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Feed-borne exposure to zearalenone leads to advanced ovarian development and limited histopathological changes in the liver of premarket size rainbow trout

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ABSTRACT

To gain insight into the possible implications for aquaculture of the presence of ZEN in fish feed at the EC guidance level, we determined the effects of over two months of feeding of premarket size rainbow trout (~250 g) with ZEN-contaminated feed at a dose of 1.81 mg·kg^{-1} feed (90.5% of the current guidance value) under aquaculture conditions. After 37 and 71 days of the experiment, both control and ZEN-challenged fish were weighed and measured to assess their growth performance. To examine the health-related effects of ZEN exposure, fish blood was collected for biochemical measurements of plasma markers, and their livers and gonads were fixed for histopathological analysis. Additionally, to examine the effect of ZEN on the molecular background of fish reproduction, the mRNA level of vitellogenin (vtg) in the fishes' liver was determined. Finally, exposed fish tissues were analyzed for residual concentrations of ZEN and its metabolites in order to estimate the potential health risks for the fish consumers. Our results indicate that when premarket size fish are fed for two months with ZEN-contaminated feed at a concentration just below the EC guidance value, the growth of the fish is not affected. The analysis of residual concentrations of ZEN indicated that there was no threat to the health of potential consumers of the meat from the exposed fish. However, the residuals accumulated in the fishes' intestine and liver, and trace amounts of ZEN were also found in their ovaries. Although histological analysis of the liver cross-sections revealed structural irregularities in the treated group, including necrotic areas, disorders of polygonal hepatocytes, cytoplasm vacuolization, and macrophage aggregates, these developments were not accompanied by alterations in levels of plasma biochemical markers. This shows that ZEN had only a limited influence on the liver structure of the exposed fish. Although the experimental groups did not differ in terms of vtg mRNA levels, our results suggest that ZEN in feed may accelerate sexual maturation of the female fish, as ovarian development was more advanced in the exposed group. Our findings suggest that the presence of ZEN in fish feed may have consequences for aquaculture and warrant the need for further research in order to reassess the recommended limits.

Statement of relevance

Current feed guideline may have consequences for aquaculture.

1. Introduction

Zearalenone (ZEN) is a mycotoxin commonly occurring in plant material which results in worldwide contamination of products intended for animal and human consumption. The most studied aspect of ZEN is its biological property of inducing structural disorders and/or dysfunction in the reproductive tract of farm animals, i.e., pigs, cattle, and poultry (Minervini and Aquila, 2008; Zinedine et al., 2007). The ability of ZEN to mimic the action of natural hormones (e.g., estradiol) gives rise to a number of reproductive disorders in exposed livestock mammals, including decreased libido, anovulation, infertility, or neoplasmic lesions (Jakimiuk et al., 2009; Minervini and Aquila, 2008; Zinedine et al., 2007). Importantly, the estrogenic activity of ZEN has also been shown to affect reproduction in fish. For example, water-borne exposure of zebrafish (*Danio rerio*) to ZEN can reduce

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spawning frequency (Schwartz et al., 2010) or induce transgenerational changes in fecundity (Schwartz et al., 2013). Although experimental data on toxicological aspects of ZEN action in fish models are constantly increasing, the influence of ZEN and other *Fusarium* mycotoxins on the growth and reproduction of economically important fish species has been incompletely evaluated (Manning, 2010).

The notable growth rate in farmed fish production in recent decades has made aquaculture an important component of the modern food supply (Tacon et al., 2011). The development of aquaculture has created a huge demand for raw materials to produce fish feeds. This has forced the producers to search for "economically-relevant" alternative inputs of nutrients, such as plant material, which constitutes the second largest (and growing) source of dietary proteins and lipids after fishmeal and fish oil for European high trophic level fishes, i.e., trout or salmon (Tacon et al., 2011). These salmonid species are carnivorous and thus it is reasonable to think that they may be more susceptible than herbivores to the detrimental effects (e.g., endocrine disruption) of contaminated feed because the latter are evolutionarily adapted to the occurrence of undesirable substances of plant-origin in their feed (Wynne-Edwards, 2001).

Since contamination of commercial fish feed (or their plant components) with molds is a potential source of mycotoxins (like ZEN) in aquaculture, such a situation may have a negative impact on the productivity and health of fish, and thus reduce the profitability of fish farming (Manning and Abbas, 2012). Moreover, if the mycotoxins carry-over into the meat and eggs of the farmed fish, the contaminated feed may pose an additional health risk to the consumers of the aquaculture products. Indeed, the results of our recent study have shown ZEN contamination in different samples collected from rainbow trout farms in north-eastern Poland (Woźny et al., 2013). Although ZEN was not found in trout meat (which suggests no health risk to the fish meat consumers), the results showed that the mycotoxin concentrated in the fishes' ovaries. Additional analyses of surface (system) water and fish pellet samples from the farms indicated that the animal feed, rather than the water, may be a vector of the contamination (Woźny et al., 2013). Thus, the common presence of ZEN in fish feed, together with the observed accumulation of this compound (known for endocrine disruptive action) in the fishes' reproductive tract may be of concern for aquaculture production.

The presence of ZEN in fish feed and feed material has been reported previously (Nácher-Mestre et al., 2013, 2015; Pietsch et al., 2013; Woźny et al., 2013). For example, ZEN was found in all tested samples of commercial fish feed for cyprinids collected from Central Europe, with an average of 67.9 $\mu g \cdot kg^{-1}$ (Pietsch et al., 2013). In our study, the concentration of ZEN reached 81.8 $\mu g \cdot kg^{-1}$ in fish pellets for rainbow trout that were collected from Polish farms (Woźny et al., 2013). According to the recommendation of the European Commission (EC, 2006), the concentration of ZEN in products intended for animal feed should not exceed 2 $mg \cdot kg^{-1}$ (with an exception for maize by-products). Importantly, the EC guidance value was mostly based on the European Food Safety Agency's opinion (EFSA, 2004), which does not contain any information on the occurrence of ZEN in fish feed or its influence on fish livestock. The lack of such data, especially on the long-term effects of food-borne exposure of economically relevant fish species to ZEN at the concentrations observed in our and other studies (Pietsch et al., 2013; Woźny et al., 2013), leaves a serious gap in our knowledge concerning possible threats to aquaculture.

Taking into account that the optimal procedure for raising rainbow trout involves feeding them approximately 1% of their body mass per day (From and Rasmussen, 1984), the guidance value of 2 $mg \cdot kg^{-1}$ of feed would result in an intake of ZEN at 20 $\mu g \cdot kg^{-1}$ of their body mass. It is reasonable to think that such a daily intake of a hormone-mimicking compound (like ZEN) in contaminated fish feed may exert an influence if continued for long enough or given at a susceptible developmental stage. Thus, although the mycotoxin has so far been found in commercially available fish feed at concentrations lower than the current recommended limit (Woźny et al., 2013), the EC guidance value may still be a cause for concern. To our knowledge, there has been no systematic research which demonstrates what concentration of ZEN may be considered safe for the welfare (health status) and reproduction (quality of gametes, fecundity) of economically relevant fish species.

Therefore, to gain insight into the possible implications for aquaculture of the presence of ZEN in fish feed at the EC guidance level, we determined the effects of over two months of intensive feeding of premarket size rainbow trout (~250 g) with ZEN-contaminated feed at a dose of 1.81 $mg \cdot kg^{-1}$ feed (90.5% of the current EC guidance limit) under aquaculture conditions. After 37 and 71 days of the experiment, the growth performance of both the control and ZEN-challenged fish was assessed. Additionally, to investigate the health-related effects of ZEN exposure, biochemical markers were analyzed in the fishes' blood and their livers and gonads were examined for histopathological changes. Finally, residual concentrations of ZEN and its metabolites were analyzed in the exposed fish tissues to confirm the intoxication and to estimate the health risks related to the fish consumers.

2. Material and methods

2.1. Feed contamination

In this study, we used a commercially available fish feed designed for trout feeding (4 mm pellet size; 43% protein and 25.5% fat as major analytical constituents). The purchased batch of (blank, non-contaminated) trout feed pellets was analyzed for the presence of background contamination, and found to be free of ZEN and other mycotoxins (aflatoxin B_1, deoxynivalenol, ochratoxin A). In order to prepare a contaminated batch of feed, an analytical sample weight of ZEN (Sigma-Aldrich; Germany) was dissolved in methanol, then atomized onto a single layer of the feed pellets, and left to evaporate overnight. All batches of contaminated feed were thoroughly mixed, and then the concentration of ZEN was analyzed using ZearalaTest™ immunoaffinitive columns combined with HPLC (see below for methodological details). The analysis of independent samples confirmed the contamination with ZEN at a concentration of 1.81 $mg \cdot kg^{-1}$ (± 0.09; $n = 3$), which is 90.5% of the EC guidance limit for animals (2 $mg \cdot kg^{-1}$ feed) except for pigs, cows, sheep, and goats (EC, 2006).

2.2. Fish maintenance and exposure

The procedures related to fish breeding, maintenance and exposure were conducted at the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn; Poland). All fish were housed and handled in compliance with widely accepted guidelines of laboratory animal care. The experiment was approved by the Local Ethical Commission (resolution No. 49/2013 of 23rd October 2013). In order to examine the effects of feed contamination with ZEN on the fishes' welfare and on the concentration of ZEN in the meat as an estimate of the potential health risks for its consumers, fish at a late developmental stage were selected for this study (2+ years old, ~250 g body mass; premarket size — nearly ready for sale). Since a unisexual fish population brings advantages in aquaculture production (Mylonas et al., 2010), the experimental individuals were selected from a female population of rainbow trout that was obtained by spawning natural females with sex-reversed females, which at least theoretically should contain 100% female individuals.

The fish were tagged with passive integrated transponders which allowed for further automatic identification during sampling procedures. After tagging, the fish were acclimated for two weeks in 2 · 2 m flow tanks supplied with system (surface) water at a flow rate of 600 $L \cdot h^{-1}$. In the beginning of the experiment (0 d), all fish were sorted by their individual masses and divided into 2 groups