An egg is always an adventure: anthropogenic impacts on Culex pipiens population dynamics

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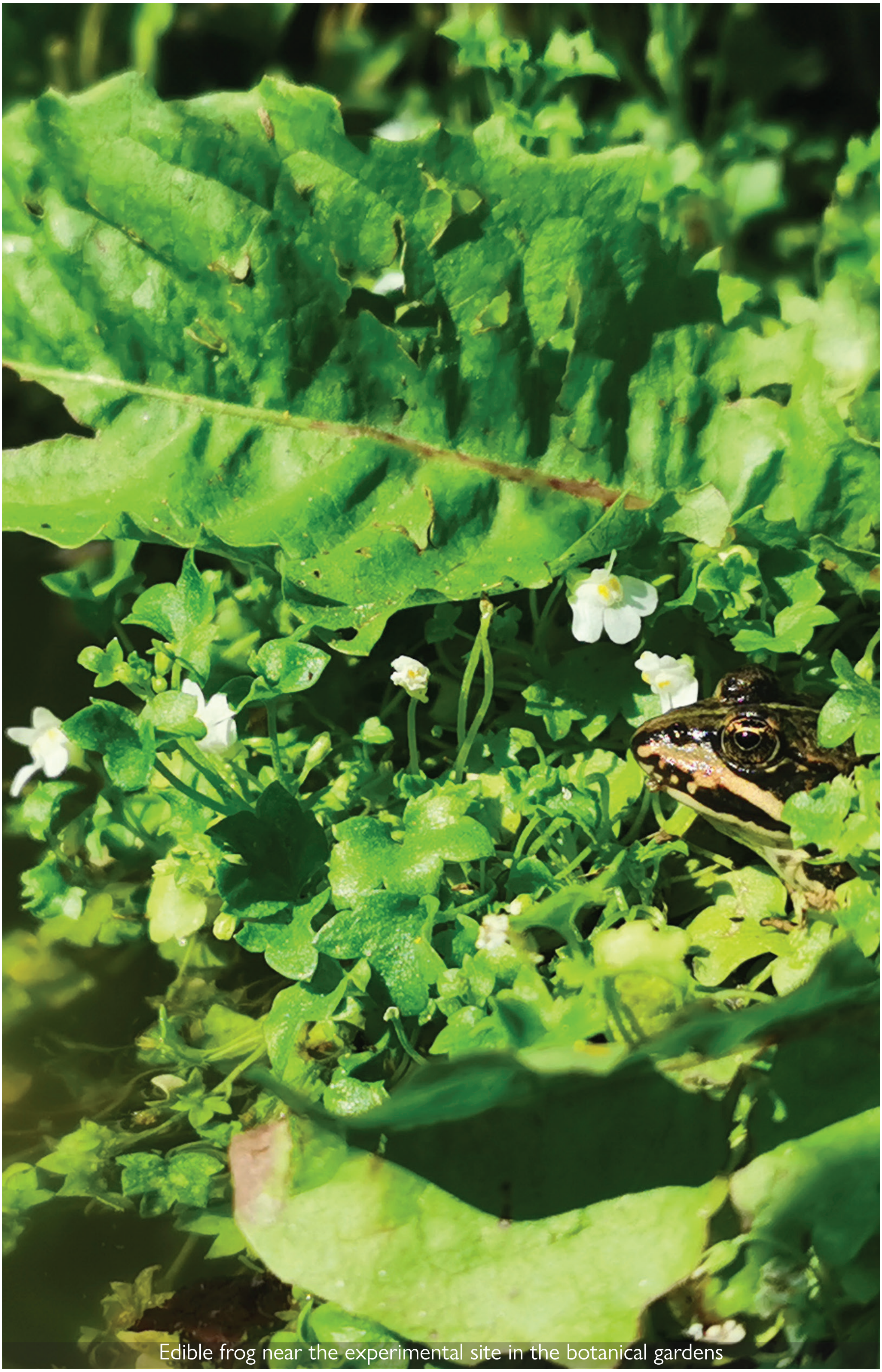

Edible frog near the experimental site in the botanical gardens

Chapter 5

Clashing in murky waters:

on amphibian mosquito suppression

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Abstract

Mosquito-borne diseases are on the rise globally due to the shifting distribution of key mosquito vector species. One Health approaches, like natural control, are gaining attention as a potential solution, especially given the link between increasing mosquito abundances and the biodiversity crisis. However, the effectiveness of different natural predators and their role in an ecological context remain poorly understood. This study compares the predator effectiveness of the common European amphibian species *Lissotriton vulgaris* and *Pelophylax kl. esculentus* to that of the common invertebrate predators *Agabus bipustulatus* and *Notonecta glauca*. We used the cosmopolitan mosquito *Culex pipiens*, known for transmitting pathogens like the West Nile virus, as a model species. We assessed predation rates, sex-specific effectiveness in amphibians, the interaction with eutrophication in *Lissotriton vulgaris* and the impact of amphibian presence on mosquito oviposition behavior. The tested amphibians proved to be effective mosquito predators, consuming between 4-8 times as many larvae per individual as compared to the invertebrates in this research. No difference in effectiveness was detected between the two amphibian species, nor between their sexes or the levels of eutrophication. Predator presence deterred oviposition behavior across the entire experimental setup, thus suggesting the ability of mosquitoes to react to a (purported) landscape of fear. The combined effect of deterring egg laying and lowering mosquito survival highlight the potential of natural predation, and particularly of amphibian species as natural larval control agents. Overall, our results highlight the importance of conserving these –globally threatened – species and facilitating them in urban and rural environments.

Keywords: Amphibian, biological control, control agent, *Culex pipiens*, *Lissotriton vulgaris*, *Pelophylax kl. esculentus*, urban green spaces

5.1 Introduction

In recent decades, distributions of several mosquito species that are key vectors of diseases have been shifting rapidly (Neiderud, 2015; Steiger et al., 2012). This has contributed to an expansion of pathogen distributions and associated increases in disease risk globally (Colón-González et al., 2021; Kraemer et al., 2015; Roche et al., 2015). Despite extensive mosquito control efforts by governments and healthcare sectors in the global south, and increasing efforts in the global north (Medlock et al., 2012), mosquito-borne diseases continue to emerge and re-emerge across the world (Ferguson, 2018).

Control of larval mosquitoes is strongly geared towards chemical interventions. In general, these measures are expensive, and their long-term effectiveness has been widely questioned (Hamdan et al., 2005; Nazni et al., 2005; Paris et al., 2011). Furthermore, direct or indirect effects on non-target organisms pose additional threats for biodiversity loss (Antwi & Reddy, 2015; Lawler, 2017; Moura & Souza-Santos, 2020; Thompson et al., 2020). As this decline in biodiversity may cause alleviation in predation pressure, mosquitoes may thrive on the long-term. Promoting ecologically healthy systems, i.e., ecosystems with an abundance of natural predators, as part of a One Health approach, has therefore gained increasing attention. With this came an increasing interest in natural control (Benelli et al., 2016), but more information is needed on which natural predators effectively suppress mosquito populations (in ephemeral water bodies), or how (neighboring) habitats could be adapted to facilitate them (Carlson et al., 2004).

The importance of natural control agents, and how their effectiveness differs in an ecological context, remains poorly understood (Shaalan & Canyon, 2009). Even though, predator-prey relationships are generally well-studied, information in the context of mosquitoes is relatively scarce. This is particularly worrisome as large-scale decreases in potential predator populations were recently reported (Eisenhauer et al., 2023; Kehoe et al., 2021). Impacts on public health are therefore hard to predict, as loss of natural control agents may necessitate alternative forms of mosquito control.

To gain insight into the role of different species of mosquito predators, species specificity is likely to be a key factor. In general, predator effectiveness – i.e. kill rate or consumed prey over time – is a species-specific interaction, as anti-predator behavior differs across species and may enhance or impair kill rates

(Ohba & Ushio, 2015; Sih, 1986). Meta analyses of predation efficiency exist for groups such as dragonflies and damselflies (Priyadarshana & Slade, 2023) and mayflies (Dasrat & Maharaj, 2021), which prey on mosquitoes during their aquatic life stages (Dasrat & Maharaj, 2021; Priyadarshana & Slade, 2023). However, these analyses are limited to specific groups of (often invertebrate) predators (Benelli, 2015; Lacey & Orr, 1994; McDonald & Buchanan, 1981), while vertebrate predators have been hypothesized to be far more efficient predators (DuRant & Hopkins, 2008).

Particularly amphibians are a poorly understood mosquito predator group (Benelli, 2015), while incidentally been proven to be potentially effective natural agents, because like mosquitoes, they often depend on ephemeral water bodies (Brodman & Dorton, 2006a; DuRant & Hopkins, 2008). Aside from lowering mosquito survival, there may be other direct or indirect inter-specific interactions that adversely impact the development of mosquito larvae in shared habitats (Alto et al., 2012; Fischer et al., 2012; Meadows et al., 2017). Tadpoles, for instance, may prey on mosquito eggs, compete with larvae, predate on mosquito larvae during their later developmental stages and affect habitat choice (Weterings, 2015). Competitive effects with mosquitoes have been shown to prevent successful recolonization by mosquitoes in crustacean competitors (Kroeger et al., 2013), and as such, predator species with herbivore immature stages like frogs, may deter, limit and prevent mosquitoes from establishing. A broad scale inventory of predatorial capacity including vertebrate taxa is hitherto lacking and therefore it is not well known how predation by different predators compare across different ecological contexts.

As such, we aimed to compare the predator effectiveness of two common European amphibians — the smooth newt (Caudata: Lissotriton vulgaris) and the edible frog (Anura: Pelophylax kl. esculentus) — against two common European invertebrate mosquito predators found in ephemeral habitats: the two-spot water beetle (Coleoptera: Agabus bipustulatus; Culler & Lamp, 2009) and backswimmer (Hemiptera: Notonecta glauca; Saha et al., 2010) across a range of ecologically realistic conditions. Experiments were conducted to assess i) the predator effectiveness of selected invertebrate and vertebrate species across different levels of eutrophication, ii) whether there is a sex specificity of predator effectiveness of selected amphibian species, and iii) the effect of predator presence on mosquito oviposition behavior.

To address these aims, we conducted a series of experiments determining and comparing the predation rate on Culex pipiens. We focus on Culex pipiens s.l., a common and cosmopolitan species with a wide tolerance to temperature and eutrophication, known to occupy almost every type of water body (Becker et al., 2010a). Culex as a genus represents the predominant vectors of West Nile virus, Usutu, Avian malaria and Dirofilariasis amongst other pathogens, of which the Culex pipiens species group is the most widespread (Harbach, 2012). Wild populations of Culex pipiens have been confirmed to transmit Avian malaria, Batai, Sindbis and Usutu (Ibañez-Justicia et al., 2015). Due to its locally high abundance, wide distribution range and its aptitude for transmission of a variety of pathogens, it is an important vector and nuisance species. Culex pipiens thrives in small aquatic systems (Buxton et al., 2020),, especially under hypertrophic and subsequently anoxic conditions (Boerlijst et al., 2023).

Predatory behavior on the mosquito genera Aedes and Anopheles was validated for L. vulgaris and Pelophylax kl. esculentus to determine whether our results could translate to other mosquito taxa. Adult frogs were used as development of the mouthparts allow for carnivorous diet only during the short window between Gosner stages 42 and 46 (Gosner, 1960; Johansson et al., 2010).

5.2 Methods

5.2.1 Experimental setup

The experiments consist of i) a comparison in predator effectiveness of *L. vulgaris*, *A. bipustulatus* and *N. glauca*, ii) an assessment of *L. vulgaris* predator effectiveness across different eutrophic levels, iii) a large-scale comparison of sex-specific predator effectiveness of *L. vulgaris* and *P. esculentus*, iv) an assessment of amphibian presence on mosquito oviposition, and v) a comparison of amphibian predator effectiveness on *Aedes* and *Anopheles* larvae. All experiments were performed in a full factorial setup under outdoor conditions.

5.2.1.1. Pre-experimental conditions

For each experiment, we used a set of similar pre-experimental conditions. A series of mesocosms (Table 5.1) was placed in a randomized full-factorial grid. The mesocosms are representative of the artificial containers that *Cx. pipiens* is known to colonize (Koenraadt & Harrington, 2008). Each mesocosm was filled with dechlorinated tap water and a standardized community of algae and bacteria, collected with a plankton net (250 µm with a 53 µm collector) from the lake next to the Living Lab field station (Leiden, The Netherlands), where all selected predator species naturally occur. The filtered algae and bacteria obtained were divided equally across all mesocosms so that one liter of water in the set-up contained as much microbes as a liter of ditch water (Dellar et al., 2022). Eutrophic levels representative of Dutch ditches (4-10mg/L N-total; Loeb and Verdonschot 2008) were created, using cow manure pellets (2,4% N; 1,5% P_2O_5; 3,1% K_2O) (Boerlijst et al., 2023). As such, 4 and 8 mg N-total was used for experiment 2 and 4mg N-total for all other experiments. After a day of acclimation, the contents of the mesocosms were stirred and strained through a 300µm sieve to remove any large particulate matter. The mesocosms were then covered with a 0.1mm mesh to prevent natural colonization by mosquitoes and predators and to prevent the emerged mosquitoes from flying out. The bacterial community was thereafter left to acclimatize for one week.

Table 5.1 *Mesocosm set-up for each of the experiments*

Experiment	Mesocosm	Replicates (treatment/control)
1	16L white polypropylene	3/3
2	16L white polypropylene	8/4
3	48L black polypropylene	11 /14
4	48L black polypropylene	11/14
5	48L black polypropylene	8

Evaporated water was replenished daily using dechlorinated tap water stored at ambient temperature. To maximally mimic field conditions and to limit the amount of stress of the predators, natural shelter for salamanders in the form of a handful of Canadian waterweeds (*Elodea vulgaris*), a stone to climb out of the water, and an air stone connected to an air pump (Vt AP-10) were provided in each mesocosm. Prey densities of 50 third/fourth instar mosquito larvae were added to each mesocosm.

5.2.1.2 Experiment 1: comparing predator effectiveness

Kill rates of different vertebrate vs invertebrate predators were assessed in May 2020. The experiment consisted of four predator treatments, with either one individual of *L. vulgaris*, *A. bipustulatus*, *N. glauca*, or no predator (control). Each treatment combination had three replicates. The experiment took place on 6 May 2020 and had a duration of one day. The number of mosquito larvae, pupae and adults were counted 1, 2 and 3 hours after the mosquito larvae were placed in the mesocosm.

We focused on predator species that are able to survive such conditions by i.e. their ability to breath air and migrate over land, like true bugs (Fischer et al., 2012), beetles (Lundkvist et al., 2003a) and amphibians (Brodman & Dorton, 2006a). Similarly, we focused on larger predator species as they are less temperature dependent (Van Der Have & De Jong, 1996), and as food intake increases with size of the animal (DuRant & Hopkins, 2008; Jennings et al., 2002). Both *L. vulgaris* and *P. esculentus* are known to feed while in and under water (Anamaria et al., 2011; Blommers-Schlösser, 1992; Covaciu-Marcov, 2010; Sas et al., 2007, 2009; Tyler, 1958)2010; Sas et al., 2007, 2009; Tyler, 1958 and Nematocera larvae have been incidentally described as a food source (Anamaria et al., 2011; Covaciu-Marcov, 2010; Sas et al., 2007).

5.2.1.3 Experiment 2: Predation across eutrophication

The impact of eutrophic conditions on predator effectiveness was assessed by taking two eutrophication treatments (4mg/L N-total and 8mg/L N-total) and two predation treatments (*L. vulgaris*, control), with eight *L. vulgaris* replicates and four control replicates. The experiment took place at the beginning of May 2020 and had a duration of 5 days. The number of mosquito larvae, pupae and adults were counted 2, 4, 6, 12, 14, 16, 18, 36, 38, 40, 42, 52 and 62 hours after *L. vulgaris* had been placed in the mesocosm. We performed daily chlorophyll a and turbidity measurements using an Aquafluor 8000-010 using manufacturers protocols, as these are indicators for both visibility and resource competition due to their relation with bacterial and algal metabolism (Ansa-Asare et al., 2000; Coolidge, 2017).

5.2.1.4 Experiment 3: sex-specific Amphibian predator effectiveness

Amphibian predator effectiveness was assessed by imposing three predation treatments (*L. vulgaris*, *P. esculentus*, control Due to limitations in availability, we used two male, and nine female replicates for *L. vulgaris*, seven male, and four female replicates for *P. esculentus*, and fourteen control replicates. The experiment took place in June 2021. A terrestrial resting spot was included in each mesocosm in the form of a 180x87x41mm brick placed vertically in the water (Figure 5.1). The number of mosquito larvae, pupae and adults were counted after 1, 2, 4, 8, 24, 28, 32, 48, 56 and 72 hours, starting 40 hours after the predators were put into the mesocosms.

The experiment was repeated directly after the first round to determine whether the 40-hour food deprivation affected predatory behavior. This second round was performed in triplicate, for which a selection of the predators was used, with two male and one female replicate for *P. esculentus* and three female replicates for *L. vulgaris*. The other predators were removed from the setup and placed in experiment 5.

Figure 5.1 *Overview of experimental setup for amphibian predator effectiveness; experiment 3 (left). Contents of the mesocosms are shown on the right including the stone as resting spot and air stone for oxygen (top right), and waterweeds as natural shelter (bottom right).*

5.2.1.5 Experiment 4: Oviposition behavior

Mosquito oviposition behavior was assessed in the experimental setup of experiment 3 After the second round of the experiment, all lids were taken off and all remaining predators were removed. The water from each mesocosm was strained through a 300µm sieve to remove any remaining larvae. The mesocosms were then left open for two weeks during which egg rafts were counted and removed daily. The water in each mesocosm was filtered daily using a 300µm sieve to prevent colonization by other (predator) species.

5.2.1.6 Experiment 5: Comparison with other mosquito genera

Predatory behavior on the mosquito genera *Anopheles* and *Aedes* was assessed using a glass 40x30x30cm aquarium filled with 20L low eutrophic (4mg/L N total) water. Two *L. vulgaris* or two *P. esculentus* were placed in the aquarium and left to acclimate for five minutes. Three larvae of either *Aedes sp.* or *Anopheles maculipennis* were then added, and feeding was recorded for five minutes after which the remaining mosquito larvae were removed using plastic pipettes. The predators were then collected and released at their capture location. This was repeated until all larvae had been eaten. Due to limited availability, only seven *Aedes* larvae and five *Anopheles* larvae were used in total.

5.2.2 Rearing of mosquito larvae

Egg rafts of *Cx. pipiens* were collected during two weeks prior to the start of an experiment at the experimental site. To this end, three 8L black polypropylene buckets were filled with three liters hypertrophic water, which has shown to be an attractive oviposition environment to female *Cx. pipiens* (100mg N-total/L; Boerlijst et al., 2023), after which they were placed under tree cover. The larvae were subsequently allowed to hatch in the buckets, where they were kept at ambient temperature until the start of the experiment. Previous studies indicated that the used conditions attract *Cx. pipiens* and *Culiseta annulata* only (Boerlijst et al., 2023; Dellar et al., 2022). The collected egg rafts were distinguished from those of *Culiseta annulata* by their difference in size (Chapman et al., 2020; Sames et al., 2005).

5.2.3 Sourcing predators

All predators were captured from the neighboring lake of the Living Lab field station using an aquatic net. The two invertebrate species, *A. bipustulatus* and *N. glauca*, were collected on the day of the experiment and were identified using the Freshwater Life field guide (Greenhalgh & Ovenden, 2007) whilst making sure all individuals were of similar size. The two vertebrate species *L. vulgaris* and *P. kl. esculentus* were collected during the two days prior to the experiment. All predators were kept individually in the experimental setup until the start of the experiment to prevent cannibalistic behavior.

5.2.4 Statistical analysis

All data were analyzed in R version 4.3.2 (R Core Team, 2018). Linear (mixed effects) models were used to test for differences in predator effectiveness across the experiments. Boxcox transformation was applied when applicable (Supplementary Table S5.1). All models, including random effects, were optimized by Akaike information criterion (Table 5.2). Dependent variables were tested for normality and assessed using Quantile Quantile-plots and a Levene's test ($P=0.05$). Absolute mortality rates were used for statistical analysis, whereas proportion of mortality due to predation were used for visualization purposes. The proportions of mortality due to predation were calculated by subtracting the background mortality, here defined as the mean mortality in the control group per time point. As such, variance in control is not visualized, but was evaluated and thus accounted for.

Table 5.2 *Linear model after selection per experiment*

Experiment	Linear model
Relative effectiveness	Mortality ~ Predator
Predation across eutrophication	Mortality ~ Eutrophication + Hours
Amphibian predator effectiveness	Mortality ~ Predator * Hours + Temperature + Error(Cosm / (Predator * Hours))
Oviposition behavior	Egg rafts ~ Predator * Day + Error(Cosm / Day)

5.3 Results

5.3.1 Experiment 1: Assessing predator effectiveness

All predators successfully captured and consumed mosquito larvae (Figure 5.2; Supplementary Table S5.1). No difference in predator effectiveness was detected between *A. bipustulatus* and *N. glauca* ($t(3,8) = -0.985$, $p>0.05$, partial $\eta^2 = 0.173$, power = 1). However, differences between the vertebrate and invertebrate predators were found as *L. vulgaris* consumed on average four times as many larvae as *A. bipustulatus* ($t(3,8) = -4.924$, $p<0.01$, partial $\eta^2 = 0.909$, power = 1) and eight times as many larvae as *N. glauca* ($t(3,8) = -5.909$, $p<0.001$, partial $\eta^2 = 0.876$, power = 1).

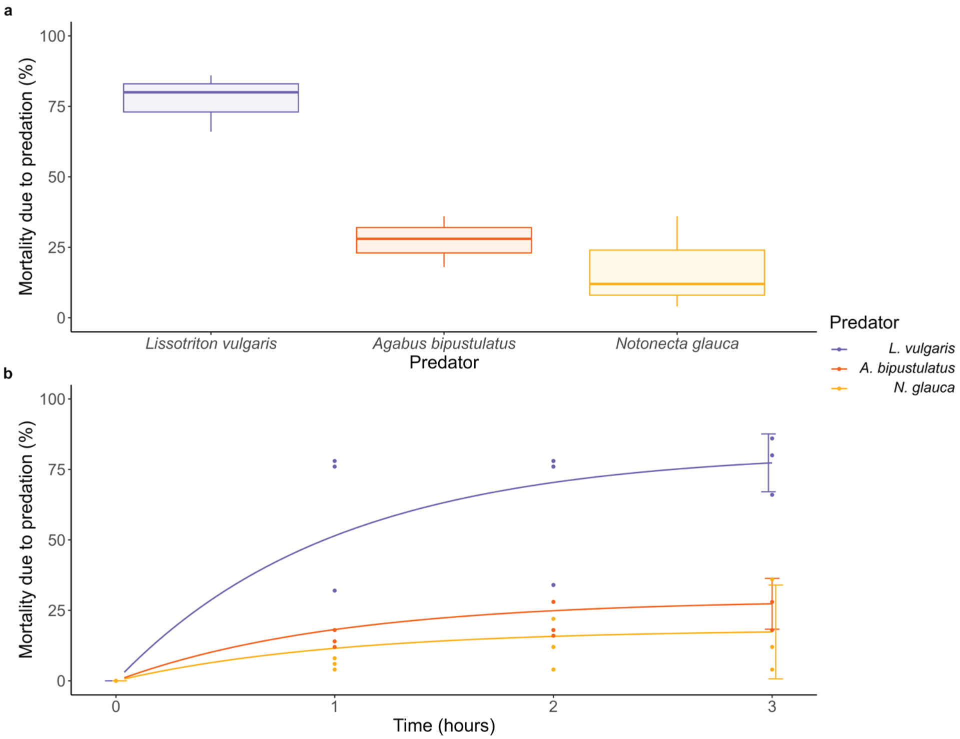

Figure 5.2 *Proportion of mortality due to predation (%) per predator species at the end of the experiment (a), and over time (b).*

5.3.1.2 Experiment 2: Predation across eutrophication

Lissotriton vulgaris was successful in capturing mosquito larvae irrespective of eutrophication level (t(3,92) = --1.814, $p>0.05$, partial $\eta^2 = 0.008$, power = 0.136; Supplementary Table S5.2), and their kill rate increased over time (t(9,92) = 13.545, $p < 0.001$, partial $\eta^2 = 0.666$, power = 1).

5.3.2 Experiment 3: sex-specific amphibian predator effectiveness

5.3.2.1 Short-term food deprivation

After a 40-hour food deprivation, approximately half of the (50) mosquito larvae within the experiment were eaten after 1-2 hours, which steadily progressed during subsequent hours (f(10,266) = 200.948, $p <0.001$, partial $\eta^2 = 0.875$, power = 1; Figure 5.3; Supplementary Table S5.3) and differed between predator treatment (f(20,266) = 5.253, $p< 0.001$, partial $\eta^2 = 0.282$, power = 1). A minor effect of temperature was detected (f(1,266) = 8.279 , $p < 0.01$, partial $\eta^2 = 0.031$, power = 0.835). Post-hoc analysis showed higher mosquito mortality for *P. esculentus* (t = -4.016, $p < 0.01$) and *L. vulgaris* (t = -2.667, $p < 0.05$) compared to the control from 2 hours onwards. No difference between the two predator species was detected (t = 1.195, $p > 0.05$). No difference between sexes was detected (Supplementary Figure S5.1).

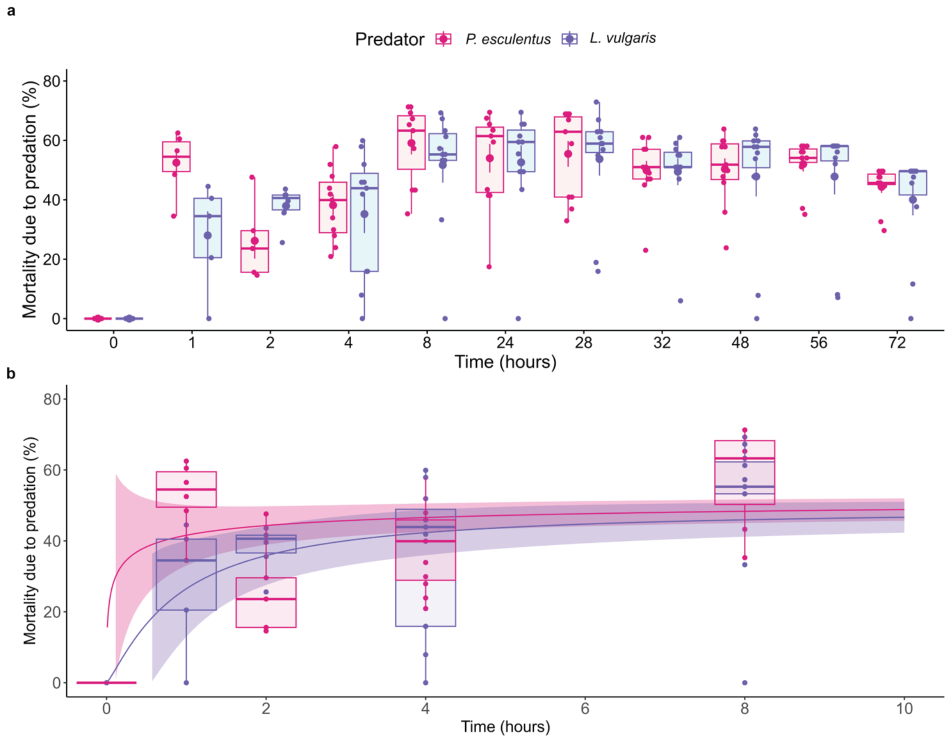

Figure 5.3 *Proportion of mortality due to predation (%) over time (after 40-hour food deprivation) per predator type depicted as boxplot with outliers as dots (a) and dose-response curve with standard error up until the asymptote is reached (b).*

5.3.2.2 No short-term food deprivation

Similar kill rates were found without 40-hour food deprivation, with the majority of the prey consumed during the first two hours. Mosquito mortality increased over time (f(8,35) = 147.777, $p < 0.001$, partial $\eta^2 = 0.965$, power = 1; Figure 5.4; Supplementary Table S5.4) per predator treatment (f(16,35) = 8.977, $p < 0.001$, partial $\eta^2 = 0.763$, power = 1). No effect of temperature was detected (f(13,35) = 0.647, $p > 0.05$, partial $\eta^2 = 0.744$, power = 1). Post-hoc analysis showed higher mosquito mortality for *P. esculentus* (t = -15.251, $p < 0.001$) and *L. vulgaris* (t = -15.163, $p < 0.001$) compared to the control from 1 hour onwards. No difference between the two predator species was detected (t = 0.406, $p > 0.05$). No difference between sexes was detected (Supplementary Figure S5.2).

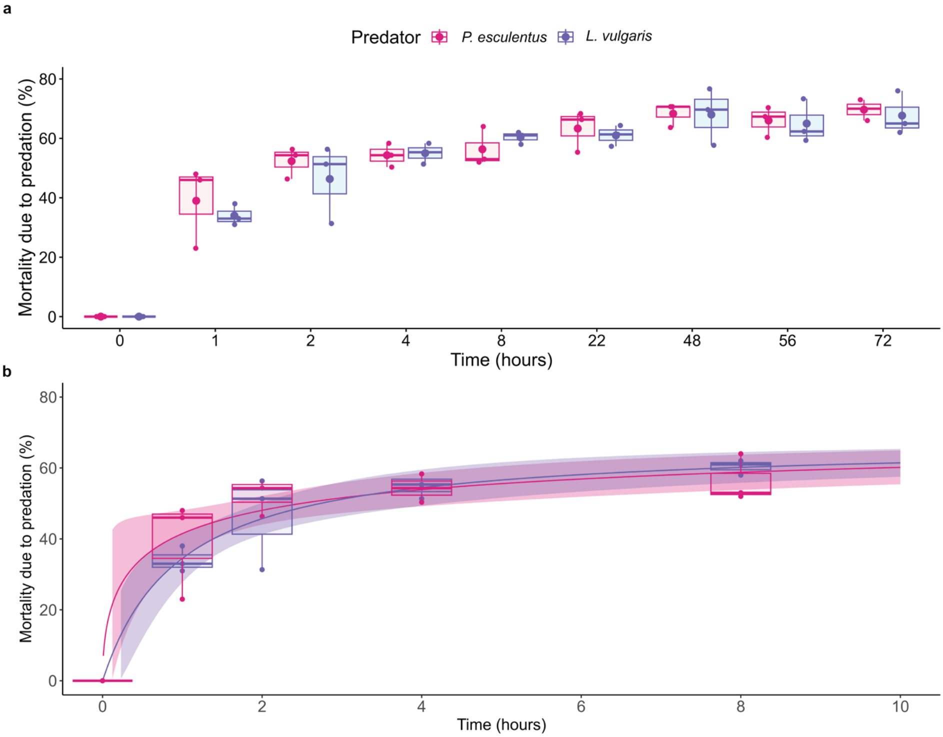

Figure 5.4 *Proportion of mortality due to predation (%) over time per predator type without food deprivation, depicted as boxplot with outliers as dots (a) and dose-response curve with standard error up until the asymptote is reached (b).*

5.3.3 Experiment 4: Oviposition behavior

During the two-week period, a total of 32 mosquito egg-rafts were laid, of which 27 in the control treatment (Supplementary figure S5.3; Supplementary Table S5.5). All rafts were deposited in the mesocosms located at the border of the experimental setup. Differences were detected across the predator treatments ($X^2 = 24.86$, df = 6, $p < 0.001$, Kramers-v = 0.19, power = 0.82). Post-hoc analysis revealed differences between the control and *L. vulgaris* ($X^2 = 8.63$, df = 3, $p < 0.05$) and between the control and *P. esculentus* ($X^2 = 18.95$, df = 3, $p < 0.001$).

5.3.4 Comparison other genera

The experiment to confirm whether other representative species from other mosquito genera were also consumed by the same predators was successful and confirmed that all 7 *Aedes* and 5 *Anopheles* larvae were eaten (Supplemental video).

Discussion

The aim of this study was to assess the potential of amphibians to control mosquitoes, as part of a One Health approach, by determining the predator effectiveness of a range of relevant mosquito predators in temperate regions. Here, we report effects on different mosquito life stages, both during larval development and oviposition. Both amphibians (L. vulgaris and P. esculentus) exhibited similar and notably high larval consumption of Cx. pipiens, regardless of preceding food deprivation. Lissotriton vulgaris consumed four times more than A. bipustulatus and eight times more than N. glauca. Additionally, breeding sites with prior amphibian presence deterred egg-laying, also for neighboring breeding sites. These effects appear more significant and ecologically diverse than previously reported, adding to the evidence that ephemeral and permanent water bodies with an abundance of natural predators might effectively reduce mosquito populations without the need for ecologically harmful larvicides (Dale & Knight, 2012).

To date, most interventions to suppress mosquito populations involve the use of chemicals like biocides. However, these chemical methods are short-term solutions, leading to resistance (Hamdan et al., 2005; Li et al., 2002) and creating a pesticide treadmill undermining ecosystem health. As competing species are often impacted similarly, chemical control may result in rapid recolonization (Meyabeme Elono et al., 2018) and overcompensation (Juliano, 2007; Neale & Juliano, 2019) by mosquitoes and ecological imbalances (Allgeier et al., 2019; Brühl et al., 2020; Meyabeme Elono et al., 2018), posing risks, especially to conservation areas. This combined with the limited information on food-web effects (Brühl et al., 2020), and the subsequent unknown fate of the substances, makes its use in conservation areas risky. In contrast, biological control, using naturally occurring predators, presents a less problematic approach. By actively releasing or facilitating predators, it may offer a more sustainable and preventive strategy. This method deters egg-laying (Rubbo et al., 2011; Sougué et al., 2021) and limits immature survival whilst lowering growth rates, fecundity and delaying reproduction (Fischer et al., 2012; Lundkvist et al., 2003b; Schrama et al., 2018). Additionally, stressful conditions – including predator presence –¬ promote a more heterogeneous mix of developmental stages, as a result of different growth strategies among the mosquito larvae (Fischer et al., 2012; Knight et al., 2004), which could enable cannibalistic behavior (El Husseiny et al., 2018; Koenraadt & Takken, 2003). Indeed, results from our study highlight the importance of seriously considering such measures, not only because they are likely cost-effective, but also because they may be far more sustainable than chemical alternatives.

An important remaining question is how these effects translate into more ecologically complex settings. Natural ephemeral ecosystems may be more biologically and physicochemically complex than the mesocosm setup used, which might lower real world impacts. For instance, the current experiments did not consider alternative prey, such as chironomids, which are commonly found alongside mosquito larvae (Dinithi & Hemantha, 2020; Leisnham et al., 2007; Talaga et al., 2020). Therefore, this study does not account for effects of prey preference. However, both currently assessed amphibian species hunt opportunistically (Kovács et al., 2014; Roşca et al., 2013). As mosquito larvae spend most time at the top of the water column (Becker et al., 2010b), consistently dive as anti-predator behavior (Awasthi et al., 2012), and often occur at high densities, they are considered easy prey. It can therefore be assumed that they would provide a prominent food source if available, even in complex communities, which is confirmed by their relative abundance in the stomach contents of both amphibian species (Brodman & Dorton, 2006b; Tyler, 1958). Moreover, Cx. pipiens often selects for breeding habitats with minimal competition and predation (Alcalay et al., 2019; Dhileepan, 1997), which further reduces potential effects of prey preference on predator behavior.

Similarly, the current study evaluated predator effectiveness at the individual level, without considering the effect of relative predator densities. Invertebrate predators may be present in much higher densities than amphibians. Consequently, although L. vulgaris and P. esculentus individuals consume more larvae, this effect may be less pronounced at the community level. Especially when re-evaluating predator effectiveness across average predator biomass, i.e., larvae consumed per gram (Supplementary Table S5.6), invertebrate predators seem much more effective in the short term. However, amphibians, which can readily move between water bodies and have stomachs capable of digesting large amounts of prey simultaneously (Bissattini et al., 2021; Brodman & Dorton, 2006b; Tyler, 1958), may still prove to be important predators in influencing mosquito populations over longer periods.

Our results suggest that tested amphibians are highly effective mosquito predators, irrespective of species, without discernible differences in kill rates between sexes or among similarly sized individuals of amphibians or invertebrate predators.

When applying these findings to other mosquito groups, it is essential to consider that mosquito larvae exhibit species-specific feeding behaviors that influence their position in the water column (Dadd, 1975; Merritt et al., 1992). Understanding these behaviors is crucial for assessing predator effectiveness, particularly in scenarios of static predatory behavior, necessitating comprehensive testing across diverse mosquito groups and predator species. However, it is noteworthy that a predominant anti-predator response among most mosquito larvae is diving (Awasthi et al., 2012; Sih, 1986). Notably, we found that L. vulgaris effectively preys on larvae of Culex, Aedes, and Anopheles, primarily in proximity to the container bottom, to which the larvae flee upon disturbance. Pelophylax esculentus was found to sit and wait floating at the water surface, we hypothesize until the larvae re-emerge after diving, consistent with previous literature (Anamaria et al., 2011; Kovács et al., 2014). As such, it is probable that our findings also translate to similar predation rates for other mosquito species.

When considering the effectiveness of predation under different abiotic conditions, it is important to recognize their interconnected nature (Krol et al., 2019). Typically, murky and nutrient-rich waters have a positive impact on larval mosquito feeding behavior (Dadd, 1975; Merritt et al., 1992), thus benefitting Cx. pipiens' survival (Boerlijst et al., 2023). Hence, it was expected that eutrophication would affect the capacity of predators to kill mosquito larvae (Schmutzer et al., 2008), which could operate either through lowered oxygen acquisition (Coffin et al., 2018), lowered hunting efficiency due to increased anti-predator behavior (Tuno et al., 2004), or reduced vision-based hunting (Abrahams & Kattenfeld, 1997). Surprisingly, eutrophication levels did not affect predation levels, with L. vulgaris successfully capturing mosquito larvae regardless, suggesting unaffected predation behavior, possibly mediated due to alternative olfactory or tactile cues (Ranta et al., 1990).

The observed effects of predators on ovipositioning may ultimately be even more important than the direct effects of larval killing, suggesting that mosquito predators are successful in establishing a mosquito landscape-of-fear. Ovipositing rates were relatively low, as adult female mosquitoes mostly refused to deposit eggs anywhere near our experimental setup. This effect was far stronger than the deterrence by abiotic factors such as salt or nutrient availability (Boerlijst et al., 2023, 2024), or relative to some invertebrate predators (Eitam & Blaustein, 2004; Why et al., 2016), indicating that these predators remain in control, even in between meals and during temporary absence.

Overall, our results suggest that amphibian predators may have important long term negative effects both on mosquito larval and egg stages. However, relative abundances of predator species play a crucial role for their effectiveness, meaning that despite the amphibians' higher effectiveness, their overall impact could be mitigated by their lower densities when compared to invertebrates. Still, given their substantial impact on ovipositioning rates, and their ability to readily move over land to colonize new ephemeral habitats, amphibians are likely relevant actors in controlling mosquito larval populations for relatively small urban habitats. As such, facilitating endemic amphibians, and endemic mosquito predators at large, in anthropogenic landscapes may prove to be a valuable and effective component of One Health approaches to mosquito control.

Ethical consideration

All experiments were performed under supervision of the RAVON foundation. The Defense Safety Inspectorate deemed the experiments to be exempt of the animal experiment legislation as the discomfort inflicted was negligible since i) the displacement was within 6 meters for less than 10 days, ii) there was no (medical) intervention, iii) there was no long-term food deprivation, and iv) they were released into their original habitat.

Acknowledgements

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Author contributions

AS, JS and MS conceived the general idea for the experiments. SB set up the experiments, and SB and AU carried out the measurements. SB performed interpretation together with AS, JS, EB, PB, RB, and MS. SB carried out all statistical analyses, together with PB and MS. All the authors contributed critically to the drafts and gave final approval for publication.

Conflict of interest

The authors report no conflict of interest.

Availability of data and materials

The original datasets used and analyzed during the present study are freely and openly available within the Zenodo repository 10.5281/zenodo.11128264.

Electronic appendix

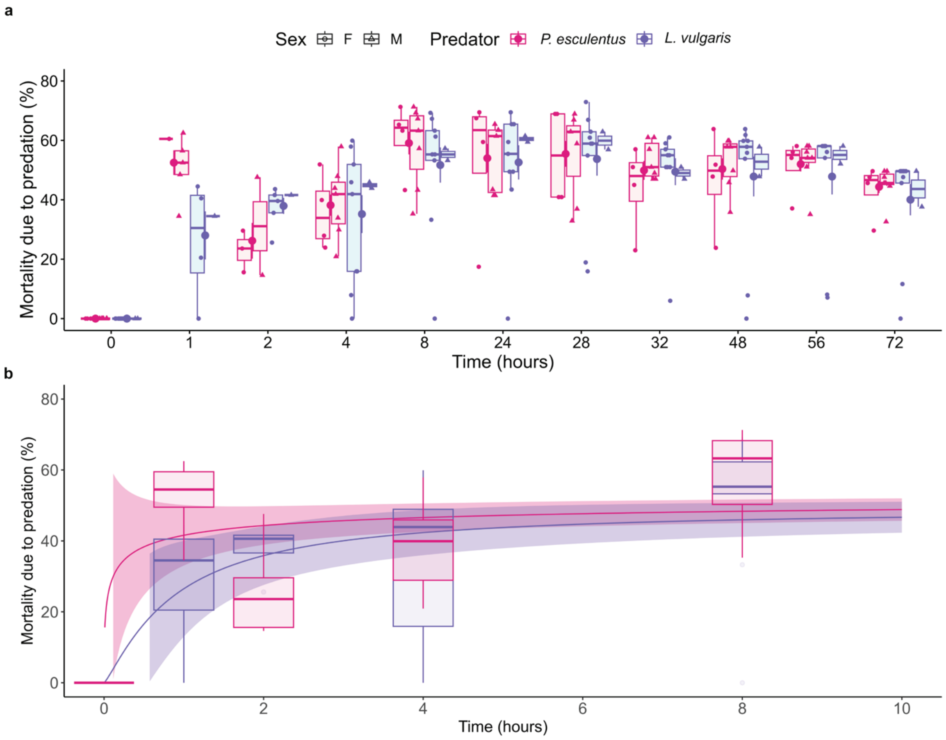

Figure S5.1 *Proportion of mortality due to predation (%) over time (after 40h fast) per predator type depicted as (a) boxplot per sex with outliers as dots and (b) dose-response curve with standard error up until the asymptote is reached. Predator sex is indicated by a circle for female, triangle for male.*

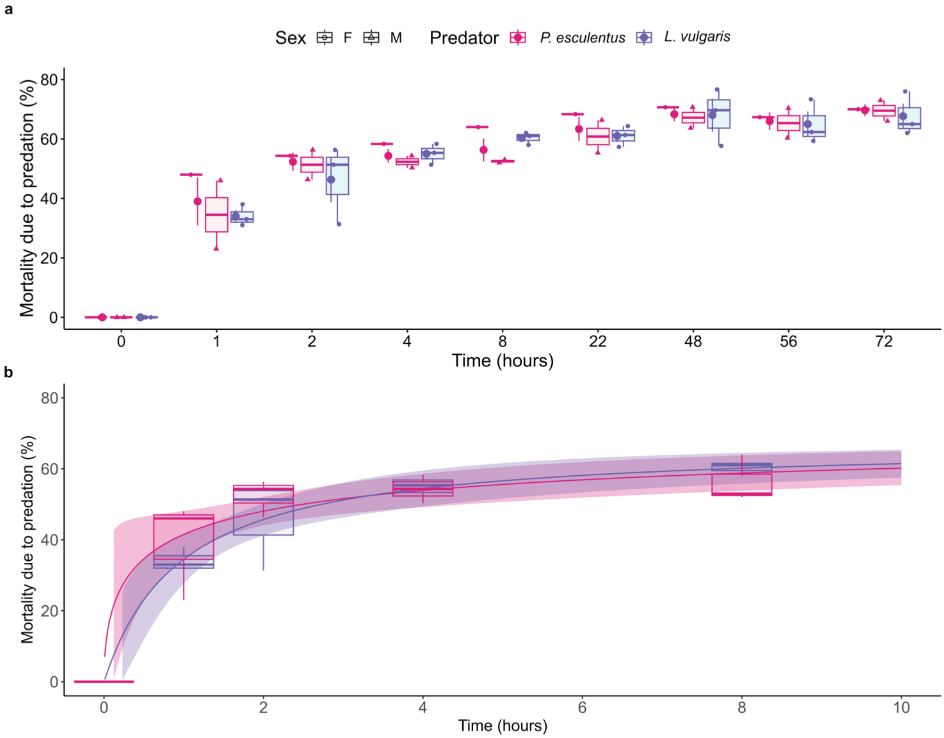

Figure S5.2 *Proportion of mortality due to predation (%) over time per predator type without fast depicted as (a) boxplot per sex with outliers as dots and (b) dose-response curve with standard error up until the asymptote is reached. Predator sex is indicated by a circle for female, triangle for male.*

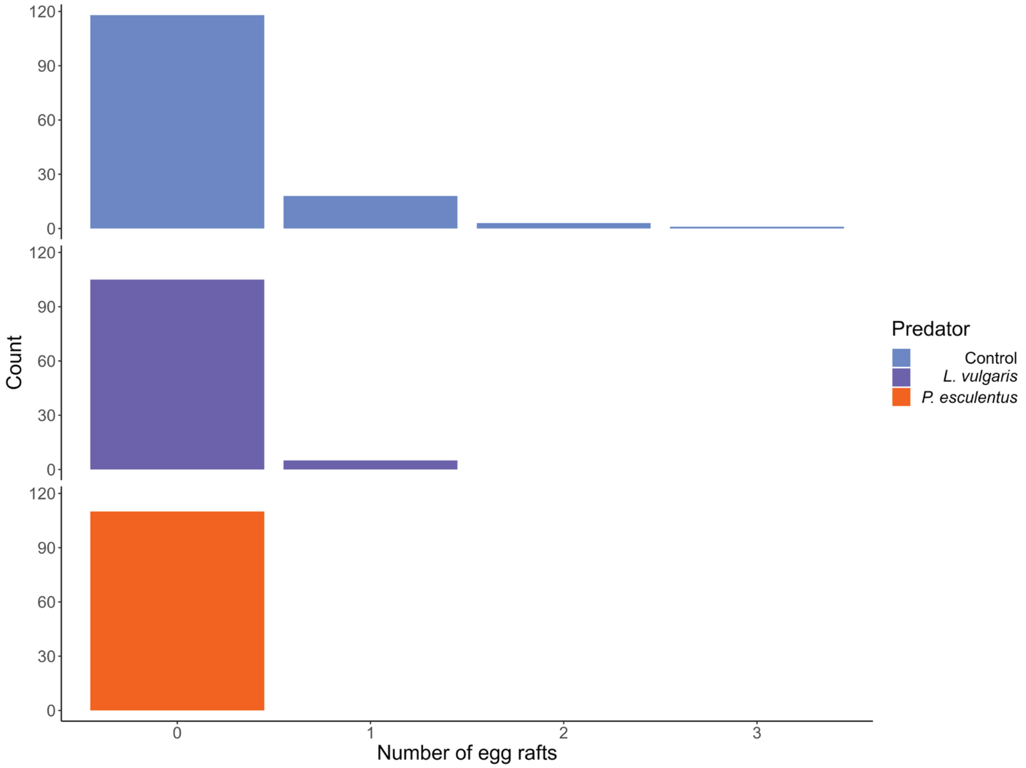

Figure S5.3 *Oviposition behavior depicted as the number of egg-rafts counted per day per predator treatment over the two weeks.*

Table S5.1 *Anova table experiment 1*

	Estimate	Std. Error	t value	Pr(>|t|)	Signif
(Intercept)	77.33	7.18	10.770	3.79e-05	***
PredatorAgabus bipustulatus	-50.00	10.15	-4.924	0.00265	**
PredatorNotonecta glauca	-60.00	10.15	-5.909	0.00105	**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

***Table S5.2** Anova table experiment 2*

	Estimate	Std. Error	t value	Pr(>|t|)	Signif
(Intercept)	-4.27542	13.15893	-0.325	0.7488	
Control + extra eutrophic	-1.62077	2.07676	-0.780	0.4448	
Smooth newt + eutrophic	-26.25201	1.72704	-15.201	4.36e-12	***
Smooth newt + extra eutrophic	-28.55619	1.73145	-16.493	1.03e-12	***
Eutrophication	0.11022	0.04109	2.682	0.0147	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table S5.3 *Anova table experiment 3 round 1*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Signif
Time	10	47.07	4.707	200.948	< 2e-16	***
Temperature	1	0.19	0.194	8.279	0.00434	**
Predator:Time	20	2.46	0.123	5.253	4.15e-11	***
Residuals	266	6.23	0.023			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table S5.4 *Anova table experiment 3 round 2*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Signif
Time	8	10.702	1.3377	147.777	< 2e-16	***
Temperature	1	0.006	0.0059	0.647	0.425	
Predator:-Time	16	1.300	0.0813	8.977	1.8e-09	***
Residuals	47	0.425	0.0091			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table S5.5 *Ovipositioning counts depicted as egg-rafts per day per predator treatment.*

	Predator		
Rafts	control	frog	newt
0	118	110	105
1	18	0	5
2	3	0	0
3	1	0	0

Table S5.6 *Calculations average weight per predator species and corresponding maximum kill rates per gram of predator*

Species	Length (mm)	Weight (mg)	kill rate/ gram	Reference length	Reference weight	Formula
A. bipustulatus	6.5-7.5	1.90-2.71	2215	(Ohba & Takagi, 2010)	(Smock, 1980)	0.019*L^2.46
N. glauca	15-16	14.86-17.42	747	(Reynaldi et al., 2011)	(Smock, 1980)	0.019*L^2.47
L. vulgaris	66.1-81.8	7070.97-13611.03	3	(Bozkurt et al., 2016)	(Santini et al., 2018)	10^-4.375 * L^3.215
P. esculentus	35.3-48.6	22812.96-64797.45	1	(Socha & Ogielska, 2010)	(Santini et al., 2018)	10^-4.744 * L^3.073

References accompanying Table S5.6

Bozkurt, E., Tural, M., Ulutaş, G., Üzüm, N., & Olgun, K. (2016). Two New Paedomorphic Population Records of the Smooth Newt, *Lissotriton vulgaris* schmidtleri (Raxworthy, 1988) (Urodela, Salamandridae), from Western Turkey. *Russian Journal of Herpetology*, *23*(2), Article 2. https://doi.org/10.30906/1026-2296-2016-23-2-158-162

Ohba, S.-Y., & Takagi, M. (2010). Predatory Ability of Adult Diving Beetles on the Japanese Encephalitis Vector *Culex* tritaeniorhynchus. *Journal of the American Mosquito Control Association*, *26*(1), 32–36. https://doi.org/10.2987/09-5946.1

Reynaldi, S., Meiser, M., & Liess, M. (2011). Effects of the pyrethroid fenvalerate on the alarm response and on the vulnerability of the mosquito larva *Culex pipiens molestus* to the predator *Notonecta glauca*. *Aquatic Toxicology*, *104*(1), 56–60. https://doi.org/10.1016/j.aquatox.2011.03.017

Santini, L., Benítez-López, A., Ficetola, G. F., & Huijbregts, M. A. J. (2018). Length–mass allometries in amphibians. *Integrative Zoology*, *13*(1), 36–45. https://doi.org/10.1111/1749-4877.12268

Smock, L. A. (1980). Relationships between body size and biomass of aquatic insects. *Freshwater Biology*, *10*(4), 375–383. https://doi.org/10.1111/j.1365-2427.1980.tb01211.x

Socha, M., & Ogielska, M. (2010). Age structure, size and growth rate of water frogs from central European natural Pelophylax ridibundus-Pelophylax esculentus mixed populations estimated by skeletochronology. *Amphibia-Reptilia*, *31*(2), 239–250. https://doi.org/10.1163/156853810791069119