Bixafen, a succinate dehydrogenase inhibitor fungicide, causes microcephaly and motor neuron axon defects during development

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Running title: Neurodevelopmental defects in bixafen-exposed embryos

Abstract

Succinate dehydrogenase inhibitors (SDHIs), the most widely used fungicides in agriculture today, act by blocking succinate dehydrogenase (SDH), an essential and evolutionarily conserved component of mitochondrial respiratory chain. Recent results showed that several SDHIs used as fungicides not only inhibit the SDH activity of target fungi but also block this activity in human cells in *in vitro* models, revealing a lack of specificity and thus a possible health risk for exposed organisms, including humans. Despite the frequent detection of SDHIs in the environment and on harvested products and their increasing use in modern agriculture, their potential toxic effects *in vivo*, especially on neurodevelopment, are still under-evaluated. Here we assessed the neurotoxicity of bixafen, one of the latest-generation SDHIs, which had never been tested during neurodevelopment. For this purpose, we used a well-known vertebrate model for toxicity testing, namely zebrafish transparent embryos, and live imaging using transgenic lines labelling the brain and spinal cord. Here we show that bixafen causes microcephaly and defects on motor neuron axon outgrowth and their branching during development. Our findings show that the central nervous system is highly sensitive to bixafen, thus demonstrating *in vivo* that bixafen is neurotoxic in vertebrates and causes neurodevelopmental defects. This work adds to our knowledge of the toxic effect of SDHIs on neurodevelopment and may help us take appropriate precautions to ensure protection against the neurotoxicity of these substances.

Keywords: Succinate dehydrogenase inhibitors (SDHIs), Fungicide, Zebrafish, Neurodevelopment, *In vivo* imaging

41 **Introduction**

42 Bixafen, a methyl-pyrazole carboxamide, is a fungicide widely used on cereal and
43 rapeseed crops. It was initially approved and marketed in 2011 by Bayer (Ravichandra
44 2018). Its high efficiency and rapid penetration resulted in a significant rise in usage in
45 Europe and the US. Currently, nearly 30 different products are sold on the French market
46 alone that contain bixafen as sole active substance or mixed with other fungicides. Bixafen
47 belongs to the succinate dehydrogenase inhibitor (SDHI) family, the fungicides most
48 widely used in agriculture to fight a broad range of fungal diseases (Lalève et al. 2014;
49 Zhang et al. 2019). Bixafen, which is derived from carboxin and categorized as a latest-
50 generation SDHIs, acts through inhibition of mitochondrial respiration chain complex II,
51 also known as succinate dehydrogenase (SDH). However, the inhibitory effect of SDHIs is
52 not restricted to fungi: they also block the mitochondrial respiration chain in many other
53 species, including human cells (Bénit et al. 2019). Additionally, the latest-generation
54 SDHIs have been shown to also inhibit mitochondrial respiration complex III (Bénit et al.
55 2019), suggesting more critical effects of these new fungicides. SDH activity is essential
56 for proper mitochondrial respiration and metabolism, and defects in its activity impair
57 the cellular metabolome and functions (Bénit et al. 2014). Many human diseases are
58 related to SDH deficiency, including neurodegenerative disorders, such as Leigh
59 syndrome, an early-onset neurodegenerative disease characterized by developmental
60 delay, ataxia and seizures (Birch-Machin et al. 2000; Bourgeron 2015; Van Coster et al.
61 2003; Finsterer 2008; Horváth et al. 2006; Parfait et al. 2000), and cancers, such as
62 familial paraganglioma syndrome, infantile leukoencephalopathy, and neuroblastoma
63 (Ghezzi et al. 2009; Martin et al. 2007; Perry et al. 2006; Ricketts et al. 2009; Stratakis and
64 Carney 2009; Timmers et al. 2009). The Norwegian scientific committee for food safety
65 (VKM) has assessed the health and environmental risk of Aviator Xpro EC225, a fungicide

containing bixafen, and concluded that the effects of bixafen in animals should be considered relevant for humans (VKM Report 2014). Also, M44, a bixafen metabolite, which has the potential to contaminate groundwater, causes abnormalities in the rabbit foetus, suggesting that similar effects in humans cannot be excluded (VKM Report 2015). However, despite the increasing use of SDHIs in modern agriculture and their frequent detection in the environment and on harvested products (Abad-Fuentes et al. 2015; Añasco, Koyama, and Uno 2010; Tanabe and Kawata 2009; Tsuda et al. 2009; Vu et al. 2016), the potential toxic effects of these substances, especially on neurodevelopment, have so far been poorly evaluated.

To fill this gap and assess the neurotoxicity of these latest-generation SDHI fungicides *in vivo,* we tested the potential toxic effect of bixafen *in vivo* on zebrafish embryos. We observed that embryos exposed to low or medium bixafen concentrations, LOAEL and LC30, developed a series of defects, including microcephaly and disorganized motor neuron axons and branches. This study demonstrated the toxicity of bixafen on CNS development and gives insight into the environmental risk of this SDHI on the nervous system in vertebrates.

83 **Results**

84 **1. Toxicity of bixafen *in vivo* in zebrafish embryos**

85 To assess the potential toxicity of bixafen, an SDHI belonging to the pyrazole group of
86 fungicides, zebrafish embryos were exposed to increasing concentrations of bixafen
87 ranging from 0.1 μM to 4 μM, from 6 hours post-fertilization (hpf) onward up to 96 hpf
88 (supplementary Figure 1A). As no differences in survival rates or embryo morphology
89 could be detected between the 0.1% DMSO and fish water control groups (data not
90 shown), embryos incubated in 0.1% DMSO were used as controls. Exposure of zebrafish
91 embryos to 0.5 μM bixafen and above induced gross morphological defects and
92 significantly increased mortality, compared with that seen in controls (supplementary
93 Figure 1). Moreover, we observed a dose-dependent effect, with increasing
94 concentrations of bixafen inducing increased mortality rates and increased phenotypic
95 defects in surviving embryos (Supplementary Figure $1B_1$-K).

96 Results indicated that the median lethal concentration of bixafen was 2.7 μM for wild-
97 type zebrafish embryos (AB line) after 96 hours of incubation, i.e. 96 hpf LC50, and closely
98 similar values were observed with the two other zebrafish lines used in this study, the
99 transgenic Tg[*Olig2:eGFP*] and Tg [*HuC:RFP*] (Supplementary Figure 1K).

100 To investigate the potential toxicity of bixafen, and particularly the effect of this SDHI
101 on the morphology of the brain and spinal cord, we chose 0.2 μM, the lowest-observed-
102 adverse-effect-level (LOAEL), and 0.5 μM, the 96 hpf LC30, two concentrations that induce
103 mild and moderate phenotypic defects in 96 hpf embryos, respectively, except for small
104 heart oedema in embryos exposed to 0.2 μM bixafen (Figure 1C), and large heart oedema
105 and curved tail in embryos exposed to 0.5 μM bixafen (Figure 1D). We next carefully
106 examined the morphological parameters of embryos exposed to 0.2 and 0.5 μM bixafen,

107 namely body length, head size, eye diameter and eye-otolith distance. Results indicated
108 that following exposure to either 0.2 or 0.5 μM bixafen, surviving embryos show reduced
109 body length and eye diameter, and decreased head size and eye-otolith distance,
110 compared with that observed in age-matched controls (Figure 1E-I).

111 **2. Brain defects in embryos exposed to bixafen**

112 Because several investigations have reported that exposure to SDHIs may induce
113 neurotoxicity (Wang et al. 2020; Yao et al. 2018), we took advantage of the
114 Tg[*elavl3:Gal4/5xUAS:RFP*] double transgenic line (Akerboom et al. 2012; Asakawa et al.
115 2008), hereafter referred to as Tg[*HuC:G/U:RFP*], a line in which all post-mitotic brain
116 neurons are labelled by the fluorescent RFP protein, to precisely examine the morphology
117 of the brain of 36 and 66 hpf embryos, following exposure to 0.2 and 0.5 μM bixafen. Live
118 imaging of the brain of 36 and 66 hpf embryos, clearly indicated that exposure to both 0.2
119 and 0.5 μM bixafen, induced smaller brain size (Figure 1J-O). Precise quantification of
120 brain volumes (Imaris software, Bitplane), indicated that 36 hpf embryos exposed to 0.2
121 ($3.07 \pm 0.13 \times 10^6$ μm^3) or 0.5 μM bixafen ($1.60 \pm 0.13 \times 10^6$ μm^3), displayed a significantly
122 smaller brain compared with that seen in age-matched controls ($3.57 \pm 0.07 \times 10^6$ μm^3)
123 (Figure 1P). Similarly, 66 hpf embryos exposed to 0.2 ($7.45 \pm 0.24 \times 10^6$ μm^3) or 0.5 μM
124 bixafen ($3.64 \pm 0.15 \times 10^6$ μm^3), also displayed small brains compared to age-matched
125 controls ($8.51 \pm 0.25 \times 10^6$ μm^3) (Figure 1Q). Our data thus showed that exposure of
126 zebrafish embryos to low-dose bixafen corresponding to LOAEL induced microcephaly, a
127 phenotype exacerbated in individuals incubated in 0.5 μM bixafen.

128 **3. Impaired mobility and spinal motor neuron axon outgrowth defects in**
129 **embryos exposed to bixafen**

130 The small size of the brain observed in embryos exposed to bixafen suggests a
131 neurotoxic effect of this SDHI. To further investigate this issue and precisely assess the
132 consequences of bixafen exposure on brain functioning, we studied and compared the
133 locomotor activity of 0.2 and 0.5 μM bixafen-exposed and control embryos by measuring
134 the distance swum by these individuals over a 25-min period, using a ZebraBox tracking
135 automaton (ViewPoint®). Results indicated that embryos treated with 0.2 or 0.5 μM
136 bixafen, displayed reduced mobility, as shown by the distance traveled over a 25 minute
137 period 77.3 ± 5.7 mm (N = 4, n = 72) and 52.7 ± 4.4 mm (N = 4, n = 77), respectively,
138 compared with the distance travelled by controls (97.5 ± 4.9 mm, N = 4, n = 85) (Figure
139 2B-C). Interestingly, the hypoactive behavior of embryos exposed to 0.2 μM bixafen was
140 markedly worsened in those exposed to 0.5 μM, which were almost motionless.

141 Because bixafen exposure induces brain defects, we next determined whether the
142 behavioral deficits observed in bixafen-exposed embryos was a consequence of defects in
143 spinal cord motor neurons or their axons. To address this issue, we next analyzed the
144 morphology of the spinal cord in 36 and 66 hpf embryos exposed to 0.2 and 0.5 μM
145 bixafen, using the Tg[*Olig2:eGFP*] transgenic line (Shin et al. 2003), in which all motor
146 neuron progenitors and their axons are labelled by eGFP. In particular, we focused on
147 caudal primary motor neurons (CaPMN) and their axons, which are the most accessible.

148 Live imaging indicated that in 36 hpf controls, primary motor neuron axons extended
149 ventrally to contact target muscles, forming a highly regular pattern (Figure 2D). By
150 contrast, motor neuron axons were markedly shortened in 36 hpf embryos treated with
151 0.2 bixafen (Figure 2E), a phenotype markedly exacerbated in age-matched embryos
152 exposed to 0.5 μM, bixafen (Figure 2F). Three-dimensional reconstruction of CaPMN and
153 their axons in 66 hpf control embryos revealed a highly stereotyped pattern with axons
154 extending ventrally and branches projecting dorsally and laterally along the dorsal

155 myoseptum and forming a regular pattern in the space between the notochord and the
156 myotome (Figure 2G, G'). By contrast, following exposure to 0.2 μM bixafen, 66 hpf
157 embryos displayed significantly shorter axons (Figure 2H, H'), a phenotype significantly
158 worsened in embryos exposed to 0.5 μM bixafen (Figure 2I, I'), which also showed a
159 marked disorganization of motor neuron axons and their branches, which extended in all
160 directions.
161 Measurements of the length of CaPMN axons confirmed that bixafen exposure induced a
162 marked shortening of these axons. Whereas in control embryos, the length of CaPMN
163 axons was 167.9 ± 2.4 μm and 368.3 ± 9.3 μm, at 36 and 66 hpf, respectively, in embryos
164 exposed to 0.2 μM, these values were reduced to 148.6 ± 3.1 μm at 36 hpf and 296.4 ± 9.0
165 μm at 66 hpf. Moreover, these defects were exacerbated in embryos exposed to 0.5 μM
166 bixafen, with a mean CaPMN axon length of 61.8 ± 3.7 μm at 36 hpf and 223.7 ± 9.6 μm at
167 66 hpf (Figure 2J, K). Thus bixafen is neurotoxic and impairs the outgrowth and patterning
168 of spinal motor axons and their branches.

169 **Discussion**

170 It is well-known that the developing CNS in vertebrates is especially sensitive and
171 vulnerable to toxic chemicals, including phytosanitary agents, which are increasingly
172 present in the environment. More specifically, in recent years, bixafen, a latest-generation
173 succinate dehydrogenase inhibitor (SDHI), has become one of the most widely-used
174 fungicides worldwide. However, the toxicity of bixafen has been under-appraised *in vivo*
175 in vertebrate models so far, and especially its neurotoxicity for the developing CNS, an
176 especially serious issue because of the complete lack of species-specificity of this latest-
177 generation SDHI (Bénit et al. 2019). In the present work, we used zebrafish embryos as
178 an *in vivo* model and demonstrate that bixafen exposure during embryogenesis induces a
179 series of CNS defects.

180 In close agreement with previous investigations (Li et al. 2020; Wu et al. 2018), we first
181 observed that zebrafish embryos exposed to bixafen showed clear developmental delays,
182 as shown by morphological examination of embryos exposed to 0.5 μM bixafen and above,
183 but also by a decrease in the eye-otolith distance and hypopigmentation, both observed
184 in embryos incubated in 0.2 and 0.5 μM bixafen, corresponding to LOAEL and 96hpf LC30,
185 respectively. Moreover, at the brain level, we observed that these two same
186 concentrations induced brain defects and microcephaly, an observation in close
187 agreement with the markedly reduced expression of the neuronal *neuroD* gene observed
188 in zebrafish embryos treated with bixafen (Li et al. 2020). Microcephaly has been
189 repeatedly observed in embryos exposed to many pesticides, including Maxim® XL
190 (Svartz, Meijide, and Pérez Coll 2016) and glyphosate (Paganelli et al. 2010), emphasizing
191 the high vulnerability of the brain to these chemicals, and our study shows that SDHI
192 bixafen also induces microcephaly, even at low doses.

193 In addition, using an automaton that quantifies the speed and distance traveled by
194 embryos, we also observed that embryos exposed to 0.2 and 0.5 M bixafen displayed
195 reduced motility and almost complete paralysis, respectively, at 96 hpf. The quantification
196 of swimming behavior clearly showed motor deficits in embryos exposed to 0.2 μM of bixafen
197 which worsens in embryos exposed to 0.5 μM of bixafen (Figure 2C), that likely results of
198 neuron abnormalities, not developmental delay. As it is known that the motor neuron is a
199 major cell type regulating swimming behavior in zebrafish during early life (Brustein et
200 al. 2003), the motor defects seen in bixafen-exposed embryos prompted us to investigate
201 the differentiation of motor neuron axons and branches in embryos exposed to 0.2 and
202 0.5 μM bixafen. In control embryos, motor neurons extend their axons in a highly
203 stereotyped manner during development. In particular, caudal primary motor neuron
204 (CaPMN) axons exit the spinal cord and extend toward the ventral muscle. Interestingly,
205 in embryos exposed to bixafen, we observed a reduction in axon outgrowth and defects
206 in their branches, even in those exposed to 0.2 μM bixafen corresponding to LOAEL,
207 indicating that the development of motor neuron axons is highly sensitive to bixafen.
208 Although the toxic effects of bixafen on the CNS are probably due at least in part to the
209 developmental delay, the disorganization of motor neuron axons and branches clearly
210 reveals a neurotoxicity of bixafen in zebrafish embryos.

211 Bixafen is a methyl-pyrazole, one of the SDHI fungicides used to manage plant diseases
212 and inhibit the respiration of pathogenic fungi by blocking succinate complex II in the
213 mitochondrial respiratory chain. SDH is an enzyme involved in both oxidative
214 phosphorylation and the tricarboxylic acid cycle, two processes that generate energy. This
215 enzyme has been shown to be irreplaceable in mitochondrial and cell metabolism and also
216 to be highly conserved among all fungi, plants and animal species (Rehfus et al. 2016;
217 Veloukas, Markoglou, and Karaoglanidis 2013; Yamashita and Fraaije 2018). Any adverse

218 change or inhibition of SDH activity can therefore lead to many diseases, including those
219 that have been linked to mitochondrial dysfunction (Birch-Machin et al. 2000; Bourgeron
220 2015; Van Coster et al. 2003; Finsterer 2008; Horváth et al. 2006; Parfait et al. 2000).
221 Several epidemiological studies published in the last two decades suggest harmful
222 effects of pesticides on human health (Merhi et al. 2007; Weichenthal, Moase, and Chan
223 2010). Pesticide poisoning is a serious health problem that disproportionately affects
224 infants and children (Rauh et al. 2012). Pesticides are known to cause millions of acute
225 poisoning cases per year (Bertolote et al. 2006). Human exposure to pesticides can occur
226 environmentally, through consumption in food and water (van den Berg et al. 2012).
227 Bixafen is considered to be highly persistent in the environment (EFSA 2012). The half-
228 lives of bixafen were 105 days in loam, 316 days in sandy loam, 1235 days in slit loam,
229 and 82 days in water (EFSA 2012). A number of studies show that prenatal and early
230 childhood exposure to some pesticides is associated with neurodevelopmental and
231 cognitive disorders (Muñoz-Quezada et al. 2013; Ross et al. 2013). Aside from SDH
232 inhibition, very little is known about the consequences of SDHI exposure on human
233 health, and it is not known how bixafen affects the development of the CNS. As it is the
234 case for other SDHIs, the toxic effect of bixafen observed in this study may be linked to the
235 basic disruption of mitochondrial respiration chain complex II activity and subsequent
236 accumulation of reactive oxygen species and oxidative DNA damage. Results showed that
237 a high concentration of bixafen induces reactive oxygen species production and
238 subsequent DNA damage in human cell lines (Graillot et al. 2012). However, a recent study
239 also demonstrated that bixafen exposure did not cause oxidative stress in zebrafish (Li et
240 al. 2020), suggesting that induction of oxidative stress may not be the main mechanism
241 underlying bixafen-induced neurotoxicity in zebrafish embryos. Thus the brain and spinal
242 cord defects observed in embryos exposed to low-medium bixafen concentrations may be

243 associated with the metabolic abnormalities reported in SDHI-exposed animals (Graillot
244 et al. 2012; Qian et al. 2018; Wu et al. 2018; Yang et al. 2018).
245 In summary, our study provides new evidence of bixafen toxicity on
246 neurodevelopment in a vertebrate model, and may help us take appropriate precautions
247 to ensure protection against the neurotoxic effects of these substances.

248 **Materials and Methods**

249 **1. Ethics statement**

250 All the animal experiments described in the present study were conducted at the French
251 National Institute of Health and Medical Research (INSERM) UMR 1141 in Paris in
252 accordance with European Union guidelines for the handling of laboratory animals
253 (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). They were
254 approved by the Direction Départementale de la Protection des Populations de Paris and
255 the French Animal Ethics Committee under reference No. 2012-15/676-0069

256 **2. Zebrafish lines and maintenance**

257 Zebrafish were maintained at 26.5 °C in 14 h light and 10 h dark cycles. Embryos were
258 collected by natural spawning, and 0.003% 1-phenyl-2-thiourea (PTU) was added at 1 dpf
259 (day post-fertilization) for embryos which were imaged *in vivo* to avoid pigmentation. The
260 following transgenic lines were used: Tg[*elavl3:Gal4*]^{zf349} (Akerboom et al. 2012),
261 T[*5xUAS:RFP*]^{nkuasrfp1a} (Asakawa et al. 2008), Tg[*Olig2:eGFP*] (Shin et al. 2003).

262 **3. Chemicals**

263 Dimethyl sulfoxide (DMSO) hybrid-max sterile (Sigma, D2650) was diluted in fish water
264 to a final concentration of 0.1% (v/v). Bixafen, (*N*-(3′,4′-dichloro-5-fluorobiphenyl-2-yl)-
265 3-(difluoromethyl)-1-methylpyrazole-4-carboxamide) (Bixafen, (Sigma-Aldrich, St.
266 Louis, MO, USA, code: 32581, CAS-No: 581809-46-3, Lot# BCBS9732V, purity > 98%).)
267 was dissolved at a stock concentration of 400 mM in pure DMSO (100%) and conserved
268 at −20 °C in aliquots until use.

269 **4. Toxicity assay**

270 Wild-type (AB line) and transgenic lines, Tg[*HuC:G/U:RFP*] or Tg[*Olig2:eGFP*] embryos
271 were collected after spawning and washed twice with clean fish water. Fertilized and
272 normal 6 hpf embryos were selected, distributed randomly in 6-well microplates, 30
273 embryos per well, following OECD TG 236 fish acute toxicity test (OECD 2013). Each well was
274 filled with 6 mL of solution for each condition, with 12 conditions in all: fish water, 0.1%
275 DMSO, 0.1 µM, 0.2 µM, 0.3 µM, 0.5 µM, 1 µM, 1.5 µM, 2 µM, 2.5 µM, 3 µM and 4 µM bixafen
276 in 0.1% DMSO. Each day, dead embryos were selected under OECD guidelines
277 (coagulation, no heartbeat or no somite) and discarded.

278 **5. Locomotor activity**

279 96 hpf larvae were distributed individually in a 96 well plate in 200 µl of fish water. The
280 plate was then placed in the recording chamber of the zebrabox system for 30 min
281 habituation before 25 min recording of larvae locomotion (Brenet et al. 2019). The results
282 indicate the distance swum by the larvae during the recording.

283 **6. Embryo imaging**

284 36 hpf or 66 hpf embryos were anesthetized with 112 µg/mL 3-aminobenzoic acid ethyl
285 ester (tricaine, Sigma), immobilized in 1% low melting-point agarose in the center of a
286 35 mm glass-bottomed dish (Corning), and covered with fish water containing 112 µg/mL
287 tricaine. For embryo morphology analysis, bright field images were captured using a
288 stereomicroscope (Zeiss). Live imaging of transgenic lines Tg[*HuC:G/U:RFP*] and
289 Tg[*Olig2:eGFP*] was done using a Leica SP8 confocal scanning laser microscope equipped
290 with a Leica 20x/0.75 multi-immersion objective.

291 **7. Image analysis**

292 Body length, head size, eye diameter and eye-otolith distance measurements from bright
293 field embryo images were made using the ruler tool in ImageJ. Brain volume and spinal
294 neuron CaPMN axon length three-dimensional reconstructions and quantifications were
295 analyzed using Imaris Measurement Pro (Bitplane Inc.).

296 **8. Statistics**

297 All statistics were obtained on Prism5 (GraphPad) and assessed using a one-way ANOVA
298 test followed by a Tukey post-test. All data are represented as means ± SEM.

299 **Author Contributions:**
300 A.B. and R.H.A. performed the experiments and the analysis, designed the figures, and
301 wrote the original draft. N.S.Y. supervised the project and wrote the manuscript.

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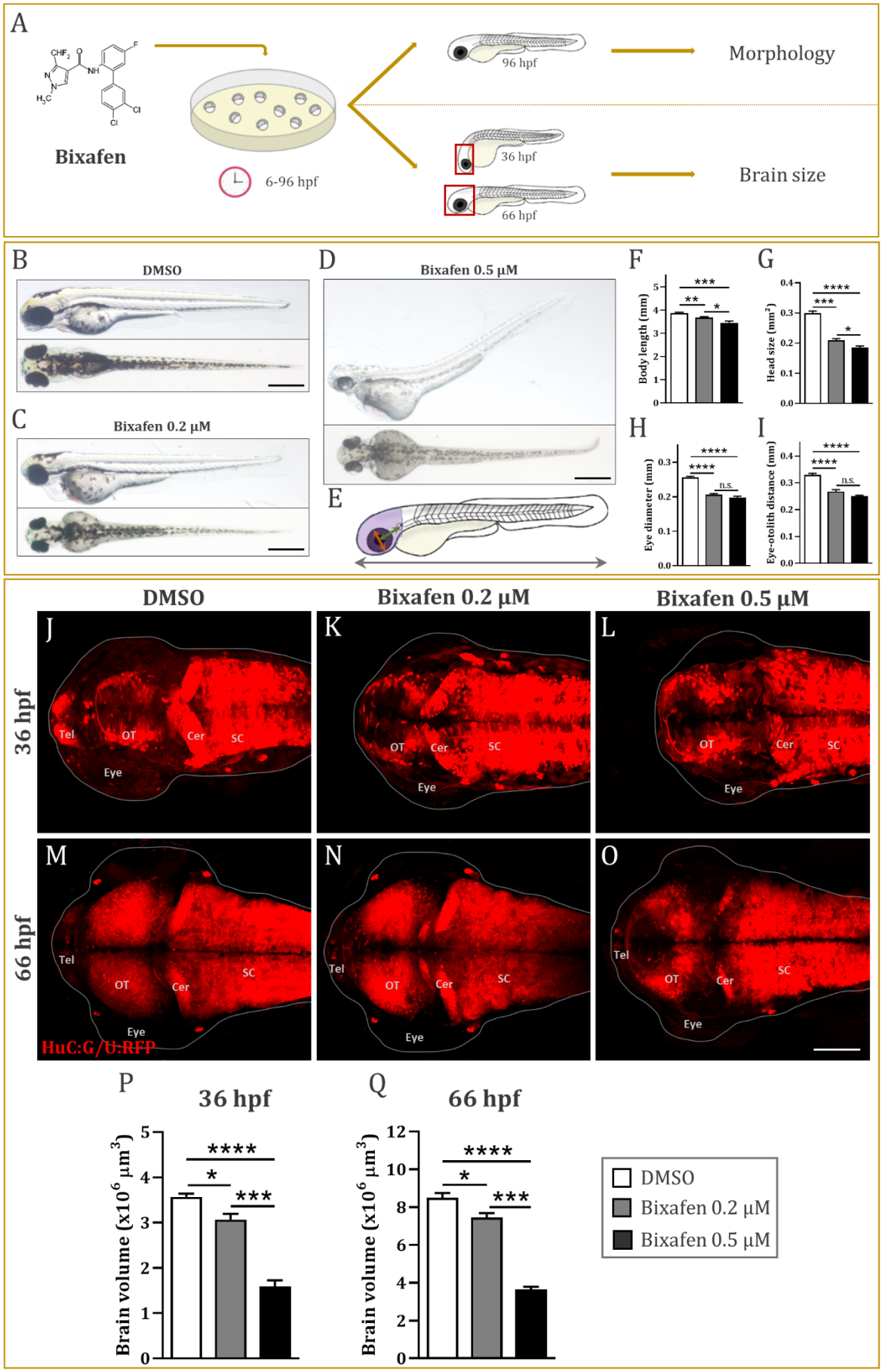
309

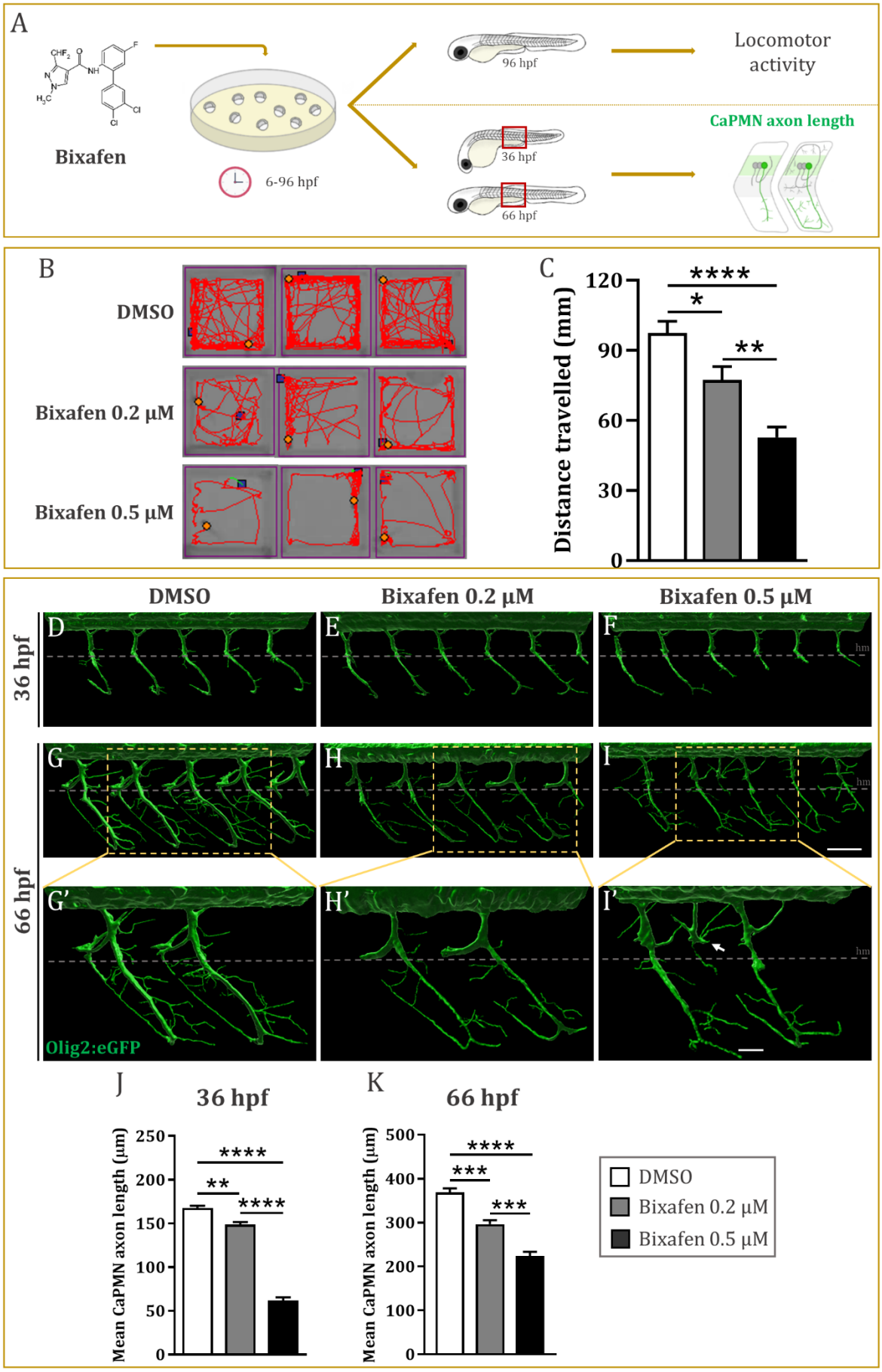
310 **Conflicts of Interest:** The authors declare that the research was conducted in the absence
311 of any commercial or financial relationships that could be construed as a potential conflict
312 of interest.


A
Bixafen
6-96 hpf
96 hpf
Morphology
36 hpf
66 hpf
Brain size
B
DMSO
C
Bixafen 0.2 µM
D
Bixafen 0.5 µM
E
F
Body length (mm)
G
Head size (mm²)
H
Eye diameter (mm)
I
Eye-otolith distance (mm)
n.s.
DMSO
Bixafen 0.2 µM
Bixafen 0.5 µM
36 hpf
66 hpf
J
K
L
M
N
O
Tel
OT
Cer
SC
Eye
HuC:G/U:RFP
P
36 hpf
Q
66 hpf
Brain volume (x10⁶ µm³)
DMSO
Bixafen 0.2 µM
Bixafen 0.5 µM

313

314 **Figure 1: Bixafen exposure causes microcephaly.**

315 (**A**) Experimental setup used to analyze the body morphological parameters and brain
316 size of zebrafish embryos exposed to 0.2 or 0.5 μM bixafen, from 6 hpf onward up to 36
317 or 66 hpf (brain analysis), or 96 hpf (morphological analysis). (**B-D**) Lateral and dorsal
318 views of 96 hpf larvae treated with 0.1% DMSO (**B**) or 0.2 μM (**C**) or 0.5 μM bixafen (**D**).
319 (**E**) Scheme depicting the different morphological parameters measured: body length
320 (black double-arrow), head size (purple area), eye diameter (red double-arrow) and eye-
321 otolith distance (green double-arrow). (**F-I**) Body length (**F**), head size (**G**), eye diameter
322 (**H**) and eye-otolith distance (**I**), of 96 hpf larvae exposed to 0.1% DMSO (n = 14), or 0.2
323 μM (n = 15) or 0.5 μM (n = 12) bixafen. (**J-O**) Dorsal views of the brain of 36 (**J-L**) or 66
324 hpf transgenic Tg[*huC:G/U:RFP*] embryos (**M-O**), treated with 0.1 % DMSO (**J**, **M**), or 0.2
325 (**K**, **N**) or 0.5 μM bixafen (**L**, **O**), and showing the microcephaly of embryos exposed to
326 low/medium doses bixafen. (**P-Q**) Measurements of brain volume of 36 (**P**) or 66 hpf (**Q**)
327 embryos exposed to 0.1% DMSO (n_{36hpf} = 8; n_{66hpf} = 5), or 0.2 μM (n_{36hpf} = 6; n_{66hpf} = 5) or
328 0.5 μM bixafen (n_{36hpf} = 8; n_{66hpf} = 4), further illustrate the microcephaly of embryos
329 exposed to bixafen at 96 hpf LC30 and LOAEL concentrations. ****, $p < 0.0001$; ***, $p <$
330 0.001; **, $p < 0.01$; *, $p < 0.05$; n.s., non-significant. Scale bar: (**B-D**) = 0.5 mm; (**J-O**) = 100
331 μm. Abbreviations: Tel, telencephalon; OT, optic tectum; Cer, cerebellum; SC, spinal cord.


A
Bixafen
6-96 hpf
96 hpf
36 hpf
66 hpf
Locomotor activity
CaPMN axon length
B
DMSO
Bixafen 0.2 µM
Bixafen 0.5 µM
C
Distance travelled (mm)
120
90
60
30
0
****
*
**
DMSO
Bixafen 0.2 µM
Bixafen 0.5 µM
36 hpf
66 hpf
D
E
F
G
H
I
G'
H'
I'
Olig2:eGFP
J
36 hpf
Mean CaPMN axon length (µm)
250
200
150
100
50
0
****
**
****
K
66 hpf
500
400
300
200
100
0
****
***
***
DMSO
Bixafen 0.2 µM
Bixafen 0.5 µM

332

333 **Figure 2: Bixafen exposure induces motor deficit and motor neuron axon defects.**
334 (**A**) Experimental setup used to analyze the motor activity and patterning of caudal
335 primary motor neuron (CaPMN) axons and branches, in zebrafish embryos exposed to 0.2
336 or 0.5 μM bixafen, from 6 hpf onward up to 36 or 66 hpf (analysis of CaPMN axon
337 morphology), or 96 hpf (analysis of motor activity). (**B**) Tracking plots illustrating the
338 distance traveled by 96 hpf larvae treated with 0.1% DMSO (n = 85), or 0.2 (n = 72) or 0.5
339 μM bixafen (n = 77), during a 25 min time period. (**C**) Quantification of the distance
340 traveled by 96 hpf larvae exposed to 0.1% DMSO, or 0.2 or 0.5 μM bixafen, during a 25
341 min time period. (**D-I**) Three-dimensional reconstruction of the organization of CaPMN
342 axons and branches in 36 (**D-F**) or 66 hpf transgenic Tg[Olig2:eGFP] embryos (**G-I'),**
343 exposed to 0.1% DMSO (**D**, **G**), 0.2 (**E**, **H**) or 0.5 μM bixafen (**F**, **I**), showing the
344 disorganization of CaPMN axons and branches, following exposure to 0.2 and, even more,
345 0.5 μM bixafen (white arrow). (**G'-I'**) Higher magnifications of the morphology of CaPMN
346 axons and branches, in embryos exposed to 0.1% DMSO (**G'**), or 0.2 (**H'**) or 0.5 μM bixafen
347 (**I'**). (**J**, **K**) Quantification of the length of the primary CaPMN axons in 36 (**J**) and 66 hpf
348 embryos (**K**), treated with DMSO (n_{36hpf} = 13; n_{66hpf} = 7), or 0.2 (n_{36hpf} = 8; n_{66hpf} = 6) or 0.5
349 μM bixafen (n_{36hpf}= 22; n_{66hpf} = 9), showing significantly shorter axons following exposure
350 to bixafen at LOAEL (0.2 μM) and, even more, 96hpf LC30 (0.5 μM). ****, $p < 0.0001$; ***,
351 $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. Scale bar: (**D-I**) = 100 μm; (**G'-I'**) = 50 μm.

352 **References**

353 Abad-Fuentes, Antonio, Eric Ceballos-Alcantarilla, Josep V. Mercader, Consuelo Agulló,
354 Antonio Abad-Somovilla, and Francesc A. Esteve-Turrillas. 2015. "Determination of
355 Succinate-Dehydrogenase-Inhibitor Fungicide Residues in Fruits and Vegetables by
356 Liquid Chromatography–Tandem Mass Spectrometry." *Analytical and Bioanalytical*
357 *Chemistry* 407(14).

358 Akerboom, J., T. W. Chen, T. J. Wardill, L. Tian, J. S. Marvin, S. Mutlu, N. C. Calderon, F.
359 Esposti, B. G. Borghuis, X. R. Sun, A. Gordus, M. B. Orger, R. Portugues, F. Engert, J. J.
360 Macklin, A. Filosa, A. Aggarwal, R. A. Kerr, R. Takagi, S. Kracun, E. Shigetomi, B. S.
361 Khakh, H. Baier, L. Lagnado, S. S. H. Wang, C. I. Bargmann, B. E. Kimmel, V.
362 Jayaraman, K. Svoboda, D. S. Kim, E. R. Schreiter, and L. L. Looger. 2012.
363 "Optimization of a GCaMP Calcium Indicator for Neural Activity Imaging." *Journal of*
364 *Neuroscience* 32(40):13819–40.

365 Añasco, Nathaniel C., Jiro Koyama, and Seiichi Uno. 2010. "Pesticide Residues in Coastal
366 Waters Affected by Rice Paddy Effluents Temporarily Stored in a Wastewater
367 Reservoir in Southern Japan." *Archives of Environmental Contamination and*
368 *Toxicology* 58(2):352–60.

369 Asakawa, Kazuhide, Maximiliano L. Suster, Kanta Mizusawa, Saori Nagayoshi, Tomoya
370 Kotani, Akihiro Urasaki, Yasuyuki Kishimoto, Masahiko Hibi, and Koichi Kawakami.
371 2008. "Genetic Dissection of Neural Circuits by Tol2 Transposon-Mediated Gal4
372 Gene and Enhancer Trapping in Zebrafish." *Proceedings of the National Academy of*
373 *Sciences of the United States of America* 105(4):1255–60.

374 Bénit, Paule, Agathe Kahn, Dominique Chretien, Sylvie Bortoli, Laurence Huc, Manuel
375 Schiff, Anne Paule Gimenez-Roqueplo, Judith Favier, Pierre Gressens, Malgorzata
376 Rak, and Pierre Rustin. 2019. "Evolutionarily Conserved Susceptibility of the

377 Mitochondrial Respiratory Chain to SDHI Pesticides and Its Consequence on the
378 Impact of SDHIs on Human Cultured Cells." *PLoS ONE* 14(11).
379 Bénit, Paule, Eric Letouzé, Malgorzata Rak, Laetitia Aubry, Nelly Burnichon, Judith
380 Favier, Anne Paule Gimenez-Roqueplo, and Pierre Rustin. 2014. "Unsuspected Task
381 for an Old Team: Succinate, Fumarate and Other Krebs Cycle Acids in Metabolic
382 Remodeling." *Biochimica et Biophysica Acta - Bioenergetics* 1837(8):1330–37.
383 van den Berg, F., A. Tiktak, G. B. M. Heuvelink, S. L. G. E. Burgers, D. J. Brus, F. de Vries, J.
384 Stolte, and J. G. Kroes. 2012. "Propagation of Uncertainties in Soil and Pesticide
385 Properties to Pesticide Leaching." *Journal of Environmental Quality* 41(1):253–61.
386 Bertolote, José M., A. Fleischmann, M. Eddleston, and D. Gunnell. 2006. "Deaths from
387 Pesticide Poisoning: A Global Response." *British Journal of Psychiatry*
388 189(SEP.):201–3.
389 Birch-Machin, Mark A., Robert W. Taylor, Bruce Cochran, Brian A. C. Ackrell, and
390 Douglass M. Turnbull. 2000. "Late-Onset Optic Atrophy, Ataxia, and Myopathy
391 Associated with a Mutation of a Complex II Gene." *Annals of Neurology* 48(3):330–
392 35.
393 Bourgeron, Thomas. 2015. "From the Genetic Architecture to Synaptic Plasticity in
394 Autism Spectrum Disorder." *Nature Reviews Neuroscience* 16(9):551–63.
395 Brenet, Alexandre, Rahma Hassan-Abdi, Julie Somkhit, Constantin Yanicostas, and Nadia
396 Soussi-Yanicostas. 2019. "Defective Excitatory/Inhibitory Synaptic Balance and
397 Increased Neuron Apoptosis in a Zebrafish Model of Dravet Syndrome." *Cells*
398 8(10):1199.
399 Brustein, Edna, Louis Saint-Amant, Robert R. Buss, Mabel Chong, Jonathan R.
400 McDearmid, and Pierre Drapeau. 2003. "Steps during the Development of the
401 Zebrafish Locomotor Network." Pp. 77–86 in *Journal of Physiology Paris*. Vol. 97.

402 Van Coster, Rudy, S. Seneca, J. Smet, R. Van Hecke, E. Gerlo, B. Devreese, J. Van Beeumen,
403 J. G. Leroy, L. De Meirleir, and W. Lissens. 2003. "Homozygous Gly555Glu Mutation
404 in the Nuclear-Encoded 70 KDa Flavoprotein Gene Causes Instability of the
405 Respiratory Chain Complex II." *American Journal of Medical Genetics* 120A(1):13–
406 18.
407 EFSA. 2012. "Conclusion on the Peer Review of the Pesticide Risk Assessment of the
408 Active Substance Bixafen." *EFSA Journal* 10(11):2917.
409 Finsterer, Josef. 2008. "Cognitive Decline as a Manifestation of Mitochondrial Disorders
410 (Mitochondrial Dementia)." *Journal of the Neurological Sciences* 272(1–2):20–33.
411 Ghezzi, Daniele, Paola Goffrini, Graziella Uziel, Rita Horvath, Thomas Klopstock, Hanns
412 Lochmüller, Pio D'Adamo, Paolo Gasparini, Tim M. Strom, Holger Prokisch, Federica
413 Invernizzi, Ileana Ferrero, and Massimo Zeviani. 2009. "SDHAF1, Encoding a LYR
414 Complex-II Specific Assembly Factor, Is Mutated in SDH-Defective Infantile
415 Leukoencephalopathy." *Nature Genetics* 41(6):654–56.
416 Graillot, Vanessa, Florence Tomasetig, Jean Pierre Cravedi, and Marc Audebert. 2012.
417 "Evidence of the in Vitro Genotoxicity of Methyl-Pyrazole Pesticides in Human
418 Cells." *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*
419 748(1–2):8–16.
420 Horváth, Rita, A. Abicht, E. Holinski-Feder, A. Laner, K. Gempel, H. Prokisch, H.
421 Lochmüller, T. Klopstock, and M. Jaksch. 2006. "Leigh Syndrome Caused by
422 Mutations in the Flavoprotein (Fp) Subunit of Succinate Dehydrogenase (SDHA)."
423 *Journal of Neurology, Neurosurgery and Psychiatry* 77(1):74–76.
424 Lalève, Anaïs, Stéphanie Gamet, Anne Sophie Walker, Danièle Debieu, Valérie Toquin,
425 and Sabine Fillinger. 2014. "Site-Directed Mutagenesis of the P225, N230 and H272
426 Residues of Succinate Dehydrogenase Subunit B from Botrytis Cinerea Highlights

427 Different Roles in Enzyme Activity and Inhibitor Binding." *Environmental*
428 *Microbiology* 16(7):2253–66.
429 Li, Wenhua, Mingrui Yuan, Yaqing Wu, and Xuan Liu. 2020. "Bixafen Exposure Induces
430 Developmental Toxicity in Zebrafish (Danio Rerio) Embryos." *Environmental*
431 *Research* 189(June):109923.
432 Martin, Caroline, Hervé Dubouchaud, Laurent Mosoni, Jean Michel Chardigny, Alexandra
433 Oudot, Eric Fontaine, Catherine Vergely, Christiane Keriel, Luc Rochette, Xavier
434 Leverve, and Luc Demaison. 2007. "Abnormalities of Mitochondrial Functioning Can
435 Partly Explain the Metabolic Disorders Encountered in Sarcopenic Gastrocnemius."
436 *Aging Cell* 6(2):165–77.
437 Merhi, M., H. Raynal, E. Cahuzac, F. Vinson, J. P. Cravedi, and L. Gamet-Payrastre. 2007.
438 "Occupational Exposure to Pesticides and Risk of Hematopoietic Cancers: Meta-
439 Analysis of Case-Control Studies." *Cancer Causes and Control* 18(10):1209–26.
440 Muñoz-Quezada, María Teresa, Boris A. Lucero, Dana B. Barr, Kyle Steenland, Karen
441 Levy, P. Barry Ryan, Veronica Iglesias, Sergio Alvarado, Carlos Concha, Evelyn Rojas,
442 and Catalina Vega. 2013. "Neurodevelopmental Effects in Children Associated with
443 Exposure to Organophosphate Pesticides: A Systematic Review." *NeuroToxicology*
444 39:158–68.
445 OECD. 2013. "Test No. 236: Fish Embryo Acite Toxicity (FET) Test." *Guidelines for the*
446 *Testing of Chemicals*.
447 Paganelli, Alejandra, Victoria Gnazzo, Helena Acosta, Silvia L. López, and Andrés E.
448 Carrasco. 2010. "Glyphosate-Based Herbicides Produce Teratogenic Effects on
449 Vertebrates by Impairing Retinoic Acid Signaling." *Chemical Research in Toxicology*
450 23(10):1586–95.
451 Parfait, Béatrice, Dominique Chretien, Agnès Rötig, Cécile Marsac, Arnold Munnich, and

452 Pierre Rustin. 2000. “Compound Heterozygous Mutations in the Flavoprotein Gene
453 of the Respiratory Chain Complex II in a Patient with Leigh Syndrome.” *Human*
454 *Genetics* 106(2):236–43.
455 Perry, Colin G., William F. Young, Sarah R. McWhinney, Thalia Bei, Sotirios Stergiopoulos,
456 Ryan A. Knudson, Rhett P. Ketterling, Charis Eng, Constantine A. Stratakis, and J.
457 Aidan Carney. 2006. “Functioning Paraganglioma and Gastrointestinal Stromal
458 Tumor of the Jejunum in Three Women: Syndrome or Coincidence.” *American*
459 *Journal of Surgical Pathology* 30(1):42–49.
460 Qian, Le, Feng Cui, Yang Yang, Yuan Liu, Suzhen Qi, and Chengju Wang. 2018.
461 “Mechanisms of Developmental Toxicity in Zebrafish Embryos (Danio Rerio)
462 Induced by Boscalid.” *Science of the Total Environment* 634:478–87.
463 Rauh, Virginia A., Frederica P. Perera, Megan K. Horton, Robin M. Whyatt, Ravi Bansal,
464 Xuejun Hao, Jun Liu, Dana Boyd Barr, Theodore A. Slotkin, and Bradley S. Peterson.
465 2012. “Brain Anomalies in Children Exposed Prenatally to a Common
466 Organophosphate Pesticide.” *Proceedings of the National Academy of Sciences of the*
467 *United States of America* 109(20):7871–76.
468 Ravichandra, N. G. 2018. *Agrochemicals in Plant Disease Management*.
469 Rehfus, Alexandra, Simone Miessner, Janosch Achenbach, Dieter Strobel, Rosie Bryson,
470 and Gerd Stammler. 2016. “Emergence of Succinate Dehydrogenase Inhibitor
471 Resistance of Pyrenophora Teres in Europe.” *Pest Management Science*
472 72(10):1977–88.
473 Ricketts, Christopher, Maurice P. Zeegers, Jan Lubinski, and Eamonn R. Maher. 2009.
474 “Analysis of Germline Variants in CDH1, IGFBP3, MMP1, MMP3, STK15 and VEGF in
475 Familial and Sporadic Renal Cell Carcinoma.” *PLoS ONE* 4(6).
476 Ross, Sarah Mac Kenzie, I. C. McManus, Virginia Harrison, and Oliver Mason. 2013.

477 "Neurobehavioral Problems Following Low-Level Exposure to Organophosphate
478 Pesticides: A Systematic and Meta-Analytic Review." *Critical Reviews in Toxicology*
479 43(1):21–44.
480 Shin, Jimann, Hae Chul Park, Jolanta M. Topczewska, David J. Madwsley, and Bruce
481 Appel. 2003. "Neural Cell Fate Analysis in Zebrafish Using Olig2 BAC Transgenics."
482 *Methods in Cell Science* 25(1–2):7–14.
483 Stratakis, C. A. and J. A. Carney. 2009. "The Triad of Paragangliomas, Gastric Stromal
484 Tumours and Pulmonary Chondromas (Carney Triad), and the Dyad of
485 Paragangliomas and Gastric Stromal Sarcomas (Carney-Stratakis Syndrome):
486 Molecular Genetics and Clinical Implications." Pp. 43–52 in *Journal of Internal*
487 *Medicine*. Vol. 266. J Intern Med.
488 Svartz, Gabriela, Fernando Meijide, and Cristina Pérez Coll. 2016. "Effects of a Fungicide
489 Formulation on Embryo-Larval Development, Metamorphosis, and Gonadogenesis
490 of the South American Toad Rhinella Arenarum." *Environmental Toxicology and*
491 *Pharmacology* 45:1–7.
492 Tanabe, Akiko and Kuniaki Kawata. 2009. "Daily Variation of Pesticides in Surface Water
493 of a Small River Flowing through Paddy Field Area." *Bulletin of Environmental*
494 *Contamination and Toxicology* 82(6):705–10.
495 Timmers, Henri J. L. M., Anne Paule Gimenez-Roqueplo, Massimo Mannelli, and Karel
496 Pacak. 2009. "Clinical Aspects of SDHx-Related Pheochromocytoma and
497 Paraganglioma." *Endocrine-Related Cancer* 16(2):391–400.
498 Tsuda, T., T. Nakamura, A. Inoue, and K. Tanaka. 2009. "Pesticides in Water, Fish and
499 Shellfish from Littoral Area of Lake Biwa." *Bulletin of Environmental Contamination*
500 *and Toxicology* 82(6):716–21.
501 Veloukas, Thomas, Anastasios N. Markoglou, and George S. Karaoglanidis. 2013.

502 "Differential Effect of SdhB Gene Mutations on the Sensitivity to SDHI Fungicides in
503 Botrytis Cinerea." *Plant Disease* 97(1):118–22.
504 VKM Report. 2014. "Health Risk Assessment of the Fungicide Aviator Xpro EC 225 -
505 Vitenskapskomiteen for Mat Og Miljø." 1–19.
506 VKM Report. 2015. "Risk Assessment of the Metabolite M44 of Bixafen, One of the Active
507 Substances in Aviator Xpro EC 225 - Vitenskapskomiteen for Mat Og Miljø." 1–20.
508 Vu, Hung T., Michael J. Keough, Sara M. Long, and Vincent J. Pettigrove. 2016. "Effects of
509 the Boscalid Fungicide Filan® on the Marine Amphipod Allorchestes Compressa at
510 Environmentally Relevant Concentrations." *Environmental Toxicology and*
511 *Chemistry* 35(5):1130–37.
512 Wang, Honglei, Zhen Meng, Fasheng Liu, Liqun Zhou, Meile Su, Yunlong Meng, Shouhua
513 Zhang, Xinjun Liao, Zigang Cao, and Huiqiang Lu. 2020. "Characterization of
514 Boscalid-Induced Oxidative Stress and Neurodevelopmental Toxicity in Zebrafish
515 Embryos." *Chemosphere* 238.
516 Weichenthal, Scott, Connie Moase, and Peter Chan. 2010. "A Review of Pesticide
517 Exposure and Cancer Incidence in the Agricultural Health Study Cohort."
518 *Environmental Health Perspectives* 118(8):1117–25.
519 Wu, Siyu, Lili Lei, Mengting Liu, Yang Song, Shibo Lu, Dan Li, Huahong Shi, Kathleen M.
520 Raley-Susman, and Defu He. 2018. "Single and Mixture Toxicity of Strobilurin and
521 SDHI Fungicides to Xenopus Tropicalis Embryos."
522 Yamashita, Masao and Bart Fraaije. 2018. "Non-Target Site SDHI Resistance Is Present as
523 Standing Genetic Variation in Field Populations of Zymoseptoria Tritici." *Pest*
524 *Management Science* 74(3):672–81.
525 Yang, Yang, Fengshou Dong, Xingang Liu, Jun Xu, Xiaohu Wu, Suzhen Qi, Wenxian Liu,
526 and Yongquan Zheng. 2018. "Thifluzamide Affects Lipid Metabolism in Zebrafish

527 (Danio Reio)." *Science of the Total Environment* 633:1227–36.

528 Yao, Hongzhou, Jianping Yu, Ying Zhou, Qingqing Xiang, and Chao Xu. 2018. "The

529 Embryonic Developmental Effect of Sedaxane on Zebrafish (Danio Rerio)."

530 Zhang, A., Ying Yue, Y. Yang, Jian Yang, Ke Tao, Hong Jin, and Taiping Hou. 2019.

531 "Discovery of N-(4-Fluoro-2-(Phenylamino)Phenyl)-Pyrazole-4-Carboxamides as

532 Potential Succinate Dehydrogenase Inhibitors." *Pesticide Biochemistry and*

533 *Physiology* 158:175–84.

534

535