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Short communication

Confirmation that pulse and continuous peracetic acid administration does not disrupt the acute stress response in rainbow trout

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Abstract

Peracetic acid (PAA) is considered an eco-friendly alternative to other antimicrobial agents of common use in aquaculture. The literature suggests that fish can habituate to PAA exposure based on a reduction of the fish corticosteroid response to PAA administration after repeated exposures. If that is true, PAA would also be a good option from the point of view of fish physiology. However, stronger evidence is needed to confirm that the use of PAA is welfare-friendly to fish. Besides habituation, other hypothetical factors such as desensitization, physiological exhaustion or PAA-mediated endocrine disruption could potentially explain the reduction in the corticosteroid response after repeated/prolonged PAA exposure. In this study, rainbow trout that had been exposed to PAA for several weeks were challenged with a secondary chasing stressor: fish were pursued with a dipnet for 1 min and their acute response was evaluated by measuring plasma cortisol, plasma glucose, plasma lactate and brain serotonergic activity. All fish were equally able to mount a normal physiological stress response to the secondary stressor independent of previous exposure to PAA. This suggests that the decrease in the cortisol response after repeated exposure to PAA, as seen in previous studies, is a true habituation to PAA administration, which supports the use of PAA as a welfare-friendly antimicrobial agent in aquaculture.

Keywords: Peracetic acid, disinfection, fish welfare, stress

1. Introduction

Peracetic acid (PAA) has proven to be an efficient antimicrobial agent for aquaculture purposes (Meinelt et al., 2015; Liu et al., 2017a). In addition to its demonstrated effectiveness against fish pathogens (Farmer et al., 2013; Jussila et al., 2011; Smail et al., 2004; Lilley and Inglis, 1997), its degradation time and kinetics make it a good eco-friendly alternative to other disinfectants of common use in aquaculture such as formaldehyde, iodophors, phenolic compounds, chlorine or quaternary ammonium compounds (Danner and Merrill, 2005; Pedersen et al., 2009, 2013; Lahnsteiner and Kletzl, 2016; Liu et al., 2017a). Peracetic acid has been demonstrated to be acutely toxic to the following typical fish pathogens *in vitro*. Toxic concentrations of PAA were found to be < 0.3 mg L^{-1} against *Ichthyophthirius multifiliis* theronts, 0.8 mg L^{-1} against *I. multifiliis* tomonts, 1 mg L^{-1} against *Flavobacterium columnare* and 4 mg L^{-1} against *Saprolegnia parasitica* (see Meinelt et al., 2007, 2009; Straus and Meinelt, 2009; Marchand et al., 2012). Recent research has shown that fish are able to tolerate PAA at low concentrations. The 24-h no observed effect concentration (NOEC) for channel catfish (*Ictalurus punctatus*) yolk-sac fry was 2.2 mg/L PAA and 1.3 mg L^{-1} PAA for swim-up fry (Straus et al., 2012). The 24-h NOEC was 1.9 - 5.8 mg L^{-1} PAA for a range of juvenile fish (Straus et al., in press). Several attempts of treating pathogens with PAA in the presence of fish were successful (Rintamaki-Kinnunen et al., 2005; Sudova et al., 2010; Jussila et al., 2011). To avoid the recurrence of pathogens, it is however necessary to use continuous or repeated exposures to PAA. In these cases, a welfare issue may emerge because the fish may suffer from chronic stress induced by the repeated exposures to PAA.

The stress response in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) was recently examined when fish were exposed to repeated applications of PAA; this was done in an attempt to identify welfare-related issues regarding the use of PAA in aquaculture facilities (Liu et al., 2017a, b). In these studies, exposed fish exhibited an increase in the levels of plasma cortisol (the most common stress marker in fish) after the initial exposure to PAA, which indicated that the exposure was stressful. After repeated exposures to PAA, the cortisol response of the fish was lower, suggesting that the fish might have become habituated to the exposure, which would support the use of PAA as a welfare-friendly antimicrobial agent. However, lower levels of released cortisol are not necessarily the result of a process of habituation (Cyr and Romero, 2009). Alternative explanations to habituation for a cortisol response of smaller magnitude could be related to desensitization or exhaustion of the physiological stress response without habituation, or to potential PAA-induced alterations of the normal functioning of the hypothalamus-pituitary-interrenal (HPI) axis.

The present study determined whether these alternative explanations could be excluded, thus confirming that the fish are truly able to habituate to PAA exposure. According to our hypothesis, fish that are apparently habituated

to PAA would be able to mount a normal physiological stress response (evaluated by measuring plasma cortisol, plasma glucose, plasma lactate and brain serotonergic activity) upon exposure to a different stressor.

2. Materials and methods

2.1. Fish, experimental design and sampling

Rainbow trout utilized during the study of Liu et al. (2017b) were used; their study evaluated how different types of PAA applications affected fish performance and system water quality in a flow-through aquaculture system. The applications were either repeated single dose (Pulse) or continuous via a peristaltic pump (Continuous). In brief, the following treatments were applied (in triplicate) to 180 L tanks containing 18 juvenile rainbow trout each: Control (no PAA exposure), Pulse (1 mg L^{-1} PAA, twice a week) and Continuous (0.2 mg L^{-1} PAA in the water inflow). This protocol was maintained for 6 weeks, after which the exposure experiment was completed. At that time, the average mass of the fish was 190.6 g (SD = 29.9 g) with no differences among treatment groups (Liu et al., 2017b).

The same experimental treatment groups and conditions were maintained for the present study; 48 h after the end of the exposure experiment described above, the fish were further exposed to a stress challenge of being pursued with a dipnet (chasing stress) for 1 min to evaluate the performance of the neuroendocrine stress pathways. The day of the experiment, 2 fish were quickly netted from one of the tanks and sampled as stress controls (time 0 treatment group). The net was then used to chase the remaining fish in the tank for 1 min. This procedure was repeated with the rest of the tanks. Therefore, 6 fish in total (2 per tank) were sampled as controls, for each of the treatments. At 1 h, 2 h, and 4 h after this stress, 8 fish from each treatment group were quickly netted and sampled. To minimize netting-induced stress, a single tank per treatment was dedicated exclusively to a particular sampling time. The fish were not fed for the 48 h prior to the experiment.

The sample procedure was as follows: fish were anesthetized in a 200 mg L^{-1} benzocaine solution; blood was collected from the caudal peduncle using 1-mL ammonium-heparinized syringes; the fish was then decapitated and the telencephalon was immediately dissected out, frozen rapidly in dry ice and later stored at -80 °C. The blood samples of each batch of fish were immediately centrifuged (6000 x g, 4 °C, 10 min) and the plasma was collected and stored at -80 °C for subsequent analyses of cortisol, glucose and lactate.

2.2. Biochemical analyses

Plasma cortisol was measured by means of a commercial ELISA kit (product #402710, Neogen Europe, Ayrshire, Scotland, UK), following the manufacturer's instructions. Plasma glucose and plasma lactate were analyzed with colorimetric kits from Sigma (#MAK013, #MAK064, St. Louis, MO, USA).

The levels of serotonin (5-HT) and its main oxidative metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the telencephalon were analyzed using high performance liquid chromatography with electrochemical detection (HPLC-EC) as described by Gesto et al. (2017).

2.3. Data analysis

The differences in the response dynamics of the different stress markers among the three experimental treatment groups were assessed by two-way ANOVA using treatment group (Control, Pulse or Continuous) and time post-stress (0 h, 1 h, 2 h or 4 h) as factors. The ANOVA was followed by Holm-Sidak post-hoc tests to identify significant differences among treatments and among time groups. SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA, USA) was used for all statistical analyses and the significance level was set at $P \leq 0.05$.

3. Results and discussion

There were no differences in the plasma cortisol response to the chasing stressor (pursuit with a net) among the different treatment groups (Fig. 1 and Table 1). An increase in cortisol levels was observed at 1 h and a progressive recovery was evident at 2 h and 4 h after the chasing protocol. The cortisol response dynamics were consistent with other studies on chasing stress in rainbow trout (Gesto et al., 2013, 2015). The magnitude of the cortisol increase in the present study was smaller than in these other studies, most likely due to the limited duration of the stress protocol which was of only 1 min for the present study versus 3 min (Gesto et al., 2015) or 5 min (Gesto et al., 2013).

Plasma glucose also increased after the acute stress (Fig. 1). Plasma glucose levels usually increase upon stress exposure due to the action of cortisol and catecholamines (Wendelaar Bonga, 1997). The glucose increase is directed to provide the animal with energy to help it overcome the threat of the stressor (Wendelaar Bonga, 1997). Although the plasma glucose increase in the Pulse treatment group took longer to occur than in the Control and Continuous treatment groups, the ANOVA demonstrated no treatment-induced differences (Table 1). The dynamics of the glucose response was consistent to other studies with the same species and stressor type (Gesto et al., 2013, 2015). Plasma glucose levels did not recover within the time frame of the experiment. This is consistent with the typical dynamics of post-chasing stress glucose response in rainbow trout (Ings et al., 2012; Gesto et al., 2013), since it can take 8 h or more for the fish to recover pre-stress glucose levels.

Plasma lactate usually increases on exposure to different stressors (Milligan and Girard, 1993; Vijayan et al., 1997). This occurs as a result of a stress-induced increase in activity, with a corresponding higher degree of anaerobic glycogenolysis in the muscle (van Ham et al., 2003; Iwama, 2006). We observed that the lactate response to stress showed the same trend than that of cortisol (Fig. 1). Plasma lactate increased 1 h after the stress and sequentially recovered to levels similar to those of the pre-stress state. At 2 h post-stress, lactate in all treatment groups was not different from the pre-stress levels. The magnitude of the increases and the dynamics of the lactate response (similar among all treatment groups) were analogous to the dynamics observed by Ings et al. (2012) and Gesto et al. (2013, 2015).

Habituation is a simple, nonassociative learning process by which animals reduce the magnitude of their response to a potentially deleterious stimulus after repeated exposures when the stimulus is recognized to be harmless (Grissom and Bhatnagar, 2009). Animals can generally habituate to stressors of low intensity (McCarty, 2016). A decrease in the cortisol response to PAA administration was observed after repeated exposures in rainbow trout and common carp (Liu et al., 2017a, b). It was suggested that the decrease was the result of habituation of the fish to mild stress generated by PAA administration. A reduction in the cortisol response to stress stimuli could, however, be due to different reasons and does not necessarily reflect a process of habituation. For example, the physiological stress responses can become desensitized or exhausted after repeated or chronic exposure to stress (Cyr and Romero, 2009; McKenzie et al., 2012; Barton et al., 2005; Madaro et al., 2015; Moltesen et al., 2016), even when the animals have not habituated to the stressor. Furthermore, different aquatic pollutants are capable of disrupting the normal function of the neuroendocrine pathways involved in the vertebrate stress response, altering the normal synthesis and release of stress hormones such as catecholamines and corticosteroids (Gesto et al., 2008, Ings et al., 2012; Hontela et al., 1997). A potential effect of PAA on these pathways cannot be excluded.

Altogether, the assessed plasma stress markers showed that when exposed to a secondary acute stressor, rainbow trout from different treatment groups (Pulse and Continuous) were equally able to develop a typical physiological stress response as opposed to fish that were never exposed to PAA (Control treatment group). This strongly suggests that the decrease in the cortisol response after repeated exposure to PAA (Liu et al., 2017a, b) was a true process of habituation (Grissom and Bhatnagar, 2009) and was not the result of damage or disruption of the neuroendocrine cascade leading to the release of corticosteroids. This was further supported by the brain serotonergic activity. Serotonergic activity usually increases upon acute stress exposure in fish and other vertebrates (Winberg and Nilsson, 1993; Emerson et al., 2000; Gesto et al., 2013). The serotonergic system is believed to participate at a high hierarchical level in the organization of the neuroendocrine response to stress in vertebrates (Winberg and Nilsson, 1993; Gesto et al., 2013, Lanfumey et al., 2008). The chasing stress challenge

induced a transient increase in telencephalic serotonergic activity (Table 2), similar to previous studies in rainbow trout exposed to acute stress (Gesto et al., 2013; Moltesen et al., 2016). Although this transient increase was only significant in the Control treatment group, the dynamics and the magnitude of the response were similar among treatment groups (Table 1) which supports an equal stress response for all fish in the experiment.

The dynamics of the different stress markers were as expected for a mild stressor in rainbow trout, with plasma cortisol, plasma lactate and brain serotonergic activity showing a rapid recovery. Fish from all treatment groups were able to mount a normal physiological stress response, independent of the previous exposure to PAA administration. Therefore, the decrease in plasma cortisol response after repeated exposure to PAA, as seen in previous studies, seems to be a true habituation to PAA administration, which supports the use of PAA as a welfare-friendly antimicrobial agent in aquaculture.

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Figure captions

Figure 1. Plasma levels of cortisol, glucose and lactate after 1 min of chasing stress in rainbow trout that were previously exposed to peracetic acid for 6 weeks. Columns represent the averaged values (and SEM) of n = 6 - 8 fish. Different letters represent statistically significant differences among time points for a given parameter and treatment group.

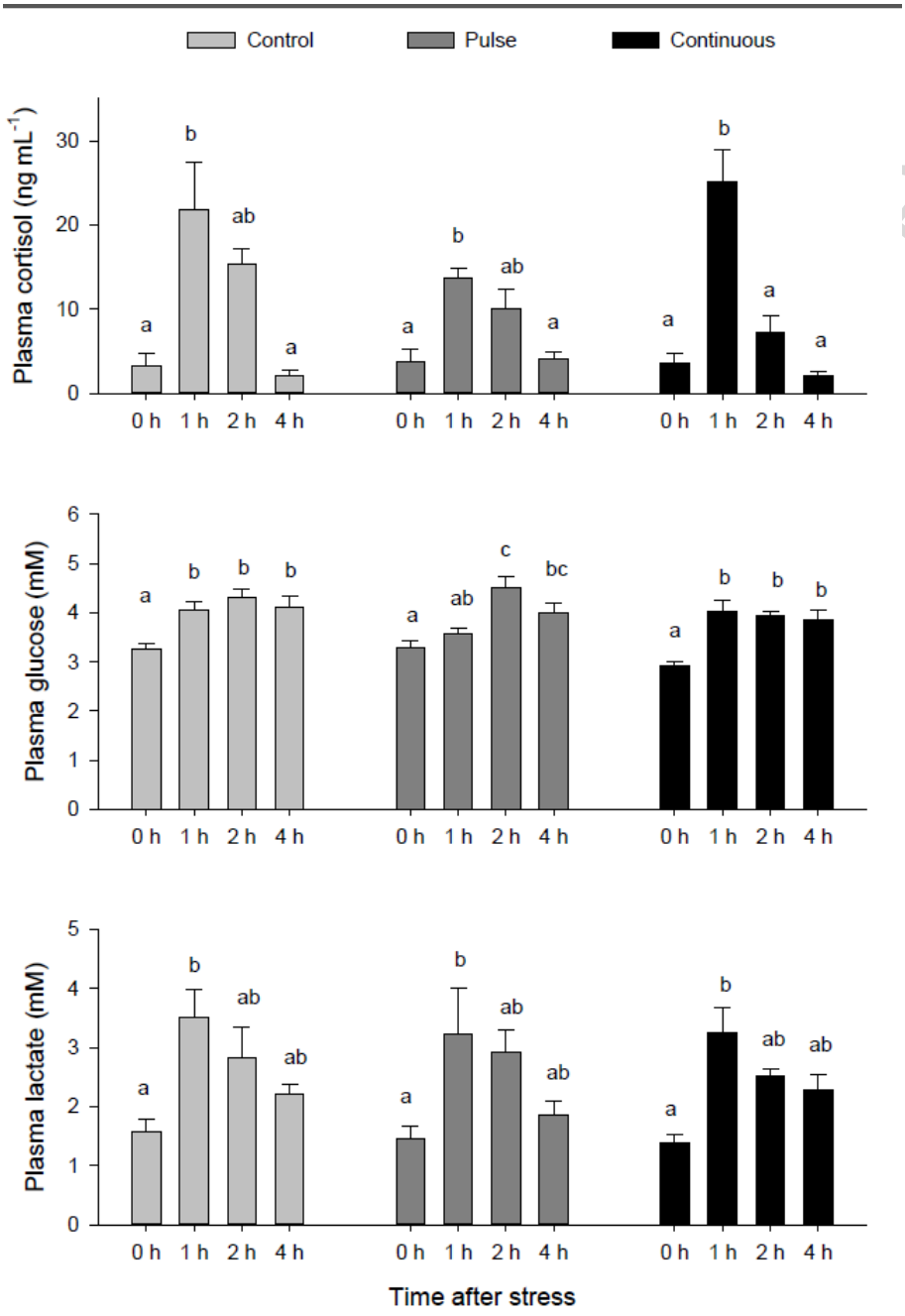

Table 1. P-values corresponding to the two-way ANOVAs performed for each parameter. Treatment group (Control, Pulse or Continuous) and time post-chasing stress (0 h, 1 h, 2 h or 4 h) were used as main factors. Significance level was set at $P < 0.05$.

	Time	Treatment group	Time x treatment group
Plasma			
Cortisol	< 0.001	0.853	0.256
Glucose	< 0.001	0.127	0.105
Lactate	< 0.001	0.800	0.976
Brain			
Serotonin (5-HT)	0.631	0.210	0.841
5-hydroxyindoleacetic acid (5-HIAA)	< 0.001	0.843	0.575
% 5-HIAA/5-HT	< 0.001	0.096	0.539

Table 2. Telencephalic content of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and values of the % 5-HIAA/5-HT mass ratio, after 1 min of chasing stress in rainbow trout that were previously exposed to peracetic acid for 6 weeks.

	control	pulse	continuous
5 hydroxyindoleacetic acid (ng g^{-1})			
0 h	122.77 ± 5.23[a]	143.63 ± 13.13	130.30 ± 6.88
1 h	171.47 ± 9.15[b]	153.31 ± 4.02	154.41 ± 11.38
2 h	159.73 ± 13.87[bc]	160.36 ± 8.31	160.81 ± 5.83
4 h	136.87 ± 8.03[ac]	128.27 ± 10.83	130.66 ± 5.62
Serotonin (ng g^{-1})			
0 h	472.68 ± 31.51	544.51 ± 19.40	491.14 ± 40.36
1 h	468.41 ± 19.67	518.14 ± 18.35	520.65 ± 32.28
2 h	500.16 ± 39.12	529.16 ± 23.74	509.16 ± 22.72
4 h	483.52 ± 18.10	478.31 ± 43.26	488.53 ± 20.79
% 5-HIAA/5-HT			
0 h	26.55 ± 2.38[a]	26.57 ± 1.92	26.79 ± 1.14
1 h	36.63 ± 1.32[b]	30.16 ± 1.43	32.01 ± 1.71
2 h	31.99 ± 1.23[ab]	30.56 ± 1.79	31.88 ± 1.43
4 h	28.41 ± 1.75[a]	25.17 ± 1.80	27.05 ± 1.51

Values represent the average (and SEM) of n = 6-8 fish. Different letters represent statistically significant differences ($P < 0.05$) among time points for a given parameter and experimental group.

Highlights

- Previous research showed a decreased response of trout plasma cortisol to repeated exposure to the antimicrobial agent peracetic acid
- After a 6-week exposure to peracetic acid, the stress response of the exposed trout to a secondary acute stressor (chasing) was evaluated.
- The results demonstrated that the ability of the fish to respond to further stressors was not compromised after peracetic acid exposure.
- The present data supports the use of peracetic acid as a welfare-friendly antimicrobial agent in trout farming.