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Abstract

In aquaculture, the many chemicals used as therapeutics, prophylactics and growth promoters, can accumulate in fish and spread in the environment through effluent water. Nowadays, the focus of zootechnical productions has shifted towards the search for possible natural alternatives, considered safer for animals and consumers, with lower environmental impact. Herbal products are made of natural compounds, but "natural" is not necessarily synonymous of harmless, thus toxicity needs to be tested for each composition at different doses. Indeed, since herbal products' action is often dose-dependent, negative effects may be prevented by studying the posology according to the species and life stage. In this study, the effects of two different doses (0.05 and 0.1 mL/L) of a natural-based complementary feed, Gill Fish®, were assessed on the early stages of life of farmed rainbow trout (*Oncorhynchus mykiss*). The product was added daily to tanks containing rainbow trout eggs during the pre-hatching period, from 30 to 36 days post fertilization. Embryos mortality (evaluated three times during the six days of treatment; t0-t2) and zootechnical indices such as biomass, weight, length and number of alevins (all evaluated 3 and 4 weeks after the end of treatment; t3-t4), were analysed to assess both direct and indirect toxicity. Furthermore, whole body cortisol (from embryos and alevins; t0-t4) was measured to highlight potential stressful effects of the treatment. For each parameter, time was a statistically significative factor, while no differences were noticed between the three groups within the same time point. These results demonstrate that Gill Fish® had no harmful nor stressful effects, both immediately and short term; this herb-

based product could therefore be a good candidate for future studies with different doses and/or longer administration, to evaluate its possible beneficial effects as an alternative to chemicals in aquaculture.

Keywords

Rainbow trout; natural-based products; cortisol; toxicity; early stage development.

1. Introduction

Fish constitute an excellent food choice for a healthy diet in developing world, high in protein and essential ω-3 fatty acids (Parian and Mullin, 2016). Nowadays, aquaculture is one of the fastest agricultural growing businesses (Cataudella et al., 2001), with farmed fish accounting for nearly 50% of the global fish consumption (FAO, 2018). However, since all farming systems, be it on land or in water, have a strong environmental impact, the challenge is to minimise the negative effects of intensive production on ecosystem and biodiversity (De Silva, 2012). The rainbow trout (*Oncorhynchus mykiss*) is a carnivorous Salmonid species, very demanding in terms of water quality, which must be relatively cold (6-20°C), clear, transparent, free of pollutants and well oxygenated ($O_2 \geq$ 5-6 mg/L) (Cataudella et al., 2001). The spawning season, in wildlife, occurs between late autumn and early winter months, with each female producing eggs over a 6-8 week period (Bromage et al., 1992). Under farming conditions, oocytes and semen (both obtained by manual abdomen pressing) are incubated in small water tanks for about 10-15 minutes (Pennell and Barton, 1996). Starting from the embryonic eyed-stage, which occurs about 18 days after fertilization, the eggs can be handled gently for cleaning, selection, counting and transfer to the hatching frames, which happens approximately 31 days post-fertilization (Ghaedi et al., 2015).

In the last decades, as consumers are getting more conscious regarding the health and environmental effects of chemical additives in aquaculture, scientists have intensified their efforts to find good alternatives to limit disease, pests and spoilage side effects (Syahidah et al., 2015). Moreover, such efforts perfectly fit with the ever-growing issue of antimicrobial resistance and animal production, where aquaculture plays an important role (Santos and Ramos, 2018). In such scenario, natural compounds and natural-based products are getting into the spotlight, due to their potential beneficial effects and popular demand. When it comes to fish production, various herbal additives have been already studied and tested as growth promoters, antimicrobial, antiparasitic, antioxidant and immunostimulants (Chakraborty and Hancz, 2011; Citarasu, 2010; Harikrishnan et al., 2010; Lee and Gao, 2012; Turan, 2006; Zilberg et al., 2010). Nonetheless, the use of natural compounds and natural-based products has several drawbacks including lack of standardization in the production phase, fluctuations in constituents depending on cultivation of the plants and, most importantly, lack of scientific data regarding efficacy, posology and toxicity (Elmi et al., 2019a). Indeed, when looking for example at essential oils, it is well acknowledged how they are capable of exerting toxic effects on all cells, not only prokaryotes, usually in a concentration-dependent manner (Elmi et al., 2017, 2019b; Vigan, 2010). Moreover, in the

particular case of aquaculture, assessing toxicity is extremely important as natural compounds may not only have direct effects on the fish, as for example membrane disruption, but also indirect ones by alteration of water quality. Some studies on the use of natural products as stress-modulating agents have also been reported (Saccol et al., 2017, 2018; Souza et al., 2017). This additional potential application is extremely interesting, as aquaculture deals with several stressful situations, such as eggs transfer and handling, which can compromise well-being and survival (Ashley, 2007; Sampaio and Freire, 2016; Sneddon et al., 2016), and usually induce, as primary stress response, cortisol and catecholamines increase (Barton, 2002; Tort, 2011).

Therefore, in the light of the above-mentioned reasons, the aim of this study was to preliminary assess the acute and short term effects of the exposure of rainbow trout embryonated eggs during the pre-hatching period to two different doses of an herbs-based commercial product (Gill Fish®, APA-CT srl, Forlì, Italy). Analysed parameters included zootechnical indices, as egg viability, alevins growth and biomass, and cortisol production both during the treatment and after hatching.

2. Materials and methods

2.1. Experimental design

The study was performed in the hatchery section of a rainbow trout farm located in Rivoli Osoppo (UD, Italy), part of the Eredi Rossi trout consortium, from November 8th to December 11th 2019. The eggs used in the study were shipped from the Colli sul Velino broodstock farm (RI, Italy), which belongs to the same Eredi Rossi consortium. Breeding females were born in 2015, while males were from the previous year, 2014. Each male can fertilize up to 3-4 females, which lay 3000-3500 eggs each. Therefore, eggs from about 40 females and 10 males were mixed and randomly used in the present study. Gill Fish® is a natural product, commonly used in aquarium as complementary feed, composed mainly of *Melaleuca alternifolia*, *Citrus limon* and *Origanum vulgare* essential oils. Due to its rich content in essential oils, the product is advertised as capable of producing antimicrobial and antifungal effects in aquariums. Ten California incubation tanks (N=10), containing 75 L of water each, were included in the trial: 3 were treated with a low concentration of Gill Fish® (0.05 mL/L; n=3), 3 with a high concentration (0.1 mL/L; n=3), and 4 were used as controls (CTR; n=4). Dosages tested were chosen on the basis of the product's manufacturer instruction, that suggest a maximum dose of 10 mL per 100 L (0.1mL/L). Tanks were supplied by a flow-through system from an elevated water reservoir, connected to an underground aquifer, monitored for temperature (T°) twice a day. Upon arrival to the facility, 30 days post fertilization, 17500±250 eggs were placed in each incubation tank, and the trial immediately started. The number of incubated eggs per tank was almost half when compared to what is usually done in this facility (30000 eggs) in order to obtain a monolayer of eggs, allowing for a more uniform exposure to the treatment. The product was added for 6 consecutive days at 2 p.m., according to the individual tank, upon water flow closure. Gill Fish® doses were first dissolved in 1 L of tank water and then poured back into it, while the eggs

were gently swung manually. After 1 h, the water flow was re-opened at the standard rate of 34 L/min. Flow was stopped to allow the product a standardized contact time with the eggs. The experimental design together with the sampling times are represented in Figure 1.

The pH of the experimental tanks water was measured both before and after the Gill Fish® treatment using dedicated test strips (pH 4.5-10.0, resolution: 0.5 pH unit; Sigma Aldrich P4536, St. Louis, Missouri, USA). Sampling for the different analytical approaches were performed on the day of arrival (t0; pool of eggs), on the third day of treatment (t1; at least 30 eggs per tank), on the last day of treatment (t2; at least 30 eggs per tank), two weeks after the end of treatment (t3; at least 20 alevins per tank) and then one week afterwards (t4; at least 20 alevins per tank). All specimens were immediately frozen at -20°C until analyses.

2.2. Egg mortality

During the treatment (at t0, 1 and 2), the experimental tanks were photographed during the water flow interruption, in order to assess viability of the eggs. To facilitate counting and allow for better visualization, from each California tank five pictures were acquired: upper right corner, upper left corner, lower right corner, lower left corner and middle part; as symbolically represented in Figure 2. For each field, at least 200 eggs were counted: orange translucent eggs were identified as viable, white and opaque ones as non-viable. The results of the five pictures of each tanks were summed and percentage of mortality was calculated.

2.3. *Zootechnical indices*

To evaluate potential effects on the growth of the animals, 10 thawed alevins for each tank were singularly weighed and measured in length at the last 2 experimental time points (t3 and t4); data were averaged per each tank. Additionally, at the end of the trial (t4), the entire amount of alevins for each tank was weighed before being transferred to larger pools, to quantify biomass. Simultaneously, the total number of alevins per tank was approximated by dividing the weight of the biomass per the average weight of the alevins.

2.4. Cortisol extraction and quantification

Cortisol was extracted following a previously described method, upon partial modification (Hwang et al., 1992). Immediately after thawing, 1.5 g of eggs and 500 mg of alevins were weighed, corresponding approximately to a pool of 15 eggs and 5 alevins. Each sample was homogenised in 500 µL PBS (phosphate buffer saline, pH 7.2) by means of an Ultra-turrax T25 for 3 min, and then the homogenate solution was extracted twice with 7 mL (eggs) or 5 mL (alevins) of diethyl ether (Sigma Aldrich, St. Louis, Missouri, USA) in a vortex for 5 min. After centrifugation (30 min, 3000 xg) the ether phase was collected, transferred into a glass tube and evaporated to dryness under an air-stream suction hood. Recovery of steroids was assessed on five replicates by addition of 3H-cortisol (~ 1000 c.p.m.), incubation for 2 h at room temperature and extraction as described above. The mean percentage of recovery was 75.5±1.1% for eggs and 78.3±0.9% for alevins. The

dry extracts were stored at -20 °C until reconstitution in 1 mL assay buffer, then 0.2 mL (eggs) or 0.05 mL (alevins) were used for measurement of cortisol by radioimmunoassay. Tritiated cortisol (30 pg/0.1mL; 94.6 Ci/mmol; PerkinElmer inc. Boston, MA, USA) was added followed by rabbit anti-cortisol serum (0.1 mL, 1:20000; produced in our laboratory). After incubation and separation of antibody-bound and –unbound steroid by charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer), tubes were centrifuged (15 min, 3000 xg), the supernatant was decanted and radioactivity immediately measured using a β-scintillation counter (Packard C1600, Perkin Elmer, USA). The sensitivity of the assay was 3.9 pg/tube and the intra-assay coefficients of variation was 4.9%. Cross reactions of various steroids with antiserum raised against cortisol were: cortisol (100%), corticosterone (9,5%), cortisone (5,3%), 11α-deoxycortisol (5,0%), prednisolone (4,60%), 20α-dihydrocortisone (0.4%), progesterone and testosterone (<0,001%). In order to determine the parallelism between hormone standards and endogenous hormone in eggs or alevins, a pool sample containing a high concentration of cortisol was serially diluted (1:1-1:8) with assay buffer. A regression analysis was used to determine parallelism between the two hormone levels in the same assay. A high degree of parallelism was confirmed by regression test (r^2 =0.98). The results are given as pg/mg tissue.

2.5. Statistical analysis

The statistical analysis was performed using the software GraphPad Prism v.8 (GraphPad Software Inc., San Diego, CA, USA). Descriptive statistics were calculated and expressed as means and standard error of the mean (SEM). Normal distribution was assessed by means of Shapiro-Wilk test; homoscedasticity by mean of the Levene test. Repeated measures ANOVAs, followed by Tukey post-hoc test, were performed to evaluate the impact of the treatments on parameters assessed at different time points, while one-way ANOVA was used for parameters measured only once. For all tests, significance was set at $p<0.05$.

3. Results

Water temperature, measured twice a day in the water reservoir supplying the California tanks, remained stable throughout the entire experimental trial (11.7 ± 2°C). The treatment, regardless of the concentration, did not alter the pH, that was consistent in all 10 tanks at 7.5.

3.1. *Egg mortality*

Egg mortality, assessed 1 h after the first treatment on the day of arrival of eggs in the hatchery (t0) and after 3 (t1) and 6 (t2) days of treatment, is represented in Figure 3.

The overall increase in non-viable eggs over time was statistically significant ($p<0.0001$), while there were no differences between the three groups (CTR, 0.05 mL/L and 0.1 mL/L) within the same time point.

3.2. Zootechnical indices

Alevins weights and lengths were recorded on a sample population (10 alevins per tank) two weeks after the end of treatment (t3) and then one week afterwards (t4). The yolk-sac was absorbed in all sampled alevins. Data are represented in Figure 4. For both parameters, no differences between the three groups (CTR, 0.05 mL/L and 0.1 mL/L) were recorded. As for egg mortality, time significantly influenced both weight ($p<0.0023$) and length ($p<0.0015$).

Data regarding biomass weight and the estimated number of alevins per tank at the end of the trial did not show any difference between groups.

3.3. Cortisol quantification

Levels of cortisol for the 3 experimental groups at the different time points are represented in Figure 5. In this case, the sample at t0 refers to a pool of eggs sampled immediately at arrival to the hatchery, before allocation and incubation to the different experimental tanks. No differences were recorded between groups at the same time point; again, also for cortisol production, time was a statistically significative factor ($p< 0.0001$). Mean cortisol values slowly increased up to hatching (from t0 to t2), with a following significant increase of more than 6 times in just one week (between t3 and t4).

4. Discussion

In aquaculture, as in all farming systems, some of the many chemicals either degrade slowly or do not degrade at all; with some potentially being carcinogenic (De Silva, 2012). Nonetheless, the constant development of fish farming and the enhancement of its production in the system of quality food, should only take place in full respect of the environment and consumers' health.

Despite plants and their extracts being considered traditionally safe, there are different examples of their negative impacts also on aquaculture, especially during the early stages of life of the fish (Abdel-Hadi et al., 2008). Some studies have for example assessed acute toxicity of essential oils on the rainbow trout (Keene et al., 1998; Velíšek et al., 2005), zebrafish (*Danio rerio*) (Doleželová et al., 2011; Mácová et al., 2008), catfish (*Rhamdia quelen*) (de Lima Silva et al., 2012) and guppy (*Poecilia reticulata*) (Doleželová et al., 2011). The cytotoxic effects of essential oils are primarily attributable to the disruption of cell membranes and alteration of their permeability, as confirmed by electron microscopy (Elmi et al., 2017, 2019b). The toxicity of herb-based products varies according to plants composition, which is itself highly variable due to different factors such as soil quality; moreover it varies depending on the chosen route of administration and the species, according to its level of development (Vigan, 2010). The potential of a commercial herbal product was tested in this trial: on the basis of the results, the two concentrations of Gill Fish® (0.05 and 0.1 mL/L) can be considered harmless for rainbow trout embryos close to hatching. Indeed, both egg mortality and growth rate (biomass and number of alevins) did not differ in the treated groups when compared to the data recorded in control tanks. Egg mortality was chosen as an acute, direct indicator of toxicity, and was indeed assessed

during the 6 days of treatment in all tanks. On the other hand, the other analysed zootechnical parameters can be considered as indirect toxicity indicators providing information regarding a "longer" time span. Since this was the first study assessing the effects of Gill Fish® on rainbow trout eggs, the use of the product was not extended past the first larval stages, to avoid damaging newborns. Nonetheless, on the basis of this preliminary study, Gill Fish® could be used in other trials, to evaluate the potential beneficial effects of longer administration.

As for cortisol in teleosts, its levels have already been measured in ovulated eggs (unfertilized oocytes), newly fertilized eggs, embryos, new-hatched larvae and alevins (Barry et al., 1995; Ghaedi et al., 2013, 2015; Hwang et al., 1992). Cortisol has different roles during the early development of these animals: it is indeed involved in hatching, growth and metamorphosis (de Jesus et al., 1991). The results clearly indicate how this steroid significantly increases overtime, yet without any difference between the three experimental groups; cortisol levels goes from a mean of 0.153 pg/mg at t0 to a mean of 49.382 pg/mg at t4. An interesting data, unfortunately missing in the present study due to its design, is the level of cortisol in eggs right after being laid. Indeed, according to literature, cortisol is initially extremely high due to maternal transfer, and subsequently lowers up to activation of the HPI axis, that confers embryos the capability to produce the hormone themselves (Barry et al., 1995; Ghaedi et al., 2013; Hwang et al., 1992). Since the present trial started 30 days after fertilization, it is safe to assume that the used embryos were already capable of producing cortisol on their own. Therefore, the data seem to confirm the hypothesis that the synthesis of this hormone by embryos could be functional for hatching as all groups (both treated and controls) showed a significant increase in cortisol levels. For the same reason, our results demonstrate that the used concentrations of Gill Fish® (0.05 mL/L and 0.1 mL/L) did not induce any negative stressful impact when looking at embryos stage and at the first weeks after hatching (alevins stage). This outcome represents the foundation for future studies with this product in the early stages of rainbow trout farming, without harming animals. Since there are many stressful events in fish farming, new "environmentally friendly substances", such as Gill Fish®, may be tested to mitigate the potential sources of stress during early life stages and potentially improve the productive performances, keeping animal welfare as a major goal. Moreover, it has to be acknowledged that some reports have documented the effect of herbs as appetite and growth promoters in aquatic species (Lee and Gao, 2012; Turan, 2006); certain of these have rated commercial herbal products with promising results (Dada, 2012; Rawling et al., 2009). Since consumers, an in particular citizens of industrialized countries due to constant media exposure, are increasingly interested to the safety and guarantee of food products, particularly those of animal origin, obtaining high production but with respect for animal and environmental well-being is pivotal.

5. Conclusions

In conclusion, the present studies suggests how the chosen herbal product, Gill Fish®, does not induce any adverse nor toxic effects of farmed rainbow trout when used up to 0.1 mL/L. Future studies could extend the proposed treatment to the subsequent growth stages of farmed rainbow trout and evaluate the antibacterial, antifungal, immunostimulant and anti-inflammatory properties, even with different doses; with the ultimate goal of a greater environmental sustainability, since it is human responsibility to respect natural balances with more sustainable production activities, aquaculture included.

Authors contributions:

Camilla Aniballi: Data curation, Investigation, Methodology, Writing - original draft **Alberto Elmi:** Data curation, Formal analysis, Methodology, Validation **Martina Bertocchi**: Investigation, Methodology, Data curation **Albamaria Parmeggiani**: Supervision, Resources **Nadia Govoni**: Supervision, Investigation, Resources, Methodology **Maurizio Scozzoli**: Resources, Investigation **Domenico Ventrella**: Formal analysis, Methodology **Maria Laura Bacci**: Conceptualization; Supervision; Funding acquisition, Resources **All authors**: Writing - review & editing

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. APA-CT srl (Forlì, IT) did not play any role in the design of the study or in the acquisition/interpretation of the results despite supplying the used compound.

Figure Legend

Figure 1. Representation of the experimental design.

Figure 2. Schematic representation of the California tank: upper right corner, upper left corner, lower right corner, lower left corner and middle part where the pictures for assessment of egg mortality were taken.

Figure 3. Effects of Gill Fish® on egg mortality (t0, t1 and t2, respectively day1, day3 and day6 of treatment). Error bars represent the standard error of the mean (SEM) (CTR $n = 4$; 0.05 mL/L $n = 3$; 0.1 mL/L $n = 3$). The overall increase in non-viable eggs over time was statistically significant ($p<0.0001$), while there were no differences between the three groups within the same time point. Different letters indicate differences between time points.

Figure 4. Effects of Gill Fish® on alevins weight (a) and length (b) at t3 and t4 (respectively day20 and day27). Error bars represent the standard error of the mean (SEM) (CTR n = 4; 0.05 mL/L n = 3; 0.1 mL/L n = 3). Both weight ($p<0.0023$) and length ($p<0.0015$) were significantly influenced by the time; no differences between the three groups were recorded. Different letters indicate differences between time points.

Figure 5. Eggs (at arrival or t0, t1, t2 respectively day1, day3 and day6) and alevins (t3, t4 respectively day20 and day27) cortisol (pg/mg). Error bars represent the standard error of the mean (SEM) (CTR n = 4; 0.05 mL/L n = 3; 0.1 mL/L n = 3). Time was a statistically significative factor ($p< 0.0001$); cortisol production increased by more than 6 times in just one week (between t3 and t4). No differences were recorded between groups at the same time point. Different letters indicate differences between time points.

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