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Assessment of Biosafety and Fillet-residues After Florfenicol Exposures in *Trachinotus blochii* to Ensure Safe-applications in Disease Incidences

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Abstract

Trachinotus blochii is a promising mariculture fish species. Scientific data on biosafety and fillet residues of florfenicol exposure, one recommended amphenicol antimicrobial for aquaculture use, remains unknown in *T. blochii*, despite its criticality for prudent application. Accordingly, the paper evaluated the safety (regarding mortality, symptoms, weight gain, and histopathology) of dietary florfenicol after therapeutic (10 mg Kg-1 for ten days) and excessive (three, five, and ten times the therapeutic dose for 10, 20, and 30 days) exposures. There was no mortality in any group. The clinical abnormalities were noted only in 10X group from the 25th exposure day, which disappeared on the fourth day after withdrawal. Reduced growth was recorded at 5X and 10X groups from 20 and 30 exposure days, respectively. Histological lesion's severity was in the liver > kidney > gill > spleen > muscle > intestine. The lesion severity relied on the quantity and duration of exposures, with maximum severity in 5X and 10X groups on the 30th day. After recommended therapeutic exposure, fillet residues were below the maximum residual limit accepted by the European Union (1000 µg Kg-1) from day three of the withdrawal, showing a minimum three-day is necessary to reach a safe, acceptable level.

Introduction

Aquaculture caters to the nutritional demands of the global population and global development by contributing ~17% of the worldwide population intake of animal protein and employing nearly 41 million people (Schar et al. 2020). However, intense farming practices and climate change have led to several infectious diseases in aquaculture, especially those caused by bacteria, resulting in substantial economic losses, which in turn made the application of antimicrobials an inevitable option (Bardhan et al., 2022). Conversely, inappropriate doses and duration of antimicrobial application in aquaculture are often associated with developing antimicrobial resistance (AMR)F in microbes and antibiotic residues in aquatic products. The environmental safety concerns of antimicrobial treatment in aquaculture require more

serious emphasis since aquatic animals are less efficient in metabolizing them than their terrestrial counterparts (Romero et al. 2012). A thorough understanding of the efficacy, biosafety, and withdrawal period of each recommended drug in each targeted species is critical in the safe clinical application of antimicrobials, minimizing the adverse effect on the target species, consumers, and the environment. The information on the drug residues in the fillet is critical in estimating the time lapse between the cessation of the treatment and human consumption, thus essential for consumer safety (EMA 2013). While many reports on the biosafety and withdrawal period for approved antimicrobials are available for temperate fish species, corresponding information on tropical fishes, especially marine fishes, is very scarce (Manna et al. 2021; Sharma et al. 2021).

Food and Drug Administration has approved four antimicrobial drugs, *viz.* florfenicol (FFC), oxytetracycline, sulfadimethoxine and ormetoprim combination, and sulfamerazine for aquaculture use in controlled conditions to combat bacterial diseases (USFDA 2011). The pharmacokinetic properties, biosafety, immunological responses, and tissue residues of FFC have been studied in European seabass (*Dicentrarchus labrax*) (Kogiannou et al. 2020), *Litopenaeus vannamei* (Fang et al., 2013) and *Oreochromis niloticus* (Reda et al. 2013; Bardhan et al., 2022) following exposure to FFC. Further, the efficacy of FFC for control of mortality caused by different aquatic pathogens had been demonstrated through both *in vitro* and *in vivo* experiments (Gaunt et al., 2010; Soto et al., 2010; Sumithra et al., 2022).

Snubnose silver pompano (*Trachinotus blochii*) is a promising aquaculture species due to its merits like fast growth, tolerance to wide salinity range, good protein quality, adaptability to higher stocking density, and consumer preference (FAO, 2022). The studies on the fatty acid profiles showed that *T. blochii* is an excellent dietary addition for human nutrition and health (Cao et al., 2019). The muscle of fish contains the appropriate ratios of n-6/n-3 polyunsaturated fatty acid (PUFA) and PUFA/SFA (saturated fatty acid), making it conducive to a balanced human diet. Further, the high proportion of sn-2-palmitic acid (>50%), similar to breast milk makes the fish suitable for infants. The current annual aquaculture production of silver pompano is >0.11 million metric tons globally, with China as the primary producer (FAO 2022). The aquaculture practices of *T. blochii* are mainly focussed on coastal ponds, sea, brackish, and backwater cages and recirculatory aquaculture systems of China, India, Indonesia, Malaysia, Philippines, Taiwan, Thailand, and Vietnam (FAO 2022). Despite its significance in aquaculture, the data on the biosafety and tissue residues of antimicrobial drugs are not known in snubnose pompano except for one report on oxytetracycline (Sharma et al. 2021). Hence, the present study was principally focused on generating data on the biosafety of FFC in snubnose pompano through the evaluation of growth, behavior, and histopathological responses after oral administration with various doses (therapeutic and excessive) for different durations. The residue concentration of FFC and its metabolite florfenicol amine in the fillet of snubnose pompano exposed to the therapeutic dose of FFC was additionally studied to ensure consumer safety. In brief, the paper generates interesting insights that can be directly applied for the safe clinical application of this approved antimicrobial drug under controlled conditions during the occurrence of bacterial diseases in aquaculture practices of one high-value marine fish species.

Materials and Methods

Biosafety Studies

Experimental Design

For the biosafety experiments, hatchery-produced pompano three-month-aged fingerlings (weight: 12.03±0.11 g; length: 8.96±0.12 cm) were maintained at the Mandapam Regional Centre of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), India. During the acclimation period of one week, the fish were fed with a commercial 1.8 mm sized pellet feed (Growel Nutrila) at a feeding rate of 5% of the biomass at a frequency of three times a day. The physicochemical water quality parameters were observed as temperature: 25.98±0.27°C, pH: 8.04±0.19, salinity: 35±0.5‰, dissolved oxygen: 5±0.2 mg/L). Water was exchanged daily (30%) and ensured that total ammonia-nitrogen, nitrite-nitrogen, and nitrate-nitrogen did not exceed the safe thresholds. Following the acclimation period, 225 pompano juveniles were randomly divided into four test groups and one control group with triplicate tanks in each group. The fish were maintained in 250 L FRP tanks, with 15 fish in each tank. The four test groups were fed with pellets top-dressed with FFC (Sigma Aldrich, USA). In detail, the first test group (Group A) was provided with FFC at the general recommended therapeutic dose of 10 mg Kg-1 biomass (1X) for fish (USFDA, 2011). The second group (Group B) was fed with 30 mg Kg-1 (3X) FFC, the third group (Group C) was fed with 50 mg Kg-1 (5X) of FFC, and the fourth group (Group D) was provided with 100 mg Kg-1 (10X) FFC. The medicated feeding in all the groups was continued for 30 days. After this period, the fish were fed with the feed without FFC for ten days. The fifth group (Group E), provided with un-medicated pellet feed, served as control. The required dose of the drug was calculated for each group, uniformly mixed with fish oil (5 mL Kg-1 feed), and the emulsion was evenly top-dressed on the feed pellets. Following drying at 50°C for two hours, the medicated feed was stored at room temperature in sealable plastic containers at a cool, dark place till they were used (Gaikowski et al. 2012). The medicated feed was prepared in batches to accommodate the required amount for every ten days.

The fish were fed at 2% body weight during the feeding experiments in two equally divided feeding schedules, and a 30% water exchange was done daily. The dosage of FFC was adjusted to the required concentration in the pellets following each sampling.

Observations on Behavior, Feed Intake, Weight Gain, and Survival

The extent of feed intake, mortality, and the presence of any abnormal behavior were recorded twice daily. For the extent of feed intake measurements, the leftover feed was siphoned out after one hour, dried at 50°C for 12 h, and weighed. Residual feed was calculated using the formula [(W0-W1)/W0] × 100 (Maltby et al. 2002); where W0 represented the initial weight of the feed and W1 described the weight that remained after one hour of feeding. Average feed residue in each group was noted down, and the scores 4, 3, 2, and 1 were assigned to the mean feed residue of ~0%, 1-25%, 26-50, 51-75%, and 76-100%, respectively. Further, the survival percentage and the average body weight gain (WG) in each group [(Final Weight-Initial weight) were recorded once every ten days (Manna et al. 2021).

Histopathology

Six fish per group, including three replicates (two fish from each tank), were randomly collected once at 10 days intervals. After recording the gross abnormalities, representative tissue samples of gills, liver, kidney, spleen, muscle, heart, eyes, and intestine were preserved in 10% neutral buffered formalin. The samples were processed for routine histopathological investigations and hematoxylin and eosin (H&E) staining. Histological lesions were scored following the semi-quantitative system (Bernet et al. 1999), considering circulatory, regressive, progressive, and inflammatory alterations. The lesions in each reaction pattern were given a score of 0, 2, 4, or 6 based on the severity and extent of damage, where 0 indicated no change and 6 indicated diffuse occurrence. Each alteration was given an importance factor, a constant value ranging from 1 to 3 depending on the pathological importance, which included minimum (1), moderate (2), and marked (3) (Table 1). The reaction index was calculated as the sum of the values obtained by multiplying the alteration and importance factor for any given reaction pattern. Similarly, the organ index was also calculated as the sum of all the reaction indices of a particular organ. Accordingly, the higher value of reaction index and organ index indicated a more significant severity/damage.

Evaluation of Tissue Residues of Florfenicol After Therapeutic Exposure

Evaluation of tissue residues of FFC following therapeutic exposure was conducted in the wet laboratory facility of ICAR-CMFRI, Kochi, India. One hundred eighty hatchery-produced pompano juveniles (weight: 12±0.62 g) were brought from Vizhinjam Regional Centre of ICAR-CMFRI and acclimatized for seven days. The fish were then randomly divided into two groups with triplicate tanks for each group. During experiments, fish were maintained in 500 L FRP tanks containing 250 L seawater (temperature: 28.88±0.54°C, pH: 7.5±0.07, salinity: 20±1.4‰) with 30 fish in each tank and daily 50% water exchange. Fish in the first group (treatment) were fed with FFC medicated feed (10 mg Kg-1 biomass day-1) in two equally divided feeding schedules. After the end of medicated feeding for ten days, the unmediated feed was given and monitored for 20 days. Fish in the second (control) group were fed with unmediated pellet feed throughout the experimental period. Three fish from each tank were sacrificed on 5, 11, 13, 15, 17, 19, 21, 23, 25, 30, 35, and 45 days post-initiation of the medicated feeding, as described earlier. Approximately 5 g of muscle tissue with intact skin collected from each fish was preserved at -20°C. To evaluate FFC and FFA (florfenicol amine, the major metabolite of FFC in animals) residue, the tissue was

Table 1. Histopathological scoring of tissue responses in snubnose pompano exposed to FFC.

Reaction pattern	Tissue lesions	Importance factor
Circulatory changes	Haemorrhage/hyperaemia/aneurysm	1
	Intercellular oedema	1
Regressive changes	Architectural & structural alterations	1
	Nuclear alterations	2
	Atrophy	2
	Necrosis	3
	Deposits	1
	Vacuolar degeneration	3
Progressive changes	Hypertrophy	1
	Hyperplasia	2
	Hypertrophy	1
	Hyperplasia	2
Inflammation	Activation of RES	1
	Infiltration	2
	Exudate	1

finely ground, homogenized, and extracted with 10 mL of ethyl acetate containing 2% ammonium hydroxide for analyzing FFC and FFA content in triplicates following the guideline of Commission Decision 2002/657/EC in the document SANCO/2004/2726 revision 4 (European Union, 2008). For LC-MS/MS, the Exion HPLC system coupled to AB Sciex 4000 QTRAP Mass spectrometer installed at the ICAR-Central Institute of Fisheries Technology (ICAR-CIFT), Kochi, India, was used.

Statistical Analysis

Differences between different experimental groups on each sampling were determined with one-way ANOVA followed by Tukey's post hoc analysis / Kruskal-Wallis test based on data normality. The results on residue concentration were analyzed through repeated measures ANOVA with a Greenhouse-Geisser correction, followed by posthoc analysis with a Bonferroni adjustment. In all tests, P values less than 0.05, 0.01, and 0.001 were considered statistically significant, very significant, and highly significant, respectively (IBM SPSS Statistics Version 16).

Results

Observations on Behavior, Feed Intake, Weight Gain, and Survival

No conspicuous abnormal changes in the behavior were observed in all the groups during the initial 20 days of the experiment. Fish in the group exposed to 10X concentrations (100 mg Kg-1 biomass) showed lethargy, loss of equilibrium, and abnormal pigmentation from day 25 after initiating FFC feeding. Nevertheless, the behavioral changes were absent from the fourth day after the cessation of the medicated feeding. Similarly, fish in the three test groups and control group consumed all the feed (0% feed residue) within 10-20 seconds during the entire experimental period. However, there was a reduction in the feed consumption within the group exposed to 10X concentration (100 mg Kg-1 biomass) from day 25 after the initiation of FFC feeding, with an average score of 2.95±0.46. Still, the feed consumption started reversing after the medicated feeding cessation and returned to the normal level (score=4) from the fourth day (34th day of experimentation). There was 100% survival during the entire experimental period in all the groups. Further, there were no significant differences (P>0.05) in weight gain of fish belonging to different treatment groups when compared to the control group during the first ten days of medicated feeding (Figure 1). However, fish exposed to 10X concentration of FFC showed significantly reduced weight (P<0.05) at 20th day of exposure. There was a further reduction in the weight gain of both 5X (P<0.01) and 10X group (P<0.001) on the 30th day of FFC exposure. The weight gain remained at a significantly lower level (P<0.01) in both these higher exposure groups compared to other groups, even ten days after termination of medicated feeding (Figure 1).

Histopathology

The microscopic lesions following exposure to FFC were predominantly circulatory alterations, including hyperemia and hemorrhage. The other histopathologic alterations included increased melanomacrophage center (MMC) in the kidney (regressive alterations), fatty changes in the liver (regressive alterations), necrosis in the liver and kidney (regressive alteration), and hyperplasia of secondary gill lamellae (progressive alteration) (Figure 2, 3).

Circulatory Alterations After FFC Exposure

There were no significant circulatory alterations (P>0.05) in the muscles and gills of the various treatment groups relative to the control (Figure 4a). The most significant circulatory alterations compared to the control group were detected in the kidney, liver, and spleen following oral administration of FFC, especially at higher doses and during extended exposures. These alterations remained even after ten days of the

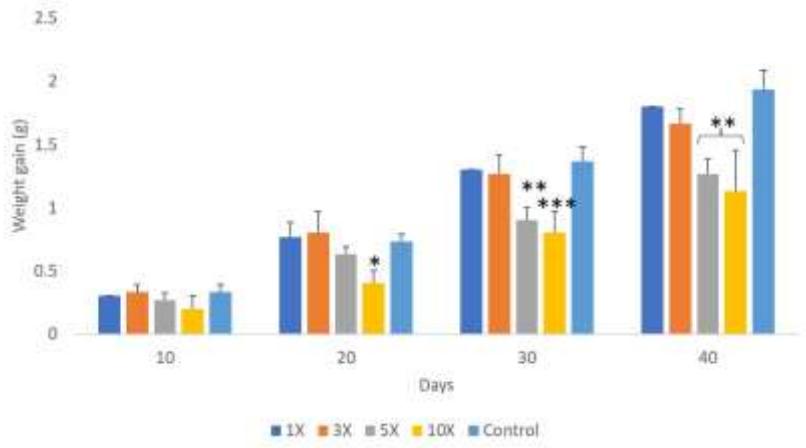

Figure 1. Weight gain in *T. blochii* after different florfenicol exposures.

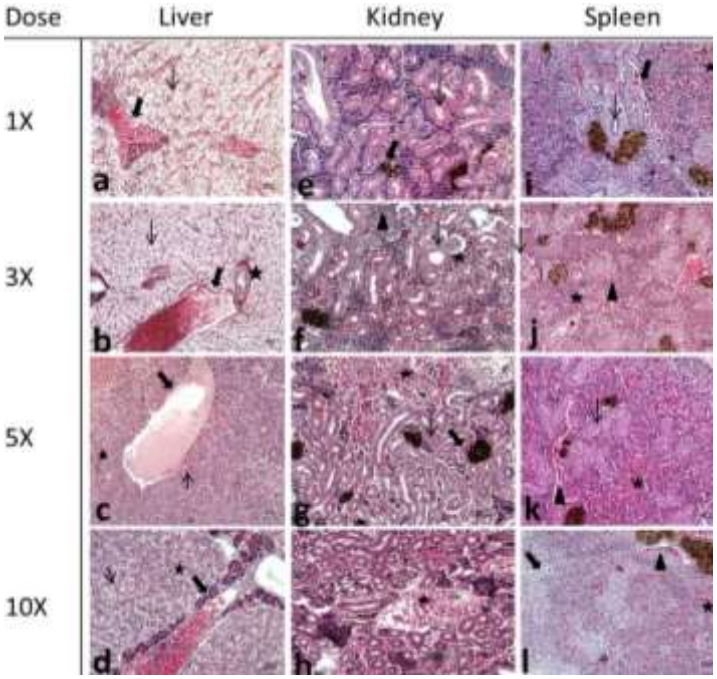

Figure 2. Sections of vital organs of snubnose pompano after different FFC exposures (H&E): a. 30 days-diffuse cloudy swelling of the hepatocytes with clear cytoplasm and pyknotic nuclei (thin arrow) and congested hepatic vein within the exocrine pancreas (thick arrow); b. 20 days-hepatocyte swelling with absence of nucleus (thin arrow), acute congestion (thick arrow) and hyperplastic bile ducts (star); c. 40 days-acute congestion (thick arrow), glycogen-type vacuolation of the hepatocytes (thin arrow) and haemorrhage (arrowhead); d. 40 days- severe congestion of hepatic vein in exocrine pancreas (thick arrow), diffuse glycogen-type vacuolation (thin arrow) and hepatocellular necrosis (star); e. 30 days- tubular degeneration (thin arrow) and aggregates of MMCs (thick arrow); f. Distension of bowman's space (star), tubular degeneration (arrow) and focal haemorrhage (arrowhead); g. 20 days-extensive hemorrhage into interstitium (star), increased aggregates of MMCs (thick arrow), tubular necrosis (thin arrow) and decrease in the lymphoid cells in the head kidney; diffuse hemorrhage (star) and tubular necrosis (arrow); h. 30 days- extensive hemorrhage into the interstitium (star) and tubular necrosis with presence of hyaline droplet (thin arrow); i. 30 days-congested splenic vessels (thick arrow) and haemorrhage (star) and decrease in white pulp; j. 10 days- increased MMC aggregates (thick arrow), prominent splenic ellipsoids (thin arrow), hemorrhage (star) and congestion of the splenic vessels (arrowhead); k. 20 days-Prominent splenic ellipsoids (thin arrow), congestion (arrowhead) and diffuse hemorrhage (star); l. congestion (arrowhead), hemorrhage (star) and sparsely distributed white pulp (thick arrow).

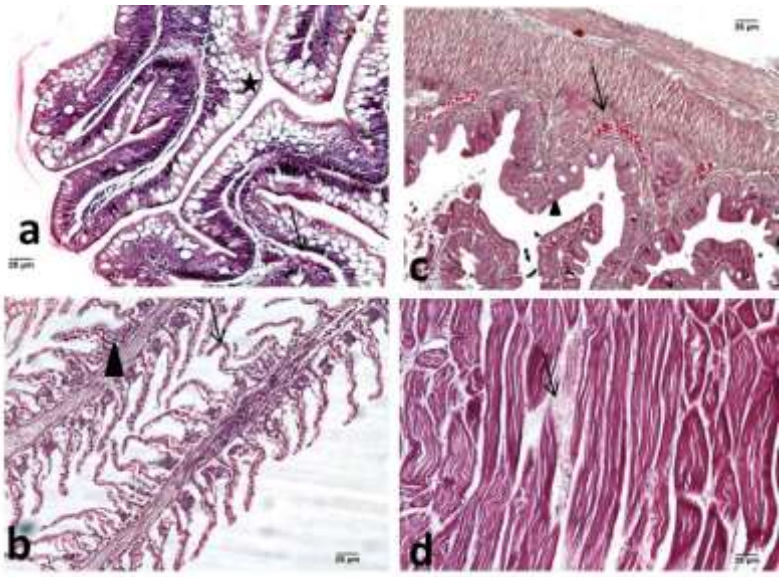

Figure 3. Sections of intestine, gills and muscle of snubnose pompano exposed to florfenicol (H& E): a. congestion of the intestinal mucosa (arrow) and Increased goblet cells (star) in 2X group on day 20; b. congestion of the intestinal mucosa (arrow) and shortening of the villi (arrowhead) in 3X exposed group on day 10; c. gills of pompano on 10X group on day 30 showing lamellar lifting (arrow) and increased thickness at the secondary lamellar base (arrowhead); d. muscle tissue in 10X group on day 40 showing Zenker's degeneration (arrow) of the muscle fibers.

cessation of medicated feeding. Significant circulatory alterations were noticed only in the kidney ($P<0.05$) and intestine ($P<0.01$) during the therapeutic exposure (10 mg Kg-1 for ten days).

Inflammatory Alterations in the Organs After FFC Exposure

Inflammatory reactions indicated by the infiltration of inflammatory cells were observed only in the kidney of the 10X treatment group on days 30 and 40 of the commencement of FFC exposure (Figure 4b).

Regressive Alterations in the Organs After FFC Exposure

Significant regressive alterations included degeneration, necrosis, and increased MMC in the kidney, fatty changes, vacuolation, cell swelling, increased MMC, and necrosis in the liver, depletion of white pulp in the spleen, lifting of lamellae in gills, and Zenker's degeneration and necrosis in the muscle (Figure3a). There were no significant regressive alterations in the intestine throughout the experimental period. In the muscle, gills, and spleen the regressive alterations were evident only in the higher exposures (Figure 5a). In the case of the liver and kidney, highly significant ($P<0.001$) regressive alterations compared to the control group were evident in all the treatment groups throughout the experimental period.

Progressive Alterations in the Organs After FFC Exposure

There were no significant progressive alterations ($P>0.05$) in any of the organs studied, except the intestine, during the therapeutic exposure (10 mg Kg-1 for ten days) (Figure 5b).

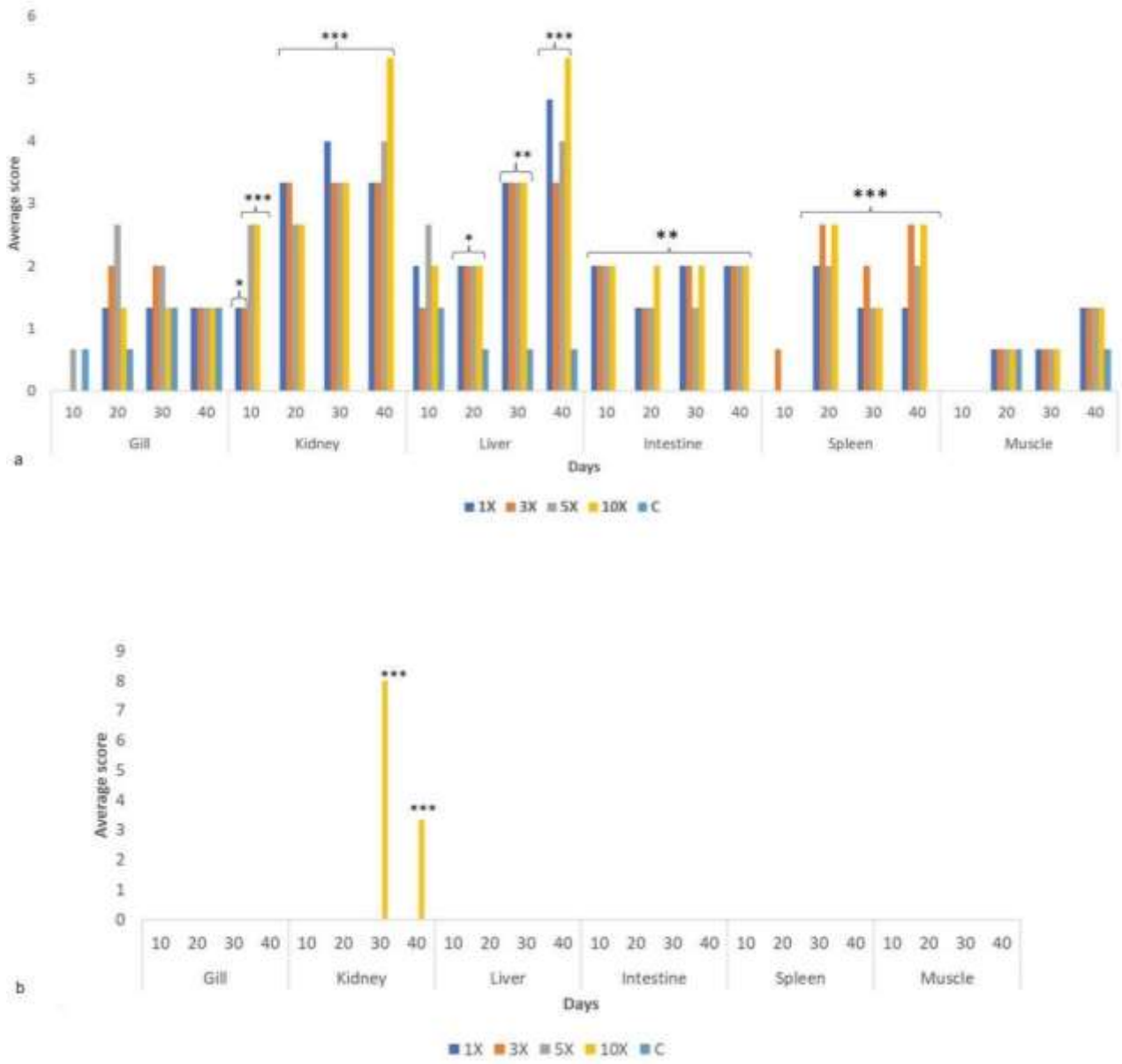

Figure 4. Reaction index-based scoring of florfenicol-induced tissue alterations in *T. blochii* (a: circulatory; b: inflammatory).

Organ Index (OI) After FFC Exposure

The OI showed that the order of susceptibility to histopathological alterations after FFC exposure was liver > kidney > gill > spleen > muscle > intestine. The alterations were depended on both dose and duration of exposure. The liver and kidney were the most severely affected organs during excessive FFC exposures (Figure 6).

Tissue Residues of Florfenicol in Snubnose Pompano Juveniles

There was a significant difference in residue concentration between the control and the treatment group ($P<0.05$). Further, the mean residue concentration differed significantly between time points ($P<0.05$) (Figure 7). The mean FFA concentration was the highest (221.33 µg Kg-1) on the 11th day of the withdrawal. The sum of FFC and FFA concentration in the muscle with skin in natural proportions was 1028.37±127.65 and 238.23±29.05 µg Kg-1 on days zero and three days after the termination of medicated feeding. In other words, the mean tissue residue concentration was below the maximum residue limit (MRL) (1000 µg Kg-1) for FFC from day three post-termination of medicated feeding.

Discussion

The present study showed that FFC exposure to both therapeutic and excessive doses did not cause any mortality in *T. blochii* during the entire experimental period, providing the safety feature of this drug in broader terms. In consonance with these results, no mortality attributable to FFC exposure was reported in tilapia, rainbow trout, and sunshine bass exposed to both therapeutic and excessive doses (Straus et al. 2012; Gaikowski et al. 2013; Bardhan et al. 2022). Nevertheless, the fish in the group exposed to 10X concentration (100 mg Kg-1 biomass) showed clinical abnormalities from day 25 after feeding. Similar to our results, a reduced feed intake in a dose-dependent fashion after FFC oral exposure has been reported in *O. niloticus, P. mesopotamicus,* and channel catfish (Gaikowski et al. 2003; Gaikowski et al. 2013; Bardhan et al. 2022). The observed abnormal clinical signs in the higher dose can be attributed to the damage to vital organs as evidenced by marked histopathological alterations induced by the FFC medicated feed. It is

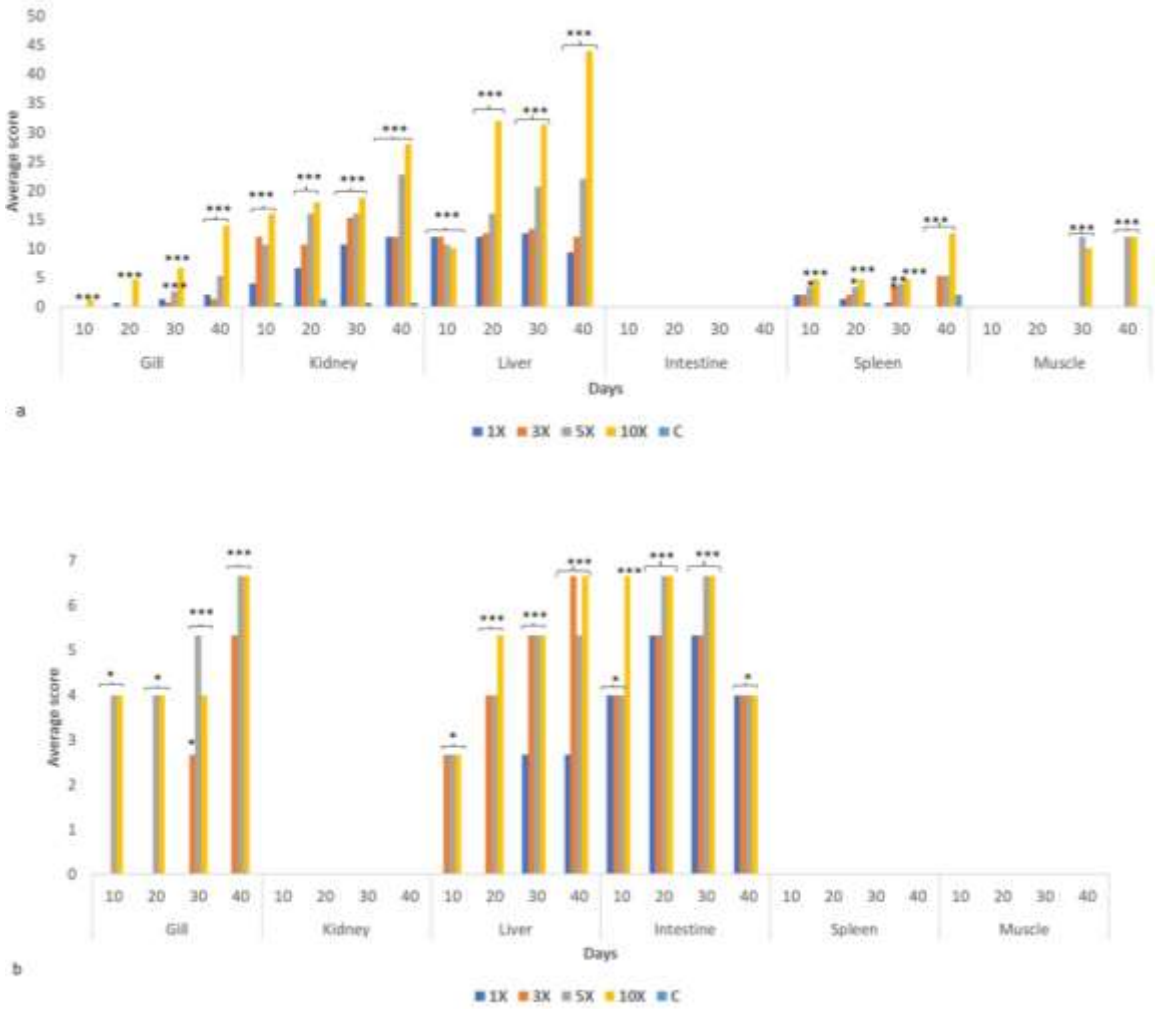

Figure 5. Reaction index-based scoring of florfenicol-induced tissue alterations in *T. blochii* (a: regressive; b: progressive).

important to note that all these clinical abnormalities were transient. Simultaneously, self-limiting the exposure to severe FFC intoxication by reduced feed intake can reduce the probability of FFC overdose in silver pompano and other fishes. The results showed that only the fish exposed to higher doses (5X and 10X) of FFC showed reduced weight gain from 30 and 20 days, respectively. The scientific data on the influence of FFC on the growth of different fishes are conflicting. FFC exposure in hybrid striped bass (Straus et al. 2012) did not alter the growth. Nevertheless, a significant dose-dependent reduction in the body size of Tilapia, *P. mesopotamicus*, and brown trout during different antibiotic exposure, including FFC, was reported (Gaikowski et al. 2013; Limbu et al. 2018). The clinical significance of the abnormalities and decreased weight gain observed will be minimal as these were observed only at five or ten times higher than the proposed dose, especially after more prolonged exposure.

Histopathological responses of various tissues serve as biomarkers for assessing and monitoring the toxic effects of different xenobiotics in the fish (Rodrigues et al. 2019). The histological lesions following FFC exposure in silver pompano were predominantly circulatory, even though certain regressive, progressive, and inflammatory alterations were also present. More importantly, all the alterations were both dose and duration-dependent. During therapeutic exposure (10 mg kg-1 for ten days), there were no significant alterations in the gill and muscle. The revealed hepatic damage might be the reason for the reduced feed intake observed in the study. Considerable inconsistencies have been observed in the histopathology of various fish species after FFC exposure. Straus et al. (2012) and Bowker et al. (2013) did not observe any significant lesions in different vital organs of Atlantic salmon, striped bass, and Yellow perch, respectively, even after exposure to higher doses (100 mg Kg-1 for ten days in salmon and 75 mg Kg-1 for 20 days in other two species). Dose-dependent pathological alterations of different vital organs, especially in the liver, kidney, and gills, were reported in *Oreochromis* sp. (Gaikowski et al. 2013; Reda et al. 2013; Bardhan et al. 2022) in support of our findings. The final score indicated that the order of susceptibility after FFC exposure was liver > kidney > gill > spleen > muscle > intestine. As scoring various organs based on histopathology after FFC exposure has not been attempted so far, comparing the results on the order of organ susceptibility was impossible. However, the marked hepatic and nephrotoxicity of FFC have been proved in terrestrial animals (Wang et al. 2021) in consonance with the present findings.

The results revealed that the liver was the most severely affected organ due to FFC exposure, which was expected, as the liver is the primary organ involved in the detoxification, biotransformation, and excretion of xenobiotics. It is important to note that after therapeutic exposure, there were only regressive alterations in the liver, while the lesions extended to circulatory and progressive alterations at higher exposures. The regressive alterations, especially cytoplasmic vacuolation of the hepatocytes, are the most reported toxicological response in the fish liver. Even though hepatocytes in fish are more vacuolated than mammals due to higher glycogen or lipid content, the control fish liver did not exhibit cytoplasmic vacuolation in the present study, pointing out FFC exposure as the probable reason. In support of our findings, regressive alterations have been reported in different fish species exposed to oxytetracycline (Sharma et al. 2021), FFC (Reda et al. 2013; Bardhan et al. 2022) and erythromycin (Rodrigues et al. 2019). Further, FFC is known to be cytotoxic (Bardhan et al. 2022), and the observed regressive changes in the liver support this hypothesis. Hyperplastic bile ducts indicated progressive alterations in the liver. Bile duct hyperplasia in fish is usually associated with contaminant exposure and parasitic infestation. Since parasites were not detected in the histological sections and hyperplastic bile ducts were found only in the FFC exposure groups, these lesions can be conveniently related to FFC exposure.

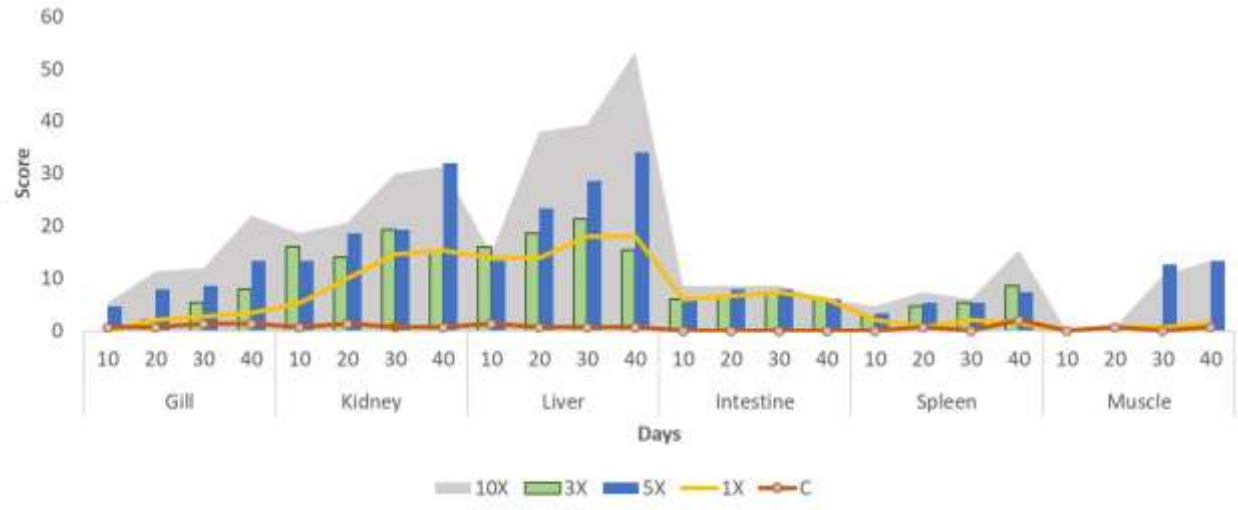

Figure 6. Organ index-based scoring of florfenicol-induced tissue alterations.

In the kidneys, the alterations were dependent on the dose and duration of exposure, similar to the observations of Gaikowski et al. (2013). The regressive lesions were evident in all the treatment groups throughout the experimental period, which revealed the FFC-induced nephrotoxicity in silver pompano, even at therapeutic exposures. Such nephrotoxic injuries indicated by the regressive alterations were also observed in *T. blochii* exposed to dietary oxytetracycline (Sharma et al. 2021). While the regressive alterations indicated the cytotoxicity of FFC to the kidneys, the persistence of these lesions from day ten might have elicited an inflammatory reaction in the interstitium indicated by the infiltration of inflammatory cells. Notably, the kidney was the only organ in which inflammatory lesions were observed, even though it was evident only in the higher exposure. In support of our results, mononuclear infiltration in the kidney was not noticed in channel catfish exposed to the therapeutic dose of FFC (Gaikowski et al. 2003), while dose-dependent inflammatory alterations at higher doses were noted in *O. niloticus* after FFC exposures (Gaikowski et al. 2013; Bardhan et al. (2022).

The general cytotoxic nature of FFC might cause regressive and progressive alterations in the gills. Similar histological gill responses have been reported in *Oreochromis* sp. exposed to FFC (Gaikowski et al. 2013). In the intestine, the alterations were also dependent on the dose and duration of exposure. The alterations observed in the present study are likely to affect the secretion of digestive enzymes; this can be a probable reason for the inhibition of digestive enzymes observed in European seabass (*D. labrax*) after FFC exposures (Zhang et al. 2021). Further, the impairment in the structure and function of the intestine following exposure to sulfamethoxazole and oxytetracycline, has been reported in zebrafish (Zhou et al. 2018) in support of the present results. The circulatory and the regressive alterations in the spleen were contributed by depletion of white pulp with increased MMC, respectively, similar to the report in Channel Catfish (*I. punctatus*) (Gaikowski et al. 2003). The only significant histological lesions observed in the muscles were regressive type, including Zenker's degeneration and necrosis. Similarly, Gaikowskiet et al. (2013) and Bowker et al. (2013) reported no detectable alterations in the muscle of *Oreochromis* sp. and yellow perch on exposure to antibiotics. The mild tissue lesions observed at higher doses after a more prolonged duration might be due to the production of reactive oxygen species that lead to tissue necrosis or direct cytotoxic effects (Bardhan et al. 2022).

Knowledge of the tissue residue level of each drug after therapeutic exposure is crucial in setting the guidelines for using these compounds in fish farming, ensuring consumer safety. Factors like dose, duration, host species, mode of drug administration, and water temperature can influence the elimination of the drug. In this context, it is very pertinent to establish the tissue residue concentrations for each drug under local environmental conditions, even within the same species. As the skin and muscle are the edible portions of the tissues in fish, these were only considered for evaluating the residue concentration. The marker residue in the target tissue of florfenicol is usually calculated as the sum of FFC and FFA. The results showed that the mean tissue residue concentration was below the MRL (1000 µg Kg-1) for FFC (EMA, 2013) from day three post-termination of medicated feeding, suggesting a withdrawal period of three days for snubnose pompano after therapeutic exposure. In consonance with our findings, a residue concentration below MRL was observed on day three post-withdrawal of medicated feeding in *Oncorhynchus mykiss* after oral exposures (Türe et al. 2019). However, in European sea bass, the drug concentrations declined below the MRL at six days post-treatment (Kogiannou et al. 2020). In *Piaractus mesopotamicus*, the level of FFC and FFA were below the MRL on day five of termination of FFC feeding (Marques et al., 2017) and in *I. punctatus*, FFA and FFC were below the MRL on day four (Wrzesinski et al. 2006). In short, a comparatively shorter period for the

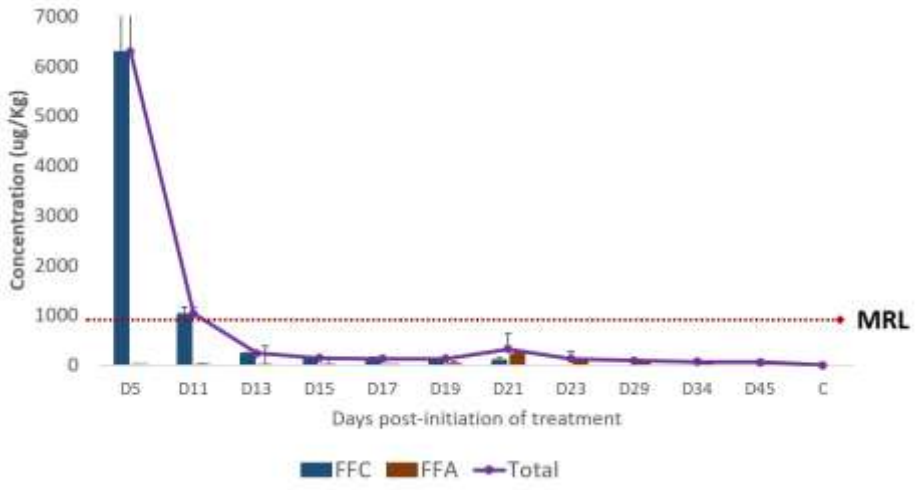

Figure 7. Tissue residue concentrations of florfenicol and florfenicol amine in muscle with skin in snubnose pompano exposed to the therapeutic dose of florfenicol.

residue to reach below MRL than in other studies might be due to the younger fish used compared to the other reports, as younger fish can clear the drug due to a higher metabolic rate. While FFA is the metabolite of FFC in many fish species, all the fish species cannot metabolize FFC into FFA, like *Paralichthys olivaceus* and *Gadus morhua* (Lim et al., 2010). As the present study could detect FFA in silver pompano, it was clear that the fish species can metabolize FFC. As there are no studies on FFC residue in *T. blochii* after therapeutic exposure, a detailed comparison with earlier studies was impossible. The results also warrant future research exploring the withdrawal period of FFC following therapeutic exposure in adult silver pompano fish.

Another noteworthy observation was that a negligible amount of FFC residue (>100 µg Kg-1) contributed by FFA was present in the muscle of *T. blochii* even after 19 days post-cessation of the medicated feeding. Similarly, several previous research reported the persistence of FFA in the muscle for varying periods, *viz*., 14 days (Kosoff et al. 2009) and 21 days (Bardhan et al. 2022) in *O. niloticus*, and 28 days in *Sander vitreus* and *Culter alburnus* (Kosoff et al. 2009) post-cessation of therapeutic exposure. Nevertheless, the presence of antibiotics far below the MRL levels has minimal practical relevance and does not affect the safe nature of flesh for human consumption (Manna et al. 2020). It was found that there was no significant difference between the residue concentration between sampling points (once every two days) after the 7th-day post-cessation of medicated feeding. The results suggested that FFC elimination from the muscle of *T. blochii* happened in two phases; a rapid initial elimination phase and a slow secondary elimination phase. A similar FFC depletion profile in two phases has been elucidated by Bardhan et al. (2022) in *C. alburnus* and *O. niloticus*, respectively.

Conclusion

In conclusion, the residue levels, growth data, and pathological investigations suggested that the dose rate of 10 mg kg-1 for ten days was well tolerated by *T. blochii*. A withdrawal period of three days after this therapeutic exposure is necessary to ensure consumer safety. Altogether, the paper will aid in designing and implementing the safe clinical application of this approved antimicrobial drug under controlled conditions during the incidences of bacterial diseases in aquaculture practices of high-value marine fish species.

Ethical Statement

All the investigations involving live animals were done in compliance with ARRIVE guidelines. The live fish were handled as per the UK Animals (Scientific Procedures) Act (1986) and EU Directive 2010/63/EU for animal experiments (2019). The followed experimental protocols were sanctioned by the ICAR-CMFRI, India (CIBA/AINP-FH/2020-21).

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Author Contribution

KSSR conceptualized the presented idea, analysed the results, supervised the project, wrote the manuscript, and acquired financial support for the project leading to this publication. STG helped in analyzing the results, and drafting the manuscript. RP, AK, SG, and VP conducted the experiments, sampling, and sample processing. APG, TG, AJ, RKK, RR, PSK, and AMK provided technical support to carry out the experiments. PKP provided critical feedback while drafting the manuscript

Conflict of 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

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