EFFECTS OF EXPOSURE TO EURASIAN MILFOIL (*MYRIOPHYLLUM SPICATUM*) ON THE GROWTH AND DEVELOPMENT OF *XENOPUS LAEVIS* AND THE COLUMBIA SPOTTED FROG (*RANA LUTRIVENTRIS*)

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of Kimberly L.P. King find it satisfactory and recommend that it be accepted.

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Chair

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Dedication

This thesis is dedicated to Finnegan and Nicholas King

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Abstract

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Chair: Paul A. Verrell

Concern is growing over the consequences of an ever-increasing number of alien plant invasions. It is commonly assumed that the presence of alien plants has negative impacts at the ecosystem level, although quantitative data to support such an assumption largely are lacking. Eurasian Milfoil (*Myriophyllum spicatum*) is one of the most widely distributed of alien aquatic plants in the United States; it is particularly prolific in freshwater aquatic habitats of the Northwest, interfering with wildlife and degrading water quality. Recent research on the negative impact of other alien plant species on the development and survival of larval amphibians led us to hypothesize that Eurasian milfoil may exert similar effects. To test this hypothesis, we conducted exposure trials with *Xenopus laevis*, the model amphibian for laboratory studies of environmental impacts. Recognizing that *Xenopus* has no ecological significance in the Northwest, we repeated our work, albeit at a reduced scale, with the Columbia spotted frog (*Rana luteiventris*), a native species suggested to co-occur with Eurasian Milfoil. Larvae of both species were exposed to a range of ecologically relevant densities of Eurasian

milfoil and the Northwest native, whorled milfoil (*Myriophyllum verticillatum*), in the laboratory. Exposure to milfoil resulted in compromised survivorship and an increased frequency of morphological abnormalities. In addition, exposed individuals exhibited changes in head width growth pattern.

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CHAPTER ONE

INTRODUCTION

The Pacific Northwest has one of the most diverse herpetofaunas in North America. Recent declines in western amphibian populations may be the result of habitat alteration, destruction, and/or introduction of exotic species (Monello & Wright 1999). Concern is growing over the consequences of an ever-increasing number of alien plant invasions. It is commonly assumed that the presence of alien plants has negative impacts at the ecosystem level, but quantitative data to support such an assumption largely are lacking. Eurasian milfoil (*Myriophyllum spicatum*) is one of the most widely distributed alien aquatic plants in the United States. Since its introduction in the 1940s, it has aggressively spread to 44 U.S. states and three Canadian provinces (Creed 2000; Balci & Kennedy 2003). Eurasian milfoil is of great concern in the northwestern United States because it interferes with wildlife, often causing a reduction in native species diversity, and degrades water quality (Ervin & Wetzel 2003; Cornelissen *et al.* 2004; Brown *et al.* 2006). There are several mechanistic hypotheses currently being investigated in order to determine the cause for its damaging effects. The most popular of which concerns its high tannin concentration (Cheruvelil *et al.* 2001). Secondary compounds in Eurasian milfoil leaves may affect the productivity or composition of tadpole food resources or act directly on the tadpoles themselves (Maerz *et al.* 2005). Studies investigating the effects of macroinvertebrate exposure to Eurasian milfoil tannin extracts showed significant increases in mortality rates (Cheruvelil *et al.* 2001). These findings along with previous research on the effects of Eurasian milfoil exposure to a variety of aquatic species (Table 1), as well as our own pilot studies (King

and Verrell, unpublished data), led us to hypothesize that Eurasian milfoil may have adverse effects on larval amphibians.

Macrophyte invasions, like that of Eurasian milfoil, are transforming once healthy ecosystems of diverse vegetation into less productive monocultures. Surveys have shown that with Eurasian milfoil invasion comes a decrease in number and biomass of native macrophytes (Creed 2000; Ali & Soltan 2006). Other studies have shown a significant reduction in macroinvertebrate biomass (Keast 1984; Smith & Barko 1990; Cheruvelil *et al.* 2001). Dense milfoil beds can also cover fish spawning sites and disrupt natural food chain dynamics by excluding predators from highly vegetated areas (Creed 2000).

We predict that the invasion of Eurasian milfoil was impacting native amphibian populations, although currently it is unclear what the specific effects are and what threats they pose. Changes in amphibian prey and predator composition, light regimes, and soil and water chemistry each have the potential to exacerbate the already precarious state of many Northwest amphibian populations.

We determined the first logical investigative step is to examine the direct effects of Eurasian milfoil on larval amphibian survival and development. The purpose of our study was to determine whether exposure to a range of ecologically relevant densities of Eurasian milfoil would (1) compromise survival and/or development to a greater degree than exposure to native milfoil and a water control; (2) compromise survival and/or development to a greater extent at higher than lower milfoil densities; and (3), compromise survival and/or development to a greater extent in longer rather than shorter durations of exposure.

Experimental organisms

Eurasian milfoil

Eurasian milfoil is a highly competitive (Smith & Barko 1990) submersed herbaceous perennial (Madsen 1998; Balci & Kennedy 2003) capable of photosynthesis at temperatures as low as 10° C (Smith & Barko 1990; Jacono & Richerson 2003). This species develops a dense canopy in early spring that can exclude competitors when inter- and intra-specific competition is possibly strongest. Eurasian milfoil also produces phenolic compounds that can account for up to 300mg · g^{-1} of its dry matter (Leu *et al.* 2002). These compounds are effective at reducing the biomass of competing submerged macrophytes (Pip 1992; Leu *et al.* 2002; Hilt *et al.* 2006), cyanobacteria and heterotrophic bacteria (Gross 2003), green algae and duck weed, as well as insect herbivores, mosquitoes, and midges (Glomski *et al.* 2002).

Whorled milfoil

We chose whorled milfoil (*Myriophyllum verticillatum*), a less thoroughly investigated native species, which to compare the alien species because of their habitat overlap and shared production of phenolic compounds (Gross 2000; Hilt *et al.* 2006). It emerges in late spring, reaching the water's surface by early summer where it thrives in shallow, still waters. In addition, it is capable of producing phenolic compounds at concentrations of 123 mg · g^{-1} dry matter (Gross 1996; Leu *et al.* 2002; Hilt *et al.* 2006), approximately half that of the alien milfoil.

Xenopus laevis

We choose *Xenopus* as our first study species as it is the model amphibian taxon of laboratory studies involving the effects of environmental change (Burkhart & Gardner 1997). *Xenopus* tadpoles are obligate suspension feeders (Seale 1982), a foraging mode also employed by several native amphibian larvae. In addition, the large size of these tadpoles, their rapid rate of development, and their translucent bodies make them an excellent animal for analyzing early vertebrate growth and development (Sive *et al.* 2000).

Columbia spotted frog

We studied the Columbia spotted frog (*Rana luteiventris*), a native amphibian, for three reasons: it sometimes resides in wetlands where Eurasian milfoil is present, it potentially uses Eurasian milfoil canopy mats as oviposition sites, and tadpoles may forage on milfoil (Pearl *et al.* 2005; Pearl *et al.* 2007). This species, which is quickly gaining conservation attention (Welch & MacMahon 2005), breeds in ponds or lakes with low water-flow rates and midday summer water temperatures ranging from 6 - 24 ºC (Reaser 2000). Breeding occurs in mid-April to early June (Davis & Verrell 2005), oviposition occurs in areas with >75% vegetative cover (Pearl *et al.* 2007), and hatched larvae feed on nearby vegetation (Welch & MacMahon 2005).

METHODS

Species Collection/Housing

Both Eurasian and whorled milfoil stock plants were collected from the public access dock of Silver Lake, Spokane County, Washington, in May of 2007. The plants were transported from Silver Lake to Washington State University in coolers filled with lake water. They were

then separated, rinsed, and placed into buckets containing aged tap water and local soil, and maintained at 20 ºC under a 12:12 L:D cycle.

Xenopus embryos were obtained from *Xenopus* I (http://www.Xenopusone.com). Embryos hatched within 24 hours of arrival and were immediately placed into either an experiment or aquarium containing aged, treated, and aerated tap water to await later use. *Xenopus* were feed powdered Purina Rabbit Chow *ad libitum*.

Columbia spotted frog eggs were collected from Pond 9, Moscow Mountain, Latah County, Idaho, in May of 2007. Eggs were transported in coolers filled with pond water to Washington State University. Eggs were kept in the transportation cooler for 24 hours before being placed in aquaria containing aged, treated, and aerated tap water allowing for acclimation. Eggs and tadpoles were maintained in aquaria awaiting exposure treatments at 18 ºC under a 12:12 L:D cycle. Larvae were fed lettuce leaves *ad libitum*.

Experiments

Exposures

Data provided by the Coeur d'Alene Tribe Ecology Department from a two-year aquatic vegetation survey in 2004 and 2005 enabled us to estimate mean local milfoil density. The lakes surveyed are commonly referred to as Chatcolet, Round and Benewah, and the estimated mean local density in those lakes was 0.52 g wet milfoil /200ml water.

Xenopus tadpoles were exposed to 25, 50, 75, 100, 125, 150, 175, or 200 percent of estimated local mean milfoil density (Fig. 1). Each of the eight densities within a treatment exposure to either Eurasian or whorled milfoil consisted of 20 larvae; the water control consisted of 60 larvae.

Because we possessed a limited number of Columbia spotted frogs, our exposures with this species were limited to 50, 100 and 150 percent of the estimated local mean milfoil density. Each of the three densities within each milfoil treatment consisted of 15 larvae; the control treatment consisted of 19 larvae.

Each tadpole was haphazardly assigned to a 200ml cup, which represented a treatment of exposure to native or alien milfoil, or a tap water control. Milfoil samples were obtained from stock plants collected in May of 2007 and maintained in the Washington State University School of Biological Sciences. Scissors were used to cut the upper two-thirds of each milfoil stalk into pieces approximately 20 mm in length. Cut pieces were then pooled into a container from which they were haphazardly pulled and weighed to within +/- 0.02 grams of the prescribed treatment mass. Weighed milfoil was then agitated using the convex side of a spoon and placed into the appropriate treatment cup. Both prepared treatment and control cups were allowed to sit for 24 hours before tadpoles were added. Every three days, cups were topped off with treated tap water in order to maintain them at a volume of 200 ml and food was provided *ad libitum*.

Measurements

Xenopus cups were visually inspected after 1, 5, 10, 15 and 20 days for survival, and on days 10, 15 and 20 for the occurrence of malformations by viewing each tadpole under 10x magnification using a dissecting microscope. Malformations observed consisted of bent axis of the tail; enlarged and/or flattened head; and inverted development of the opercular fold (see fig. 2).

Head width also was measured to provide a nonlethal measurement of linear body size. Measurements were made by placing each tadpole under 10x magnification, and recording the

width of the head directly behind the eyes to the nearest 1mm (Fig. 3a, 3b). This degree of precision was the smallest at which we could obtain the highest degree of consistent measurement, as larvae move often and rapidly. Measurements were recorded on day 15 for *Xenopus* in 1-20 day exposures, on day 20 for *Xenopus* in 10-20 day exposures, and on day 20 for Columbia spotted frogs in 15-30 day exposures.

Statistical analysis

We employed a GENMOD model, using a Pearson Chi-Square test to test for differences of the effect of milfoil density on survival and malformations within treatments (Eurasian milfoil- whorled milfoil- water control). We found no differences in occurrence of malformations and/or survival within treatment groups by milfoil density (see below); therefore, all data within treatments were pooled for subsequent analyses. We employed a Pearson chi-square test to detect differences in survival and malformations among treatment groups and controls. We used the Tukey Method for all pairwise comparisons to analyze differences in head width among treatment groups and controls.

RESULTS

Experiment 1: *Xenopus laevis*

Effect of Treatment Density on Survivorship and Malformations

As previously stated, we found no significant effect of treatment density on survivorship in exposures days 1-20 (χ^2 = 21.6751, df = 173, P = 0.1253), days 1-10 χ^2 =15.3395, df = 173, P = 0.0887)or 10-20. χ^2 = 54.0481, df = 358, P = 0.1510). In addition, we found no significant effect of treatment density on the occurrence of malformations in days 1-20 (χ^2 = 17.8033, df = 358,

P= .0597), 1-10 (χ^2 = 30.597, df = 345, P = 0.0887) or 10-20 (χ^2 = 7.6684, df = 173, P= 0.0543). We then pooled the data among treatment for all subsequent analysis.

Exposure Days 1-20

We found a decrease in survivorship when larvae were exposed to either Eurasian (χ^2 = 505.40, df = 1, $P < 0.0001$) or whorled (χ^2 = 494.20, df = 1, $P < 0.0001$) milfoil compared to a water control, although there was no difference between the two milfoil treatment groups (χ^2 = 0.15, df = 1, P = 0.6952) (Fig. 4). There was a significant increase in the number of larvae presenting with malformations in exposures to both Eurasian (χ^2 = 1148.7 , df = 1, $P < 0.0001$) and whorled (χ^2 = 1111.8, df = 1, $P < 0.0001$) milfoil compared to a water control. However, there were no differences between milfoil treatments (χ^2 = 0.74, df = 1, P = 0.3898) (Fig. 5). After finding an overall significant effect of treatment group on head width ($F_{2, 356}$ = 9.37, $P < 0.001$, Fig. 6a), we continued with pairwise comparisons. We found that, although there was no difference between the milfoil treatments ($F_{2, 356}$ = 9.37, $P < 0.0890$), there were differences between both Eurasian ($F_{2, 356}$ = 9.37, P = 0.0191) and whorled ($F_{2, 356}$ = 9.37, $P < 0.0001$) milfoil exposures when compared to a water control.

Exposure Days 1-10

Exposure to either Eurasian (χ^2 = 906.92, df = 1, $P < 0.0001$) or whorled (χ^2 = 916.92, df = 1, $P < 0.0001$) milfoil compromised survival relative to a water control. However, there were no differences in survival between milfoil treatments (χ^2 = 0.07, df = 1, $P < 0.7976$) (Fig.4). Frequency of malformations was greater with exposure to either Eurasian (χ^2 = 10.21, df = 1, P = 0.0014) or whorled (χ^2 = 10.21, df = 1, P = 0.0014) milfoil relative to the control. However,

there were no differences between the two milfoil treatments (χ^2 = 0.00, df = 1, P < 1.0000). Head width measurements were not made during this exposure period.

Exposure Days 10-20

Exposure to either Eurasian (χ^2 = 204.83, df = 1, P < 0.0001) or whorled (χ^2 = 239.33, df = 1, P < 0.0001) milfoil decreased survivorship relative to a water control. Similar to our results from days 1-10, we detected no significant differences in survival between the two milfoil treatments (χ^2 = 3.25, df = 1, P < 0.0716); (Fig. 4). We found the number of frogs exhibiting malformations to be greater with exposure to either Eurasian (χ^2 = 407.30 , df = 1, P < 0.0001) or whorled milfoil (χ^2 = 398.60, df = 1, P < 0.0001) relative to a water control. However, there was no difference between the two milfoil treatments (χ^2 = 0.11, df = 1, P < 0.7355); (Fig. 5). In addition, as we detected an overall effect of treatment group on head width ($F_{2, 167}$ = 8.04, P < 0.0005, Fig. 6b) and therefore we continued with analysis of pairwise comparisons. We found significant differences between Eurasian ($F_{2, 167}$ = 8.04, P < 0.0049) and whorled ($F_{2, 167}$ = 9.37, P < 0.0890) milfoil relative to a water control treatment although, no difference was found when comparing the two milfoil treatments ($F_{2, 167}$ = 9.37, P < 0.0890).

Experiment 2: Columbia spotted frog

Effect of Treatment Density on Survivorship

Similar to the analysis of *Xenopus* we found no significant effect of treatment density on survivorship (χ^2 = 22.2923, df = 102, P = 0.2186) and therefore the data was pooled within treatment.

Exposure Days 15-30

We determined that exposure to either Eurasian (χ^2 = 70.43, df = 1, $P <$ 0.0001) or whorled (χ^2 = 61.77, df = 1, $P <$ 0.0003) milfoil resulted in decreased survivorship relative to a water control (Fig. 7). There were no differences in survivorship between the two milfoil treatments (χ^2 = 0.63, df = 1, $P <$ 0.4284). No malformations were observed during this exposure period. We found no significant difference in head width measurements between individuals as an effect of treatment ($F_{2, 106}$ = 0.10, P = 0.9012).

DISCUSSION

We tested the hypothesis that exposure of amphibian larvae to an alien aquatic plant compromises important components of individual fitness: survivorship, developmental integrity and growth. We found that larvae of two frog species exposed to milfoil, alien or native to the northwestern U.S., experienced a reduction in survivorship, an increased occurrence of external anatomical malformations and, in one, changes to body size, relative to water controls. Contrary to our hypothesis, we found few differences in the magnitude of effects between the two milfoil species, although we did find numerous differences between the two milfoil species relative to water controls. In addition, and again contrary to our hypothesis, the magnitude of effects was not dependent on the density of plant material present. Given the ever-increasing number of threats amphibians are facing today, even seemingly minor changes to survivorship, development and/or growth could compromise further the ability of a population to persist (likely most true for small and/or geographically isolated populations).

We conclude that milfoil's effects on survivorship, development and growth are a result of its presence or absence during larval development. Despite our expectation, higher densities of milfoil did not result in effects of greater magnitude, i.e., effects were not dose-dependent. Because previous studies suggest that high tannin concentrations are the primary cause of mortality for aquatic species, we determined tannin concentrations in both Eurasian and native milfoil in preliminary work (details not presented here). We found that our Eurasian milfoil stock contained tannin concentrations nearly one-and-a-half times greater than that of our whorled milfoil stock: 41 mg BSA ppt/gm dry plant matter versus 26 mg BSA ppt/gm dry plant matter. Despite this pronounced difference in tannin concentrations between milfoil species, we found no effect of species treatment on larval survival, development and growth. Although tannins may be a factor in inducing mortality (Gross 1996; Leu *et al.* 2002; Ervin & Wetzel 2003), we suggest that they are not the primary agent and that their effects are not dose-dependent.

Increased frequencies of deformed amphibians found in nature has led to much research on how exposure to chemical contaminants, ultraviolet radiation, parasites and predators may affect anatomical development (Blaustein & Johnson 2003). Our data indicate that exposure to plants also may induce anatomical abnormalities in larval frogs. Although the number of individuals affected was small, only in larvae exposed to milfoil, Eurasian or native, did we observe disturbed development; no individuals in the water control developed malformations. Furthermore, our data suggest that even relatively brief exposures (as short as 10 days in duration) may be sufficient to elicit effects on development. Also, we found that malformations only arose if exposure to milfoil occurred 10 days or later after hatching. It may be that there exists a critical stage of development, or sensitive period, during which larvae are most susceptible. Further research is needed to test between this and a more trivial alternative

explanation – that gross malformations simply are easier to detect visually in larger larvae of 10 days or older post-hatch.

Previous studies have demonstrated that exposure of young amphibians to environmental toxicants can compromise locomotor ability even in the absence of gross malformations (Carey & Bryant 1995; Verrell 2000; Ingermann *et al.* 2002). However, it seems likely that such behavioral effects may be exacerbated if accompanied by malformations, even though the latter may not be directly lethal to larvae. Mortality during the larval stage of life can be considerable under natural conditions, and so anything that adds to this to decrease the recruitment of individuals into the breeding pool could impact population persistence. From a conservation perspective, it matters little whether individuals die because of direct poisoning or through indirect effects on behavioral components of fitness, such as predator avoidance capabilities (Johnson *et al.* 2001). Malformations of the tail or body clearly may affect spontaneous activity, responsiveness, endurance and sprint speed (Hopkins *et al.* 2000; Johnson *et al.* 2001), all of which are important components of predator avoidance tactics. In our study we observed a variety of malformations that could result in avoidance capability, such as bent tail axes. In addition, we found abnormal head shapes that likely increase drag and impair mobility; slowing an individual's swim speed and increasing its likelihood of predation (Blaustein & Johnson 2003).

We hypothesized that exposure to Eurasian milfoil alters normal patterns of larval growth; indeed, younger larval amphibians are most susceptible to external factors that may affect growth (Carey & Bryant 1995). However, we found that larval *Xenopus* exposed to either Eurasian or native milfoil developed wider heads relative to water controls. Given the strong correlations between head width and both total body length and body mass, we conclude that

exposure to milfoil accelerates the growth rate of larval *Xenopus*. Because body size impacts many aspects of fitness in amphibians (for example, survivorship, female fecundity and male mating success), changes in individual growth rates can have consequences for population growth, size and persistence (Werner 1986; Carey & Bryant 1995). It is commonly accepted that larger body size confers greater fitness on individuals, and in some species, an individual's body size at metamorphosis is positively related to its body size in adulthood (e.g., North American ranids: (Werner 1986). Larger individuals are more capable of overcoming periods of resource scarcity (Pechenik *et al.* 1998) and are also more likely to obtain reproductive success in adulthood (Snodgrass *et al.* 2004).

Thus, we must consider the possibility that, for *Xenopus*, exposure to milfoil results in enhanced growth rates which may be advantageous for individual survival and reproduction. If this is true, then it provides considerable motivation for further work to examine how such a positive influence may interact with negative effects on short-term survival and anatomical development. We must note here that we found no significant differences in head widths between Columbia spotted frog larvae exposed to milfoil versus water controls. Either our tadpoles were not sensitive during the exposure period we used, or head width is not a phenotypic trait affected in this species.

Our study draws attention to the important, although understudied, effects that macrophyte communities may exert on amphibians. Frogs use vegetation in shallow water for egg laying, refuge, and feeding, and so spend much of their aquatic phases in direct contact with macrophytes. Despite the scarcity of significant differences between milfoil species treatments, we hypothesize that the presence of Eurasian milfoil still may result in a greater number of detrimental effects in the wild relative to that of whorled milfoil. Eurasian milfoil shares many

habitat and physical attributes with its native relatives, but what defines Eurasian milfoil as a nuisance species is its apparent competitive superiority. When Eurasian milfoil is present in an aquatic habitat it develops a dense canopy and occupies a greater portion of water column compared to native milfoils; indeed it may come to monopolize a habitat both in terms of species richness and abundance (Valley & Newman 1998). Thus, Eurasian milfoil may be detrimental to an ecosystem (including larval frogs) not because it is especially toxic but because the probability that it will be encountered is greater than that for native milfoils (which typically are dispersed more patchily in aquatic habitats). It would seem possible that Eurasian milfoil and other alien plants are among the multiple stressors that are resulting in amphibian populations becoming ever more threatened and vulnerable to extinction.

Future Directions

We suggest that future work should proceed in two complementary directions, both involving a variety of amphibian species. It is now recognized that reactions to environmental perturbations can differ among anuran families (Relyea 2003; Drake *et al.* 2007).

First, the mechanistic basis of the effects of exposure to milfoil that we have found need to be addressed. For example, can the effects produced by exposure to whole plants be induced by exposure to extracted tannins only? If tannins exert negative effects, by what physiological mechanisms do they act? In addition, while we have evidence that exposure to milfoil may affect the development of certain organs and impact growth rate, we do not know if it impacts developmental rate. We must stress that, in larval amphibians, growth and developmental rates can be decoupled.

Second, the effects of exposure to milfoil should be addressed at a more complex and realistic level, allowing both direct and indirect effects to become revealed, through the use of mesocosm studies that mimic natural ecosystems (albeit simplified). For example, does milfoil affect the species richness and/or abundance of microphyte that are used by larval amphibians for food? Does milfoil alter water chemistry (hardness, pH, etc) in ways that may have negative impacts?

Management Implications

While laboratory experiments provide excellent opportunities for control and precise quantification, they are highly artificial relative to conditions in nature. Thus, caution is required in using laboratory data to develop guidelines for the management of natural habitats. A stronger case for such extrapolation could be made if our findings were replicated in mesocosm studies, as outlined above. Our data suggest that exposure to Eurasian milfoil may have negative effects on larval survival and anatomical development, but no or even positive effects on larval growth. However, these effects appear to be no or only a little greater than those observed when larvae are exposed to native milfoil. To the extent that amphibians may be more likely to encounter Eurasian milfoil due to its growth habits in aquatic habitats, then we suggest that attempts to control this alien should be continued.

Table 1. Effects of Eurasian milfoil exposure on a variety of aquatic species

Species	Observational/ Experimental	Field/ Laboratory	Results	Reference
Algae and Cyanobacteria	Information not provided	Lab	Phenolics interfere with alkaline phosphatase, an exoenzyme used to overcome inorganic phosphorus limitation	Gross et al., (1996) Gross, (1999); Gross, (2003)
Cyanobacteria, chlorophytes, and diatoms	Information not provided	Lab	Extracts and exudates from Eurasian milfoil inhibit photosynthesis, growth and the protein complex photosynthesis II (starch synthesis)	Gross, (2003); Leu et al., (2002)
Prickly Waternymph	Observational	Field	Shading by the taller Eurasian milfoil may be the cause of reduced growth	Ali, (2005)
Sediment-associated invertebrates	Observational	Field	Less abundant beneath Eurasian milfoil beds compared to stands of native macrophytes	Creed, (2000)
Macroinvertebrates	Observational	Field	As milfoil biomass increases macroinvertebrate biomass decreases	Cheruvelil, (2001)
Inshore Fish	Information not provided	Information not provided	Dense stands of Eurasian milfoil obstruct swimming space, shelter too many juvenile fishes, disrupts foraging movements and depletes dissolved oxygen inshore that can cause fish kills when shoots decay	Creed, (2000); Engle, (1995)

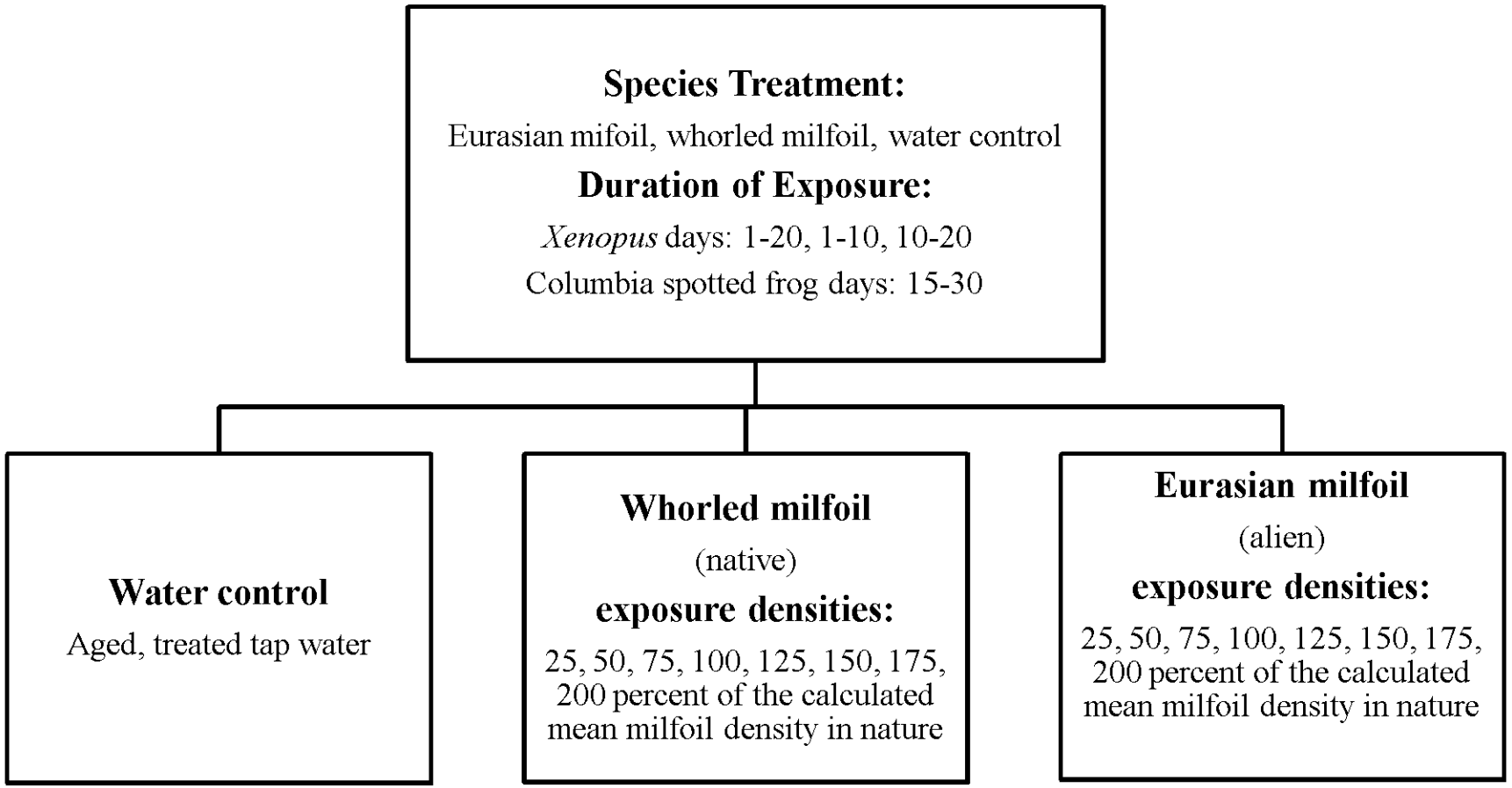

Figure 1. Experimental design for the treatment, duration and density of exposures for *Xenopus* and Columbia spotted frogs

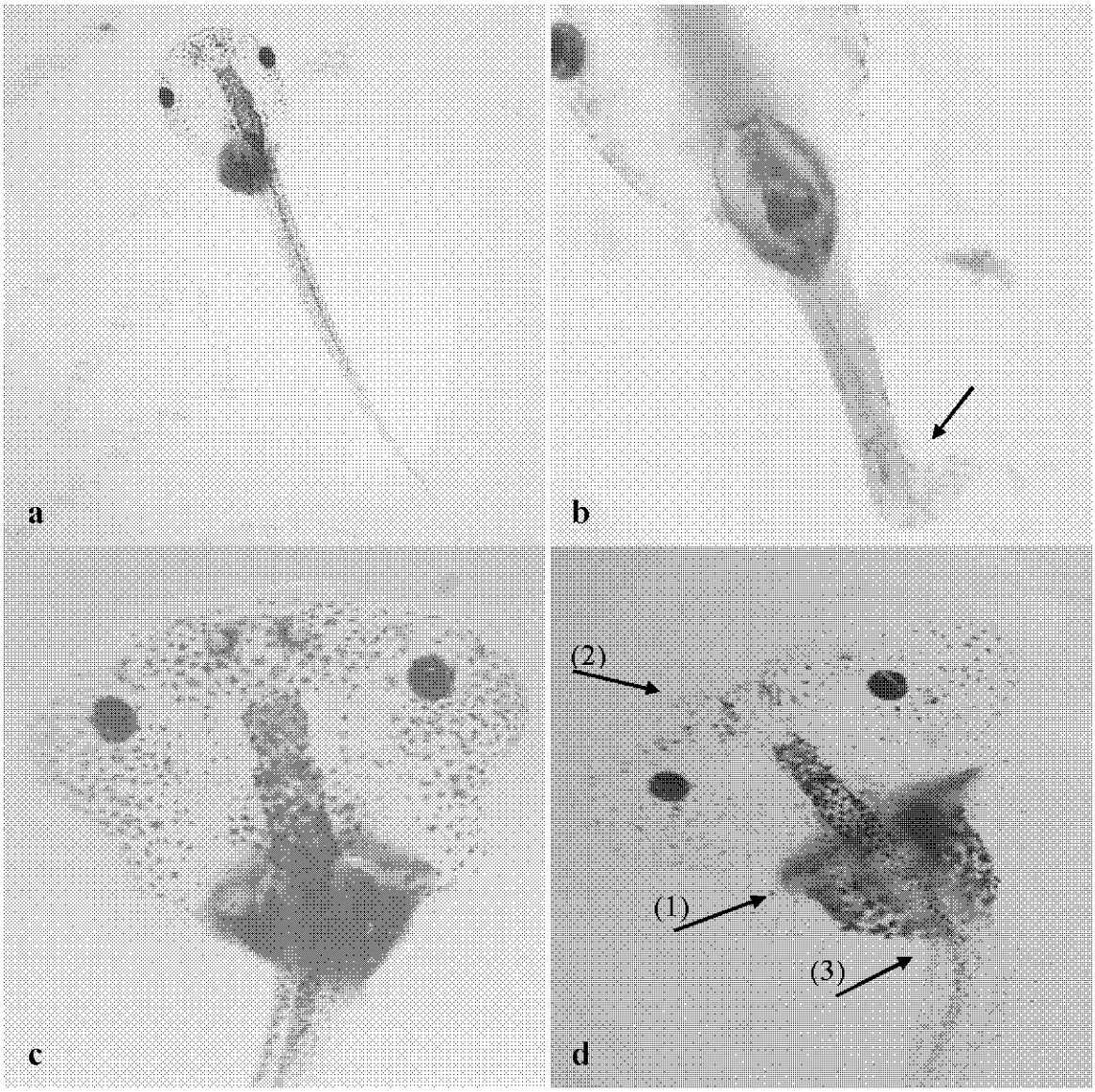

Figure 2. (a) Dorsal view of normally developed *Xenopus* larvae (b) Ventral view of *Xenopus* larvae with a bent axis (skeletal or muscle kinking) along the tail (arrow here) (c) Dorsal view of *Xenopus* larva with enlarged head and inverted opercular fold (d) Dorsal view of *Xenopus* larvae with an inverted opercular fold (arrow 1), and widened-flattened head (arrow 2) and bent tail axis (arrow 3)

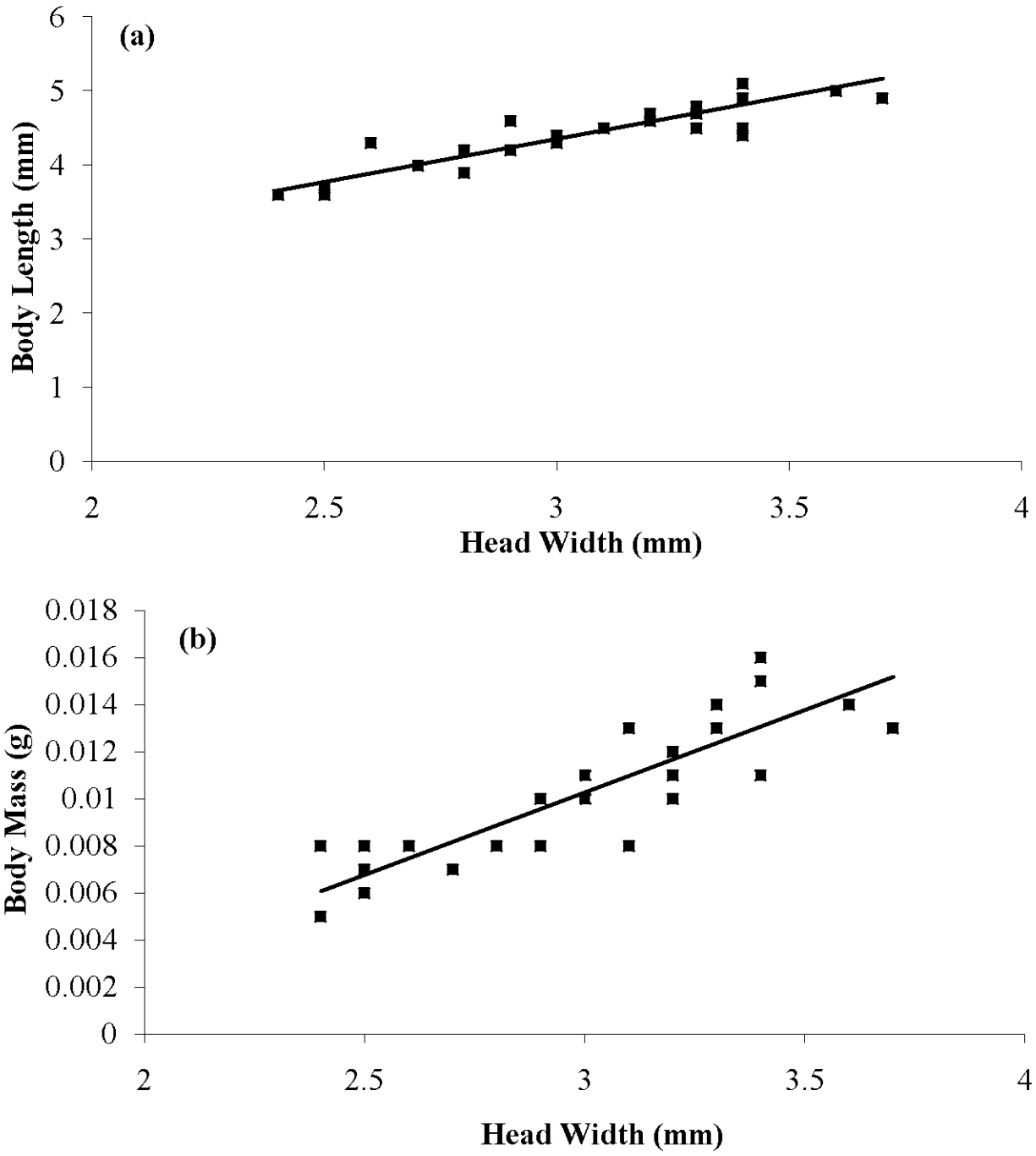

Figure 3. Linear relationship between (a) head width and body length (y= 1.15x + 0.892, R^2= 0.839) and (b) head width and body mass (y= 0.007x + 0.010, R^2= 0.758), for larvae of *Xenopus*.

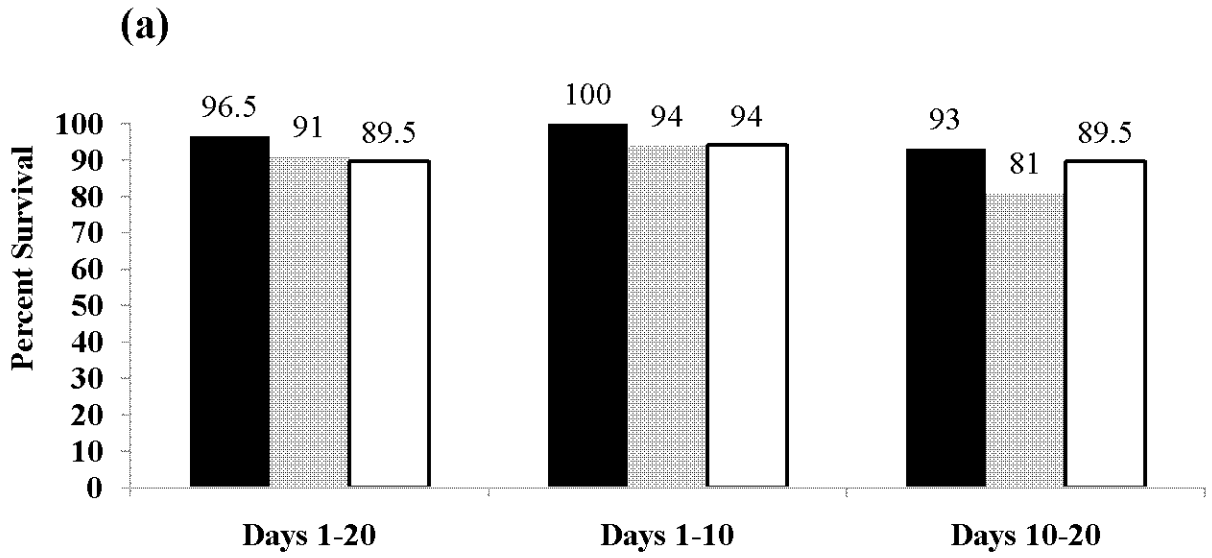

Figure 4a. Percent survival of *Xenopus* across exposure period (n = 380) further subdivided within treatment, water control (black bars), whorled milfoil (gray bars), Eurasian milfoil (white bars). Numbers above bars are percent survival.

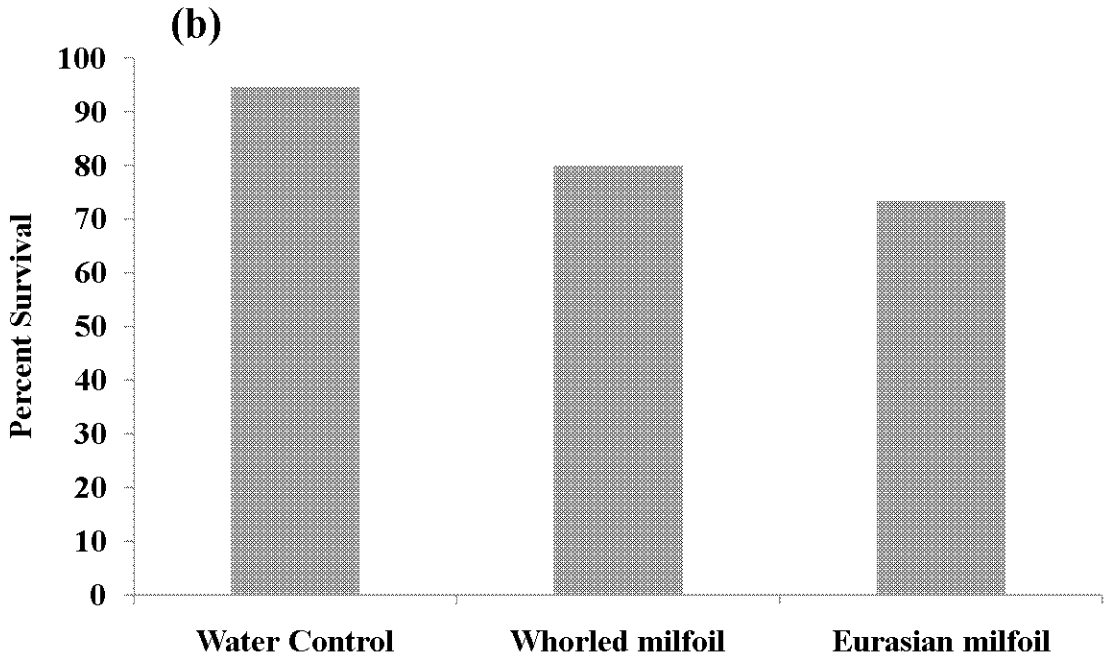

Figure 4b. Percent survival of Columbia spotted frog across treatment (n = 109).

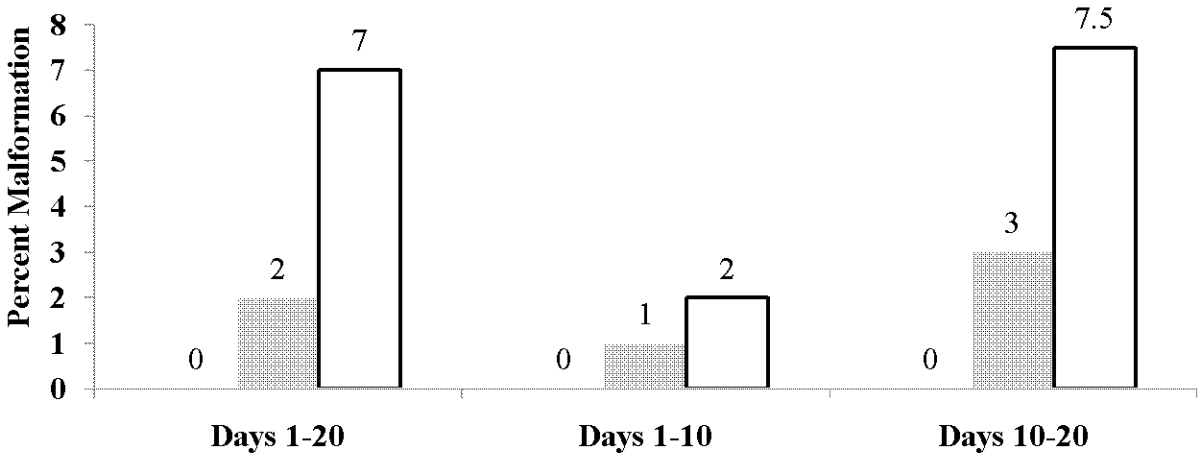

Figure 5. Percent *Xenopus* larvae exhibiting one or more malformations across treatment (n = 380) subdivided by treatment, water control (black bars), whorled milfoil (gray bars), and Eurasian milfoil (white bars). Numbers above bars are percent malformed.

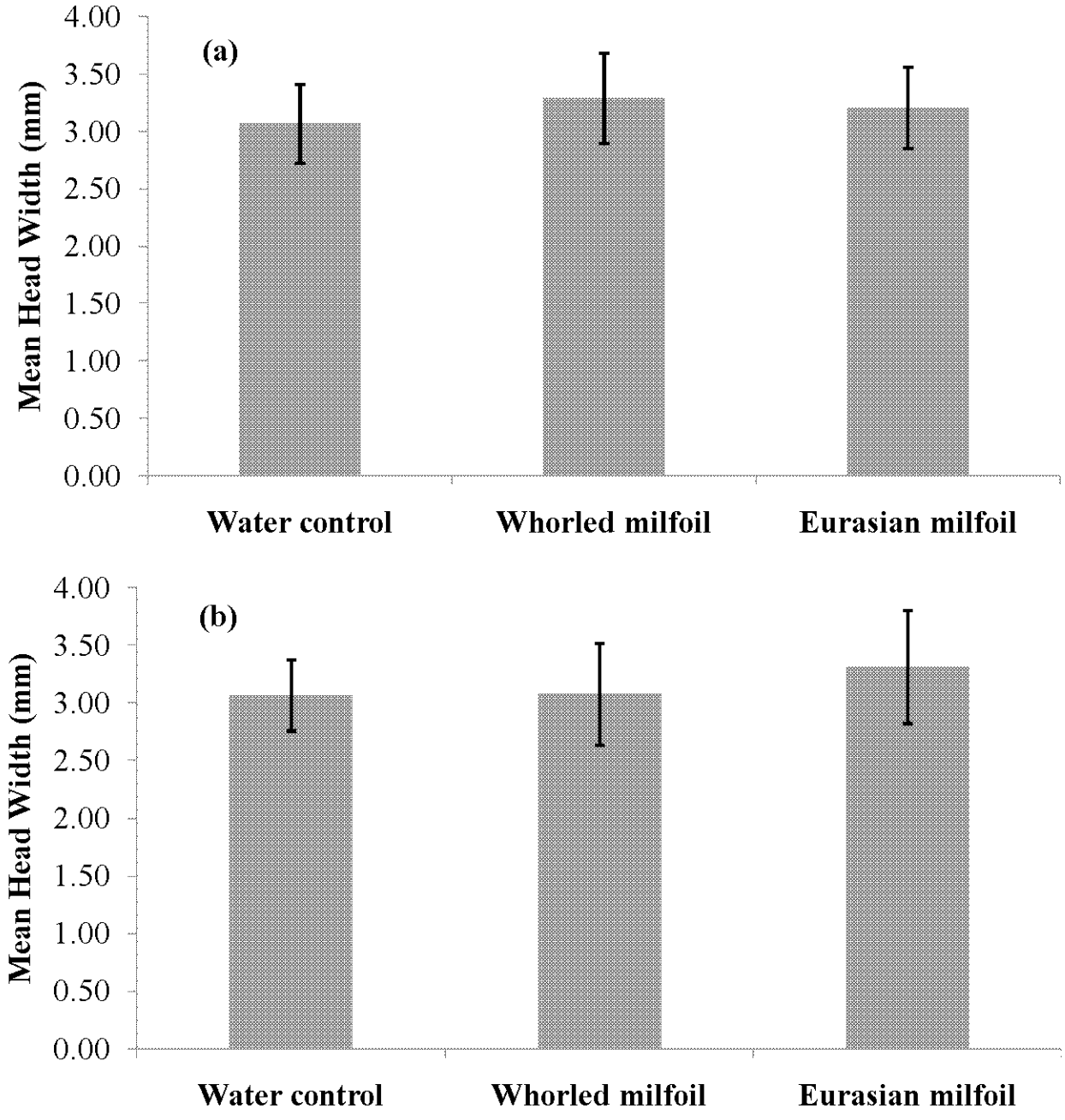

Figure 6. Mean head width of *Xenopus* in relation to treatment type (n= 380) (a) larval exposure days 1-20, and (b) larval exposure days 10-20. Bars denote standard deviation.

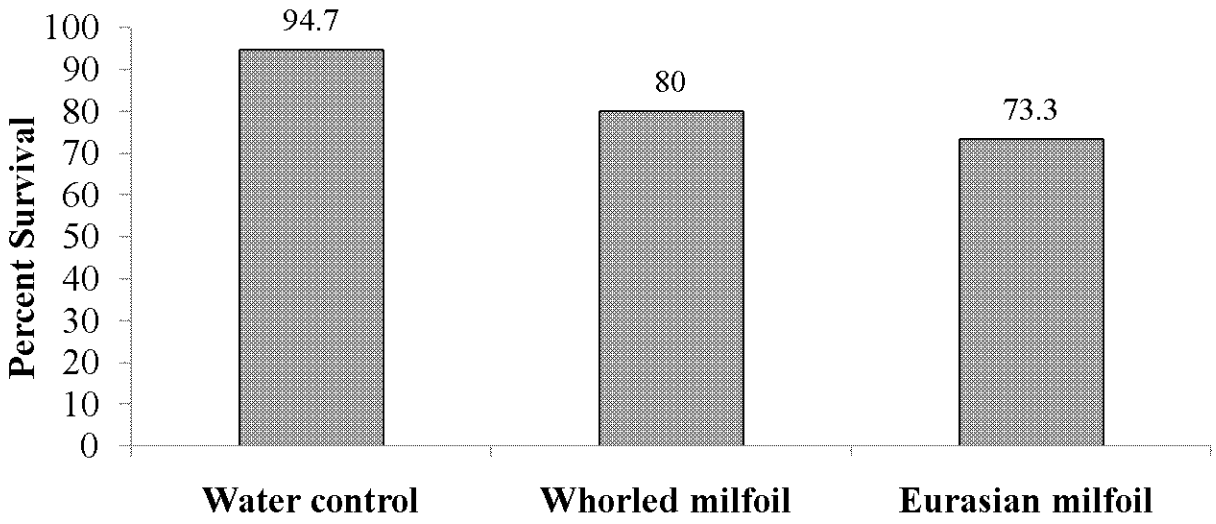

Figure 7. Percent survival during an exposure period of days 15-30 for Columbia spotted frog across treatment (n = 109 (total)).

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