Sveriges lantbruksuniversitet
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Faculty of Veterinary Medicine and Animal Science
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Maria Blomberg

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Veterinärmedicinska antiparasitära läkemedel – effekter på beteende hos fiskyngel

Maria Blomberg

Supervisor: *Gunnar Carlsson, Department of Biomedical Sciences and Veterinary Public Health*

Assistant Supervisor: *Johannes Pohl, Department of Biomedical Sciences and Veterinary Public Health.*
Stefan Örn, Department of Biomedical Sciences and Veterinary Public Health

Examiner: *Eva Tydén, Department of Biomedical Sciences and Veterinary Public Health*

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SUMMARY

Antiparasitic pharmaceuticals are important groups of pharmaceuticals in veterinary medicine and are used in large quantities. Antiparasitic pharmaceuticals and their metabolites enter the environment via excretion in urine and/or faeces from treated animals and by run off to surface water these compounds reach aquatic environments and thereby present a risk for aquatic organisms. The goal of this master thesis is to investigate if veterinary antiparasitic pharmaceuticals have any effects on the behaviour of zebrafish larvae. In addition, adding of a behavioural assay could increase sensitivity in the fish embryo toxicity test. There are some studies of toxicity of veterinary pharmaceuticals in aquatic animals, but they focus mainly on general toxic effects. Several of the antiparasitic pharmaceuticals has a neurotoxic mechanism of action, and may therefore affect behaviour of zebrafish larvae, which in turn can affect its responses to stimuli and the survival of the larvae in its natural environment.

In this study zebrafish larvae were exposed, from day 0 until day 6, to three different antiparasitic pharmaceuticals: doramectin, flumethrin and toltrazuril. An additional study was performed with doramectin, in order to study the effects of short time exposure. In the short term study the embryos were exposed to doramectin at day 6 for 1 h before behaviour recording. In the long time exposure experiments a behavioural assay was performed at day 6 using a video recording device (Viewpoint Zebrabox®) that recorded the swim activity of the larvae. Directly after the behavioural assay the larvae were assessed in stereo microscope for abnormalities and abnormal body posture. Only data from individuals that were considered as normal according to the macroscopic examination were included in the data set and used in the statistical analyses. Because of the large amount of data that were received from the recording device, four responses were chosen for statistical analysis: mean activity during the first period of darkness, mean activity during the first period of darkness, increase in activity during the first 10 seconds at the first period of darkness and difference between mean activity during the first and the last period of darkness.

Significant results were found for doramectin and flumethrin. The activity in the first five minutes dark period and the first five minutes light period were both significantly reduced in larvae exposed to the high concentration (0.58 mg/L) of doramectin compared with the control. Larvae exposed to the high concentration (0.11 mg/L) of flumethrin had a significant higher reduction in activity from the first to the last dark period as compared with the reduction in control larvae. The conclusion is that it is possible to detect altered behavioural response, in individuals that could not be classified as affected based on visual features. This finding supports that behavioural assays are potential tools to increase the sensitivity in toxicological studies in zebrafish.

SAMMANFATTNING

Antiparasitära läkemedel är en läkemedelsklass som används i stor mängd inom veterinärmedicinen och som sannolikt kommer ut i miljön via urin och faeces från behandlade djur. Via bland annat avrinning från åkrar där gödsel och urin från djuren spridits så kan dessa substanser, och dess eventuella metaboliter, nå vattendrag och därmed utgöra en risk för vattenlevande organismer. Syftet med studien är att studera hur zebrafiskyngels beteende påverkas av tre olika läkemedels substanser ur denna läkemedelsklass. Ett annat syfte var även att undersöka om beteendestudier kan öka känsligheten i toxiska tester. Det finns en del data gällande generella toxiska effekter av veterinärmedicinska läkemedel på vattenlevande organismer, däremot få studier om hur de påverkar beteendet hos dessa djur. Flera antiparasitära läkemedel är neurotoxiska och antas därigenom kunna påverka beteende, vilket i sin tur antas kunna påverka dessa djurs respons på olika stimuli och därigenom deras överlevnad i naturen.

I denna studie exponerades zebrafiskembryon från dag 0 till dag 6 för tre olika läkemedelssubstanser, doramektin, flumetrin och toltrazuril enligt en standardiserad metod. Det gjordes även ett försök med korttidsexponering där embryona exponerades för doramektin dag 6, under en timme innan avläsning. I långtidsexponeringarna utfördes en beteendeanalys dag 6 avseende påverkan på aktivitetsnivån (simaktiviteten) med hjälp av ett videoanalyssystem (Viewpoint Zebrabox®). Direkt efter videoanalysen gjordes en visuell bedömning av embryona avseende fysiologiska och morfologiska avvikelser samt avvikelser i kroppspositionering. Endast data från embryon som inte uppvisade några förändringar inkluderades i de data som analyserades statistiskt. Ur den stora mängd data som erhölls valdes fyra responser ut för statistisk analys: aktivitet under de 10 första sekunderna under första mörkerperioden, medelaktivitet under första mörkerperioden, medelaktivitet under första ljusperioden, samt skillnad i medelaktivitet mellan första och sista mörkerperioden.

Signifikant resultat erhölls för högsta koncentrationen av flumetrin och doramektin. Flumetrin i koncentrationen 0,11 mg/L gav en signifikant sänkning i medelaktivitet mellan första och sista mörkerperioden hos de exponerade ynglen jämfört med kontrollen. Yngel som exponerats för doramektin i koncentrationen 0,58 mg/L hade lägre aktivitet både under första mörkerperioden och under första ljusperioden. Det var således möjligt att med metoden detektera beteendeförändringar hos yngel som vid en visuell bedömning klassificerades som normala och detta talar för att beteendeanalys kan vara användbart för att öka känsligheten i toxikologiska studier på zebrafisk.

CONTENT

INTRODUCTION

Antiparasitic drugs are important groups of pharmaceuticals that are widely used for veterinary purposes. They are administrated to animals to treat or prevent parasitic infections and can be administrated topically, orally or injected intramuscular/subcutanous. The major route of entry into the environment is probably via excretion to urine and/or faeces. The antiparasitic compounds and/or their metabolites are then released in the in the environment either directly from grazing livestock or by the disposal of manure and slurry to agriculture land. By run off to surface water the antiparasitic compounds can reach aquatic environments and thereby present a risk for aquatic organisms.

There are some studies (Wollenberger *et al.*, 2000; Oh *et al.*, 2006; Carlsson *et al.*, 2013) where the toxicity of veterinary pharmaceuticals in aquatic animals have been examined. These studies focusing mainly on general toxic effects, not on behavioural effects. Carlsson *et al.*, (2013) investigated the toxicity of 15 different veterinary pharmaceuticals. Several substances in that study were antiparasitic pharmaceuticals, some of which are known to have neurotoxic mechanisms of action in their target organisms. It is possible that drugs acting on the nerve system may lead to altered behaviour in fishes and there are some studies done (Beauvais *et al.*, 2000; Chen *et al.*, 2012; Domingues *et al.*, 2016), but there are currently few studies done that focus specifically on the effects veterinary pharmaceuticals. An altered behaviour, either caused by direct neurotoxicity or due to developmental abnormities may have an impact of the survival of the animal (Tierney, 2011). Behaviour are responses to cues, either from the animal itself or from its environment, with the intention to increase the animal's fitness. Detecting a deviant or impaired behaviour is therefore an interesting and relevant endpoint in toxicity tests.

The goal of this master thesis is to investigate if three different veterinary antiparasitic compounds have any neurotoxic effects on zebrafish larvae (*Danio rerio*). In addition, if a behavioural assay could increase sensitivity in fish embryo toxicity tests.

LITERATURE REVIEW

Pharmaceuticals in the environment

Low levels of veterinary pharmaceuticals have been detected worldwide in soils, surface water and groundwater (Boxall *et al.*, 2003). The consequences of this has been widely studied for some groups of pharmaceuticals such as anthelmintics and some antibacterial compounds, but remains unclear for many other substances.

Veterinary drugs can enter the environment via several different pathways (Halling-Sørensen *et al.*, 1998; Boxall *et al.*, 2003). They can be released as emissions from pharmaceutical factories during the manufacture, be released from pharmaceuticals and their containers that are disposed after usage or be released from the animal during the treatment process. How the drug is emitted during the treatment process will depend on way of administration and on the methods of animal husbandry. The most important routes of entry into the environment are likely the discharge of aquaculture products, the wash-off of topical treatments and the

excretion of substances in urine and faeces of livestock (Boxall *et al.*, 2003). Contributions from the manufacturing process are likely low in the US and EU due to strict regulatory controls of manufacturing and formulation of pharmaceuticals. Another possibility is that veterinary drugs enter the environment as aerosols or dusts, but the significance of these releases is unknown. Also the impacts of emissions from treating pets and disposing of unused or expired products cannot be established but researchers consider emissions via these routes less relevant than emissions from treatment of aquaculture and livestock animals.

When released into the environment veterinary compounds will be distributed to air, water, soil, or sediment (Boxall *et al.*, 2003). The distribution pattern will be influenced by the physicochemical properties of the substance, the extent of degradation, its propensity to partition to soil and sediment and the characteristics of the receiving environment. Chemical properties such as water solubility, pH of the matrix, volatility, and sorption potential will influence its behaviour. Also the level of degradation will play a major role for how much of the pharmaceutical that will reach the environment and how persistent it will be. Rate of degradation of pharmaceuticals can vary significantly across chemicals. The environment, in which the pharmaceutical ends up, also effects the rate of degradation due to environmental conditions such as temperature, soil type, and pH. The same component can have different degradation rates in manure, slurry, soil and water. Even smaller differences, such as various manure types can have a significant effect. The degradation process generally reduces the potency of the pharmaceutical but there also exist degradation products that have similar toxicity to their parent compound (Boxall *et al.*, 2003).

Antiparasitic pharmaceuticals

According to the 2015 annual report of veterinary pharmaceutical sales a total of 20353 kg (active substance) of antiparasitic pharmaceuticals were sold during 2015 (Jordbruksverket, 2015). This was an increase from the previous year by 4218 kg. A big part of the sales consisted of sulfonamides and triazins sold as feed additives for production animals i.e. poultry. The following antiparasitic substances were on the list as the most sold antiparasitics in Sweden during 2015: sulphonamides, triazins, quinolones, benzimidazoles, tetrahydropyrimidines, pyretrins and pyretroids, avermectines and milbemycines.

Sulphonamides are used as a food additive to prevent coccidiosis in poultry (Siddiki *et al.*, 2008). It is a competitive antagonist of para-aminobenzoic acid (PABA), which is a precursor of folic acid in protozoa and bacteria. Folic acid is a coenzyme necessary for the synthesis of nucleic acid. Triazins are used to treat coccidiosis in several species. It affects the perinuclear space, mitochondria, and the endoplasmatic reticulum of the parasites and leads to an reduce of enzymes in the respiratory chain such as succinate-cytochrome C reductase, NADH oxidase and succinate oxidase (Harder & Haberkorn, 1989). Quinolones such as praziquantel, are used to treat tapeworm infections. They increase calcium permeability of parasite muscle and/or tegumental membranes (Martin, 1997). The increased Ca^{+} influx across the tegument cause a rapid muscle contraction of the parasite. Benzimidazols are also used as anthelmintics for treatment of nematode infections (Lacey, 1990). They bind to microtubulin, a component of the cytoskeleton of eukaryotic cells, and thereby inhibit the polymerisation. Tetrahydropyrimidines, such as pyrantel, another anthelmintic for treatment of nematode

infections, acts by disrupting neuromuscular transmission in nematodes (Rayes *et al.*, 2001). It causes depolarization of the muscle membranes in nematodes by acting as an agonist of nicotinic receptors (AChRs). Pyrethrins and pyrethroids are used to control ectoparasites in animals (Valentine, 1990). They act on voltage-dependent sodium channels in nerves by slowing down the closing of the sodium activation gate which result in neural dysfunction. Some pyrethroids has a second mechanism of action, they disrupting the nerve signals by inhibiting the peripheral gamma amino butyric acid (GABA) receptors, which are unique to arthropod muscles. Avermectines and milbemycines are anthelmintics, belonging to macrocyclic lactones, belong to endectocides and are used for treatment of both nematode infections and ectoparasities. Avermectines and milbemycines increase the muscle Cl^- permeability by opening glutamate-gated Cl^--channels and thereby cause paralysis (Martin, 1997). They also act at lower concentrations by potentiating the effect of glutamate.

Few of the anthelmintics and endectocides are completely metabolised to inactive molecules within the host (McKellar, 1997). This means that antiparasitic pharmaceuticals with biological activity are normally excreted in the urine or faeces of the treated animals and that way may present a risk for non-target organisms. The amount of an antiparasitic compound that will enter the environment depends on the husbandry systems and stocking densities of the treated animals. The ecotoxicological effects may also be diminished if non-treated animals within the herd contribute with uncontaminated faeces. For the individual treated animal, the contribution will depend on amount of the substance, method of delivery (faeces, urine, wash off), the extent of metabolism and level of excretion of biologically active molecules.

After being excreted some antiparasitic compounds can cause harm to non-target organisms (McKellar, 1997). It has been proven that members of the avermectin/milbemycin group effects on non-target organisms that are utilising the faeces of the treated animals. Ivermectin residues in the dung has reduced, or even prevented, breakdown of pats by insect activity (Strong *et al.*, 1996). Antiparasitic pharmaceuticals, such as benzoylphenylurea insecticides and pyrethroids, such as pyrethrins and pyrethroids, are also used in aquaculture to treat parasitic infections in fishes kept for food production. In a Norwegian study (Langford *et al.*, 2014) antiparasitic compounds were detected in wild shrimps at levels at which chronic effects are seen in experimental studies on this species. This suggest that antiparasitic pharmaceuticals from aquaculture is a potential risk to these animals.

Toxicity of antiparasitic pharmaceuticals to water living or organisms have been tested in several studies. In a study by Yoshimura & Endoh, (2005) acute toxicity of five antiparasitic pharmaceuticals used in the veterinary field were tested. The five pharmaceuticals that were included in the study were amprolium hydrochloride, bithionol, levamisole hydrochloride, pyrimethamine and trichlorfon. They were tested for acute toxicity on three aquatic species: *Oryzias latipes*, *Daphnia magna*, and *Brachionus calyciflorus*. The least toxic substance to these organisms were amprolium hydrochloride, were the LC_{50} concentration ranged between 227 mg L^{-1} for the most sensible species to >600 mg L^{-1} for the most tolerant one. Bithionol was the most toxic substance for *O. latipes* (LC_{50} of 0.24 mg L^{-1}). *D. magna* was the most susceptible species to trichlorfon (EC_{50} 0.00026 mg L^{-1}) while *B. calyciflorus* was the most susceptible species to bithionol (EC_{50} 0.063 mg L^{-1}). Carlsson *et al.* (2013) tested the toxicity

of 15 veterinary pharmaceuticals by using the Fish Embryo Toxicity (FET) test on zebrafish. Ten of the tested substances were antiparasitic compounds and they were able to determine EC_{50}-values for eight of them. Four benzimidazoles were tested: albendazole (EC_{50} of 0.042 mg/L), febantel (EC_{50} of 0.34 mg/L), fenbendazole (EC_{50} of 0.024 mg/L) and oxfendazole (EC_{50} of 6.8 mg/L). Two avermectines, doramectin and ivermectin, were tested and were found to have EC_{50} values of 0.58 mg/L respectively 0.44 mg/L. The two remaining substances were flumethrin (a pyrethroid) with an EC_{50} of 0.11 mg/L and toltrazuril (a triazin) with an EC_{50} of 1.1 mg/L.

Fish Embryo Toxicity (FET) test

The zebrafish embryo toxicity test is a popular model used to study toxicity. The advantages are many; zebrafishes are easy to keep and handle, they are relatively inexpensive, the produce a large number of progeny and they have a rapid embryo development. Another advantage is that they are egg laying and their eggs are transparent which makes it easy to follow the developmental process (Goldsmith, 2004; Lieschke & Currie, 2007). The embryos can be kept in microtiter plates in a very small volume of water and because of their yolk sac they do not have to be fed. Only a small amount of the toxic compound, that are going to be tested, are needed. The embryos develop organs that are similar to their mammalian counterparts at an anatomical, physiological and molecular level.

The principle for the test is that newly fertilized embryos are exposed for the substance for a total of 96 h (OECD, 2013) . The embryos are then observed every 24 h for four lethal indicators: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail bud from the yolk sac, lack of heartbeat. A somite is a division of the body of an animal or embryo and an indication of that they have been formed is that the embryo shows spontaneous movements (typically side-to-side contractions). Hatching rates of the embryos are recorded from 48 h and onward. The test is terminated after 96h. Based on the recordings of those four lethal indicators, acute toxicity is determined and the LC_{50} is calculated. Experiments in fish embryos do not need approval from an Ethical Committee on Animal Experiments, if the experiment is ended before the larvae reaches the age when they start eating (2 cap. 5 § Statens jordbruksverks föreskrifter och allmänna råd [SJVFS 2012:26] om försöksdjur, L 150).

Behaviour of zebrafish larvae

Zebrafish larvae has also several advantages when it comes to behavioural studies. Newly-hatched zebrafish larvae have a rich behavioural repertoire (Budick & O'Malley, 2000; Burgess & Granato, 2007). At the same time they have less diversity of their locomotor repertoire compared to vertebrates with more complex behaviour. This, together with their small size, makes it easier to assess behaviour using objective quantification methods. Fish larvae has a nervous system of limited complexity but at the same share many features with the mammalian neuroanatomy.

Several locomotor behaviours has been described in zebrafish larvae such as optomotor response, prey tracking, phototaxis and escape response (Burgess & Granato, 2007). Optomotor response is a behaviour in which the larvae turns and swim in the direction of a perceived motion, after being presented with a whole-field moving stimulus, (Portugues & Engert, 2009).

This behaviour can at earliest be detected 5 days post fertilization shall be present in the larvae at day 7. The behaviour persists throughout adulthood. Prey tracking is a complex behaviour consisting of several parts. Firstly, the larvae have to spot and identify the prey. Then it starts to track the prey and swims and turns slowly. This part of the prey tracking behaviour demands very fine motor control. Finally, the larvae starts the swim to capture the prey. The larval escape or visual startle response are fully developed in zebrafish at 79 hours post fertilization (Easter & Nicola, 1996). The locomotor activity of zebrafish larvae are affected by exposure to dark or light conditions (Padilla *et al.*, 2011). When zebrafish larvae (6d hpf) were exposed to light they showed an increase in locomotor activity when the light was switched off and they were left in completely darkness. Also if the light was switched to a lower level, but not to complete darkness, they showed an increase in activity. Padilla *et al.* (2011) tested switching between different light levels. They found that the larger difference in light level between the lighter and the darker period was, the more locomotor activity in the larvae. This implies that the changes in activity is caused by the contrast between the different light levels and not by the simple change from light to darkness. In the present study we will use response to dark and light as a tool to access altered behavioural response in larvae exposed to three different antiparasitic pharmaceuticals.

MATERIAL AND METHODS

Pharmaceuticals

Three antiparasitic compunds were tested in the present study: doramectin, flumetrine and toltrazuril (supplier: Sigma–Aldrich). The selection of substances was based on a previous study by (Carlsson *et al.*, 2013) in which 15 veterinary pharmaceuticals were tested for toxicity in zebrafish embryos. Doramectin and fluethrin were chosen because of their potential neurotoxic effects which make them highly interesting in a behavioural assay. Toltrazuril was included because it, in high concentrations, reduced early spontaneous movements in the zebrafish embryos. The concentrations used in the present study were based on the EC_{50} values in (Carlsson *et al.*, 2013) with the EC_{50} representing the highest concentration. The low concentration was set to 10 times less than EC_{50} and the medium concentration to be in the middle of the high and the low concentration based on a logarithmic scale. Stock solutions were prepared by dissolving the three substances in dimethyl sulfoxide (DMSO). The medium used for the larvae were carbon filtered and aerated tap-water with a temperature of 26°C and a pH of 8.2.

Rearing of parental fish

Adult zebrafish were kept in two tanks with a flow-through system of carbon filtered tap-water. The photoperiod was 12 h darkness and 12 h light. Water temperature was kept at 26 °C and the fishes were feed twice a day with commercial flakes (SERA Vipan).

Egg collection

In the afternoon, the day before egg laying, adult zebrafish were moved from their original tanks and placed in cages in four 20L tanks with 6-7 females and 5-6males in each. The cages were then moved to new tanks, same number and size, in the morning to ensure that the eggs collected were freshly laid. The fishes were allowed to spawn for 1 h from onset of light and

the eggs were then collected and transferred to a petri dish. Before starting the test procedure, the eggs were checked to have developed at least to the four cell stage to ensure fertilization and acceptable egg quality.

Exposure design

The exposure design is visualized in table 1. In the present study 0.1 % DMSO were used in the final solutions. OECD (2013) recommend that if a solvent is needed for the solution preparation, the final concentration of the solvent should not exceed 100 µL/L (0.01%). Because the solvent in this study was used in 10 times higher dose than OECD guidelines recommend we did an additional behavioural assay investigating if DMSO in the concentration of 0.1 % could cause changes in locomotor activity. Water without any additives were used as control.

Table 1. *Design of exposure in the different tests. Measurements of malformations and behaviour were performed at day 6 post fertilization (dpf). DMSO (0.1 %) were always included in both controls and all exposure groups. The groups exposed at day 6 were free from DMSO before exposure and exposure occurred one hour before start of behaviour recording*

Test	Compound	Concentration (mg/L)			
Normal test		Control	Low (L)	Medium (M)	High (H)
	Doramectin	0	0.058	0.18	0.58
	Flumethrin	0	0.011	0.035	0.11
	Toltrazuril	0	0.11	0.35	1.1
Short exposure					
	Doramectin	0			0.58

Embryo test and behaviour assay

The embryos were placed in petri dishes with different concentrations of the pharmaceutical according to the exposure design (see table 1). Each embryo was then transferred, together with 750 µl of the solution, to wells in 48-well plates. Each exposure group included 24 embryos. The plates were covered with parafilm and incubated for 6 days in a room with a consistent temperature of 26 ° C and a photoperiod of 12 h darkness and 12 h light. Embryos undergoing the short time exposure were placed in the wells together with 750µl of water and incubated under the same conditions as described above. At the morning day six, before the light was turned on in the room and 60 minutes prior to the locomotor assay, 7.5 µl of the different stock solutions were transferred to the wells.

The larval behavioural assay was performed using the Viewpoint Zebrabox® behavioural recording device (ViewPointLife Sciences, Lyon, France). This equipment consists of a multi-well plate holder (Zebrabox) in which a plate with zebrafish larvae can be placed. Inside the

Zebrabox is an automated camera system that records the activity of the larvae. The Zebrabox is equipped with internal LED lights and infrared illumination so recordings can be performed under light as well as dark conditions.

On the day of recording, the light in the room was turned off. Hence, all zebrafish larvae were kept in entirely darkness until they were placed in the device and recorded. Each 48-well plate was transferred to the Zebrabox where the larvae were allowed to acclimatize in light for 15 minutes. After the acclimation phase, the locomotor activity was recorded in 18 consecutive 5-min phases of alternate dark and light.

Macroscopic evaluation of the larvae

Directly after the behavioural assay all larvae were assessed in stereo microscope for abnormalities. Because the main purpose of this study was to test if a behavioural assay increases the sensitivity of the FET-test only individuals that was visual healthy (no anomalies, deformities or larvae that showed abnormal body posturing at rest) were included in the data set. Abnormal body posturing can be a sign of neuro toxicity. Dorso-ventral position (fig 1) was considered as the normal body position of a zebrafish larvae. The following abnormities in body posturing were found: side laying (fig 2a), tilting to one side (fig 2b), laying in a normal dorso-ventral position but with lateral flexed back (fig 2c), tilting to one side with a lateral flexed back (fig 2d). The individuals that tilted to the side did this to various extent, from close to the normal dorso-ventral position to almost side laying. The ones that were close to dorso-ventral position often seemed to wobble and trying to correct themselves to a dorso-ventral position, but fell back into the tilted position immediately after they finished the attempt.

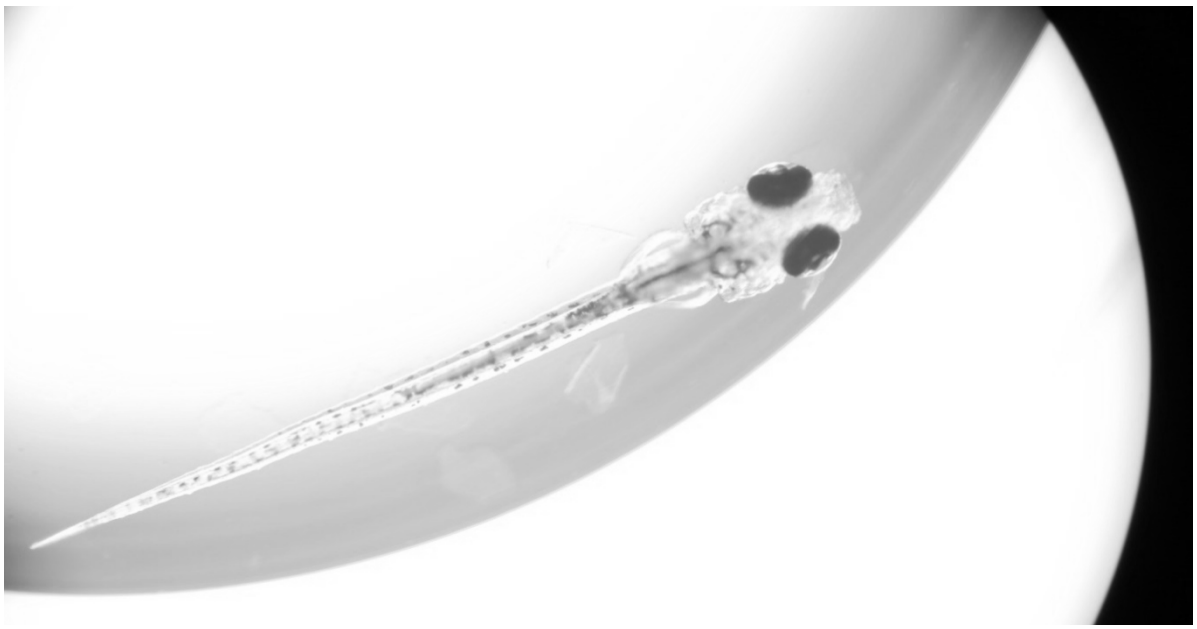
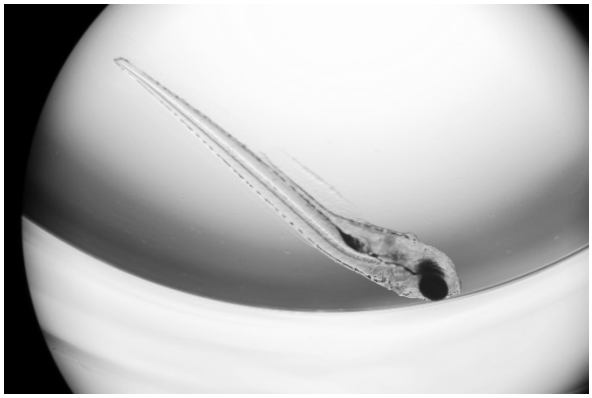
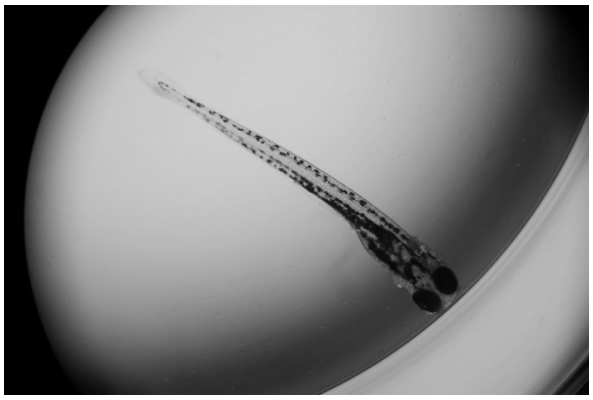


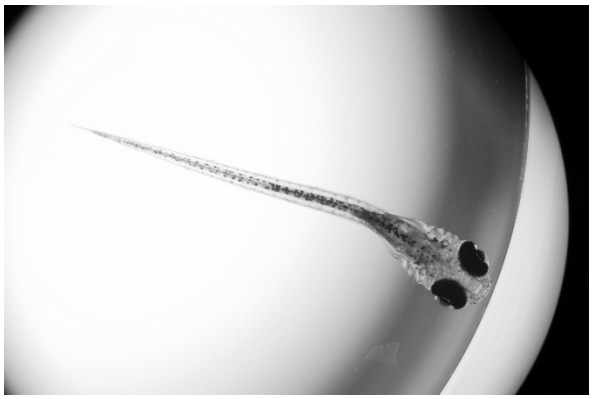
Figure 1: *The normal dorso-ventral body posturing of a zebrafish larvae.*



2a



2b



2c

2d

Figure 2a-d. *Abnormal body postures that was observed in the larvae: side laying (1a), tilting (1b), lateral flexed back (1c), tilting to one side with lateral flexed back (1d).*

Data analysis

Individuals that did not met the previous mentioned criteria, visual healthy and no abnormal body posturing at rest, and dead individuals were excluded from the data set. The mean locomotor activity where then plotted as a function of time for each of the three pharmaceuticals. Based on these graphs four responses were chosen for statistical analysis: mean activity during the first period of darkness, mean activity during the first period of light, increase in activity during the first 10 seconds at the first period of darkness and difference between mean activity during the first and the last periods of darkness. These four responses were statistically analysed using ANOVA with Dunnett's post-hoc test.

RESULTS

Visual inspection

The result of the visual inspection of the embryos exposed to doramectin is presented in figure 3a. The mortality rate was found to be 4.2 % in the control group (this represents one individual) and 8.3 % in the highest concentration. There was no mortality in the low and the medium concentrations. There were affected animals in all four groups including the control. The number of affected animals increased with the increasing concentration of doramectin, from 12.5 % in the control group to 33.3 % in the highest concentration. Figure 3b shows the percentage of visually affected individuals in the flumethrin toxicity test. All 96 embryos were alive at day 6 so the overall mortality rate was 0 %. Figure 3b also shows that it was a quite

high number of affected animals in the control group (29.2 %). All of these were considered as affected based on abnormal body posturing. However, these individuals were considered as less severe affected than the affected individuals in the higher concentrations. All but one of the affected individuals in the control group showed tilting whereas the affected larvae in the highest concentration showed side laying and some had malformations. The total percentage of affected larvae in the highest concentration was 41.7 %. The percentage of affected larvae in the toltrazuril test is visualized in fig 3c. The number of affected animals were low in all three groups, ranging between 8.3-16.7 %. In the DMSO test the number of affected or dead larvae was approximately the same: 21 % for DMSO and 25 % for the control.

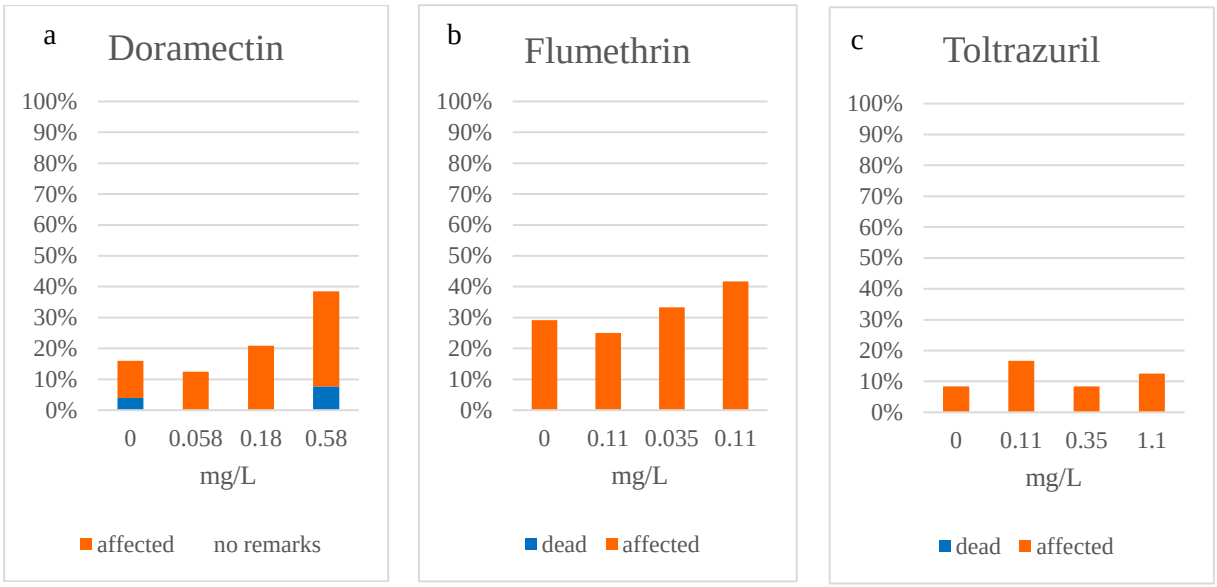
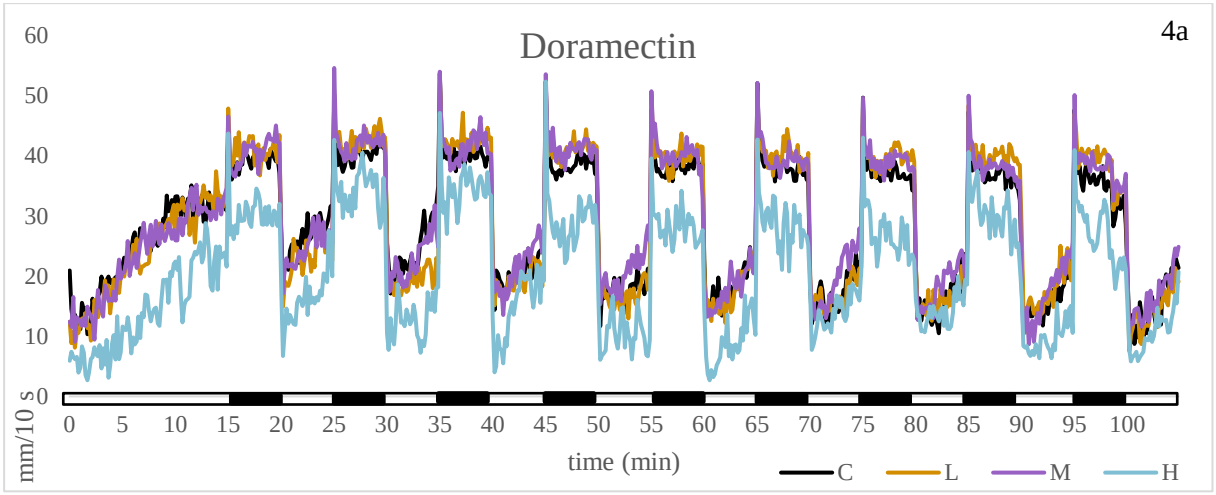
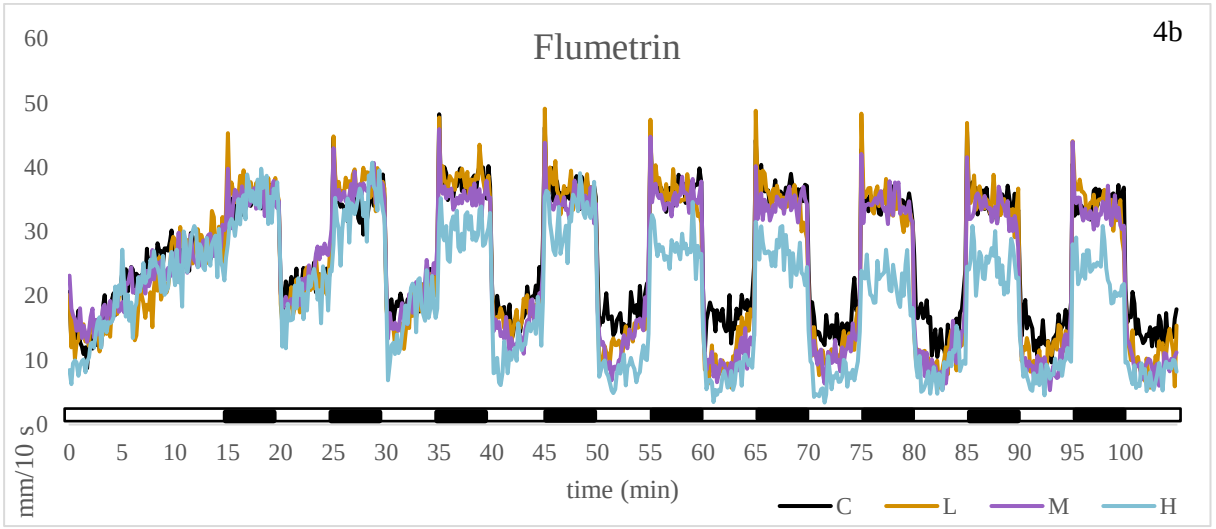


Figure 3a-c. *The percentage of dead and affected larvae in the different concentrations for the three tested pharmaceuticals.*

Behavioural assay

The mean locomotor activity as a function of time for larvae treated with doramectin was plotted and can be seen in figure 4a. What is notable in the figure is that the highest concentration seems to have a consistent lower activity compared to the other groups. Figure 4b shows the mean locomotor activity over time for larvae treated with flumethrin. Interestingly, the highest concentration seems to lack the initial peak, during the first seconds of darkness, which can be seen in the controls and in the other two concentrations. The overall activity in the highest concentration is strongly reduced over time while the activity of control and the other concentrations is approximately the same during the entire test period. The mean locomotor activity over time for treated larvae treated with toltrazuril can be seen in figure 4c. There were no obvious differences between the control and the different concentrations visualized in the graph. Figure 5 visualise the short exposure test with doramectin. Larvae that received doramectin 1h prior behavioural recording showed heavily reduced activity and almost none activity at all during the last 20 minutes of the recording. In the additional test with DMSO there were no differences in the graph between the larvae that received 0.01% DMSO and the control.





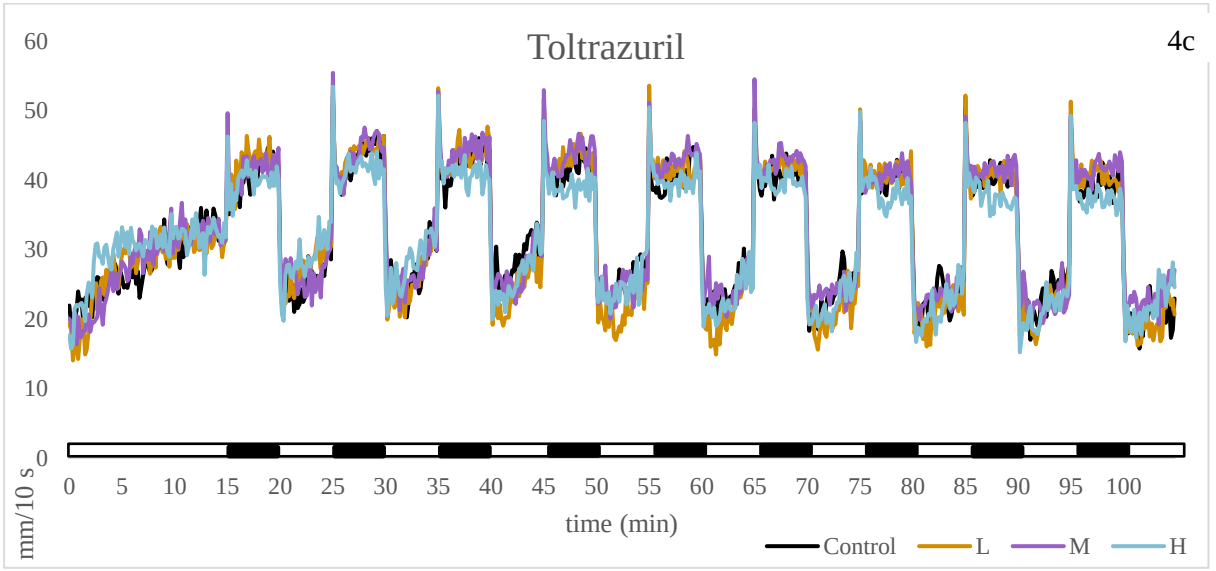


Figure 4. Locomotor activity means (mm per 10 s) in zebrafish larvae exposed to different veterinary pharmaceuticals in different concentrations: low (L), medium (M) and high (H) as well as controls (a-c). The black line represents the control group and the coloured lines the different concentrations of the test substance: orange line for the low concentration, purple for the medium concentration and blue for the high concentration. The bars below each graph shows the five minute consecutive light and dark periods; white represents light and black represent darkness.

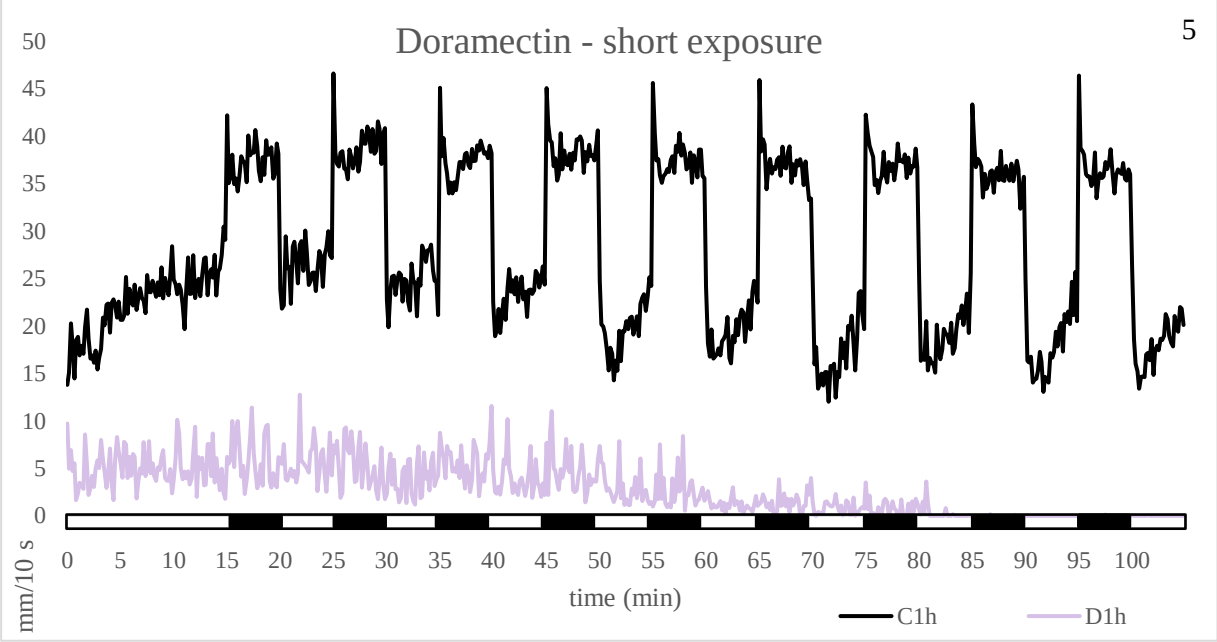


Figure 5. Locomotor activity means (mm per 10 s) in zebrafish larvae that received doramectin 1h prior behavioural recording (D1h) as well as the corresponding control (C1h), the black line in the graph represents the control and the grey line represents the tested substance. The bars below each graph shows the five minute consecutive light and dark periods; white represents light and black represent darkness.

Statistical analysis

The result of the statistical analyses of the four selected responses revealed some effects in larval activity (table 2). The activity in the first five minutes dark period and the first five minutes light period were both significantly reduced in larvae exposed to the high concentration of doramectin compared with the control. Larvae exposed to the high concentration of flumethrin had a significant higher reduction in activity from the first to the last dark period as compared with the reduction in control larvae. The toltrazuril exposed larvae had no significantly difference in activity compared with the control group. In the control study of the impact of 0.1% DMSO on locomotor behaviour, the larvae that received 0.1 % DMSO did not differ from the controls.

Table 2. *Results from the statistical analysis of the four selected responses in the behaviour test: increase in activity during the first 10 seconds at the first period of darkness (First 10 s of D1), mean activity during the first period of darkness (D1), mean activity during the first period of light (L1) and difference between mean activity during the first and the last periods of darkness (D9-D1)*

		mean distance (mm (±SD))				
		Control (C)	Low (L)	Medium (M)	High (H)	P-value ANOVA
Doramectin	First 10 s of D1	12.91 (14.33)	14.56 (20.41)	11.85 (17.38)	22.14 (17.59)	0.870
	D1	39.37 (7.14)	41.09 (5.16)	40.82 (7.43)	30.45 * (14.93)	0.002
	L1	26.66 (9.42)	23.17 (10.90)	24.57 (9.79)	16.25 * (14.49)	0.043
	D9-D1	-4.82 (8.46)	-2.04 (12.24)	-3.11 (8.78)	-0.99 (19.13)	0.806
Flumethrin	First 10 s of D1	6.61 (12.74)	11.87 (16.77)	8.86 (19.12)	2.20 (18.92)	0.443
	D1	34.65 (7.23)	36.98 (6.80)	35.05 (10.49)	34.43 (11.22)	0.833
	L1	22.08 (7.80)	20.83 (10.01)	22.93 (11.40)	19.92 (11.46)	0.843
	D9-D1	0.18 (5.66)	-2.45 (7.05)	-2.45 (5.13)	-10.60 ** (15.22)	0.006
Toltrazuril	First 10 s of D1	9.50 (9.19)	12.48 (16.64)	18.25 (12.85)	14.49 (13.14)	0.171
	D1	40.66 (7.69)	42.56 (6.36)	41.54 (7.84)	40.08 (8.46)	0.746
	L1	25.43 (11.45)	27.11(10.95)	26.44 (10.46)	28.50 (7.38)	0.796
	D9-D1	-1.11 (4.98)	-1.66 (4.72)	0.25 (5.15)	-2.25 (4.71)	0.389

		mean distance (mm (±SD))		
		Control (H_2O)	DMSO (0.1%)	P - value
DMSO	First 10 s of D1	28.67 (22.70)	36.77 (18.66)	0.243
	D1	42.35 (9.2)	42.7 (9.0)	0.392
	L1	29.6 (13.4)	29.8 (13.1)	0.539
	D9-D1	-8.0 (4.9)	-5.0 (5.3)	0.960

Dunnett's test: * = $p \leq 0.05$ ** = $p \leq 0.01$

DISCUSSION

Overview

Because one aim of the present study was to test if adding a behavioural assay could increase the sensitivity of the fish embryo toxicity (FET) test, only larvae that had normal appearance and body posture at visual inspection were included in the data set. These criteria were set because if possibly toxic effects could be seen on forehand by visual inspection, a behavioural assay would not be necessary to make a statement about the toxicity of the substance. Three substances were tested in this study: doramectin, flumethrin and toltrazuril by using a

behavioural recording device that records the locomotor activity of the larvae. Because of the large amount of data produced, it was difficult to analyse all possible effects. Therefore, four measurements were chosen based of the graph of mean locomotor activity (fig. 4): mean activity during the first period of darkness, mean activity during the first period of light, increase in activity during the first 10 seconds at the first period of darkness and difference in mean activity between the first and the last period of darkness. These four measurements were tested statistically for differences between the control group and the different concentrations. Significant results were found for doramectin and flumethrin but not for toltrazuril. These behaviour effects were observed in relatively high concentrations which already have been determined as toxic corresponding to EC_{50} (Carlsson et al., 2013). However, the behavioural effects were observed in larvae which were considered as normal which implies that the activity recording may reveal effects not detected in the normal embryo toxicity test and that this gives additional information of the modes of action of the tested pharmaceuticals. This study successfully showed that it is possible to use behavioural measurements to increase sensitivity in the FET test which makes behavioural assays highly interesting for further development of the test.

Doramectin

Doramectin belongs to the avermectine family of veterinary drugs and its mechanism of action is that it binds to the glutamate-gated chloride (GluCl) receptor and GABA, which cause an increase in the membrane permeability, which leads to an influx of chloride ions into the cells (Martin, 1997). This leads to hyperpolarisation in motor neurons and muscle cells of nematodes and arthropods and thereby paralysis. GluCl receptors are only found in invertebrates, so fishes and mammals does not have these (Zemkova *et al.*, 2014), this explains why the drug can affect the parasite but not the host. In this study we found significant results for two of the analysed behaviour parameters between the control and the highest concentration. The mean activity during the first periods in the high concentration were significantly reduced both in light and darkness. There was no significant difference in activity increase during the first 10 second or between first and last period of darkness compared with the controls.

A second test was performed with doramectin to evaluate if the reduced activity seen in the first test was an effect of developmental changes or if it was a result of a direct neurotoxic effect on the nerve system of the larvae. This test showed that larvae exposed to doramectin one-hour prior reading were more severely affected than larvae that were continuously exposed from day 0. This implies that the effects of doramectin is a result from a direct acting mechanism and not from developmental disturbances. It is reasonable to think that the effect that was seen in this test, were caused by the same mechanisms as in the target parasite. In the study by Carlsson *et al.* (2013) the exposure medium was tested for doramectin concentrations before the FET test were performed and at 144 hpf. The result suggested that there is a large reduction of doramectin concentration throughout the test period both due to embryo metabolism and abiotic breakdown. This implies that the embryos that were exposed one-hour prior to reading probably were exposed to a higher concentration during recording than the ones that were exposed from day 0. A study by Gellert & Heinrichsdorff (2001) investigated if susceptibility to chemical pollutants could be affected by the egg age. They found that eggs younger than 1h were more susceptible than older eggs and drew the conclusion that this was due to changing of

permeability of the chorion. In the present study the eggs were about 1-2.5 h old when exposed to the pharmaceuticals so the actual concentration that reached the embryos during the first two days may have been reduced. Another possible explanation is that the long term exposed embryos had time to adapt by upregulate important cell mechanisms such as the P-glycoprotein efflux pump. P glycoprotein is present in most organisms and mediate the transport of xenobiotics out of the cell. Fischer *et al.* (2013), showed that the *abcb4* gene that encodes for a P-glycoprotein in zebrafishes is a major component of the multidrug resistance in zebrafish embryos. Long *et al.* (2011) exposed zebrafish embryos for heavy metals and analysed for transcriptional expression of another P-glycoprotein gene, *abcb1*. They found that toxic heavy metals stimulated the expression of the *abcc1* gene in the embryos. Induction of the *abcc1* expression is a cell defensive mechanism.

Flumethrin

Flumethrin is a pyrethroid insecticide that acts on voltage-dependent sodium channels in nerves by slowing down the closing of the sodium activation gate (Valentine, 1990). This leads to delayed repolarisation of the nerve resulting in neural dysfunction in the parasites. For the highest concentration of flumethrin, a significant difference in mean locomotor activity between first and last period of darkness were found. The activity in the highest concentration of flumethrin decreased significantly more between the first and the last period of darkness compared to the control. A reduction in activity over the course of repeated dark cycles is considered normal. Ašmonaitė *et al.* (2016) also saw decrease in response after repeated cycles when they used behavioural assays to study the effect of silver ions and silver nanoparticles in zebrafish embryos. Their theory about the background of this change in response over time was that it could be an effect of habituation. Habituation is defined as "a behavioral response decrement that results from repeated stimulation and that does not involve sensory adaptation/sensory fatigue or motor fatigue" (Rankin *et al.*, 2009). Wong *et al.* (2010) studied habituation in adult zebrafish and found that some substances, caffeine and pentylenetetrazole (an anxiogenic agent), impaired habituation. Ašmonaitė *et al.* (2016) also mentioned motor and sensory fatigue as an alternative explanation to the observed reduction of locomotor response. In the graph (fig 4b) the initial peak during the first 10 seconds of darkness seemed to be absent in the embryos exposed to the highest concentration of flumethrin. This effect was however not statistically significant.

Toltrazuril

Toltrazuril is a triazinonderivate that affects the perinuclear space, mitochondria, and the endoplasmatic reticulum of the parasites and leads to a reduction of enzymes in the respiratory chain (Harder & Haberkorn, 1989). It was included in this study because in the study by Carlsson *et al.* (2013) it reduced early spontaneous movements in the zebrafish embryos in a very high concentration. However, in the present study no behavioural effects of toltrazuril was found. The simplest explanation for this is that toltrazuril do not induce any behavioural changes, but an alternate explanation could be that the concentration used was too low. We used the concentration 1.1 mg/mL whereas in the study by Carlsson *et al.* (2013) toltrazuril showed an "all-or-nothing" pattern; at 1.0 mg/mL there was a low percentage of affected embryos but at the nearest higher level of 2.2 mg/mL, almost all embryos were affected. The concentration used in the present study were chosen based on the calculated EC_{50} value from Carlsson *et al*

(2013) study, so it is possible that the concentration became slightly too low and therefore we could not see any effects at all.

Behavioural assay as a tool to access toxicity

We were able to detect altered behavioural response in individuals that had been treated with doramectin or flumethrin, and that were considered as normal according to the macroscopic examination. Therefore, we believe that behavioural assays have a great potential as a tool to increase the sensitivity in toxicological studies in zebrafish. An important part of this study was the fact that the individuals contributing to affected locomotory endpoints could not be classified as affected based on visual features. This excluded all individuals with malformations or other physical anomalies such as oedemas. Considering that two of the antiparasitic pharmaceuticals in the study have a neuro toxic mechanism of action in their target organisms we also decided to exclude all larvae that showed abnormal body posture, which can be interpreted as a sign of neuro toxicity. In this study we decided to have protocol with several cycles in order to be able to detect changes in activity over time. By using a protocol with many cycles and a longer total recording time (1.5 h) we were able to detect a change in response over time for flumethrin. The protocol is identical to the one used in a study by Ašmonaitė *et al.* (2016).

Other comments

The concentrations used in this study were selected based on the study by Carlsson *et al.* (2013) in which 15 veterinary pharmaceuticals were tested for toxicity in zebrafish embryos. The highest concentration in our study corresponds to the EC_{50}-value determined in that study. According to the definition of EC_{50}, 50% of the individuals would be affected at the given concentration. For doramectin 8.3 % died and 33.3 % were alive but affected in the concentration that responded to EC_{50} in the study by Carlsson et al. This gives a total of 41.6 % which can be considered as similar results. In the flumethrin test none of the embryos died but 41.7 % were affected in the highest concentration which is also quite close to 50 %. For toltrazuril the percentage of affected larvae were low in all four groups. As earlier mentioned there is a possible explanation for this by the sudden shift in toxicity that could be seen in the study by Carlson et al (2013); from few affected to almost all affected when the substance reaches a certain concentration.

The pharmaceuticals used in this study are fat soluble substances and therefore dissolves poorly in water. Therefore, we had to use a solvent, dimethyl sulfoxide (DMSO). The same solvent were used in the study by Carlsson *et al.* (2013) and in order to make both studies comparable, the same concentration of DMSO, 0.1% was used in the present study. The results from the comparisons between clean water and DMSO on zebrafish larval behaviour in the present study suggests that this concentration of DMSO does not affect the activity.

CONCLUSIONS

For two of the pharmaceuticals, doramectin and flumethrin, we were able to detect altered behavioural response in individuals that could not be classified as affected based on visual features. Therefore, we believe that behavioural assays have a great potential as a tool to increase the sensitivity in toxicological studies in zebrafish.

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