Agrichemicals chronically inhibit the cortisol response to stress in fish

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Highlights

- We studied the stress response of Rhamdia quelen following exposure to agrichemicals.
- Acute exposure of fingerling-aged fish to agrichemicals chronically inhibits stress response.
- The stress axis of fish exposed to MPBI and to TBF were fully recovered after a 180 d.
- The acute exposure to the tested agrichemicals impairs fish growth and survival.

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Abstract

We studied the stress response of Rhamdia quelen fingerlings at 45, 90, 135 and 180 d following acute exposure to agrichemicals. Herein, we report the novel observation that acute exposure of fingerling-aged fish to a methyl parathion-based insecticide (MPBI) and to a tebuconazole-based fungicide (TBF) induced chronic inhibition of the stress response. In contrast, fish exposed to an atrazine–simazine-based herbicide (ASBH) recovered the stress response on day 45, and fish exposed to a glyphosate-based herbicide (GBH) did not present stress response inhibition. Additionally, fish exposed to MPBI, GBH and ASBH showed lower survival rates and attained lower final weights. In the case of TBF, the presence of the stressful stimulus more strongly influenced the changes in the performance parameters than did the agrichemical exposure itself. An impairment of the cortisol response may seriously hamper the adaptive response and the ability to promote the necessary metabolic and ionic adjustments to respond to environmental stress.

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1. Introduction

Exposing fish to various stressful situations, such as environmental changes, prey-predator interactions and, in the case of aquaculture, several different management procedures (such as the transfer of fish to a different tank or biometrics measurements), triggers a cascade of adaptive alterations. These events have been classified as a stress response (Barton and Iwama, 1991; Wendelaar Bonga, 1997) and are coordinated by the hypothalamus–pituitary-interrenal (HPI) axis (Barton, 2002). The end product of activation of the HPI axis is the glucocorticoid cortisol, which is a regulator of the necessary metabolic and ionic adjustments for coping with stress (Mømmsen et al., 1999). Thus, an impairment of the cortisol response may seriously hamper the overall adaptive response and the ability to maintain metabolic and osmoionic homeostasis.

Both chronic and acute exposures to environmental contaminants might disrupt the stress axis and consequently affect stress reactivity in fish (Hontela et al., 1997; Girard et al., 1998; Norris et al., 1999; Pacheco and Santos, 2001; Dorval et al., 2005; Gravel and Vijayan, 2007; Horii et al., 2008), including in jundiá (Cericato et al., 2008, 2009). However, the long-term effects on fish lifespan and stress...
reactivity after acute exposure to agrichemicals during an early life stage have not been reported in the literature. This type of exposure is plausible because most natural and constructed water bodies are located near agricultural areas or have been filled with water that ran through cultivated soil. Significant amounts of the products used in crop production, such as herbicides, pesticides and fungicides, could reach these water bodies and affect non-target organisms (van der Oost et al., 2003).

To address this potential exposure scenario, we posed three questions. First, does an impairment of the cortisol stress response occur following exposure to the test agrichemicals? Second, can the fish restore their ability to trigger the response? Third, could this initial exposure affect survival rates and performance parameters? To answer these questions, fingerlings were acutely exposed to agrichemicals and then monitored for 6 months to assess the long-term effects on both the cortisol response to new stressors and growth performance parameters. This exposure and recovery paradigm was evaluated with fish fingerlings that were stocked in aquaculture ponds at the time of agrichemical application to nearby agricultural fields.

2. Material and methods

2.1. Ethical note

This study was approved by the Ethics Commission for Animal Use (CEUA) of Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol#3/2011-CEUA, July 2009) and met the guidelines of the Brazilian College for Animal Experimentation (COBEA; http://www.cobea.org.br).

Stress response evaluation based on only peak cortisol measurements (Koakoski et al., 2012; Barcellos et al., 2012a) is derived from established animal welfare science. Several previous studies have used this methodology to evaluate the stress response in Rhamdia quelen (Cericato et al., 2008, 2009). Thus, we selected this single point evaluation methodology to prevent the use and sacrifice of more experimental fish than necessary to draw conclusions regarding agrichemical effects on cortisol profiles.

2.2. Location and study subjects

Experiments were conducted from September 2011 to March 2012 at the facilities of the Universidade de Passo Fundo in Rio Grande do Sul, Brazil. We used 90-day-old, mixed-sex R. quelen (Heptapteridae, Teleostei) juveniles from Jundiaí municipality with an average weight of 11.2 ± 0.32 g (mean ± SEM, n = 1440, 360 exposed to agrichemicals). The fish were kept in a 6200-L plastic tank prior to being transferred into experimental tanks under a natural photoperiod. The fish were fed twice a day, at 10:00 and 16:00 h, with commercial extruded food provided at 5% of body weight (42% crude protein, 3400 kcal kg⁻¹ digestible energy, DE).

The mean water temperature in all of the tanks was maintained at 24 ± 2°C, and the dissolved oxygen concentrations varied from 5.6 to 7.2 mg L⁻¹ (both measured using a YSI model 550A oxygen meter; Yellow Springs Instruments, USA). The pH values ranged from 6.2 to 7.4 (measured using a Bernanuer pH meter). The total ammonia–nitrogen concentration was less than 0.5 mg L⁻¹ in each of the tanks (measured using a colorimetric test), the total alkalinity was 60 mg L⁻¹ of CaCO₃, and the hardness was 65 mg L⁻¹ of CaCO₃ (both measured using colorimetric tests).

2.3. Agrichemicals tested

Four experiments were conducted, each with one specific agrichemical used to expose the fish. The agrichemicals used were a methyl-parathion-based insecticide (MPBI, Folisuper600™, 600 g L⁻¹ of O,O-dimethyl O-4-nitrophenyl phosphorothioate), a tebuconazole-based fungicide (TFB, Folicur200CE™, 200 g L⁻¹ of RS-1-p-chlorophenyl-4,4-dimethyl-3-{1H-1,2,4-triazol-1-ylmethyl} pentan-3-ol), a glyphosate-based herbicide (GBH, Roundup Original™, 360 g L⁻¹ of N-phosphonomethylglycine) and an atrazine–simazine-based herbicide (ASBH, Herbinix™, 450 g L⁻¹ of 6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine + 450 g L⁻¹ of 6-chloro-N2,N4-diethyl-1,3,5-triazine-2,4-diamine). The exposure concentration used for each agrichemical was based on previously reported results (Cericato et al., 2008, 2009; Kreutz et al., 2008; Ferreira et al., 2010), and corresponded to 16.6% of the calculated lethal concentration for 50% of animals (LC₅₀) at 96 h (MPBI = 0.80 mg L⁻¹; TBF = 0.88 mg L⁻¹; GBH = 1.21 mg L⁻¹ and ASBH = 1.74 mg L⁻¹). All chemicals were obtained from commercial sources.

2.4. Study design

In each experiment, fish were divided into four treatment groups with three replicates per group, i.e., a total of 12 tanks. Each tank contained 900 L of chlorine-free, well-aerated tap water and 30 fish. Treatment 1 was the control (C) group, in which the fish were kept in water without agrichemical exposure and were not subjected to stress. In treatment 2, the stressed (St) group, the fish were kept in water without agrichemical exposure but were subjected to an acute stress stimulus after 96 h. In treatment 3, the fish were kept in water containing a sub-lethal concentration of the specific agrichemical for 96 h. Finally, in treatment 4, the fish were kept in water contaminated with the same sub-lethal concentration of an agrichemical for 96 h and were subjected to an acute stress stimulus, i.e., being chased with a pen net for 60 s (Barcellos et al., 2004). The elapsed time between the stress application and sampling for all fish was 30 min because previous results indicated that cortisol peaks at this time in fingerling R. quelen (Koakoski et al., 2012; Barcellos et al., 2012a).

After this initial sampling, the fish in all treatment groups were maintained in water for a 180-d recuperation period, in the absence of exposure. During this period, fish from treatment groups 2 and 4 were subjected to a stress test on days 45, 90, 135 and 180, and all of the fish were sampled at these times. A schematic representation of the experimental design is depicted in Fig. 1.

All experiments were conducted using a static-test design during the exposure period. Because cortisol is a glucocorticoid that might be influenced by starvation (Barcellos et al., 2010), the fish were fed daily during the 96 h exposure (24, 48, and 72 h after the beginning of exposure) at a rate of 0.75% of their biomass. During the 180-d recuperation period, the fish were fed twice daily at 5% of their biomass. Food residues and feces were not removed during the exposure period to prevent any stress caused by introducing a cleaning siphon. During the recuperation period, a water change rate of 100% per day (in open circulation system) helped keep the tanks clean, as the tanks had a conical bottom. To ensure optimal conditions for viability, water quality parameters were accessed daily to verify whether parameters ranged within normal concentrations.

The fish were closely observed to identify potential declines in individual and/or group health over the entire exposure period. Abnormal swimming behavior, skin darkening, anorexia and body lesions were observed to detect fish at a moribund stage. Fish in this situation were immediately captured, anesthetized with buffered (NaH₂CO₃) MS222 (300 mg L⁻¹) and euthanized by spinal section.
2.5. Blood sampling and cortisol analysis

For blood sampling, two fish from each tank (n = 6) were captured and anesthetized with buffered (NaH₂CO₃) MS222 (300 mg L⁻¹). After a loss of orientation and complete immobilization, blood samples (0.1–0.30 mL) were drawn from the caudal peduncle using sterile syringes. For the initial sampling, when the fish were very small, the blood collection was drawn from the severed caudal peduncle using heparinized microhematocrit tubes. Fish were euthanized immediately after blood collection by spinal section and discarded in a biological waste collector. The elapsed time from anesthesia to blood collection never exceeded 1 min. Blood was then transferred to 1.5-mL microcentrifuge tubes that were centrifuged at 3000 g for 10 min, and the resultant plasma was stored at -25 °C until analysis. Following blood withdrawal, the remaining fish were transferred to pure water and returned to their original tank after recuperation.

Cortisol was measured in duplicate in the unextracted plasma samples, using fully validated, commercially available EIA kits (EIAgenTM Cortisol, Adaltis Italy S.p.A).

2.6. Performance parameters

During the 180-d experimental period, we evaluated the survival rates and the final weight of the fish in order to calculate the final biomass of each tank. Performance parameters were used to assess the effects of acute exposure to agrichemicals on the growth and performance of fingerling fish.

2.7. Agrichemical concentrations in the water

The water concentration of each tested agrichemical was monitored immediately after application, as well as at 48 and 96 h after application. The agrichemical concentration in the water was analyzed by high-pressure liquid chromatography (HPLC) using the general methodology described by Zanella et al. (2003), along with specific methodologies for MPBI (Sabharwal and Belsare, 1986), TBF (Tang et al., 2010) and GBH (Hidalgo et al., 2004). The water concentration of ASBH was not measured because a determination methodology is currently unavailable.

2.8. Statistics

Data are presented as the mean ± SEM values for each group, calculated using the GraphPad InStat 3.00 statistical package (GraphPad Software, San Diego, CA, USA). The cortisol concentration in each stress test, survival rates and performance parameters were compared using an analysis of variance (ANOVA), followed by Tukey's test. The homogeneity of variance was determined using Hartley's test, and normality was determined using the Kolmogorov–Smirnov test. Differences with p values <0.05 were considered statistically significant.

3. Results

All of the agrichemicals tested were persistent in the water after 96 h after inoculation, with the percentages of the inoculated nominal concentration varying from 55% to 101% (Table 1).

3.1. Cortisol concentrations and performance parameters

3.1.1. MPBI-exposed fish

In the first stress test, after a 96 h exposure to MPBI (Fig. 2), the cortisol levels increased only in the stressed group (St), whereas the St + MPBI group demonstrated cortisol values similar to the control (C) and to the MPBI-exposed fish (P < 0.0001). Similar results were observed following stress tests conducted on day 45 (P < 0.0001). In tests conducted on days 90 and 135, the cortisol concentrations of the St + MPBI group were higher than the concentrations of the C group, but lower than those of the St group (P < 0.0001). In the stress test conducted on day 180, the cortisol concentrations measured in the St + MPBI group were similar to those of the St group, and both group means for cortisol concentrations were higher than those of the C and MPBI groups (P < 0.0001). Treatment of fish with St or MPBI resulted in lower survival rates than those observed in the C group or the St + MPBI group (Fig. 3A1). Fish treated with St, MPBI or St + MPBI attained lower final weights (Fig. 3A2). A similar pattern was observed for the final tank biomass (Fig. 3A3).

3.1.2. TBF-exposed fish

After 96 h of TBF exposure (Fig. 2), the cortisol levels increased only in the stressed group (St), whereas the St + TBF group demonstrated cortisol values similar to the control (C) and to the MPBI-exposed fish (P < 0.0001). Similar results were observed following stress tests conducted on day 45 (P < 0.0001). In tests conducted on days 90 and 135, the cortisol concentrations of the St + TBF group were higher than the concentrations of the C group, but lower than those of the St group (P < 0.0001). In the stress test conducted on day 180, the cortisol concentrations measured in the St + TBF group were similar to those of the St group, and both group means for cortisol concentrations were higher than those of the C and TBF groups (P < 0.0001). Treatment of fish with St or TBF resulted in lower survival rates than those observed in the C group or the St + TBF group (Fig. 3A1). Fish treated with St, MPBI or St + MPBI attained lower final weights (Fig. 3A2). A similar pattern was observed for the final tank biomass (Fig. 3A3).
Table 1
Agrichemical concentrations (mg L\(^{-1}\)) measured in the water immediately after the inoculation and after 48 h and 96 h post agrichemical inoculation.

<table>
<thead>
<tr>
<th>Agrichemical</th>
<th>Nominal concentration (mg L(^{-1}))</th>
<th>Concentrations measured (% mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After inoculation</td>
<td>48 h After</td>
</tr>
<tr>
<td>MPB(^a)</td>
<td>0.80</td>
<td>97%, 0.776</td>
</tr>
<tr>
<td>TEB(^b)</td>
<td>0.88</td>
<td>97.3%, 0.856</td>
</tr>
<tr>
<td>GBH(^c)</td>
<td>1.21</td>
<td>101%, 1.222</td>
</tr>
</tbody>
</table>

\(^a\) MPB = methyl-parathion-based insecticide; TEB = tebuconazole-based fungicide; GBH = glyphosate-based herbicide.

Fig. 2. Plasma levels of cortisol in \textit{Rhamdia quelen} acutely exposed to a methyl-parathion-based insecticide (MPBI, A), a tebuconazole-based fungicide (TBF, B), a glyphosate-based herbicide (GBH, C) or to an atrazine-simazine-based herbicide (ASBH, D), immediately after exposure and at 45, 90, 135 and 180 d of recuperation in uncontaminated water. Small letters above the bars indicate significant differences between the treatment means for that sampling event, as determined by an ANOVA followed by Tukey’s range test.
The C- and TBF-exposed fish demonstrated higher survival rates than those of the St- or the St + TBF-exposed fish (Fig. 3B1). Fish in the C group attained a higher final weight, whereas the St groups showed decreased final weights (Fig. 3B2). The final tank biomass varied by group (Fig. 3B3).

3.1.3. GBH-exposed fish

The presence of GBH (Fig. 2) in the water did not influence the cortisol response to stress. When sampled, the St and St + GBH groups presented similar cortisol peaks; both group means were increased in relation to the C and GBH groups ($P < 0.0001$).

The survival rates of the fish in the groups treated with St, GBH or St + GBH were lower than the survival rate of the fish in the C group (Fig. 3C1). The GBH and St + GBH groups demonstrated greater mean growth than the St group, but these fish were still smaller than the C fish (Fig. 3C2). The final tank biomass displayed a similar pattern (Fig. 3C3).
3.1.4. ASBH-exposed fish

After 96 h of exposure, cortisol concentrations of fish exposed to ASBH (Fig. 2) were increased compared to fish in the C group. The fish in the St + ASBH group also demonstrated increased cortisol concentrations, but not to the same extent as the St group (P < 0.0001). The results of the stress tests performed at days 45, 90, 135 and 180 demonstrated that the cortisol values of fish from the St + ASBH group were similar to the concentrations measured in the St group and that both group means were higher than those of the C and ASBH groups (P < 0.0001). The measurements conducted on days 90 and 135 revealed higher cortisol values for the ASBH group than those measured in the C group.

The C group presented a higher survival rate than the St, ASBH or St + ASBH groups (Fig. 3D1). The lowest final mean weight was obtained for the St group, but the ASBH and St + ASBH group mean weights showed decreases relative to the C group weight (Fig. 3D2). A similar pattern was observed for the final tank biomass.

4. Discussion

We demonstrate for the first time that an acute contaminant exposure during the fingerling stage induced chronic inhibition of cortisol release in response to stress in fish. The stress axis returns to a fully responsive state only after a minimum recovery period of 135 d in fish exposed to MPBI or to TBF. We also show that stress exposures during the juvenile fingerling stage impaired fish growth and survival because all of the exposed fish, independent of the agrichemical, presented worse growth in relation to the control, contaminant-free fish.

We hypothesize that MPBI and TBF damaged the HPI axis, perhaps at hypothalamic or pituitary regulatory levels or the interrenal tissue directly, resulting in long-term impairment of stress-induced cortisol release by MPBI and TBF exposure. Previous studies conducted in our laboratory showed that impairment of the cortisol release by MPBI was not reversed by an injection of adrenocorticotropic hormone (ACTH) (Cericato et al., 2009), suggesting that the disruptive effect occurs in the hypothalamus and/or the pituitary gland. Additionally, we recently showed that MPBI impairs the zebrafish (Danio rerio) HPI axis (Rosa et al., 2013), most likely by inhibiting the gene expression of steroidogenic acute regulatory protein (StAR) and heat shock protein 70 (hsp70) (Rosa et al., in preparation).

Similar results were found with TBF exposure (Cericato et al., 2009), but the HPI impairment caused by this compound was likely also related to its strong oxidative potential, both in R. quelen (Ferreira et al., 2010) and Cyprinus carpio tissues (Toni et al., 2011).

Despite uncertainty regarding the exact mechanism by which MPBI and TBF exert their deleterious effects on the HPI axis, fish exposed to these agrichemicals recovered their HPI-responsivity only in stress tests performed 180 d after the acute exposure. The affected parts of the HPI axis likely regenerate this functioning capacity via growth compensation. At this time, we do not have an alternate hypothesis for this intriguing finding, and it will be the focus of our subsequent research.

Chronic impairment of cortisol release by the HPI axis, which was observed for fish exposed to MPBI or TBF, may indicate serious obstruction of the adaptive function of the neuroendocrine stress axis in promoting restoration of homeostasis following stress (Hontela, 1998), which was reflected in the lower survival rates we observed.

Unlike fish exposed to MPBI or TBF, fish exposed to GBH or ASBH did not demonstrate impairment of cortisol release (except after 96 h of recovery from ASBH exposure). However, these chemicals did exert effects on fish survival and growth parameters. Both GBH and ASBH have been proven to induce oxidative stress in fish liver (Ferreira et al., 2010). This effect appears to be a plausible mechanism of lethality for fish exposed to these agrichemicals (Ferreira et al., 2010; Toni et al., 2011). Because the liver is the primary organ involved in metabolism, prolonged damage in the liver cells might have caused the observed poor growth and survival of the fish. Independent of the cause and mechanisms, our data demonstrated that both GBH and ASBH negatively affected fish survival and growth. Thus, the four agrichemicals tested, exert adverse effects in fish, under both environmental and aquaculture conditions.

The exposure to MPBI produces lower survival rates than those observed in the control fish. Surprisingly, the survival rates of fish exposed to a combination of MPBI and stress were similar to those of control fish. In contrast, the survival rates of fish exposed to the combination of stress and TBF, GBH or ASBH were at least as low as those found in exposure to the agrichemical alone. In terms of final weight, except for MPBI – where the fish exposed to the agrichemical showed lower weights similar to those found in the MPBI + St and St groups – the occurrence of stress was a stronger influence on weight impairment than the exposure to the agrichemical per se. As expected, the survival rate of control fish in laboratory conditions was greater than that obtained for pond-reared R. quelen, whereas the final weight of laboratory fish was lower than that of the pond-reared fish (Silva et al., 2006, 2008; Barcellos et al., 2012b).

Finally, three important aspects underscore the environmental relevance of our data: one, the concentrations to which the fish were exposed are low (less than 20% of the LC50), two, MPBI (0.25–12.5 mg L−1, Williams and Jones, 1994) and GBH (100 mg L−1, Monsanto, 2003) are used directly in water bodies to control predatory insects and aquatic macrophytes, respectively. Even TBF and ASBH, which are not used directly in water, can easily enter water bodies in small concentrations as a result of leaching by rain or as a result of accidents (as postulated by Soumis et al. (2003)). Thus, the concentrations used in the present study were very plausible in terms of the potential entry of the agrichemicals into natural water bodies and fish ponds; and three, all of the tested agrichemicals reduced the survival rates as well as growth parameters.

These intriguing results demonstrated for the first time that an acute sub-lethal exposure to an agrichemical during juvenile development, which might not be perceived or detected, may impair the long-term function of the HPI axis, at least in terms of cortisol release. A fish with an impaired HPI axis capacity to increases cortisol may lose the ability to stimulate the metabolic and ionic adjustments necessary to cope with stress.

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