonic-treated and control tadpoles before and after infusion of water containing kairomone (postnatal kairomone treatment) at 10 and 30 days after hatching. We used tadpole

movements as the response variable, embryonic kairomone treatment and postnatal kairomone treatment as fixed factors, strain and tadpole identity as random intercept effects, and time of day and Gosner stage as covariates. A similar model was run on pooled data from both age groups, but in this case we included age (day 10 or 30) as a fixed effect instead of Gosner stage. Interaction terms between fixed factors were included in the models.

Finally, LMMs were performed to compare various electrophysiological parameters recorded in MCs according to embryonic kairomone treatment, accounting for strain identity as a random intercept effect. For measures recorded both before and after a kairomone perfusion on the same MCs (sEPSCs and firing frequency), models were run by including strain and cell identity as random intercept effects. The reported *n* values refer to the number of cells analysed (one cell for each tadpole).

LMMs were fitted using the lme4 library (ver. 1.0-5) of R 3.0.2 (R Core Team 2013) and SAS 9.3 PROC MIXED. Degrees of freedom were estimated according to the Kenward-Roger method. Parameter estimates and mean values are reported with their associated s.e.m..

RESULTS

Embryonic kairomone exposure modifies life-history traits and post-hatching behaviour of tadpoles

Hatching time was significantly affected by embryonic kairomone exposure ($F_{1,9} = 33.96$, $P=0.0003$) and by embryo developmental stage at collection ($F_{1,8}=50.16$, $P=0.0001$). As expected, late-staged clutches hatched earlier than early-staged clutches (estimate: -14.66 ± 2.07 ; Fig. 1A), but embryonic-treated embryos always hatched later than their control siblings ($419.1 \text{ h} \pm 14.4$ vs. $373.5 \text{ h} \pm 13.3$, c.a. 2 days of difference; Fig. 1A). Most strains exhibited a similar response to environment (Fig. 1B).

Developmental stage at hatching did not differ between control and embryonic-treated siblings or among strains (Table 1). However, embryonic-treated hatchlings were significantly smaller than their control siblings in all morphological measures but body mass (Table 1). The morphological effects of embryonic kairomone exposure were persistent, as similar differences in body traits between control and exposed siblings were still detectable at 40 days of age, when embryonic treated tadpoles showed also a lower developmental stage compared to controls (Table 1). At hatching, we detected statistically significant genotype by environment interactions in most body traits, which however disappeared at 40 days of age (Table 1). A close scrutiny revealed that such genotype by environment effects on body traits at hatching were due to a single strain (strain L) showing a stronger response to embryonic kairomone treatment than the other strains.

Tadpole activity at 10 days of age significantly differed according to both embryonic treatment and postnatal kairomone exposure (Fig. 1C, Table 2), but the effect of postnatal kairomone exposure did not significantly vary according to embryonic treatment (no significant embryonic \times postnatal treatment interaction, Table 2). In this model, we accounted for the confounding effects of time of day (daily tadpoles' activity regularly decreased from morning to evening) and developmental stage (Table 2). Embryonic-treated tadpoles exhibited lower motility than their control siblings both before and after kairomone infusion, which had however a significant suppressive effect on the activity of both groups (Fig. 1C, Table 2). Similarly, at day 30, activity dramatically decreased following kairomone infusion, while the effect of the embryonic kairomone exposure disappeared (Fig 1D, Table 2). Again, there was no significant embryonic \times postnatal treatment interaction (Table 2). The global model run on pooled data from both age groups showed that the overall effect of embryonic kairomone exposure on tadpole movements significantly differed between day 10 and day 30 (age \times embryonic treatment interaction in Table 2), corroborating the results of the tests run separately

on each age group (Table 2). In addition, the effect of postnatal kairomone infusion significantly differed between day 10 and day 30 (age \times postnatal treatment interaction in Table 2), with movements after kairomone infusion decreasing more markedly at day 30 than at day 10 (Fig 1C,D).

In short, ontogenetic behavioural effects of embryonic kairomone exposure were detectable within the first ten days from hatching, diminishing rapidly over time thereafter, while the intensity of the contextual behavioural response to postnatal kairomone exposure increased with age in both tadpole groups.

Embryonic kairomone exposure induces variation in MCs voltage-dependent currents during *in vivo* whole cell recording experiments

The image of a typical *in vivo* recording preparation is shown in Fig. 2 A, where the olfactory pit (OP), the olfactory epithelium (OE), the OB, and the telencephalon can be seen. Mitral cells (MCs) were located below the granule cells, GCs, in a single lamina, the mitral cell layer, with their dendrites spanning the OB, and showed more depolarized resting potentials compared to granule cells (GCs) (-53.2 ± 1.12 mV, $n = 13$ vs. -66.5 ± 1.55 mV, $n = 4$; mixed model, $F_{1,15}=35.86$, $P<0.0001$, Fig. 2B,C) in agreement with previous studies (Chen and Shepherd 1997, Heyward *et al.* 2001, Scheidweiler *et al.* 2001, Arruda *et al.* 2013). In MCs, action potentials evoked by threshold current injections occurred with a long delay of up to 300 milliseconds ($n = 13$, Fig. 2B). Moreover, a voltage clamp analysis of passive membrane properties obtained by -10 mV hyperpolarizing voltage steps from a holding potential of -70 mV (Fig. 2D, see the “*In vivo* preparation for patch-clamp recordings” section in Materials and Methods for further details) showed that MCs (of control tadpoles) had an input resistance of 184.7 ± 17.7 M Ω ($n = 13$), which is significantly lower than that of the GCs (315.5 ± 7.9 M Ω ,

n = 4; mixed model, $F_{1,14}=9.13$, $P=0.009$), according to previous studies (Chen and Shepherd 1997, Scheidweiler *et al.* 2001, Arruda *et al.* 2013, Heinbockel *et al.* 2004).

Resting potential of MCs did not differ between control and embryonic-treated tadpoles (respectively, -53.2 ± 1.12 mV, n = 13 and -54.2 ± 1.03 mV, n = 13, mixed model, $F_{1,24}=0.43$, $P=0.52$, Fig. 2C). Similarly, the input resistance of MCs of embryonic-treated tadpoles did not exhibit a significant change compared to controls (210.7 ± 13.7 M Ω , n = 16; mixed model, $F_{1,26} = 1.20$, $P = 0.28$).

Responses to +10 mV depolarizing voltage steps from -70 mV to -10 mV revealed an inward rectifier current and a deactivating outward current in MCs (Fig. 2E). The initial inward current was significantly smaller in control tadpoles (37.6 ± 5.6 pA; n = 13) than in embryonic-treated ones (107 ± 13.9 pA; n = 16; mixed model, $F_{1,25}=16.97$, $P=0.0004$; Fig. 2F). The outward current was also significantly greater in amplitude in embryonic-treated tadpoles compared to controls (40.2 ± 4.6 pA; n = 16 vs. 18 ± 1.1 pA; n = 13; mixed model, $F_{1,27}=17.70$, $P=0.0003$, Fig. 2F). Thus, embryonic kairomone exposure did not modify the passive electrophysiological properties of MCs, but affected their active properties.

Embryonic and postnatal kairomone exposure elicits an increase in the frequency of spontaneous activity of MCs

When perfused with bath solution as a control, MCs of the embryonic-treated tadpoles showed a significantly higher baseline frequency of spontaneous excitatory postsynaptic currents (sEPSCs) compared to control MCs (4.84 ± 0.28 Hz n = 16, vs 1.47 ± 0.09 Hz, n = 13; Fig. 3A,B and Table 3). When bath solution with dragonfly kairomone was puffed close to the OP during the recording section (see Methods section), the frequency of spontaneous activity significantly increased in both groups (control cells: 2.4 ± 0.11 Hz, n = 11; embryonic treated cells: 7.8 ± 0.24 Hz, n = 15, Fig. 3C and Table 3), but this increment was much higher for

embryonic-treated MCs than for the control group (embryonic \times postnatal treatment interaction in Table 3). However, both embryonic-treated and control olfactory receptor neurons (ORNs) expressed kairomone-activated receptors, indicating that tadpoles possess intrinsic sensitivity to predator chemical cues.

To investigate the mechanism responsible for the increased frequency of sEPSCs among embryonic-treated MCs, we performed a quantal analysis (Nusse et al., 2001; Granseth and Lindstrom, 2003; Sola et al, 2004). Higher frequency of sEPSCs among embryonic-treated MCs may depend on different mechanisms, including: 1) a greater number of synaptic contacts between olfactory receptor neurons (ORNs) (presynaptic neurons) and MCs (postsynaptic neurons); 2) an increase in the number of neurotransmitter release sites per synapse;. The mean amplitude of these sEPSCs was 5 pA, determined by the peak of a Gaussian curve fitted to the size distribution (Fig. 3D,E). A few large sEPSCs could be multiples of this unitary amplitude. The histograms exhibited similar distributions in both control and embryonic-treated MCs, but with a significantly higher peak (i.e. a higher frequency at which the events occur) in the latter cells (Fig. 3E). In response to puff application of bath solution with dragonfly kairomone, the Gaussian distribution was maintained, but the peaks increased for both control and embryonic-treated cells (Fig. 3D,E). The fact that the frequency of events, but not the amplitude, was enhanced by kairomone perfusion was consistent with an increase in the number of synaptic contacts per MC or a possible increase in the firing frequency of the presynaptic ORNs. An increase in the number of neurotransmitter release sites per synapse would instead be associated with a Gaussian multipeak, with peaks approximately multiples of each other (Paulsen and Heggelund, 1996).

Embryonic and postnatal kairomone exposure elicit an increase in MCs firing rate

MCs of the embryonic-treated tadpoles showed a significantly higher baseline firing frequency than MCs of control tadpoles (0.74 ± 0.09 Hz, $n = 15$ vs. 0.12 ± 0.07 Hz, $n = 13$; Table 3, Fig. 4A,C), as was expected from the difference in network activity observed in the sEPSCs analysis. With local puff application of bath solution with dragonfly kairomone, firing rate increased markedly in both groups of MCs (control cells: 0.96 ± 0.14 Hz, $n = 11$; treated cells: 5.32 ± 0.21 Hz, $n = 13$, Table 3, Fig. 4A,B), but again the difference in firing rate between control and embryonic-treated cells widened further (embryonic \times postnatal treatment interaction in Table 3, Fig. 4C). Kairomone solution also induced frequent bursts of action potentials in the embryonic-treated group (5.2 ± 0.9 APs, $n = 12$, Fig. 4B), but not in the control group (Fig. 4A). Two minutes after the last application was terminated, the kairomone solution was fully washed-out, so that bursting behaviour ceased and the firing frequency returned to values not significantly different from those recorded before kairomone application (control cells: 0.25 ± 0.04 Hz, $n = 5$; embryonic-treated cells: 1.15 ± 0.12 Hz, $n = 8$, Fig. 4A,B; mixed model with wash-out vs. baseline firing frequency and embryonic treatment as a fixed factors, run on the subsample of cells for which we were able to record both baseline and post wash-out firing frequency; effect of wash-out: $F_{1,17} = 3.04$, $P = 0.10$; effect of embryonic treatment: $F_{1,20} = 18.6$, $P = 0.004$; interaction: $F_{1,17} = 1.26$, $P = 0.28$).

DISCUSSION

We demonstrated that agile frog embryos exposed to larval predator cues markedly modified various phenotypic traits, including hatching time, body size and post-hatching motility, in response to the perceived risk of future predation. Remarkably, embryonic exposure to larval predator cues altered also the activity of both pre- and post-synaptic olfactory neurons: we found a significant difference in the baseline sEPSCs and in firing rate between mitral neurons

of control and embryonic-treated tadpoles, and this difference was magnified after a local puff kairomone perfusion. Thus, the contextual effect of predator stimulus was strongly affected by the embryonic predator experience. Similarly, embryonic-treated tadpoles showed a lower baseline motility than control siblings at 10 days of age, but noteworthy, at this age both tadpole groups responded to postnatal kairomone exposure in a similar way. In addition, in behavioural trials at 30 days of age, we detected a similar baseline motility between embryonic-treated and control tadpoles, which strongly decreased after postnatal kairomone exposure, irrespective of embryonic treatment.

A decrease in tadpole activity after exposure to predator cues is consistent with an adaptive antipredator response because dragonfly larvae can most likely detect and capture actively moving prey (Skelly, 1994; Mathis et al., 2003, 2008). The contextual behavioural response of agile frog larvae to dragonfly kairomone might thus be, to a large extent, innate (Lima and Dill, 1990; Scheurer et al., 2007; Epp and Gabor, 2008), since control tadpoles displayed the typical antipredator behaviour upon their first postnatal experience with the odour of the predator (Fig. 2C,D).

MCs of embryonic-treated tadpoles were more sensitive and responsive compared to those of control siblings and, though neuronal firing frequency strongly increased in both tadpole groups after kairomone local application, the firing pattern switched from a single-spike to a robust bursting pattern (Cang and Isaacson, 2003) in neurons from embryonic-treated tadpoles only. The strong increase in the sEPSCs frequency and in firing rate of MCs of embryonic-treated tadpoles was consistent with an increase in the number of synaptic contacts between olfactory receptor neurons ORNs (presynaptic neurons) and MCs (postsynaptic neurons) or an increase in the firing frequency of the synaptically connected ORNs, rather than with an increase in neurotransmitter release sites. In addition, the permanently increased baseline activity of kairomone-primed mitral neurons and their higher sensitivity/response to postnatal

experiences with predator cues revealed how strong the effects of embryonic experience on CNS (central nervous system) were, and how persistently the information obtained during early stages of development could be retained by OB neurons. In fact, MCs of embryonic-treated tadpoles responded differently to kairomone application compared to those of control tadpoles up to one month after their exposure to the kairomone during egg development.

In short, we documented an overall decline of ontogenetic effects on antipredator behaviour during tadpole development, while ontogenetic effects on neuronal activity apparently persisted over time. Moreover, we never observed a difference in contextual behavioural plasticity according to embryonic kairomone treatment, while the contextual neuronal response was strongly modified by prenatal predator exposure. The decline of ontogenetic behavioural effects over time and the lack of differential contextual behavioural response to predator cues according to embryonic treatment may be due to the fact that, differently from neural activity, the defensive behaviour should be continuously adjusted to the current level of predation risk. If predator chemical cues are removed from the environment, the antipredator behavioural response (freezing) should disappear over time (Gonzalo et al., 2009), because there would be important costs to pay in maintaining activity, and hence foraging, at low levels in the absence of predators. However, the memory of the embryonic dragonfly treatment was retained by the SNC, as revealed by the neuronal activity changes in MCs of embryonic-treated tadpoles at 30 days, suggesting that behaviour was a more labile trait than neurophysiology (Dalesman et al., 2013) or morphology (Saino et al., 2003). Indeed, it remains to be elucidated whether and how the observed altered activity patterns in MCs actually translates in the inhibition of muscular fibers resulting in tadpole behavioural freezing. Admittedly, amphibians present strong synaptic connections between the olfactory bulb and the amygdala (Herrick, 1921; Moreno et al., 2005), which plays a crucial role in their fear behaviour as described for mammals (Roseboom et al., 2007).

In fact, relatively little is known about the neurophysiological processes that underlie the plastic responses of prey to their predators. A link between neurophysiological changes and the parallel behavioural effects induced by predator cue detection, was demonstrated in molluscs (*Lymnea stagnalis*, Orr et al., 2007); in this case however, an increase of breathing and overall defensive behaviour was associated to a decrease in firing and bursting activity of a key neuron. In crustaceans (e.g. *Daphnia pulex*), cholinergic-and GABAergic-dependent pathways are involved in the perception and transmission of different predator cues, suggesting that nervous system mediates the development of specific defences against a particular predator species (Weiss et al., 2012). In fish, exposure to a putative alarm substance enhances optical alertness suggesting an action on CNS that affects visual acuity (Pfeiffer et al., 1985). In mammals, exposure to predator odour causes behavioural inhibition (freezing), activation of the neuroendocrine stress axis and correlated changes in CNS limbic circuitry associated with fear and anxiety (Figueiredo et al., 2003; Halpern and Martinez-Marcos, 2003; Beny and Kimchi 2014). In light of our results, we speculate that a similar mechanism can be at work in anuran embryos chronically exposed to a predator kairomone (Denver, 2009; Maher et al., 2013). Recent findings show that stress hormones play a central role in the timing of life-history transitions and can have organizational effects on the developing embryo, since embryonic exposure to corticosteroids results in widespread effects on growth and development that can permanently alter physiology and morphology (Denver, 1987; Denver and Crespi, 2006). The altered activity in MCs of the OB, as well as the other phenotypic changes we observed in embryonic-treated tadpoles, could be attributed to the elevation of corticosteroid levels in response to the predator stressor during embryonic development, to a self-remodelling of the neuronal circuitry, or a combination of the two.

Plasticity in hatching time and the reduced baseline motility at 10 days may be regarded as a tadpole direct short-term response to predation threat experienced as embryos (Ferrari and

Chivers, 2010; Ferrari and Chivers, 2013). By delaying their hatching time, embryonic-treated embryos may have matured their brain and brain performances earlier than control siblings, thus showing a consistently higher neuronal sensitivity and responsiveness to predator cues in a later life-stage. This implies also a long-term neuronal memory of embryonic experiences. Although experienced individuals may improve survival chances (Mathis and Smith, 1993), growing a more efficient antipredator response in early life, by e.g. increasing the neurotransmission between ORNs and MCs, may incur energetic costs, because deviations from developmental norm impose trade-offs in resource allocation (Van Buskirk and Steiner, 2008; Callahan et al., 2008; Auld et al., 2010). Actually, we observed a significantly smaller body size in embryonic-treated hatchlings than in their control siblings, though development stage at hatching was almost identical for both. This may suggest that embryos exposed to the predator kairomone responded to the ‘ghost of predation future’ (Mathis et al., 2008; Ferrari and Chivers, 2010) by allocating their energy reserves to memorize environmental information and improve their sensory system rather than to grow up. Thus, neuronal plasticity may have entailed non-trivial costs during embryonic development, mainly because the neural tissue is one of the most metabolically expensive tissues (Laughlin, 1998; Attwell and Laughlin, 2001). Moreover, the inhibiting effect of embryonic kairomone exposure on growth and development was maintained and even exacerbated during ontogeny, at least until the age of 40 days, even in the absence of further exposure to predator cues. This could result in a delayed metamorphosis and/or in a smaller adult size for embryonic-treated tadpoles as compared to their control siblings, entailing possible fitness costs (Altwegg, and Reyer, 2003). However, all clutches showed a similar response to embryonic kairomone exposure (delayed hatching time and reduced body size at hatching, as well as a higher neuronal activity at tadpole stage), implying little genotype by environment interactions in defensive phenotypic responses and suggesting a general adaptiveness of the ontogenetic plasticity in the population.

In conclusion, our findings, by revealing extensive neuronal plasticity induced in tadpoles by predator chemical cues experienced during the embryonic stage, provide a novel insight into predator-induced patterns of ontogenetic phenotypic plasticity in anuran larvae.

List of abbreviations

OP: olfactory pit, OE: olfactory epithelium, OB: olfactory bulb, MCs: mitral cells, GCs: granule cells, LMMS: linear mixed models, EPSCs: spontaneous excitatory postsynaptic currents, ORNs: olfactory receptor neurons, CNS: central nervous system, APs: action potentials

Ethical note: The study was carried out with permissions by the Italian Ministry of Environment (Prot. 0035817/PNM, validity 2013-2015) and the Italian Ministry of Health (D.M. n° 68/97-A, permanent validity, to the Physiology Lab, Dept. of Biology and Biotechnology, University of Pavia). The study was conducted in conformity with the Italian current laws for amphibian collection and detention and adhering to the Animal Behaviour Society Guidelines for the Use of Animals in Research.

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Table 1. Developmental stage and morphology of tadpoles that were exposed during the embryonic phase to well water (control) or kairomone (treated). Values represent mean (s.e.m.). Estimated treatment effects (slope and s.e.m.) from linear mixed models are also shown, with corresponding *P*-values from likelihood ratio tests. $P_{G \times E}$ corresponds to the *P*-value of a likelihood ratio test of the genotype by environment interaction (by-strain random slope for treatment effect; see Statistical Analysis in the Methods section). Sample size is 50 individuals per treatment for all traits (5 individuals \times 10 half clutches).

Variables	Control	Treated	Estimate	χ^2	<i>P</i>	$P_{G \times E}$
Hatching						
Stage (Gosner)	23.78 (0.07)	23.61 (0.08)	-0.17 (0.14)	1.48	0.22	0.06
Total length (mm)	11.29 (0.06)	10.80 (0.11)	-0.48 (0.14)	8.35	0.004	0.0002
Body length (mm)	3.96 (0.02)	3.79 (0.04)	-0.17 (0.07)	5.34	0.021	0.0003
Mass (mg)	10.26 (0.22)	9.23 (0.26)	-1.03 (0.54)	3.35	0.07	0.0009
Tail length (mm)	7.33 (0.06)	7.02 (0.09)	-0.31 (0.08)	9.31	0.002	0.06
Tail depth (mm)	2.35 (0.03)	2.15 (0.05)	-0.19 (0.07)	5.70	0.017	0.0001
Tail length:body length	1.86 (0.02)	1.86 (0.02)	-0.01 (0.01)	0.01	0.97	0.36
Tail depth:length	0.32 (0.01)	0.31 (0.01)	-0.01 (0.01)	2.57	0.11	0.0002
Day 40						
Stage (Gosner)	34.53 (0.22)	32.79 (0.25)	-1.81 (0.47)	24.78	< 0.0001	0.54
Total length (mm)	33.81 (0.40)	31.74 (0.45)	-0.21 (0.07)	7.19	0.007	0.71
Body length (mm)	11.68 (0.12)	10.80 (0.14)	-0.88 (0.25)	8.48	0.004	0.33
Body depth (mm)	6.66 (0.09)	6.11 (0.10)	-0.47 (0.16)	6.43	0.011	0.18
Body depth length	0.56 (0.01)	0.57 (0.01)	0.01 (0.01)	0.18	0.67	0.99
Mass (mg)	344.80 (10.4)	276.70 (10.8)	-68.0 (22.2)	7.15	0.008	0.07
Tail length (mm)	22.14 (0.31)	20.93 (0.32)	-1.20 (0.45)	5.92	0.015	0.85
Tail depth (mm)	7.39 (0.11)	6.65 (0.12)	-0.74 (0.19)	10.01	0.002	0.25
Tail length:body length	1.89 (0.02)	1.94 (0.02)	0.04 (0.02)	3.50	0.06	0.87
Tail depth:length	0.33 (0.01)	0.32 (0.01)	-0.02 (0.01)	9.69	0.002	0.39
Muscle depth (mm)	2.91 (0.04)	2.64 (0.04)	-0.27 (0.06)	10.51	0.001	0.15
Eye size (mm ²)	5.67 (0.22)	4.51 (0.22)	-1.15 (0.34)	8.23	0.004	0.34

Table 2. Mixed models of tadpole activity (movements) in relation to embryonic kairomone treatment and postnatal kairomone exposure. Strain and individual identity were included as random intercept effects. Sample sizes were 34 tadpoles at day 10 and 60 tadpoles at day 30.

Variables	<i>F</i>	<i>df</i>	<i>P</i>
Day 10			
Embryonic treatment	5.25	1,22	0.032
Postnatal treatment	7.38	1,32	0.010
Embryonic × postnatal treatment	0.49	1,32	0.49
Time of day (h)	4.22	1,29	0.049
Gosner stage	2.65	1,21	0.12
Day 30			
Embryonic treatment	2.60	1,47	0.11
Postnatal treatment	74.18	1,58	< 0.0001
Embryonic × postnatal treatment	0.13	1,58	0.72
Time of day (h)	0.21	1,29	0.65
Gosner stage	1.29	1,55	0.26
Global model			
Age	1.74	1,88	0.19
Embryonic treatment	0.05	1,80	0.83
Postnatal treatment	54.23	1,90	< 0.0001
Age × embryonic treatment	8.31	1,80	0.005
Age × postnatal treatment	19.18	1,90	< 0.0001
Embryonic × postnatal treatment	0.02	1,90	0.89
Age × embryonic × postnatal treatment	0.41	1,90	0.53
Time of day (h)	0.51	1,88	0.48

Table 3. Mixed models of spontaneous excitatory postsynaptic currents (sEPSCs) and firing frequency of mitral cells (MCs) in relation to kairomone application (postnatal treatment) and embryonic treatment. Strain and individual cell identity were included as random intercept effects.

Variables	<i>F</i>	<i>df</i>	<i>P</i>
sEPSCs			
Embryonic treatment	381.4	1,51	< 0.0001
Postnatal treatment	76.43	1,51	< 0.0001
Embryonic × postnatal treatment ^a	18.95	1,51	< 0.0001
Firing frequency			
Embryonic treatment	329.48	1,27	< 0.0001
Postnatal treatment	441.63	1,26	< 0.0001
Embryonic × postnatal treatment ^b	209.80	1,26	< 0.0001

a: *post-hoc* test; all pairwise differences statistically significant at $P < 0.005$

b: *post-hoc* test; all pairwise differences statistically significant at $P < 0.001$, except the comparison between control cells after kairomone puffing and baseline embryonic-treated cells ($P = 0.25$).

Figure legends

Fig. 1. Embryonic exposure to kairomone affected embryo and tadpole phenotypic traits.

(A) Hatching time (hours when 50% of eggs in each half clutch hatched) of control and kairomone-treated embryos according to their developmental stage at collection time. R^2 of regression lines were 0.84 for control and 0.75 for kairomone-treated embryos. (B) Hatching time of control (well water) and treated (kairomone) embryos according to strain (i.e. genotype). (C) Activity of control and embryonic-treated tadpoles at the age of 10 days before and after kairomone infusion (see Table 2 for statistics). (D) Activity in the same experimental set-up at the age of 30 days (see Table 2 for statistics). Bars represent s.e.m..

Figure 2. Recording set-up and electrophysiological characterization of mitral cells in experimental tadpoles.

(A) The head of a tadpole immobilized in a recording chamber with the olfactory epithelium (OE) and the olfactory bulb (OB) exposed for patch clamp recording. The puff pipette, filled with kairomone solution, is placed in front of the olfactory pit (OP) (B): A typical firing response of a mitral cell to depolarizing steps of increasing amplitude. Note the delay in the occurrence of the first spike, especially with low current injections, as compared to a granule cell response. (C) Average resting potential for mitral cells of control and embryonic-treated tadpoles, and granule cells. (D) Transient currents elicited by -10 mV voltage steps from a holding potential of -70 mV were induced to analyze passive properties and to assess the stability of the patch at the beginning and at the end of the recording. No significant difference was detected between control and embryonic-treated mitral cells. (E) Depolarizing voltage steps from a holding potential of -70 mV to -10 mV were delivered to assess active membrane currents in control and embryonic-treated mitral cells. (F) Mean amplitudes of inward and outward currents between mitral cells of control vs. embryonic treated tadpoles (bars represent s.e.m.).

Fig. 3. Embryonic exposure to kairomone increased the amplitude and frequency of spontaneous excitatory post synaptic currents (sEPSCs) and sensitivity of tadpole mitral cells to local puff of kairomone perfusion.

(A) Activity of a representative mitral cell from a control tadpole before (bath solution) and after kairomone solution puff applications (bath solution with dragonfly kairomone). Note that cells from previously unexposed tadpoles also

responded to kairomone solution. (B) Activity of a representative mitral cell from an embryonic-treated tadpole before (bath solution) and after kairomone solution puff applications (bath solution with dragonfly kairomone). Note that the sEPSC frequency was higher than in the cell from a control tadpole even before kairomone solution applications. Puff applications of kairomone enhanced sEPSCs to a greater extent in embryonic treated than in control cells. (C) sEPSCs of recorded mitral cells from both control and embryonic-treated tadpoles in the two experimental settings (bath solution, bath solution with dragonfly kairomone; bars represent s.e.m.). (D) Size distribution of sEPSCs binned at intervals of 1 pA in control mitral cells before and after kairomone solution puff application. Note that data could be fitted with a single Gaussian that increases its amplitude (frequency of sEPSC) when kairomone was applied. (E) Quantal analysis for the same experiments as in (D), but in mitral cells from the embryonic-treated group. Again, data could be fitted with a single Gaussian that increased its amplitude (frequency of sEPSC) in response to kairomone solution.

Fig. 4. Kairomone applications induced a bursting firing pattern in mitral cells of embryonic- treated tadpoles only . (A) A representative trace of action potential firing recorded in current clamp mode from a mitral cell of a control tadpole before (bath solution) and after kairomone applications (bath solution with dragonfly kairomone). Note that in baseline conditions the cell rarely reached the threshold for spiking. When kairomone was puffed, the firing frequency significantly increased, but was composed mostly of single spikes (see expanded time scale below the trace). In most cells, baseline activity recovered after kairomone solution washout. (B) Same as in (A), but for the group of embryonic-treated tadpoles. Note that either before and after kairomone solution applications, the firing frequency was considerably higher than in the control group. In the representative cell shown, activity recovered after kairomone washout. (C) Firing frequency of recorded mitral cells from both control and embryonic-treated tadpoles in the two experimental settings (bath solution, bath solution with dragonfly kairomone; bars represent s.e.m.).

Figure 1

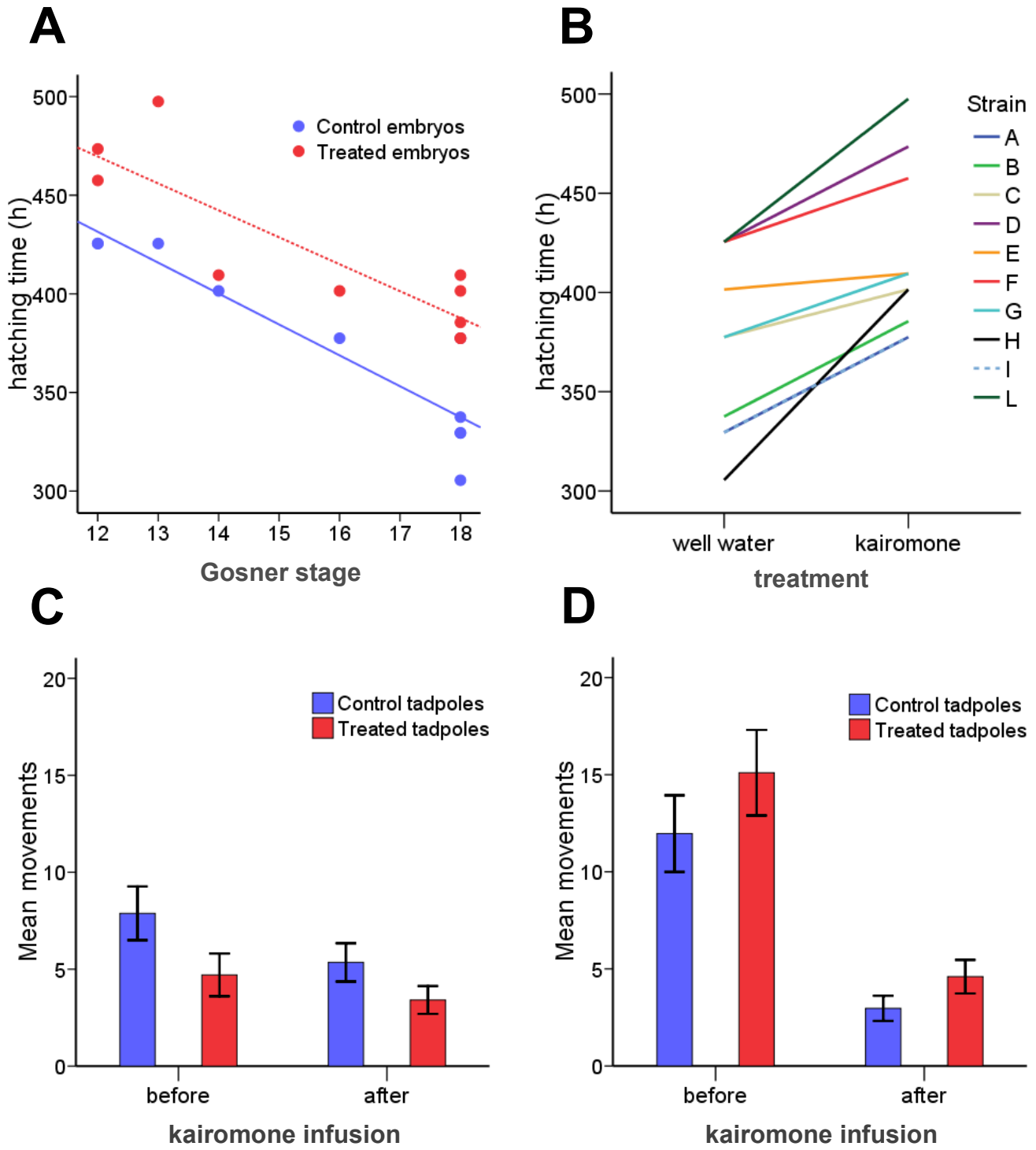


Figure 2

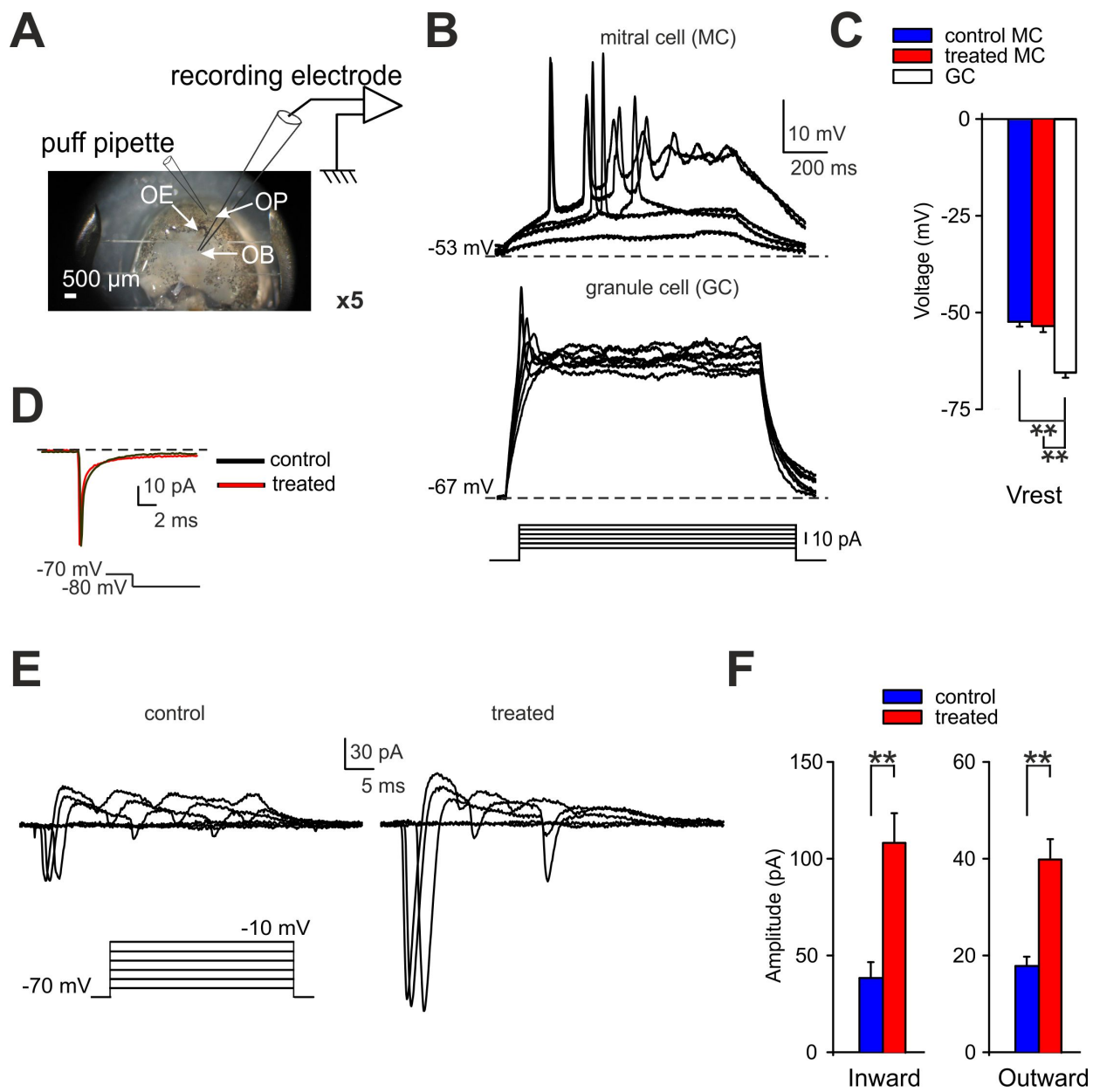


Figure 3

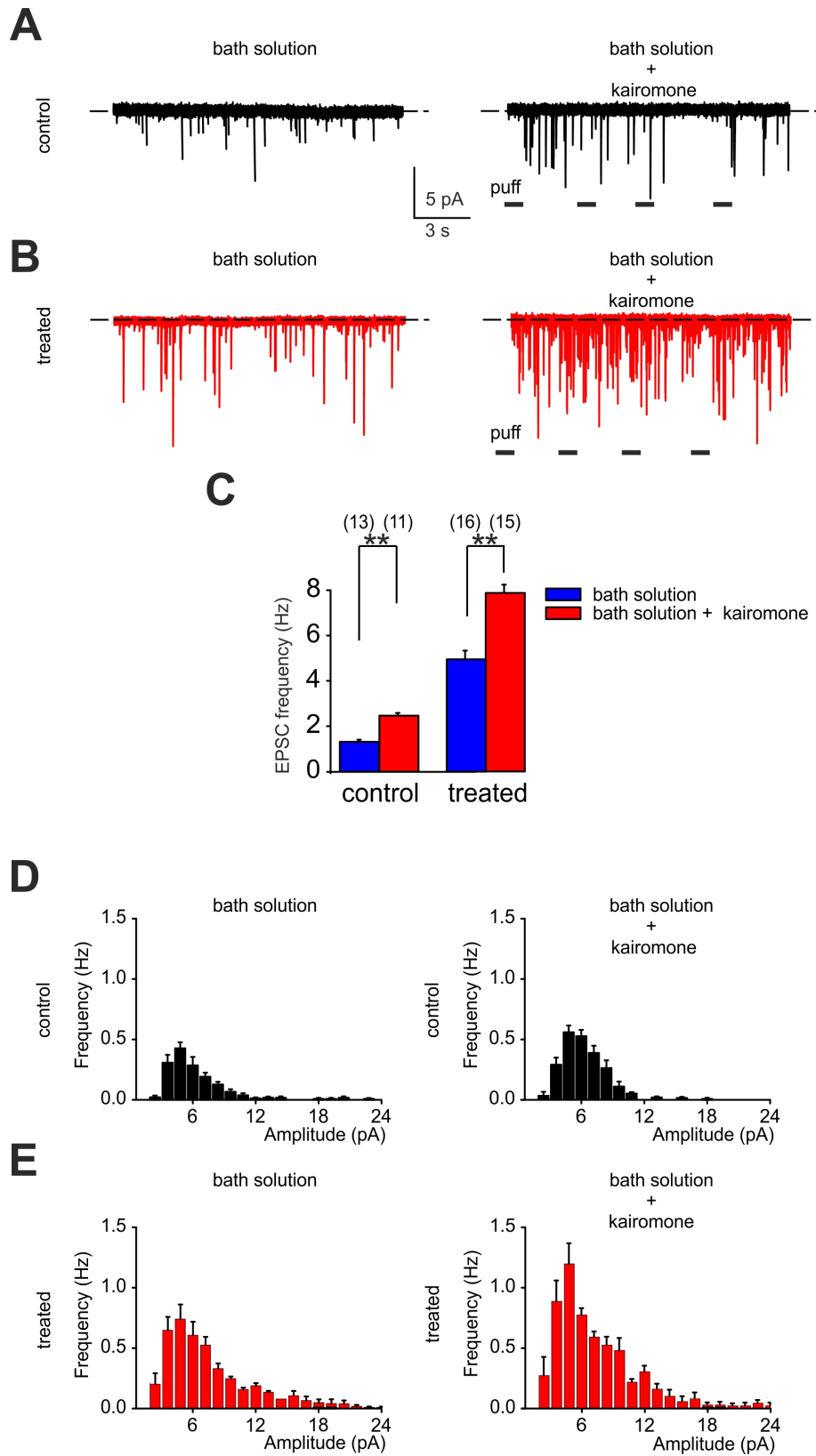
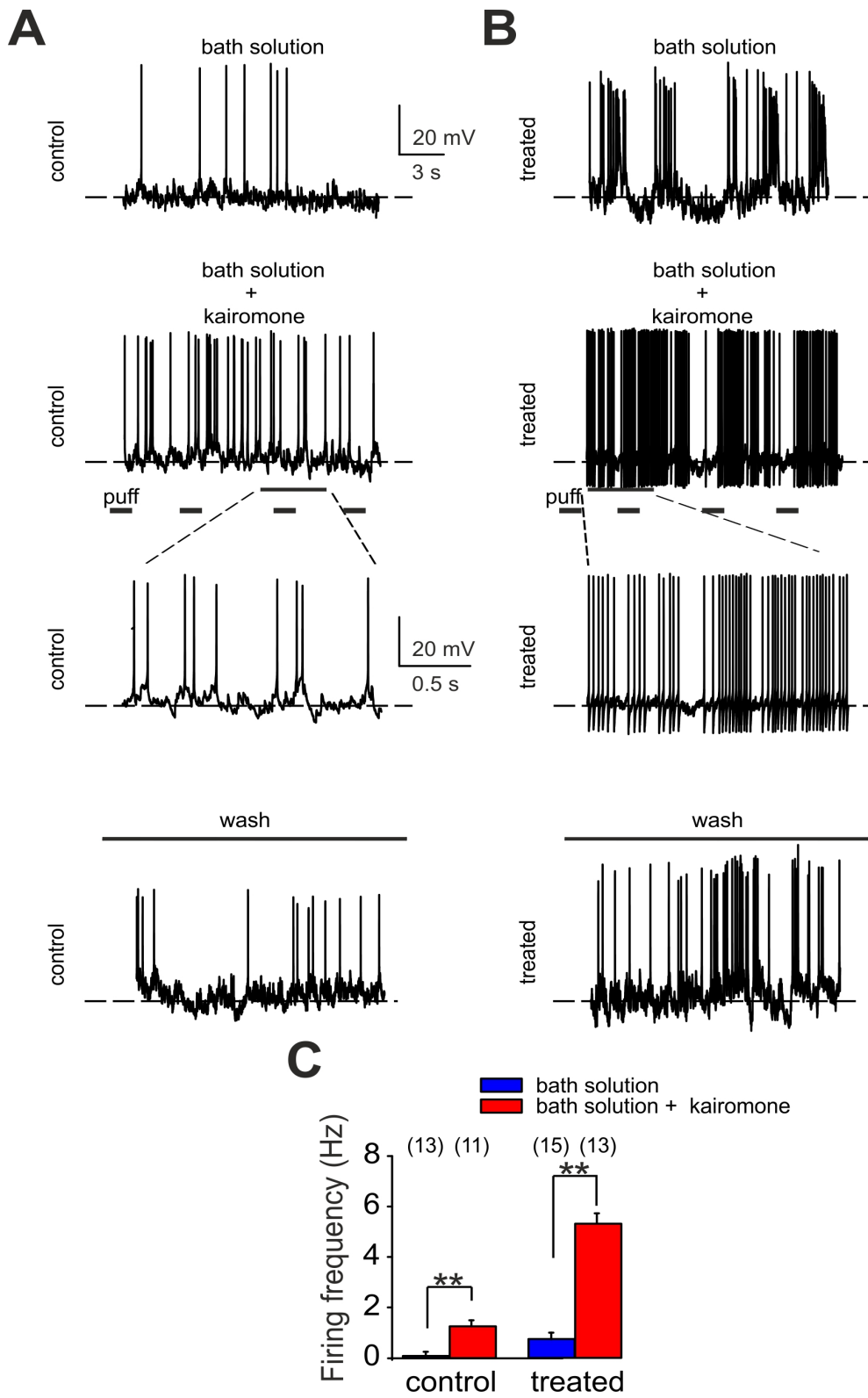


Figure 4



CHAPTER 2

Behavioural and life history responses to predation risk by common frog tadpoles in a narrow window of time

(Acta Ethologica, under review)

Behavioural and life history responses to predation risk by common frog tadpoles in a narrow window of time

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Abstract

Risk of predation can modify behavioural and life history traits of prey, and these changes can be tuned to specific responses to different predator species. The advantage to behave differently, according to predator species, can have a positive effect on prey's fitness. To be optimized, the strategy adopted by prey needs to be flexible during development, and refined according to current dangerousness posed by predators. In this study we exposed common frog (*Rana temporaria*) tadpoles, for a period of two weeks, to non-lethal presence of dragonfly larvae and backswimmer adults. Changes in behaviour (hiding and activity level), development and growth were measured. Overall, tadpoles increased hiding behaviour and decreased level of activity when predators were present in the experimental containers. The rate of development significantly slowed down for predators' treatments in comparison to control, while growth was just slightly enhanced by predators' presence.

Keywords Predators · Development · Tadpoles · Defensive behaviour

Introduction

Predators have been demonstrated to induce behavioural, physiological, morphological and life-history responses in several freshwater prey species, *e.g.* planktonic rotifers (Stemberger and Gilbert 1987), water fleas (*Daphnia pulex*, Dodson and Havel 1988), backswimmers (*Notonecta hoffmanni*; Sih 1982), snails (*Physella virgata virgata*, Crowl and Covich 1990), toads (*Bufo americanus*, Skelly and Werner 1990), frogs (*Rana* spp., Werner and Anholt 1996; *Hyla* spp., Shulse et al. 2013) and fish (*Lepomis macrochirus*, Werner and Hall 1988). These responses can affect both resource acquisition and overall fitness of prey species (West-Eberhard 1989).

Several Anuran species show high inter-population variation in developmental rates and body size at metamorphosis, depending on environmental conditions (Wilbur and Collins 1973; Loman 2003). Generally, if biotic and abiotic conditions allow rapid growth (*e.g.* low density, food availability), developmental rate will be slower and tadpoles will reach metamorphosis at a larger body size than in unfavourable environmental conditions (Wilbur and Collins 1973). The presence of predators is a common biotic factor, which can induce changes in tadpole development (Benard 2004; Relyea 2007; Steiner 2007). Anuran larvae have been shown to be sensitive to water borne chemical cues and respond to predators' odour by varying their behaviour and morphology (Van Buskirk 2001; Relyea 2001; Freeman et al. 2009). These defensive responses are costly and affect both tadpole size and timing of metamorphosis. As both traits are closely related to the fitness of adult individuals (Smith 1987), these parameters are considered an effective tool for evaluating the costs of adaptive responses to predator's threat (Smith 1988).

Tadpole behavioural defences mainly coincide with hiding behaviour and reduction of activity,

to minimize the probability of encountering predators (Steiner 2007). The investment in behavioural defences depends on both food availability and post-metamorphic fitness costs: with low resource availability, when exposed to predators tadpoles are expected to reach metamorphosis earlier, albeit at a smaller size, while when resource availability is high tadpoles can reduce the time devoted to foraging activity and metamorphose later (Higginson and Ruxton 2009). As an example, the growth of common frog *Rana temporaria* tadpoles has been reported to be slightly affected by predation risk (dragonfly *Aeshna juncea* larvae), while their development is significantly slowed down (Laurila and Kujasalo 1999). In contrast, bullfrog *Rana catesbeiana* tadpoles exposed to dragonfly larvae (*Aeshna* sp.) grow bigger than controls (Ferland-Raymond and Murray 2008; but see Peacor and Werner 1997 for an opposite, although not-significant, trend).

There are also indications that tadpoles are able to distinguish among predator species and respond differently (Van Buskirk 2001; Relyea 2001). Van Buskirk (2001) showed that common frog tadpoles are able to differentiate their behavioural responses in relation to predator's identity. After standardizing each predator's diet (feeding all predators with the same amount of food), Van Buskirk found that young tadpoles (14-16 days old) seemed to be more sensitive to backswimmers (*Notonecta glauca*), while older tadpoles (41-46 days old) showed a stronger response to dragonfly larvae (*Aeshna* sp. and *Anax* sp.), suggesting that tadpoles, during development, can assess each predator's relative dangerousness and tune their behaviour accordingly. These findings seem to support a prey-size functional response to predators (McCoy et al. 2011). In fact, notonectids are size-limited, preying only small tadpoles (Cronin and Travis, 1986; Ramos and Van Buskirk 2012), while dragonfly larvae can prey on a wide range of size classes, although Aeshnid larvae select small tadpoles in feeding trials (Brodie and Formanowicz 1983). Accordingly, Jara and Perotti (2010), by comparing the proportion of active tadpoles of different size and species in the presence of four common

tadpole predators, found that behavioural responses were driven by the risk imposed by each predator, which, in its turn, largely depended on the prey to predator size ratio. These data support the hypothesis that behavioural ontogenetic plasticity (Stamps 2015) shown by common frog tadpoles in response to predation risk is generally adaptive (Auld et al. 2010).

Although several empirical tests have been performed to investigate behavioural predator-specific responses in a variety of anuran species (Relyea 2001; Van Buskirk 2001; Kishida and Nishimura 2005), further experiments in controlled environments are needed to test the consistency of phenotypic changes for different species and to assess whether experimental conditions can bias behavioural and developmental responses (Winkler and Van Buskirk 2012). Moreover, different populations can activate different responses in relation to specific evolutionary processes, such as exposure to different selection pressures or genetic drift (Relyea 2002).

Phenotypic antipredator responses of tadpoles are generally studied by conditioning animals with chemical cues of predation over 24-40 days long periods, as to allow morphological and life history traits changes to be large enough to be easily recorded (e.g. Steiner 2007; Van Buskirk 2001). In this study, we measured hiding behaviour, reduction of activity and consequent changes in life history (development rate and mass) induced by predation risk to common frog tadpoles, during two weeks of non lethal exposure to two common syntopic predators: backswimmers (*Notonecta glauca*) and dragonfly larvae (*Anax imperator*). These predator species show different feeding behaviours and hunting efficiency (backswimmers are visual predators of the open water, while dragonflies wait for their prey hidden inside aquatic vegetation), which we assumed to correspond to different degrees of dangerousness during development, as perceived by tadpoles (Van Buskirk 2001).

The experiment was performed between Gosner stages 28-30 and 34-36 (Gosner 1960), corresponding to mean weights ranging between 43 and 380 mg, respectively. Measures were

then taken in a short span of time, i.e. two weeks, exactly in the developmental phase included between the two stages previously measured by Van Buskirk (2001). In this transitional larval-size time we expected tadpoles to perceive both backswimmers and dragonfly larvae as potential predators and then respond similarly to their exposure. Moreover, as treatments were carried out in a short time interval, we expected exposure to predation risk to have negligible effects on tadpole development and weight.

Methods

Experimental design and data collection

We conducted the experiment in 80 L plastic mesocosms (0.28 m²) placed outdoors in a field inside the campus of the University of Zurich, Switzerland. Mesocosms were arranged in the field within a single grid in a completely randomized design. Mesocosms were filled with tap water 10 days before the experiment onset, provided with 40 g dried leaf litter and 2 g of commercial rabbit food, and covered with lids, constructed of 37% shade cloth, to prevent colonization by predators. Aliquots of pond water containing zooplankton and phytoplankton were added to each mesocosm, to assure a long-lasting healthy freshwater environment (Van Buskirk 2001). We added further 2 g of rabbit food to each experimental container at the onset of the experiment. The experiment began on 25 April 2012, three weeks after hatching, and ended on 9 May, when common frog tadpoles were weighed and their developmental stage assessed according to Gosner (1960). At the beginning of the experiment tadpoles' mean weight (\pm SD) was 0.043 ± 0.003 g (mean was calculated from 25 individuals, 5 from each clutch), and developmental stage ranged between 28 and 30.

Tadpoles came from five egg masses previously collected in a pond 30 km north of Zurich.

Predator species - dragonfly *Anax imperator* larval forms and backswimmer *Notonecta glauca* adults -, were collected from artificial ponds inside the campus. Both species are known to feed on tadpoles and are sensitive to movements of prey (Van Buskirk 2001). Fifteen tadpoles, 3 individuals per egg mass, were randomly assigned to each experimental tub and exposed to individually caged predators or to an empty cage as control. Cages consisted of a plastic tub 12 cm in diameter (1 L volume), covered at both ends with mesh netting to prevent predators from preying on tadpoles. Each treatment was replicated 5 times, for a total of 15 tubs and 225 tadpoles. Both predators were fed with two *Rana temporaria* living tadpoles (200-300 mg wet mass) every other day throughout the experiment, while control cages were manipulated in the same way to equalize disturbing effects on tadpoles. In the feeding days all cages were also checked to assess the health status of predators, and rotated among tanks of the same treatment to avoid the confounding effect due to differences among individual predators. The behaviour of tadpoles was recorded every two days, at the same time (12 a.m.), slowly approaching the tubs and counting all visible individuals and those, among them, showing some form of active behaviour (i.e. moving the tail, swimming or eating). Counts were repeated five times within each sampling day, ca. every 15 min. With this method there is no way to completely exclude the disturbance effect caused by the observer, and to minimize this bias we discarded every count for which interference seemed to have suddenly modified the behaviour of tadpoles. At the end of the experiment all individuals were removed from the experimental mesocosms to assess their developmental stage and measure body weight by a digital balance (accuracy ± 0.1 mg).

Statistical analyses

Dependent continuous variables stage and mass at the end of the experiment were analyzed

using linear mixed models (LMMs). We fitted the full model and then compared it with lower order models by the likelihood ratio test (LRT). Treatment (predator species or control) was considered a fixed factor and experimental tank a random effect. The model was fitted with nlme package (Pinheiro and Bates 2000). To analyse behavioural response variables (number of visible or active tadpoles) we used generalized linear mixed models (GLMMs) with binomial family error distribution. Treatment (fixed factor), date (covariate), to account for daily treatment-dependent variation in activity and hiding behaviour, and treatment x date were included as predictor variables; tank was treated as random intercept. All analyses were performed with R 3.0.1 (R Development Core Team, 2013).

Results

Behavioural responses were strongly affected by treatment, date and their interaction (Tab. 1), the latter indicating that behavioural responses to the different predator species changed as tadpoles grew larger. Overall, tadpoles were less visible when dragonfly or backswimmer were present than in control cages ($t_{12} = 12.27$, $p < 0.001$, and $t_{12} = 11.84$, $p < 0.001$, respectively), but there was no significant difference between predator species ($t_{12} = -0.50$, $p = 0.62$). During the first five recording days only 13% ca. of predator-exposed tadpoles were, on average, visible with respect to ca. 53% of controls; this pattern reversed in the last phase of the experiment, when tadpoles exposed to both backswimmers and dragonfly larvae were, on average, more visible than controls (Fig. 1).

As expected, exposure to predators caused a decrease in tadpole activity in comparison to controls (dragonfly: $t_{12} = -9.00$, $p < 0.001$; backswimmer: $t_{12} = -6.38$, $p < 0.001$), with tadpoles

exposed to backswimmers being slightly more active than those exposed to dragonfly larvae during the first two thirds of the experiment ($t_{12}=2.99$, $p=0.011$) (Fig. 2).

No tadpole died during the experiment. Rate of development was significantly, although slightly, affected by predator presence (L-Ratio_{5,3}=6.14, $p=0.04$), with tadpoles from both predator treatments less developed (mean Gosner stage \pm SE = dragonfly: 34.06 ± 0.02 ; backswimmer: 34.69 ± 0.028) than controls (35.58 ± 0.022) (Fig. 3). The final mass of predator-exposed tadpoles was higher than that of controls (mean weight \pm SE = dragonfly: 0.38 ± 0.001 g; backswimmer: 0.38 ± 0.001 g; control: 0.34 ± 0.001 g), but the difference was not statistically significant (L-Ratio_{5,3}=4.23, $p=0.12$) (Fig. 4).

Discussion

There is evidence that for several species behavioural responses to predator-exposure depend on the magnitude of the risk perceived by the potential prey (e.g. slimy sculpin *Cottus cognatus*: Chivers et al. 2001; *Rana temporaria* larvae: Laurila et al. 2004; *Ochlerotatus triseriatus* mosquito larvae: Kesavaraju et al. 2007). Ontogeny is likely to alter predator-prey relationships, either because increasing in mass lowers prey vulnerability (Van Buskirk and Relyea 1998; Van Buskirk 2001) or as a consequence of changes in the predator community (Relyea 2003). Consistently with previous research (Hossie and Murray 2012; Relyea 2003), our results show that strong behavioural responses (hiding behaviour and reduced activity) were constrained to the early phase of the ontogeny and were no longer used when tadpoles reached a threshold size. As tadpoles grew, large size and, possibly, morphological defences (Relyea 2003), seemed sufficient to lower the perceived risk of predation, making behavioural defences unnecessary.

As expected, in the narrow window of time considered behavioural responses of common frog tadpoles were affected by exposure to predator but differed only slightly between backswimmers and dragonfly larvae. This result suggests that between three and five weeks after hatching tadpoles perceived both predators as posing the same threat, but, as we did not measure morphological traits that may exhibit predator-induced plasticity (e.g. body length and depth of the tailfin; Smith and Van Buskirk 1995), we cannot exclude the use by tadpoles of predator-specific morphological defences. Anyway, the previous study by Van Buskirk (2001) showed that different predators induce similar morphological responses in common frog tadpoles. In the same study, about 80% of two-week old common frog tadpoles tended to hide also in control tanks (Van Buskirk 2001). Accordingly, Relyea (2003) reported that treefrog (*Hyla versicolor*) hatchlings respond to predators by hiding and reducing their activity, while morphological defences predominate later in ontogeny. We may argue that in the early phases of ontogeny hiding behaviour is an effective and highly reversible defence for a wide range of predators and is consequently maintained by natural selection imposed by predators.

Whether hiding and reduced activity are likely to reduce the probability of encountering predators, these antipredator behaviours involve a reduction in the time devoted to foraging, which, matched with increasing energy demand for morphological defences, may be expected to lead to slower growth and development (Semlitsch 1993; Relyea and Werner 1999; Van Buskirk 2000; Stoks et al. 2005) and prevent timely metamorphosis (Relyea 2001).

Nonetheless, similarly to previous studies (Laurila et al. 1998; Laurilia and Kujasalo 1999; Steiner 2007), tadpole survival and mass were not affected by predator presence, although slightly lower development scores were recorded for predator-treatments with respect to controls. Given the short time length of exposure to predators it is possible that the difference in Gosner stage between predator-induced tadpoles and control treatments would increase at metamorphosis.

Food availability has been reported to be a major factor affecting both tadpole body size and development (Laurila and Kujasalo 1999); as all mesocosms were provided with the same amount of food at the beginning of the experiment and food ingestion by predator-exposed tadpoles seems not to be affected by behavioural responses (Steiner 2007), the slight developmental delay of predator-exposed tadpoles may have been the consequence of energetic investment in unrecorded morphological defences.

The variation in the number of visible or active tadpoles recorded in our experiment seems to disagree with the strong response found by Van Buskirk (2001) for large tadpoles exposed to dragonfly larvae, unless a second threshold mass reverses once again the pattern of behavioural responses of *Anax*-exposed tadpoles. Unfortunately we did not get ahead with the experiment until seven weeks after hatching, a task that it would be worth to accomplish in the future.

Variation in environmental or laboratory conditions - e.g. water temperature and volume (Harkey and Semlitsch 1988; Sanuy et al. 2008), light intensity (Ding et al. 2014), tadpole density (Loman 2003) -, uncontrollable random variables, the choice of independent variables and the way they are measured (e.g. number of active tadpoles, in this study, vs. proportion of time active, in Van Buskirk 2001), can make it hard to compare the results of different experiments. We then recommend that future comparative studies adopt experimental protocols as similar as possible to those applied by previous research.

Acknowledgements

We are grateful to Josh Van Buskirk for hosting the experiment and helpful advice.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. Ethics permits were provided by the Veterinary Office of Canton Zurich, and permits to collect the animals came from the Canton's Office for Landscape and Nature.

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Tab. 1 Effects of treatments and date on common frog tadpole hiding behaviour (a) and activity (b).

Effects		Test statistic	df	Significance
<i>(a) Visible</i>	Treatment	$\chi^2 = 27.36$	2	P < 0.001
	Date	$\chi^2 = 182.79$	1	P < 0.001
	Treatment × Date	$\chi^2 = 392.20$	2	P < 0.001
<i>(b) Active</i>	Treatment	$\chi^2 = 150.37$	2	P < 0.001
	Date	$\chi^2 = 2.02$	1	P = 0.15
	Treatment × Date	$\chi^2 = 25.42$	2	P < 0.001

Figure legends

Fig. 1 Mean number (\pm SE) of visible tadpoles for the entire the experiment.

Fig. 2 Mean number (\pm SE) of active tadpoles for the entire experiment.

Fig. 3 Mean developmental stage (\pm SE) of tadpoles at the end of the experiment.

Fig. 4 Mean body weight (\pm SE) of tadpoles at the end of the experiment.

Fig. 1

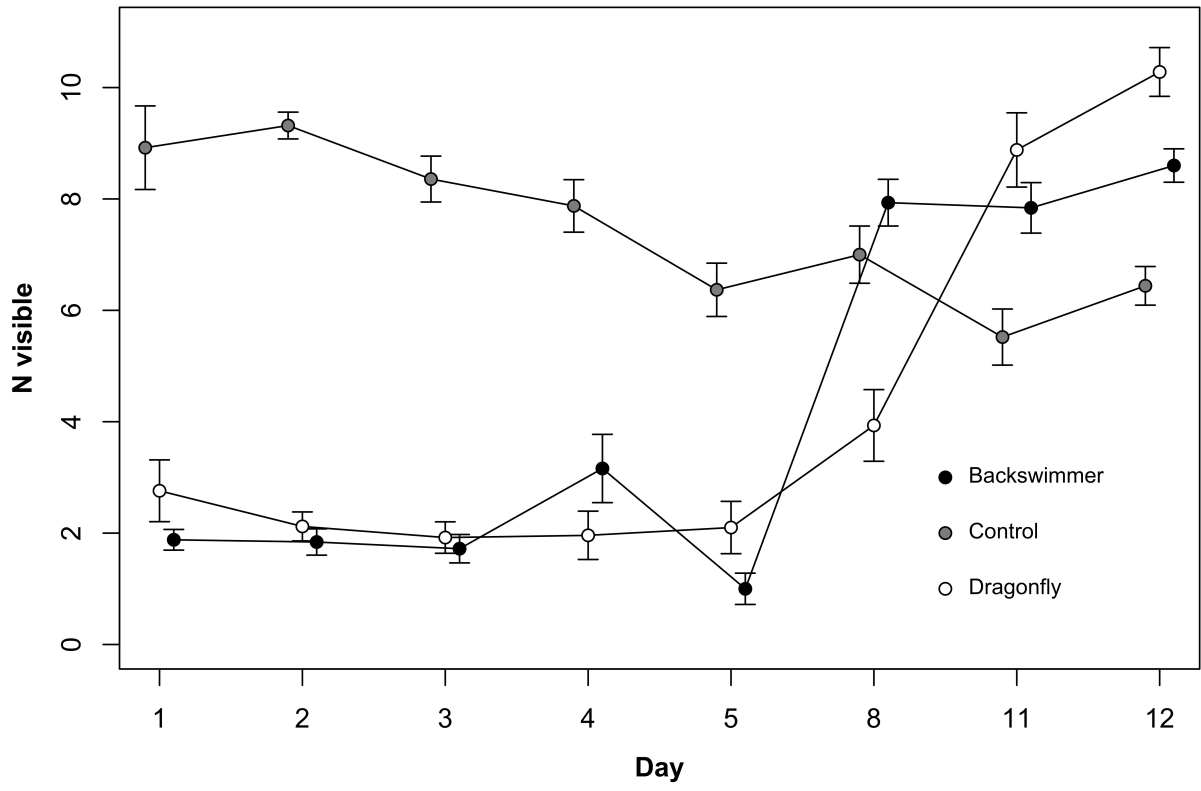


Fig. 2

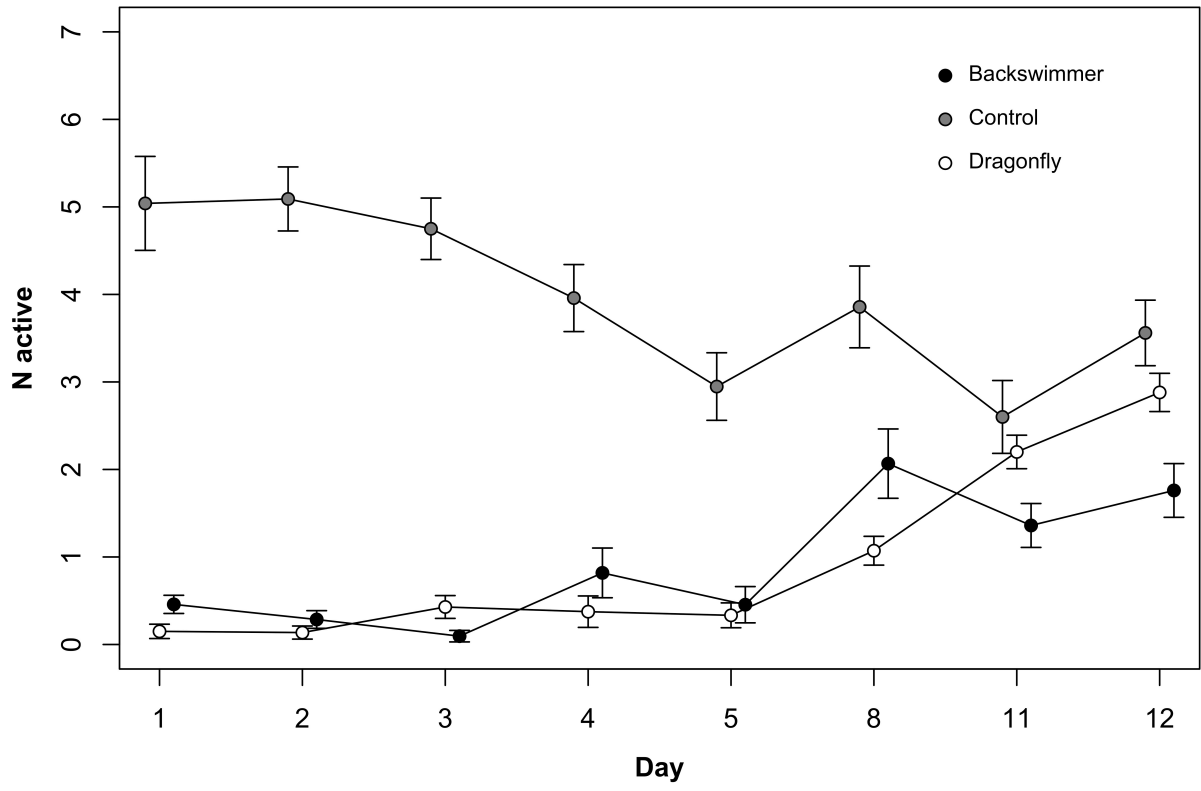


Fig. 3

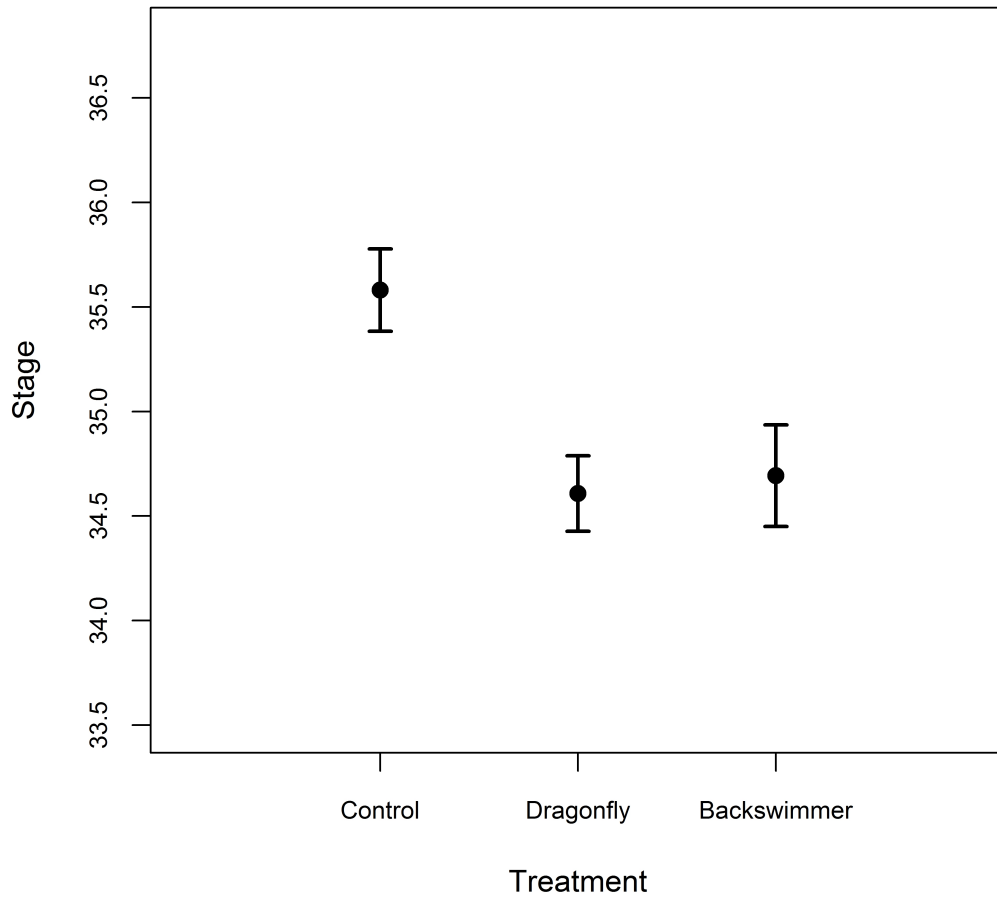
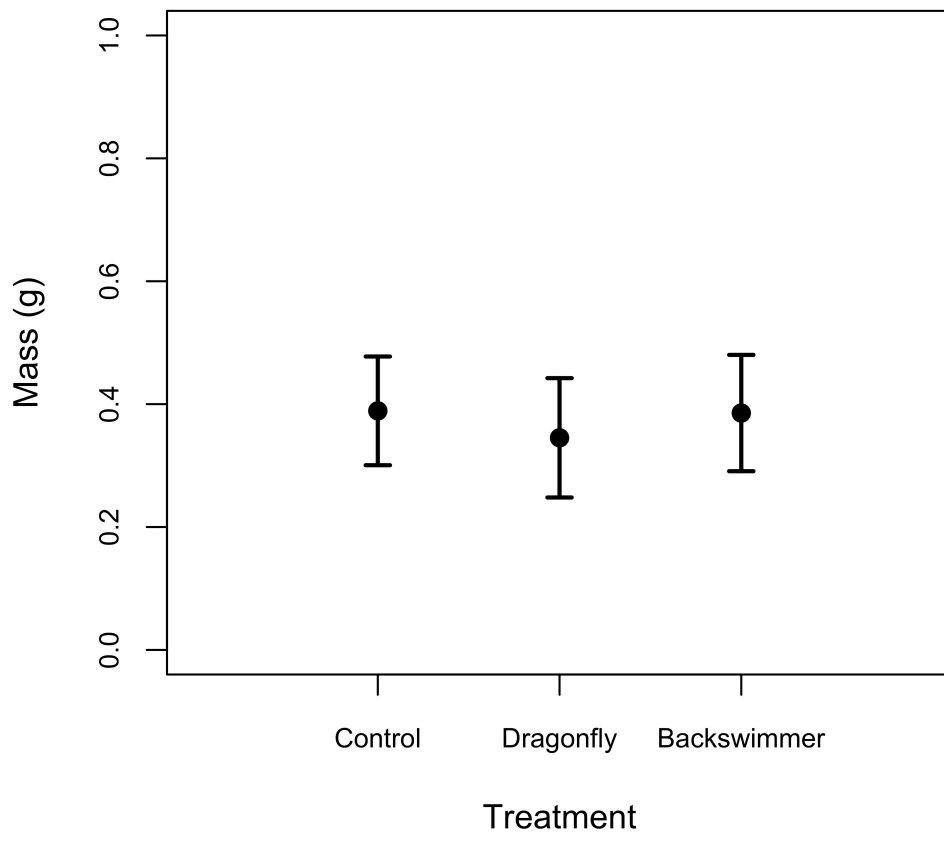


Fig. 4



CHAPTER 3

The effect of thinning and cue:density ratio on tadpoles' risk perception

(Manuscript)

The effect of thinning and cue:density ratio on risk perception by *Rana dalmatina* tadpoles

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Abstract

The information on which animals rely to assess the risk of predation is a crucial issue to figure out how prey defences have evolved. An understanding of the information used by prey to assess the risk is essential to the proper modelling of antipredator decision making. The “risk assessment hypothesis” considers prey density as an important variable to properly assess the actual level of risk, and predicts that, when the ratio between predator cue concentration and prey density is constant the level of risk perceived by prey is the same. Thinning is a direct effect of predation and is closely related to the reduction of population size of prey, and, reasonably, can be considered as an indication of the current level of risk. In this study we combined thinning (by manually removing tadpoles over time) with a constant cue:density ratio (fixed over time) to evaluate how these two types of information affected the level of activity of *Rana dalmatina* tadpoles, and using dragonfly larvae (*Aeshna cyanea*) as predators. Our results showed no effect of density reduction on prey level of activity, and thinning did not interact with ratio to modify tadpoles’ behaviour. However we observed no differences between treatments with the same ratio, and this provide a sound support of the RAH.

Keywords

Predation risk - Conspecific density - Activity - Removal

Introduction

Predation is a primary selective force influencing many phenotypic traits of prey species, including morphology, life history and behaviour (Lima and Dill 1990; Lima 1998) and can shape the structure and dynamics of ecological communities (Peacor and Werner 2004).

Since, to grow and reproduce, animals need to accomplish a number of activities while trying to avoid predators (Brown and Kotler 2004; Creel and Christianson 2008), it is fundamental for them to be able to properly assess the risk of predation (Lima and Dill 1990). The extent to which defensive responses can be properly matched to the real level of risk, optimizing the growth/predation risk trade-off, has major fitness consequences (Skelly and Werner 1990; Ball and Baker 1996; Lima 1998).

Decisional strategies of predator-exposed animals are a function of both the kind and reliability of available information about the risk of predation and prey capacity to use this information properly (Lima and Steury 2005). Prey can adjust defensive responses according to predator identity (Van Buskirk 2001; Touchon and Warkentin 2008), predator abundance (Van Buskirk and Arioli 2002) or diet (Schoeppner and Relyea 2005; Hettyey et al. 2015), and mass of prey consumed (Van Buskirk and Arioli 2002; Fraker 2008; McCoy et al. 2012). A further variable that can affect prey responses is conspecific density (Roberts 1996). Several studies have demonstrated that many species are able to distinguish between groups of conspecifics that differ in size (Krause and Ruxton 2002). Consequently, group size can influence individuals'

perception of risk and contribute to tune antipredatory behaviours (Bohlin and Johnsson 2004; McCoy 2007).

The classic “many eyes” theory (Lima 1990, 1995) suggests that, as group size decreases, individuals should increase their personal commitment to vigilance. This is consistent with the so-called “dilution effect” (Wrona and Dixon 1991; Lehtonen and Jaatinen 2016), which predicts that the larger the group the lower the chance that one particular individual will be the one attacked.

Particularly, the “cognitive dilution effect” mechanism (Pitcher and Parrish 1993) predicts that a predator’s attack to one member of the group provides information about the position, state and capability of the predator itself, which can be used by the other members of the group to reduce the probability of predation. In aquatic environments, this information is mainly propagated by chemical cues (Laurila 2000; Dicke and Grostal 2001), which can be produced by prey (stress-, attack- or capture-released prey cues), predators (kairomones) or both (digestive-released cues; Hetteyey et al. 2015).

Peacor (2003) proposed that organisms, to adaptively respond to predation risk signalled by chemical alarms, should consider both conspecific density and the amount of cue dissolved in the medium (risk assessment hypothesis); this prediction basically states that prey response to predation risk should be a function of the *per capita* amount of cue in the surrounding environment.

As an example, the alarm signal from a killed prey should be considered as a potential higher threat in a group of 10 individuals than in a group of 100 individuals, stimulating a more intense defensive response in the individuals belonging to the former. At the same time, if a group of 20 individuals is brought into contact with double the amount of cue perceived by a group of 10 individuals, the *per capita* intensity of the signal being equal, responses are expected to be similar.

Both conspecific density and cue level have been reported to affect behaviour in the larvae of several anuran species. Several studies have shown that group size influences the level of activity of tadpoles, with tadpoles in groups being more active than tadpoles in pairs or alone (e.g. *Bufo bufo*, Griffiths and Foster 1998; *Rana temporaria*, Niecieza 1999; *R. pipiens*, Golden et al. 2001; Awan and Smith 2007; *R. sylvatica*, McClure et al. 2009). Thinning has been reported to lower tadpole activity in *R. sylvatica* tadpoles (Van Buskirk and Yurewicz 1998), and to affect both growth and behaviour in gray treefrog (*Hyla versicolor*) tadpoles (Relyea 2002a).

Many studies have demonstrated that chemical cues induce antipredator defences in tadpoles, determining both the type and intensity of responses (Chivers and Smith 1998; Kishida and Nishimura 2005; Hettyey et al. 2010). The time an individual spend active is, generally, inversely proportional to the amount of cue released from predation events (Van Buskirk and Arioli 2002; Bennett et al. 2013).

Despite this plenty of information, very few studies have tried to test the predictions of Peacor's model by analyzing the combined effects of density and predator exposure on tadpoles' behaviour. Van Buskirk et al. (2011) compared the level of activity of *R. temporaria* tadpoles kept at three different levels of density crossed with four levels of cue concentration, finding strong support for the risk assessment hypothesis. In contrast, Guariento et al. (2015) did not find evidence of the effect of conspecific density on *Lithobates catesbeianus* tadpoles' behavioural responses, while the interaction between predation risk and prey conspecific density affected their morphological traits, as predicted by Peacor's model. Modulation of morphological responses in function of both the density of conspecifics and chemical cues from predators had been previously reported for *Hyla femoralis* tadpoles by McCoy (2007).

All these studies manipulated density by placing tadpoles into experimental tubs at three fixed group sizes (e.g.: N = 3, 10 and 30 in Van Buskirk et al. 2011). As, in the real world, predation

is a process that produces a progressive decrease of prey density, it is feasible that prey adapt their defensive behaviour to the real rate of reduction rather than the absolute number of conspecifics in a given place at a given time. Nonetheless, to our knowledge, the effects of thinning over time on the defensive responses of predator-exposed tadpoles have not been investigated.

To test if tadpoles perceive the progressive decrease in population density due to predation and are able to use this information to fine-tune anti-predator responses we (i) kept constant the cue:density ratio, (ii) controlled density by removing tadpoles at a constant rate, and (iii) recorded and compared variations in tadpole activity levels. Reduction of activity, to minimize the probability of encountering predators, is a common behavioural response to predation risk (Steiner 2007), which, moreover, can be expressed much promptly than morphological changes (West-Eberhard 1989).

Assuming removal rate (i.e. density reduction) as an effective informational signal, we expected tadpoles to progressively decrease their level of activity, possibly even in the absence of predatory cues. Moreover, according to the risk assessment hypothesis, we expected tadpoles activity to decrease with increasing cue:density ratios, regardless of their potential interaction with thinning.

Materials and methods

Experimental design

The prey model were tadpoles of agile frog *Rana dalmatina*. Five clutches of eggs were collected from a pond 30 km east of Pavia (San Colombano, Milan) and individually placed in 40 l plastic containers filled with well water until the beginning of the experiment. Predators - dragonfly *Aeshna cyanea* larvae -, were collected by dip netting in a nearby pond. When

tadpoles were at Gosner (1960) stage 27-29, four randomly selected tadpoles from each egg mass were assigned to an experimental tank (30×25×15 cm) for a total of 20 tadpoles per tank. Tadpoles were introduced into each tank 48h before the onset of the experiment and provided with food ad libitum.

The experimental design included four treatments replicated eight times, for a total of 32 experimental tanks. Treatments were as follows (Fig. 1, 2):

- A) control (well water, with removal),
- B) low risk ($[\text{cue}]/n = x$, without removal),
- C) low risk ($[\text{cue}]/n = x$, with removal),
- D) high risk ($[\text{cue}]/n = 5x$, with removal),

where n = number of tadpoles, and $x = 0.5$ ml of predatory cue per tadpole.

Treatment B served as control for C, as they had the same cue:density ratio but removal was applied only to the latter; D had a five times higher ratio than B or C, representing a stronger *per capita* risk of predation.

Cues for the four treatments at the onset of the experiment consisted of: A) 50 ml of water, B, C) 10 ml of cue + 40 ml of water, D) 50 ml of cue (Fig. 1). For treatments C and D the amount of cue was successively corrected (i.e. reduced) to take into account the effective number of tadpoles per tank and keep constant the cue volume *per capita* during the whole experiment. The cue was inoculated into the experimental containers using a 50 ml volume plastic syringe and, whenever necessary, a 5 ml syringe to accurately dose lower amounts of signal.

The experiment was performed with a two-days schedule: 1) during the first day we added the cue into the experimental containers (Fig. 1) and recorded tadpole behaviour one hour later; 2) in the second day we changed all the water in all experimental containers and provided an excess of food to control for the potential effects of competition and hunger. Water change

prevented carryover effects due to the accumulation of the cue, which was expected to have a lifetime of ca. 72 hours (Van Buskirk et al. 2014). To ensure the fast change of water and reduce disturbance, a fiberglass net was made to adhere to the inside walls of all tanks.

A linear decline of tadpole density (i.e. survivorship), from 20 to 4 individuals per container, was obtained by removing every first day (starting from day three from the onset of the experiment), four randomly selected tadpoles immediately before adding the cue (except for treatment B; Fig. 1). Tadpoles removal was conducted by a small fish net and lasted a few seconds for each container; a similar disturbance effect was produced for treatment B containers.

All experimental tanks were arranged in a room in four spatial blocks and each tank was randomly assigned to a treatment, with each block including two replicates per treatment (balanced block design). This design allowed us to test for treatment×block interaction (generalized randomized block design; Quinn and Keough 2002).

Chemical cue

The cue used for the assessment of predation risk was obtained by feeding 10 dragonfly larvae, individually maintained in separate tubs (250 ml), with living agile frog tadpoles. To obtain a uniform concentration of the cue, tadpoles were accurately weighed as to provide each predator with 2-3 similar-sized larvae (100-250 mg in total). Knowing tadpole mass consumed by each predator, we could then mix the water contents of a subset of tubs to obtain the desired amount of cue throughout the experiment (~500 mg/(1×day) of tadpoles consumed). The cue was collected two hours after having exposed tadpoles to predators. Each time, tubs were then re-filled with aged tap water.

Behavioural data

Risk-averse behaviour was assessed one hour after the perfusion of the cue, by counting the number of inactive individuals (neither swimming nor even moving the tail) during five, 10s long observations separated each other by a period of 10 minutes. Behavioural data were always recorded by the same observer, moving carefully among the experimental containers.

To assess the effects of treatment, day and their interaction on the level of tadpoles' activity (proportion of inactive tadpoles), we applied generalized linear mixed models (GLMM) with a binomial distribution. Block was included as a random factor. We run a separated GLMM for the first day of the experiment, with treatment as fixed factor and block as random factor, to further investigate the differences among treatments at the start of the experiment. Statistical analyses were conducted using R (version 3.2.1; R Core Development Team 2015) and lme4 package (Bates et al. 2015). Significance of fixed effects was assessed with a type III test for fixed effects analysis of variance in the R statistical package "car" (Fox and Weisberg 2011).

Results

We found a highly significant interaction between treatment and day ($\chi^2 = 67.4$, d.f. = 3, $P < 0.0001$), suggesting that the level of tadpoles' activity changed over time according to treatments. Except for the control group A ($\beta = 0.38 \pm 0.32$, $z = 1.15$, $P = 0.24$, Fig. 3) inactivity level significantly increased over time (treatment \times day interaction; B: $\beta = 0.14 \pm 0.04$, $z = 3.42$, $P = 0.0006$; C: $\beta = 0.18 \pm 0.05$, $z = 3.57$, $P = 0.0003$; D: $\beta = 0.49 \pm 0.06$, $z = 8.16$, $P < 0.0001$; Fig. 3, Fig. S2). The slopes of groups B and C did not differ ($\beta = 0.03 \pm 0.04$, $z = 0.795$, $P = 0.42$), while both differed from D (respectively: $\beta = -0.35 \pm 0.05$, $z = 6.13$, $P < 0.0001$; $\beta = -0.31 \pm 0.06$, $z = 4.93$, $P < 0.0001$; Fig. 3). The separated model for the first day showed that, at the onset of the experiment, the proportion of inactive individuals of A was not

significantly different from B ($\beta = 0.14 \pm 0.09$, $z = 1.44$, $P = 0.14$; Fig. S1), but differed from C and D (respectively: $\beta = 0.21 \pm 0.10$, $z = 2.15$, $P = 0.03$; $\beta = 0.35 \pm 0.10$, $z = 3.50$, $P = 0.0004$; Fig. 4). Finally, activity did not differ between C and B and C and D (respectively: $\beta = 0.07 \pm 0.10$, $z = 0.71$, $P = 0.47$; $\beta = 0.14 \pm 0.10$, $z = 1.36$, $P = 0.17$), while differed between D and B ($\beta = -0.21 \pm 0.10$, $z = -2.07$, $P = 0.03$; Fig. 4).

	A	B	C
B	=		
C	≠	=	
D	≠	≠	=

Discussion

The first day of the experiment, which can be considered a direct test of Peacor’s model (Van Buskirk et al. 2011), did not provide a sound confirmation of the risk assessment hypothesis, although the proportion of inactive tadpoles showed a tendency to increase with cue level.

However, throughout the experiment behavioural responses did not vary between treatments with the same per capita cue level (B and C) despite variation in tadpole density, supporting the

hypothesis that tadpoles assessed the risk of predation evaluating both group size and cue intensity. Moreover tadpole response increased in magnitude for the treatment with the highest cue level (D), confirming that behavioural responses were adjusted according to the perceived per capita predation risk, consistently with Peacor's predictions (2003).

Contrary to our expectations, thinning seemed to have no effect on behaviour neither in cue-exposed tadpoles nor in controls, suggesting that removal per se did not represent an effective source of information on predation risk. These results are in contrast with those reported by Relyea (2002a), who recorded a reduction in the proportion of active tadpoles as a consequence of thinning also in the absence of predator threats. Intraspecific competition for available resources is a major factor determining the level of activity of individuals (Wilbur 1977; Van Buskirk and Yurewicz 1998; Glennemeier and Denver 2002), with activity levels increasing with density. As in our experiment food was not a limiting resource, it is likely that the reduction in tadpole density did not influence the amount of time invested for feeding.

Living in a group can confer many anti-predator benefits to group members (reviewed by Krause and Ruxton 2002). In the case of Anuran larvae, these may range from a greater effectiveness in detecting approaching predators ("many eyes" theory), predator confusion (predators' inability to single out and attack individual prey in a group), and lowered predation risk by means of predator avoidance (i.e. increased time needed by predators to encounter clumped prey), the "dilution effect" (i.e. reduced per capita chance to be captured), or abatement (i.e. lowered overall risk to be detected and selected by a predator).

Individual reaction to the per capita intensity of the cue together with the apparently negligible effect of thinning suggest that dilution is the main anti-predator benefit of grouping for *Rana dalmatina* tadpoles. In addition, pre-consumption cues (Hettyey et al. 2015) play also a role in information transfer between individuals (Chivers and Smith 1998). Further studies are needed to assess the importance of dilution for both the larvae of other Anuran species with different

degrees of propensity to form aggregations (Blaustein and Waldman 1992) and agile frog tadpoles in different developmental stages (Niecieza 1999).

Despite the cue:density ratio was fixed, the proportion of inactive tadpoles increased over time, except for control groups, suggesting that predator-exposure played some role in shaping this trend.

As predation risk was manipulated as to be constant, the increase in defensive behaviour could be interpreted as an increase in prey sensitivity to predation cues (i.e. risk as perceived by tadpoles).

Tadpoles have been reported to be sensitive to the number of cue pulses, which may provide information on the number of predation events irrespective of *per capita* cue concentrations (Schoeppner and Relyea 2009; McCoy et al. 2015). Inoculation of chemical cue on alternate days, could have thus triggered a progressively stronger defensive response. Moreover, Gazzola et al. (2015) have recently demonstrated that embryonic exposure to predation risk has long-term effects on the activity of olfactory bulb's neurons, enhancing predator-specific sensitivity in the early stages of tadpole development. A similar mechanism may be at work in tadpoles repeatedly exposed to predator cues.

Decreasing tadpole activity may also partially depend on a mechanical or “domino” effect: the sudden departure of an individual often sets off a chain of similar responses in the other group members and the frequency of these movements increases with group size (Cresswell et al. 2000; Yamaguchi et al. 2016). As such movements were recorded as “activity”, they are likely to have lowered the proportion of inactive individuals during the first phases of the experiment. Interestingly, activity was constant for the control group, suggesting that predator exposure was the mechanism which triggered of the domino effect rather than disturbance by the observer.

To our knowledge, the current understanding of tadpole responses to predator-exposure does not include the comprehension of how animals actually perceive the risk (Lima and Steury

2004). Information in this direction would provide a much deeper understanding of tadpole behavioural ecology and would allow formulating explanations much more fitted to experimental data. This is a basic issue, and future advances will need joint efforts by behavioural ecologists and neurobiologists to shed light on the physiological bases of behavioural responses to predation risk.

Ethical note: The study was carried out with permissions by the Italian Ministry of Environment (Prot. 0035817/PNM, validity 2013-2015). The study was conducted in conformity with the Italian current laws for amphibian collection and detention and adhering to the Animal Behaviour Society Guidelines for the Use of Animals in Research.

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Figure legends

Fig.1. Example of the experimental design for the days of removal/testing (schedule first day) for treatment D. With the exception of B (no removal treatment) all treatments we conducted following this procedure. In the upper label is reported the cue-concentration % present in the experimental container, and in the lower the *per capita* amount of cue.

Fig. 2. Illustration of the experimental design for all treatments during removal/testing days. The circle size is proportional to the density of tadpoles for each treatment along the experiment. On the left vertical axis are reported treatments while on the right the amount of cue per tadpole (i.e cue:density ratio or per capita amount of cue), which was held constant for each day of testing.

Fig. 3. Visualization of the means \pm SE of the proportion of inactive tadpoles for the whole experiment for the days of removal/testing (schedule first days)

Fig. 1

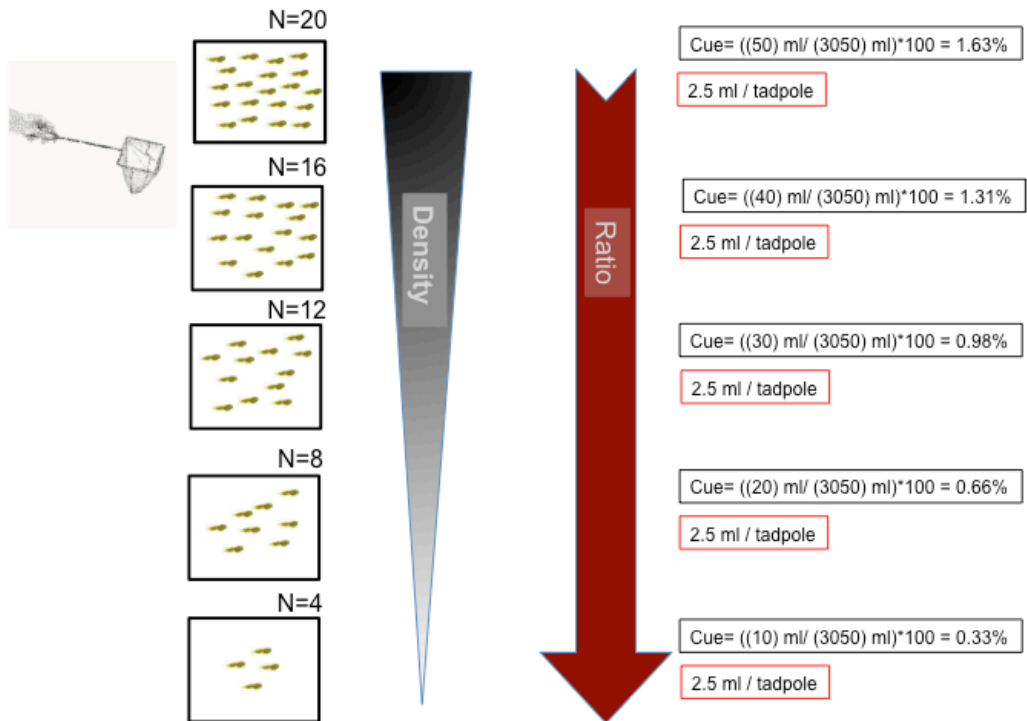


Fig. 2

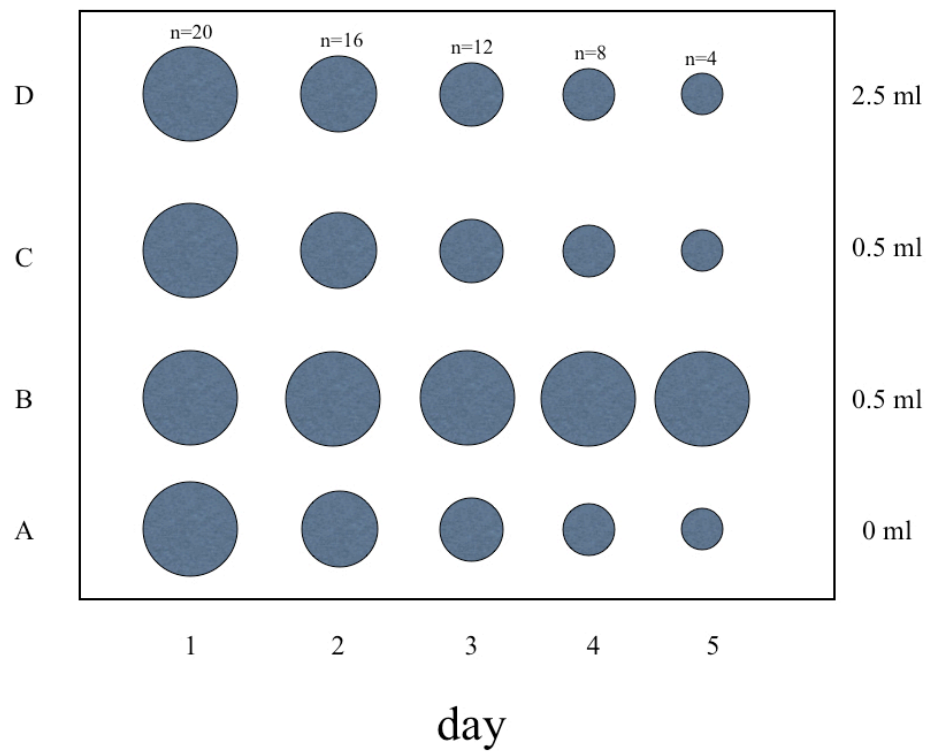
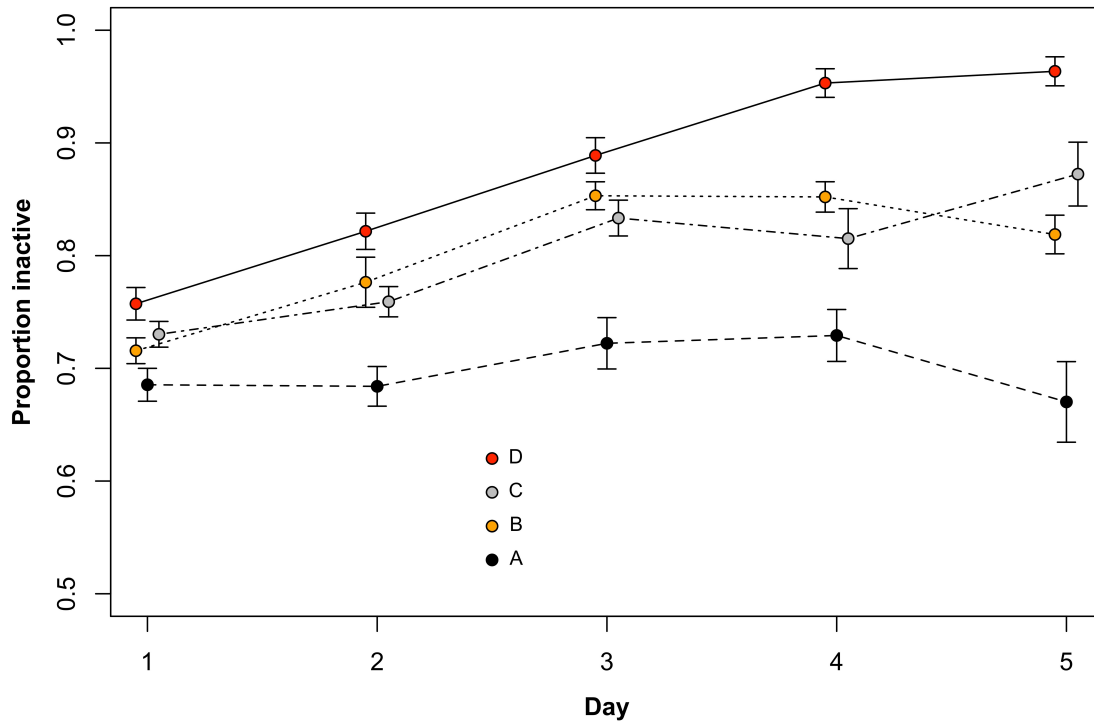


Fig. 3



Supplementary material

Tab. ST1. Means \pm SE of the raw data for all treatments during the entire experiment. Upscript letters show differences among treatments, after post hoc comparison, for each day based on fitted value of the full model.

Days	Treatments A-D			
	A	B	C	D
1	0.68 ^a \pm 0.01	0.71 ^b \pm 0.01	0.73 ^c \pm 0.01	0.75 ^d \pm 0.01
2	0.68 ^a \pm 0.01	0.77 ^b \pm 0.02	0.75 ^c \pm 0.01	0.82 ^d \pm 0.01
3	0.72 ^a \pm 0.02	0.85 ^b \pm 0.01	0.83 ^b \pm 0.01	0.88 ^c \pm 0.01
4	0.72 ^a \pm 0.02	0.85 ^b \pm 0.01	0.81 ^c \pm 0.02	0.95 ^d \pm 0.01
5	0.67 ^a \pm 0.03	0.81 ^b \pm 0.01	0.87 ^c \pm 0.02	0.96 ^b \pm 0.01

Fig. S1. Boxplot representing the proportion of inactive tadpoles, for the four treatments, during the first day (day 1) of the experiment.

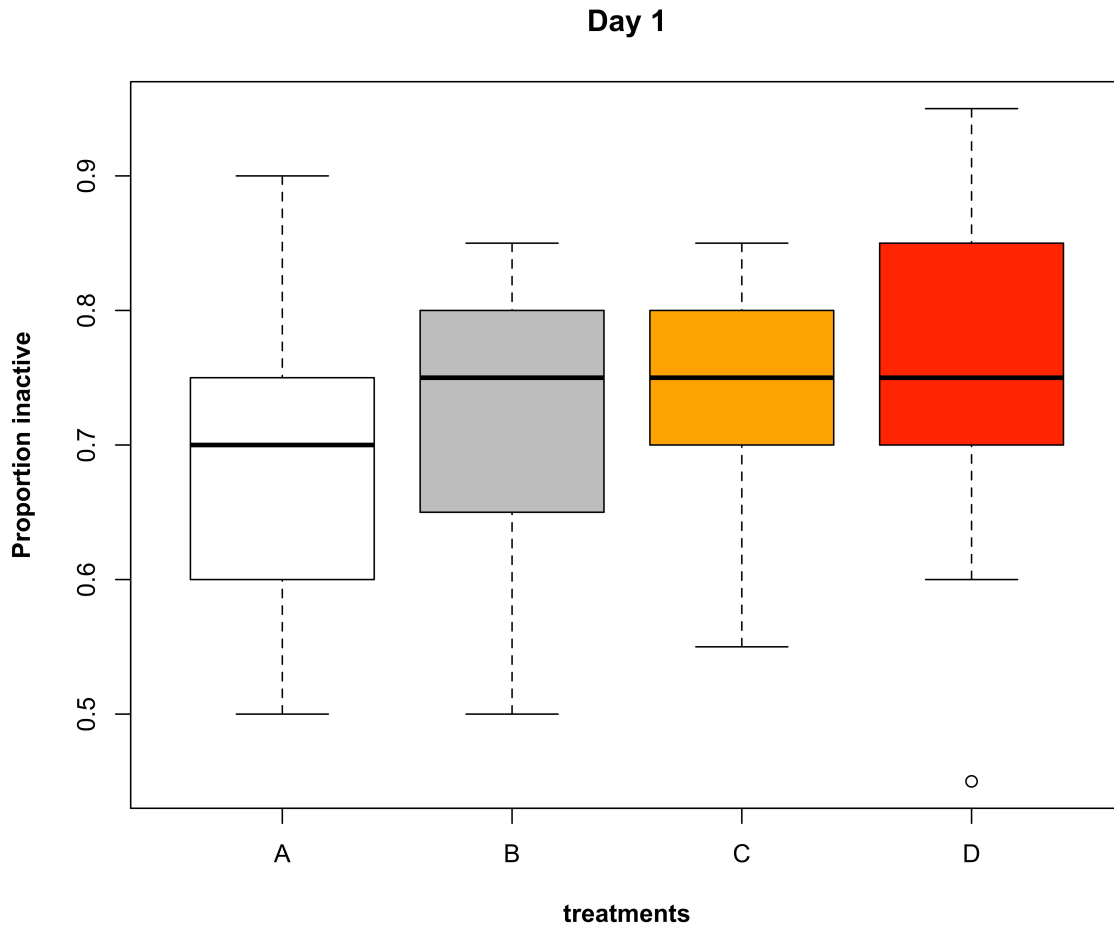
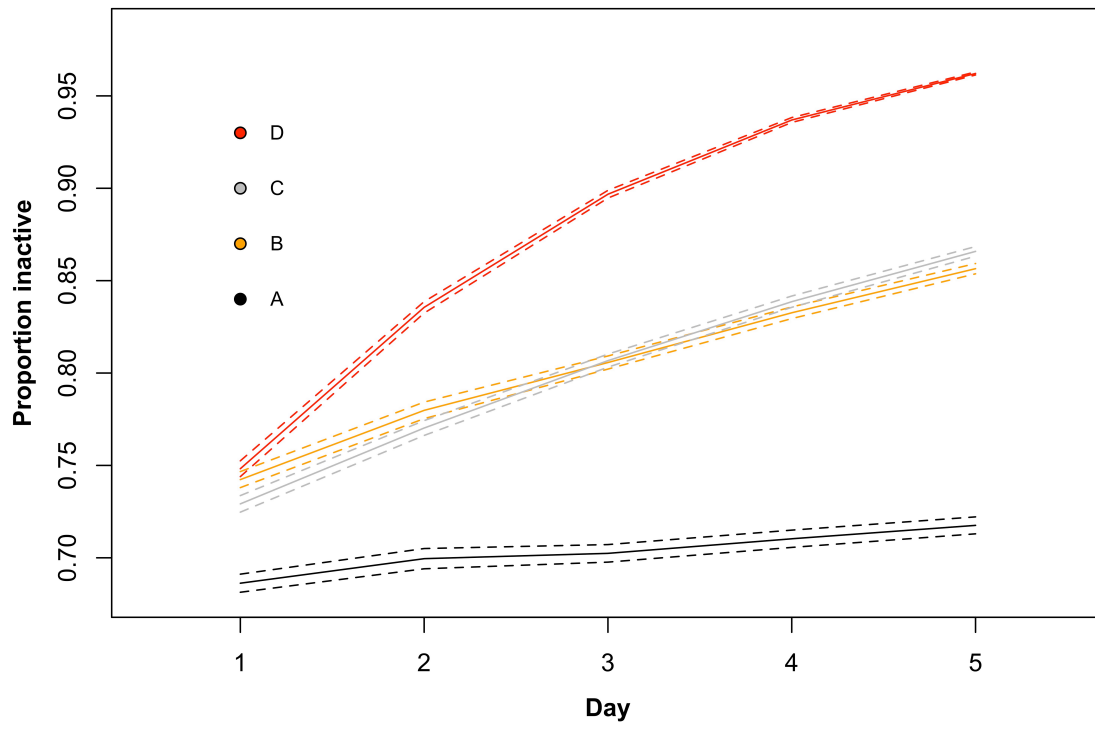


Fig. S2. Predictive graph showing means (solid lines) and 95% CI (dashed line) of the model (GLMM) for the proportion of inactive tadpoles.



CHAPTER 4

Is it worth the risk? Food deprivation affects anti-predatory responses in *Rana esculenta* tadpoles

(Manuscript)

Is it worth the risk? Food deprivation affects anti-predatory responses in *Rana esculenta* tadpoles

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Abstract

Predation risk has been demonstrated to affect foraging behavior and, more generally, the amount of time devoted by animals to the search for food. Food deprived animals should be risk prone and relax behavioral defenses to a wider extent than well fed individuals, when exposed to the risk of predation (context-dependent effects of predation risk). To test for this prediction we used a 2×2 factorial design experiment, manipulating both the energetic state (fed vs. fasted) and exposure to an attack-released cue (injured vs. uninjured conspecifics) of water frog tadpoles (*Rana esculenta*). Food deprivation significantly lowered the level of activity of predator-exposed tadpoles. As, in our experiment, no food resource was added to test containers, energy conserving behavior both delayed starvation and lowered the probability of encountering the potential predator. Tadpoles belonging to the fed-injured group tended to swim nearer to the cage containing the stimulus than other treatments. Possibly, prey-borne cues are interpreted by conspecifics as the potential presence of both a predator and a food source and fed tadpoles may be more confident than fasted tadpoles in their ability to escape predation.

Key words: alarm cues, defensive behavior, foraging activity, hunger, predation risk

Introduction

A clear feature of life is that, in order to survive and reproduce, animals must search for food in the surrounding environment. Foraging effort and diet selection are generally considered functions of either energy maximization (MacArthur and Pianka 1966) or macronutrient optimization (Simpson and Raubenheimer 2012). Nonetheless, foragers are potential meals themselves, or, in the case of top-predators, have to face with intra-guild interference competition (Linnell and Strand 2000). Predation risk has been demonstrated to affect foraging behavior and, more generally, the amount of time devoted by animals to the search for food (Lima and Dill 1990) and is expected to be a further selective force driving the way by which wild animals properly balance the benefits of nutrition and the costs of a premature death at the hands of predators or by starvation.

Theoretical models and empirical studies on the trade-off between starvation and predation risk predict that, when the risk of starvation increases, prey should be more prone to face the costs of exposure to predators (Kohler and McPeck 1989; Petterson and Bronmark 1993; Heithaus et al. 2007). On the opposite, when resources are abundant, prey can adopt a stronger defensive strategy, devoting less time to food consumption in the presence of predators (Werner and Anholt 1993; Steiner and Pfeiffer 2007).

The role played on by predation risk strongly depends on the context; to enhance an individual's fitness, behavioral choices need to take in account three major factors: 1) the physical state of the organism, 2) the value of the resources and 3) the reliability of the cues from predators (Matassa et al. 2016). Animals' physiological conditions are affected by both biotic and abiotic environmental factors (Blaustein et al. 2012; Seebacher and Franklin 2012), including predation risk (Creel et al. 2007; Janssen and Stoks 2013), and can be a function of reserve levels, body mass and resource availability (Lima and Dill 1990; Houston et al. 1993; Lima 1998). The value of food resources differs among different species and can vary

according to the different life stages of any single animal (Raubenheimer and Simpson 2003). High quality foods are expected to yield benefits that outweigh the costs of predation risk during foraging and relax antipredator responses (Fraser and Huntingford 1986). The wide variety of morphological, physiological and behavioral responses evolved by prey to cope with predation risk can be induced by external signals such as chemicals released by predators. Adaptive responses require that prey are able to detect cues and that cues are reliable indicators of the actual risk of predation (Moran 1992; Ghalambor et al. 2007).

Anuran tadpoles are an excellent and well-studied system to test the effect of interacting environmental variables on behavioral anti-predator responses (Relyea 2003). The trade-off between growth and mortality rates can be a major factor in determining both the length of the larval period and size at metamorphosis (Werner 1992; Werner and Anholt 1993). Moreover, tadpoles rely primarily on olfaction to detect their predators (Saidapur et al. 2009) and can evaluate the intensity of predation risk by detecting and responding to several chemical cues present in the water (Brönmark and Hansson 2000), such as continually released predator-borne cues (kairomones), pre-consumption prey-born cues (stress-, attack-, capture-released cues) or post-consumption borne cues (kairomones and digestion released cues) (Chivers and Smith 1998; Hettyey et al. 2015).

When exposed to predation risk, tadpoles generally respond with schooling, spatial avoidance, reduced activity and release of alarm substances (Lawler 1989, Laurila et al. 1997, Relyea 2001, 2003; Hagman 2008), although some species have been reported to exhibit opposite responses (Hews and Blaustein 1985), suggesting that behavioral responses can be fine-tuned depending on environmental (experimental) conditions.

Energetic state has already been shown to affect activity levels and anti-predator responses in the tadpoles of several species (*Rana lessonae* and *R. esculenta*, Horat and Semlitsch 1994; *Pseudacris triseriata*, Bridges 2002; *R. clamitans*, Fraker 2008; *Lithobates sylvaticus*, Carlson

et al. 2015), suggesting that hunger increases the activity of tadpoles and, sometimes, the duration of their antipredator response. Nonetheless, no interaction between predation risk and hunger has been observed in *Rana* spp. (Horat and Semlitsch 1994).

In this study, we investigated how food deprivation (i.e. energetic state) affects *R. esculenta* tadpoles' ability to respond to predation risk. We combined a food regime (fed vs. fasted tadpoles) with a cue treatment (absence of presence of attack-released cue) and assessed the effect of their interaction on tadpoles' behavior. Prey-borne attack-released cues, which should inform on a very recent attack to a conspecific, are suitable to evaluate tadpole short-term behavioral responses (Fraker 2008).

According to the current models we expected risk-proneness, as assessed by measuring both the level of activity (Van Buskirk and MCCollum 2000) and stimulus avoidance, to be higher in fasted tadpoles than in fed conspecific exposed to predation cues.

Materials and methods

We collected *Rana esculenta* tadpoles in a pond within the University Campus (Pavia, Northern Italy) by dip netting. After capture, tadpoles were immediately introduced in 2 plastic containers (150 l) and the day after (31 May 2015) we selected 80 similar-sized individuals (Gosner stage. 30-34; Gosner, 1960) that were individually located in 0.8 l tubs filled with 0.5 l of well water. To uniform the feeding status of tadpoles, we provided food ad libitum for 3 days, checking every day for an ever-present excess of food in each tub. On 3 June we made a total water change in each tub and randomly assigned tadpoles to one of the two food treatments. Tadpoles of the “fed” treatment were provided with rabbit food in excess, while for the “fasted” treatment we removed every particle of food or feces that could be seen in the water (checking 3 times a day for particles removal) and maintained these conditions for 24

hours, until the beginning of the experiment. A 24 h food deprivation period is enough to induce different behavioral responses in predator-exposed tadpoles (Carlson et al. 2015).

By keeping each animal in separate containers we could properly exclude the possible effects arising from competition for food. Each tadpole was then randomly assigned to a cue treatment and housed in a 30×20×20 cm transparent plastic containers (test containers) filled with 3 l of aged tap water. We applied the same procedure for three consecutive days, recording the behavioral activity of, respectively, 28, 28 and 24 tadpoles per day. The 80 tadpoles tested were equally divided among the four treatment combinations (fed-fasted × uninjured-injured). Each test container was provided with 2 fiberglass-net cylindrical cages (3.5 cm in diameter), of which one was used for submitting the stimulus (injured or uninjured tadpole) and the other to control for the effect of the cage on tadpole behavior (Fig. 1). We let the focal tadpole to acclimatize for 20 minutes in a cage placed in the center of the test container, and then we carefully removed the cage and started to record tadpole activity by a digital camera (Sony HDR-CX240; 60 frame s⁻¹) for 15 min. Five minutes before the beginning of recordings, we added either a pocked or an uninjured tadpole in one of the two cages placed at the opposite corners of the test container, while the other cage was left empty. The pocked tadpole was used to simulate a predation attempt and was intended to trigger a defensive response. To prepare the pocked tadpole stimulus we followed a procedure similar to that used by Fraker et al. (2009). From our stock tank, we selected an individual slightly larger than the focal tadpole and submerged it in 50 ml of well water, within a small net. We employed a hypodermic needle (0.5 mm outer diameter) to poke the tadpoles tail muscle 3-4 times for 10-15 seconds each poke (i.e. the amount of time the needle remained in the tail muscle). Poked tadpoles were kept in the water for 10 minutes and then released in the cage inside the test container. The healthy status of pocked tadpoles was checked during and after the whole experiment, and no

tadpoles either died or seemed to be anyway affected by the treatment. Once the experiment ended, all animals were released at the site of capture.

We measured two kinds of behavioral variables: activity and avoidance. The level of activity, i.e. explorative behavior, was considered as a measure of tadpoles' risk-proneness, and was assessed by recording tadpoles' behavior (moving vs. inactive) every 30 seconds for the whole duration of the videoclip. At each snapshot, tadpoles were recorded to be active whenever we could observe any kind of movement, such as swimming or moving the tail. Avoidance was assessed by measuring the distance of the focal tadpole from the stimulus, using a 4×6 grid (5 cm in side) laid under the test container (Fig.1). At each snapshot, the centre of the square where the focal tadpole was observed was recorded as its actual position at that moment.

Statistical analysis

We used linear mixed models (LMMs) to evaluate the main effects of food treatment (fed vs. fasted), cue of predation (pocked vs. uninjured tadpole) and their interaction on behavioral responses (activity: total number of times each tadpole was seen to be active; avoidance: distance of the focal tadpole from the stimulus averaged on the 30 observations). Day of testing was inserted as random factor and its effect was evaluated comparing models with or without the random factor by likelihood ratio test (LRT).

All analyses were performed in R (version 3.2.1; R Core Development Team 2015) using the libraries nlme and lme4 (LMMs; Pinheiro et al. 2016) and lsmeans (Lenth 2016; we adjusted the p-values for multiple comparisons using Holm's correction). Diagnostic plots of residuals were examined to validate the model.

Results

Food treatment significantly affected tadpole level of activity, with fasted tadpoles being overall less active than fed tadpoles ($P=0.011$, Tab. 1, Fig. 2). A significant fasting \times cue interaction showed that the level of activity depended jointly on the stimulus provided and whether tadpoles were fed or fasted ($P=0.026$, Tab. 1). Fasted tadpoles exposed to injured conspecific were recorded as active in 26% of observations vs. 39% for the fasted-uninjured group. Fed tadpoles showed a very similar level of activity (respectively 40% for the uninjured group and 43% for the injured group). On the whole, as showed by the contrasts of the model, activity was significantly lower in the fasted-injured group compared to all the other treatments (fed-injured: $\beta=-5.24\pm 1.53$, $t_{74.1} = 3.409$, $P = 0.0063$; fed-uninjured $\beta=-4.30\pm 1.53$, $t_{74} = -2.799$, $P=0.0327$; fasted-uninjured $\beta=-3.95\pm 1.53$, $t_{74} = -2.571$, $P=0.0486$; Fig. 2), whilst the level of activity of the remaining groups did not differ (lower estimate: $\beta=-0.35\pm 1.53$, $t_{74} = -0.228$, $P=0.99$). Tadpoles did not show any clear tendency to avoid the stimulus cue, although tadpoles belonging to the fed-injured group stayed closer to the stimulus compared to the other treatments (Fig. 3). Day of testing did not significantly affect either level of activity (LRT: $\chi^2 = 1.08$, d.f.=1, $P=0.298$) or avoidance (LRT: $\chi^2 = 0.011$, d.f.=1, $P=0.915$).

Discussion

Activity was the only behavior affected by both predation risk and hunger, with fasted tadpoles exposed to alarm signals from pocked conspecifics significantly reducing their activity compared to the other experimental groups. This outcome apparently disagrees with current theoretical model of starvation-predation risk trade-off, which predict that, to lower predation risk, fed individuals should be able to decrease their activity level without affecting their energetic state, while fasted individuals should be more prone to face predator threats to prevent starvation.

Since in our experiment no food resource was added to test containers, predator elusion was probably the main priority for fasted tadpoles. Animals have been shown to respond to declining levels of food availability by two opposite behavioral mechanisms that can delay the risk of starvation (Abrams 1984; Sogard and Olla 1996). On one side, animals can increase searching activity to improve the probability of finding prey; alternatively, at very low food levels, animals can lower energetic costs by hiding in refuges or reducing foraging activity, thus postponing the depletion of their energy reserves. The latter response has been observed in several taxa, from tubeworm *Serpula vermicularis* (Dill and Fraser 1997) to fish (*Perca fluviatilis*, Mehner and Wieser 1994; walleye pollock *Theragra chalcogramma*, Sogard and Olla 1996) and Iberian rock lizard *Lacerta monticola* (Martín et al. 2003).

In the real world, energy conserving behavior has the surplus benefit of decreasing the probability of encountering predators. In the highly dangerous, as presumably perceived by tadpoles, and contemporary food deprived area of our test containers, fasted tadpoles chose to reduce their level of activity as the most favorable behavioral response.

Neither cue-exposed fed tadpoles nor unthreatened fasted tadpoles show any reduction in activity with respect to controls (fed-unthreatened tadpoles), supporting the hypothesis that it was the interaction between energetic state and predation risk to trigger a strong behavioral response. Our results are consistent with the findings by Pueta et al. (2016), who, testing *Pleurodema thaul* tadpoles, recorded an increase in the activity of food-deprived tadpoles only when exposed to both an alarm cue and food ration.

Testing four hunger levels of *Rana esculenta* tadpoles, Horat and Semlitsch (1994) concluded that hunger and predation risk generate conflicting demands – increased activity and feeding effort vs. reduced activity and energy intake -, and rejected the balancing hypothesis, i.e. that the response to one demand is influenced by the other one. In the absence of food resources, it is plausible that the effects of predation threat predominated.

As reported for fish (Martínez et al. 2003; Yan et al. 2015), fasting may also lower the swimming performance of tadpoles, increasing their vulnerability to predators, and/or enhance their olfactory detection ability, as shown for laboratory rats (Pager et al. 1972; Aimé et al. 2007), both factors affecting their defensive response in the same direction.

Other studies have obtained contrasting results: as an example, Carlson et al. (2015) observed that fasted wood frog (*Lithobates sylvaticus*) tadpoles became more active when exposed to conspecifics alarm cues, while fed tadpoles did not respond. In contrast, a previous study had recorded a reduction in activity in wood frog (*Rana sylvatica*) tadpoles exposed to alarm cues (Ferrari et al. 2007). Unfortunately, it is not clear whether in the experiment of Carlson and collaborators test arenas contained algal and microbial food, which would have stimulated fast tadpoles to feed even in the presence of alarm cues (see Horat and Semlitsch 1994).

Although avoidance behavior did not significantly differ among treatments, tadpoles belonging to the fed-injured group tended to swim nearer to the cage containing the stimulus. It is hard to explain why these tadpoles should have been more prone to risk predation. It is possible that chemical cues from poked tadpoles are interpreted by conspecifics in two contrasting ways, i.e. as the potential presence of both a predator and a food source, the injured conspecific itself. In fact, as occurs in many other Anuran species, *R. esculenta* tadpoles are known to consume dead conspecifics, both larvae (Tyler 1958; Dushin 1975) and adults (Sas et al. 2007).

Fed tadpoles, which should maintain high physiological functioning, such as escape ability, may be more confident than fasted tadpoles in their ability to escape predation and then more prone to explore the cue-source as to possibly get access to a protein-rich food source (Crump 1990).

Foraging and avoiding predators are conflicting demands (Lima and Dill 1990), and there still is a lack of consensus in available data as to how tadpoles kept at different levels of hunger respond to cues of injured conspecifics. Responses may vary among species or even among

different populations of a single species; moreover, we suspect that experimental results are greatly influenced by laboratory conditions, since variation in only one factor (in this specific case: food availability inside test containers) may lead to contrasting outcomes. Sound estimates of the relative importance of predation- and starvation-risk for behavioral responses in Anuran larvae will then depend on the accuracy with which they will be measured.

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Tab. 1. Main effects of food treatment (fed or fasted), cue treatment (injured or uninjured conspecific) and their interaction on tadpoles' activity and avoidance behavior (d.f. = 1.74 for all tests). Linear mixed model fit by REML t-tests with Satterthwaite approximations to degrees of freedom.

	Variable	$F_{1,74}$	P
(a) Activity	Food	6.72	0.011
	Cue	1.83	0.179
	Food \times Cue	5.16	0.026
(b) Avoidance	Food	1.44	0.233
	Cue	0.052	0.818
	Food \times Cue	0.73	0.390

Figure legends

Fig. 1. Experimental container used for measuring tadpoles' behavior. The focal tadpole, on which behavioral variables are recorded, is located in the center of the container. Tadpole stimulus (injured or uninjured) is located in one of the cage in the corner of the container.

Fig. 2. Behavioral activity of tadpoles for food (fed and fasted) and cue treatment (injured and uninjured conspecific). Bars represent means \pm SE. Bars under the same dashed line are not significantly different ($P>0.05$).

Fig. 3. Avoidance behavior of fed and fasted tadpoles to the chemical stimulus (injured or uninjured conspecific). On the y-axis is represented the mean distance (cm) from the stimulus. Bars represent means \pm SE. Bars under the same dashed line are not significantly different ($P>0.05$).

Fig.1

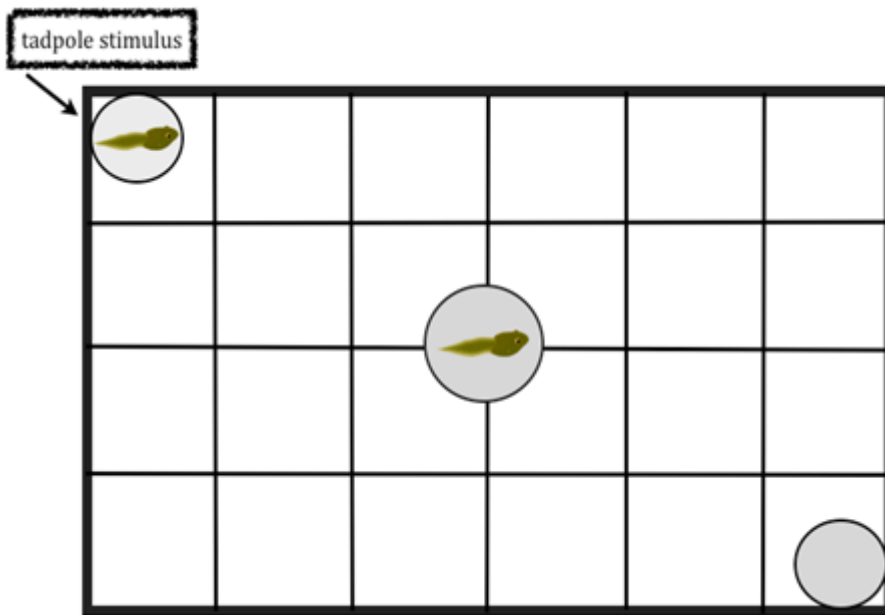


Fig. 2

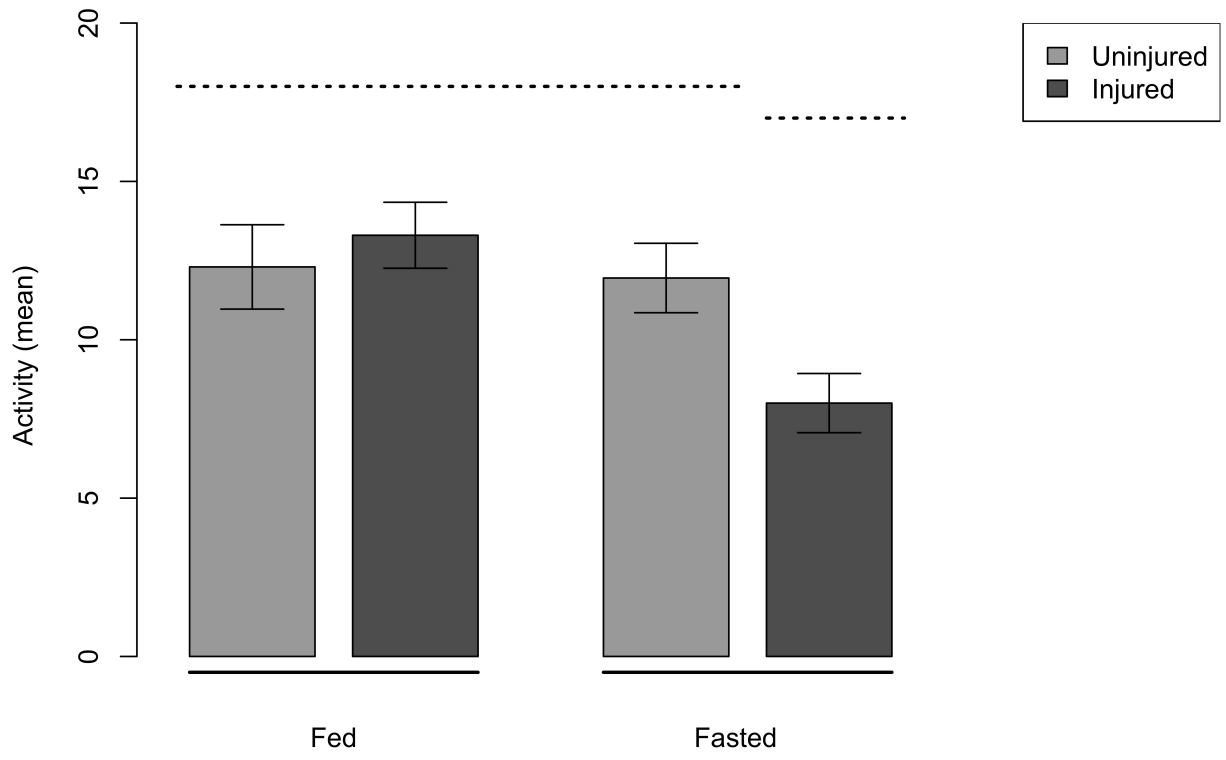
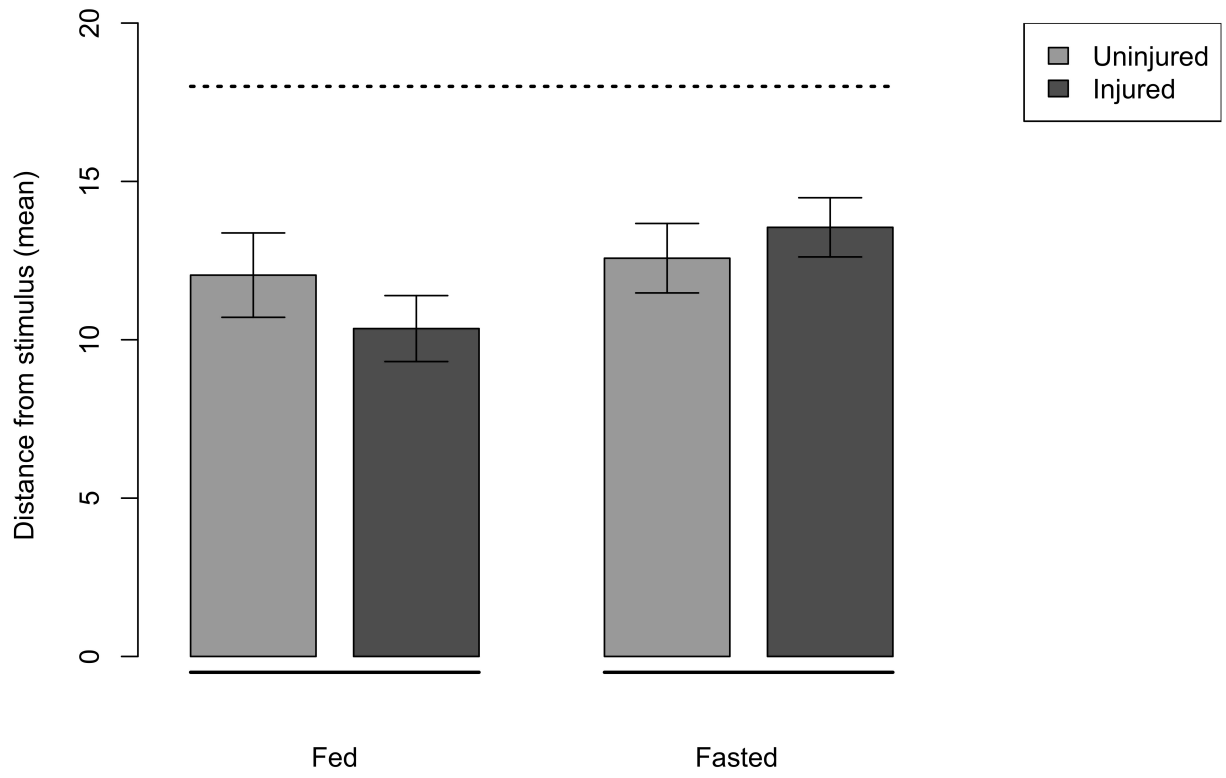


Fig. 3



CHAPTER 5

Phenotypic responses to alien and native predators by agile frog embryos and tadpoles

(Preliminary manuscript)

Phenotypic responses to alien and native predators by agile frog embryos and tadpoles

Introduction

Invasive predators species represent a serious ecological threat, and can have a deep impact on native prey populations (Kats and Ferrer 2003, Salo et al. 2007). Anti-predator defences of native prey may be useless to face the danger of new predators, and natural selection need a certain amount of time to produce effective defensive traits (Strauss et al. 2006, Sih et al. 2010). The possible outcome of this scenario it that prey can go extinct or decline. However, prey may also be able to express defensive responses through plasticity (induced defences), and coexist with the predator. These plastic modifications have been observed to enhance survival in presence of free hunting predators. Proper predator recognition and effective defences responses are generally observed when prey and predator share a common evolutionary history (Strauss et al. 2006, Sih et al. 2010). Native prey may not be able to recognize a new predator as a threat, and this situation may lead to a dramatic reduction of prey population.

In this study, I examined predator-prey interactions between embryos or tadpoles of the native agile frog (*Rana dalmatina*) and the introduced red swamp crayfish (*Procambarus clarkii*) in the provinces of Pavia and Milan.

P. clarkii (Girard, 1852), has been introduced, for aquaculture purposes, into several states of the continental USA and into other countries; this massive translocation has made *P. clarkii* to be the most cosmopolitan crayfish today (Scalici and Gherardi 2007).

In Europe, the crayfish was first imported into Spain in 1972 (Ackefors 1999) and then was introduced in many countries (Portugal, Cyprus, England, France, Germany, Mallorca, Netherlands (Hobbs et al. 1989) and recently Switzerland (Stucki 1997). In Europe, it was first

imported into Spain (1972)(Ackefors 1999), and then was introduced in many countries (Portugal, Cyprus, England, France, Germany, Mallorca, Netherlands, Switzerland). Since 1990, *P. clarkii* has been found in numerous ponds and streams of northern and central Italy provinces (Gherardi et al. 1999). In northern Italy, *P. clarkii* had a great expansion in some areas of the Po River drainage basin in Piedmont region and in the Reno River drainage area in eastern Emilia-Romagna (Barbaresi and Gherardi 2000).

In this study I considered two anuran populations in relation to the presence of *P. clarkii*; the one located in San Colombano (Milan, Lombardy) has a well established crayfish population, while the other, inside Bosco Castagnolo nature reserve (Pavia, Lombardy), probably lack or has a scarce presence of *P. clarkii*.

Materials and methods

I collected 6 agile frog clutches from each of two anuran populations located in as many ponds about 30 km far from each other (Fig. 1). One location (San Colombano, Milan province) has an established *P. clarkii* population, with evidence of crayfish reproduction (juveniles and brooding females). Predators - sub-adult crayfish and late instar dragonfly larvae - were collected from the same ponds using dip-nets. All predators were transferred to the laboratory and kept individually in 1l plastic tubs until the onset of the experiment.

I extracted three subsamples (50 eggs each) from every single egg mass (18 subsamples from each population) and inserted them in separated experimental plastic tanks (30×20×20 cm) containing 6 l of aged tap water. All tanks were arranged in three spatial blocks inside a room (36 tanks), where window were left open to uniform light and temperature, as much as possible, to external conditions. The daily chemical treatments, randomly assigned to each experimental tank at the beginning of the experiment, were as follows: 1) 50 ml of well water

(control group), 2) 50 ml of fed gammarids dragonfly cues and 3) 50 ml of fed gammarids crayfish cues. Each subsample of one clutch was assigned for a different chemical cue. Treatments (well water and predators' cues) were stopped as soon as the first egg in whichever kairomone (i.e. predators' cues) tank hatched. I recorded the time of hatching for each experimental tank (i.e. when 50% of tadpoles were detached from the jelly) and morphological measurements of hatchlings individuals (tail length, tail depth, body length, body depth, total length; these data are not reported in this preliminary draft) for all experimental tanks.

By a second experiment I conditioned tadpoles with chemical cues coming from crayfish (*Procambarus clarkii*) or dragonfly larvae (*Anax imperator*) fed with either gammarids or tadpoles. I used a 2×5 full factorial design combining populations (two levels) and chemical treatments (five levels). All experimental tanks were arranged in 6 spatial blocks inside the laboratory, and treatments were randomly assigned to each tank. Tanks (30×20×20 cm) were filled with 8 l of aged tap water; a total change of water was made twice a week. I had six replicates for each combination, and a total of 60 experimental tanks.

The daily chemical treatments, randomly assigned to each experimental tank at the beginning of the experiment, were as follows: 1) 100 ml of well water (control group), 2) 100 ml of fed gammarids dragonfly cues, 3) 100 ml of fed gammarids crayfish cues, 4) 100 ml of fed tadpoles dragonfly cues and 5) 100 ml of fed tadpoles crayfish cues. In both experiments chemical cue was obtained keeping each individual predator in separated tubs (0.8 l) filled with 0.5 l of aged tap water, and providing food (tadpoles or gammarids) every day.

I provided chemical treatments to experimental tanks four days a week for five weeks, and recorded behaviour every day at the same time (2 p.m.). During behavioural observations, consisting of a 30 seconds period for each tank, I recorded the number of tadpoles that could be seen (visible) and the number of active individuals (swimming, moving the tail or eating). I recorded a further behavioral variable named "burst", consisting of observed number of

tadpoles' sudden accelerations. After five weeks I collected four tadpoles from each experimental tank for assessing morphological measurements.

Preliminary results and discussion

Hatching time was not affected by chemical treatments (control vs. predators' cues) and the difference between populations was fully captured by developmental stage at collection, as shown by the lack of difference between models with and without population factor ($LR\chi^2 = 5.3$, d.f.=3, $P=0.16$; Fig. 2-3, Tab.1-2). Tail depth showed to be affected by both treatment and population of origin (Tab. 3); in particular population 1 (Bosco Castagnolo) responded to tadpole-fed dragonfly significantly increasing tail depth on tadpole fed dragonfly, while population 2 (San Colombano) showed a tail depth decrease on tadpole fed crayfish treatment (Fig. 4, 7). Activity and hiding behaviour were affected both by treatment and population of origin (Tab. 5-6, Fig. 9-10); overall, tadpoles were less visible and active when in presence of cue coming from dragonfly larvae fed with tadpoles. The number of burst (sudden acceleration) strongly depended on treatment (Tab. 6, Fig. 11), with tadpole-fed dragonflies triggering the strongest response; population 1 showed a higher number of burst compared to population 2 for tadpole-fed dragonflies treatment.

The preliminary results of this experiment show some general differences for predator-induced traits between populations in relation to predator's species, however further analyses must be conducted to confirm these explorative findings.

Note: if not differently specified the data reported in figures represent mean \pm standard errors. For body measures, treatments are identified with the followings codes: c=control, P1=P.

clarkii fed gammarids, P2=*P. clarkii* fed tadpoles, A1=*A. imperator* fed gammarids, A2=*A. imperator* fed tadpoles. Elsewhere, predator-1 represents predator fed gammarids, and predator-2 refers to predator fed tadpoles. In figures and tables Bosco Castagnolo population is referred as population 1, San Colombano as population 2.

Ethical note: The study was carried out with permissions by the Italian Ministry of Environment (Prot. 0035817/PNM, validity 2013-2015). The study was conducted in conformity with the Italian current laws for amphibian collection and detention and adhering to the Animal Behaviour Society Guidelines for the Use of Animals in Research.

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Tab. 1. Main effects of LMM for **hatching time** as response variable (time ~ Treatment× Population+Stage at collection + (random=clutch)).

	Variable	χ^2	<i>d.f.</i>	<i>P</i>
	Treatment	0.67	2	0.71
<i>Hatching time)</i>	Population	6.29	1	0.01
	Stage at collection	0.007	1	0.92
	Treatment×Population	0.32	2	0.85

Tab. 2. Main effects of separated LMM for each population and **hatching time** as response variable for (time ~ Treatment×Stage at collection + (random=clutch)).

<i>Hatching time</i>	Variable	χ^2	<i>d.f.</i>	<i>P</i>
	Treatment	1.61	2	0.44
<i>Population1)</i>	Stage collection	0.41	1	0.51
<i>(Castagnolo)</i>	Treatment×Stage collection	1.51	2	0.46
		0.71	2	0.69
	Treatment	0.34	2	0.84
<i>Population2)</i>	Stage collection	0.13	1	0.71
<i>(S.Colombano)</i>	Treatment×Stage collection	1.22	2	0.54

Tab. 3. Main effects of LMM for body measurements (measure ~ treatment×population + total length + (random = block)).

	Variable	χ^2	<i>d.f.</i>	<i>P</i>
<i>Tail depth</i>	Treatment	6.58	4	0.159
	Population	7.01	1	0.008
	Treatment×Population	11.83	4	0.001
	Total length	1337	1	<0.0001
<i>Tail length</i>	Treatment	3.26	4	0.51
	Population	1.74	1	0.18
	Treatment×Population	4.19	4	0.380
	Total length	11634	1	<0.0001
<i>Body length</i>	Treatment	3.26	4	0.51
	Population	1.74	1	0.18
	Treatment×Population	4.19	4	0.380
	Total length	2570	1	<0.0001
<i>Tail muscle depth</i>	Treatment	12.05	4	0.016
	Population	0.26	1	0.605
	Treatment×Population	10.41	4	0.034
	Total length	540	1	<0.0001

Tab. 4. Main effects of GLMM for proportion of visible tadpoles as dependent variable. (prop visible ~ treatment × population + treatment × week + (random=block)).

	Variable	χ^2	<i>d.f.</i>	<i>P</i>
	Treatment	18.22	4	0.001
	Population	1.10	1	0.292
<i>Visible</i>	Week	9.96	4	0.041
	Treatment×Population	9.40	4	0.051
	Treatment×Week	34.56	16	0.004

Tab. 5. Main effects of GLMM for proportion of active tadpoles as dependent variable (prop active ~ treatment × population + week + (random=block)).

	Variable	χ^2	<i>d.f.</i>	<i>P</i>
	Treatment	42.10	4	<0.0001
	Population	9.08	1	0.002
<i>Active</i>	Week	382.04	4	<0.0001
	Treatment×Population	19.66	4	0.0005

Tab. 6. Main effects of GLMM for proportion of bursting tadpoles as dependent variable (prop burst ~ treatment × population + (random=block)).

	Variable	χ^2	<i>d.f.</i>	<i>P</i>
	Treatment	133.47	4	<0.0001
<i>Burst</i>	Population	2.18	1	0.13
	Treatment×Population	9.90	4	0.04

Fig. 1. Populations' location.

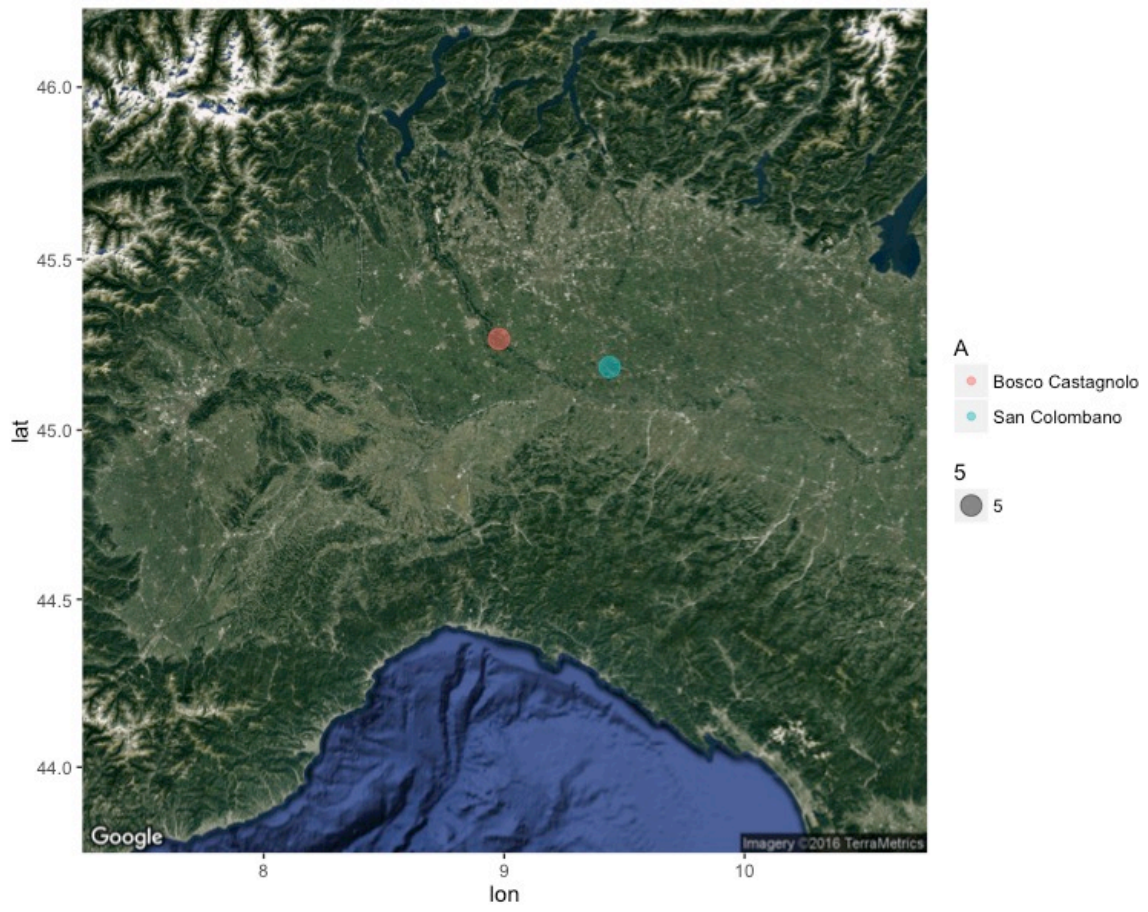


Fig. 2. Regression of hatching time (hours) against stage at collection.

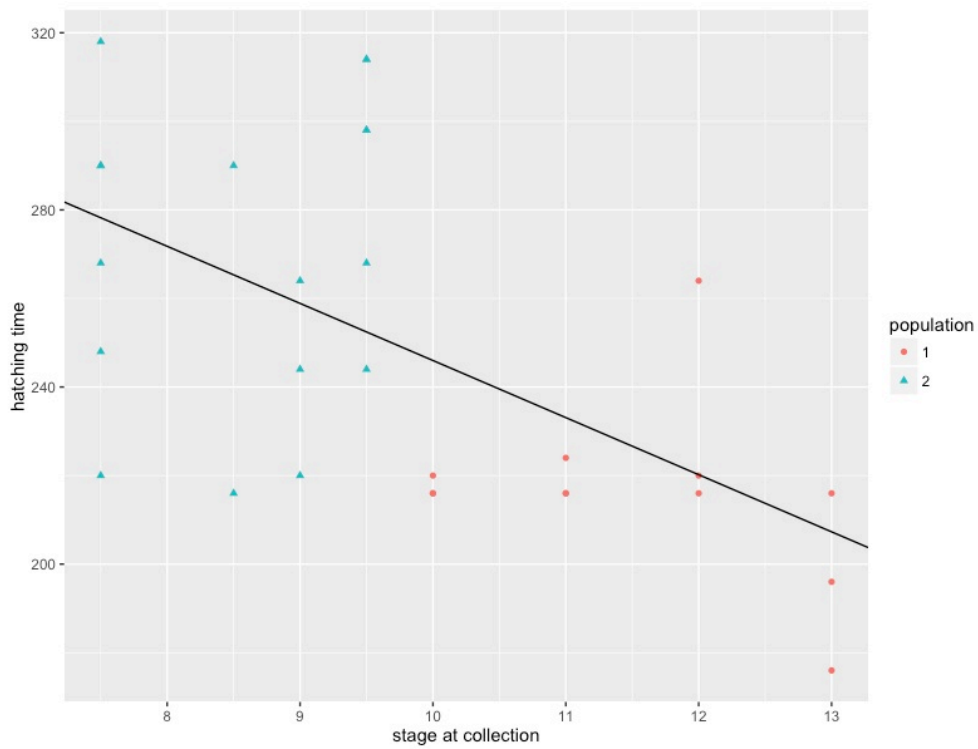


Fig. 3. Means \pm se of hatching time from the onset of the experiment.

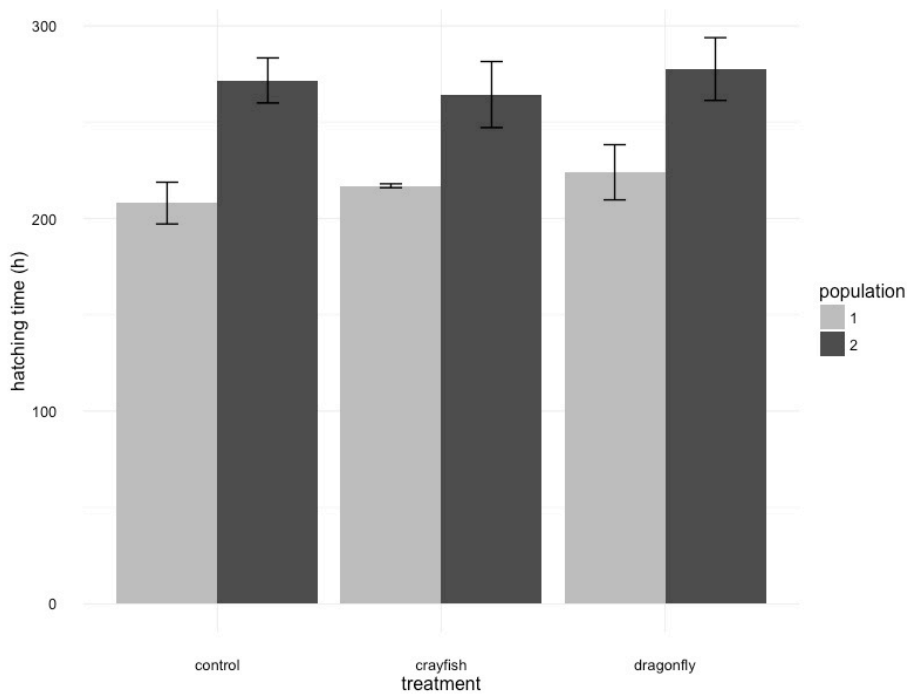


Fig. 4. Tail depth and tail length means (\pm se) for all treatments.

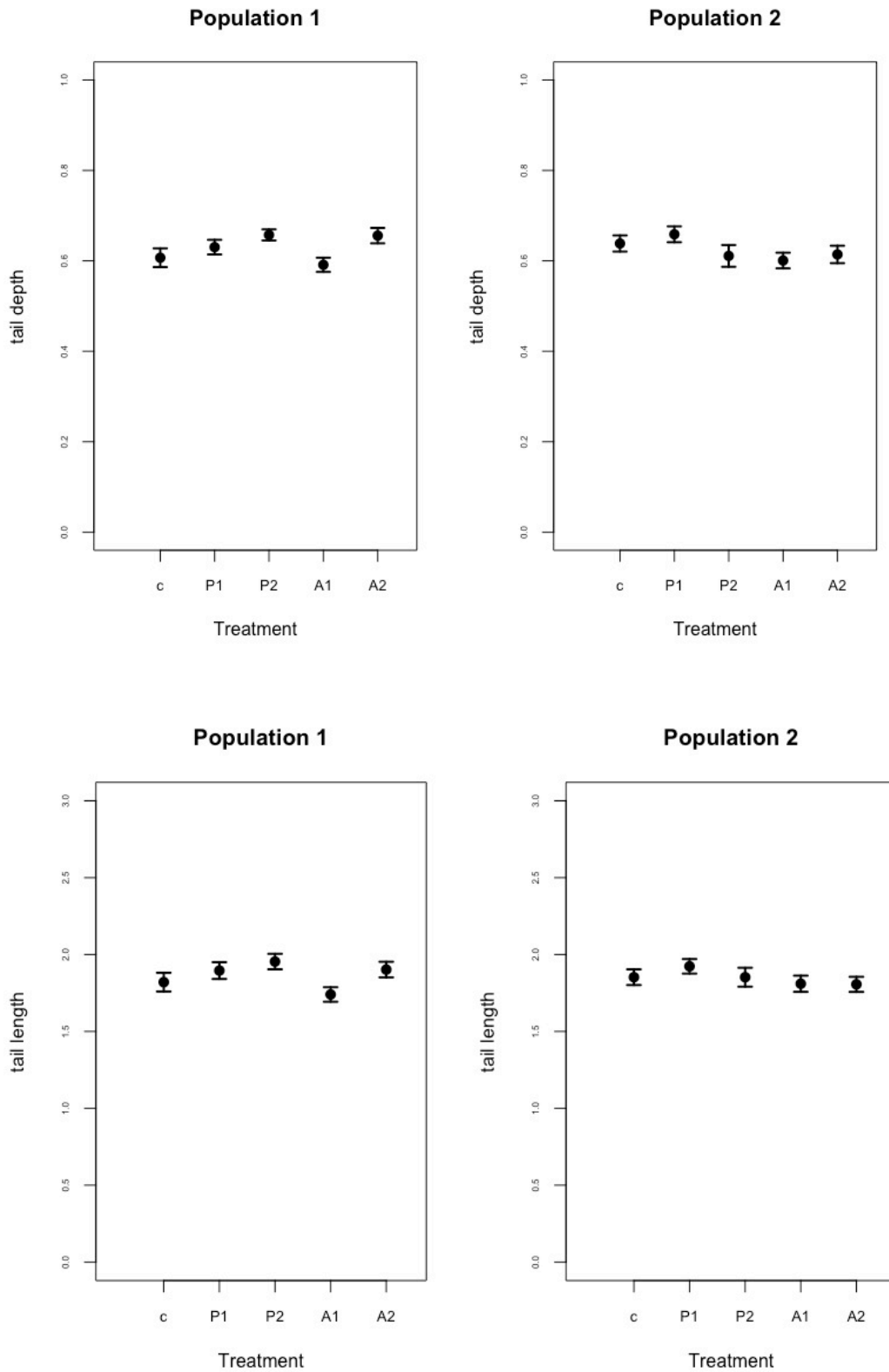


Fig. 5. Body length and body depth means (\pm se) for all treatments.

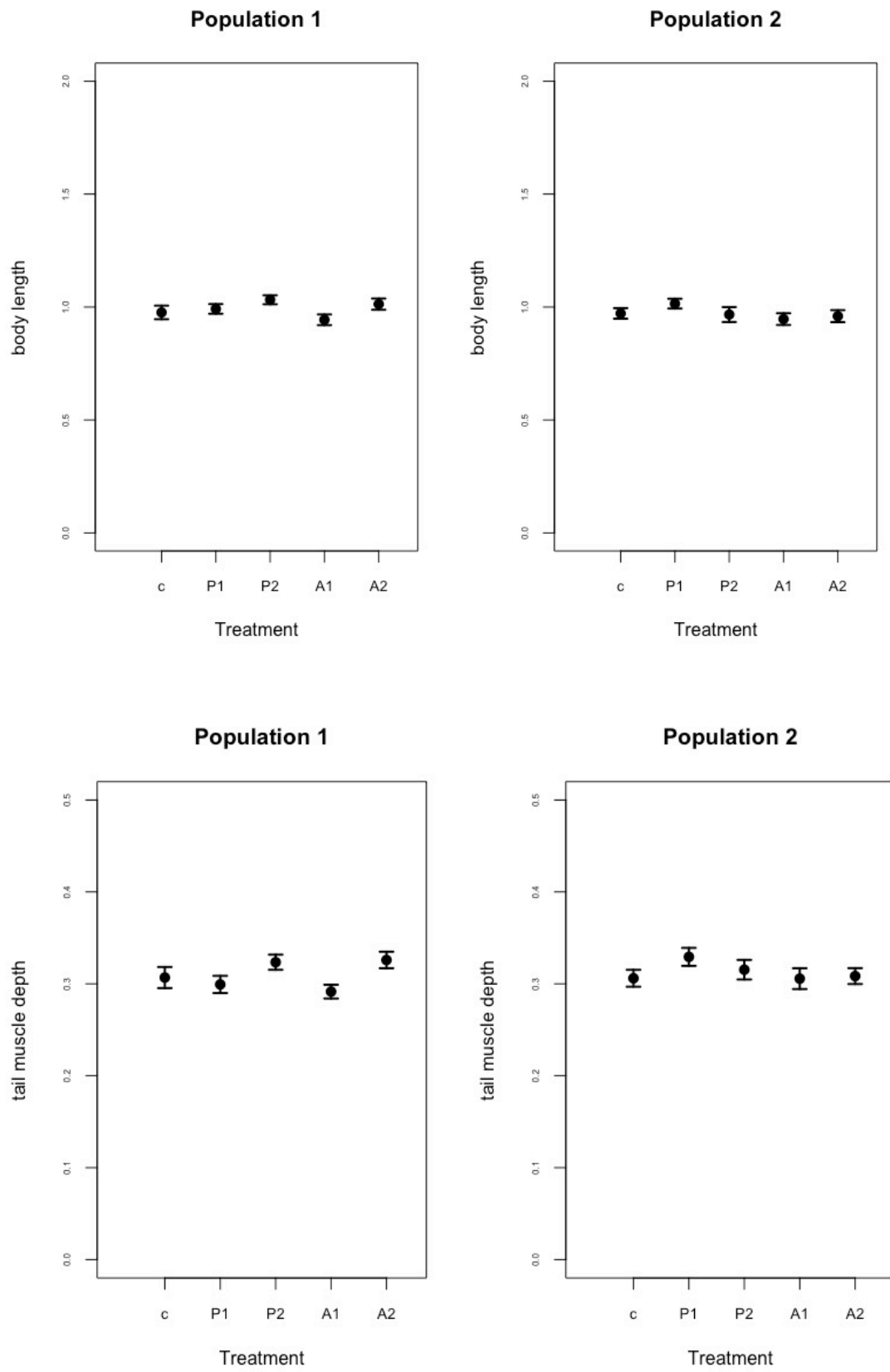


Fig. 6. Body measures against total length.

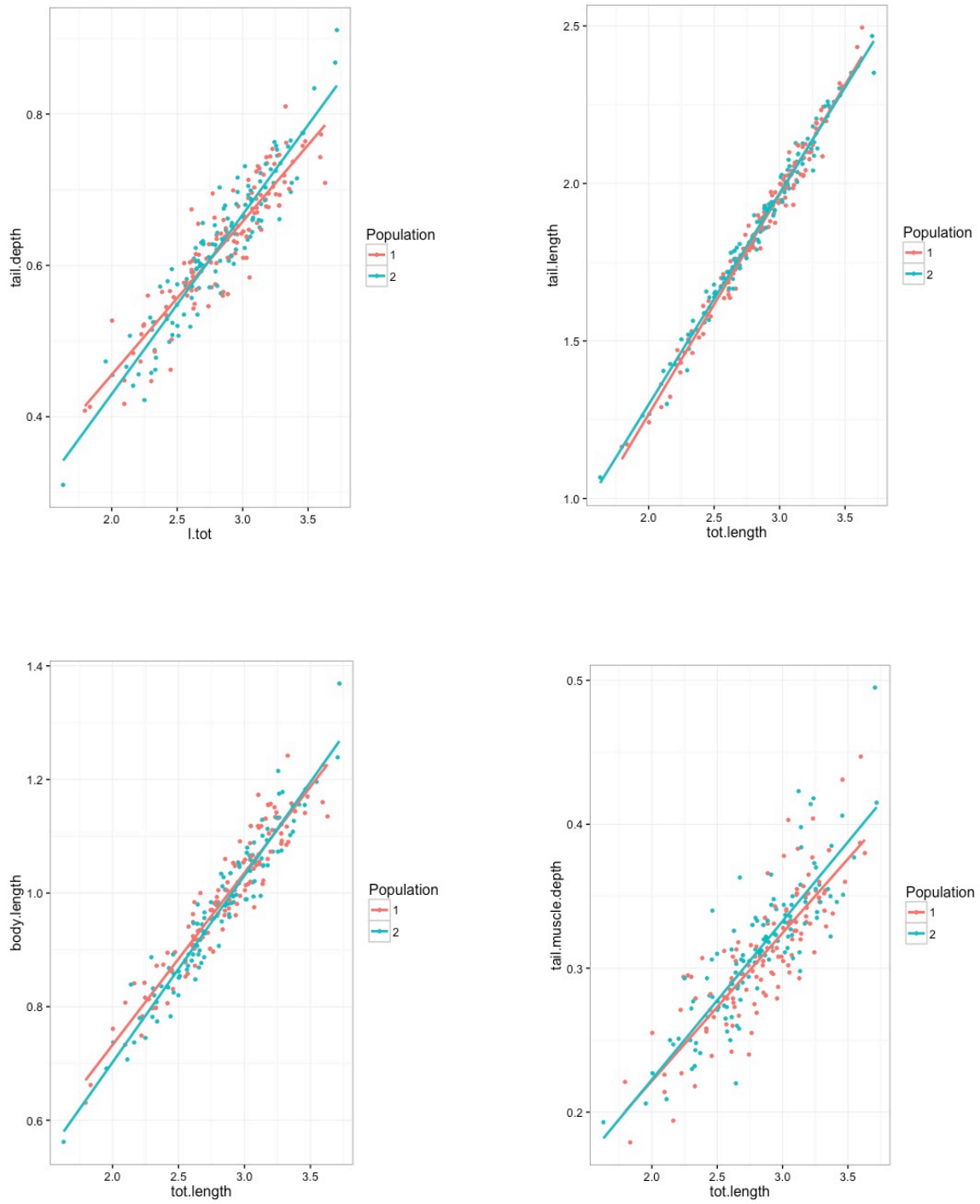


Fig. 7. Tail depth against total length for all chemical treatments.

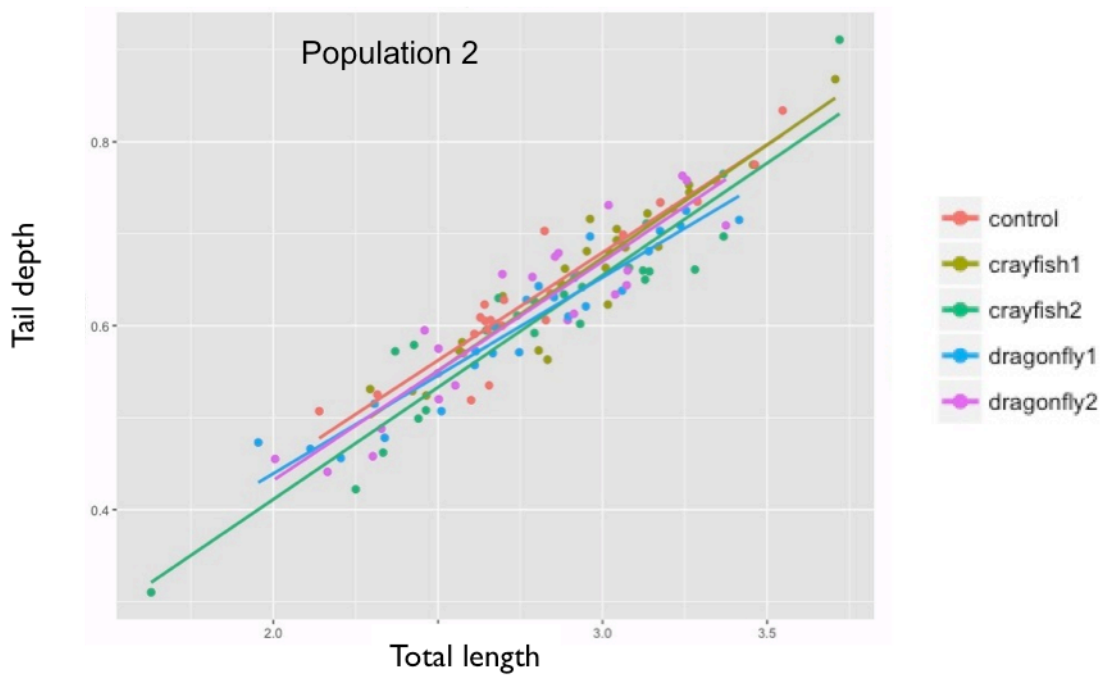
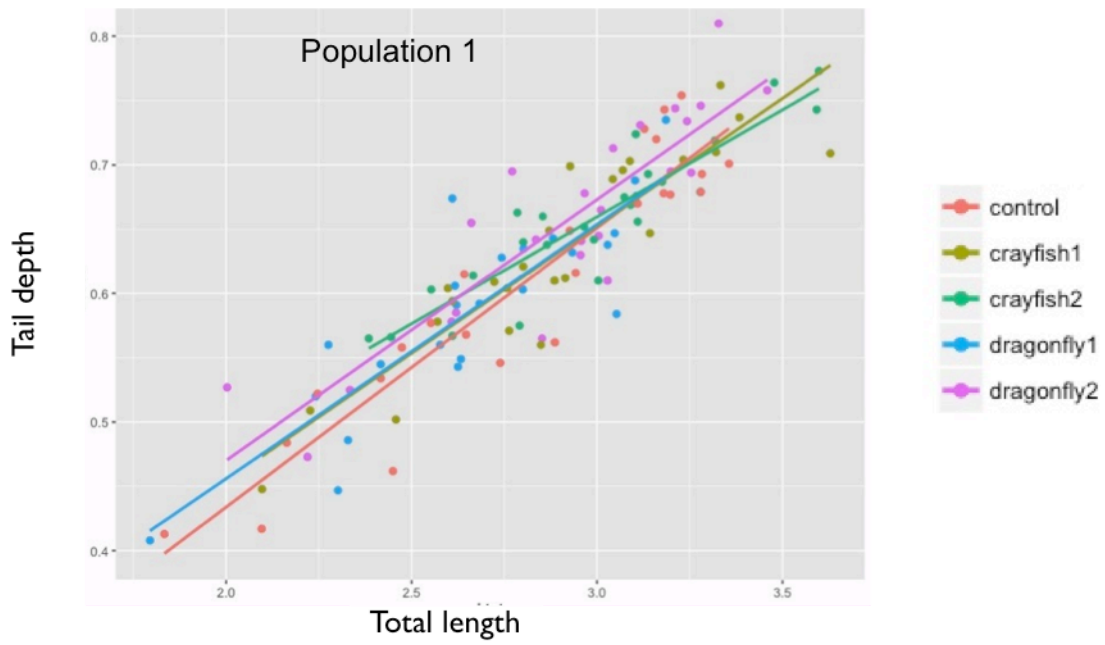


Fig. 8. Tail depth residuals (means \pm se) for all treatments from Bosco Castagnolo (population1) and San Colombano (population 2).

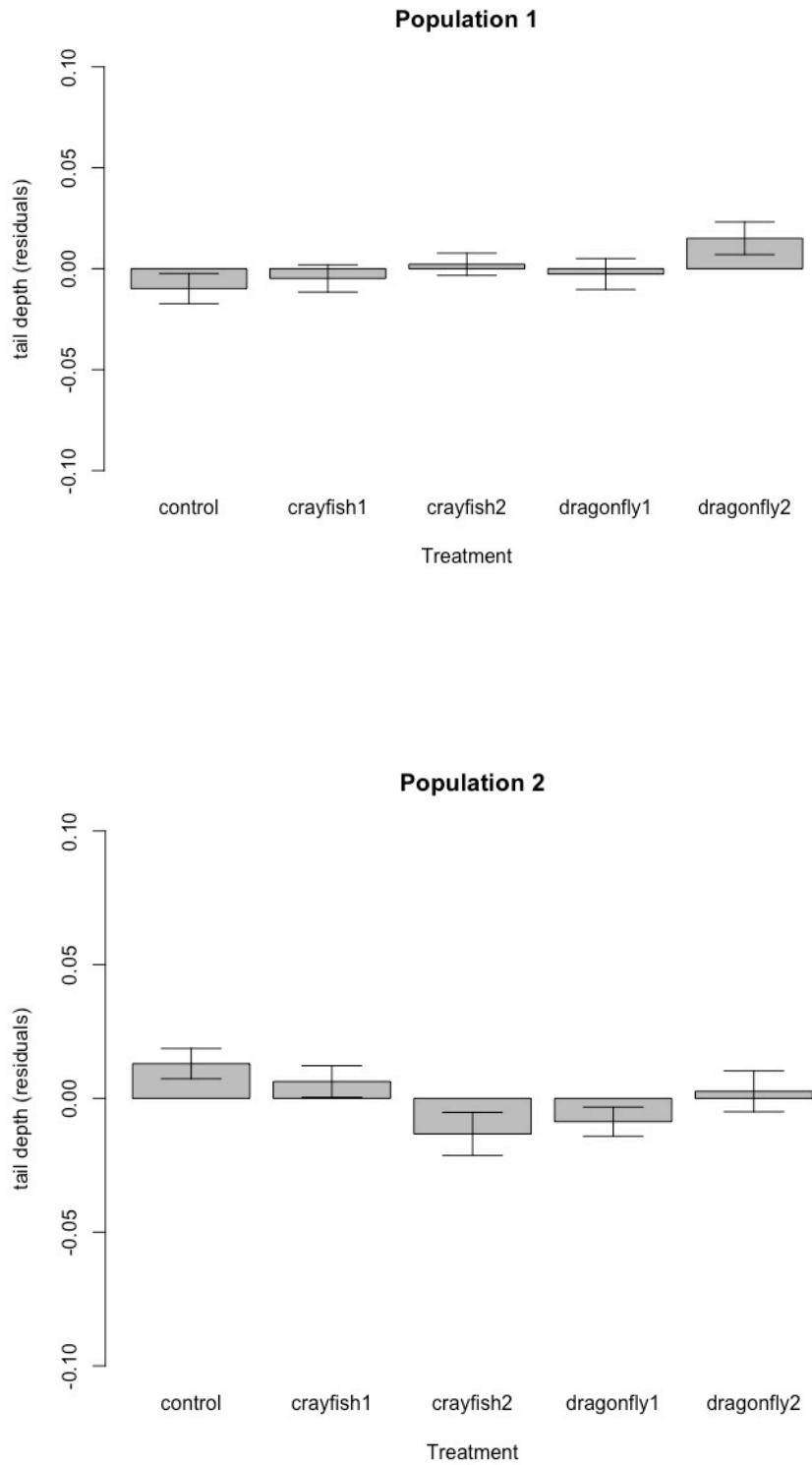


Fig. 9. Proportion of tadpoles visible (means \pm se).

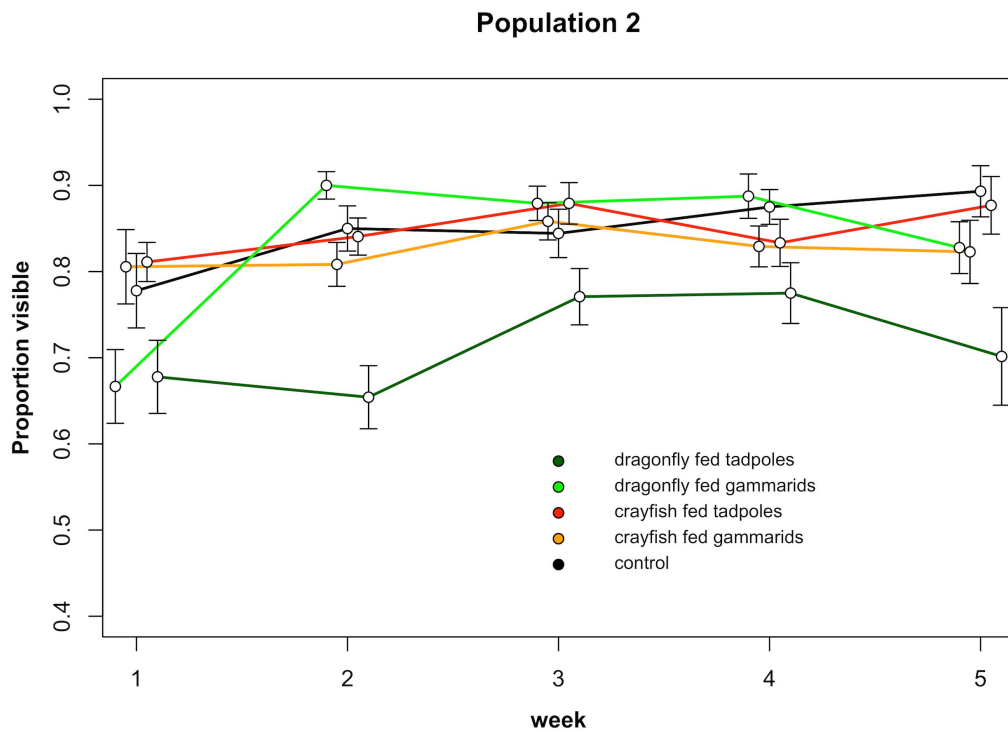
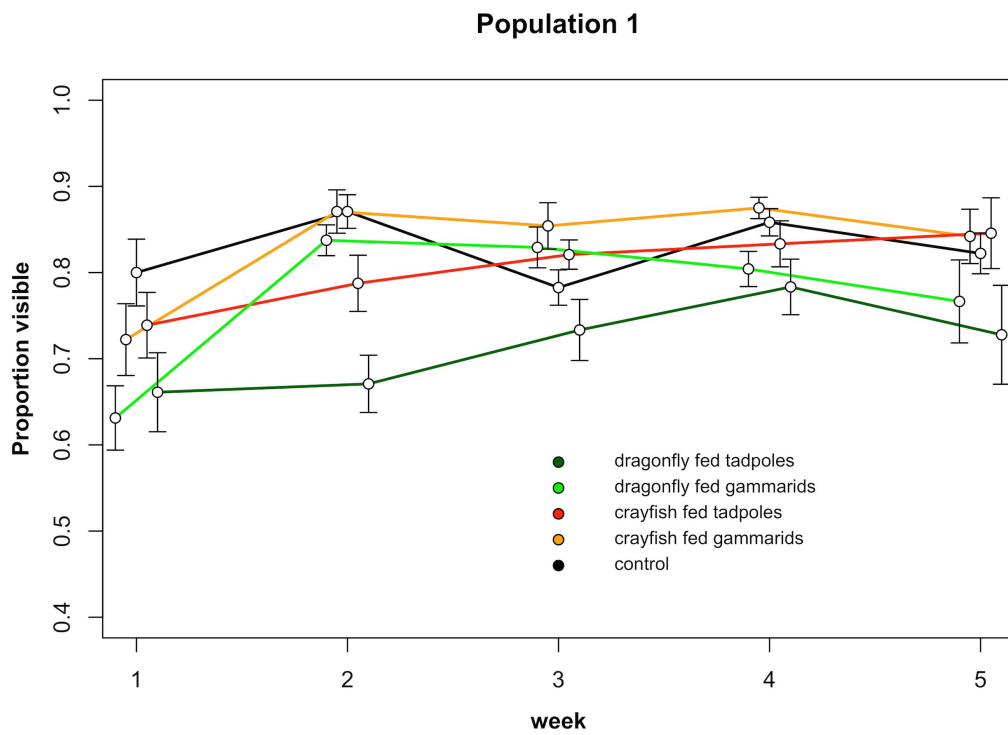


Fig. 10. Proportion of tadpoles active (means \pm se).

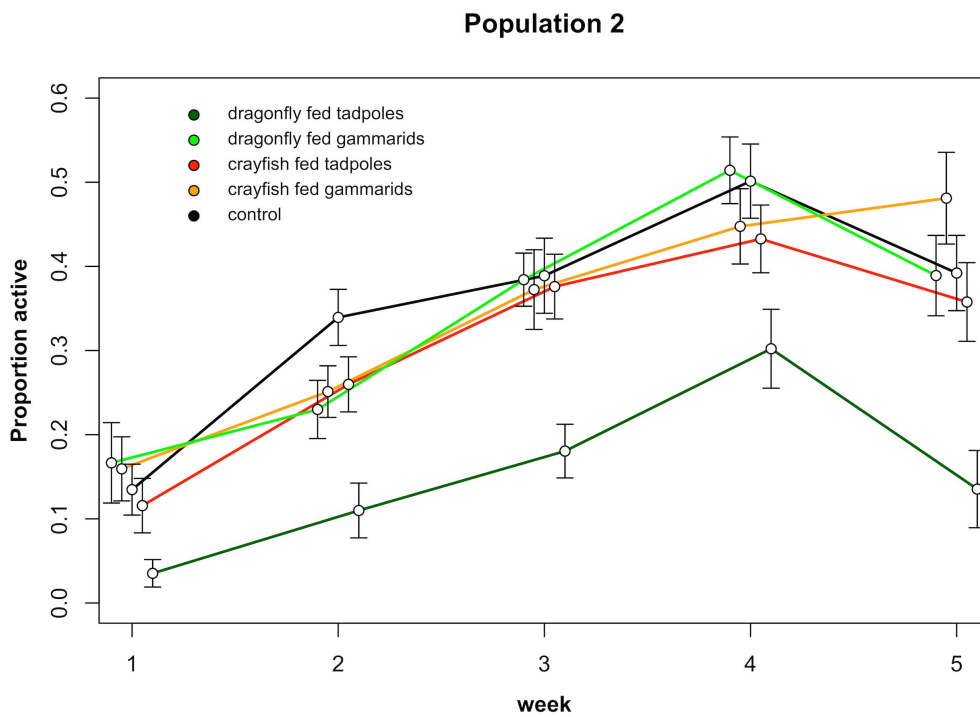
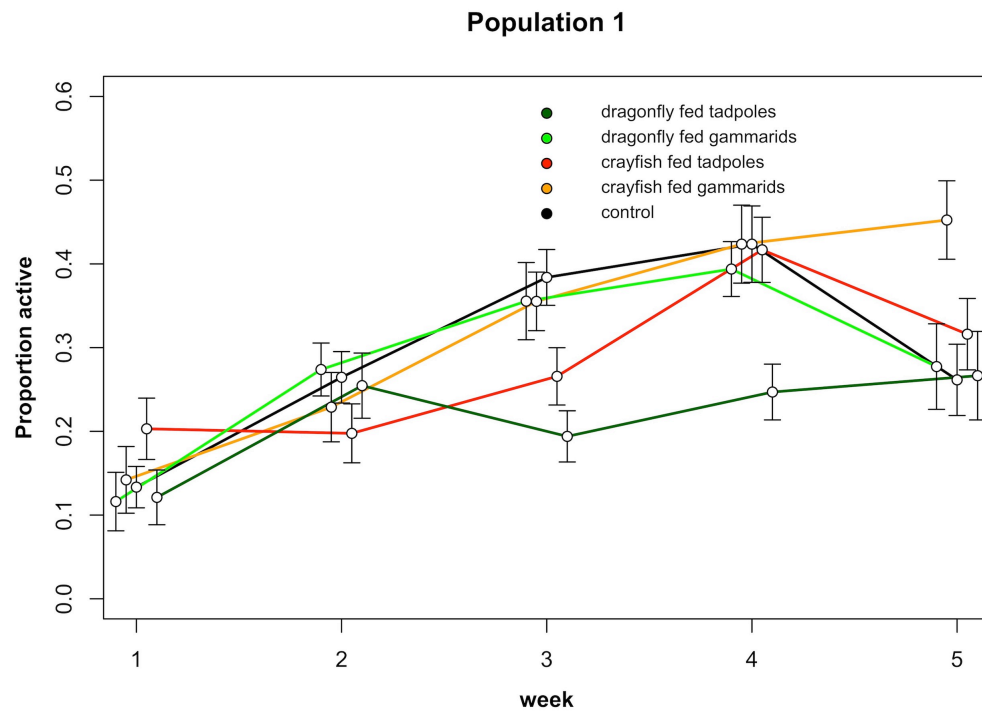
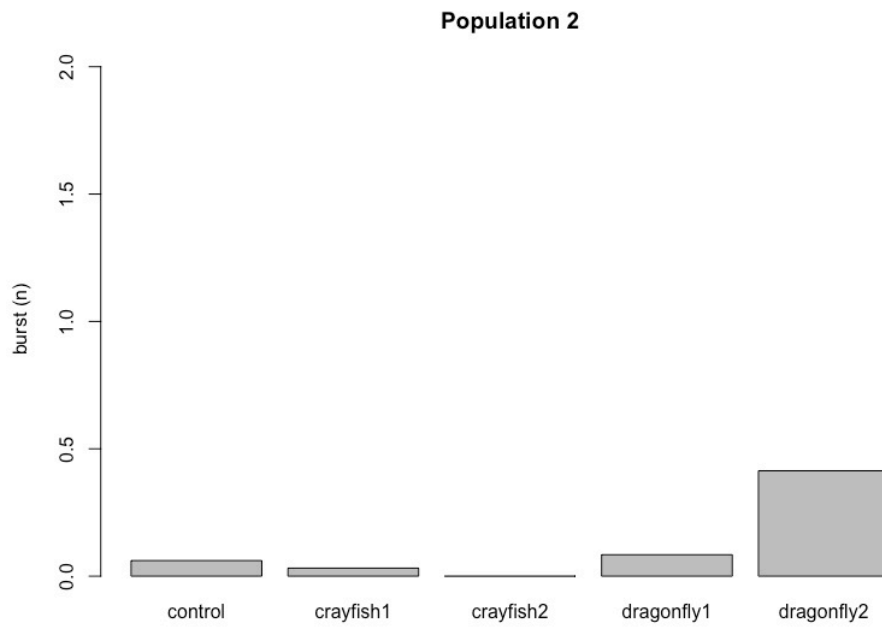
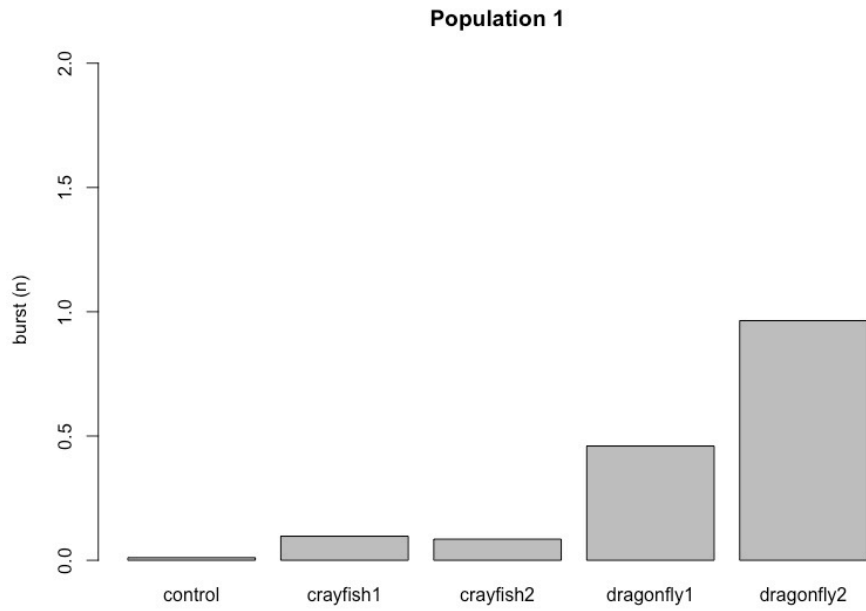


Fig. 11. Mean number of bursts (sudden acceleration events) recorded during behavioural observations for the whole experiment.



Conclusions

The main aim of my research was to study phenotypic plasticity, induced by predators, in anuran tadpoles and embryos. In the first part of my work I explored, along with behavioural and life-history traits, the activity of olfactory neurons (mitral cells). Collaboration with neuroscientists gave me the possibility to obtain the first data linking phenotypic plasticity and the activity of the cells that are at the basis of water-borne cues perception in larval anurans. Unfortunately, the experimental design of this study (chapter 1) did not allow obtaining any conclusion about the proximate causes of plasticity. However, some interesting findings emerged. The basal activity of tadpoles' mitral cells was altered by treatment with predator scent and this clearly support the innate ability of embryos to recognize a specific cue. Moreover kairomones-treated tadpoles showed a stronger increase in the level of activity of olfactory neurons when newly exposed to predator kairomones. This last result shows how embryonic exposition to predators can modify neuronal responses, and how these can endure during larval development. We recorded neuronal activity one month after hatching, and we did not observe any parallel change in tadpoles' behaviour (i.e. reduction of activity level) for individuals exposed to predator cues during embryonic phase in comparison to control. I observed a strong difference in contextual neuronal response (short time exposure to cue) according to embryonic treatment, but contextual behavioural plasticity was not affected by prenatal kairomones exposure. This result demonstrates that, as pointed out by Lima and Steury (2005), a lack of behavioural response may not indicate the lack of a perceived change in the current level of risk, and also that similar behaviours can be triggered of by different physiological changes (e.g. caused by previous experiences).

In the second part of my study I found the unexpected result that a low energetic state can trigger a stronger defensive behaviour in *Rana esculenta* tadpoles than a high energetic state (Chapter 4). This is surprising, because current theoretical models predict the opposite (i.e. well fed individuals should invest more in defensive behaviour, while fasting ones should be more risk prone). A possible explanation emerges considering the experimental design; no food was provided during behavioural recordings and the cue used to represent the risk of predation was obtained from pocked tadpoles. The absence of food could have reversed the expected behaviour in fasted tadpoles; in fact, the risk of predation was not counterbalanced by a potential reward. On the other hand, fed tadpoles could have considered cue coming from pocked tadpoles as a possible food source and, being more confident in their energetic state, were more prone to explore the surrounding environment. Further experiments, including food and predator's kairomones, will provide a better understanding of this unexpected result.

In another experiment (Chapter 3), I focused on the risk assessment hypothesis (Peacor 2003), which considers prey density as a necessary environmental factor to properly evaluate the actual level of risk in the current environment. The main coming out prediction is that prey should assess the level of risk on the base of the cue:density ratio (i.e. per capita amount of cue). I tested the effect of prey thinning (density reduction), and its potential interaction with risk assessment hypothesis, by applying a constant removal rate of individuals but holding the cue:density ratio fixed. This experiment provided two main results: 1) thinning did not affect agile frog tadpoles' level of activity; 2) comparison among treatments supported the risk assessment hypothesis. The effect of thinning on prey behavioural defences is an interesting and overridden topic; despite it may provide new insight to refine current theoretical models on prey decision-making under the risk of predation. Although in my experiment agile frog tadpoles seemed insensitive to density reduction, other studies showed that other species are not. In this study, the risk assessment hypothesis seems capable to predict prey behavioural

responses, but globally very few experiments have been conducted to validate this model. Moreover, I think it could be worth to repeat the experiment with other anuran species, possibly more sensitive to conspecific density decrease.

APPENDIX

Parallel lines of research

(In parallel to my research I also enjoyed collaborating to other studies)

**Isocline analysis of competition predicts stable coexistence
of two amphibians**

(Oecologia 178:153-159)

Isocline analysis of competition predicts stable coexistence of two amphibians

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Abstract

We experimentally investigated the interaction between larvae of two amphibian species (*Rana temporaria* and *Bufo bufo*) to test models of two-species competition. Our response surface experimental design had four replicates each of 24 density combinations. Larval performance – and, by assumption, change in population size – was defined by a linear combination of survival, growth, and development. We fit six competition models from the literature, and discovered that density dependence was strongly non-linear with the highest support for the Hassel and Comins (1976) model. *Rana temporaria* was competitively superior to *B. bufo*; the impacts of both species on growth and development were about 5-10 times greater than those on survival. Isocline analysis predicted a stable configuration, which agrees with the observation that these two species are syntopic in nature. This study confirms competition theory by identifying a model structure that agrees with data and making predictions that are broadly supportive of observations.

Key words: amphibians, *Bufo*, coexistence, competition, *Rana*, response surface models

Introduction

In a classic study, Ayala et al. (1973) illustrated how interspecific competition models could be tested by fitting them to experimental data. Nevertheless, the great majority of experiments on competition have not been designed to address theory directly. Much experimental and comparative evidence demonstrates the importance of competition in diverse communities (Connell 1983, Schoener 1983, Simberloff and Dayan 1991, Wootton 1994, Kaplan & Denno 2007, Hibbing et al. 2010), but the data from most of these studies cannot be used to test models of interspecific competition (Inouye 1999, 2001). A better connection between experiments and theory would enable us to evaluate the validity of existing models, including their structure, assumptions, and predictions. In the end, direct comparison between theory and data may confirm the adequacy of existing theory (Vandermeer 1969) or might justify developing more sophisticated models (Ayala et al. 1973).

Here we describe a model-based approach inspired by Ayala et al. (1973) and Inouye (2001) to investigate competition between two species of amphibian, *Rana temporaria* and *Bufo bufo*. These two species occupy freshwater wetlands across much of western Eurasia during the larval stage of the life cycle, and are usually in the terrestrial environment during the juvenile and adult stages. The species coexist both at local spatial scales within breeding sites (Fig. 1) and at larger landscape scales (Babik and Rafinski 2001, Van Buskirk 2005). Therefore, any successful model of the interaction between *R. temporaria* and *B. bufo* must predict that they coexist stably. Because tadpoles of these species occur at relatively high densities in discrete ponds, it is reasonable to expect that competition is most severe during the larval stage (Wilbur 1980, Pechmann 1995, Altwegg 2003). We therefore conducted an experiment to estimate competition within and between tadpoles of the two species. We determined which of several competition models was best supported by the data and then asked whether the estimated parameters predicted stable coexistenc

Methods

The experiment included four replicates each of 24 density combinations of *Rana temporaria* and *Bufo bufo*. For each species, the design included a 4×5 complete factorial design (5, 10, 20, and 40 individuals of the target species, crossed with 0, 5, 10, 20, and 4 individuals of the other species). The treatment with 0 individuals of both species was not included. One replicate of the treatment with 10 *Bufo* and 0 *Rana* was lost.

We conducted the experiment in 80-L plastic mesocosms (0.28 m²), placed outdoors in a field on the campus of the University of Zurich, Switzerland. The 96 mesocosms were arranged in two blocks according to spatial proximity in the field. Mesocosms were filled with tap water two weeks before the experiment began, provided with 40 g dried leaf litter and 2 g of commercial rabbit food, and covered with lids constructed of 43% shade cloth. Many features of natural ponds are obviously not present in mesocosms established in this way, and these conditions might influence the outcome of interspecific competition (refs). However, our mesocosms supported diverse communities of microorganisms, zooplankton, and periphyton, and provided sufficient food for anuran larvae to feed and grow.

The tadpoles came from five clutches of each species, collected from a pond 30 km N of Zurich, Switzerland. The experiment began on 5 April 2012, when the tadpoles were 6-7 days old, weighed 25.4 ± 6.6 mg (mean \pm standard deviation; *R. temporaria*) and 9.5 ± 3.0 mg (*B. bufo*), and were at Gosner (1960) stage 25. Tadpoles were drawn haphazardly from different clutches in equal proportions, and groups were assigned to mesocosms at random. The experiment continued until 8 May, at which point all tadpoles were removed and we recorded the number of survivors of each species, along with their mass and developmental stage. In mesocosms with more than 10 survivors, we assessed stage in a randomly-chosen sample of ten individuals of each species. The staged tadpoles were preserved in 10% formalin.

Statistical analyses

First, we compared the ability of each of six competition models to describe the interaction between *R. temporaria* and *B. bufo* tadpoles. The models [listed in Electronic Supplementary Material (ESM) Table A1] consist of difference equations that describe how the number of individuals of a particular species present in the next time step (X_{t+1}) change with the numbers of individuals of the two competing species present in the current time step (X_t and Y_t). Parameters of the models describe the population growth rates and carrying capacities of both species, competition between species, and the functional form of density dependence. Data can be used to differentiate these models by fitting the two surfaces that they describe: X_{t+1} as a function of X_t and Y_t , and the corresponding surface for Y_{t+1} (Ayala et al. 1973, Inouye 2001). Of course, changes in population size are not accessible in this study because of the complex life cycles and long generation times of the organisms under study. We therefore assumed that changes in population size are proportional to a composite measure of individual fitness, defined below. The coefficients that scale individual fitness to change in population size cannot be estimated from our data, and these are integrated into the model parameters for growth rate and carrying capacity; estimates of competition coefficients are unaffected by scaling (Inouye 2001, Hart and Marshall 2012).

“Fitness” was defined as survival to reproduction, which we predicted for each individual in the experiment using data from a different species of frog (*Pseudacris maculata*; Smith 1987). Smith (1987) estimated that survival to reproduction at age two years was related to size at metamorphosis (SM) and date of metamorphosis (DM) in the following way: $\text{logit}[\text{survival to age 2}] = -1.16 + 0.601 \times \text{SM} - 0.198 \times \text{DM}$. Both SM and DM are standardized [mean = 0, (SD) = 1]. The intercept scales mean survival and is therefore arbitrary. We substituted final

mass for SM and final stage for DM, both standardized so that the units of measurement were the same as in Smith's study. Individual fitness was estimated for each tadpole in the experiment; those that did not survive were assigned a value of 0. The exact coefficients are certainly different in *P. maculata* than in our study organisms, but we are confident of the general form of this relationship because coefficients relating size and age at emergence with one-year survival in European waterfrogs (Altwegg and Reyer 2003) are similar to those estimated by Smith (1987), and because the connection between larval and adult performance has been confirmed in other amphibian species (Berven and Gill 1983, Semlitsch et al. 1988, Berven 1990, 2009, Scott 1994).

We fit the six competition models using mean values of relative fitness at the level of the mesocosm, ignoring spatial blocks; models were compared using Akaike's Information Criterion (AIC; Burnham and Anderson 2002, p. 46). The best model for *R. temporaria*, with an AIC value 15 units better than the next-best model, was that of Hassell and Comins (1976). The best model for *B. bufo* was Leslie (1958), but the next-best model – separated by just 0.6 AIC units – was Hassell and Comins, and all other models were > 10 AIC units worse. Therefore, we hereafter consider only the Hassell and Comins competition model, because this model was well-supported by the data for both species. The modified model was

$$w = X \cdot \lambda \cdot [1 + c \cdot (X + \beta \cdot Y)]^b, \quad (\text{Eqn 1})$$

where w = relative fitness (= fitness / mean fitness), X and Y are the numbers of conspecific and heterospecific tadpoles entering the experiment, respectively, λ and c are parameters related to population growth and carrying capacity, β is the competitive effect of species Y on species X , and b controls the functional form of density dependence.

The probable outcome of competition in this system was evaluated in several ways. As shown by Hassell and Comins (1976), coexistence is possible when the product of the two competition coefficients is < 1. Also, the zero isocline for species A (plotted on the horizontal

axis) must be steeper than the isocline for species B near their point of intersection (Fig. 1 in Hassell and Comins 1976). Because we do not know how population growth scales with fitness, we drew what we call “mean-fitness isoclines” connecting all points for which predicted $w = 1$. The isoclines intersect where individuals of both species have equal relative fitnesses. Mean-fitness isoclines correspond to zero-growth isoclines only if population sizes of the two species remain constant when individuals have average fitness (i.e., only if $R_0 = 1$ when mean $w = 1$). More often, the transition between population growth and decline will occur above or below the mean-fitness isoclines. This will influence their point(s) of intersection, but should not affect the general configuration of isoclines. Note that zero-growth isoclines are linear in the Hassell and Comins model, but mean-fitness isoclines are not because increasing conspecific density has both positive and negative effects; isoclines become linear after division on both sides by X (Eqn 1). We explored the dynamical behavior of the system in two ways. First, we superimposed onto the phase diagram “displacement vectors” (Ayala et al. 1973), representing changes in population size between time steps. Because population change was not measured in our study, the length of displacement vectors was proportional to $(w - 1)$. This again assumes that the population declines when $w < 1$ and increases when $w > 1$. We also simulated dynamics over 1000 generations under the same assumption about population change, starting at all two-species combinations of density between 0 and 40 at intervals of 1.

We also explored the influence of conspecific and heterospecific density on the separate components of individual performance assessed at the end of the experiment: survival, mass, and developmental stage. Using mixed-effects linear models, we regressed each component against \log_{10} -transformed numbers of *Rana* and *Bufo* + 1, and their interaction. Models included random effects for variation between blocks and heterogeneity between blocks in the effects of *Rana* density and *Bufo* density. In one set of models, performance components were

transformed to improve the distributions of residuals (arcsin-sqrt-transformation for survival, ln-transformation for mass, and no transformation for stage). We used these models to evaluate significance, which was determined by inspecting profile likelihood confidence intervals (Venzon and Moolgavkar 1988). In a second set of models, performance traits were not transformed and the slopes were scaled by the pooled SD of the trait among individuals. These models were used for evaluating the magnitude of density effects because they produced estimates in comparable and easily interpretable units (SD units per ten-fold change in density). All analyses were implemented in R 3.0.1, using nls and lme4 (R Core Team 2014).

Results

Competition was strongly asymmetric. The competition coefficient representing the per capita effect of *R. temporaria* on *B. bufo* (0.166, 95% CI: 0.109 – 0.235) was nearly four times greater than that of *B. bufo* on *R. temporaria* (0.044, CI: 0.027 – 0.063). The fitted models suggest that the two species should coexist. First, the product of the competition coefficients was well below 1, a condition for coexistence in the original Hassell and Comins (1976) model. The mean-fitness isoclines intersected at two points (Fig. 2). The upper intersection was characterized by relatively steep mean-fitness isoclines for both species in a configuration that implies stability; the lower intersection implies damped cycles in the abundance of the two species. Displacement vectors originating at each of the 24 initial density combinations pointed toward the intersections of the isoclines (Fig. 2). This is a necessary consequence of high individual fitness at low density and lower-than-average fitness at high density, but the specific arrangement of vectors implies that the system is strongly attracted to the upper intersection. Indeed, simulations confirmed that the system always converges upon the upper intersection, at 15.9 *R. temporaria* and 16.9 *B. bufo*, assuming that change in population size from one time interval to the next is proportion to $(w-1)$.

Analysis of the separate performance measures indicated that competitive effects were stronger on tadpole size and development than on survival, and confirmed that the per capita impact of *R. temporaria* was usually higher than that of *B. bufo*. Tests of significance on the left side of Table 1 show that, for both species, mass and stage were negatively influenced by increases in both conspecific and heterospecific density; survival was never significantly affected by density and interactive effects were weak (On-line Appendix B). The right side of Table 1 confirms that density-dependent declines in growth and development were about 4-8 times greater than the declines in survival. A ten-fold increase in density caused a decline in survival of roughly 0.0 – 0.3 SD units, a reduction in mass of 1.3 – 3.8 SD units, and a delay in developmental stage of 0.2 – 2.0 SD units. Competition affected the mass of *R. temporaria* at least twice as strongly as it affected development rate, and the impact of conspecific density was more than double that of heterospecific density. For *B. bufo*, mass and stage responded strong and about equally, and the impacts of conspecific and heterospecific competitors were similar.

Discussion

The observation that natural populations of *Rana temporaria* and *Bufo bufo* coexist during larval and adult stages implies that their interaction is stable. A variety of models have been proposed to describe the outcome of competition among two or more species and specify conditions under which coexistence is possible. This study favors non-linear rather than linear forms of density dependence, and specifically supports the model of Hassell and Comins (1976), especially for *R. temporaria*. Many studies agree that linear density dependence represented by the Lotka-Volterra model does not match empirical results (e.g., Wilbur 1977, Goldberg and Landa 1991, Hart and Marshall 2012). When data are compared explicitly with competition models, the models of Ricker (1954) or Hassell and Comins (1976) – with the

parameter $b \geq 1$ – are often well supported (Inouye 1999, Paine et al. 2008, Hart and Marshall 2012, Kim et al. 2013).

Given an appropriate structure of the underlying competition model, our experiment then estimated values of its parameters and suggested dynamic behavior of the system. The competition coefficients suggest that the interaction between tadpoles of the two species was asymmetric, with *R. temporaria* roughly four times as strong as *B. bufo* in interspecific competition and twice as strong in intraspecific competition. Although asymmetric competition can cause competitive exclusion of a competitively inferior species (May 1975, Hassell and Comins 1976), this was not predicted in our system because interspecific competition was relatively weak in both directions (both β s well below 1). This result agrees with a sizeable literature indicating that asymmetric competition among coexisting species is widespread (Lawton and Hassell 1981, Connell 1983, Schoener 1983, Kaplan and Denno 2007), including in amphibians (Morin and Johnson 1988, Pearman 2002, Richter-Boix et al. 2004, Behm et al. 2013). The mechanism generating asymmetry in this case might be related to the difference in body size, as has been noted elsewhere (Richter-Boix et al. 2004). *Rana temporaria* tadpoles were about 2.5 times larger than *B. bufo* at hatching and throughout the experiment, and may therefore have consumed more resources per capita. An alternative explanation is that the two species have settled upon different evolutionary solutions to the trade-off between competitive ability and resistance to predators. This trade-off, observed in a wide variety of taxa, arises because phenotypic traits that enable success in competitive interactions also confer high mortality from predators (Woodward 1983, McPeck 1990, Smith and Van Buskirk 1995, Wellborn 2002, Yoshida et al. 2004). Indeed, *B. bufo* tadpoles appear to be relatively weak competitors and they typically occur in permanent wetlands with high densities of aquatic predators, especially fish (Van Buskirk 2003, 2005).

The locally syntopic occurrence of *R. temporaria* and *B. bufo* across much of Europe and

the abundance patterns of larvae in our field sites are mostly consistent with the predictions of theory. Indeed, their completely independent occurrences imply that the two species pay little attention to each other (Fig. 1). The approximately equal densities of the two species in ponds where they co-occur is also consistent with the model (although this prediction depends entirely on how population growth rate scales with relative fitness). However, the fact that *R. temporaria* and *B. bufo* larval densities were somewhat positively correlated in our field survey seems incompatible with the finding that tadpoles compete strongly. For *B. bufo*, the predicted equilibrium density in the absence of competition was well above the density under coexistence (Fig. 2), but the observed density in ponds without *R. temporaria* competitors was lower (Fig. 1). An obvious explanation is that wetlands vary in overall quality so that both species occur together at higher larval density in sites of higher quality; this produces a positive density correlation in spite of a negative interspecific interaction (Gascon 1992, Schmitz and Suttle 2001, Thurnheer and Reyer 2001). More generally, our experimental and field data highlight challenges associated with using non-experimental field data to test predictions about abundance from theory and experiment (Werner 1998). In this case, it is clear that intra- and interspecific competition among larvae are not the only processes affecting occurrence and abundance of these two species. Many environmental factors are known to influence amphibian larval abundance (Van Buskirk 2005, Werner et al. 2007a), and processes taking place during the terrestrial stage of the life cycle certainly influence occurrence and abundance (Joly et al. 2001, Werner et al. 2007b, Hamer and Parris 2011).

The value of linking experimental data and models in the study of species interactions is that explicit predictions about system dynamics can be made and tested. Applying this approach to organisms that have complex life cycles – such as amphibians – is difficult because of the uncertain connection between individual performance and population dynamics. This issue can be overcome by studying model species with relatively accessible life cycles,

such as *Drosophila* (Ayala et al. 1973, Prout and McChesney 1985). In non-model organisms, the issue will have to be overcome by studying multiple life stages at once, better connecting the performance of individuals with their contributions to population growth, and developing appropriate models of species interactions in multi-stage organisms.

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Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. Ethics permits were provided by the Veterinary Office of Canton Zurich, and permits to collect the animals came from the Canton's Office for Landscape and Nature.

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Table 1. Two assessments of the competitive effects of *R. temporaria* and *B. bufo* tadpoles on survival, body size, and developmental stage measured on the last day of the experiment. Both methods estimate the effects of log₁₀-transformed density of conspecific and heterospecific tadpoles; slopes therefore represent the change in response with a ten-fold increase in competitor density. All models included random effects for differences between blocks and heterogeneity between blocks in the effects of *Rana* density and *Bufo* density, but these effects were never significant in likelihood ratio tests. The left side is appropriate for evaluating significance: survival was arcsin-sqrt-transformed, mass was ln-transformed, and stage was untransformed. Asterisks indicate significance judged from profile likelihood confidence intervals (* P < 0.05, ** P < 0.01). The right side is appropriate for comparing effect sizes: responses were untransformed and slopes were rescaled to units of SD among individual tadpoles averaged across treatments.

Source	Original/transformed units			SD units		
	Survival	Mass	Stage	Survival	Mass	Stage
A. Responding species: <i>Rana temporaria</i>						
<i>Rana</i> density	-0.066	-0.934**	-3.152**	-0.316	-3.839	-1.928
<i>Bufo</i> density	-0.040	-0.295**	-0.338	-0.235	-1.581	-0.207
<i>Rana</i> × <i>Bufo</i>	0.017	0.145	0.119	0.103	0.902	0.073
B. Responding species: <i>Bufo bufo</i>						
<i>Rana</i> density	-0.141	-0.464**	-2.844*	-0.239	-1.317	-2.022
<i>Bufo</i> density	-0.058	-0.497**	-1.951*	-0.031	-1.456	-1.387
<i>Rana</i> × <i>Bufo</i>	0.076	0.066	0.757	0.124	0.435	0.539

Fig. 1. Average densities of *Rana temporaria* and *Bufo bufo* tadpoles in pond samples collected mid-way through the larval period of both species, near Zurich, Switzerland. The data include 321 sampling occasions of 79 ponds between 1997 and 2003 (ponds were sampled an average of 4.1 years each). Samples sizes are the number of pond-sampling occasions. Field methods are in Van Buskirk (2005). Both species were at higher density when they occurred together with the other species than when alone. The two species were not associated in their presence/absence across all years ($G = 0.41$, $P = 0.52$, $df = 1$, $N = 79$ ponds; Sokal and Rohlf 1981, p. 695). Nevertheless, larval densities of *R. temporaria* and *B. bufo* were somewhat positively correlated among ponds ($r = 0.17$, $P = 0.13$, $N = 79$ ponds) and among years within ponds ($r = 0.06$, $N = 14$ ponds with at least four years of data and both species present).

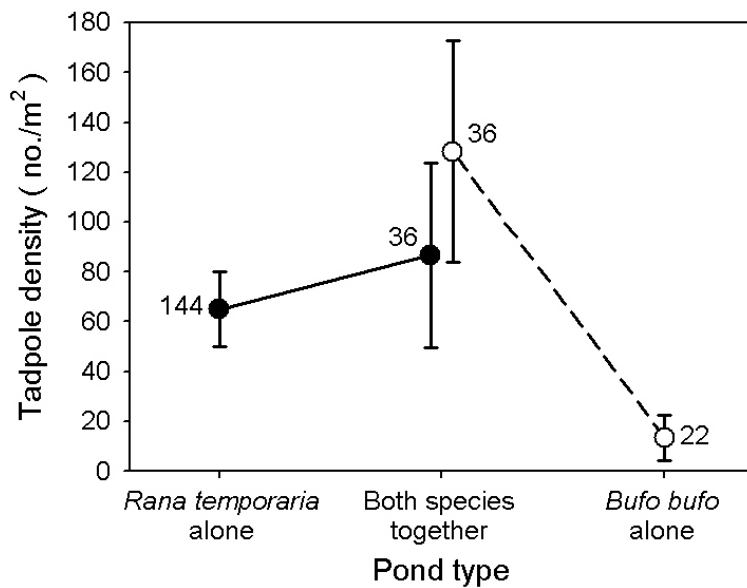
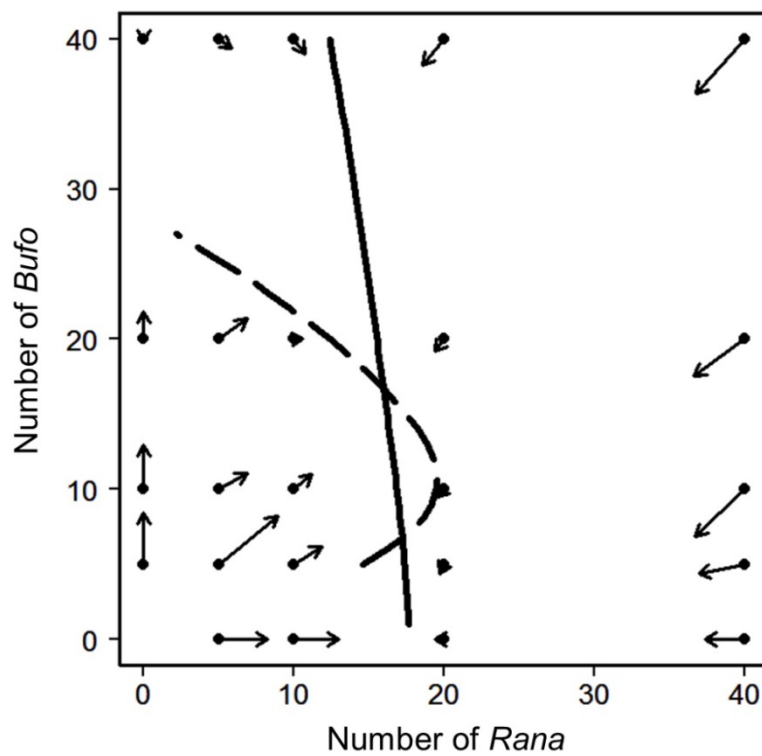


Fig. 2. Mean-fitness isoclines of the Hassell and Comins (1976) model estimated for competition between tadpoles of *Rana temporaria* (solid line) and *Bufo bufo* (dashed line). Isoclines occur when relative fitness (w) is 1. Small solid points are the 24 density combinations in the experimental design; the axes represent the number of hatchling tadpoles of the two species added to each 0.28 m² mesocosm. Arrows show the direction and relative magnitude of change that the system is predicted to experience in the next time step under the assumption that population size increases when $w > 1$; arrow lengths are proportional to $(w - 1)$.



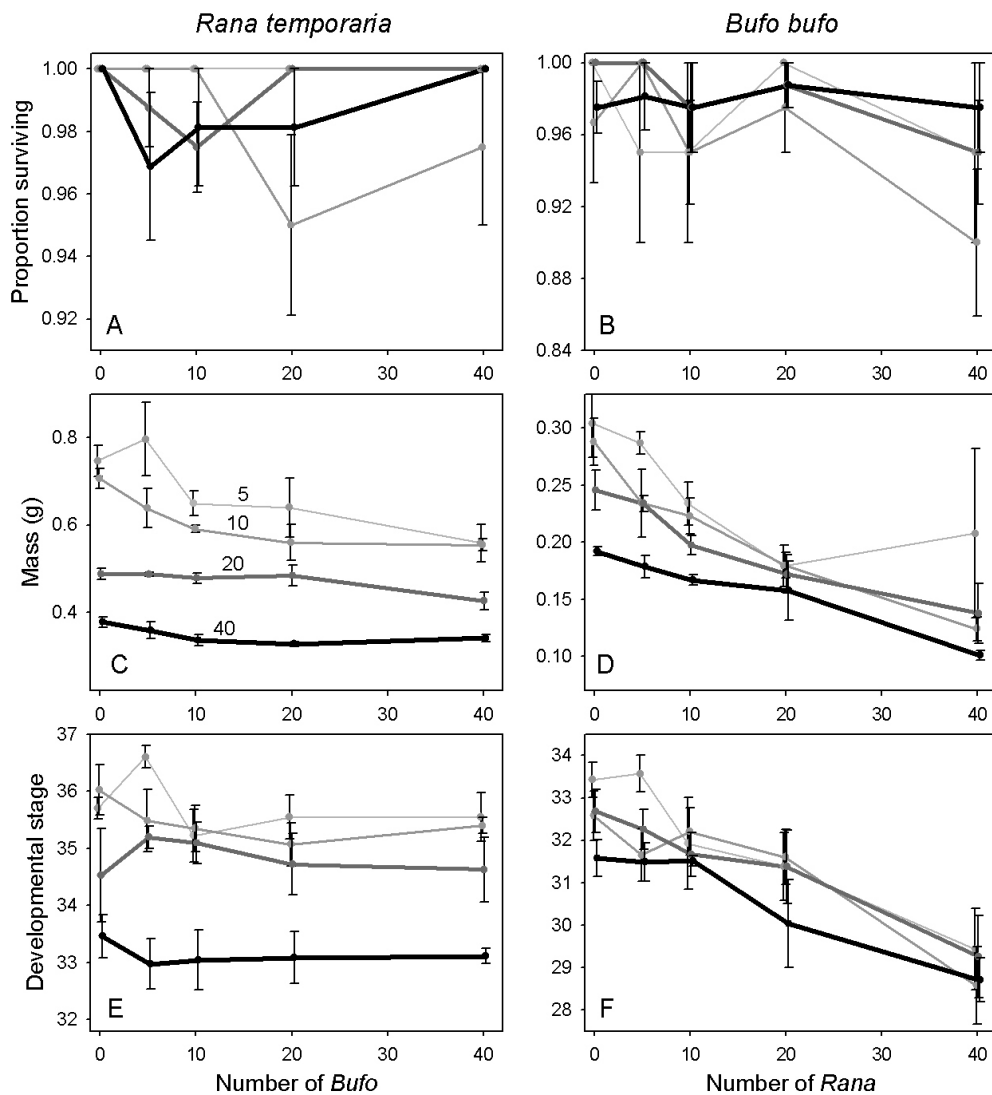
Appendix A. Six models of interspecific competition that were fitted to the experimental data. All models consist of two difference equations – one each for species X and species Y – of the form: $X_{t+1} = X_t \cdot f(X_t, Y_t)$, where X_t is the population size of species X at time t, and Y_t is the population size of species Y at time t. Density dependence is described by the function $f(X_t, Y_t)$. β is a competition coefficient representing the effect of species Y on species X. The parameter λ is the maximum rate of population growth for species X (at low density). The carrying capacity and/or functional form of density dependence are controlled by b and c .

Model	$f(X_t, Y_t)$	Reference
1	$1 + \lambda \cdot [1 - (X_t + \beta Y_t) / K]$	May and Oster 1976
2	$\lambda \cdot \exp[-c (X_t + \beta Y_t)]$	Ricker 1954
3	$\lambda \cdot [1 + c (X_t + \beta Y_t)]^{-1}$	Law and Watkinson 1987
4	$\lambda \cdot [1 + (X_t + \beta Y_t)^{-b}]^{-1}$	Leslie 1958
5	$\lambda \cdot [1 + c (X_t + \beta Y_t)]^{-b}$	Hassell and Comins 1976
6	$\lambda \cdot (1 + X_t^b + Y_t^c)^{-1}$	Law and Watkinson 1987

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Appendix B. Responses of three measures of tadpole performance to variation in conspecific and heterospecific density, measured on the last day of the experiment. Panels A, C, and E are data from *Rana temporaria*; B, D, and F are *Bufo bufo*. Developmental stage is according to Gosner (1960). Lines connect treatments with the same conspecific density (defined in panel C: from narrow-pale through heavy dark representing 5, 10, 20, and 40 individuals). Density is the number of tadpoles per 0.28-m² mesocosm. Error bars are ± 1 SE.



**Distribution and habitat use by pine marten *Martes martes*
in a riparian corridor crossing intensively cultivated
lowlands**

(Ecological Research 30:153-162)

Distribution and habitat use by pine marten *Martes martes* in a riparian corridor crossing intensively cultivated lowlands

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Abstract

The location of pine marten records in northern Italy suggests that main rivers may play the role of natural corridors favouring this species' colonisation of cultivated lowlands. We assessed the distribution and habitat use by the pine marten on a 35 km long stretch of the River Ticino. Surveys were carried out between October 2011 and June 2012 along linear transects in a 2x2 km grid. Using the variation in marking intensity as an indicator of habitat use, habitat selection was assessed at two landscape levels – at transect-scale by the χ^2 test with Bonferroni's confidence intervals for the proportion of use, and at grid-scale by multiple linear regression. By a PCR-RFLP method, 91 faecal samples were assigned to the pine marten. Faeces were mainly located in wooded areas, while fields were avoided. At the grid-scale of analysis, marking intensity was positively related to the mean area of wooded patches and negatively to their mean perimeter-area ratio. This suggests that pine marten relative abundance may partially depend on the degree of fragmentation and structure of residual woods. The survey protocol allowed to assess the probability of detection. Occupancy models outlined that heterogeneity in detection probability may arise as a result of

variation in marking intensity, *i.e.* the number of marking individuals. Our results suggest that the availability of both woodland corridors and wood patches are major factors shaping pine marten distribution in intensively cultivated plains and that non-invasive genetic surveys are a cost-effective method for future studies at a broader scale.

Key words: Non-invasive genetic sampling, detectability, faecal DNA, stone marten, Northern Italy.

Introduction

In the Mediterranean region, human population growth, agricultural intensification and the consequent loss of natural habitat have led to a general decline of biodiversity in both plain and coastal areas (Matson et al. 1997; Benton et al. 2003; Lepers et al. 2005). Plains show the lowest species richness, with the exception of some small wet areas and residual riparian woods, which support a more diverse fauna than surrounding habitats (*e.g.* Warkentin et al. 1995; Bentley and Catterall 1997; Hilty and Merenlender 2004).

A major biological effect of habitat fragmentation caused by anthropogenic modification is the decline of species that need large areas of connected natural habitats to meet their ecological requirements (Beier 1993; Mortelliti et al. 2010). Mammal distribution is particularly affected by the increased isolation and reduction in area of habitat patches (Bright 1993; Waldron et al. 2006) and habitat fragmentation is considered a major cause of the decline of forest-dwelling species (Reed 2004).

The pine marten (*Martes martes*) has been long considered a forest-specialist and its generalized decline has been imputed to the combined effects of large individual home ranges and deforestation (Buskirk 1992; Buskirk and Zielinski 2003). Nonetheless, recent studies have shown that the pine marten can colonise agricultural landscapes with highly fragmented woods (Balestrieri et al. 2010; Mergey et al. 2011; Caryl et al. 2012), suggesting that, as already observed in Mediterranean Italy (De Marinis and Masseti 1993), it is not such a strict forest-dweller as previously believed (see Virgós et al. 2012).

In Italy, the pine marten occurs sympatrically with the closely related stone marten (*Martes foina*) in mountainous areas, while in plain areas only the stone marten has been reported (Genovesi and De Marinis 2003a, b). Currently, the pine marten is colonising the western sector of the intensively cultivated Po plain (N Italy), where it probably went extinct at the end of the 1960s (Mantovani 2010), and pine marten expansion seems to coincide with the

contraction of the stone marten range (Remonti et al. 2012).

The pattern of pine marten expansion seems to suggest that water systems may play the role of natural corridors favouring the dispersal of the pine marten from subalpine areas and the colonization of agricultural lowland (Fig. 1).

Riparian zones are important for maintaining landscape connectivity (Naiman et al. 1993; Taylor et al. 1993) and biological connections for wildlife (Clerici and Vogt 2013). The use by dispersing mammals of both natural and man-made linkages has been reported for several species (see Bennett 2003 for a comprehensive review). Corridors can assist the range expansion of both scarcely and highly mobile mammals, from meadow vole *Microtus pennsylvanicus* (Getz et al. 1978) to mink *Neovison vison*, which, following its introduction, has spread in Great Britain through river corridors (Harris and Woollard 1990).

As in most European lowlands (Coles et al. 1989; Bennett 2003), in northern Italy main rivers form linear habitats covered with remnant riparian vegetation, crossing clearly distinct, heavily disturbed farmland and urban areas. Lowland woods cover only 1750 km², of which about 70% are located in the western and central plain of the River Po (Camerano et al. 2010) as small residual fragments (mean area 4.5 ha; Lassini et al. 2007). The plain of the River Ticino includes the largest and best conserved riparian woods of northern Italy and represents the less altered river corridor of the western basin of the River Po, a critical European region from a conservation perspective (Clerici and Vogt 2013).

Records of road-killed pine marten suggested that the valley of the River Ticino may represent a suitable dispersal route for the pine marten (Balestrieri et al. 2010). Accordingly, since 2005 a stable pine marten population has dwelt in a small agricultural area, 5 km to the west of the River Ticino, about 20 km upstream its confluence in the River Po (Remonti et al. 2012). Thus, we aimed to draw the actual distribution of both pine and stone marten in the downstream, 35 km long section of the River Ticino valley and assess the pattern of pine marten habitat use in a

fragmented landscape. To account for martens elusiveness and the difficulty of distinguishing its tracks from those of other medium-sized carnivores, we applied a non-invasive genetic sampling method based on mtDNA extracted from faecal samples (Ruiz-González et al. 2008). As the survey protocol involved repeated surveys, we also modelled the effects of both habitat variables and marking intensity on the probability of pine marten detection, in order to evaluate the cost-effectiveness of the survey method a major factor to consider in the design of occupancy surveys for carnivore mammals (Slauson et al. 2009; Long et al. 2011).

Study area

The Italian stretch of the River Ticino flows southwards from the southern edge of Lake Maggiore to the median course of the River Po, forming a 110 km long and, on average, 7 km wide valley.

The valley is partly protected by two Regional Parks: the Park of the Ticino Valley (Lombardy), covering 906.4 km² and the Natural Park of the Ticino Valley (Piedmont), 62.5 km².

The river crosses an intensively cultivated and urbanized plain, where mesophilous - *Fraxino-carpinion* - and hygrophilous - *Alno-Ulmion*, *Alnion glutinoso-incanae*, *Salicion albae* - woods are still widespread inside the weave of meanders, streams, canals and oxbow lakes which characterise the downstream stretch of the river. On the whole, water-bodies cover an area of about 48 km², while wet woods account for 87 km² (Prigioni 1995).

Pine marten monitoring focused on the lower part of the valley, from the towns of Vigevano and Abbiategrasso (Milan, Lombardy) in the north to Gropello Cairoli village (Pavia, Lombardy) in the south (Fig. 2). This *ca.* 35 km long stretch of the river has a mean annual discharge of about 300 m³s⁻¹ and a catchment area of more than 7000 km². Mean percent riparian vegetation cover, as assessed in a 100 m large belt on both river banks for seven 5 km

long river stretches, is 47.8% (min-max: 12-86%; Prigioni and Balestrieri 2011).

The climate is temperate, mean annual values being 13°C for air temperature and *ca.* 700 mm for rainfall.

Methods

Collection and genetic identification of faecal samples

Surveys were carried out within a 2x2 km grid (Fig. 2), superimposed on the kilometric grid of digitalized, 1:10000 Regional Technical Maps. Grid size was chosen as to broadly correspond to pine marten mean home range in Tuscany (370 ha; Del Fante 2012).

Sampling was conducted between October 2011 and June 2012 along linear transects drawn along wood/field margins, paths and country roads to cover both open and forested habitats. Transects were surveyed 1-3 times each (mean \pm SD = 2.44 \pm 0.87).

A portion (*ca.* 1 cm) of each “marten-like” faeces (*i.e.* less than 10 mm large and then suspected to belong to the pine marten; for more *details* on faeces identification, see Remonti et al. 2012) was picked up using sticks stored in autoclaved tubes containing ethanol 96% and frozen at -20°C until processed (Ruiz-González et al. 2008).

Bi-monthly variation in marking intensity was checked by comparing the observed number of faecal samples to that expected based on the overall transect length covered during each period (N = 4).

DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer’s instructions. The specific identification of faecal samples was accomplished by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, according to Ruiz-González et al. (2008). Two primers amplify the mtDNA from *Martes martes*, *M. foina* and four *Mustela* species, of which only the European polecat *Mustela putorius* had been previously reported for our study area, whilst red fox (*Vulpes vulpes*) faeces,

which can be easily mistaken for those of martens (Davison et al. 2002), render no amplicons. The simultaneous digestion of amplified mtDNA by two restriction enzymes (RsaI and HaeIII), generates different restriction patterns for each mustelid species (*i.e.* DNA fragments differing in both number and length), allowing the unambiguous identification of faecal samples (see Ruiz-González et al. 2008 for further details).

Habitat use

Based on CORINE Land-Cover maps, satellite images and ground surveys, a land-cover, digitalized, 1:10.000 map was produced by a Geographic Information System (QGis®).

Habitat selection was assessed by a use-availability design (Garshelis 2000) at two landscape levels, using faeces relative distribution as an indicator of habitat use.

Faecal counts have been widely used for assessing the abundance and habitat preferences of many mammal species (Putman 1984; Kohn and Wayne 1997; Gese 2001). Their reliability has been long debated (*e.g.* Kruuk et al. 1986; Messenger and Birks 2000), the interpretation of survey results involving several assumptions about marking activity and dropping identification (Sadlier et al. 2004). Nonetheless, faecal counts have been recently reported to be effective estimators of both relative abundance and habitat use by other carnivores (Clavero et al. 2006; Guter et al. 2008; Lanszki et al. 2008; Rosalino et al. 2008; Balestrieri et al. 2009; Kauhala and Salonen 2012).

To assess habitat use at transect-scale, faecal samples were georeferenced and assigned to a habitat type. Three main habitat types were considered: woods, poplar plantations and cultivated fields (mainly maize and rice). Samples located within a 5-m wide strip on each side of the border between two different habitat types were assigned to both habitats with a 0.5 score. The chi-squared (χ^2) test was performed to test for the goodness of fit of used habitat to available habitat types (White and Garrot 1990). Expected frequencies were calculated based

on the overall transect length covered for each habitat. To determine whether a habitat was selected or avoided Bonferroni's confidence intervals for the proportion of use of each habitat were checked:

$$P_i - z_{\alpha/2k} \cdot \sqrt{\frac{P_i(1-P_i)}{N}} < P_i < P_i + z_{\alpha/2k} \cdot \sqrt{\frac{P_i(1-P_i)}{N}},$$

where: P_i is the proportion of use of the i^{th} habitat, N is sample size, z is the percentage point of the standard normal distribution corresponding to an upper tail probability of $\alpha/2k$, α is the level of significance and k is the number of habitat types (Neu et al. 1974; Byers et al. 1984).

To assess habitat use at grid scale, the following variables of potential importance to pine marten distribution were measured for each square:

- a) W: percentage of woods,
- b) U: percentage of urbanised areas,
- c) C: percentage of cereal crops,
- d) P: percentage of poplar plantations,
- e) H: Shannon's index of habitat diversity,
- f) minDr: minimum distance from the River Ticino (with minDr = 0 for all grid squares including a river stretch),
- g) MWA: mean area of wooded patches, as to account for the degree of fragmentation of pine marten potentially preferred habitat,
- h) MPAR: mean perimeter-area ratio of wooded patches, representing their compactness.

The influence of the measured variables on pine marten marking intensity, (MI = number of genetically identified faeces found in each grid square / total transect length per square) was tested by means of a multiple linear regression, using two different approaches.

First, we used a stepwise (forward selection) method to identify which variables were related to pine marten marking intensity (SPSS 12.0.1; SPSS, Chicago, IL, USA). Deviation from

normality of the residual distribution was tested by Shapiro-Wilk's test.

Then we performed a linear multiple regression for each subset of uncorrelated (Pearson's test, $p > 0.05$) explanatory variables, entering all independent variables simultaneously. The obtained models were ranked by second order Akaike Information Criterion (AICc; Sugiura 1978), selecting those showing ΔAICc values < 2 ($\Delta\text{AICc} = \text{AICc}_i - \min \text{AICc}$, where $\min \text{AICc}$ is the AIC value of the 1st-ranked model; Burnham and Anderson 2002; Posada and Buckley 2004; Mazerolle 2006). The analysis was performed by the software SAM 4.0 - Spatial Analysis in Macroecology (Rangel et al. 2010).

Before the analyses, all variables were tested for normality. To achieve normality, U and MPAR were rank transformed, C was square-root transformed, while MWA, P and the length of linear transects were $\log(x+1)$ transformed. Being a frequency of occurrences, MI was $\log(x+1)$ transformed and modelled as a linear function of independent variables (Quinn and Keough 2002).

Detection probability

The influence of habitat variables on pine marten probability of site occupancy (Ψ) and detection probability (p) was analysed by the likelihood-based method for modelling occupancy data proposed by MacKenzie et al. (2002). This modelling is analogous to traditional capture-recapture methods, but uses the proportion of sites occupied by the target species as a state variable (rather than individuals). The goal is to assess Ψ knowing the species is not always detected, even when present. As the method requires multiple surveys in a demographically closed system (*i.e.* closed to changes of the occupancy state during the sampling interval; MacKenzie et al. 2003), in a sub-sample of 17 grid squares 1 transect (mean length: 3.6 ± 1.38 km) was surveyed three times between November 2011 and April 2012 (*i.e.* they were suspended before the time the cubs-of-the-year are supposed to start to scent mark;

Genovesi and De Marinis 2003b), as to compile replicate observations into a sequence of 1's (detections) and 0's (non-detections). As grid size was almost equal to the size of home ranges and each home range was not occupied by more than two individuals of same-sex because of territorial behavior (Balharry 1993), we could assume detections at different sites to be independent.

As an example, the likelihood for site i with history 010 would be:

$$\Psi_i (1 - p_{i,1})p_{i,2}(1 - p_{i,3}),$$

with Ψ_i : probability of site i occupancy; and $p_{i,n}$: detection probability for each visit. Assuming independence of sites, the product of all terms (one for each site) constructed in this manner creates the model (MacKenzie et al. 2002). Ψ may be some function of site characteristics and p may vary with certain variables; using a logistic model this information can be introduced to the model as, respectively, site- and survey covariates, (MacKenzie et al. 2002).

Analyses were run by the software PRESENCE (Hines 2006), using single-season models. As pine marten presence was expected to depend on wood availability and connectivity (Zalewski and Jedrzejewski 2006), 10 habitat covariates were measured: the length of the transect (L), the percent length of the transect covered by wood (Wt), the overall area of the wood patch crossed by each transect (Wa), wood patch perimeter (Wp), the minimum distance between the wood patch covered by the transect and the nearest $>2 \text{ km}^2$ large wood patch (Dnw), the mean distance between each transect and the river (Dr), the percent cover of woods (W), urban areas (U) and crops (C) into each grid square; the mean distance between each transect and the nearest urban area (Du) was considered as an index of human disturbance. To avoid multicollinearity, Spearman's correlation (with $\alpha = 0.01$) test was used to check for any relationship between the covariates. Five covariates (L, Wa, Dnw, %Wt and W) were then selected for modelling.

Variation in marking intensity has been reported as a potential cause of heterogeneity in

detection probabilities (Balestrieri et al. 2011a). As any non-invasive genetic survey for *Martes* species is a two-step process – the collection of “marten-like” faeces in the field and their later identification by genetic analyses in the laboratory -, we included as a survey covariate the abundance of “marten-like” faeces (FA = number of “marten-like” faeces per km), recording each survey as either 0 or 1 according to the results of genetic analyses.

Before the analyses, all covariates were standardised by the Z transformation (Donovan and Hines 2007). Models were first ranked according to Akaike Information Criteria values, excluding those showing delta values ≥ 2 (Burnham and Anderson 2002). The goodness of fit of the models was then assessed by Mackenzie and Bailey’s test using 1000 simulated bootstrap detection histories (MacKenzie and Bailey 2004). To assess fit, the test use a chi-square approach, while the c-hat statistic is used for adjusting the standard errors for the model parameters (c-hat > 1 indicates a lack of fit).

Naïve probabilities (*sensu* MacKenzie et al. 2003) were calculated as the number of transects or sites positive for pine marten over the total number of transects or sites surveyed.

The minimum number of surveys required to statistically establish the occurrence of the pine marten was assessed by the probability model: $N_{min} = \log(\alpha) / \log(1 - p)$, where $\alpha = 0.05$ sets the confidence level (McArdle 1990; Reed 1996). To account for modelling results, mean p was calculated for three classes of faeces abundance - FA < 0.5 (N=5), 0.51 < FA < 1 (N=6), FA > 1.1 (N=6) – using top model estimates.

Results

Overall 160 “marten-like” faecal samples were collected inside 21 of the 2x2 km large sampling squares (84 km²; Fig. 2), on a total length of 273.9 km of transects (mean transect length \pm SD = 3.33 \pm 1.45 km, min-max = 1.1–8.0 km), corresponding to 1.9 faeces/km² and 0.58 faeces/km, respectively. No time-related variation in marking intensity was recorded ($\chi^2 = 3.81$, d.f. = 3, $p = 0.28$).

DNA was extracted from 123 (76.9%) faecal samples, out of which 91 were assigned by our PCR-RFLP method to the pine marten and nine to the stone marten, corresponding to an overall species identification of 81.3% of analysed samples.

The pine marten occurred in all surveyed squares, averaging 4.3 ± 3.15 samples per square (min-max = 1-10; Fig. 2), while stone marten samples were found in only two squares. Grid squares overlapping the river showed 30-50% of woodland, while inside neighbouring squares the percentage of woodland decreased down to 0.9% (Tab. 3 in the Appendix).

Most pine marten faecal samples (78.8%) were collected inside or at the margins of woods, whilst 16 (17.6%) were found in poplar plantations or crops. Along surveyed transects, faeces were not distributed according to habitat availability ($\chi^2 = 15.6$, d.f. = 5, $p < 0.001$), being mainly sited in wooded areas, whilst fields were avoided (Fig. 3).

At the grid-level of analysis, the stepwise regression of the index MI on the habitat variables yielded a significant model ($R^2 = 0.28$, $F = 7.49$, d.f. = 20, $p = 0.013$), which included only the mean area of wooded patches (MWA), positively related to pine marten marking intensity ($\beta = 0.57$). Shapiro-Wilk's test showed a normal distribution of residuals ($W = 0.93$, $p = 0.11$). The AICc selected three models (Tab. 1). The best model included the mean area of wooded patches (MWA), positively related to pine marten marking intensity, while the second model included the mean perimeter-area ratio of wooded patches, negatively related to marking intensity ($\beta = -0.46$; Fig. 4).

On the first sampling occasion, the pine marten was genetically ascertained for 15 out of the 17 sampling squares which were surveyed three times (Tab. 4 in the Appendix). One new site was identified during the second survey and an additional one in the third survey. On average, 80.4% of squares yielded genetically identified pine marten samples from a single survey.

Two occupancy models showed to be supported by the data (ΔAIC scores < 2 ; Tab. 2). The 2nd-ranked model was the one which best fitted the occupancy framework (MacKenzie and

Bailey's test: $\chi^2 = 0.031$, $p = 0.18$; $c\text{-hat} = 0.26$). Both models supported the inclusion of only FA as a covariate, positively related to the probability of detection. According to the best model, pine marten detection probability was similar in the first and third surveys ($p \pm SE = 0.88 \pm 0.034$ and 0.80 ± 0.012 , respectively) and decreased in the second one ($p \pm SE = 0.71 \pm 0.041$). These values almost coincided with naïve probabilities (0.88, 0.82 and 0.7, respectively), as so as for the probability of site occupancy ($\Psi = 1$ for both raw data and top model).

According to the probability model, the minimum number of surveys needed to ascertain pine marten occurrence is 3.9 (CI = 3.0-5.2) where $FA < 0.5$ and 1.67 (1.66-1.69) where $0.51 < FA < 1$, while only one survey is needed where, on average, more than one “marten-like” faecal sample is found per kilometre of transect.

Discussion

Although in the last two decades of the 20th century sampling in plain areas was probably biased towards the stone marten, indirect signs of presence having been assigned to this species due to its well-known anthropophilia (Sacchi and Meriggi 1995; Lanszki 2003; Herr et al. 2009), our results draw a quite different picture, with the pine marten as the most widespread *Martes* species on the River Ticino (in terms of the number of both genotyped samples - 91 vs. 9 -, and positive squares - 21 vs. 2).

As supported by the exponential increase in road-kill reports (Balestrieri et al. 2010), the spreading of the pine marten in the valley may have occurred during the last 15 years. This rapid expansion stresses the role of riparian woods as a suitable corridor for pine marten colonisation of a landscape largely dominated by agriculture and artificial land-cover. Unfortunately, information about the status of the pine marten in Italy is insufficient to determine the causes of pine marten expansion, although, on the Alps, increased forest cover

may have favoured the growth of pine marten populations (O'Mahony et al. 2012), as occurred for other forest species (e.g. Jepsen et al. 2004).

In our study area, the distribution of pine marten samples confirmed its preference for woodlands. Although signs were found not only in large woods but also in small, isolated wood plots and on wooded slopes and canal banks surrounded by agricultural land, the relation between marking intensity and mean wood size and perimeter-area ratio suggests that pine marten relative abundance depends on the structure and degree of fragmentation of residual woods (see also Pereboom et al. 2008).

Neither marking intensity nor occupancy rates decreased with the distance of the sampling transects from the river. As the main aim of the study was to assess pine marten distribution and habitat use along a potential river corridor, transect distance from the river never exceeded 5 km. Thus, our survey-scale may have been inadequate to detect the effect of this habitat variable.

Our results are consistent with those obtained by radiotelemetry in rural areas of France (Pereboom et al. 2008) and Scotland (Caryl et al. 2012), suggesting that the pine marten is more generalist in terms of habitat preferences than previously reported (Virgós et al. 2012). Such ecological generalization should increase the likelihood that individuals will find suitable resources in a new area (Mettke-Hofmann et al. 2002; Echeverría et al. 2006; Moritz et al. 2008; Pöyry et al. 2009), including man-altered habitats (Musiani et al. 2003). Accordingly, diet analyses suggested that in agricultural habitats the pine marten can cope with the reduced availability of small mammals by relying on introduced Eastern cottontail *Sylvilagus floridanus* (Balestrieri et al. 2011b).

The aims and spatial scale of occupancy surveys drive the choice of survey protocols. For large scales, the need to maximise the cost-effectiveness of the surveys may lead to lower the chance of detecting the target species (Slauson et al. 2009). By the non-invasive genetic method

adopted, species identification was obtained for a rather high percentage of faecal samples with respect to other available approaches (*e.g.* 58%, Lucentini et al. 2007; 53.4%, Pilot et al. 2006), confirming the results of previous studies carried out by the same method (Rosellini et al. 2008; Ruiz-González et al. 2008, 2013; Balestrieri et al. 2010, 2011b). Although sample size was small, multiple surveys allowed us to point out that, as previously reported for the Eurasian otter *Lutra lutra* (Balestrieri et al. 2011a), heterogeneity in detection probability and hence the effort (*i.e.* the minimum number of surveys) needed to ascertain the presence of the target species may arise as a result of variation in the number of marking individuals (see also Kéry 2002 and Balestrieri et al. 2011), which should be accounted for by occupancy models (Royle and Nichols 2003).

Variation in p over the three surveys suggests that it may partially depend on covariates different from those tested, such as rain or cloud cover, which can affect the ability of surveyors to detect faecal samples (Olson et al. 2011). We suggest that future survey protocols have to involve multiple surveys, adjusting the overall effort per sample unit on the basis of the number of “marten-like” faeces recorded per km of transect.

In regions exposed to intense anthropogenic pressure, riparian areas are severely threatened, in spite of their crucial role for landscape connectivity (Clerici and Vogt 2013). Although further studies are needed to assess the actual pine marten distribution in the lowlands of northern Italy, on the basis of our results we can hypothesise the availability of both woodland corridors and wood patches spread in the crop matrix to be major factors shaping the distribution of this marten species in the Po-Venetian plain. The PCR-RFLP method adopted in this study, if combined with the search for faecal samples by trained surveyors (see also Ruiz-González et al. 2013), represents a cost-effective tool for future investigations on pine marten distribution at a broader scale.

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Figure captions

Figure 1. Distribution of pine marten road kill records (1988-2012; grey dots) in the western and central River Po plain (a) (Balestrieri et al., 2010; Mantovani, 2010) and with respect to northern Italy (b). Main rivers and lakes are shown in dark grey, while the light grey line is the 300 m a.s.l. contour line, which broadly marks the upper limit of the plain.

Figure 2. Genotyped faecal samples into 21, 2x2 km grid squares surveyed in the valley of the River Ticino (a), and location of the study area in Italy (b).

Figure 3. Habitat selection by pine marten as assessed at transect-scale by the comparison of the observed (“use”) and expected (“availability”) frequencies (p) of faecal samples (statistical significance according to Bonferroni’s confidence intervals; * $p < 0.01$, ** $p < 0.001$).

Figure 4. Relationship between pine marten marking intensity (MI) and both the mean area (MWA) (a) and mean perimeter-area ratio (MPAR) (b) of wooded patches into 21, 2x2 km wide grid squares. The two linear regressions selected by second order Akaike Information Criterion are shown (see methods).

Table 1. Models resulting from linear multiple regressions of pine marten marking intensity MI, ranked according to the Akaike Information Criterion (AICc) (MWA: mean area of wooded patches; MPAR: mean perimeter-area ratio of wooded patches; P: Percent cover of poplar plantations; Δ AICc: delta AICc, see methods).

Explanatory variables included in the model	R ²	AICc	Δ AICc	AICc weight
MWA	0.282	-52.166	0.000	0.14
MPAR	0.226	-50.573	1.5935	0.07

Table 2. Top occupancy models obtained by PRESENCE (Hines 2006) and ranked according to Akaike Information Criteria (AIC) ($p_{1,2,3}$: detection probability during each survey; int: intercept; Ψ : probability of site occupancy; FA: marten-like faeces km⁻¹; Δ AICc: delta AICc, see methods).

Model	AIC	Δ AIC	AIC weight	Model likelihood	-2Log (likelihood)
$\Psi(\cdot), p(\text{int}) p(\text{FA})$	14.49	0.00	0.3353	10,000	8.49
$\Psi(\cdot), p(\text{int}) p_1(\text{FA}) p_2(\text{FA}) p_3(\text{FA})$	14.79	0.30	0.2886	0.8607	4.79

Table 3. Habitat variables of potential importance to pine marten distribution, total length of surveyed linear transects and pine marten marking intensity (MI) in 21, 2 x 2 km wide grid squares (W, U, P, C: percent cover of woods, urban areas, poplar plantations and crops, respectively, into each grid square; H: Shannon's index; minDr: minimum distance to the river; MWA: mean area of wooded patches; MPAR: mean perimeter-area ratio of wooded patches; TL: transect length; MI: marking intensity, expressed as number of faeces km⁻¹; see methods).

Grid square	W (%)	U (%)	P (%)	C (%)	H	minDr (km)	MWA (km ²)	MPAR	TL (km)	MI
1	17.7	1.7	1.0	79.5	0.29	4.61	0.70	0.009	7.92	0.76
2	8.9	6.8	2.7	81.6	1.24	3.96	0.06	0.031	4.19	0.24
3	13.5	7.9	9.1	69.3	1.57	3.05	0.13	0.032	6.77	0.15
4	11.0	0.7	6.7	81.3	1.18	1.97	0.11	0.027	9.34	0.43
5	5.8	1.5	5.5	86.5	1.22	3.22	0.05	0.094	15.27	0.07
6	0.9	0.5	9.7	88.8	0.71	1.64	0.01	0.062	10.07	0.10
7	13.8	1.7	22.0	62.1	1.72	2.88	0.11	0.036	11.64	0.34
8	3.9	0.8	12.7	82.4	1.17	0.89	0.07	0.038	7.48	0.53
9	42.2	0.3	7.4	42.7	1.96	0.00	0.51	0.021	37.93	0.21
10	52.5	1.6	0.0	36.7	1.81	0.00	0.70	0.024	8.96	1.01
11	22.1	5.0	21.4	51.4	1.88	1.16	0.10	0.031	13.37	0.52
12	54.4	0.8	18.4	26.4	1.67	0.41	1.09	0.024	29.78	0.34
13	47.5	0.1	16.8	21.5	2.53	0.00	0.33	0.034	15.02	0.60
14	53.7	0.0	15.9	28.2	2.29	0.00	0.19	0.046	23.04	0.30
15	26.2	22.2	23.0	20.7	2.87	0.00	0.17	0.021	6.30	0.48
16	57.8	0.0	17.7	8.5	2.22	0.00	0.22	0.098	26.31	0.27
17	12.3	0.7	15.6	70.3	1.44	0.19	0.10	0.030	9.73	0.21
18	20.6	1.7	0.0	68.6	1.99	0.00	0.08	0.031	6.55	0.15
19	36.5	1.2	5.9	31.7	2.73	0.00	0.10	0.048	11.45	0.09
20	30.0	18.4	5.4	29.1	2.49	0.00	0.28	0.034	3.16	0.32
21	59.3	0.0	2.7	35.1	1.23	0.00	2.21	0.007	9.61	0.42

Table 4. Detection histories and survey- and site covariates (S: survey; L: length, km; Wa: wood area, km²; Wp: wood perimeter, km; Dr: distance to the river, km; Dnw: minimum distance between wood patches, km; Du: mean distance to urban areas, km; Wt: percent length of wood covered by each transect; W, U, C: percent cover of woods, urban areas and crops, respectively, into each grid square; see the methods for details) for each sampling site.

Site	Detection history			FA (marten like faeces km ⁻¹)			L	Wa	Wp	Mean Dr	Dnw	Du	Wt	W	U	C
				S 1	S 2	S 3										
1	0	0	1	0.00	0.00	0.25	4.0	0.60	1.70	4.63	2.80	1.23	10.75	6.13	1.63	91.54
2	0	1	1	0.00	0.23	0.69	4.34	2.29	20.56	0.41	0.05	2.50	74.10	28.50	17.53	37.80
3	1	0	0	0.53	0.00	0.00	3.74	6.96	62.47	0.62	0.05	3.45	82.20	54.38	0.80	44.77
4	1	0	0	0.72	0.00	0.00	2.77	0.60	1.70	5.05	3.60	0.34	37.10	6.13	1.63	91.54
5	1	1	1	0.12	0.62	0.25	8.01	6.96	62.47	0.56	0.05	0.99	41.10	38.47	0.30	54.42
6	1	0	1	0.88	0.00	0.88	2.26	0.05	1.90	3.71	0.43	2.28	39.10	13.60	1.65	84.33
7	1	0	1	1.34	0.00	0.69	3.72	0.06	17.58	3.03	2.40	1.20	13.70	0.92	0.52	98.50
8	1	1	1	0.25	1.31	0.65	3.06	2.29	20.56	0.49	0.05	3.39	92.00	48.93	0.00	37.54
9	1	1	1	0.97	0.38	1.12	4.13	6.96	62.47	2.23	0.05	1.99	53.40	54.38	0.80	44.77
10	1	1	1	0.62	1.40	0.83	4.80	2.29	20.56	0.55	0.05	2.20	63.20	52.82	0.00	44.95
11	1	1	0	0.61	2.46	0.00	1.63	6.96	62.47	2.18	0.05	2.34	53.30	3.72	0.75	95.28
12	1	1	1	1.99	0.66	0.66	3.01	0.60	2.90	5.34	4.50	1.02	58.70	17.74	1.72	80.53
13	1	1	1	0.29	0.61	2.45	3.26	1.20	8.12	1.48	0.25	1.20	98.20	52.82	0.00	44.95
14	1	1	1	1.03	0.54	2.15	2.93	0.27	3.96	2.94	0.03	0.61	32.30	23.02	5.17	71.67
15	1	1	1	1.39	2.71	1.16	2.58	6.96	62.47	0.60	0.05	2.39	79.10	40.88	0.09	46.90
16	1	1	1	0.56	3.61	2.72	3.57	2.84	10.82	0.42	0.75	1.33	68.60	52.48	1.59	36.73
17	1	1	1	2.87	1.91	1.59	3.54	0.99	15.22	0.25	0.04	2.18	95.00	12.34	0.69	85.86
Mean				0.83	0.98	0.95	3.61	2.88	25.76	2.03	0.89	1.80	58.34	29.84	2.05	64.24
SD				0.75	1.09	0.84	1.38	2.84	25.32	1.79	1.46	0.90	26.86	20.53	4.17	23.57

Figure 1

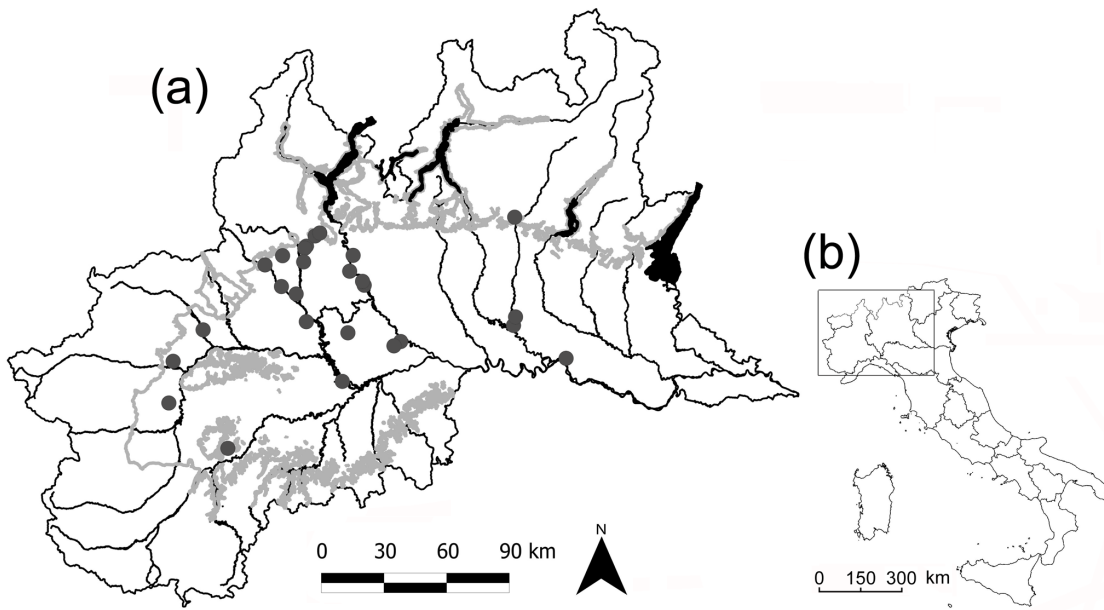


Figure 2

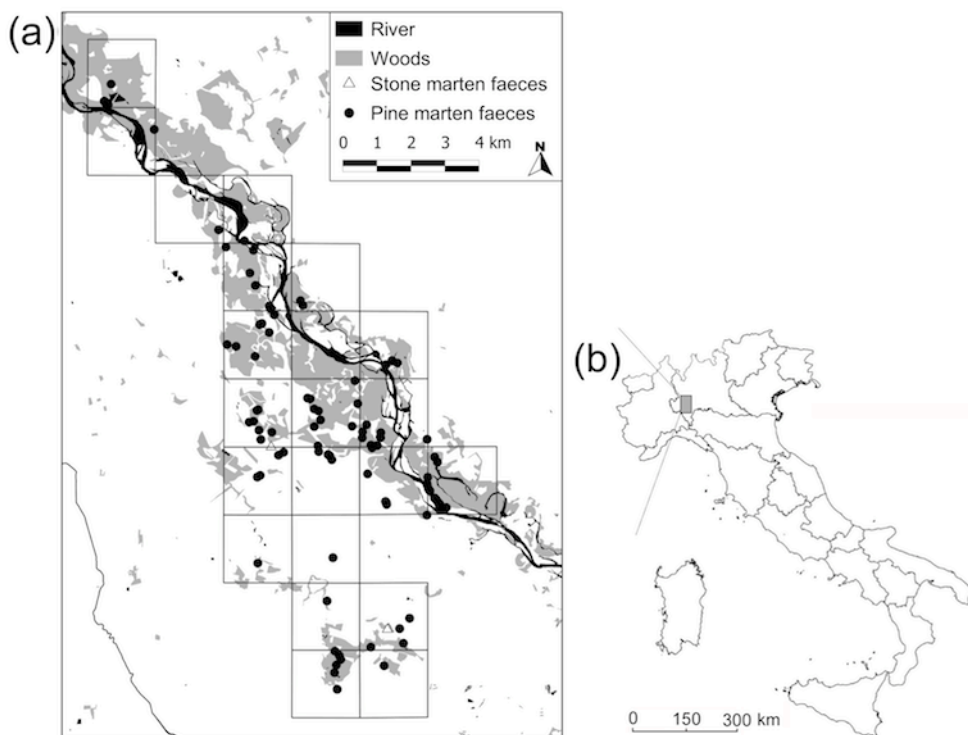


Figure 3

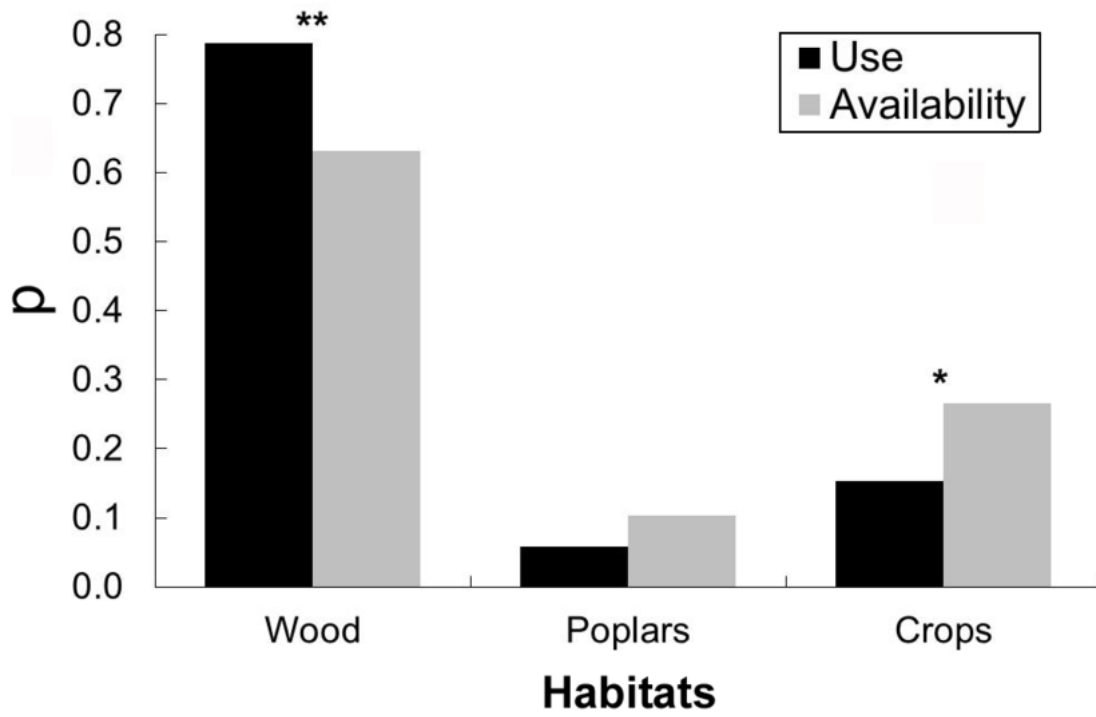
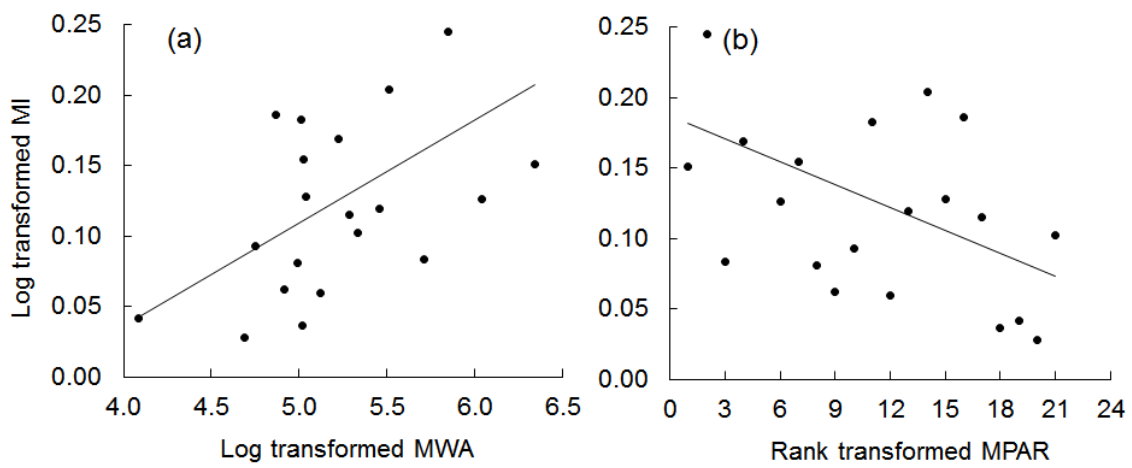


Figure 4



In memory of Professor Konrad Lorenz

In memory of Professor Werner Kallebaut

“This storing, like any retention of information, of knowledge, is achieved by the formation of structure. Not only in the little double helix, but also in the programming of the human brain, in writing, or any other form of “memory bank”, knowledge is laid down in structures”

Konrad Lorenz, 1973