



Exposure to teflubenzuron negatively impacts exploratory behavior, learning and activity of juvenile European lobster (*Homarus gammarus*)



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ARTICLE INFO

Keywords:

Lobster
Behavior
Aquaculture
Salmon farming
Teflubenzuron
Sub-lethal concentration

ABSTRACT

Infestations with salmon lice, a parasitic copepod, is a major problem in the salmon farming industry. Teflubenzuron is an in-feed pharmaceutical applied to control lice outbreaks; the standard medication is 10 mg per kg fish per day for seven days. Surveys reveal that teflubenzuron accumulates and persists in the sediment around fish farms and causes deformities and mortality in juvenile European lobster (*Homarus gammarus*), a species commonly found in the vicinity of salmon farms in Norway. To date, there is no information on sub-lethal effects of teflubenzuron on, for example, behavior. We conducted an experiment to assess possible difference in the shelter seeking behavior of teflubenzuron-exposed (N = 19) vs. not exposed (N = 19) *H. gammarus* juveniles. The teflubenzuron-exposed juveniles had been given very low concentrations, 1.7 µg per pellet twice per week for 113 days prior to this experiment. The concentration of teflubenzuron was estimated to be less than 1 ng/g lobster when they were tested in the behavior experiment. Animals were placed in a lane with a shelter at one end. Once a lobster had found and entered the shelter, they were repeatedly displaced back to the opposite end of the lane, for a total of 3 repeated runs per animal. Three of the exposed juveniles failed to settle in the shelter, and the remaining teflubenzuron-exposed animals took significantly more time to explore the environment and to find and recognize shelter. Furthermore, exposed lobsters also exhibited slower walking speed compared to the controls. These results demonstrate that teflubenzuron significantly reduces exploratory behavior, learning and activity of juvenile *H. gammarus*. Thus, exposure to teflubenzuron could increase predation mortality of juvenile lobsters in the wild.

1. Introduction

Salmon lice (*Lepeoptheirus salmonis*) infestation is a major problem in the salmon farming industry (Igboeli et al., 2014; Thorstad et al., 2015; Vollset et al., 2016). Since the industry uses open net-pens, the infective stages of salmon lice can move between neighboring farms and can also have a negative impact on local populations of sea trout (*Salmo trutta*) and migrating wild post smolts of Atlantic salmon (*Salmo salar*) (Costello, 2009; Skaala et al., 2014; Vollset et al., 2016; Wagner et al., 2008). The Norwegian authorities have, therefore, set a threshold of infestation of 0.2–0.5 mature female lice per fish (depending on geographic location and time of the year) (<https://www.lovdata.no/dokument/SF/forskrift/2012-12-05-1140>) above which delousing treatment is mandatory. To meet this requirement, most farmers apply antiparasitic agents. The antiparasitic agents approved in Norway are either dissolved in water and used for bath treatment (hydrogen

peroxide, azamethiphos, deltamethrin, cypermethrin) or administered orally via the feed (diflubenzuron, teflubenzuron, emamectinbenzoate). The total use of teflubenzuron and diflubenzuron increased from 7690 to 9033 kg active compound from 2014 to 2016, and decreased to 2096 kg in 2017 (www.fhi.no). These compounds are chemical pesticides that are not found naturally in the environment. The standard medication with teflubenzuron when treating salmon is 10 mg/kg day, over a period of 7 days. During medication on fish farms, teflubenzuron-containing organic material (excess food pellets, fecal material) settles on the bottom and the compound is found in sediment samples for many months after treatment (Langford et al., 2014; Samuelsen, 2016; Samuelsen et al., 2015; Selvik et al., 2002). Residues of teflubenzuron were found in organic particles sampled at distances of up to 1100 m from a farm. The concentration in the sediment decreased with distance from the farm and time from the treatment (Langford et al., 2014; Samuelsen et al., 2015). Based on field data, a, half-life of

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170 days for the persistence of teflubenzuron in the sediment was estimated. The highest concentration measured in a sediment sample was 40 µg/g wet weight. These observations demonstrate that the benthic community is exposed to teflubenzuron for a prolonged period after a medication.

Flubenzurons act by interfering with the synthesis of chitin, disrupting molting in sea lice (Campbell et al., 2006; Ritchie et al., 2002), and causing deformities in European Lobster (*Homarus gammarus*) (Samuelsen et al., 2014). Residues of teflubenzuron and diflubenzuron have been found in several crustacean species captured in the vicinity of fish farms, for example Norway lobster (*Nephrops norvegicus*), brown crab (*Cancer pagurus*), squat lobster (*Munida* sp.) and deep-water shrimp (*Pandalus borealis*) (Langford et al., 2014; Samuelsen et al., 2015). The highest concentration found was in squat lobster (*Munida* sp.); 393 ng teflubenzuron/g soft parts. No European lobster was captured in either of these studies.

The European lobster is a species at risk of teflubenzuron exposure since its distribution overlaps with salmon farms in Norway and lobsters can also potentially ingest farm waste and feed spillage. Samuelsen et al. (2014) reported 40% mortality in lobster juveniles after short-term exposure to doses of teflubenzuron (10 and 20 µg/g) that simulated a 7-day treatment of salmon in farms. The whole-animal concentration of teflubenzuron in the juveniles that died during the experiment varied from 8175 ng/g (day 5) to 14 ng/g (day 53). The mean concentration in the first 12 lobsters that survived molting was 152 ng/g. However, the large individual variation made it impossible to determine a threshold concentration for survival. At the end of the experiment (day 95), sub-lethal effects were observed; one third of the surviving individuals had developed deformities in the carapace, peripods, cheliped and/or second antenna, i.e. exoskeletal components. There is currently little information about sub-lethal effects of teflubenzuron on lobster.

The antennae in lobster is associated with their sense of touch, smell and taste (Atema and Voigt, 1995). Sheltering is an important anti-predator mechanism in lobster, especially in European lobster juveniles, and the antennae are important for detecting and exploring potential shelters (Agnalt et al., 2017; Aspaas et al., 2016; Linnane et al., 2001; Wahle and Steneck, 1991). The European lobster is nocturnal, moving out of their shelter at night to feed, returning to the shelter at dawn (Cooper and Uzmans, 1980). Juvenile European and American lobster (*H. americanus*) display a natural exploratory and shelter-seeking behavior when placed in an unfamiliar environment (Agnalt et al., 2017; Bayer and Bianchi, 2017; Van der Meeren, 2001). Furthermore, they can memorize the position of a previously-experienced shelter under laboratory conditions (Bayer and Bianchi, 2017). Upon first experiencing a shelter, and acclimating to it, juvenile *Homarus* spp. require less and less time to find and enter the same shelter after being displaced repeatedly (Bayer and Bianchi, 2017; Van der Meeren, 2001). Shelter-seeking behavior in juvenile lobsters has been studied using tanks with open spaces (Van der Meeren, 2001) or in maze-like arenas (Bayer and Bianchi, 2017). Juvenile European lobster tend to seek shelter by moving along the edges of the tank, maintaining physical contact with rigid surfaces (Van der Meeren, 2001). When introduced into an open arena, juvenile lobsters move towards the sides, establishing physical contact with the walls of the arena, and only after do they search for a shelter (van der Meeren, 2001). In this context, we designed a behavioral assay to test if exposure to low doses (sub-lethal) of teflubenzuron over a period of three months have an effect on learning, exploratory behavior and activity level of juvenile European lobsters.

2. Methods

2.1. Animals

European lobster juveniles used in this study were purchased from The National Lobster Hatchery, Padstow UK (www.nationallobsterhatchery.co.uk)

in September 2014 as newly metamorphosed stage IV and Vs. The juveniles were housed in separate units. The exposure treatment to teflubenzuron was conducted over a period of 113 days, starting on 18 January 2016 and ending on 19 May 2016. The control lobsters (N = 14 in each of two replicates) were given one pellet (Spirit S HH 150-70A 4,5; Skretting AS Norway) twice a week on Monday and Thursday. This was to ensure good appetite and consumption of the pellet at each feeding. Although this administration method does not allow for perfect control of the dose delivered to individual lobsters, we only observed three individuals that had not consumed their pellet on one occasion. The exposed lobsters (N = 14 in each of three replicates) were fed using the same procedure as the controls except that the pellets contained teflubenzuron. The medicated pellets contained 1.8 µg teflubenzuron, corresponding to a dose of 1 µg per gram lobster (the average weight of lobsters at the beginning of the experiment was 1.77 ± 0.32 g). This was the highest dose in a dose-response study the objective of which was to determine no-effect concentration (NEC) after a long-term exposure that simulated environmental conditions (Samuelsen, unpublished data). Analysis of 10 pellets per sample (5 samples) was conducted following the method described by Samuelsen et al. (2014) and yielded a concentration of teflubenzuron of 1.72 ± 0.15 µg per pellet. After 113 days, 28 exposed lobsters had survived and were used in this study. Six of these were sacrificed for whole animal residue analysis of teflubenzuron following the analytical method described in Samuelsen et al. (2014). The mean concentration of teflubenzuron in those lobsters was 12.55 ± 7.60 ng/g. This is lower than the highest concentration reported in various wild-captured crustaceans such as squat lobster (393 ng/g), Norway lobster (319 ng/g) and deep-water shrimp (200 ng/g) (Samuelsen et al., 2015).

After the exposure period, surviving lobsters (control and exposed) were housed separately in 170 ml white PVC plastic compartments (7.0 cm x 3.5 cm x 7.0 cm), with a perforated bottom (2.5 mm diameter round holes) to allow water flow. The units were held in tanks at 15 °C, in aerated water with a water exchange of approximately 5 L/min. All juveniles were given control feed until the behavioral experiments commenced, i.e. about 14 days had passed since any animal had ingested feed containing teflubenzuron. The half-life of teflubenzuron in European lobster juveniles of the size used in this experiment is 3.4 days at 15 °C (Samuelsen et al., 2014). Consequently, the average concentration of teflubenzuron in the lobster used for this behavior experiment was estimated at 0.8 ng/g and 0.1 ng/g on 1 June 2016 and 13 June 2016, respectively.

At the time of the behavior experiment, 19 lobster juveniles from the exposed group, and 19 from the control group were selected for observation (Table 1). Carapace length (CL) was recorded using calipers (+/- 0.1 mm) as the distance from the posterior rim of the eye socket to the posterior edge of carapace, total length (TL) as distance from the anterior tip of rostrum to the end of telson and wet body weight was recorded to the nearest 0.1 g. Only juveniles with two intact chelae were used in the behavior study. Two of the juveniles in the exposed group were classified as deformed (abdomen and tail-fan), although not in the antennae.

Table 1

Summary statistics of the size and weight of the European lobster juveniles (*Homarus gammarus*) used in this study. CL is the carapace length, TL is the total length of the lobsters and N is the number of individuals in each group. The row with Comparison reports the p values from the ANOVA statistical analysis used to compare CL, TL and Weight of the Exposed and the Control groups.

	CL (mm)	TL (mm)	Weight (g)	N
Exposed	17.14 ± 1.53	48.92 ± 17.15	2.56 ± 0.37	19
Control	16.86 ± 1.07	49.00 ± 8.81	2.62 ± 0.22	19
Comparison (p value)	0.44	0.94	0.59	

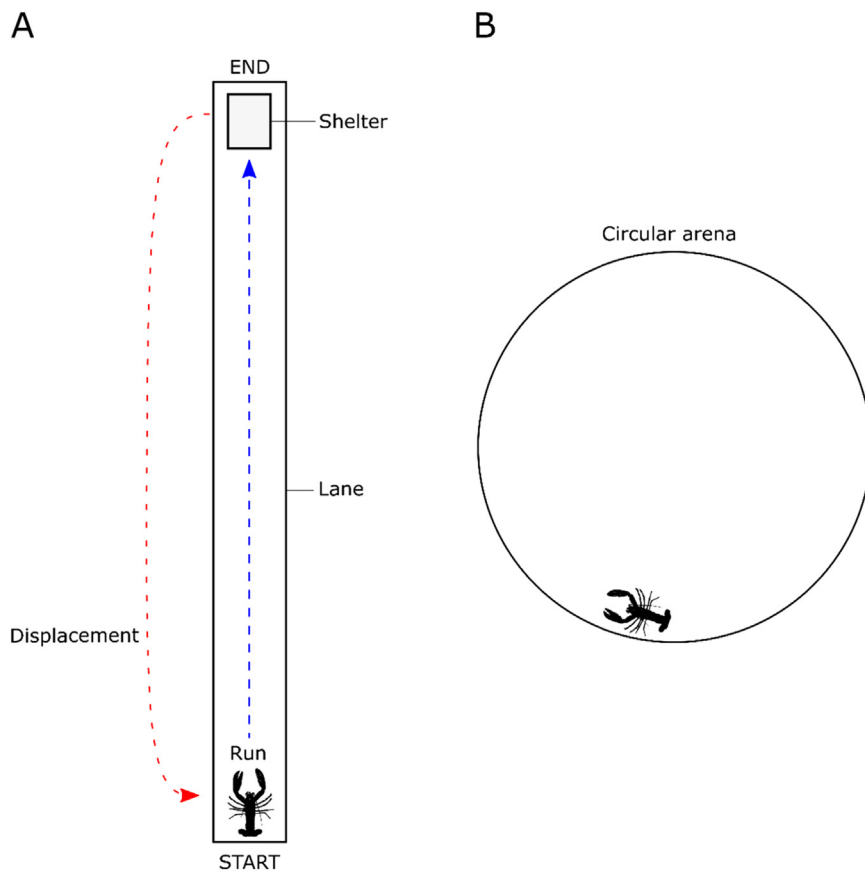


Fig. 1. Diagram of the experimental setup and the trials. A: diagram of the trials in the PVC lane (not to scale). The lane was 3 m long and 16 cm wide. At the END side of the lane there was a shelter made out of half a PVC pipe 12 cm long and 5 cm wide. One juvenile lobster (*Homarus gammarus*) was placed at the START end of the lane. During the trial, the animal walked towards the shelter (blue dashed arrow towards the grey square). After 30 min in the shelter, a small black box was used to displace the animal back to the start end of the lane (red dashed arrow). B: Walking distance during 10 min of observations in a circular arena (40 cm diameter) (not to scale). The lobsters were allowed 10 min to acclimate.

2.2. Behavioral observations

To promote immediate shelter-seeking behavior, we used long narrow lanes, where the animals' stimulus to establish physical contact with the walls while exploring the environment would not prolong the time needed to locate and settle in the shelter. We used a 3 m long PVC tube as a lane, which was cut longitudinally and filled with seawater (Fig. 1A, S1). The lane was 16 cm wide and the water in the lane was 8–10 cm deep at a temperature of 12–15 °C. A shelter 12 cm long and 5 cm wide, which was made of PVC pipe cut in half, was placed at one end of the lane. A camera was positioned above the lane to observe the behavior of the lobsters from an adjacent room.

The experiment started with the release of one lobster at the end of the lane without the shelter (START in Fig. 1A). We videotaped the animal and recorded the amount of time (seconds) from release until they found and entered the shelter (Fig. 1A; Table S1). The lobster was then left inside the shelter for 30 min, to allow for acclimation and to minimize stress (Van Der Meeren, 1993). Thereafter, the shelter was removed, and the lobster was placed in a small black box. The lobster was then displaced to the initial position at the START end of the lane. In total, each lobster performed three runs in the lane, being displaced twice. The consecutive runs in the lane allowed us to observe whether the lobsters were learning and remembering the position of the shelter. If the animals did not find the shelter within 60 min from the start of the trial, the test was considered failed. A total of 38 animals were tested in this manner.

After the trials in the lanes, each lobster was placed in a 40 cm diameter circular acrylic arena (Drifting in situ Chamber, or DISC (Paris et al., 2008)). The bottom of the arena was made of acrylic and the wall was made of rigid transparent plastic mesh that allows water to pass through (Fig. S2). A GOPRO Hero 4 camera was placed underneath the arena, looking upwards. The DISC was submerged in a larger black tank (diameter = 1.40 m; height = 0.90 m) filled with ~1000 L of seawater

(water depth of 60 cm). The tank and the lane were in the same room, with the same source of water and at the same temperature (12–15 °C). We calculated the walking speed of the animals from videos recorded with the GOPRO camera. Each animal was recorded for 20 min (1 frame per second in time-lapse mode). Data was collected only from the last 10 min; the first 10 min were considered an acclimation period (Rosson et al., 2006; Tolomei et al., 2003). Walking distance and speed was analyzed using the tracking procedure in the Drifting In Situ Chamber User Software in R (<https://github.com/jiho/discr>). All code is released under the GNU General Public License v3.0. Date of access: 13/07/2016. This tracking permitted calculation of the walking speed of the animals (cm/sec), which was considered as an indicator of their activity level. All of the trials were performed between 1/6/2016 and 13/6/2016, under laboratory conditions, during the day under artificial lights (3 LED lights).

2.3. Statistics

We tested the data for normality using the Shapiro test. When data met the requirement for normality, we used an unpaired two-sample *t*-test for comparison of walking speed. When data did not meet the requirement for normality, we used a non-parametric Mann-Whitney *U* Test (Fig. 2, time to find and enter the shelter). To test for learning in each experimental group, we used a multivariate repeated measures ANOVA. For pairwise comparisons of the shelter-seeking performance between runs, within each group, we used a repeated measures ANOVA combined with Tukey Pairwise comparisons. The statistical analyses were performed using R (Version 3.3.2 (2016-10-31) Copyright © 2016 The R Foundation for Statistical Computing).

3. Results

The control and teflubenzuron exposed lobsters did not differ in size

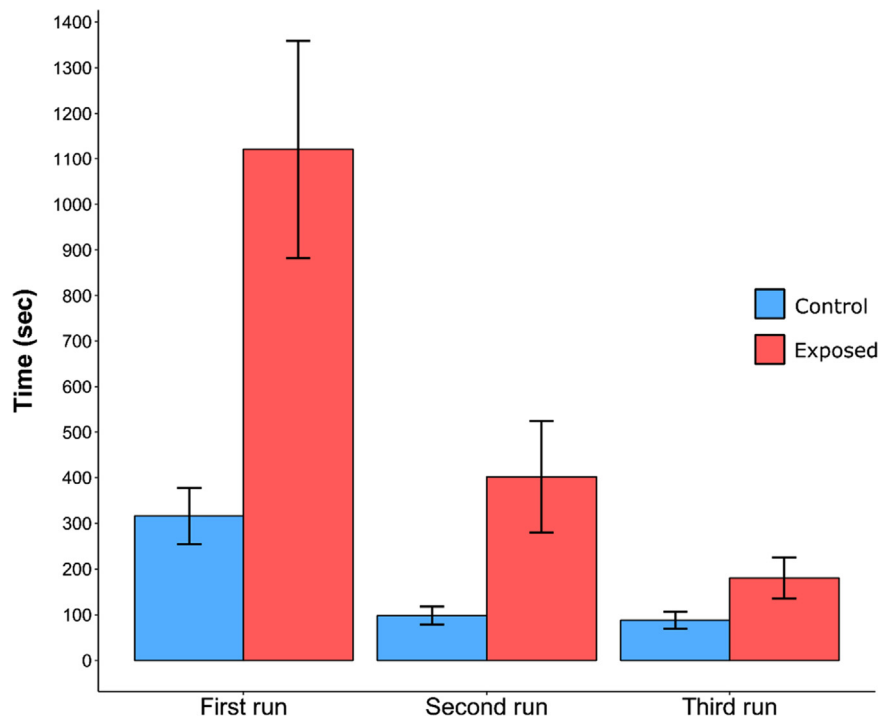


Fig. 2. Average time in seconds (\pm SD) that juvenile lobsters (*Homarus gammarus*) needed to find and enter the shelter for control (N = 19) and teflubenzuron-exposed animals (N = 16; three animals failed the test).

(Table 1). In the shelter-seeking behavioral tests, the lobsters started exploring the environment immediately. The animals maintained physical contact with the walls of the lane using their antennae and claws. The walking direction was mainly oriented along the longitudinal axis of the lane, punctuated by 180° turns. All of the control animals settled in the shelter (controls, N = 19). Three teflubenzuron-exposed lobsters failed to do so. These were, therefore, not considered for statistical analysis. Juvenile lobsters exposed to teflubenzuron needed significantly more time (mean 1120.31 ± 952.93 s, N = 16) to find and enter the shelter compared to the control animals (mean 316.11 ± 267.89 s, N = 19) (Fig. 2) when introduced to the lanes for the first time (Mann-Whitney U Test, $P = 0.0007$). Lobsters never exited the shelter after entering it. In general, the control animals moved actively, exploring all of the space available and entering the shelter upon first contact. The lobsters exposed to teflubenzuron displayed a different behavior: they moved forward in the lane for short distances, then turned back to the start end of the lane. Moreover, once the exposed lobsters came in contact with the shelter (touching or partially entering), they seemed to not immediately recognize it as a possible refuge. Instead, exposed juveniles turned without completely entering the shelter, going back in the lane in the opposite direction and only entering the shelter after 2–3 interactions. Controls never exhibited this behavior.

In the second run, the control juveniles significantly improved their shelter-seeking performance compared to the first run (Tukey Pairwise, $P = 0.0002$), quickly moving in the direction of the shelter and finding and entering it in less than 2 min (mean 98.11 ± 86.52 s, N = 19) (Fig. 2). The teflubenzuron-exposed animals also took significantly less time to settle in the shelter compared to their first run in the lane (Tukey Pairwise, $P = 0.0005$). However, these lobsters were significantly slower than the control animals (Mann-Whitney U Test, $P = 0.008$), using on average 401.81 ± 488.03 s to find shelter (N = 16). In the third run, neither the control nor the exposed animals improved their performance compared to the second run (Tukey Pairwise, $p > 0.05$). However, the control juveniles found shelter in 88.00 ± 82.21 s during the third run, significantly faster than the

exposed juveniles that spent 180.37 ± 181.12 s to find shelter (Mann-Whitney U Test, $P = 0.023$).

Learning was occurring both in the control group (repeated measures ANOVA, $p = 0.00001$) and in the exposed lobsters (repeated measures ANOVA, $p = 0.00003$). However, although the exposed juveniles in the second run were able to find the shelter faster compared to the first run (Fig. 2), they never walked directly towards the shelter, in any of the runs, and some individuals frequently turned back and forth.

In the circular arena, the animals tended to walk along the perimeter, touching the wall with their antennae and claws. Lobsters exposed to teflubenzuron moved significantly slower (2.38 ± 0.79 cm/second) compared to control animals (3.18 ± 0.77 cm/second) (t -test $P = 0.005$) (Fig. 3).

4. Discussion

Exposure to sub-lethal doses of teflubenzuron negatively affected the shelter-seeking behavior of juvenile lobsters - exposed animals took longer to find and enter shelter. The estimated concentration of teflubenzuron in the lobsters at the time this sub-lethal effect was studied, was 3.05–1.64 ng/g. Moreover, upon first contact with the shelter, exposed animals failed to recognize the PVC pipe as a potential refuge. These results suggest that juvenile lobsters exposed to sub-lethal concentrations of teflubenzuron are less efficient in exploring the environment and finding a shelter. Observations conducted *in situ* on the survival rate of juvenile European lobsters indicate that a lack of protective cover or shelter significantly lowers the probability of surviving predator attacks. Thus, exposure to teflubenzuron could increase predation mortality in juvenile lobsters in the wild.

The spatial learning abilities observed in this study are consistent with earlier findings on juvenile *Homarus spp.* (Bayer and Bianchi, 2017; van der Meeren, 2001). Both the control and the exposed groups took less time to find and enter the shelter during the second and third runs. The control animals showed rapid learning ability, settling in the shelter in less than 2 min from release, after having experienced the

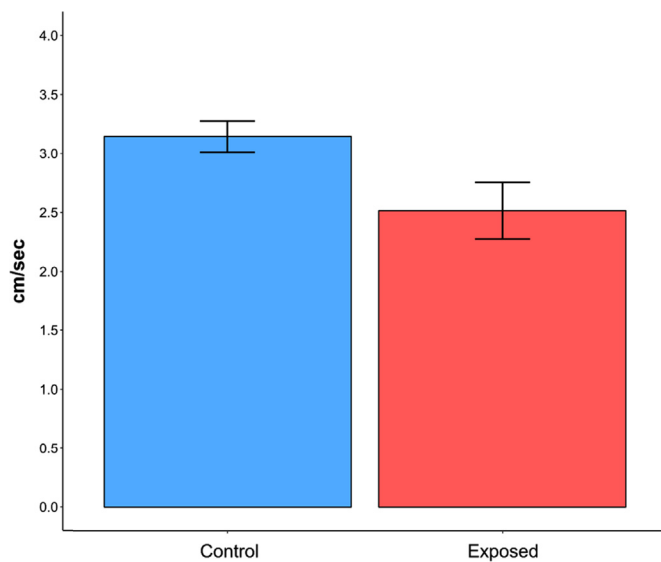


Fig. 3. Walking speed (cm per second) of juvenile lobsters (*Homarus gammarus*) in the circular arena, for the control (N = 19) and teflubenzuron-exposed animals (N = 16).

environment only once. This memory was retained through the third run. The time to find shelter in the second run was lower for teflubenzuron-exposed animals as well. However, they needed significantly longer to find and enter the shelter compared to the controls. Exposed animals also displayed difficulty in recognizing the shelter during the second and third runs, even though they had already experienced it. These results demonstrate that the learning ability of juvenile lobsters is negatively affected by exposure to sub-lethal doses of teflubenzuron.

Teflubenzuron persists in the sediment near salmon farms, with residues of $6.8 \pm 6.7 \mu\text{g/g}$ wet weight recorded 8 months after a farm was medicated (Samuelsen et al., 2015). Corresponding maximum concentrations in non-target crustaceans sampled near the medicated farm was reported to be 7.5 ng/g in squat lobster, 16.1 ng/g in deep-water shrimp and 45.2 ng/g in Norway lobster (Langford et al., 2014; Samuelsen et al., 2015). Although the experiments reported here were conducted under laboratory conditions, the results imply that wild juvenile lobsters living in the proximity of fish farms could be impacted by teflubenzuron exposure affecting their shelter-seeking behavior, which could affect recruitment and survival via increased predation risk from e.g. small benthic fish and crabs (Ball et al., 2001). The European lobster exhibits “homing” to their preferred shelter after nocturnal exploratory activity (Smith et al., 1998), has limited home range (Moland et al., 2011; Smith et al., 2001) and displays site-fidelity (especially when the availability of shelter is limited), which all serve to increase the probability of exposure to teflubenzuron and predation risk.

Movement has been used as an indicator of the activity level of lobsters (Smith et al., 1999, 1998). In this study, we observed a significant decrease in the walking speed of the juvenile lobsters treated with teflubenzuron compared to the controls. The reduced walking speed of treated lobsters could result in slower exploration of the environment or in a less efficient escape response to predator attacks, either of which could affect survival. Although significantly improving their performance over the consecutive runs, the exposed lobsters never reached the shelter as quickly as the controls; their lower walking speed might have contributed to this. However, the decreased locomotor activity of the exposed lobsters does not explain their poorer ability to recognize the shelter during the second and third runs, suggesting that teflubenzuron impacted both the locomotor activity and their ability to memorize the shelter. To date, there is no knowledge about the persistence of such sub-lethal effects of teflubenzuron.

Although the results of this experiment demonstrate that very low concentrations of teflubenzuron affects the behavior of juvenile European lobsters in laboratory conditions, further research is needed to understand the ecological consequences of the exposure to this chemical on wild populations of lobster and other benthic organisms. Additional studies should investigate whether juvenile European lobsters recover after sublethal effects caused by exposure to teflubenzuron, or whether this pesticide causes irreversible damage that persists throughout the whole life cycle. The results presented here should also be considered – in terms of their sub-lethal effects on non-target organisms – during deliberations over standard medication procedures to be applied on salmon farms.

Acknowledgements

We thank Eva Farestveit and Inger Semb Johansen for their assistance with the lobsters and Claire B. Paris for lending us the DISC used for the movement speed observations. Funding for the work was provided by the Norwegian Institute of Marine Research projects “Fine-scale interactions in the plankton”, Project # 81529, to H.I.B., and “Environmental effects of anti-sea lice drugs”, Project # 83569-01, to O.B.S. AC was supported by funds from the Paris Lab at the Rosenstiel School of Marine and Atmosphere Science of the University of Miami and by the Norwegian Institute of Marine Research project # 81529 to H.I.B.

Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2018.05.021>.

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