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Aminomethylphosphonic acid alters amphibian embryonic development at environmental concentrations

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Abstract

Despite intense societal and scientific debates regarding glyphosate toxicity, it remains the most widely used herbicide. The primary metabolite of glyphosate, AMPA (aminomethylphosphonic acid), is the main contaminant detected in surface waters worldwide, both because of the extensive use of glyphosate and because of other widespread sources of AMPA (i.e., industrial detergents). Studies on potential effects of glyphosate using environmentally relevant concentrations of AMPA on non-target wildlife species are lacking. We experimentally tested the effects of AMPA on embryonic development in a common European toad at concentrations spanning the range found in natural water bodies (from 0.07 to 3.57 $\mu\text{g}\cdot\text{l}^{-1}$). Our experimental concentrations of AMPA were 20 to 1000 times lower than official Predicted-No-Effect-Concentrations. We found that these low-level concentrations of AMPA decreased embryonic survival, increased development duration and influenced hatchling morphology. Response patterns were more complex than classical linear concentration-response relationships, as concentration responses were nonmonotonic, with greater effects at low-concentrations of AMPA than at high levels. Based on our results we recommend that investigators focus not only on effects of “parent compounds,” but also their metabolites at environmentally relevant concentrations in order to comprehensively assess impacts of anthropogenic contaminants on the environment.

Keywords: Aminomethylphosphonic acid; Glyphosate; Contamination; Herbicide toxicity; Non-monotonic dose response; *Bufo spinosus*; Sublethal effects

Introduction

Anthropogenic activities have been responsible for the creation of a multitude of synthetic chemicals that have been released into the environment (Kendall et al., 2016). Many synthetic chemicals are now (in)famous for their high levels of toxicity for organisms (Tsui & Chu, 2003; Kendall et al., 2016; Giaquinto et al., 2017).

Persistent organic pollutants (POPs) can be highly toxic and are widespread, threatening ecosystems worldwide (Ashraf., 2017). Environmental contamination by POPs and other chemicals is now recognized to play a major role in the current loss of biodiversity, along with climatic influences and habitat modifications (Kendall et al., 2016).

Among the numerous sources of environmental contamination, modern agricultural practices play a significant role because of the widespread use of agrochemicals, which span from fertilizers (that can lead to organic matter enrichment and eutrophication; Khan & Mohammad, 2004) to pesticides (i.e., herbicides, insecticides and fungicides; Prakash & Rao, 1997). Although pesticides are designed to alter specific components of ecosystems (e.g., weeds, insect pests, fungi) that can be deleterious to crop productivity, they can also affect non-target species either because of their toxicity, or because they can be transported to non-target environments (Hasenbein et al., 2017; Relyea et al., 2006; Isidori et al., 2005).

Pesticides tend to accumulate in aquatic habitats due to the transfer of agrochemicals from contaminated agricultural fields into wetlands by soil erosion during rain events (Solomon et al., 2003). In addition, many agrochemicals exhibit properties

(e.g., solubility, polarity) which promote transfer to and persistence in aquatic ecosystems (Mackay et al., 2000; Giesy et al., 2000).

Although traditional toxicity studies are critical to assess median lethal doses (LD50) and associated deleterious effects, they usually fail to detect more subtle sub-lethal effects at very low concentrations of agrochemicals (i.e., levels below Predicted-No-Effect-Concentrations [PNEC]), especially on non-target (non-model) organisms. Sub-lethal effects may influence multiple traits (e.g., physiological effects due to endocrine disruption; Hayes et al., 2010), and they may also impact the persistence of natural populations. In addition to not examining sub-lethal effects, many toxicity studies focus solely on parent compounds (i.e., the listed active substances), but ignore the potential effects of their metabolites. Yet, many metabolites can be found in natural environments more frequently and at higher concentrations than their parent compounds (Grandcoin et al., 2017; Matozzo et al., 2019).

This is the case for the most widely used non-selective herbicide – glyphosate – and its primary metabolite AMPA, which are the main pesticides detected in surface waters in North America and Europe (Grandcoin et al., 2017; Coupe et al., 2012; Bonansea et al., 2017). The great abundance of AMPA in surface waters is presumably linked to the worldwide use of glyphosate (Grandcoin et al., 2017), but also because AMPA is an organic phosphonate derived from water treatment facilities, textile industries, and industrial or household detergents (Grandcoin et al., 2017). Despite the global presence of AMPA in surface waters, surprisingly few

studies have investigated its potential effects on non-target organisms (Giesy et al., 2000), and even fewer studies have focused on actual environmental concentrations of AMPA (Matozzo et al., 2018, 2019).

Amphibians are a particularly well-suited non-target taxon to investigate the potential effects of AMPA at low, realistic environmental concentrations. First, amphibians are often very susceptible to changes in water quality because they have highly permeable skin, which allows for the exchange of gases, water and ions, but may also facilitate the diffusion of harmful substances (Quaranta et al., 2009). Second, in many species, larvae filter water or graze on submerged surfaces to feed. As a consequence, larvae can directly ingest sediment, which can accumulate pesticides even if rates of contaminant inputs are low (Hayes et al., 2010). Third, many amphibians have biphasic life-cycle, with terrestrial adults breeding in aquatic sites (ponds) where eggs and larvae develop. Early developmental (embryonic and larval) phases bear strong and long-lasting influences on life-history traits such as body size, growth, survival, and reproduction (Kashiwagi et al., 2009; Arrighi et al., 2013). As a consequence, disturbances to aquatic habitats during early development may have lifelong effects on an individual phenotypic variation. For instance, the development and metamorphosis of larvae are hormonally dependent, and amphibian larvae are particularly sensitive to xenobiotics, which can disrupt hormonal pathways (Kashiwagi et al., 2009, Chai et al., 2017; Crump et al., 2002). Accordingly, amphibians have been particularly studied to investigate the consequences of environmental contaminants (Mann et al., 2009; Hayes et al., 2006).

In this study, we experimentally investigated the effects of AMPA through environmentally relevant concentrations (i.e., actual concentrations found in surface waters, 20 to 1000 times lower than PNEC) on embryonic development in a common European amphibian, the Spined toad (*Bufo spinosus*). This non-model species is particularly well-suited to investigate the effects of AMPA for several reasons. This species can live in a variety of habitats and even persist in highly modified agricultural areas (Guillot et al., 2016), suggesting that individuals can be exposed to agrochemicals and their metabolites at multiple life stages. In some instances, eggs, tadpoles, juveniles, and adults of *B. spinosus* are found in and around ponds located in agricultural areas (Bókony et al., 2018). In addition, eggs may be intensely exposed to AMPA, as breeding/oviposition time often coincides with the timing of herbicide (glyphosate) application in agroecosystems (Lenhardt et al., 2015; Berger et al., 2013).

Material and methods

Eggs collection

Spined toad (*Bufo spinosus*) eggs strings (number of egg string=15) were collected from 29/01/2018 to 28/02/2018 in two ponds near the Centre d'Etudes Biologique de Chizé (46°090N, 0°240W) and returned to the laboratory. The two ponds were monitored twice a day to insure that the eggs were collected immediately after oviposition to minimize embryonic development in the natural environment. A subset of 120 eggs on each clutch was collected for our experiment, while the remaining eggs (i.e., 3000-5000) were released at their oviposition site.

Treatment concentrations and chemical solutions

Environmental concentrations found in aquatic environments in France range from 0.1 µg.l⁻¹ to 6.6 µg.l⁻¹ (data from Water Agencies, “Agence de l'eau Loire Bretagne” and “Agence de l'eau Adour-Garonne”) and are similar to concentrations found in aquatic environments in Northern America and Europe (Grandcoin et al, 2017; Coupe et al, 2012; Bonansea et al, 2017). Because our goal was to mimic the range of concentrations found in the wild, we produced three treatments spanning from low to relatively high concentrations (Table 1). Stock solutions (0.1g/L) of AMPA were obtained by dissolving commercial crystalline powder (Aminomethylphosphonic acid, 99% purity, ACROS ORGANICS™) in dechlorinated tap water. These stock solutions were further diluted with dechlorinated tap water to reach each of the treatment (Table 1). We produced 3 additional water samples for each treatment in order to perform analytical verifications of the concentrations. These analytical

verifications of the actual concentrations were performed by an independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Overall, these verifications showed that actual concentrations were similar to concentrations found in aquatic environments (see above) and that the differences between “low”, “medium” and “high” treatments were significant (Table 1).

Treatment	AMPA concentrations
Control	0
Low	0.07±0.01
Medium	0.32 ±0.052
High	3.57 ±0.153

Table 1. Concentrations ($\mu\text{g.l}^{-1}$) measured in the experimental tanks (Mean± SD)

For clarity, we will refer to the treatments as Low, Medium and High hereafter.

Experimental design

Eggs were subjected to different concentrations of AMPA (Low, Medium and High) and a control during the whole embryonic development until hatching. To maintain relatively constant exposure levels throughout the experiment but to avoid excessive mechanical disturbances to developing eggs, water was changed once a week according to the half-life of AMPA ranging from 7 to 14 days in water (Battaglin et al, 2015). Egg jelly was maintained throughout the experiment.

Each subset of each clutch (containing ~120 eggs) was further separated into segments containing ~30 eggs (ie. 120/4 treatments) that were randomly placed in different glass-molded tanks containing 2 L of water at one of our experimental treatments (i.e., Control, Low, Medium or High) and monitored until hatching (total N = 4 treatments * 15 clutches representing 60 tanks containing 30 eggs each).

Measurements

We determined the stage of development using Gosner stages (Gosner, 1960).

Embryos were checked twice a day. Hatching occurred at Gosner stage 22 (hereafter GS 22) after 16.10 ± 0.02 days.

At GS 22, hatching success, mortality and deformation rates were assessed as follows: undeveloped eggs and embryos that did not hatch were counted, and hatchlings (GS 22) were qualified as normal, dead or deformed. All live and undeformed tadpoles were put in a petri dish with water from their own tank and photographed above graph paper in order to measure their length (body; tail and total length, N = 1639). Morphological measurements were performed with the software ImageJ (Schneider et al, 2012). All measurements were performed by the same person throughout the experiment.

All experiments took place in a thermally controlled room with the temperature set at 17°C (both air and water) and under natural 12:12 hr day/night photoperiod.

Statistical Analyses

All data were tested for homogeneity of variance and normality with Barlett's and Shapiro-Wilks tests, respectively. We also checked normality of the residuals using diagnostics plots. The only variable that slightly diverged from normality was development duration. Yet, because the F-statistic is extremely robust to violation of the normality assumption when sample sizes are equivalent among groups and degrees of freedom are large (both conditions were met in our analyses), we also used parametric test to analyze development duration.

Clutch identity significantly influenced all response variables (all $P < 0.001$) and was kept as a random factor in all our analyses. Conversely, the sampling site never influenced our results, and it was excluded from our final analyses.

We did not use tank as the replication unit in our analytical design because each tank represented a single treatment (either control, low, medium or high) and contained only the eggs of one clutch. Balanced sample sizes across clutches and treatments (e.g., each clutch was represented by 30 eggs in each treatment) and the use of clutch identity as a random factor allow to avoid significant pseudoreplication issues.

All statistical analyses were conducted with R.Studio v1.2.1335 (R Core Team, 2019). First, we used Generalized Linear Models (GLMERs, (package *lme4*)) with a log-link function for variables following binomial distribution to test whether there were correlations between treatment and embryonic mortality. Second, we fitted two mixed-effect linear models (LMERs, package *nlme* (Pinheiro et al, 2017)) to analyze differences in development duration and total length with "Clutch" as a random

factor to control for the non- independence of individuals coming from the same clutches. Lastly, we checked for linear relationship between covariate (body length) and response variable (body proportion) using visual inspection of scatter plot, which allowed us to conduct analysis of covariance (ANCOVA) with total length as the covariate.

We performed effect size tests with statistical power analysis to assess the magnitude of the difference between treatments (Cohen, 1977). The magnitude of differences (at $P < 0.05$) between two groups is considered significant when Cohen's δ is close to or above 0.2.

Results

AMPA influenced embryonic mortality ($\chi^2 = 20.894$, $P = 0.0001$, Fig. 1). Relative to control, embryonic mortality was higher for the lowest and intermediate AMPA concentrations (all $P < 0.04$) but similar for the highest AMPA concentration ($P = 0.99$, Fig. 1).

Similarly, AMPA influenced the duration of embryonic development ($F_{3,1615} = 13.33$, $P < 0.001$, Fig. 2). Relative to control, all treated embryos took longer to develop into tadpoles (Fig. 2). Among treatments, the highest AMPA concentration took less time to hatch than the lower and intermediate concentrations (respectively $P < 0.001$ and $P = 0.004$).

Among hatchlings, the number of deformed tadpoles (overall: 6.07 ± 0.57 %), or the number of tadpoles that died upon hatching (overall 0.18 ± 0.10 %) were similar between treatments (ANOVA, all $P > 0.10$).

The morphology of viable hatchlings (i.e., not deformed) was also influenced by AMPA concentrations ($F_{3,1615} = 11.22$, $P < 0.001$, Fig 3). Relative to control ones, tadpoles exposed to the lowest AMPA concentration were larger (TukeyHSD, $P < 0.001$) while tadpoles exposed to higher AMPA concentrations were marginally smaller (TukeyHSD, $P = 0.063$, Fig. 3). Importantly, AMPA modified body proportions (Fig. 3). Although body lengths were similar between treatments (ANCOVA with the total length as a covariate, $F_{3,1615} = 0.64$, $P = 0.59$, Fig. 3), tail lengths were different (ANCOVA with the total length as a covariate, $F_{3,1615} = 3.2241$, P

=0.02, Fig. 3). Relative to the Low treatment, tadpoles exposed to the highest AMPA concentration had shorter relative tail length (TukeyHSD, $P = 0.01$).

Tests of effect sizes supported these results (Appendix 1).

Discussion

Based on a very large sample size (i.e., > 2000 eggs), our results show that chronic exposure to low-level, environmentally relevant concentrations of AMPA, a primary contaminant detected in surface waters (Grandcoin et al., 2017; Coupe et al., 2012; Bonansea et al., 2017) alters the quality of embryonic development in Spined toads. More specifically, our results show that AMPA influences embryonic mortality, development duration and body architecture in a complex way. Interestingly, the response patterns of all measured traits diverged from classical linear concentration-response relationships.

Embryonic mortality was 2 to 3 times higher in eggs that developed with the lowest and the intermediate AMPA concentrations. Focusing solely on the differences among control embryos and the embryos raised at the two lower AMPA concentrations, the pattern we found could suggest a direct lethal effect of AMPA on embryos, presumably through increased toxicity driven by increased AMPA concentration (Broomhall et al., 2003; García-Muñoz et al., 2010; Baier et al., 2016a,b). However, the results from the highest AMPA concentration do not support the hypothesis of increasing AMPA concentrations causing increased mortality. Indeed, embryos that were exposed to the highest AMPA concentration developed and survived as well as those in the control treatment. A non-linear survivorship pattern is clearly more complex than classical linear concentration-response relationships and future studies are required to assess whether this pattern is maintained with other increasing test concentrations (see also below). Development duration was

also significantly influenced by a chronic exposure to AMPA. Indeed, chronic exposure to AMPA seems to delay hatching as development duration was longer in all groups exposed to AMPA, irrespective of the concentration. The two lower AMPA concentrations yielded similar development durations, while the highest concentration of AMPA produced development duration that were significantly shorter than that of the two lower AMPA concentrations, although longer than that of control embryos. Again, this pattern seems more complex than classical concentration-responses and future studies should usefully investigate the outcome of similar approaches with higher AMPA concentrations. We found that chronic exposure to AMPA influences not only the size of tadpoles upon hatching, but also their body proportions. Embryos submitted to the lowest AMPA concentration produced longer tadpoles, with similar body length than other groups but longer tails. Conversely, embryos submitted to the highest AMPA concentration produced shorter tadpoles, with shorter tails. Similarly to the previous metrics of embryonic development we studied, these response patterns of morphology contrast from classical concentration-responses, and seem to point to a concentration-dependent effect of AMPA on body architecture. Such response clearly deserves future investigations in order to clarify the mechanisms through which exposition to AMPA during embryonic development influences overall morphology.

Whether the alteration of embryonic development we highlighted bears consequences for developing tadpoles in natural ponds remains complicated to directly assess with our experimental design. Increased embryonic mortality could

decrease the reproductive success of toads that lay their eggs in a contaminated pond (Haye et al., 2006, Richter et al., 2003). Delayed hatching may potentially increase the vulnerability of immobile embryos to predators (Zamudio et al., 2016). In addition, delayed embryonic development may also induce cascading effects on the subsequent larval (tadpole) development duration, a potentially deleterious consequence if spawning takes place in ephemeral water bodies. Finally, reduced body size may decrease mobility and thus foraging ability while increasing susceptibility to predation (Broomhall et al., 2003; Hoff & Wassersug., 2000). Although taken together all of these elements tend to suggest ecological consequences for embryos (and may be tadpoles) developing in water contaminated by environmental concentrations of AMPA, these hypotheses need to be taken with caution and will require thorough testing, especially as post-embryonic survival may be more important to population persistence than egg survival (Vonesh & De la Cruz., 2002). In addition, it is important to highlight the fact that the absolute magnitude of the effects we found appears relatively small. Indeed, although embryonic mortality was marked by a two- or three-fold increase in some of our experimental concentrations, development duration increased by ~2.5% and body size was either increased by ~2.1% or decreased by ~1.2%. Although we cannot entirely rule out that the ecological significance of these effects may be trivial they could equally induce consequences on fitness-related traits such as parent reproductive success (embryonic mortality) and offspring survival (development duration, tadpole morphology) and thus may ultimately affect population persistence (Hayes et al, 2010; Orton et al., 2015). Such results are clearly important

to take into account at a time when amphibians are dramatically declining (Hayes et al, 2010; Berger et al, 2013; Grant et al, 2016., Wake., 2012). Importantly, our results contrast with those found with the parent compound on similar species (Baier et al., 2016a,b) suggesting that AMPA can be at least as toxic as glyphosate or its formulation containing surfactants (Giesy et al., 2000; Relyea., 2009; Moore et al., 2012). Yet, we clearly lack studies regarding its toxicity at ecologically relevant concentrations in non-target species (Matozzo et al., 2018, 2019).

Perhaps more importantly than the magnitude of the effects *per se*, we emphasize the complex patterns of concentration-response curves we found. Indeed these patterns contrast with other studies that have focused on much higher test concentrations (FAO., 2013), and may indicate low-concentration effects and nonmonotonic concentration responses (Vandenberg et al., 2012). Such nonmonotonic responses were not expected, and thus we lack the power to thoroughly test for this hypothesis because verifying nonmonotonicity requires at least two concentrations on either side of the inflection point (OECD., 2014). Yet, it is important to stress that low-concentration effects and nonmonotonic responses seems to be strongly linked to endocrine disruption (Vandenberg et al., 2012), a possible underlying mechanism to the complex patterns we found for all metrics studied. If such hypothesis is true, deciphering the targets of this putative endocrine disruption will be critical to comprehensively assess the impact of glyphosate's primary metabolite. In addition, this hypothesis would add to the recently growing evidences that, for anthropogenic contaminants, nonmonotonic response curves at low concentrations are much more

widespread than previously suspected (Hill et al., 2018). Yet, nonmonotonicity, although fundamental for regulatory decision-making, occurs at minute concentrations that are usually disregarded by regulatory toxicology (i.e., in our case 4000 times lower than PNEC). Clearly, decision-making needs to go beyond the use of high-dose studies to infer official No Effect Concentrations (Hayes et al 2010; Hill et al., 2018). As a consequence, our study pleads for the investigation of parent compounds but, perhaps more importantly, also their metabolites at environmentally relevant concentrations in order to comprehensively assess impacts of anthropogenic contaminants on the environment.

More generally, our results emphasize the potential effects of AMPA on human and environmental health (e.g., Matozzo et al., 2018, 2019; Martinez & Al-Ahmad 2019). Indeed, the putative nonmonotonic responses we detected, and thus the underlying potential endocrine disruption (Vandenberg et al., 2012) can be of critical importance for both humans and wildlife. This seems especially important given that our tested concentrations are very similar to concentrations found in aquatic environments in Northern America and Europe. Future studies are required to assess whether other study models can be affected by environmental concentrations of AMPA.

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Caption to figures

Figure 1. Embryonic mortality relative to AMPA concentrations. Lowest and intermediate concentrations yielded significantly higher mortality. Letters indicate significant differences (Tukey HSD, $p < 0.05$).

Figure 2. Embryonic development duration relative to AMPA concentrations. All AMPA concentrations yielded delayed hatching. Letters indicate significant differences with controls (Tukey HSD, $p < 0.05$).

Figure 3. Hatchlings morphology at Gosner stage 22 (upper panel: total length, middle panel: head length, lower panel: tail length) relative to AMPA concentrations. Lowest AMPA concentration yielded longer tadpoles (with longer tails) while highest AMPA concentration yielded shorter tadpoles (with shorter tails). Letters indicate significant differences with controls (Tukey HSD, $p < 0.05$).

Figure 1.

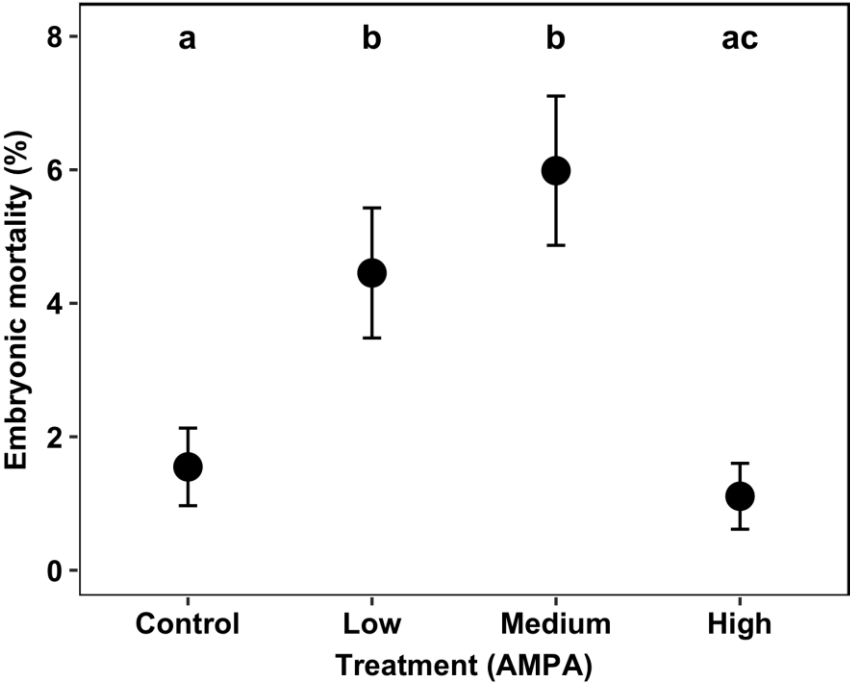


Figure 2.

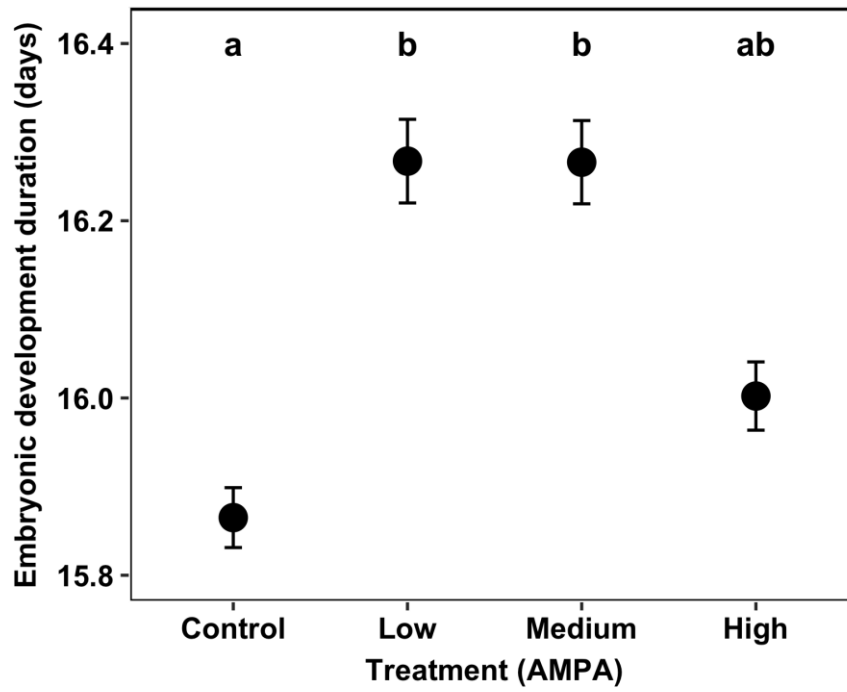
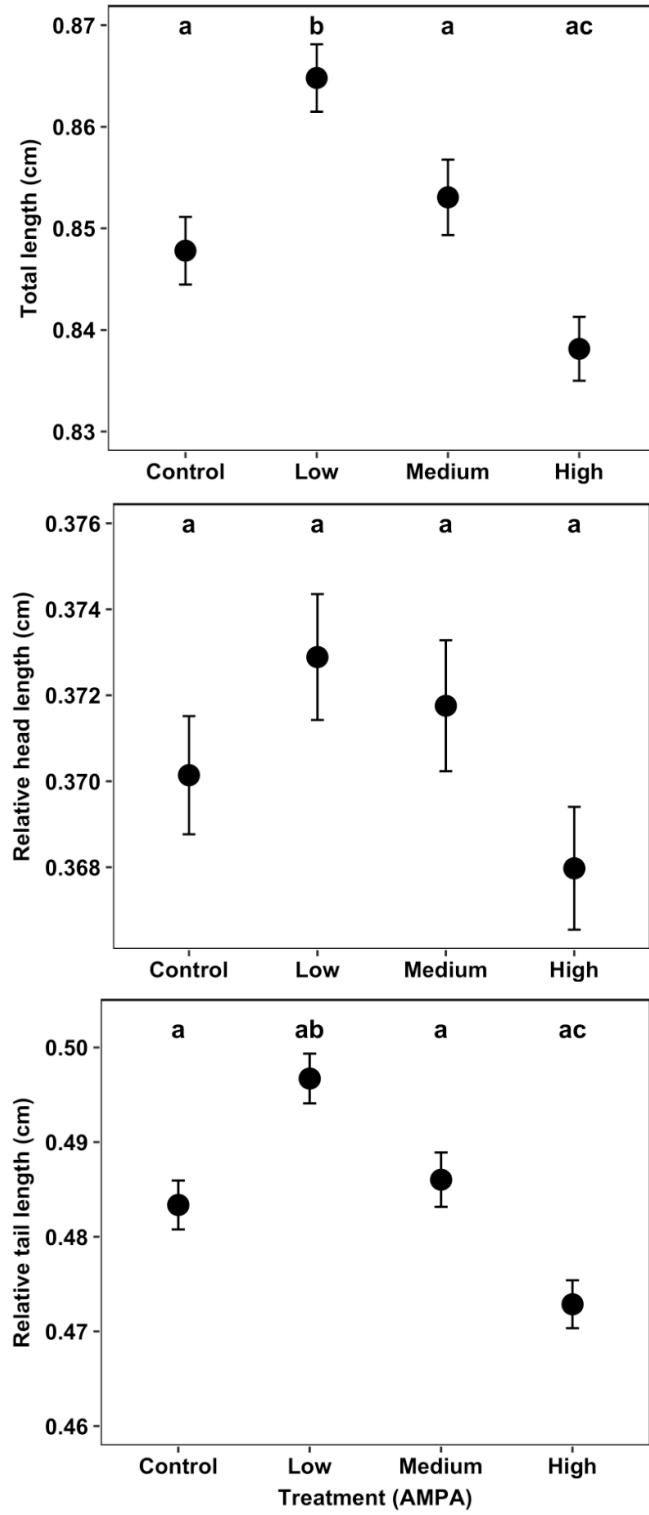


Figure 3.



Appendix 1. Effect size of pair-wise comparisons using Cohen's δ statistics. C stands for control. The magnitude of differences (at $p < 0.05$) is considered significant for Cohen's δ that are reaching values close to or above 0.2 and are indicated in bold. Cohen's δ is not indicated for non-significant differences ($p > 0.05$).

Pair-wise comparisons	Mortality	Development duration	Morphology
C - Low	0.170	0.314	0.253
C - Medium	0.234	0.230	-
C - High	-	0.164	0.147
Low - Medium	-	-	0.168
Medium - High	0.187	0.144	0.217
Low - High	0.249	0.160	0.409