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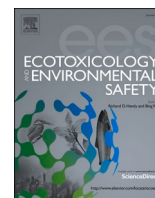
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## Fluopyram SDHI pesticide alters fish physiology and behaviour despite low *in vitro* effects on mitochondria

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### ABSTRACT

Pollution from pesticides is an increasing concern for human health and biodiversity conservation. However, there is lack of knowledge about some emerging molecules such as SDHI fungicides (succinate dehydrogenase inhibitors) that are widely used but potentially highly toxic for vertebrates. Boscalid, fluopyram, and bixafen are 3 frequent SDHI molecules commonly detected in surface waters, which may pose risks to aquatic species. This study aimed to (1) test the *in vitro* effects of SDHI on mitochondrial activities (inhibition of succinate dehydrogenase SDH, also named respiratory chain complex II) and (2) assess the *in vivo* effects of sublethal SDHI concentrations on fish physiology and behaviour over 96 hours of exposure, using *Carassius auratus* fish as a model species. Results show that bixafen and boscalid inhibited complex II activities *in vitro* as expected (bixafen > boscalid), while fluopyram had no *in vitro* effects. In contrast, *in vivo* studies showed that fluopyram strongly altered fish behaviour (enhanced activity, social and feeding behaviours), likely explained by reduced AChE enzymatic activity. In addition, fluopyram increased muscle lipid content, suggesting metabolic disruption. These findings raise serious concern about the toxic effects of SDHI pesticides, especially fluopyram, although its underpinning molecular mechanisms remain to be explored. We thus encourage further research on the long-term impacts of SDHI pesticides to improve existing regulation and prevent adverse effects on wildlife.

### 1. Introduction

Intensive agriculture and global changes have strongly increased the use of pesticides worldwide (Sharma et al., 2019). Some of these pesticides run off into surface waters, particularly following rainfall events, posing a threat to aquatic life due to their toxicity and persistence (de Souza et al., 2020; McGinley et al., 2023). Among pesticides, fungicides account for more than 40 % of pesticide use in Europe since 1990 (Duarte-Hospital et al., 2023), but their effects on wildlife are still poorly known. One mode of action of fungicides is the disruption of fungal energy production by targeting the mitochondrial respiratory chain and reducing ATP production. For instance, succinate dehydrogenase

inhibitor (SDHI) fungicides, which are widely used in agriculture, inhibit the activity of complex II of the mitochondrial respiratory chain, also known as the tricarboxylic acid (TCA) cycle enzyme succinate dehydrogenase (SDH), thereby blocking energy production in fungi. However, this enzyme is phylogenetically conserved across fungal and vertebrate taxa, raising concerns about potential off target effects in wildlife and its impacts on biodiversity, especially in aquatic systems. The SDHI pesticide family comprises 24 molecules, (most of them being discovered since 2010) which makes it the leading pesticide family in terms of new molecules introduced to the market (Umetsu and Shirai, 2020). The rapid introduction of these new SDHI molecules is thus concerning due to the complexity of assessing their environmental

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effects. In addition, *in vitro* studies have demonstrated complex II inhibition in non-target organisms, suggesting potential impacts on the respiratory chain function in humans, bees, worms and fish (Bénil et al., 2019; Huang et al., 2022), and thus potential deleterious impacts on wildlife health.

In this study, we aimed at studying the effects of SDHI pesticides on aquatic wildlife. We focused on 3 molecules from the SDHI family - boscalid, bixafen and fluopyram - due to their common application by spraying on crops, which makes them more likely to run off into surface waters. Boscalid (C<sub>18</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>2</sub>O), a pyridine-carboxamide, was introduced to the market in 2003, and was the leading SDHI until late 2010's. It has since been supplanted by newer generation SDHI like bixafen and fluopyram. Bixafen (C<sub>18</sub>H<sub>12</sub>Cl<sub>2</sub>F<sub>3</sub>N<sub>3</sub>O), a pyrazole-carboxamide, has been reported to inhibit both complex II and III activities (Bénil et al., 2019). Fluopyram (C<sub>16</sub>H<sub>11</sub>ClF<sub>6</sub>N<sub>2</sub>O), a pyridinyl-ethyl-benzamide which is also a nematicide, is the top-selling SDHI molecule since 2018 in France (BNV-D). Fluopyram could also be classified as a per- and poly-fluoroalkyl substance (PFAS), a group of extremely stable chemicals that have adverse effects on aquatic life (Lee et al., 2020), and was listed among the PFAS substances subject to a restriction proposal in a recent ECHA restriction report (ECHA, 2023). SDHI are frequently detected in various water bodies, with boscalid concentrations ranging from 22 ng.L<sup>-1</sup> in South African rivers to 30 µg.L<sup>-1</sup> in a California estuary (Curchod et al., 2019; Smalling and Orlando, 2011). Bixafen concentrations have been reported at 220 ng.L<sup>-1</sup> in small Swiss streams, while fluopyram has been found at concentrations ranging from 3.2 ng.L<sup>-1</sup> in South African rivers to 6 µg.L<sup>-1</sup> in the same Swiss study (Curchod et al., 2019; Spycher et al., 2018). However, despite their widespread use and frequent detection in aquatic environments, the effects of these molecules on wildlife in natural environments are still poorly known.

Some studies have been conducted on the effects of SDHI in domestic zebrafish (*Danio rerio*) model, at both embryo-larval and adult stages. These studies highlight various adverse effects, including organ malformations, neurotoxicity, behaviour impairments, and metabolic disruptions (Yanicostas and Soussi-Yanicostas, 2021). Previous research on boscalid has documented developmental and neurobehavioral effects on zebrafish and medaka (*Oryzias latipes*) embryo-larvae (Bedrossiantz et al., 2024; Qian et al., 2021, 2018; Wang et al., 2020). In adult zebrafish, boscalid has been shown to impact behaviour, steroidogenesis, reproduction, and metabolism (Qian et al., 2019b, 2020, 2021). Bixafen's toxicity has been studied in zebrafish embryo-larvae, revealing organ-specific toxicities during development (Li et al., 2020; Brenet et al., 2021; Yuan et al., 2021; Xiao et al., 2021; Wang et al., 2023). Fluopyram has been less studied, but the few data available showed impacts on bone development in zebrafish larvae at high exposure concentrations (> 6 mg.L<sup>-1</sup>) (Zhang et al., 2024). There is however limited knowledge on the effects of SDHI molecules on aquatic wildlife, also because of the limited diversity of fish model species used in the literature. In this study, we used the goldfish (*Carassius auratus*) as a model species due to its wide distribution and established status as a model organism in ecotoxicology and fish physiology, allowing for both behaviour and physiological analyses (Xia et al., 2013; Gandar et al., 2016; Aliko et al., 2019). In addition, goldfish are commonly found in lentic habitats, such as agricultural hill reservoirs, which are frequently exposed to chemical stressors, including SDHI pesticides.

In this study, we first evaluated the inhibition of SDH in *C. auratus* mitochondria exposed to SDHI *in vitro*. Subsequently, we assessed the impacts of boscalid, bixafen, and fluopyram after a four-day exposure on fish physiology and behaviour across biological scales. We measured several physiological traits reflecting neurotoxicity (acetylcholinesterase - AChE activity), immunity (neutrophils/lymphocytes ratio), indicator of stress response in fish, and energy management (lipids, proteins and carbohydrates reserves in muscle). Behavioural traits (feeding, activity, sociability) were used as integrative and early indicators of pollutant effects based on previous studies (Jacquin et al., 2020; Petitjean et al., 2019; Sharma, 2019).

## 2. Materials & methods

### 2.1. Chemicals

The pesticide active substances (Boscalid, CAS: 188425-85-6; bixafen, CAS: 581809-46-3; fluopyram, CAS: 658066-35-4), along with all other chemicals, were purchased from Sigma-Aldrich (St.Louis, USA).

### 2.2. Model species and animal care

Goldfish (*Carassius auratus*, Linnaeus 1758, family Cyprinidae) were obtained from the fish farm EARL CARPIO (Consac, France). Individuals had variable coloration patterns, allowing for individual behavioural tracking and monitoring. Fish size spanned between 5 and 10 cm (10.4 ± 3.6 g). Upon arrival, the fish were acclimated for two weeks in a 200 L stabilization tank with a pump to ensure oxygenation using previously validated acclimation protocols (Gandar et al., 2016; Jacquin et al., 2019). Parameters were: temperature = 18°C, pH = 7.5, NO<sub>3</sub> < 10 mg.L<sup>-1</sup>, NO<sub>2</sub> = 0 mg.L<sup>-1</sup>, conductivity = 260 µS.cm<sup>-1</sup>, with a photoperiod of 12 h L:12 h D. Half of the water was renewed every week. Fish were fed daily (about 1 % of the total biomass) with commercial fish food (TETRA®) to ensure sufficient nutritional intake (Jacquin et al., 2019; Kestemont and Philippart, 1990). No mortality was observed during acclimation.

### 2.3. Complex II activities *in vitro*

We conducted pilot tests *in vitro* to assess the inhibitory effects of boscalid, bixafen and fluopyram on fish mitochondrial complex II enzymatic activities, the primary target of commercial SDHI pesticides. Two specific enzymatic activities related to complex II were studied, as detailed in Lemarie et al. (2011). The first activity comprises the whole activity of Complex II, named Succinate coenzyme Q Reductase (SQR) activity, and concerns the four complex II subunits SDHA to SDHD, from succinate oxidation to coenzyme Q reduction. The second one measures succinate dehydrogenase (SDH) activity, supported by SDHA and SDHB subunits, from succinate oxidation to an intermediate surrogate electron acceptor (PMS, phenazine methosulfate). These two *in vitro* absorbance-based assays were performed on pools of mitochondria samples, isolated from muscles of non-exposed fish *C. auratus*, which were adjusted at 5 µg protein content. Boscalid, bixafen, and fluopyram effects were evaluated *in vitro* at concentrations of 0.1 µM, 1 µM, and 10 µM (V1, V2, and V3), solubilized in DMSO. The corresponding µg.L<sup>-1</sup> concentrations for each tested molecule are reported in Table 1.

The first step was to isolate mitochondria from fish. Ten acclimated (non-exposed) fish were anesthetized with a lethal benzocaine concentration of 150 mg.L<sup>-1</sup>. Mitochondria were then isolated from the collected white muscle samples. All steps were carried out on ice. Tissue samples were lysed using a lysis buffer (1:9 w:v), which comprised an assay buffer (20 mM Hepes pH 7.5, 1.5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, pH 7.4). Twelve mL of this buffer were supplemented with 3 mL of 1.25 M sucrose and 1 Tab Mini Complete. Tissue suspensions were processed using a Potter homogenizer with a pestle motor mixer (450 rpm). After centrifugation for 10 min at 1620 g and 4°C, the resulting supernatant was centrifuged for 20 min at 15700 g and 4°C. Resultant mitochondrial pellets were resuspended in the lysis buffer. The protein content was determined using the Bradford method, with BSA (bovine serum albumin) serving as the standard reference.

The second step was to quantify the SQR and SDH activities of fish mitochondria *in vitro*, in the presence or absence of the different SDHI concentrations added extemporaneously. SQR function was assessed through the CoQ (Coenzyme Q)-dependent reduction of DCPIP (dichlorophenolindophenol). The reduction was triggered by a reaction buffer, inclusive of 1.6 mM PMS, 10 mM succinate, 70 µM DCPIP, 5 µM rotenone, 2 µg.mL<sup>-1</sup> Antimycin A, and 2 mM KCN, all in a 35 mM potassium buffer. SDH activity was determined via the PMS-mediated

**Table 1**

Concentration used in the *in vitro* complex II activity assay (V1, V2, and V3) and the *in vivo* exposure (C1, C2, and C3) for boscalid, bixafen and fluopyram in  $\mu\text{g.L}^{-1}$ . The *in vitro* concentrations (V1, V2, and V3) correspond to 0.1, 1, and 10  $\mu\text{M}$ , respectively, with their equivalent values in  $\mu\text{g.L}^{-1}$  provided. Effective *in vivo* C1, C2 and C3 concentrations (mean (standard deviation)) ( $n=2$ ) were reported for each tested SDHI in water samples at 96 hours. ND: non-detected, LOQ: limit of quantification.

Molecule	<i>In vitro</i> concentrations in $\mu\text{g.L}^{-1}$				<i>In vivo</i> effective concentrations in $\mu\text{g.L}^{-1}$			
	Control	V1	V2	V3	Control	C1	C2	C3
Boscalid	0	34	343	3432	ND ( $< 0.025$ )	8.6 (0.3)	113 (1)	994 (54)
Bixafen	0	41	414	4142	ND ( $< 0.005$ )	0.26 (0.03)	1.7 (0.2)	12.1 (0.2)
Fluopyram	0	40	397	3967	$< \text{LOQ}$ ( $< 0.005$ )	3.5 (0.1)	29 (4)	321 (19)

reduction of DCPIP. The reaction was initiated by adding 200  $\mu\text{L}$  of a reaction buffer containing 5 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  coenzyme Q2, 25 mM succinate, 88  $\mu\text{M}$  DCPIP, 5  $\mu\text{M}$  rotenone, 2  $\mu\text{g}/\text{mL}$  Antimycin A, and 2 mM KCN in a 35 mM potassium buffer (pH 7.2). TTFA at 5 mM and malonate at 10 mM served as positive controls for SQR and SDH inhibition, respectively. Reduction of DCPIP absorbance ( $\epsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ ) was measured at 600 nm every 30 sec during 20 min (FLUOstar Optima, BMG Labtech), monitoring the extinction of DCPIP at 37°C. Results were expressed as relative activities compared to control DMSO mitochondria. Each experiment was replicated thrice, with absolute control DMSO values ranging from 111.2 to 176.0 and from 56.4 to 92.2  $\text{nmol min}^{-1} \text{ mg}^{-1}$  protein for SQR and SDH activities, respectively.

#### 2.4. Subacute SDHI exposure of fish

Three concentrations of boscalid, bixafen, and fluopyram were tested on fish *in vivo* based on the 96 h LC50 values for Rainbow trout, *Oncorhynchus mykiss* or, if not available, sheepshead minnow, *Cyprinodon variegatus* (EFSA, 2013, 2012; US EPA, 2010). The objective was to test the effects of realistic sub-lethal concentrations, with the highest concentration (i.e., C3) corresponding to approximately half of the LC50 to avoid mortality. Concentrations C2 and C1 were obtained by a 10-fold dilution cascade and environmentally realistic concentrations (Table S1). Pesticide stock solutions were prepared in acetone to facilitate dissolution. At a final volume per tank of 0.05 %, acetone has no expected effect on goldfish, making it a good control group (Gandar et al., 2016).

We tested 4 replicates per condition (2 tanks x 2 exposure series), with 20 fish per concentration (i.e., C1, C2, C3) for each molecule (i.e., boscalid, bixafen, fluopyram). Prior to the experiment, 5 randomly selected fish were placed in each tank corresponding to each condition. Fish were identified according to their colour pattern and tracked individually throughout the exposure. Tanks were made of glass to limit the adsorption of molecules and had a volume of 34 L. The tanks were visually separated by plastic panels to eliminate all visual interactions between experimental groups. Physicochemical parameters ( $\text{NO}_2$ ,  $\text{NO}_3$ , and temperature) were similar to the acclimation and were monitored during the experiment, with no fluctuation was detected. Half of the water with or without SDHI solution was renewed after 48 h of exposure, and the total duration of the experiment was 96 h. At 48 h and 72 h, behavioural tests were conducted (see below). At 96 h, the end of the exposure, fish were anaesthetized with a lethal dose of benzocaine (150  $\text{mg.L}^{-1}$ ), then weighed ( $\pm 0.1$  g), measured ( $\pm 0.1$  cm), and dissected to weigh several organs ( $\pm 0.001$  g). Sex was determined by observation of the gonads. Each muscle sample was divided into three aliquots: one for measuring *in vivo* SQR and SDH activities, another for assessing AChE activity, and the third for quantifying reserves.

##### 2.4.1. SDHI quantification in water samples

Water samples were collected at the end of the experiment (96 h), one per exposure series and per condition. Analyses of bixafen, boscalid and fluopyram were carried out using an ultra-high-performance liquid chromatograph coupled with tandem mass spectrometry (UHPLC-MS/

MS; see details in Supp Material). The precision and accuracy ranged from 5 % to 12 % (RSD) and 88–105 %, respectively. Results showed that the actual levels of boscalid, bixafen, and fluopyram in water at 96 h were slightly lower than the nominal values (see Table S1). For boscalid, the measured concentrations deviated by less than 15 % from the nominal values (14.5 %, 13 %, and 0.6 % for concentrations C1, C2, and C3, respectively). Bixafen displayed more pronounced deviations, with differences of 48 %, 66 %, and 75.8 % for the corresponding concentrations, suggesting a low solubilization of bixafen in water. Similarly, for fluopyram, the deviations were noted at 31 %, 42 %, and 35.8 % for C1, C2, and C3, respectively. Differences between target and measured concentrations were thus observed. Uncertainty over target values is high, due to the complexity of the experimental set-up. As the accuracy of measured values is always less than 20 %, the latter are considered in the remainder of this article for *in vivo* data. However, despite lower than the expected concentrations, effective concentrations remained lower than LC50, and the variation between experimental groups was stable and differed by a 10 factor between C1 and C2 and between C2 and C3 (Table 1). Therefore, C1, C2, C3 corresponded to low, medium and high concentrations as needed in our experimental design.

##### 2.4.2. SQR/SDH enzymatic activities *in vivo*

SQR and SDH activities were assessed on the mitochondria of fish exposed to SDHI molecules, in order to understand the effects of these molecules on mitochondrial activities under more physiological conditions than *in vitro* tests exposed above. Mitochondria were isolated from fish white muscle samples as described in Section 2.3. Mitochondrial SQR and SDH activities in control and SDHI-exposed fish (20 replicates/condition) were assessed as previously detailed *in vitro*. Results are presented as activities relative to control. The average SQR and SDH activities of the controls corresponded to 180.2 and 103.5  $\text{nmol min}^{-1} \text{ mg}^{-1}$  protein, respectively.

##### 2.4.3. Acetylcholinesterase (AChE) activity

White muscle samples (25 mg, 20 replicates/condition) were homogenized in 1 mL of 100 mM phosphate buffer (pH 7.8) with 0.1 % Triton X-100 using FastPrep-24® (MP Biomedicals) and centrifuged at 9000 g, 4 °C for 15 minutes. Supernatants (S9 fraction) were quarter-diluted. AChE activity was quantified using a modified colorimetric method from Ellman et al. (1961), adapted to microplate (Noury, 2016; Xuereb et al., 2007), using ATCh (acetylthiocholine iodide) as substrate. Briefly, 10  $\mu\text{L}$  supernatant was mixed with 180  $\mu\text{L}$  reaction mixture (100 mM phosphate buffer, 0.2 mM DTNB, 2 mM ATCh). The production of 5-thio-2-nitrobenzoic acid was monitored at 405 nm every 48 seconds over an 8-minute period using Clariostar microplate reader (BMG Labtech). A calibration curve was established using commercial AChE (*Electrophorus electricus*, Sigma) from 0 to 0.0084 U. Proteins were assayed via the Bradford method, using BSA as the standard. AChE activity was initially expressed as  $\text{U mg}^{-1}$  protein, with the control mean value representing 1.26  $\text{U mg}^{-1}$  protein, and then represented as a percentage relative to the control.

#### 2.4.4. Neutrophils/lymphocytes (N/L) ratio

At 96 h, immediately post blood collection, smears were prepared and subsequently stained using May-Grunwald (Sigma-Aldrich, 1:2 v:v) and Giemsa (Sigma-Aldrich, 1:20 v:v). Using an Olympus microscope with x100 magnification, 100 leukocytes – consisting of monocytes, neutrophils, and lymphocytes – were identified and counted, based on established criteria (Petitjean et al., 2020b).

#### 2.4.5. Energy reserves: carbohydrates, proteins, lipids

White muscle samples (25 mg, 20 replicates/condition) were analysed for total carbohydrate, protein and lipid contents with the methods detailed in Gandar et al. (2017). Protein concentration ( $\mu\text{g}\cdot\text{mg}^{-1}$  of tissue) was established using the Bradford assay, adding the Bradford reagent and measuring absorbance at 595 nm against a BSA standard in 0.2 N NaOH (Bradford, 1976). Carbohydrate concentration ( $\mu\text{g}\cdot\text{mg}^{-1}$  of tissue) was determined spectrophotometrically at 492 nm using a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany), with glucose in TCA as a reference. Total lipid concentration ( $\mu\text{g}\cdot\text{mg}^{-1}$  of tissue) was measured by reading absorbance at 525 nm using menhaden oil in chloroform as a standard (Welker and Congleton, 2005).

#### 2.4.6. Condition indices

At 96 h post-exposure, following anaesthesia, fish were weighed, measured, and several organs were weighed to calculate the Fulton's condition factor (FI), the hepatosomatic index (HSI), and the gonadosomatic index (GSI). Fulton's index was calculated as follows:  $\text{FI} = (\text{somatic mass} / \text{Length}^3) \times 100$  (Dutil and Lambert, 2000). The HSI corresponds to the mass of the liver relative to the total mass, while the GSI represents the ratio of gonad mass to total body mass.

#### 2.4.7. Behavioural tests

To measure feeding behaviour, fish were fed with 10 pellets after 24 h of SDHI exposure and filmed using Logitech C922 PRO STREAM WEBCAM® cameras placed above each aquarium for 10 minutes. For each individual, the time before feeding on the first pellet was recorded, as well as the cumulative proportion over time of fish that effectively fed on at least one pellet (Jacquin et al., 2019). The total number of pellets ingested was not measured because fish had eaten all the pellets at the end of the test.

At 72 h, fish were filmed in aquaria similarly to the feeding test to measure locomotion and sociability traits. A 10 minutes extract was analysed using the behavioural analysis software BORIS 7.10.3® (Friard and Gamba, 2016). As the aquaria were divided into 6 fictive zones of equal dimensions, the number of crossed zones was recorded during these 10 minutes, reflecting fish activity. The time of active swimming was also tracked during this period. Finally, the locomotion criterion corresponds to the combination of these two variables: total movement duration and number of crossed zones.

We recorded the number of contacts between fishes using the behavioural analysis software BORIS. We also measured the ANND, i.e. average nearest neighbour distance, from static images extracted from the recording at 72 h every 30 seconds and analysed using Image J 15.0.2® software. The protocol is inspired by Bartolini et al. (2015) and Colchen et al. (2017). The final endpoints are the number of contacts between individuals and the nearest neighbour distance of each individual (ANND) during the recording. Six individuals were extreme outliers (very high number of contacts), likely attributable to breeding behaviour. These individuals were identified and excluded when analysing behavioural data using the *statix* R package.

### 2.5. Statistical analysis

Statistical analyses were conducted using RStudio (version 2023.06.2) with R version 4.2.3. First, we used mixed models (*lme* function) with sex and size as fixed factors and replicate tanks as random factor to consider the non-independence of fish exposed in the same

tank. However, adding random effects of replicate tanks in models did not significantly influence the results and fish sex and size were not retained in any final models. We thus used simple Anova tests hereafter, followed by Dunnett's multiple comparison test (*DescTools* R package) based on previous studies (Almeida et al., 2010; Zhang et al., 2024). For AChE activity, reserves, condition indices and behavioural traits, data were log or square root transformed to achieve normal distribution (Lee, 2020). The proportion of fish feeding over time was analysed using a survival function, i.e. a Cox regression analysis with the *survival* R package, to compare the time before feeding among condition groups (Therneau and Grambsch, 2000). Immune data on N/L ratio, did not reach normality post-transformation, and we thus used non-parametric Wilcoxon test. P-values were adjusted based on the Benjamini-Hochberg method to account for multiple comparisons. The figures were created using GraphPad Prism (v.9.0.2) or RStudio.

## 3. Results

### 3.1. Complex II activities *in vitro*

We tested the *in vitro* inhibitory effects of SDHI pesticides on fish mitochondrial SQR and SDH activities. This assay was conducted to determine whether SDHI could exert their mode of action on fish. For SQR, boscalid at 10  $\mu\text{M}$  and bixafen at concentrations of 0.1, 1, and 10  $\mu\text{M}$  decreased the enzymatic activity compared to the DMSO control (Dunnett test,  $p < 0.001$ ; Fig. 1 A). Bixafen at its highest concentration (10  $\mu\text{M}$ ) was the only compound observed to inhibit SDH activity compared to the DMSO control (Dunnett test,  $p < 0.05$ ; Fig. 1 B). Fluopyram did not affect either SQR or SDH activities even at high

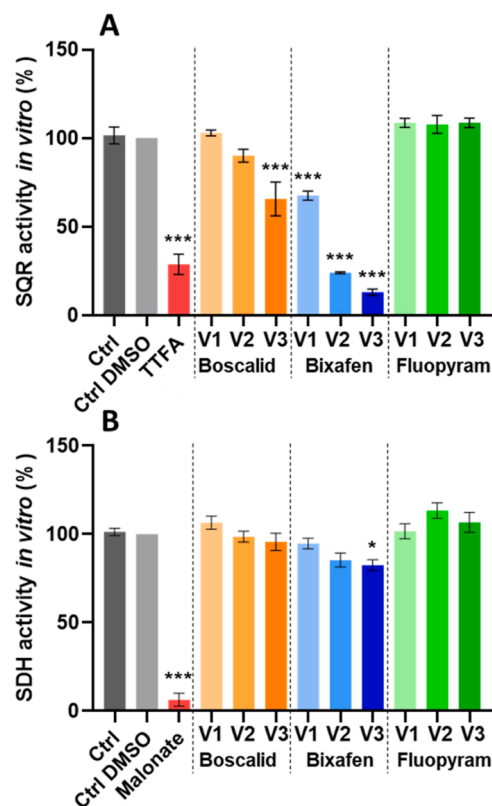


Fig. 1. *In vitro* effects of boscalid, bixafen, and fluopyram on mitochondrial SQR (A) and SDH (B) activities. Enzymatic activities in the extracted mitochondria are presented as percentages relative to the DMSO control. TTFA and malonate served as positive controls. Asterisks indicate significant differences compared to the DMSO control (Dunnett's test). \* $p < 0.05$ , \*\*\* $p < 0.001$ . Mean  $\pm$  SEM,  $n=3$ .

concentrations ( $p > 0.05$ ).

### 3.2. SDHI impact on physiological endpoints

#### 3.2.1. Enzyme activities

The SQR and SDH activities assay was then carried out under more physiological conditions, where the fish were exposed to SDHI molecules directly in the water for 4 days. After *in vivo* exposure to boscalid, bixafen and fluopyram, there was no significant inhibition of SDH and SQR mitochondrial activities in exposed fish compared to control ( $p > 0.05$ ; Fig. S1).

We then measured the impact of SDHI on AChE activity in muscle, a classic biomarker of pesticide exposure reflecting neurotoxic effects (Davies and Vethaak, 2012). Inhibition of AChE activity by pesticides can disrupt neuromuscular signaling, which can have a negative effect on locomotor behaviour (Bretaud et al., 2000; Könemann et al., 2022). The results showed that boscalid and fluopyram decreased AChE activity in the white muscle relative to the control group (Fig. 2). Specifically, the AChE activity decreased by 28.5 % in the boscalid C2 group ( $p < 0.05$ ), while decreases of 26.3 % and 30.2 % were evident in the fluopyram C1 ( $p < 0.05$ ) and C3 groups ( $p < 0.01$ ), respectively.

#### 3.2.2. Immune N/L ratio

We then investigated the impact of SDHI exposure on the immune N/L ratio, an immune marker correlated with cortisol levels, making it a reliable biomarker of stress (Gabriel et al., 2015; Sula and Aliko, 2017). Environmental stressors, including chemicals, often induce an increase in neutrophils and a decrease in lymphocytes, reflecting inflammation (Davis et al., 2008). Results showed that SDHI exposure did not significantly influence the immune N/L ratio ( $FDR > 0.05$ ; Fig. S2).

#### 3.2.3. Reserves in white muscles

We also determined the level of energy reserves available (carbohydrates, proteins and lipids) in fish muscle, which are essential for the maintenance of biological functions of fish, especially under stress (Gomes et al., 2015; Petitjean et al., 2020a). Carbohydrate and protein content in the white muscle of fish exposed to each of the 3 SDHI showed no significant deviations from the control group (Fig. 3 A and B). However, only fluopyram increased lipid reserves in white muscle tissues compared to control (Fig. 3 C). Specifically, there was a 3.7-fold increase at the C1 fluopyram concentration ( $p < 0.01$ ), a 2.9-fold increase at the C2 concentration ( $p < 0.05$ ), and a 3.3-fold increase at the C3 concentration ( $p < 0.01$ ), in comparison to the control group.

#### 3.2.4. Condition indices

The Fulton index, hepatosomatic index (HSI), and gonadosomatic index (GSI) are established indicators of the fish's overall energy

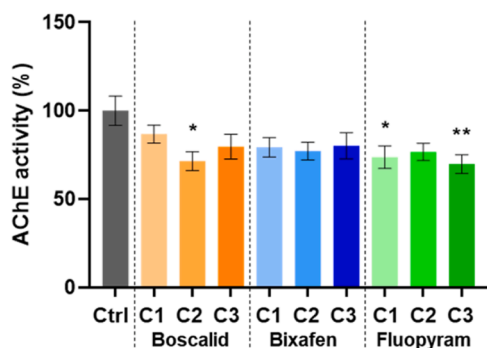


Fig. 2. Effect of SDHI on *C. auratus* acetylcholine esterase (AChE) activity at 96 h of exposure. Enzymatic activities were measured in white muscle samples and are represented as percentages relative to the control. Asterisks indicate significant differences compared to the control (Dunnnett's test). \* $p < 0.05$ , \*\* $p < 0.01$ . Mean  $\pm$  SEM,  $n=20$ .

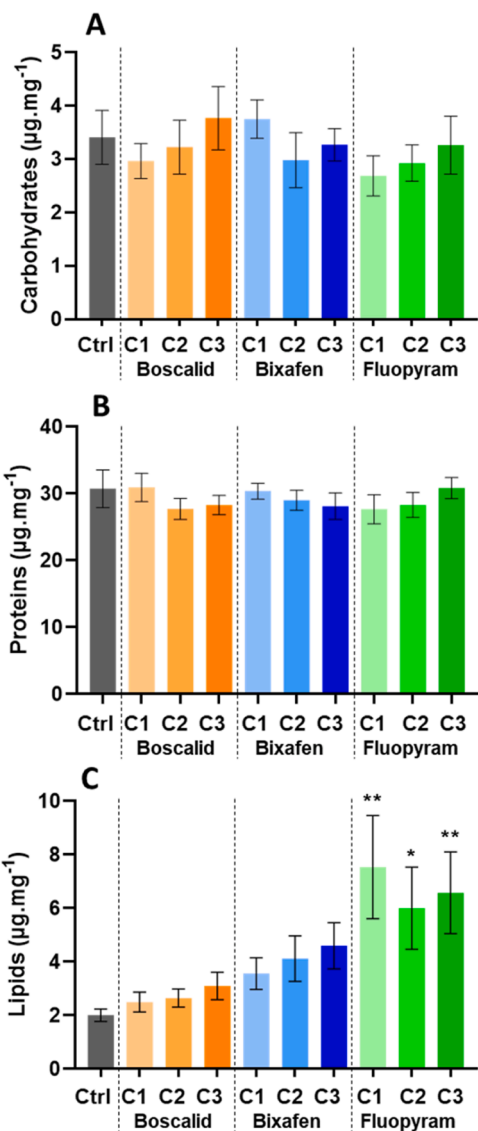


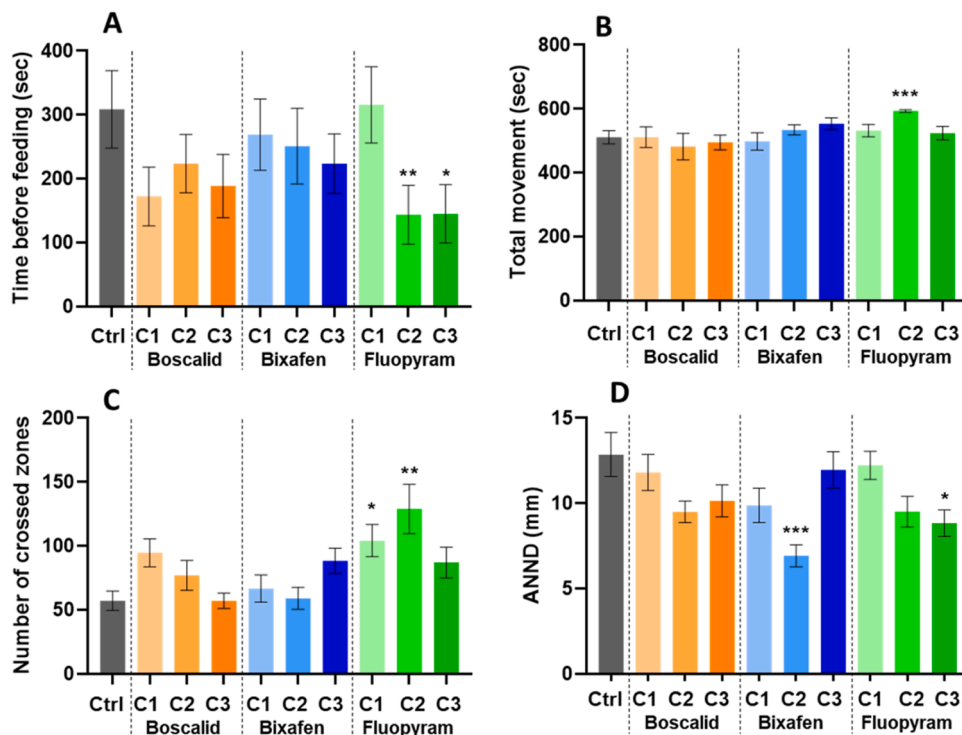
Fig. 3. Impact of SDHI on *C. auratus* white muscle reserves at 96 h of exposure. Carbohydrates (A), proteins (B) and lipids (C) levels in  $\mu\text{g.mg}^{-1}$  of tissue (fresh weight). Differences compared to control (Dunnnett's test, \* $p < 0.05$ , \*\* $p < 0.01$ ). Mean  $\pm$  SEM,  $n=20$ .

reserves, liver energy storage, and energy investment in gonads (Chellappa et al., 1995; Jacquin et al., 2019; Marentette and Corkum, 2008). These condition indices were not significantly affected by SDHI exposure ( $p > 0.05$ ; Fig. S3).

### 3.3. SDHI impact on fish behaviour

Following the assessment of physiological parameters, we investigated the impact of SDHI exposure on individual fish behaviour, focusing on feeding, locomotion and sociability. Feeding behaviour, a key indicator of fish health and energy intake, can be altered by changes in metabolism or stress responses (Assan et al., 2021). Locomotion is one of the most sensitive behavioural traits, and is commonly used to measure the effects of pollutants (Jacquin et al., 2020). Sociability in gregarious organisms is also very sensitive to stressful conditions (Colchen et al., 2017).

Only fluopyram at C2 and C3 concentrations affected fish feeding behaviour after 24 h of exposure. Indeed, fish fed more rapidly when exposed to C2 and C3 concentrations of fluopyram compared to the control group ( $p < 0.05$  &  $p < 0.01$ , respectively; Fig. 4 A). In addition,



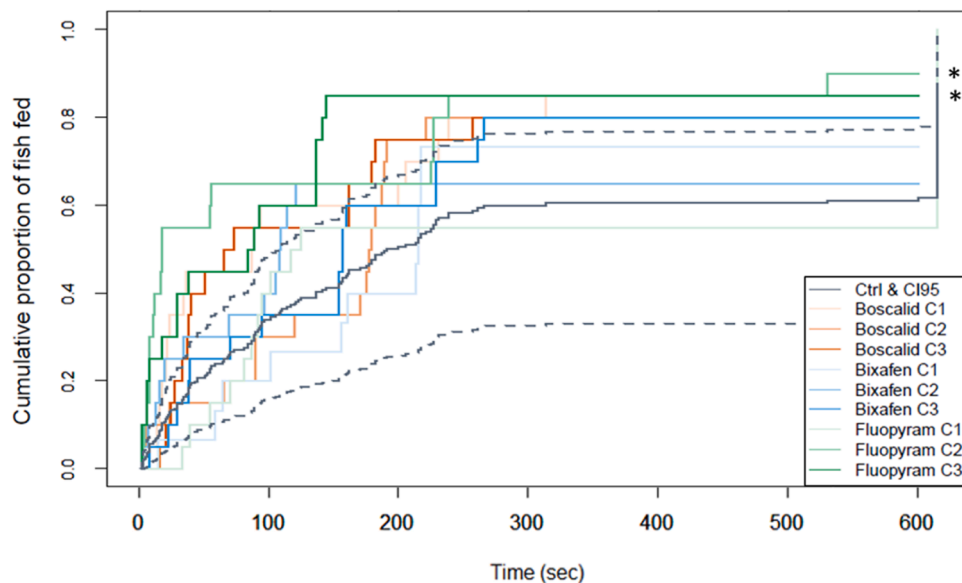
**Fig. 4.** Effect of SDHI on behaviour parameters. (A) Time before feeding at 24 h of exposure; (B) Total movement duration (sec) at 72 h of exposure; (C) Number of zones crossed at 72 h of exposure, during the 10-minute observation period and (D) Averaged nearest neighbour distance (ANND in mm), measured at 72 h of exposure, every 30 sec over 10 min. Asterisks indicate significant statistical differences compared to the control (Dunnett’s test). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Mean  $\pm$  SEM,  $n=20$ .

the proportion of fish fed over time significantly increased for fish exposed to C2 and C3 fluopyram compared to control fish ( $p < 0.05$ ; Fig. 5).

No significant changes in locomotor activity were observed in fish exposed to either boscalid or bixafen compared to control fish ( $p > 0.05$ ). In contrast, fluopyram significantly increased locomotion activity (duration of movement in sec) for C2 (but not C3) concentration of fluopyram compared to control fish (+ 16 %,  $p < 0.001$ ; Fig. 4B). Moreover, the number of zones crossed was significantly greater for fish

treated with fluopyram at both C1 and C2 concentrations, as compared to the control (+ 82.6 % and + 125.9 %,  $p < 0.05$  and  $p < 0.01$ , respectively; Fig. 4 C). Interestingly, there was no significant effect of C3 fluopyram concentration on locomotion compared to control ( $p > 0.05$ ), suggesting a non-linear response of fish locomotion to fluopyram.

The number of contacts between individuals remained unchanged across all SDHI exposure levels ( $p > 0.05$ ; Fig. S4). Fish social behaviour measured by ANND increased in fish exposed to the bixafen C2 concentration (fish stayed closer to their neighbours as indicated by -



**Fig. 5.** Cumulative proportion of fish fed over time at 24 h of exposure to SDHI. The control is represented in black, with its 95 % confidence interval (CI95) shown by dotted lines. Asterisks indicate statistically significant deviations from the control, as determined by the Cox regression model. \* $p < 0.05$ ,  $n=20$ .

46.2 % of ANND,  $p < 0.001$ ) and in the fluopyram C3 concentration (-31.3 %,  $p < 0.05$ ) compared to control fish (Fig. 4D).

#### 4. Discussion

We aimed at testing the effects of three SDHI pesticides: boscalid, bixafen, and fluopyram on fish mitochondrial activity *in vitro* and on fish physiology and behaviour *in vivo*. The three *in vivo* concentrations tested describe two decades in concentration and are clearly sublethal in terms of the results obtained.

As expected, bixafen and boscalid inhibited complex II activity in the mitochondria of *C. auratus in vitro*. Interestingly, despite fluopyram showed no inhibitory effects on mitochondrial activity *in vitro* at the chosen concentrations, it had significantly impacted goldfish behaviour and physiology *in vivo* at concentrations from 3.5 to 321  $\mu\text{g.L}^{-1}$ , with the lowest concentration being similar to levels detected in surface waters (Naiades database; Spycher et al., 2018). Indeed, fluopyram increased feeding behaviour, activity and sociability in goldfish, while inhibiting AChE activity and increasing lipid muscle content.

##### 4.1. Inhibition of complex II activities by bixafen and boscalid *in vitro* but not *in vivo*

SDHI are commercialized as inhibitors of the complex II (also known as the SDH enzyme of the TCA cycle) of the mitochondrial respiratory chain in fungal organisms, thus resulting in an efficient fungicide effect. However, this mitochondrial complex is phylogenetically rather conserved in all eukaryote organisms, making SDHI pesticides a potential threats for non-target organisms (Sun et al., 2005). Accordingly, some SDHI are commercially registered as nematicides. The SDH complex plays a dual role in mitochondrial metabolism: it participates in the metabolic TCA cycle through SDH activity, catalysing the oxidation of succinate to fumarate; and contributes to the mitochondrial respiratory chain via SQR activity (Lemarie and Grimm, 2011).

In this study, bixafen and boscalid inhibited complex II activities in goldfish mitochondria exposed *in vitro*, as expected. Bixafen exhibited a stronger inhibitory effect than boscalid, showing concentration-dependent inhibition of SQR activity from 0.1 to 10  $\mu\text{M}$  and inhibiting SDH activity at 10  $\mu\text{M}$ . Boscalid only inhibited SQR activity at the highest concentration tested (10  $\mu\text{M}$ ). Our results align more closely with findings from studies on vertebrates (Bénit et al., 2019; Huang et al., 2022). This study also highlights the greater inhibition of SQR activity compared to SDH activity by SDHI, consistent with their mode of action. Indeed, SDHI pesticides are known for their great affinity for the ubiquinone binding site within the SDH complex, thus acting as competitive inhibitors of SQR activity (Sierotzki and Scalliet, 2013; Zhu et al., 2014). In contrast, fluopyram did not inhibit complex II activities in goldfish mitochondria at the tested concentrations, suggesting that its IC50 (inhibitory concentration 50) exceeds 10  $\mu\text{M}$ , which is supported by literature reporting an IC50 of 160  $\mu\text{M}$  for fluopyram in human cells (Bénit et al., 2019).

We also assessed the effects of SDHI *in vivo* on fish complex II activities. In this experiment, we exposed goldfish for 4 days, extracted their mitochondria and tested the effects of SDHI on their mitochondrial complex II activities in muscle tissue, but we found no effects of any SDHI. This could be due to the lower concentrations used in the *in vivo* experiment compared to the *in vitro* experiment. Indeed, we chose lower exposure values to avoid fish mortality (994 and 12.1  $\mu\text{g.L}^{-1}$  for the highest concentrations of boscalid and bixafen, respectively), which are below the LOEC (lowest observed effect concentration) determined *in vitro*. Previous *in vivo* studies showing a reduction in complex II activity used either higher concentrations than ours (e.g., 4 days of boscalid exposure at 3.5  $\text{mg.L}^{-1}$  in zebrafish) (Huang et al., 2022), or longer exposure times (e.g., 28 days of boscalid at 0.1 and 1  $\text{mg.L}^{-1}$ ) (Qian et al., 2019b). It is thus possible that at the chosen concentrations tested in our study, boscalid and bixafen had no observable deleterious effect on

complex II *in vivo*. Additionally, exposure via water may have resulted in a lower reaching of target sites in fish mitochondria *in vivo*, potentially due to absorption mechanisms and to biotransformation processes. Another possibility is that our assay may not be entirely suitable for measuring *in vivo* activity, as complex II inhibition might be lost during the mitochondrial isolation process, which involves freezing and thawing steps.

Interestingly, fluopyram had no effects on complex II mitochondrial activities both *in vivo* and *in vitro*, which suggests that it has weaker SDHI activity in fish compared to its target species, despite important effects on fish physiology and behaviour.

##### 4.2. SDHI impact on fish physiology and behaviour

In the present study, boscalid and bixafen had little effects on fish behaviour. Bixafen only increased sociability (decreased ANDD) at the C2 concentration (1.7  $\mu\text{g.L}^{-1}$ ). Additionally, boscalid decreased AChE activity at C2 (113  $\mu\text{g.L}^{-1}$ ) but not at the higher C3 concentration (994  $\mu\text{g.L}^{-1}$ ). Interestingly, a decrease in AChE activity was observed in zebrafish larvae at concentrations comparable to our C3, but with a longer exposure time (8 days) (Qian et al., 2021). Unlike our findings showing no effect of boscalid-induced AChE inhibition on fish behaviour, this inhibition of AChE activity in zebrafish larvae was associated with hypoactivity. This may indicate a difference in sensitivity to boscalid between larvae and adults.

In contrast, fluopyram was the molecule with the greatest impact on fish behaviour and physiology, showing effects across all tested concentrations, including at environmentally relevant levels. Indeed, fish exposed to fluopyram showed an enhanced feeding behaviour at C2 and C3 (29 and 321  $\mu\text{g.L}^{-1}$ , respectively), as well as hyperactivity (increased time in movement at C2 and a higher number of crossed zones at C1 (3.5  $\mu\text{g.L}^{-1}$ ) and C2). They also displayed greater sociability (reduction of the distance between fish at C3). These results are consistent with a previous study in medakas exposed to chlorpyrifos pesticide (Khalil et al., 2013) and suggests increased anxiety (Collier et al., 2017). However, our behavioural results differ from most previous studies showing that various SDHI molecules (i.e. bixafen, boscalid, fluxapyroxad, penthiopyrad and thifluzamide) decrease activity in zebrafish larvae (Brenet et al., 2021; Qian et al., 2019a, 2021; Wang et al., 2020; Yang et al., 2021; Yu et al., 2022). Interestingly, the only previous study performed on adult zebrafish presents a different picture. After 21 days of exposure to 1  $\text{mg.L}^{-1}$  of boscalid, adult zebrafish had increased activity (Qian et al., 2021). This suggests that the impact of SDHI on fish behaviour might vary depending on life stage. While larvae tend to show SDHI-induced hypoactivity, adults might display hyperactivity due to different mechanisms of neurotoxicity, with contrasting effects for fish survival and fitness in the wild. In all cases, the observed increase in activity, sociability and feeding behaviours could have important effects on fish fitness, for instance through enhanced predation risk that could increase fish mortality in their natural environment.

Different underpinning mechanisms might account for these observed behavioural changes. Endocrine disruption, neurological dysfunction and/or metabolic disruption can all affect feeding, activity and sociability (Scott and Sloman, 2004). In the present study, results show decreased AChE activity in the muscles of fluopyram-exposed fish, thus supporting the neurotoxic impact hypothesis. Indeed, AChE hydrolyses and inactivates acetylcholine in the neuromuscular junction, thereby regulating acetylcholine concentration (Soreq and Seidman, 2001). When AChE is inhibited, acetylcholine levels increase, leading to the overstimulation of nicotinic and muscarinic receptor. This could induce higher muscle activity and movement (Könemann et al., 2022).

In addition, changes in muscle activity, locomotion and feeding are likely costly and are thus expected to induce changes in energy reserves. In contrast, fluopyram increased lipid content in muscle compared to control. These changes in lipid storage in muscle rather points towards SDHI-induced changes in lipid metabolism caused by exposure to



fluopyram. Such increase in lipid storage have been observed in previous studies in fish under other stressors (Gandar et al., 2017; Hurst et al., 2019), and the SDHI pesticides tend to cause changes in lipid metabolism, as has already been observed in *D. rerio* with boscalid and penthiopyrad (Qian et al., 2019b; Wang et al., 2024; Yang et al., 2018). Interestingly, lipid accumulation has also been reported in various species (both aquatic and terrestrial) exposed to other PFAS than fluopyram, such as perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) (Fragki et al., 2021; Lee et al., 2020; Lin et al., 2022). Thus, it would be interesting to consider whether the different behaviour on lipid metabolism of fluopyram might be linked to its fluorocarbon tail.

Fluopyram may also act as an endocrine disruptor. Indeed, previous studies showed an impact of thifluzamide on zebrafish leptin levels, the hormone that regulates appetite (Yang et al., 2019), and on the hypothalamus-pituitary-gonad-liver (HPGL) axis after exposure to boscalid, flutolanil and penthiopyrad (Qian et al., 2023, 2020; Teng et al., 2020). These findings suggest that SDHI may have neuronal and endocrine toxicity that translate into cascading effects at the whole organism level on key behavioural traits. Indeed, the behavioural effects observed can have consequences on foraging and antipredator behaviours, with important consequences for fish survival and fitness (Amiard-Triquet, 2009). Whatever the underlying mechanisms, this study thus raises concerns about the deleterious effects of fluopyram on aquatic species and encourages further research on the effects of SDHI on non-target organisms.

## 5. Conclusion

Taken together, our results highlight several impacts of SDHI pesticides, especially fluopyram on fish at different scales: molecular, cellular and individual. As expected, bixafen and boscalid had significant *in vitro* effects and inhibited the complex II (or SDH complex) activity in fish mitochondria, with a particularly strong effect on SQR activity. However, they had few effects on fish behaviour and physiological markers at the whole organism level.

In contrast, fluopyram had severe deleterious effects on fish behaviour and physiology at environmentally realistic concentrations. More specifically, fluopyram induced increased fish feeding behaviour, activity and sociability, alongside a reduction in AChE activity and an increase in lipid content in fish muscle. Interestingly, these changes were not associated with an inhibition of SDH complex activities both *in vitro* and *in vivo*, suggesting potential off-target effects. These effects may be explained by neurotoxic effects, metabolic and/or endocrine disruptions, that need to be investigated in future studies. Whatever the underpinning mechanisms, the behavioural and physiological effects observed at the whole organism level could have strong impacts on fish survival in their natural environment. This study highlights that recent SDHI molecules that are widely used in agriculture may have underestimated adverse effects for wildlife, including in aquatic systems, and calls for further research on pesticide effects on biodiversity across biological scales.

## Ethical standards

The experimental procedures were carried out within the framework of establishment approval for vertebrate experimentation n° B31113002 and were approved by the ethics committee n° 073 (authorization n° #36124–2023021011201494V7), in accordance with French and European legislation on animal experimentation (European directive 2010/63/EU).

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## CRedit authorship contribution statement

**Aurianne Hagimont:** Investigation. **Pascal Laffaille:** Project administration. **Bénédicte Lalot:** Investigation. **Anthony Lemarié:** Writing – review & editing, Resources, Methodology. **Lisa Jacquin:** Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Florian Chapeau:** Writing – original draft, Visualization, Investigation, Formal analysis. **Jean-Marc Bonmatin:** Writing – review & editing, Resources, Methodology. **Myriam Cousseau:** Investigation. **Séverine Jean:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Camille Pasquet:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **Laurence Huc:** Funding acquisition. **Lucie Bouly:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Formal analysis.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2024.117400](https://doi.org/10.1016/j.ecoenv.2024.117400).

## Data availability

Data will be made available on request.

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