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Behavioral effects of the neurotoxin β-N-methylamino-L-alanine on the mangrove rivulus (*Kryptolebias marmoratus*) larvae

Alessandra Carion, Julie Hétru, Angèle Markey, Victoria Suarez-Ulloa, Silvestre Frédéric

Laboratory of Evolutionary and Adaptive Physiology, Institute of Life, Earth and Environment, University of Namur, Namur, Belgium

Abstract

Mangrove rivulus, *Kryptolebias marmoratus*, is a hermaphrodite fish capable of self-fertilization. This particularity allows to naturally produce highly homozygous and isogenic individuals. Despite the low genetic diversity, rivulus can live in extremely variable environments and adjust its phenotype accordingly. This species represents a unique opportunity to clearly distinguish the genetic and non-genetic factors implicated in adaptation and evolution, such as epigenetic mechanisms. It is thus a great model in aquatic ecotoxicology to investigate the effects of xenobiotics on the epigenome, and their potential long-term impacts. In the present study, we used the mangrove rivulus to investigate the effects of the neurotoxin β-N-methylamino-L-alanine (BMAA) on larvae behaviors after 7 days exposure to two sub-lethal concentrations. Results show that BMAA can affect the maximal speed and prey capture (trials and failures), suggesting potential impacts on the organism's fitness.

Introduction

Pollution involving neurotoxic compounds (NCs) is one of the emerging issues for human health but also for wild species and ecosystems. In wild organisms, the adverse outcome of exposure to NCs is a change in behavior, which can affect an individual's fitness and potentially lead to population decline.[1] Moreover, it is well established that embryonic development and early life stages (ELS) are periods during which the organism is particularly sensitive to environmental stress, and consequently to NCs exposure. In addition to immediate effects on ELS, developmental exposure at relatively low dose can lead to adverse outcomes later in life, at the adult stage, an idea accepted under the concept of Developmental Origin of Health and Disease (DOHaD).[2] However, even if experimental and epidemiological studies support this concept, the mechanisms explaining long-term delayed effects of early life NCs exposure remain largely unclear.[3]

Nowadays, an increasing number of people suffer from neurological disorders such as Alzheimer's, Parkinson's or Amyotrophic lateral sclerosis diseases, which could be partly related to environmental influences during ELS. Such diseases are triggered by a complex interplay between genes, ageing, and environmental conditions plus a random component.[4] In this context, the need for understanding the role played by exposure to NCs has been growing in importance.[5] Of particular interest is β-N-methylamino-L-alanine (BMAA), a non-protein amino acid NC produced by the extremely ubiquitous cyanobacteria, dinoflagellates and diatoms.[6] Although the first link between BMAA and neurodegenerative diseases was established more than 50 years ago, evidence of BMAA effects on human brain is still inconclusive and remains controversial.[7] On the other hand, considering that BMAA can be found in aquatic food webs at high concentrations,[8] further studies about BMAA risk assessment on aquatic organisms are urgently required.

With this aim in mind, the present work has been developed using a strategic model organism, the mangrove rivulus fish. Together with its sister species, the mangrove rivulus (*Kryptolebias marmoratus*) is the only known vertebrate that naturally reproduce by self-fertilization.[9] In nature, this Cyprinodontiform oviparous fish is characterized by an androdioecious mixed-mating reproductive system, in which hermaphrodites coexist with a low proportion of males (between 5 and 25%). While outcrossing with males is known to happen, it is much less frequent than self-fertilization of hermaphrodites. Consistent self-fertilization is an extreme form of inbreeding and it consequently naturally produces isogenic lineages after a few generations.[10] Additionally, despite its low genetic variability, the rivulus displays a high level of phenotypic plasticity and it is capable to efficiently acclimate to the highly variable environment of mangrove forests. These features have recently put the mangrove rivulus in the spotlight of scientific research, as it constitutes an innovative and valuable model for the identification of true cause-effect relationships between the environment, the phenotype and the epigenome.

Impacts of NCs on the brain epigenome are one of the most often cited mechanisms potentially explaining neuronal disorders and DOHaD.[3] Effects on the epigenome do not involve changes in the DNA sequence but can modify gene expression patterns in a heritable manner through mechanisms such as DNA methylation (DNAme), histone modifications, and non-coding RNAs. Together, the emerging fields of neuroepigenetics and environmental epigenetics show that exposure to environmental NCs can affect the brain epigenome and consequently lead to impacts on behavior and/or cognitive faculties.[11] In this context, the rivulus constitutes an optimal model choice to investigate the role of epigenetic mechanisms in DOHaD and in transgenerational inheritance via sexual reproduction, while minimizing the confounding effects of genetic variation.[12,13]

The main objective of this study was to determine the immediate effects of BMAA exposure during ELS on locomotion and prey capture behaviors in the rivulus. Moreover, this preliminary work paves the ground towards a better understanding of the conspicuous consequences that NCs may have for populations of aquatic organisms and their link with molecular mecha-

Correspondence: Alessandra Carion, Laboratory of Evolutionary and Adaptive Physiology, Institute of Life, Earth and Environment, University of Namur, 61 Rue de Bruxelles, 5000 Namur, Belgium.
E-mail: alessandra.carion@unamur.be

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nisms, such mechanisms including modifications of the expression level of some key genes involved in behavior and in nervous cells metabolism as well as potential variations in epigenetic mechanisms.

Materials and Methods

Rivulus larvae were exposed to 2 doses of BMAA directly after hatching for 7 days: 20 µg/L and 15 mg/L, plus a control unexposed group (n = 24). Larvae were fed *ad libitum* with *Artemia salina* every day. The BMAA working solution was made from L-BMAA Hydrochloride powder (Sigma-Aldrich®) mixed with 25 ppt (± 1) water. About 2/3 of water was renewed every day. During exposure period, mortality was assessed and larvae were individually measured at 1, 3, 5 and 7 days post-hatching (dph) using a Nikon Digital Camera USB3 ½.5 15 IM/SEC mounted on a Nikon SMZ1270 stereomicroscope and the NIS-Elements® program. Larvae locomotion and thigmotaxis (test adapted from Norton, 2012) were video recorded after 7 days of exposure (10 min of acclimation followed by 5 min of test) in 6-wells microplate. Larvae behaviors were analyzed using Ethovision® software. Afterwards, 10 nauplii of *Artemia* were provided in each well to measure larvae prey capture ability during 5 min. Results are expressed as the percentage of success and failure, calculated as the proportion between successful capture or failed capture trials over the total number of trials.

Agostino and Pearson tests were performed to confirm data normality. Accordingly, one-way ANOVA or non-parametric Kruskal-Wallis tests were applied followed by Dunn's multiple comparisons test in order to evaluate an effect of BMAA on larvae locomotion, thigmotaxis or capability to capture prey. Percentages of success and failure were arcsine square root transformed. Statistical analyses were performed using GraphPad Prism 7 Software. Significance level was set at $P<0.05$.

Results and Discussion

No effect of BMAA exposure was observed on larvae growth, body shape and mortality. Locomotion test showed a significant effect of 20 µg/L BMAA exposure on larvae maximum velocity with a 61% increase compared to group controls (Table 1 and Figure 1A), while no effect has been observed at the highest concentration. The prey capture assay revealed significant effects of BMAA exposure on the percentage of failure in prey capture and on the total trials expressed by larvae to capture prey (Figure 1B-C). The 20 µg/L and 15 mg/L groups respectively expressed a 14% and 17% increases in total trials compared to group-controls. Moreover, these two exposure concentrations respectively expressed a 13% and 15% increases in percentage of prey capture failure compared to controls.

According to these results, BMAA exposure during development impacts larvae locomotion and capabilities to behave appropriately to efficiently hunt their prey. BMAA appears to mainly disrupt brain functions. Larvae maximum velocity was impacted by BMAA exposure at lowest concentration (20 µg/L) while no effect was evident at higher concentration (15 mg/L). This suggests that an environmentally relevant concentration of BMAA applied during fish development can elicit changes in maximum velocity possibly due to neuromuscular effects and/or impairments in fish perception of its environment.[14,15] However, these effects were not observed at a higher concentration suggesting a possible activation of repair mechanisms limiting brain damages.[16] Interestingly, the total number of prey capture for larvae exposed to BMAA did not differ from control group while larvae showed a higher rate of failure since they tried more to catch a prey. This indicates that larvae exposed to BMAA need more trials to achieve the same success in prey capture with probably more energy expenditure. These observations support the assumptions of movement impairments induced by BMAA exposure and are consistent with literature in behalf of assumptions that BMAA can disrupt synaptic signaling.[17] Effects observed on larvae serve as a baseline to assess possible consequences later in life. Movement impairments can lead to lower food intake and consequently impact fish growth, reproduction and therefore organism fitness.

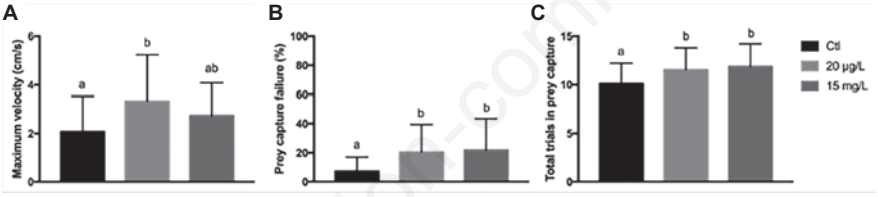

Figure 1. Immediate effects of 7 days β-N-methylamino-L-alanine (BMAA) exposure on newly hatched rivulus larvae behaviors. A) A significant effect at 20 µg/L BMAA was observed on larvae maximum velocity (P-value = 0.035); B) Larvae exposed to 20 µg/L and 15 mg/L BMAA expressed significant higher rate of failure during prey capture assay (P-value = 0.016 and 0.004, respectively); C) significant higher trials to capture prey (P-value = 0.04 and 0.027, respectively) compared to controls. Results are expressed by mean ± SD. Different letters (a-b) mean significant differences (*i.e.*, P-value <0.05) between conditions.

Table 1. Summary table including the number of biological replicates per treatment and results of locomotion and prey capture tests. Mean ± SD.

Treatments	Number of replicates	Locomotion test			Prey capture test			
		Distance moved (cm)	Thigmotaxis	Maximum velocity (cm/s)	Total trials	Prey capture success (%)	Prey capture failure (%)	Maximum velocity (cm/s)
Ctl	24	39.33±36.14	0.83±0.48	2.06±1.46	10.08±2.12	94±17	7±10	3.09±5.11
20 µg/L	23	55.92±39.13	0.92±0.34	3.32±1.90	11.52±2.27	89±26	20±19	5.47±9.41
15 mg/L	24	49.45±25.30	0.91±0.22	2.72±1.36	11 .83±2.37	97±22	22±22	4.31 ± 6.34

Epigenetics can explain long-term latent or transgenerational effects. These hypotheses will be tested in future experiments.

Conclusions

Overall, 7 days exposure of sub-lethal concentrations of BMAA on newly hatched larvae of mangrove rivulus revealed significant effects on fish behavior. BMAA showed non-monotonic effects, as 20 µg/L exposure increased maximum larvae speed, while 15 mg/L exposure had no effect. This emphasizes the need to test low concentrations when assessing the environmental risk. Moreover, both concentrations under study increased the number of trials necessary for the rivulus to catch the same amount of prey, suggesting impairment in energy expenditure and possible impacts on animal's fitness. Further molecular analyses such as gene-specific DNAme and gene expression in brain will provide new insights into the modes of action of BMAA. Further experiments on the delayed effects in adults will afford a better understanding of long-term consequences of BMAA presence in the environment and the putative roles of epigenetic mechanisms in neurotoxicity. These preliminary findings stress the importance of ecotoxicological studies about BMAA and other NCs on wild organisms, such as the mangrove rivulus, in addition to studies on human neurodegenerative diseases, as its presence in the environment could have consequences on populations' survival even at environmental concentrations, jeopardizing whole communities interconnected in trophic networks.

Research highlights

BMAA negatively impacts rivulus' capacity of prey hunting.

Needs to test low concentrations of toxicants to assess environmental risk.

The mangrove rivulus is a new valuable model species to test long-term effects of pollutants.

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