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Effects of exposure to low, ecologically relevant doses of atrazine on somatic and gonadal development in American toad (Bufo americanus) tadpoles

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Effects of exposure to low, ecologically relevant doses of atrazine on somatic and gonadal development in American toad (*Bufo americanus*) tadpoles

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Abstract

Atrazine is the most commonly used herbicide in the United States, with 80 million pounds applied annually, making it the most common contaminant of ground and surface water nationwide. It has been shown to act as a potent endocrine disruptor in amphibians, causing altered somatic and gonadal development in the ecologically relevant part per billion range; as a result, it has been hypothesized that atrazine may be a major factor behind amphibian declines. However, responses of different species to the chemical vary widely, and have made predicting susceptibility difficult. Recently, it has been shown that life history can serve as a strong predictor of vulnerability, as the speed of somatic development and the timing of gonadal differentiation may determine the effects of exposure. However, previous studies leading to these conclusions have examined atrazine under stable laboratory conditions, although it is widely accepted that chemical contaminants can interact synergistically with natural stressors in the wild, producing exaggerated effects. To test whether more stressful conditions alter the effects of atrazine with respect to existing data, we raised *Bufo americanus* tadpoles under more stressful conditions, including a high larval density and simulated pond drying, and exposure to ecologically relevant doses of atrazine (0, 0.1, 1, 25 ppb). We measured markers of somatic development (mass, time, survival at metamorphosis) and gonadal differentiation (ovarian stage in females, presence of testicular oocytes in males). Our results do not suggest that stressful conditions worsen the effects of atrazine, as only mass at metamorphosis was affected by exposure. Our results are interesting, however, in that they support the hypothesis that atrazine displays a non-monotonic dose-response curve, with very low concentrations (0.1, 1 ppb) producing the most severe effects, an important implication for any conservation policy regarding the chemical.

Introduction

Biodiversity and Amphibian Declines

The global biodiversity crisis has received a great deal of attention in recent years due to the startling rate of extinctions over the past few centuries. In fact, the extinction rate over the past 1000 years is more than 10 times the background rate over the past 540 million, indicating that species are disappearing at rates that rival those of previous mass extinctions, such as the familiar event at the end of the Cretaceous period 65 million years ago, which led to the demise of the dinosaurs and an estimated 76% of extant species at the time (Baraonosky et al. 2011). Class Amphibia is one taxon that has experienced especially steep, global declines in recent years, with 1856 species (32.5% of all known species) listed as Vulnerable, Endangered, or Critically Endangered by the IUCN in 2001, compared to 1211 species (12%) of birds and 1130 species (23%) of mammals (Stuart et al. 2004). Thus, amphibians seem to be disappearing at a much more alarming rate than other vertebrates, an especially intriguing trend given that amphibians have persisted through the previous four periods of mass extinction (Wake and Vredenburg, 2008).

Despite the vast interest in amphibian declines since their elucidation in the late 1980's, research has failed to provide a unified explanation. For example, in examining the 435 species that are experiencing especially rapid declines (i.e. those that have been elevated to a higher threat level on the IUCN Red List than in 1980), 50 are declining because of overexploitation and 183 because of habitat loss, leaving 207 species categorized as facing "enigmatic declines," defined as those cases where sufficient habitat exists, but species are still declining (Stuart et al. 2004). Many recent studies have sought to shed light on the mechanisms underlying these

poorly understood cases, and it has become clear that there are a variety of factors aside from habitat loss and overexploitation, the most significant of which being global climate change, chemical contamination of the environment, disease and pathogens, and the presence of invasive species (Semlitsch, 2003). These are all factors with the ability to affect populations even in situations where sufficient habitat exists, which means that even animals living in what may traditionally be considered unaltered environments, such as large national parks and nature preserves, are susceptible to these threats.

Chemical Contamination

Among the factors behind these "enigmatic declines", few have received attention comparable to that which has been paid to the effects of chemical contamination. Rachel Carson's *Silent Spring* (1962) first raised public attention regarding the effects of chemical contaminants on wildlife by highlighting a great deal of anecdotal evidence of their effects, including dead birds scattered across lawns, reproductive failure among carnivorous birds and farm animals, and even disease in humans after exposure (Boone and Bridges, 2003). Thankfully, modern pesticides (which include herbicides and fungicides) are more short-lived and tend to bioaccumulate to a lesser degree than those applied in the mid 20th century, due to more extensive regulation under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), which was officially adopted in 1942 (Cowman and Manzanti, 2000). However, FIFRA has done little to curb the application of pesticides in the United States, with approximately 20,000 pesticides approved for release into the environment (Boone and Bridges, 2003).

While many of these chemicals are more benign than those which were the inspiration for Carson's novel, the toxicological tests currently required under FIFRA and related legislation

are, in many ways, insufficient with regard to their ability to predict effects on communities of non-target species (such as amphibians) in the wild. This is because most standard assays for the effects of pesticides on bystander species are accomplished through acute toxicity tests; non-target organisms of representative taxa are exposed to varying concentrations of the chemical in question, and an LC 50 (the lowest concentration required to kill 50% of the sample) is determined. While this methodology presumably identifies species that are vulnerable to direct mortality as a result of a contaminant, it is not typical for amphibians to experience direct mortality in the wild, as concentrations of pesticides rarely reach levels necessary to produce this effect (Bridges and Semlitsch, 2001). Rather, there is a growing body of evidence indicating that ecologically relevant doses of chemicals, far below the thresholds determined by standard toxicity screening, have the ability to drastically affect amphibian populations due to sublethal effects which may indirectly lead to mortality (Bridges, 1997).

Sublethal effects can be either direct or indirect with respect to the amphibian in question, and vary widely depending on the species. Direct effects refer to instances in which a chemical directly alters the physiology of the organism, and are widely observed in amphibians. For example, organophosphate and carbamate pesticides are neurotoxins which inhibit acetylcholinesterase (AChE) activity in amphibians. Although not lethal at low, ecologically relevant concentrations, these low doses can impair motor activity and produce a variety of detrimental effects. Carbaryl, a common insecticide sold under the trade name Sevin, has been widely studied and can serve as a model to exhibit direct and indirect effects of sublethal contaminants. First, carbaryl has been shown to reduce the swimming capacity of tadpoles, a direct effect (Bridges, 1999a). This decreased activity may then lead to lead to non-adaptive predator avoidance behaviors (Bridges 1999a, 1999b), and decreased feeding behavior, which

may lead to smaller metamorphs, which are likely to exhibit decreased survivorship (Wilbur and Collins, 1973) and also decreased egg production in females, as size at metamorphosis and egg production are positively correlated (Semlitsch et al. 1988). Indirect effects are also common, and refer to instances in which a pesticide alters the food web in a given community. Furthermore, they can be especially relevant in natural settings. While the aforementioned carbaryl studies demonstrated negative effects such as decreased size, time, and survivorship to metamorphosis, still others have revealed the exact opposite (Boone et al., 2001; Boone and Semlitsch, 2002). In these scenarios, carbaryl is hypothesized to have negatively affected zooplankton populations, which in turn led to an increased algal population, allowing tadpoles to feed more effectively. Thus, it is clear that sublethal contaminants have the ability to drastically alter amphibian communities through both direct and indirect mechanisms, and with varying outcomes depending on not only species, but also on the structure of the entire community.

In understanding how sublethal effects can lead to population and community level disruption, it is also of critical importance to understand that organisms do not encounter contaminants in a vacuum. Rather, pesticides are an added stressor, which interact with many other factors to produce their effects. These interactions are often synergistic, with the combined stress of several components far outweighing the effects of any component in particular. Stressors that have been shown to interact with chemical contaminants to produce synergistic effects include predator-induced stress (Relyea and Mills, 2001; Relyea, 2003), hydroperiod (Boone and Semlitsch, 2002), density and intraspecific competition (Boone and Semlitsch, 2001), and additional chemical contaminants (Boone and James, 2003). Regardless of the pesticide in question, it is critical to recognize that any ill effects noted in laboratory

exposures are added stressors, with the ability to produce synergistic effects when coupled with other stressors in the environment.

Atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is one of the most broadly utilized pesticides in the world. It is primarily applied to corn, but also to sorghum and sugarcane in order to control both pre- and post-emergent, annual broad-leaf weeds and grasses. In the United States alone, more than 80 billion pounds are applied annually, making it the most common pesticide contaminant of ground, surface, and drinking water (Soloman et al., 1996). Atrazine also has the ability to travel over 1000 km from the site of application via rainfall, giving it the potential to contaminate wetlands, even in otherwise remote or pristine areas where it is not used (Thurman and Cromwell, 2000; Mast et al., 2007). Over 20 million pounds of atrazine are precipitated in rainfall each year in the United States (Thurman and Cromwell, 2000). As a result of these properties, atrazine was banned for use in the European Union in 2004 (Ackerman, 2007).

Application of atrazine in the United States has become a subject of controversy due to a wealth of evidence indicating that it acts as a severe endocrine disruptor, even in the ecologically relevant parts per billion (ppb) range. Atrazine seems to exhibit its most potent effects in amphibians, although it has been shown to produce ill effects in other vertebrate taxa as well, including disruption of osmoregulation in Atlantic salmon (*Salmo salar*), skewed sex ratios in zebrafish (*Danio rerio*), altered steroidogenesis in alligators (*Alligator mississippiensis*), altered gonadal development in chickens (*Gallus gallus domesticus*), and increased estrogenicity and the potential for reproductive cancer in human cell lines (Crain and Guillette, 1997; Fan et

al. 2007; Matsushita, 2006; Sanderson et al, 2001; Suzawa and Ingram, 2008; Waring and Moore, 2004). The mechanism by which atrazine produces endocrine disruption has only recently been revealed; atrazine binds and inhibits phosphdiesterase in the cell. Inhibition of phosphodiesterase causes an elevated level of cyclic AMP (cAMP), which in turn leads to increased transcription of the CYP 19 gene coding for the enzyme aromatase. Aromatase functions to convert testosterone to estrogen, so the ultimate effect is an elevation of estrogen levels in the organism (Fan et al., 2007).

Atrazine-induced endocrine disruption in amphibians has been extensively studied for a variety of reasons. First, endocrine disruption as a result of atrazine exposure takes place at very low concentrations in amphibians. In fact, doses as low as 0.1 ppb, well below the EPA specified maximum allowable concentration in drinking water (3 ppb), have the ability to produce severe gonadal malformations (Hayes et al. 2003). Secondly, it is likely that a large proportion of amphibian populations in the United States are being exposed to the chemical in the wild, as it is extensively used in the Midwest and has the ability to travel to other regions via rainfall (Figure 1; Mast et al., 2007; Soloman et al., 1996; Thurman and Cromwell, 2000). Lastly, the timing of application makes it of particular concern for breeding amphibians; because it is primarily a pre-emergent herbicide, atrazine is most heavily applied in the spring. Unfortunately, the times of the year during which atrazine levels are highest in surface water coincide with periods of amphibian larval development (Figure 2; Conant, 1998; Stebbins, 1995). This is significant because amphibian larvae are especially vulnerable to exogenous chemicals as a result of the thin, porous skin and gill membranes (Hall and Henry, 1992). However, it is also important because the larval period is also the time in which gonadal differentiation begins in most species; since differentiation is largely regulated by endocrine hormones such as estrogen

and testosterone, changing the concentrations of these hormones (as atrazine does) has the ability to alter the formation of reproductive tissue, an irreversible process with potentially dire implications for successful reproduction later in life.

Numerous studies have examined the effects of atrazine on amphibians, using a variety of endpoints. Atrazine exposure during the larval period has linked to a variety of effects; altered laryngeal size and morphology, as well as nuptial pad and breeding gland morphology, reduced fertility, reduced mating success in mate choice experiments, increased gonadal aromatase expression, altered plasma hormone concentrations, and increased time to metamorphosis have all been observed (Hayes et al. 2002, 2010; Miyahara et al., 2003; Storrs and Semlitsch, 2008). However, the most commonly noted effects are morphological abnormalities in male gonads; atrazine exposure can lead to underdevolped testicular tissue, testicular oocytes, ovotestes (testicles containing >30% oocytes), and even complete sex reverasal, where genotypic males develop as phenotypic females with the ability to successfully reproduce (e.g., Hayes et al., 2002, 2003, 2010; Storrs and Semlitsch, 2008). All of these malformations have the ability to negatively affect amphibian populations through a variety of mechanisms, which likely act as added stressors for wild amphibian populations; it is clear why atrazine is suggested to play a significant role in amphibian declines (Hayes et al., 2010).

However, amphibian gonad histology as it relates to atrazine exposure has become a subject of controversy (Storrs-Méndez and Semlitsch, 2009). This controversy stems largely from a suite of studies reporting testicular oocytes in unexposed testes, leading to the suggestion that perhaps testicular oocytes are a normal part of amphibian testicular development (e.g., Coady et al., 2004; Jooste et al., 2005; reviewed in Hayes, 2004). It has recently been suggested, however, that the inconsistencies in histological data can be explained

when viewed through the scope of life history. Amphibian species undergo sexual differentiation at different rates, summarized as basic, retarded, or accelerated; in species with accelerated rates, testes are likely to be fully differentiated at the time of metamorphosis, while species following the basic rate are not fully differentiated until 3.5 weeks post-metamorphosis, with retarded rate species reaching differentiation much later (Ogielska and Kosutz, 2004). Furthermore, a period of intersex (including the presence of testicular oocytes) is normal for unexposed individuals of some or all species before differentiation, but is *not* normal in fully differentiated individuals (Storrs-Méndez and Semlitsch, 2009). Since most studies noting testicular oocytes in control groups have utilized metamorphosis as an endpoint, it is likely that individuals in these experiments were passing through a natural period of intersex en route to complete sexual differentiation.

The rate of somatic development also varies widely between amphibian species and can be affected by atrazine exposure (Storrs and Semlitsch, 2008). This is important because the rate of somatic development is negatively correlated with size at metamorphosis (an important predictor of survival and reproductive fitness in the terrestrial stage) and also predicts metabolic activity, with speedier development correlated to increased metabolism in the tadpole (Beck and Congdon, 2000; Semlitsch et al., 1988; Wilbur and Collins, 1973). Recent evidence suggests that the rate somatic development may be a strong predictor of amphibian susceptibility to atrazine. Species with rapid somatic development tend to experience the most dramatic somatic effects as a result of exposure, such as a lengthened larval period or decreased mass at metamorphosis, while basic rate species experience mixed somatic effects and species with retarded somatic development seem unaffected (Storrs and Semlitsch, 2008).

Thus, developmental rates show a great deal of promise in predicting the effects atrazine will have on a given species, which would be an invaluable tool for elucidating the chemicals role in amphibian declines. However, as the aforementioned carbaryl examples demonstrate, the effects of a given contaminant are dependent on environmental factors such as competition, predation, hydroperiod, and additional chemicals, which can all be viewed as added stressors encountered by amphibians in nature. A majority of the data suggesting these correlations between the effects of atrazine and developmental rates has been under "low stress" conditions, with larvae commonly reared individually in jars with stable environmental conditions. In order to examine how these predictions may vary in nature as a result of added stressors,we reared *Bufo americanus* tadpoles with ecologically relevant doses of atrazine (0, 0.1, 1, 25 ppb) under stressful conditions, including a high larval density (intraspecific competition) and simulated pond-drying. Since *B. americanus* follows an accelerated rate of somatic development, it is predicted to be highly susceptible to disrupted somatic development. Furthermore, since it displays the retarded rate of sexual differentiation, *B. americanus* gonadal differentiation and morphology should be relatively unaffected by atrazine exposure.

Methods

Larval Exposures:

Two *Bufo americanus* egg masses were collected on May 25, 2009, at Morgan-Monroe State Forest (Morgan County, Indiana). The masses were then transferred to the laboratory, where they were placed together in a single aquarium, in water collected at the field site. Over the next five days, the eggs were allowed to hatch and develop into free swimming tadpoles. On May 30, 2009 (Day 0), tadpoles were haphazardly distributed into aquaria for treatment.

The experiment incorporated three levels of atrazine exposure (0.1 ppb, 1 ppb, and 25 ppb) and a solvent control, which received only ethanol. Within each treatment were three replicates, each consisting of 40 tadpoles placed in a size 10 aquarium (50.8 x 25.4 x 30.5 cm) with 10 L of tap water, neutralized with AquaSafe Water Conditioner (Tetra Holding Inc., Blacksburg, VA, USA). One stock solution of analytical-grade atrazine (99.9% pure; Supelco Analytical, Bellefonte, PA) was prepared, and appropriate dosing was achieved by varying the aliquot with a micropippetter. Treatments receiving less stock solution (including the control) received additional ethanol to ensure that the concentration of solvent was identical across treatments. Water was changed every third day in order to remove waste, renew chemical treatments, monitor mortalities, and rotate tanks among shelving units (to eliminate position effects). Tadpoles were also fed at this time; tanks received Vita+Plus Rabbit Formula (Sunseed Company, Inc., Bowling Green, OH, USA) in quantities sufficient to allow for *ad libitum* feeding. One tank in the 0.1 ppb group experienced severe mortalities (36 of 40 tadpoles perished) during the first three days of the experiment (Fig. 3,4); since this event was not caused by treatment with atrazine, the tank was recolonized with tadpoles from the original stock during day 3. Time to metamorphosis was adjusted to compensate for this event.

When the presence of forelimbs were noted on one individual in a tank (Gosner stage 42-46), the water level was lowered to 5 L and the gravel substrate pushed aside to create a mound such that metamorphosing individuals were offered the option to leave the water. At the completion of metamorphosis (Gosner stage 46, complete tail reabsorption), each animal was sacrificed in 1% Finquel MS-222 (tricaine methanesulfonate; Argent Chemical Laboratories, Redmond, WA), measured for mass to the nearest 0.001 g and time to metamprophosis (days),

fixed in Bouin's Solution (Ricca Chemical Company, Arlington, TX), and stored in 70% ethanol for later histological analyses

Histology:

The fixed kidney-gonadal complexes were dissected out, dehydrated, and embedded in Paraplast Embedding Medium (Fisher Scientific, Pittsburgh, PA). Sectioning was performed at either 7 or 11 µM thickness (AO 820 Microtome, American Optical, Buffalo, NY), and sections were subsequently placed on gelatin-coated slides. Mounted sections were stained with Mallory's Trichrome Stain. Photos were taken with a Spot RT camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Statistical Analysis:

All data were analyzed by one-way, fixed effects ANOVAs (Microsoft Excel 2007). For survivorship to metamorphosis, the last day of tank maintenance prior to the first day of sacrificing was used as an endpoint. Survivorship was calculated as the proportion of surviving individuals, and these values were arcsine transformed prior to statistical analysis. Time to metamorphosis was defined as the number of days from the beginning of treatment to the completion of metamorphosis; these data were inverse-transformed prior to analysis. Mass at metamorphosis was subjected to a log transformation prior to analysis. Pairwise comparisons were conducted using Tukey's test for multiple comparisons (MINITAB). Statistical significance was defined as a P-value < 0.05.

Results

Chemical Effects On Gonadal Development

Analyses of gonadal histology were confounded by histological error. Only a few slides were successfully processed to produce interpretable histological features. A majority of slides were unusable because the orientation of sections failed to properly intersect the gonads. Slides that were sectioned with proper orientation suffered from a variety of flaws, including nicks and holes in sections, blade chatter, improper staining, and insufficient definition (cells difficult to identify, with an overall blurred appearance).

Chemical Effects On Somatic Development

Survivorship to metamorphosis was not affected by treatment with atrazine, although all chemical treatments exhibited lower survivorship than the control (Fig. 3, 4, Table 1; $F_{3,11}$ = 1.08, P = 0.410). Time to metamorphosis was also unaffected by treatment with atrazine, although again in this case, all chemical treatments exhibited lengthened larval periods in comparison with the control treatment (Fig. 6, Table 4; $F_{3,11}$ = 0.469, P = 0.712). Mass at metamorphosis showed an overall significant effect with respect to treatment with atrazine, as all chemical treatments produced larger metamorphs than the control (Fig. 5, Table 2; $F_{3,11}$ = 4.20, P = 0.046). However, pairwise comparisons failed to identify statistically significant differences between treatments, although both the 1 ppb treatment and the 0.1 ppb displayed the greatest differences from the control group and were both only slight above P =0.05 (Table 3; P = 0.054 and 0.069 respectively).

Discussion

Effects On Gonadal Development

A variety of histological errors prevented any analyses of gonadal development. Initially, we modeled our histological protocol after that referenced in Hayes et al. (2003), with the exception of orientation; rather than transverse sections, we chose to begin with longitudinal sections, which we intended to intersect a large portion of the gonad proper. However, our first attempts failed to achieve this orientation. Rather, we would commonly obtain a large portion of kidney, a significant portion of the Bidders organ (an ovary-like organ of unknown function, present in both sexes, located at the anterior end of the gonad proper, and composed primarily of diplotene oocytes), and only a small portion of gonad proper (Fig. 7). Another common problem with these early attempts was heavily degraded tissue, which had a flakey or even shredded appearance (Fig. 8). A large number of samples also suffered degradation during sectioning, with nicks and holes in the sample as the most common symptom. These slides were aborted prior to staining, so photographic documentation was excluded here.

In an attempt to rectify difficulties in finding the proper orientation to intersect large areas of the gonad proper, and to address the issue of nicks from the microtome blade, we attempted several series of transverse sections. While damage caused by the microtome blade was largely averted, tissue degradation continued to prevail, and compression of sections became an issue (Fig. 9). Although we were confident in the ability of this protocol to target the gonad proper, slides were even more difficult to interpret than sections taken longitudinally.

Given the lack of success with transverse sections, we again returned to longitudinal sectioning, but altered the tissue processing protocol. Given that two of the most common flaws in slides thus far had been degradation and damage from the microtome, it was determined that insufficient infiltration of paraffin was the most likely cause; we attempted to rectify this by increasing the infiltration period, allowing the kidney-gonadal complex to incubate for up to 4 weeks in molten paraffin. Although this round of sectioning did produce slides in the proper orientation, degradation continued to occur. As a result, we increased the thickness of our sections to 12 µM. To date, we have completed one preliminary round of sectioning at this thicker setting. It appears that sectioning at 12 µM has largely rectified the degradation and microtome damage issues; although markedly understained, these slides do reveal the overall structure of the developing gonads. Adjustments to the staining procedure will likely produce slides that are clear and ready for analysis (Fig 10).

Somatic Effects

Our results indicate that atrazine exposure does alter somatic development, at least with respect to mass at metamorphosis, and that the most severe effects often occur at very low, ecologically relevant doses; mean mass and survivorship were both most different from the control mean at 1 ppb and 0.1 ppb, highlighting the importance of examining the sublethal effects of this chemical at low concentrations. Furthermore, our more stressful conditions (higher larval density, simulated pond drying) did not intensify somatic effects with respect to past work (Storrs and Semlitsch, 2008). This is surprising, given that our higher larval densities likely did lengthen the larval period with respect to the species average (Wilbur, 1977) and averages from past work (Storrs and Semlitsch, 2008; Storrs-Méndez and Semlitsch, 2009). It is

also surprising given the wealth of data suggesting synergistic effects of natural stressors and chemical contaminants (e.g. Relyea and Mills, 2001; Reylea, 2003; Boone and James, 2003).

Mass at metamorphosis is a particularly strong predictor of fitness in the terrestrial life stage (Wilbur and Collins, 1973; Semlitsch et al. 1988). In our experiment, mass was positively affected by atrazine exposure, with the largest metamorphs produced at the 1 and 0.1 ppb levels. While it is tempting to suggest that perhaps atrazine exposure is beneficial in this way, it is important to remember that even if atrazine were to increase terrestrial survivorship in *B. americanus*, it is likely that any benefits would be negated in terms of reproductive fitness; survivorship means very little if the survivors are feminized, hermaphroditic, or chemically castrated. It is also likely that atrazine may not have this effect in a community context, as indirect effects of a chemical to the community (e.g., algal growth) can sometimes outweigh direct effects on the physiology of the organism (Boone and James, 2003; Boone and Semlitsch, 2002; Relyea and Mills, 2001).

Increased mass as a result of atrazine exposure was likely a result of a phenomenon known as "density-mediated compensation." In this scenario, it is hypothesized that atrazine exposure decreased larval density in treatment tanks, reducing competition among the remaining larvae, thus allowing them to grow to larger sizes (Fig. 11). This is supported by the relationship between survivorship and mass in our experiment; 0.1 ppb and 1 ppb treatments exhibited the lowest survivorships, and also the highest masses at metamorphosis. However, food was not limiting in our study, as we fed the tadpoles to allow for *ad libitum* feeding. Thus, this should have reduced competition among larvae. Regardless, density-mediated compensation has been noted in *Ambystoma barbouri* larvae exposed to comparable doses of atrazine; however, the benefits gained from larger mass were negated by carryover effects of

chemical exposure into the terrestrial stage, as these larger individuals also exhibited higher mortality than smaller, unexposed animals months after exposure (Rohr et al., 2006).

Survivorship at metamorphosis, although not affected statistically by atrazine exposure, did display a trend, in that all treatment groups displayed lower survivorship values than the control. Furthermore, as previously mentioned, the means were most different from the control at the lowest concentrations. On one hand, our low mortality rates are consistent with other studies of amphibian survival in response to low doses of atrazine (e.g., Hayes et al., 2002). However, Storrs and Kiesecker (2004) exposed *B. americanus* tadpoles to comparable levels of atrazine (0, 3, 30, or 100 ppb), and reported extremely high mortalities (>80%) among low and medium treatments. The extremely high mortalities produced in this experiment are not typical of atrazine at these concentrations, but may be the result of the atrazine source. Studies reporting low mortalities (such as the present and Hayes et al., 2002) have used analytical grade atrazine (≈99 % pure), whereas the Storrs-Kiesecker study utilized commercial grade atrazine (=85.5% pure). Commercial grade herbicides often contain other compounds (e.g., surfactants) that may increase the deadliness of the atrazine itself.

Although time to metamorphosis was not statistically affected, atrazine did seem to lengthen the larval period, as all treatments displayed larger mean values for days to metamorphosis. Contrary to our results for survivorship and mass at metamorphosis, the dose-response relationship seemed linear here, with each increasing concentration of atrazine displaying a longer average larval period. This is consistent with previous findings, which have displayed a similar dose-specific response with respect to this parameter (Storrs and Semlitsch, 2008). However, this is not typical of atrazine overall, which tends to display a non-monotonic dose-response curve (NMDRC).

An NMDRC means that the response reverses as concentration increases, created an inverted U-shaped curve; the NMDRC of atrazine is displayed in Fig. 12. Responses such as this are typical of endocrine disrupting chemicals, and are especially alarming for amphibian populations in the wild (Storrs and Kiesecker, 2004). Given the prevalence of atrazine use, its mobility in the environment, the timing of application, and the doses required to produce harmful somatic and gonadal effects (Hayes et al., 2002, 2003), it is clear that atrazine is likely a contributor to amphibian declines in the wild. Our results confirm that the most dramatic somatic alterations occur in this species at very low concentrations, to which a large proportion of amphibians in the United States are likely exposed.

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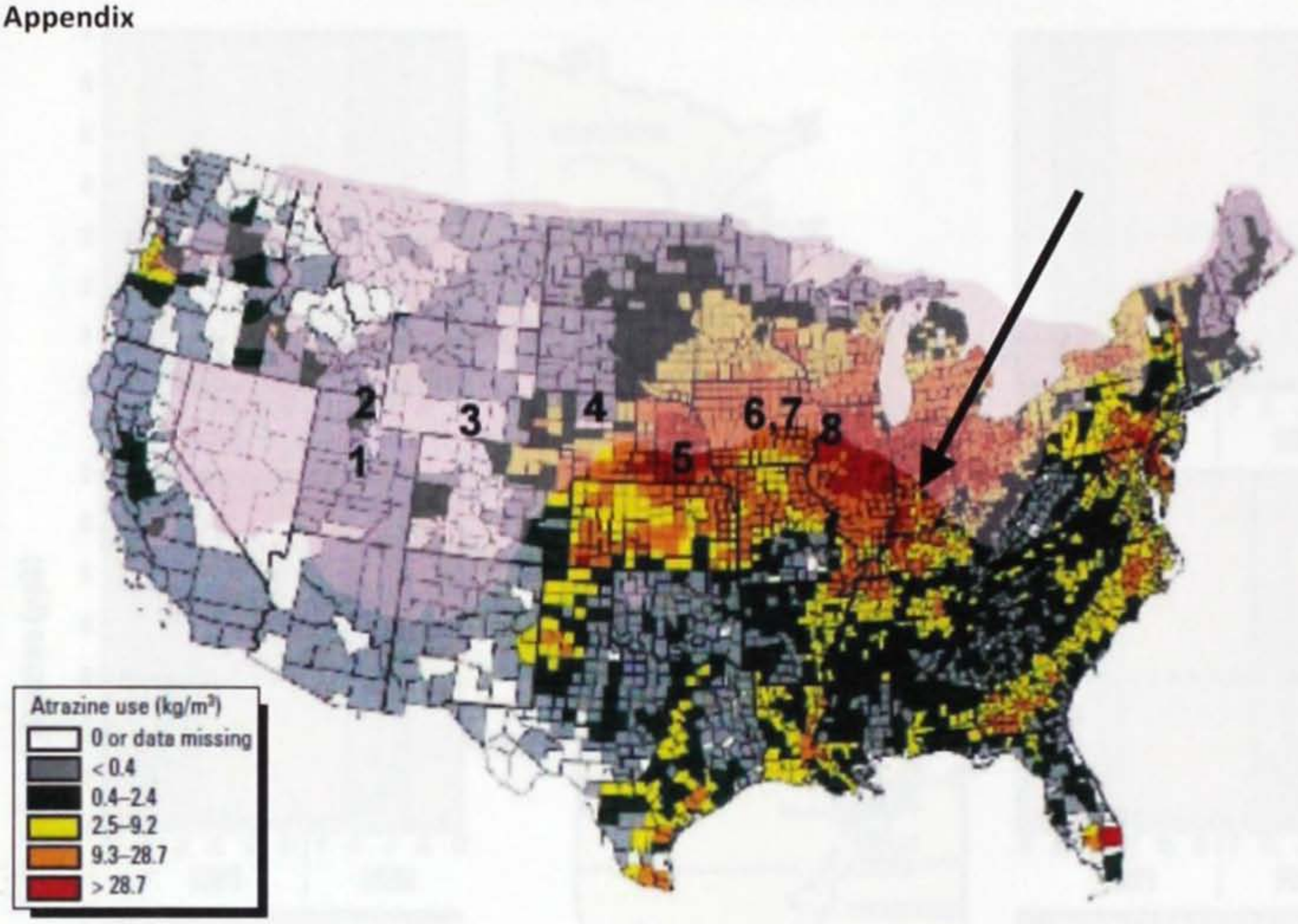

Figure 1 Atrazine use in the United States based on sales. Arrow indicates approximate location of egg-collection site. Reproduced from Hayes et al., 2003.

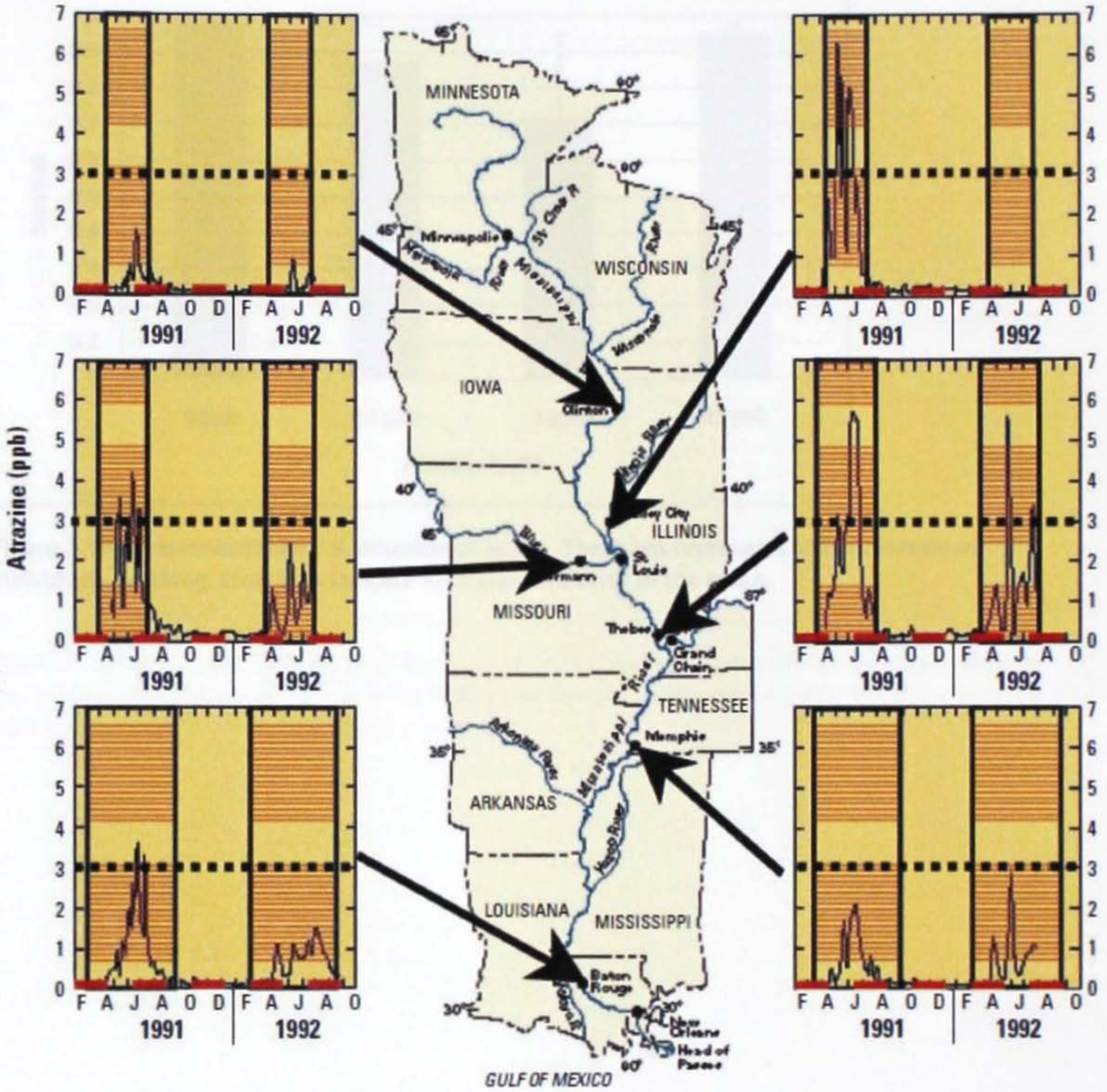

Figure 2 Atrazine contamination along the Mississippi watershed in 1991 and 1992. The horizontal axis shows months (February, April, June, August, October, December). The black dashed line indicates the EPA Drinking Water Standard, 3 ppb, while the red dotted line represents the lowest concentration necessary to produce hermaphrodites in the laboratory (0.1 ppb). Vertical black lines indicate the timing of amphibian larval metamorphosis for each region. Reproduced from Hayes et al., 2003.

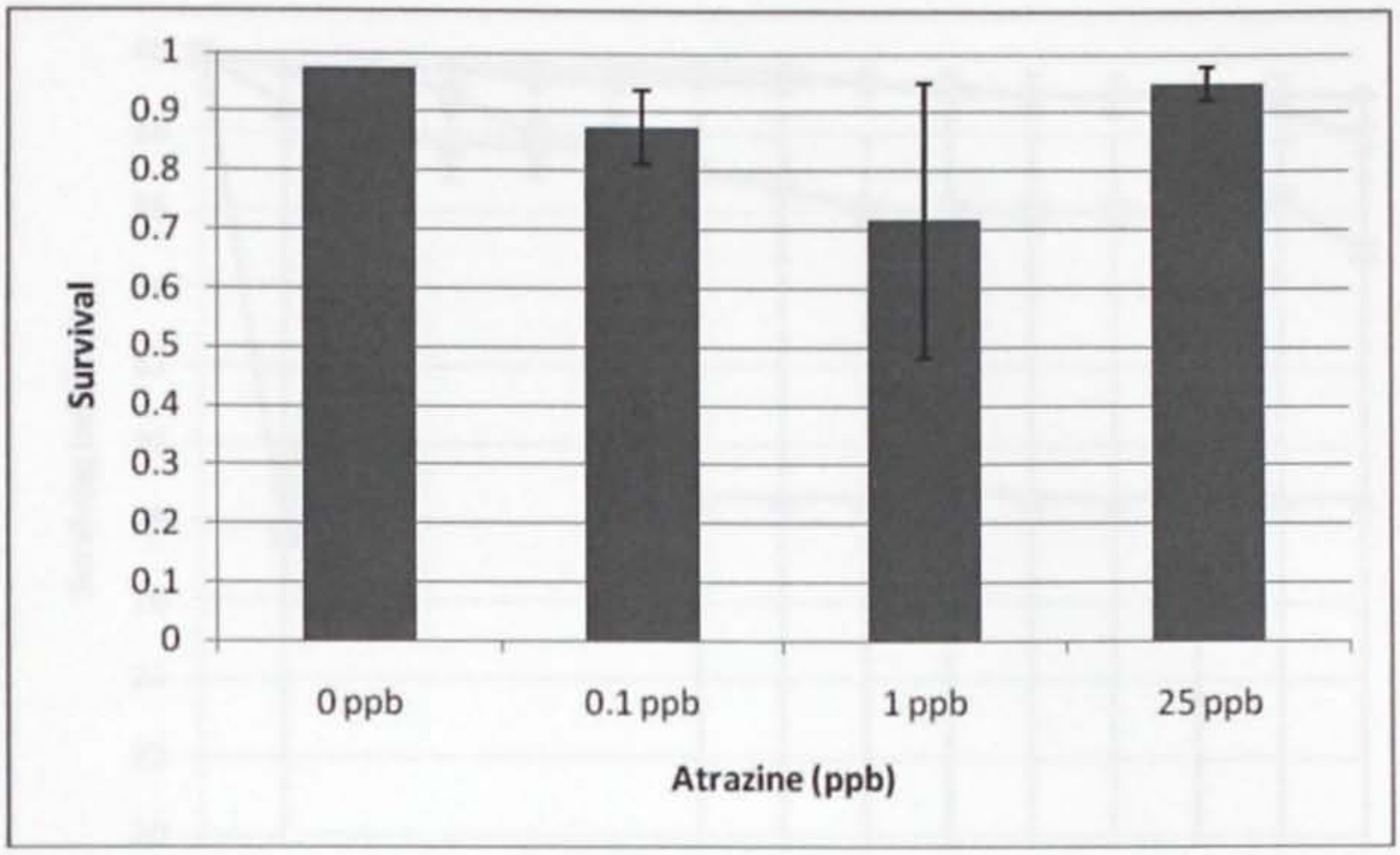

Figure 3 Mean survivorship for *B. americanus* larvae. The y-axis represents the proportion of individuals surviving. Error bars indicate +/- 1 standard error of the mean.

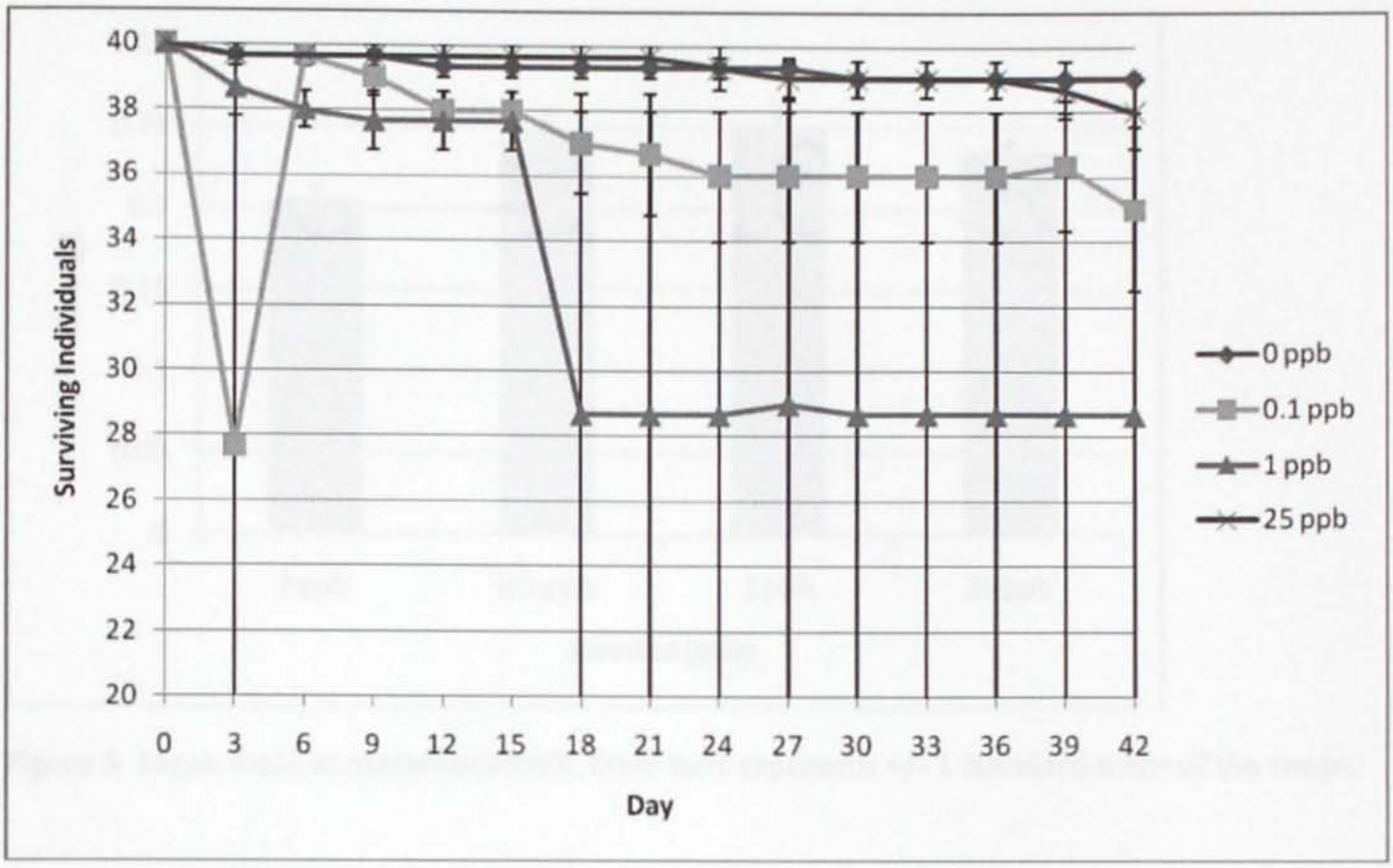

Figure 4 Survivorship over time. Days from the beginning of the experiment are indicated on the x-axis, while the number of surviving individuals is represented on the y-axis (n=40). Error bars represent +/- 1 standard error of the mean.

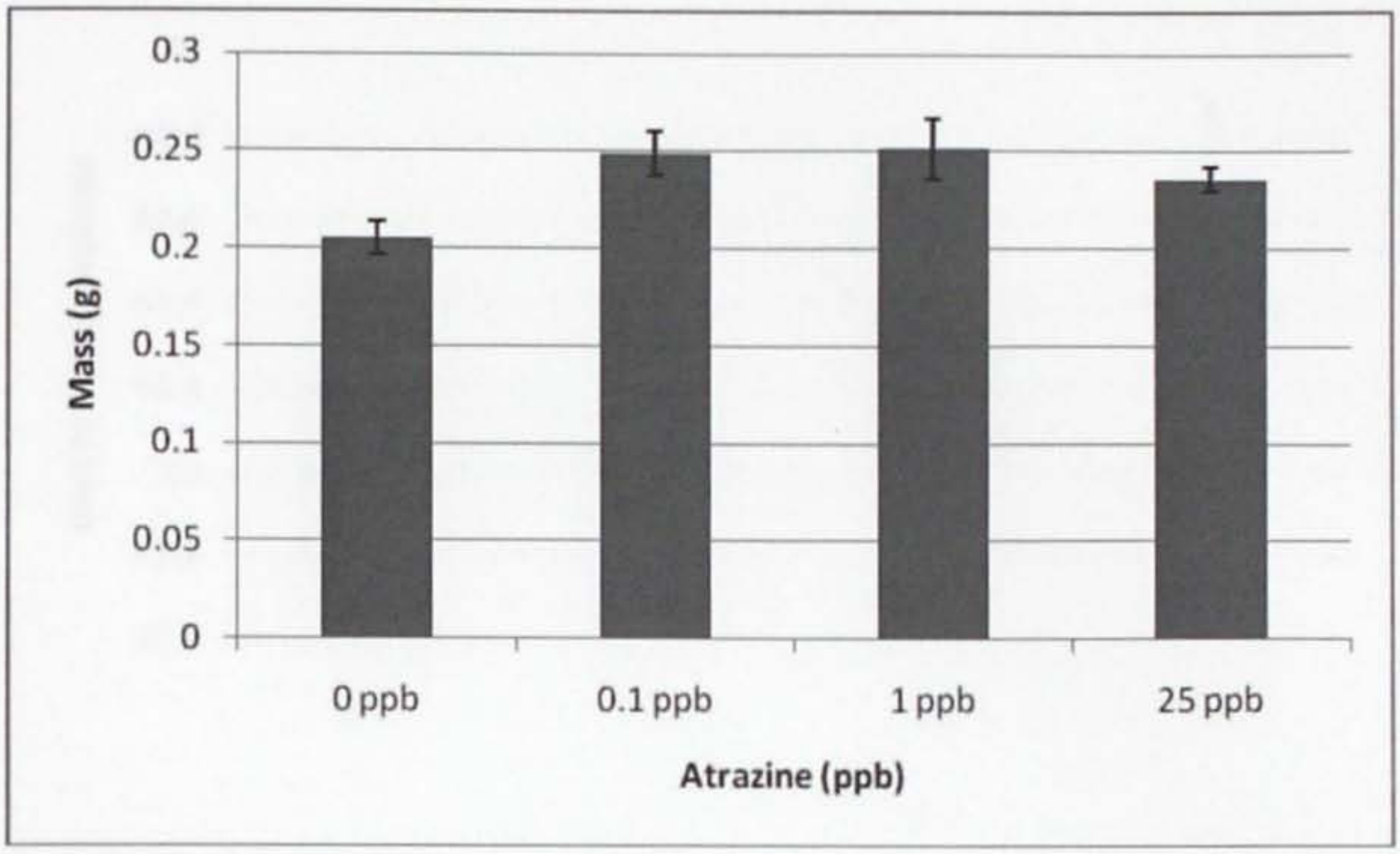

Figure 5 Mean mass at metamorphosis. Error bars represent +/- 1 standard error of the mean.

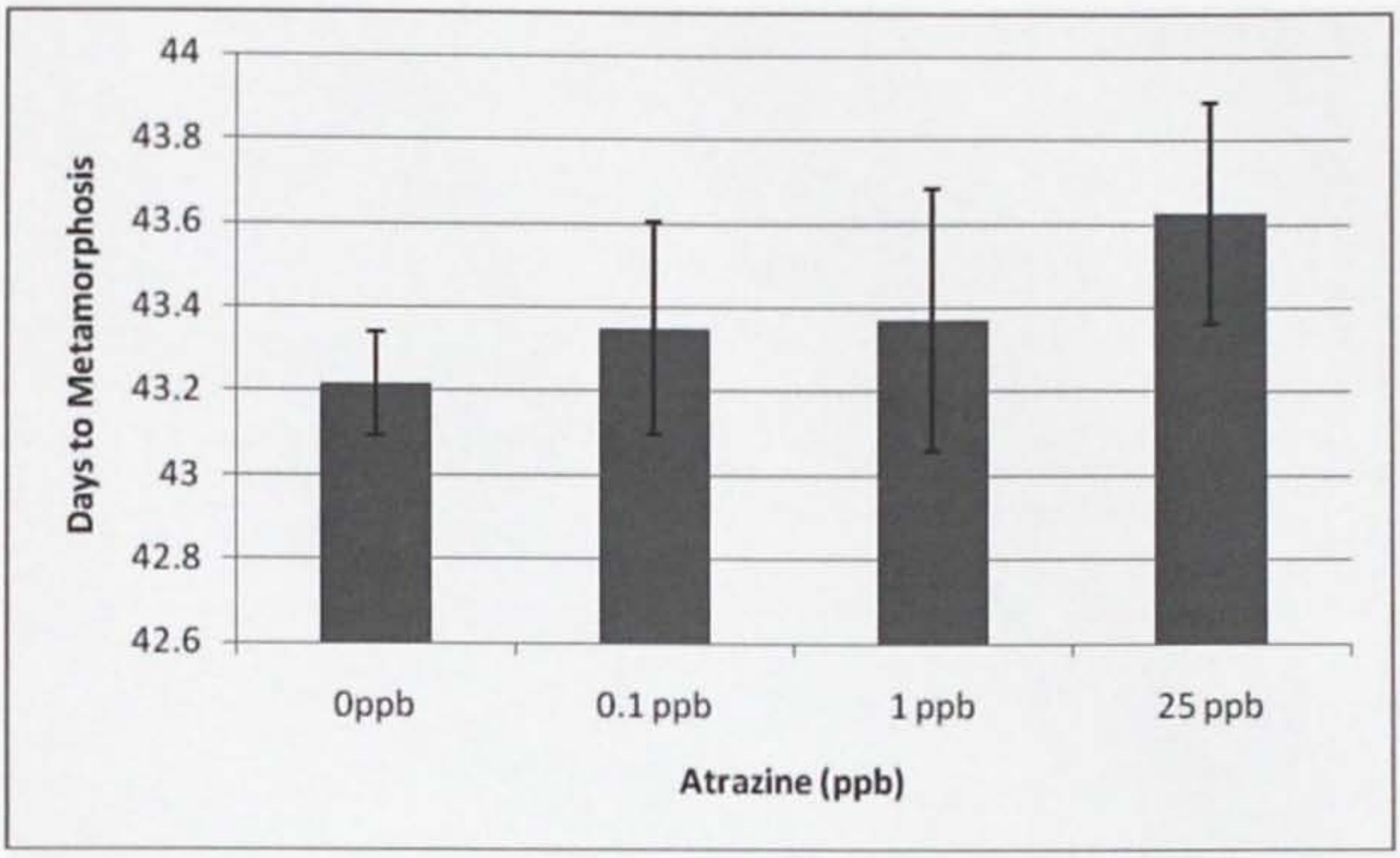

Figure 6 Mean time to metamorphosis. Error bars represent +/- 1 standard error of the mean.

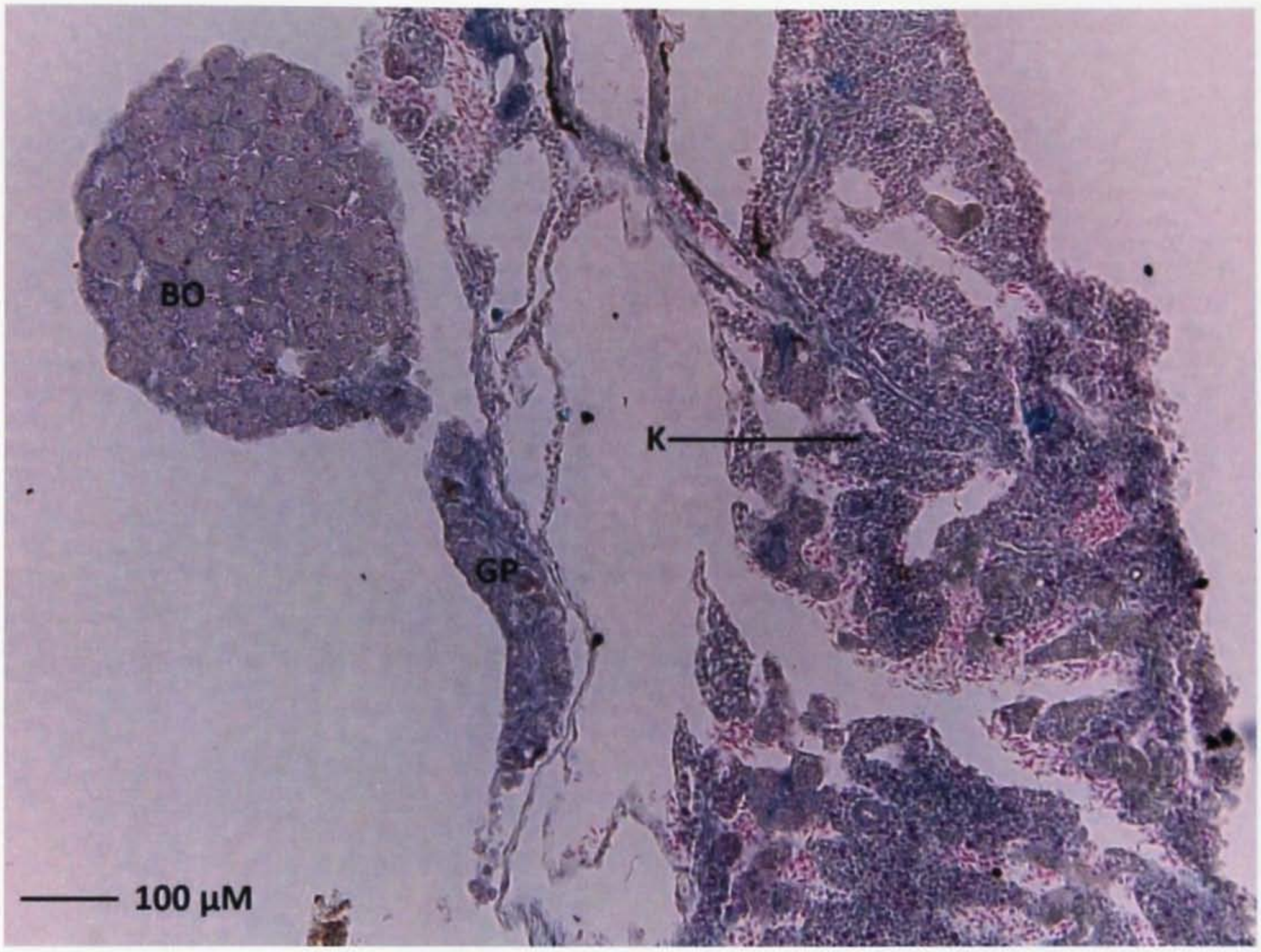

Figure 7 Longitudinal *B. americanus* kidney/gonad tissue from an individual treated with 1 ppb atrazine, viewed under 100x maginification, showing typical faulty orientation. The Bidders Organ (BO) is clearly visible, as is a large portion of the kidney (K), although only a small section of the gonad proper (GP) is present.

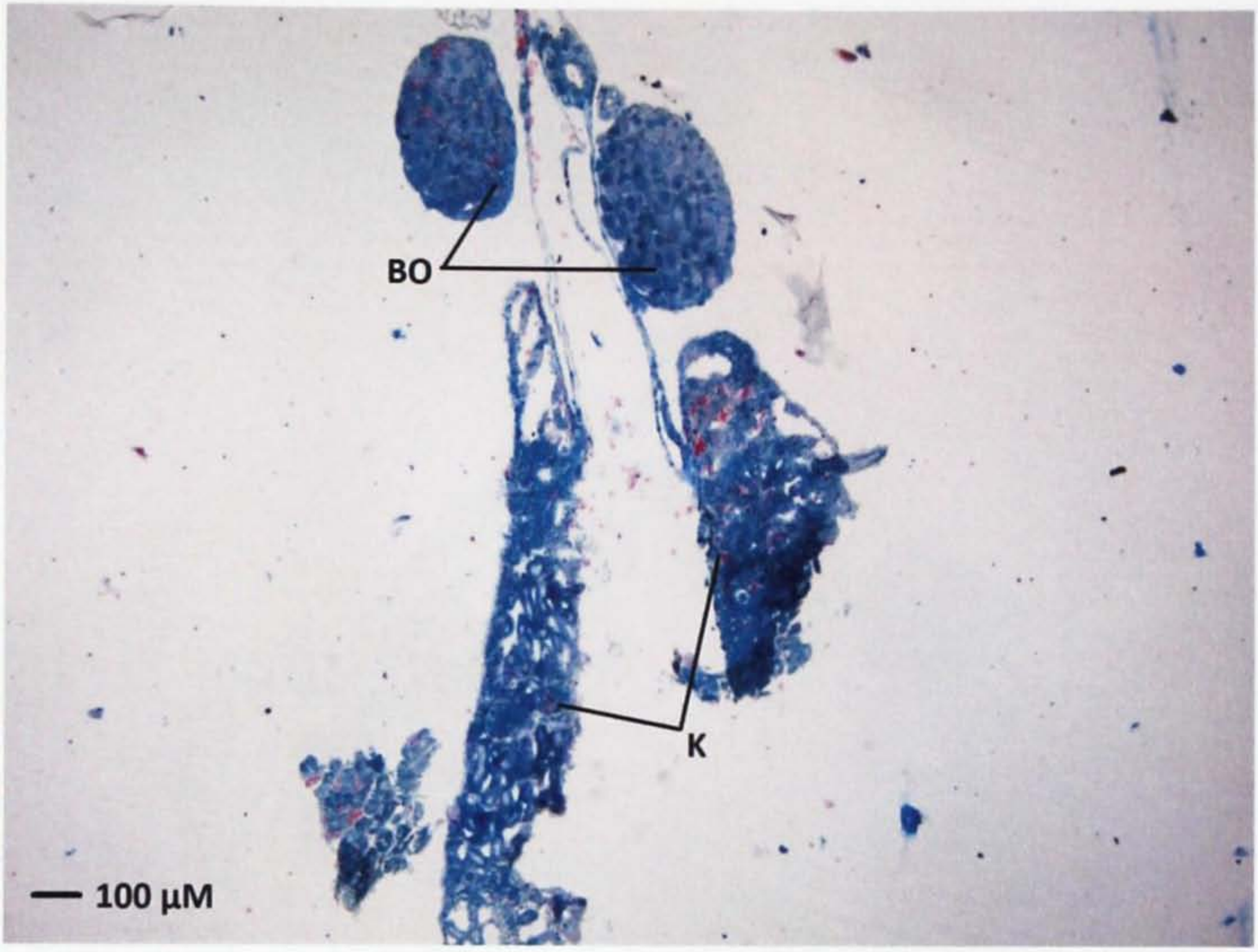

Figure 8 Longitudinal *B. americanus* slide, viewed under 50x magnification, showing heavily degraded tissue. Note the flakey appearance of the kidneys (K), as well as the relative lack of definition within the Bidders Organ (BO). The gonad proper was not intersected in this section.

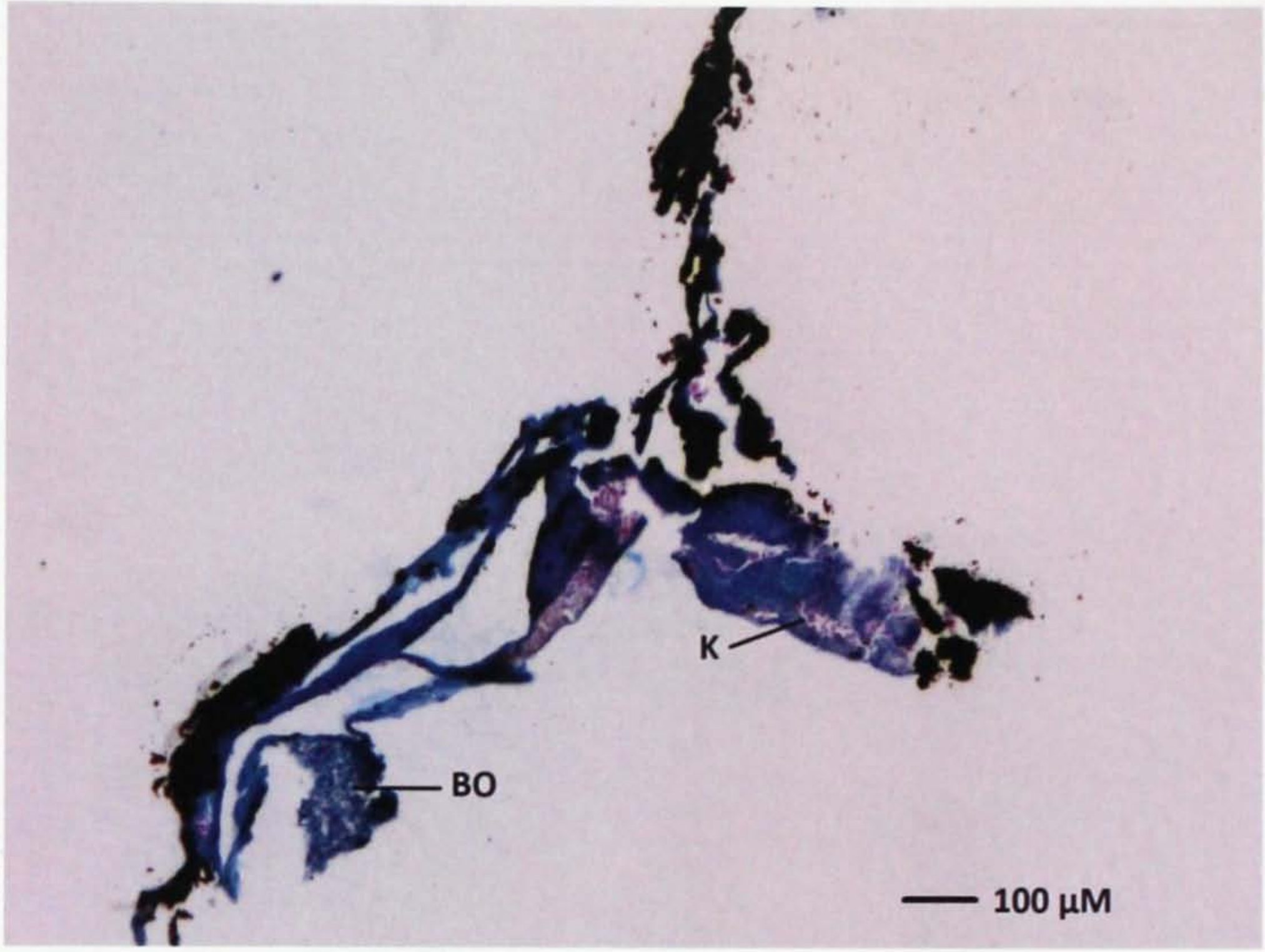

Figure 9 Transverse section of *B. americanus* kidney-gonadal complex. Note the heavy degradation of the sample, as well as the compression of the identifiable Bidders Organ (BO) and kidney (K).

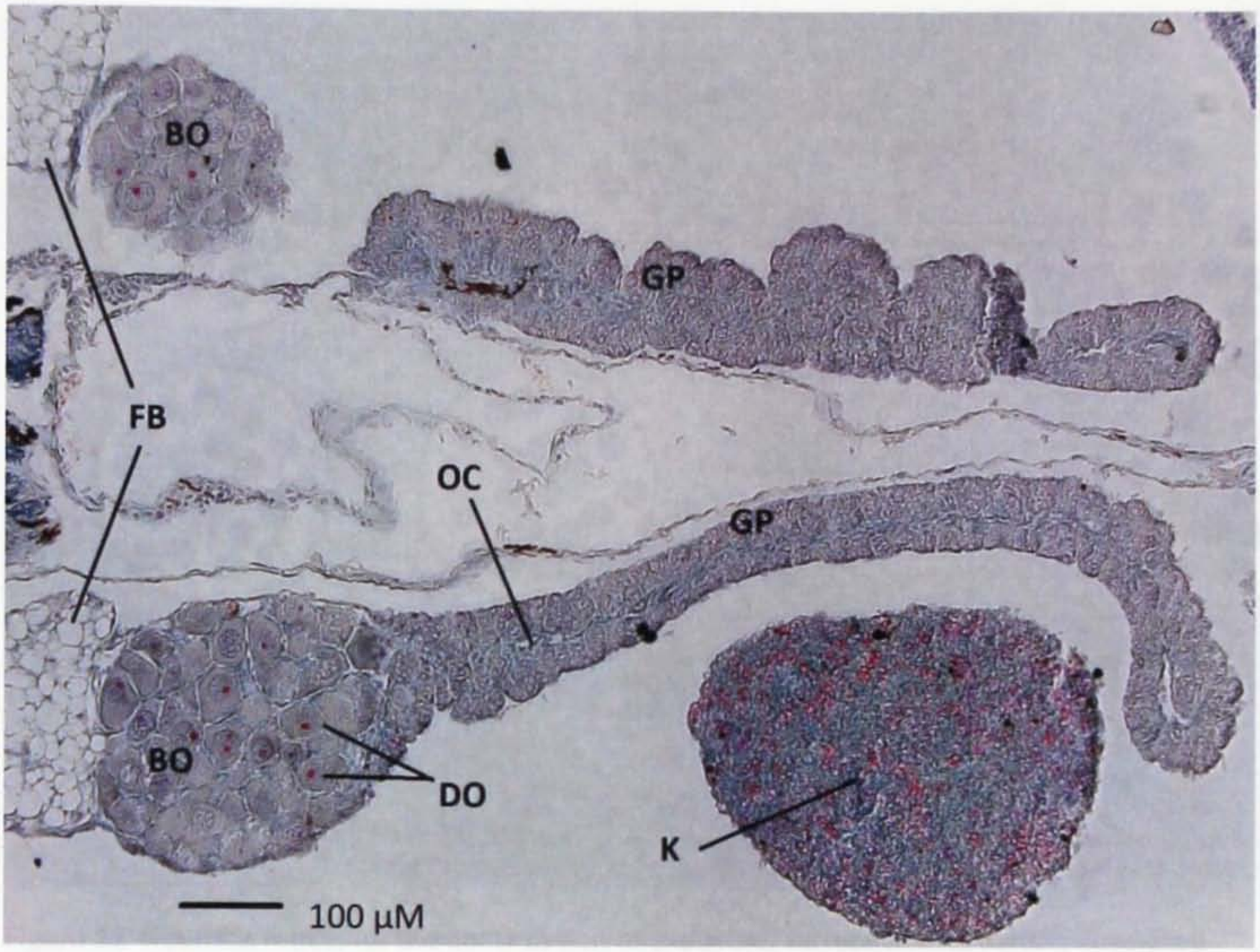

Figure 10 Longitudinal section of *B. americanus* gonads, sectioned at 12 µM and viewed under 100x maginification. These are likely a Stage V (Ogielska and Kosutz, 2004) ovaries, indicated by the appearance of the ovarian cavity (OC), fat bodies (FB), and the lobed appearance of the upper gonad proper (GP) . Note the clearly defined diplotene oocytes (DO) present in the Bidders Organ (BO).

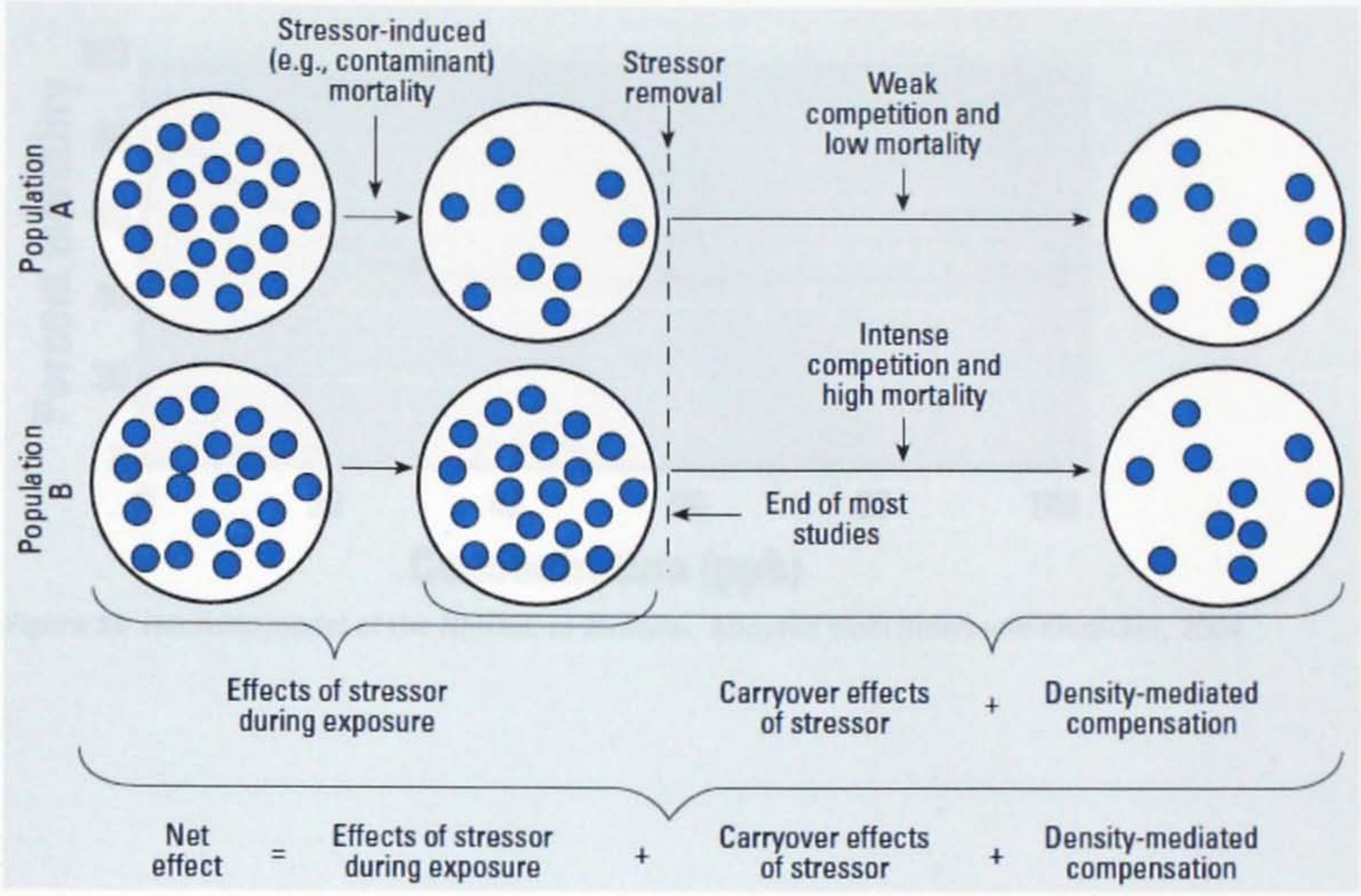

Figure 11 Heuristic model for the contribution of exposure, carryover, and density-mediated effects of a stressor, to a stressor's net effect on survival. Figure adopted from Rohr et al., 2006.

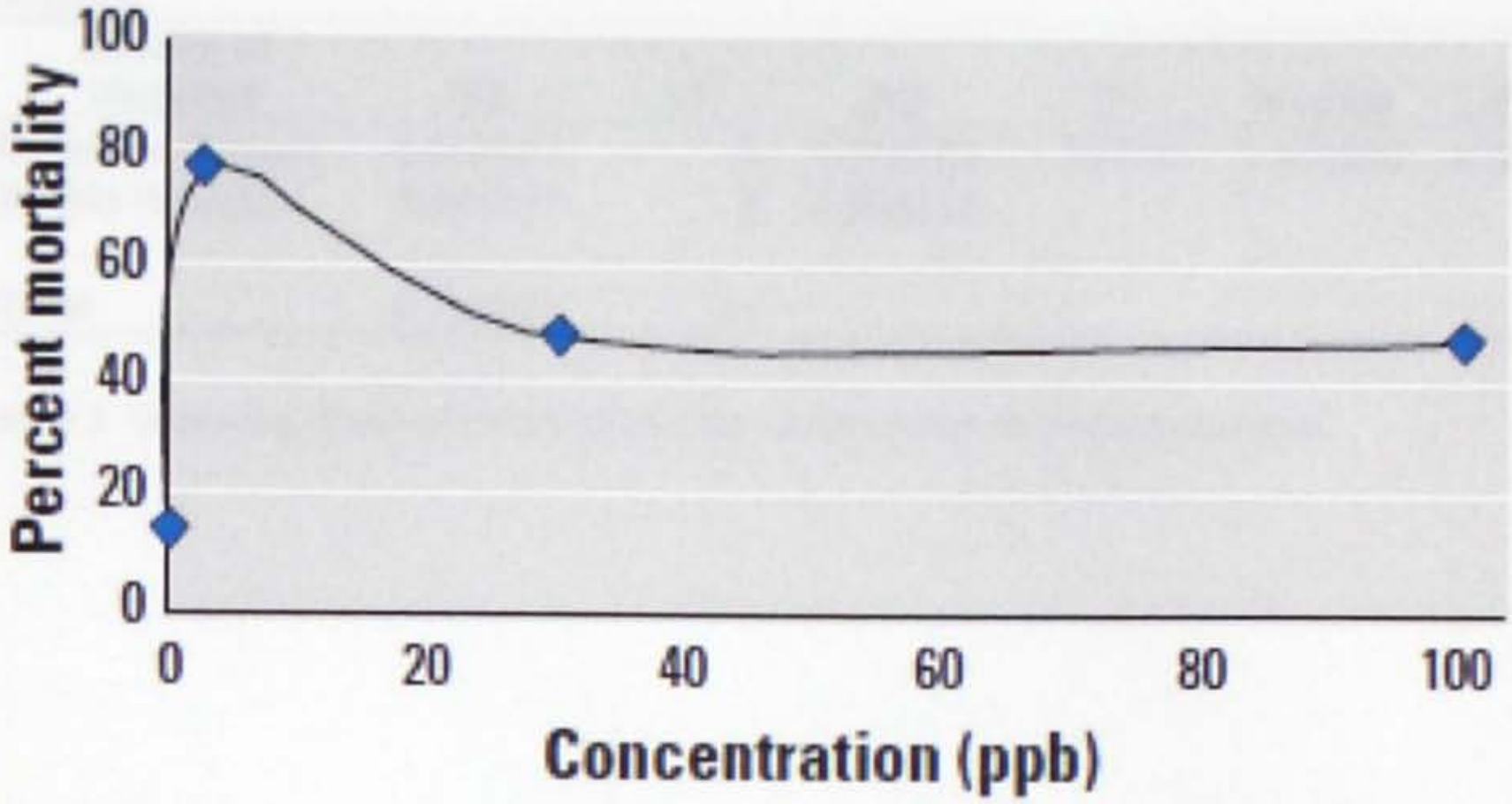

Figure 12 Heuristic model of the NMDRC of atrazine. Adopted from Storrs and Kiesecker, 2004.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.225707	3	0.075236	1.083744	0.409592	4.066181
Within Groups	0.555377	8	0.069422			
Total	0.781084	11				

Table 1 One-way, fixed effects ANOVA for survivorship at metamorphosis.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0146155	3	0.004872	4.197665	0.046486	4.066181
Within Groups	0.00928485	8	0.001161			
Total	0.02390035	11				

Table 2 One-way, fixed effects ANOVA for mass at metamorphosis.

Tukey's Mutiple Comparisons

Comparison	Difference of Means	SE of Difference	T- Value	Adjusted P-Value
0 ppb - 0.1 ppb	-0.08286	0.02783	-2.977	0.0687
0 ppb - 1 ppb	-0.08761	0.02783	-3.148	0.054
0 ppb - 25 ppb	-0.06147	0.02783	-2.208	0.2006
1 ppb -0.1 ppb	-0.004756	0.02783	-0.1709	0.9981
25 ppb - 0.1 ppb	0.021392	0.02783	0.7685	0.8664
25 ppb - 1 ppb	0.02615	0.02783	0.9394	0.7855

Table 3 Tukey's Pairwise Comparisons for mass at metamorphosis.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.86E-08	3	2.29E-08	0.468771	0.712244	4.066181
Within Groups	3.91E-07	8	4.88E-08			
Total	4.59E-07	11				

Table 4 One-way, fixed effects ANOVA for time to metamorphosis.