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Energetic endpoints provide early indicators of life history effects in a freshwater gastropod exposed to the fungicide, pyraclostrobin

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A B S T R A C T

Organismal energetics provide important insights into the effects of environmental toxicants. We aimed to determine the effects of pyraclostrobin on *Lymnaea stagnalis* by examining energy allocation patterns and life history traits. Juvenile snails exposed to pyraclostrobin decreased feeding rate and increased apparent avoidance behaviors at environmentally relevant concentrations. In adults, we found that sublethal concentrations of pyraclostrobin did not affect reproductive output, however, there were significant effects on developmental endpoints with longer time to hatch and decreased hatching success in pyraclostrobin-exposed egg masses. Further, there were apparent differences in developmental effects depending on whether mothers were also exposed to pyraclostrobin suggesting this chemical can exert intergenerational effects. Pyraclostrobin also affected protein and carbohydrate content of eggs in mothers that were exposed to pyraclostrobin. Significant effects on macronutrient content of eggs occurred at lower concentrations than effects on gross endpoints such as hatching success and time to hatch suggesting potential value for these endpoints as early indicators of ecologically relevant stress. These results provide important insight into the effects of a common fungicide on important endpoints for organismal energetics and life history.

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1. Introduction

A primary goal in ecotoxicology is to predict adverse effects of chemicals in natural systems; however, there is a widespread acknowledgment that standard toxicity assays and endpoints provide limited insight into effects in these systems (Kimball and Levin, 1985). To that end, other approaches including mechanistic modeling and various "omics" methods are being developed to better relate observed effects to ecological risk (Chapman, 2002; Beketov and Liess, 2012). Organismal energetics may be a useful framework in ecotoxicology to assess and integrate effects of chemical stressors (Sokolova et al., 2012; Congdon et al., 2001; Zonneveld and Kooijman, 1989). The use of energy or energy budgets in ecotoxicology is not a new concept (Congdon et al., 2001) but recent advances in the application of Dynamic Energy Budget theory (Jager et al., 2006) and the development of biochemical/omics measurement techniques (e.g., De Coen and Janssen, 2003) provide new opportunities for further development. Importantly, novel insights can be gleaned with perhaps only minimal additions to standardized toxicity tests that already include measures of energetic impacts via changes in primarily growth and reproduction.

Importantly, energy-based responses can be used to detect sublethal effects that may eventually manifest at higher levels of organization. Because the majority of an organism's energy budget is used for growth, reproduction, and basal metabolism, increased energy expenditure to cope with the toxic stress can lead to a reduction in energy reserves (Congdon et al., 2001; Rowe et al., 2001; Sancho et al., 2009). Toxicant-induced changes in metabolic rate (Rowe et al., 2001), lipid reserves (Sancho et al., 2009), feeding rate (Maltby et al., 2002), cellular energy assimilation (De Coen and Janssen, 2003), and other metrics provide valuable insights into sublethal effects with clear linkages to higher order processes. In the long run, for example, a depletion of energy reserves may have consequences for individual fitness (e.g., diminished growth) (Roex et al., 2003), which then translate to changes in population dynamics (Martin et al., 2013). The responsiveness of organismal energetic endpoints to a variety of chemical classes has yet to be evaluated, although it is reasonable to hypothesize that all

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chemicals are likely to impact energetics at some level. However, for chemicals with known impacts on conserved energetic systems, an organismal energetic or bioenergetics perspective may provide valuable ecotoxicological insight. Some fungicides, for example, operate on conserved energetic systems and therefore may impact the energy budgets of non-fungal species.

Increased fungicide use in recent years, specifically pyraclostrobin, is due in part to recent outbreaks of soybean rust caused by the fungus, *Phakopsora pachyrhizi* (Deb et al., 2010). Pyraclostrobin belongs to the group of respiration inhibitors classified as "quinone outside inhibitors" (QoI, BASF Corporation, 2012). The mode of action of QoIs relies on their ability to inhibit mitochondrial respiration by binding at the outer quinol-oxidation site (Q_o site) of the cytochrome bc_1 enzyme complex III ultimately preventing the production of ATP. Although designed to target fungi, QoI fungicides can show toxicity towards animals (Hartman et al., 2014; Morrison et al., 2013; Belden et al., 2010), but data are lacking for many taxa (Bartlett et al., 2002).

Because fungicides are often applied via aerial spray (BASF Corporation, 2008), aquatic environments adjacent to or imbedded in agricultural landscapes may be inadvertently exposed (Morrison et al., 2013). Given the use and exposure profile of many fungicides, it is important to understand how chemicals like pyraclostrobin impact aquatic species in order to develop better environmental management practices and to protect important ecological resources. Gastropods are ecologically important model organisms (Rittschof and McClellan-Green, 2005) and are seeing more widespread use in aqautic toxicity testing (Ducrot et al., 2010 & 2006; Bandow and Weltje, 2012; De Vaufleury et al., 2006). *Lymnaea stagnalis* is a particularly promising model organism having been identified as a prospective species for evaluating reproductive toxicity (Matthiessen, 2008). As a member of the pulmonate subclass, *L. stagnalis* have a limited ability to exploit deeper water habitats (Corr et al., 1984), increasing the risk of direct exposure to chemical stressors such as pesticides that are sprayed directly or by drift onto aquatic systems during application.

The purpose of this study was to evaluate the toxicity of environmentally relevant concentrations of pyraclostrobin to the freshwater snail, *L. stagnalis*. The specific objectives were to (1) determine acute toxicity of pyraclostrobin to juvenile and adult snails; (2) evaluate growth, feeding rate, and behavior of exposed juveniles; (3) assess the reproductive output of adult snails; (4) evaluate effects on development and the potential for intergenerational effects; and (5) analyze macronutrient content as an indicator of the effects of pyraclostrobin on bioenergetic allocation patterns. We discuss the results in light of a bioenergetics perspective on organismal responses to stress.

2. Methods

2.1. Test organisms

L. stagnalis (Linnaeus, 1785) (Gastropoda, Basommatophora) is a freshwater gastropod abundant in ponds, lakes, and ditches (Berrie, 1965). These habitat preferences and life history traits increase the risk of exposure due to direct and drifting pesticide spray. Our laboratory culture was initiated with egg masses obtained in 2013 from a culture at the University of Miami.

2.2. Laboratory conditions

Snails were raised in moderately hard water synthesized by the addition of several salts to deionized water (60 μg/L $CaSO_4$, 60 μg/L $MgSO_4$, 4 μg/L KCl, and 98 μg/L $NaHCO_3$; hereafter, referred to as lab water). Snails were housed in glass aquaria of various sizes (from 1 to 9 L) dependent on life history stage and size, with constant aeration once reaching the sub-adult phase. The culture was maintained at 20 ± 2 °C with a photoperiod of 16:8 h (light:dark). Complete water changes and refreshing of food was conducted one to two times per week. Snails were fed organic romaine lettuce *ad libitum* and deceased snails were removed immediately.

2.3. Chemical treatments

Pyraclostrobin (CAS: 175013-18-0; purity 99.9%) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved using HPLC grade acetone as a carrier. Nominal concentrations of pyraclostrobin used for the following tests are listed in Table 1. Concentrations of pyraclostrobin in surface waters are generally in the mid to high ng/L range (Reilly et al., 2012) but can be in the high μg/L range after direct runoff events (Battaglin et al., 2011; Deb et al., 2010) or in cases of a direct overspray to water bodies (e.g., Hooser et al. 2012). Hence, the pyraclostrobin exposure concentrations used in our experiments (up to 20 μg/L during longer duration exposures) are very likely on the high-end of environmentally relevant surface water concentrations.

2.4. Acute toxicity of pyraclostrobin to L. stagnalis

As an initial characterization of pyrclostrobin toxicity to *L. stagnalis*, we conducted two 96 h, acute toxicity studies on juveniles and adults. For each replicate, ten juveniles, approximately 2 weeks old, were randomly selected from cultures (Fisherbrand®, polyethylene) photographed, weighed as a group, and placed into 300 mL treatments (Table 1). Length was measured from images using ImageJ®. Each treatment was replicated 3 times ($n = 3$, 21 experimental units). Snails received 2 cm^2 of lettuce at initiation. Juveniles were provided lettuce to avoid starvation-related mortality. Water changes, concentration renewal, and food replacement were completed at 48 h. Survival was checked every 24 h and snails were removed if observed on bottom and unresponsive to gentle prodding. All removed snails were verified as dead after inspection for a heartbeat.

In the adult toxicity study, 4.5 month old snails, were selected at random from culture tanks. Five adults per replicate were measured, weighed, and placed into 600 mL treatments (Table 1). Each treatment was replicated four times ($n = 4$, 24 experimental units). Experimental conditions and water changes were as above except adult snails were not fed.

2.5. Sublethal toxicity

Twenty juvenile snails (4 weeks old) were selected, photographed, weighed as a group, and placed into 300 mL replicates (Table 1) with 6 replicates per treatment ($n = 6$, 30 experimental units). Water changes, concentration renewal, and food replacement were completed every 48 h. Survival, feeding rate, and behavior were recorded every 48 h for 22 days. To estimate feeding rate, snails received pre-weighed food (2 cm^2 lettuce) and after 48 h, ort was removed, blotted dry and weighed. Total lettuce eaten was divided by number of snails in the treatment during the 48 h period. The proportion of juveniles on lettuce and above the waterline was recorded to further estimate feeding activity and to assess potential avoidance of pyraclostrobin. Growth was estimated from digital images as the difference between initial and final shell length (lip to apex measured with ImageJ® software). Final wet mass was also used as a growth metric. Upon termination, total juveniles remaining per treatment were randomly divided and weighed for macronutrient analysis (4–10 individuals per treatment). Combined mass was used to calculate milligram of

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