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ORIGINAL RESEARCH

Effects of Diclofenac on the embryonic development of freshwater crayfish

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Abstract In recent years, there has been increasing concern about the ecotoxicological consequences of the drug Diclofenac on freshwater organisms. Influences on the largest freshwater invertebrates, the freshwater crayfish, are especially interesting in the context of cascading effects due to their important role as keystone species. In this study, lethality, influences on body weight, embryonic development and histological changes in embryos of marbled crayfish (*Procambarus virginalis*) as well as noble crayfish (*Astacus astacus*) were investigated in response to their exposure to different concentrations of Diclofenac. Additionally, the suitability of marbled crayfish as a model organism for endemic freshwater crayfish was established when studying the effects of Diclofenac. For both species, lethal effects started at concentrations of 10.24 mg/L Diclofenac, weight was not affected, embryonic development slowed down from concentrations of 0.16 mg/L and histological changes were visible from concentrations of 0.64 mg/L. The similarity of LOEC (Lowest Observed Effect Concentrations) between the two species showed that marbled crayfish can serve as a model for native crayfish when investigating the effects of exposure to Diclofenac.

Keywords Marbled crayfish . Noble crayfish . Juveniles . Diclofenac . Embryonic

Introduction

Diclofenac (DCF) is the most frequently detected drug in German surface waters. It has been measured in concentrations of up to 29.8 µg/L in 55 countries (Dusi et al. 2019; Lin et al. 2008). Different harmful effects have been described for non-target organisms. Concentrations as low as 1 µg/L have shown negative effects on liver, kidney and gills of rainbow trout (*Oncorhynchus mykiss*, Triebskorn et al. 2004), and survival, growth and reproduction of *Daphnia magna* are reduced from concentrations of 0.4 mg/L (Du et al. 2016).

As "keystone species" and "ecosystem engineers" (Weinländer and Füreder 2016) freshwater crayfish are a central element of benthic ecosystems. During development, their sensitive embryos are directly exposed to potentially harmful chemicals dissolved in surface waters for months (Khan and Nugegoda 2007). As they rely on diffusive transport for gas and nutrition exchange during embryonic development (Reiber 1997), they can be influenced by these potentially harmful substances.

On those grounds, it was hypothesized that DCF influences survival rate, embryonic development time, weight increase and histological effects on the hepatopancreas of freshwater crayfish embryos. The crayfish hepatopancreas is typically formed of numerous tubules separated by connective tissues (Abd El-Atti et al. 2019) and consists of lumen, membranes and four types of epithelial cells: resorptive lipid cells (R-cell) for nutrient intake, blister-like secretory cells (B-cell) to channel off harmful substances, fibrillar cells (F-cell) as connecting tissue and embryonic cells (E-cell). Consequently, changes in R-cells would indicate a higher or lower intake of nutrients, changes in B-cells would indicate a higher or lower outtake of harmful substances, whereas changes in the other two types would indicate problems in the individual's biosynthesis.

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The European native species *Astacus astacus* was once widespread in European surface waters until the crayfish plague as well as structural and chemical changes in surface waters nearly eradicated this species. The noble crayfish, which is well suited for this study due to its natural habitat (lower sections of streams, lakes etc.), is often influenced by agricultural drainages and sewage (Skurdal and Taugbøl 2002). The study at hand was additionally carried out using marbled crayfish embryos (*Procambarus virginalis*). One female marbled crayfish can produce up to 700 eggs every eight to nine weeks. The offspring is genetically identical to its mother due to the species' parthenogenetic reproduction strategy (Chucholl and Pfeiffer 2010; Vogt et al. 2004). These facts provide a predictable and continuous supply of clonal eggs, making this species a suitable model organism for higher invertebrates in the laboratory (Hossain et al. 2018; Vogt 2018).

Materials and methods

Experimental design

Parental noble crayfish were obtained from a commercial hatchery in Schleswig-Holstein, Germany (Oeversee crayfish farm). Fifteen females and six males were kept in three 600 litre aquaria during their mating time at 8 °C and a light regime of L:D= 10:14. They were fed frozen midge larvae and peas ad libitum. Females were checked for eggs on a daily basis from the end of November onwards. Noble crayfish embryonic development is triggered by temperatures below 4 °C. Therefore, the eggs were extracted 72 hours after a 14 days cold period. The parthenogenetic reproduction strategy of marbled crayfish made it easier to obtain a large number of eggs. Twelve animals were kept separately in 25 Litre aquaria at 23 °C with a light regime of L:D = 10:14. They were fed the same diet as the noble crayfish. These conditions allowed a collection of eggs from every individual marbled crayfish every eight to nine weeks. As for noble crayfish, experiments started 72 hours after egg deposition.

For the experiments, ten wells of 5 ml-multititer plates (Greiner bio-one, Kremsmünster, Austria) were equally filled with 0.75 mL of a mixture of tap water and deionized water at a ratio of 2:1. The mixture was autoclaved and aerated beforehand to ensure an oxygen-saturated and germ-free environment for embryos. Prior to the start of the experiment, 0.75 mL of deionized water with a specific concentration of DCF was added. Subsequently, one egg per well was transferred into each well of twelve microtiter plates. Each plate contained all of the nine concentrations of DCF used in the experiments and additionally one zero and one solvent (Ethanol: ETH) control (Table 1). These concentrations were chosen to cover a wide range from concentrations occurring in surface waters to concentrations known to have effects on other animal groups (Dietrich et al. 2010; Han et al. 2006) and are listed in Table 1.

The microtiter plates were placed on a laboratory shaker (Dual-Action shaker KL 2, Edmund Bühler GmbH, 72411 Bodelshausen, Germany) with 60 movements per minute to ensure a constant supply of oxygen and simulate parental movement of the abdomen. The experimental solutions were changed daily via pipetting to maintain optimum water quality.

Developmental stages and mortality were recorded three times per week. This was possible due to the transparent membrane of crayfish eggs that enabled visual examination of the status of the living embryo's development under a binocular. The developmental stages were transferred following the methods described by Sandeman and Sandeman (1991, noble crayfish) and Alwes and Scholtz (2006, marbled crayfish) to

Table 1 Concentrations of DCF, designation of groups and numbers of replicates/embryos per species. Due to their reproduction strategy, the experimental time included the complete embryonic development time of marbled crayfish (*P. virginalis* = Group P). For noble crayfish and their longer development time, the experiment was conducted for three different parts of their embryonic development (A1, A2, A3)

Species	Group	Number of replicates per concentration								
		0.0	ETH	0.01	0.04	0.16	0.64	2.56	10.24	40.96
P. virginalis	P	36	36	36	36	36	36	36	36	36
A. astacus	A1	16	16	16	16	16	16	16	16	16
A. astacus	A2	16	16	16	16	16	16	16	16	16
A. astacus	A3	16	16	16	16	16	16	16	16	16

standardized percentages to generate direct comparability of the embryonic development of the two species.

Differences in development time of marbled crayfish (486–540 degree days (Seitz et al. 2005)) and noble crayfish (1900 degree days (Kozák 2015; Skurdal and Taugbøl 2002)) were balanced by choosing exposure times of between 13 and 16 days for both species. The last period was carried out until the first moult of juvenile crayfishes. The embryonic development of marbled crayfish was fully completed by this time. For noble crayfish, the experiment was conducted for three different parts of their embryonic development. Hereinafter, these first, second and third periods will be referred to as A1, A2 and A3.

To avoid observer-biased data recording, the observing person did not know the respective assignments of eggs and concentrations. The experiments were terminated after the first moult of juvenile crayfish. At that time, the fresh weight of the moulted animals was measured (Sartorius Research R160 P, Sartorius GmbH, Göttingen, Germany).

Chemicals

DCF (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) in 99.5% purity was purchased for the study. Due to its low solubility in water (5 mg/L in 20 °C), DCF was first dissolved in Ethanol (ETH). To exclude any effects of ETH on the embryos a control group with this solvent was included.

Histology

For histological assessments three noble crayfish per concentration were fixated in buffered formaldehyde (3.7%) directly after the first moult. The crayfish were stored in Kristensen solution for two weeks, as described in the LR white user's handbook, to ensure a complete decalcification of the exoskeleton. The samples were embedded in LR White (LR White acrylic resin, hard, Sigma-Aldrich, Germany) and hepatopancreas sections of 2 µm thickness were made using an ultramicrotome. The sections were stained with haematoxylin and eosin (HE) with an extended exposure time, in accordance with the LR White usage instructions. These tissue samples were examined under a light microscope combined with a camera system (Leica DM1000 LED, Leica ICC50 HD, Leica Application Suite Version 3.0.0, Leica Microsystems CMS GmbH, D-35578 Wetzlar, Germany). The examination of hepatopancreas cells included the observation of membrane damage, damages in the four different cell types as well as changes in size and number of the four different cell types. For this procedure, ten sections per individual were photographed and subsequently analysed by counting and measuring cells under the microscope.

Statistical methods

All statistical analyses were performed using R, version 3.2 (R Core Team 2015). The weight and number of B-cells were tested for normality and equal variances prior to analysis. If these criteria were met, a one-way ANOVA (analysis of variance) and a post hoc Tukey's test were performed. The LC_{50}-values (medium lethal concentrations) were first corrected following Abbott's method and then estimated using the trimmed Spearman Karber method. Survival rates were analysed using the Kaplan-Meier survival analysis of Gehan Breslow and the groups were compared via the Holm-Sidak method. The embryonic development was analysed via linear regressions. Fulfilling the condition of good correlation values (> 0.8) the linear regressions were compared with an ANCOVA (analysis of covariance). Pictures were analysed using GIMP software (version 2.8, Fa. the Gimp Team).

Results

Lethal effects

Survival rates of marbled crayfish are shown in Figure 1. Overall, the survival of embryos was between 5% and 36%. Embryos of *P. viginalis* exposed to concentrations of 10.24 mg/L and higher showed a significantly lower survival rate than the control groups (Holm-Sidak, $P \leq 0.03$). The estimated LC_{50}-value over 15 days was 13.96 mg/L (SE = 3.86).

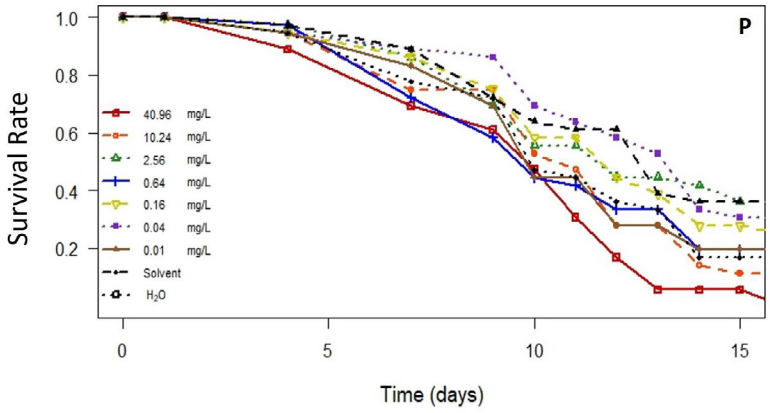

Fig. 1 Survival rates of marbled crayfish (group P) exposed to different concentrations of DCF over time

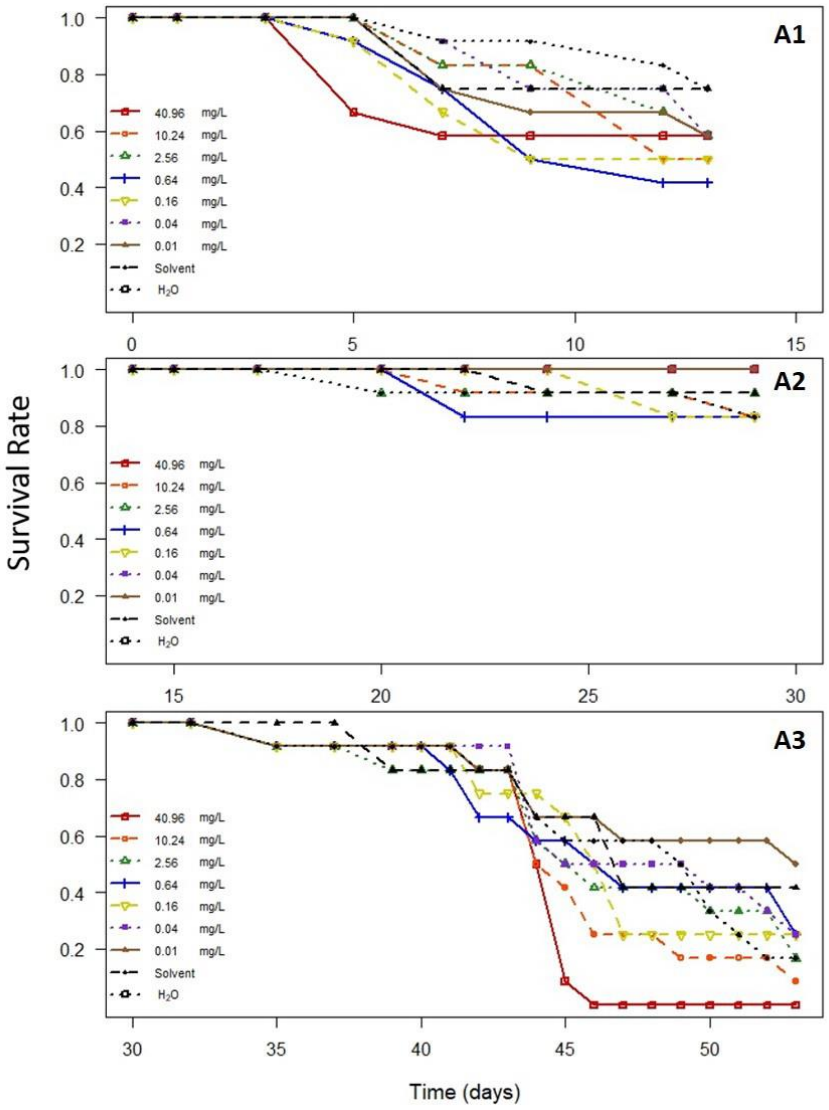

Fig. 2 Survival rates of noble crayfish exposed to different concentrations of DCF over time in first (A1), second (A2) and final (A3) third of development

Overall, the survival of noble crayfish embryos was higher than for marbled crayfish. In both crayfish species, survival rates were significantly lower for concentrations of 10.24 mg/L and higher (Holm-Sidak, $P \leq 0.007$). In noble crayfish, however, these effects were only observed in group A3 (Figure 2). During this third developmental period, hatching takes place so that the effects of DCF on the survival of noble crayfish are linked to the latest stage of embryonic development and hatching. An LC_{50}-value of 19.56 mg/L (SE = 5.22) was estimated for this group.

Sublethal effects

Comparison of weight

Weights after the first moult of both crayfish species did not reveal any differences between the different treatments ($P > 0.05$).

Embryonic development time

A comparison of development time of marbled crayfish revealed major differences between treatments. From concentrations of 0.16 mg/L and higher, embryonic development was slower than in the control group (ANCOVA, $P = 0.047$). The time until 70% of development was completed varied between 13 days for the control groups and 18 days for the treatment group exposed to 10.24 mg/L DCF. Embryos exposed to the highest concentration did not develop further than 69% on average (Figure 3).

In noble crayfish, there were no differences in development between group A1 and A2. In group A3, however, the development of embryos exposed to DCF concentrations of 0.16 mg/L and higher was slowed down compared to the control groups ($P = 0.019$) (Figure 4). By day 43, all embryos of the control groups had hatched. Embryos exposed to 0.16 mg/L DCF hatched after 46 days and embryos exposed to the highest concentration of DCF did not develop until hatching.

Histology

Figure 5 illustrates the effects of DCF on the hepatopancreas structure of *A. astacus*. The average number of B-cells in the H_2O control group was 24.6 ± 2.4 (n = 30). All sections of concentrations of 2.56 mg/L and higher showed a higher number of B-cells and enlarged B-cells compared to the control groups (post hoc Tukey, $P < 0.001$). In hepatopancreas sections of embryos exposed to concentrations of 40.96 mg/L, 41.9 ± 9.8 (n = 30) B-cells per section were recorded. These cells were estimated to be 30% larger than B-cells in the control groups. Additionally, no disruption of the membrane occurred in the control groups, whereas

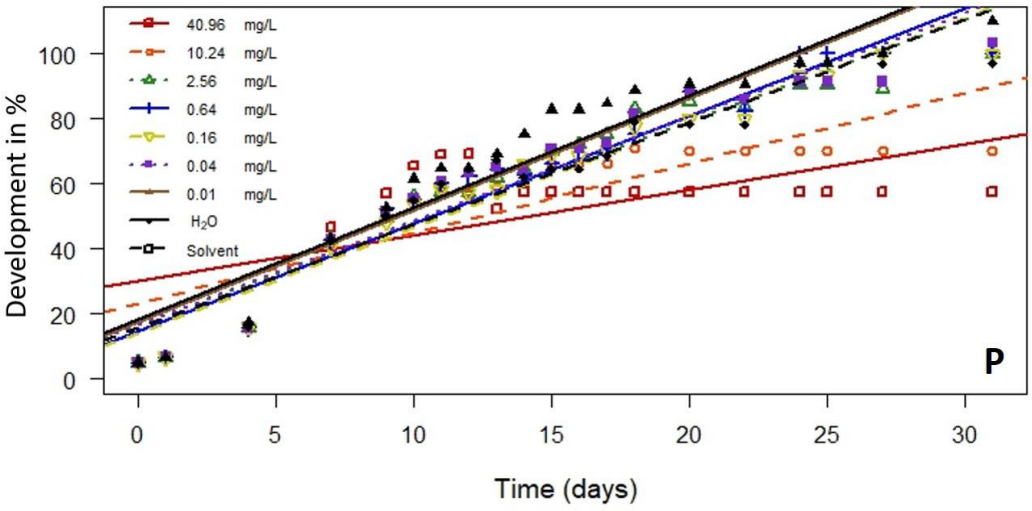

Fig. 3 Linear regression of embryonic development of marbled crayfish (group P) exposed to different DCF concentrations over time

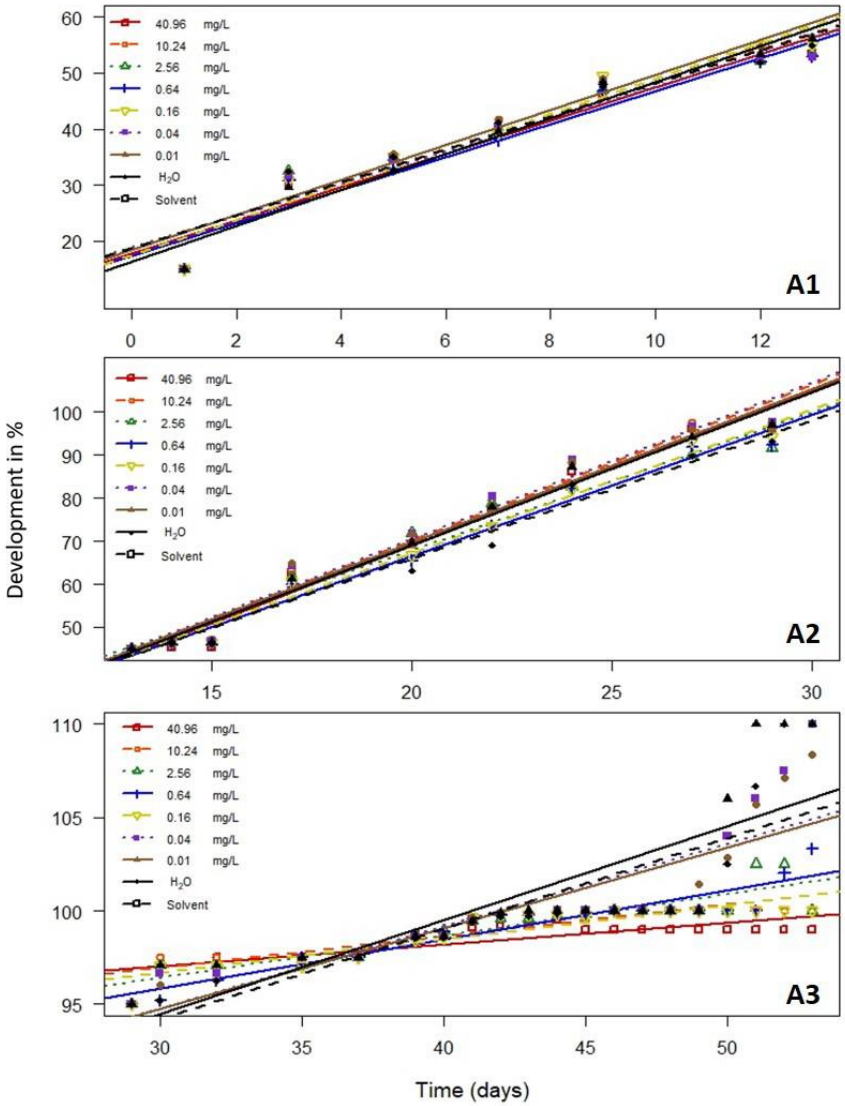

Fig. 4 Linear regression of embryonic development of noble crayfish exposed to different DCF concentrations over time in the first (A1), second (A2) and final (A3) third of development

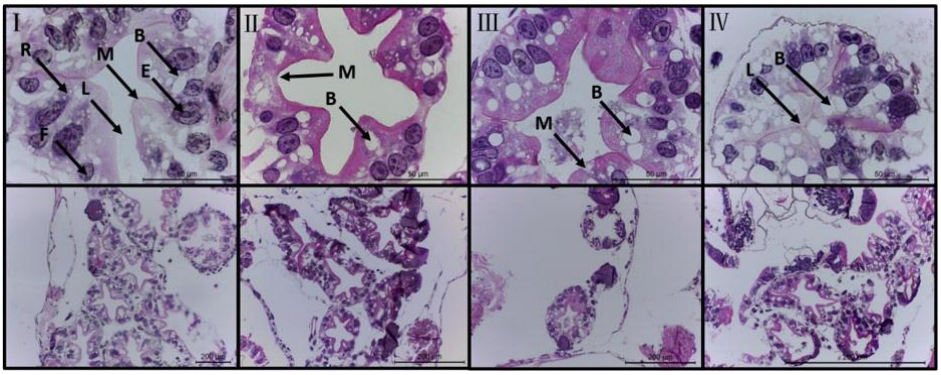

Fig. 5 Histological sections of the hepatopancreas in juvenile noble crayfish (Astacus astacus) exposed to DCF. I – control showing the lumen (L), membrane (M) and four types of epithelial cells: resorptive (R) lipid cells, blister-like (B) secretory cells, fibrillary (F) cells and embryonic (E) cells; II – group exposed to 0.64 mg/L DCF; III – group exposed to 2.56 mg/L DCF; IV– group exposed to 40.96 mg/L DCF (HE, 200 ×). Top row: close-up of one tubular. Bottom row: overview of hepatopancreas structure

disruptions at concentrations of 0.64 mg/L DCF in 20% of all sections were observed. At concentrations of 1.6 mg/L and higher, this membrane damage was present in every section.

Discussion

The purpose of all types of non-steroidal anti-inflammatory drugs (NSAIDs) including DCF is to decrease the production of thromboxanes and prostaglandins (Satoh et al. 2015) in order to reduce pain, inflammation and fever. With 3.996 positively detected environmental concentrations on the watch list of the database "Pharmaceuticals in the environment" (UBA, German Environment Agency), it is considered a "contaminant of emerging concern" and was included in the previous watch list of EU Decision 2015/495 (Li et al. 2019; Lonappan et al. 2016; Sousa et al. 2018; Sathishkumar et al. 2020). This high incidence shows the relevance of understanding the effects of this drug on non-target organisms.

Comparison of the two species

With respect to the assessed parameters, the effects of DCF on embryos of the two species were very similar. The LOEC for lethality and embryonic development were identical and LC_{50}-values were comparable. Hence, it has been demonstrated that marbled crayfish can serve as a model organism for endemic crayfish concerning the effects of DCF. The suitability of marbled crayfish as a model organism for a broad range of biological disciplines is described by Vogt (2018) and Hossain et al. (2018). Both reviews claim that marbled crayfish are organisms that can be used for studies in epigenetics and developmental biology as well as physiological, ecotoxicological and ethological research. Buřič et al. (2018) used *Procambarus fallax* to assess the effects of an opioid painkiller (tramadol) and an antidepressant drug (citalopram) on behavioural patterns. Marbled crayfish have even been used as a model for the neural and molecular mechanisms of drug addiction (Jackson and van Staaden 2019). This shows that marbled crayfish are already being used as model organisms. Nevertheless, not every observed effect on marbled crayfish is transferable to other species. Marbled crayfish can show a lower sensitivity to environmental factors, especially during embryonic development. The short embryonic development of marbled crayfish (Vogt and Tolley 2004) leads to a shorter exposition time during this vulnerable period. Additionally, Vogt (2010) described marbled crayfish as tolerant towards a wide range of environmental conditions for long periods of time. Considering the results of Rubach et al. (2011), who demonstrated that freshwater arthropod species can be highly variable in their dynamic response to a particular stressor, it is reasonable that the suitability of marbled crayfish as a toxicological model organism depends on the chemical compound used. Effects are, thus, transferable to other species, but the dose at which an effect occurs might differ.

Lethality of Diclofenac

There have been few investigations about lethal concentrations of DCF for crustaceans and, to the best of our knowledge, there was none for crayfish. Data are only available for the water flea *Daphnia magna* (LC_{50} = *56.6 mg/L–94.1 mg/L* (Quinn et al. 2011; Ra et al. 2008)), the mysid *Sirella armata* (LC_{50} = *0.01 mg/L–2.91 mg/L* (Pérez et al. 2015)) and the copepod *Tisbe battagliai* (LC_{50} = *15.8 mg/L (Schmidt et al.* 2011)). These results are within the range of the observed values for crayfish in this study. It is notable that the commonly used organism for risk assessments, *Daphnia magna,* shows the highest LC_{50} of all four organisms and, therefore, shows a very optimistic estimation of the hazardous effects of DCF. However, there are documented lethal concentrations of DCF on fish. The LC_{50}-value of DCF on zebra fish (*Danio rerio*), for instance, is 5.3 mg/L (van den Brandhof and Montforts 2010) while DCF has a 96 h LC_{50}-value of 2.6 mg/L on four weeks old African catfish (*Clarias gariepinus*) (Folarin et al. 2018). For embryos of this species, Zhang et al. (2020) showed that Diclofenac led to the inhibition of spontaneous muscle contractions and a decreased hatching rate at a concentration of 24.1 µg/L. The deviation in these lethal concentrations compared to crustaceans, despite the larger body volume, can be explained by bioaccumulation in tissue. Several authors have reported that DCF can accumulate in fish, even though reported bioconcentration factors differ greatly between species (Brown et al. 2007; Cuklev et al. 2011; Fick et al. 2010; Schwaiger et al. 2004). Transferred to freshwater crayfish, DCF would most

likely accumulate in muscle and hepatopancreas tissue and could, therefore, show even more drastic effects over time. The toxicity of DCF is additionally dependent on pH. At lower pH mortality increases (Alsop and Wilson 2019). Nevertheless, lethal concentrations of DCF are multiple times higher than the highest monitored DCF concentration worldwide (Sousa et al. 2018). Therefore, indirect effects of DCF on fitness are more likely to have an impact on population dynamics.

Sublethal effects of Diclofenac

Mohd Zanuri et al. (2017) showed effects of DCF on sperm activity of important components of the marine benthos in concentrations lower than 0.1 µg/L. In addition to the effects of DCF, mixtures with other chemicals introduced to surface waters can increase the negative effects of this analgesic (Gonzalez-Rey et al. 2014; Prokkola et al. 2015). In our study, sublethal effects of DCF on freshwater crayfish were observed from concentrations of 0.16 mg/l and higher. These low effective concentrations support the statement made by Fent et al. (2006) that DCF seems to be the compound having the highest acute toxicity within the class of NSAIDs. Even though the weight of hatched crayfish did not decrease when exposed to DCF, the increased developmental time of the crayfish due to DCF exposure can have a negative effect on population dynamics. For natural populations, late hatching can have massive influences as it leads to a later start of feeding and, therefore, slower growth of the respective cohort. These individuals have a lower survival potential due to lower feeding success and increased mortality through predation, as shown for marine fish (Franke and Clemmesen 2011). The sublethal effects described in this study are only one example of the expectable effects of DCF. Gonzalez-Rey and Bebianno (2014), for instance, showed that concentrations as low as 0.25 µg/L can lead to biomarker responses in muscles. These or other unknown effects could also be present in crayfish muscle tissue.

Histology

The hepatopancreas in decapods is the site of nutrient absorption, digestion, synthesis and secretion of digestive enzymes and reserve storage (Calvo et al. 2011; Johnston et al. 1998; Xiao et al. 2014). For this reason, the tissue is used for monitoring the health of crayfish and can indicate diseases and exposure to harmful substances (Velisek et al. 2017; Xiao et al. 2014). Changes in B-cells indicate a higher or lower outtake of harmful substances. The observed changes in B-cells can, thus, be explained by a greater need to lead off chemicals. The damaged membranes, on the other hand, can interrupt this mechanism. The overload of this system can, therefore, explain other sublethal and lethal effects of DCF on these animals. When the extrusion of harmful substances is disrupted due to hepatopancreatic damage, the harmful effects on development, growth and survival can occur unimpeded.

There are currently no literature data describing the effects of DCF on the hepatopancreas of crayfish. Nevertheless, influences on the organ by human medications have been reported before: Wren and Gagnon (2013) showed membrane damage and size changes of cells in the hepatopancreas of *Orconectes virilis* exposed to platinum group metals commonly used for industrial and biomedical purposes at 5 mg/L after ten days. They also showed a high bioaccumulation in hepatopancreas tissue, with 81.68 mg/g in a concentration of 1 mg/L platinum group metals. Marenkov et al. (2016) showed influences of the drug Albuvir on the hepatopancreas and weight of marbled crayfish at 0.01% solution. The results of the investigation of the hepatopancreas show the wide range of sublethal effects on freshwater crayfish and lead to the assumption that other effects are possible and should be investigated in future studies.

Conclusion

This study shows that marbled crayfish can be used as model organisms to investigate physiological effects of DCF on noble crayfish. Furthermore, the study could demonstrate that the non-steroidal anti-inflammatory drug has a negative influence on the embryos of freshwater crayfish. Although the reported effective concentrations are unlikely to be found in surface waters, the mixture of DCF with other introduced chemicals might reduce the effective doses of the pharmaceutical or synergise to other unknown effects. Additionally, DCF is constantly released from sewage treatment plants, which are a major source of the

pharmaceutical compound in the aquatic environment. As a result, exposure time is continuous and endless. Therefore, effects on population dynamics are possible and should be investigated in the future.

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Competing interests The authors declare that they have no competing interests.

Authors' contributions JL and KL conceived and designed the experiments. JL performed the experiments. JL analysed the data. JL, KL and HB wrote the manuscript; all authors provided editorial advice.

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