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POSSIBLE AOP FOR TBBPA IN EARLY LARVAL DEVELOPMENT OF *MYTILUS GALLOPROVINCIALIS*

MOLECULAR INITIATING EVENT

KEY EVENTS

EFFECTS ON INDIVIDUALS

* NOT DETERMINED

Highlights

- Tetrabromobisphenol A-TBBPA affects early larval development in *Mytilus*
- Effects on shell formation, organic matrix and CaCO₃ deposition
- Altered expression pattern of genes involved in shell biogenesis from 28 hpf
- From 24 hpf impairment of serotonin, GABA and dopamine systems
- TBBPA acts as a neurodevelopmental disruptor in mussel early larval stages

Tetrabromobisphenol A acts a neurodevelopmental disruptor in early larval stages
of *Mytilus galloprovincialis*

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18 **Abstract**

19 Tetrabromobisphenol A-TBBPA, a widely used brominated flame retardant detected in aquatic
20 environments, is considered a potential endocrine disruptor-ED for its reproductive/developmental
21 effects in vertebrates. In aquatic invertebrates, the modes of action of most EDs are largely unknown,
22 due to partial knowledge of the mechanisms controlling neuroendocrine functions.
23 In the marine bivalve *Mytilus galloprovincialis*, TBBPA has been previously shown to affect larval
24 development in the 48 hours larval toxicity assay at environmental concentrations. In this work, the
25 effects of TBBPA were further investigated at different times post-fertilization. TBBPA, from 1 µg/L,
26 affected shell biogenesis at 48 hours post fertilization-hpf, as shown by phenotypic and SEM analysis.
27 The mechanisms of action of TBBPA were investigated at concentrations of the same order of
28 magnitude as those found in highly polluted coastal areas (10 µg/L). At 28-32 hpf, TBBPA
29 significantly affected deposition of both the organic matrix and CaCO₃ in the shell. TBBPA also
30 altered expression of shell-related genes from 24 to 48 hpf, in particular of tyrosinase, a key enzyme
31 in shell matrix remodeling. At earlier stages (24 hpf), TBBPA affected the development of
32 dopaminergic, serotonergic and GABAergic systems, as shown by in situ hybridization-ISH and
33 immunocytochemistry. These data contribute draw adverse outcome pathways-AOPs, where TBBPA
34 affects the synthesis of neurotransmitters involved in key events (neurodevelopment and shell
35 biogenesis), resulting in phenotypic changes on individuals (delayed or arrested development) that
36 might lead to detrimental consequences on populations.

37
38 Keywords: *tetrabromobisphenol A, Mytilus, early larval stages, shell formation, neuroendocrine*
39 *disruptor*

40 1. Introduction

41 Tetrabromobisphenol A (TBBPA) is one of the most widely used brominated flame retardants in the
42 production of epoxy and polycarbonate resins employed in electronic devices, furniture, plastics,
43 textiles, and other products (Covaci et al., 2009). TBBPA is highly produced worldwide, and its
44 global market is expected to increase over the next five years (WorldAnalytics, 2019). EPA
45 designated TBBPA as a high priority chemical currently undergoing risk evaluation (EPA, 2019).
46 TBBPA is frequently detected in different ecosystem compartments, including aquatic environments
47 (Liu et al., 2016; Zhou et al., 2020) and it can be also accumulated in the food chain (Morris et al.,
48 2004; Yang et al., 2012; Shi et al., 2017).

49 TBBPA is likely to reach the marine environment largely through industrial waste waters from land-
50 based industrial activities, and coastal areas worldwide are subjected to increasing TBBPA
51 contamination, thus raising great environmental concern (Gu et al., 2019). Levels of TBBPA in
52 urbanized coastal areas range from ng to μg in both sediments ($\text{g}^{-1} \text{ dw}$) and waters (L^{-1}) (Liu et al.,
53 2016; Sühling et al., 2015; Gong et al., 2017 and refs therein). High concentrations ($>1 \mu\text{g L}^{-1}$)
54 detected at polluted sites (Liu et al., 2016; Gong et al., 2017; Li et al., 2020) are comparable to TBBPA
55 levels in urban soils and freshwater, suggesting that coastal seawater may receive discharge from
56 industrial effluents or landfill leakages without effective treatment.

57 Due to its almost ubiquitous presence, the potential impact of TBBPA on human and environmental
58 health has been widely investigated (Wang et al., 2020; Zhou et al., 2020). Studies based on cell and
59 animal models (mammals and fish) have demonstrated that TBBPA can induce pleiotropic effects on
60 liver, kidney, heart, neural and reproductive functions; in particular, TBBPA has been shown to be a
61 potential endocrine disruptor (ED), because of its reproductive and developmental effects including
62 interference with thyroid hormone signalling (Zhang et al., 2014; Zhou et al., 2020). Significant
63 effects of TBBPA on early embryo development have been observed in mammalian and zebrafish
64 models in the nmol/L range, underlying that particular attention should be paid to the potential effects
65 of TBBPA on early developmental stages (Zhou et al., 2020). Moreover, it has been shown that

molluscan shell growth may be uniquely sensitive to TBBPA, more sensitive than chronic fish or crustacean toxicity endpoints (Pittinger and Pecquet, 2018).

We have previously demonstrated that in the model marine bivalve, the mussel *Mytilus galloprovincialis*, environmental concentrations of TBBPA affected early larval development evaluated in the standard 48 h embryotoxicity assay, with a LOEC of 0.1 µg/L and an EC₅₀ of 5.52 µg/L (Fabbri et al., 2014). The effects were comparable with those observed with the parent compound Bisphenol A-BPA (Fabbri et al., 2014), that has been subsequently shown to interfere with the molecular mechanisms involved in shell biogenesis and development of the serotonergic system in early mussel larvae (Balbi et al., 2016; Miglioli et al., 2021a).

In this work, the effects and mechanisms of action of TBBPA were further investigated in mussel larvae at different times post fertilization, with special emphasis on early shell formation, the key step in the transition from the trocophora (24 hpf) to the first D-veliger stage (48 hpf), as well as on neurodevelopment. Concentrations were chosen among those encompassing the EC₅₀ values obtained in the 48 h larval developmental assay (1 and 10 µg/L, depending on the endpoint measured) (Fabbri et al., 2014), and environmental levels detected in polluted coastal areas (Liu et al., 2016, Gong et al., 2017; Li et al., 2020). Higher, embryotoxic concentrations (100 µg/L) (Fabbri et al., 2014) were also utilized to evaluate the effects on shell formation at earlier stages.

The effects of TBBPA on shell biogenesis were investigated by different techniques, following the experimental setup previously described (Miglioli et al., 2019, 2021a). Deposition of the organic matrix and of the calcified shell were evaluated at 24, 28, 32, and 48 hpf by Calcofluor/Calcein staining, and quantified by digital imaging. In addition, shell morphology at 48 hpf was investigated by Scanning Electron Microscopy-SEM. Across different developmental stages, expression of key genes involved in shell matrix deposition (chitin synthase-CS; tyrosinase-TYR) and calcification (carbonic anhydrase-CA; extrapallial protein-EP) was quantified by qPCR; the expression pattern of tyrosinase and chitinase was also evaluated by In Situ Hybridization-ISH.

Moreover, the possible effects of TBBPA on neurodevelopment were investigated focusing on the main neurotransmitters identified in bivalves (Liu et al., 2018; Joyce and Vogeler, 2018). Localization of components of the dopaminergic system (Tyrosine Hydroxylase-TH, Dopamine- β -Hydroxylase-D β H, Dopamine Receptor 1-DR1) was investigated by ISH; development of serotonergic and GABAergic neurons was evaluated by immunohistochemistry.

2. Methods

2.1 Mussels, larval development and exposure conditions

All procedures were carried out as previously described (Miglioli et al., 2019, 2021a). Sexually mature specimens of *M. galloprovincialis* were collected from a natural population in the Bay of Villefranche-sur-mer (43.682°N, 7.319°E - France) during the spawning season of 2019/20 (December-March). Animals were maintained at the Institut de la Mer de Villefranche (IMEV) by the Centre de Ressources Biologiques Marines of the institute (CRBM), transferred to the laboratory and acclimatized in flow-through vessels containing filtered natural seawater MFSW (Millipore filtered seawater) (pH 7.9-8.0, 38 ppt salinity, 15°C).

Gametes were obtained by spontaneous spawning; after checking egg quality (shape, size) and sperm motility, fertilization was carried out with an egg:sperm ratio of 1:10. After 30 min, fertilization success (n. fertilized eggs/n. total eggs x 100) was verified by microscopic observation (usually > 90%). Fertilized eggs were transferred to 24-well plates (200 larvae mL⁻¹) and exposed to TBBPA 3,3',5,5'-Tetrabromobisphenol A (Sigma-Aldrich, Milan), from stock solutions (0.5 M in Dimethylsulfoxide/DMSO) suitably diluted in filtered marine sea water-MFSW to obtain the selected final concentrations. Parallel control samples were run in MFSW added with 0.01% DMSO. In all experimental conditions, samples were represented by larvae obtained from 5 independent parental pairs (N=5), unless otherwise indicated.

TBBPA final concentrations (1 and 10 μ g/L, corresponding to 1.8 and 18 nM, respectively), depending on the endpoint measured, were chosen among those encompassing higher levels detected

117 in polluted coastal areas (Gong et al., 2017; Li et al., 2020), as well as the EC₅₀ values previously
118 obtained for TBBPA in the 48 h embryotoxicity assay (Fabbri et al., 2014). Higher, embryotoxic
119 concentrations (100 µg/L) were also utilized to confirm the effects on shell formation at early stages.
120 Concentrations in test solutions were checked by LC/MS as previously described (Fabbri et al., 2014).
121

122 *2.2 Effect of TBBPA on shell formation*

123 At 48 hpf, the effect of TBBPA (1, 10 and 100 µg/L) was evaluated by scoring larval phenotypes in
124 at least 50 larvae/sample. A larva was considered normal when the shell was D-shaped (straight
125 hinge) and the mantle did not protrude out of the shell, and malformed if the larva had not reached
126 the stage typical for 48 h (trochophore or earlier stages) or when developmental defects were observed
127 (concave, malformed or damaged shell, protruding mantle) (Fabbri et al., 2014). Data are reported as
128 % values of normal D-veligers. The acceptability of test results was based on control samples with a
129 percentage of normal D-larvae >75% (ASTM, 2012).

130 The time-course of shell formation was also investigated at different times pf between the trochophora
131 and the D-veliger stage (24, 28, 32 and 48 hpf). Larval shell components were visualized by
132 Calcofluor White Fluorescent Brightener 28 (Sigma Aldrich, Lyon, France) for organic matrix, and
133 Calcein (Sigma Aldrich, Lyon, France) for CaCO₃ deposition, respectively (Miglioli et al., 2019,
134 2021a). Larvae were imaged with a Leica SP8 Confocal Laser Scanning Microscope (CLSM - Leica,
135 France) scanning sequentially Brightfield, Calcofluor, and Calcein signals with a 0.5 µm Z-stack
136 interval. Channels were merged, 3D rendered and rotated to measure the area (in µm²) of each shell
137 component in a single valva of each larva by manual drawing using IMAGEJ software (Miglioli et
138 al., 2019, 2021a). Analyses were performed on at least 50 larvae for each sample. Data were
139 normalized with respect to controls for each parental pair and experimental condition.

140

141 *2.3 Role of thyroid hormone and dopamine signaling in early larval development*

142 The role of thyroid hormone-TH and dopamine signaling were investigated by exposure of fertilized
143 eggs to different TH agonists and antagonist: Triiodotyronine (T₃) and Tetraiodotyronine (T₄), the
144 selective and high-affinity THR antagonist 1-850 (Schapira et al., 2003), and the antithyroid drug
145 thiourea (TU), a TH depleting compound (Davidson et al., 1979) and to the selective antagonist of
146 dopamine receptor DR1, SCH 23390 (Liu et al., 2018, 2020) (See SI).

147

148 *2.4 Scanning electron microscopy (SEM) of larval shell*

149 Control and TBBPA exposed larvae (1 and 10 µg/L) at 48 hpf were fixed in 3% glutaraldehyde in
150 ASW. After fixation, samples were placed onto Whatman 22 µm filters, dehydrated in an ascending
151 ethanol series (50% - 80% - 90% - 100%) and air-dried. Samples were sputter-coated with gold and
152 observed at 20 kV with a Vega3 - Tescan scanning electron microscope. All procedures were carried
153 out as previously described (Balbi et al., 2016, 2017, 2018).

154

155 *2.5 qPCR*

156 Fertilized eggs were exposed to TBBPA (10 µg/L) in polystyrene 6-well plates (Balbi et al., 2016;
157 Miglioli et al., 2021a). Control samples were run in parallel. Larvae were collected at 24, 28, 32 and
158 48 hpf by a nylon mesh (20 µm pore-filter) and washed with filter sterilized artificial seawater (ASW)
159 (Balbi et al., 2016). Three wells for each sample were pooled in order to obtain approximately 7000
160 larvae/replicate. All procedures (RNA extraction, retro-transcription and qPCR) were carried out as
161 previously described (Balbi et al., 2016; Miglioli et al., 2019, 2021a). Primers utilized for qPCR are
162 reported in Tab. S1.

163

164 *2.6 In Situ Hybridization*

165 In situ hybridization (ISH) was used to evaluate the expression pattern of enzymes involved in matrix
166 deposition (tyrosinase and chitinase) and of components of the dopaminergic system (Tyrosine
167 Hydroxylase, Dopamine-β-Hydroxylase, Dopamine Receptor 1-DR1) following the protocol

168 developed for *M. galloprovincialis* larvae (Miglioli et al., 2019, 2021a). Primer pairs were designed
 169 to amplify approximately 1-1.5 Kb cDNA fragment of mussel Tyrosinase (F:
 170 ATGCGATTCTTTATACATGAAA; R: TTGGTGGTTTTGGTACATGT; GeneBank
 171 KV583276.1); Chitinase (F: CACCATGAATCTTAGAGGGATACAC; R:
 172 TGAACATCCGGGTACATTATA; GeneBank KP757835.1); Tyrosine Hydroxylase (F:
 173 ATGTATTCACCGACACCTCG; R: ACACCTTCAGTTCTTTTACTG; GeneBank
 174 GGUW01014444.1); Dopamine- β -Hydroxylase (F: CCTTCTGAAGTTTGCCTTTTC; R:
 175 CTGGCTCCCCGGGCACT; GeneBank GHIK01112718.1); Dopamine Receptor 1-DR1 (F:
 176 ATGATAACAAATTTTTCTATATTGG; R: ACAAAGCAGTTACCTTGTCTCCT; GeneBank
 177 Unigene27870_All). Amplicons were used to synthesize sense and antisense digoxigenin-labelled
 178 RNA probes (Miglioli et al., 2019, 2021a). Control and TBBPA-exposed larvae (10 μ g/L) were grown
 179 in 50 mL culture flasks as described above, sampled at 24, 28, 32 and 48 hpf, concentrated with a 60
 180 μ m nylon filter, fixed overnight in 4% Paraformaldehyde in PBS, washed in the same buffer and kept
 181 in 100% methanol at -20°C. Analyses were performed on larvae from 4 independent parental pairs
 182 (N=4) (at least 50 individuals were analyzed for each sample).

183

184 2.7 5-HT and GABA immunocytochemistry

185 Antibodies against the neurotransmitter serotonin (5-HT) and γ -Aminobutyric acid (GABA) were
 186 utilized to visualize the development of the serotonergic and GABAergic systems in both control and
 187 TBBPA-exposed larvae (10 μ g/L) at different times pf as previously described (Miglioli et al., 2021a;
 188 Vaasjo et al., 2018).

189 Samples were incubated with primary antibodies either against Rabbit 5-HT (1:10.000; Immunostar,
 190 Hudson, WI, USA) or GABA (1:4.000; A2052, Sigma Aldrich) for 5 days at 4°C. Subsequently,
 191 samples were incubated with Anti-Rabbit Rhodamine Red™-X (RRX) IgG secondary antibody
 192 (1:500, Ex/Em: 570/590 nm - Jackson laboratory, Bar Harbor, Maine, USA) for 3 days at 4°C; non-
 193 specific binding was evaluated in subsamples incubated only in the presence of the secondary

antibody (not shown) as previously described (Miglioli et al., 2021a). Larvae were then stained with 1 µg/mL Hoechst in PBS (UV, Ex/Em: 352/461 nm- Hoechst 33342, Invitrogen) and mounted with glycerol phosphate buffered solution containing antifading agent (CitiFluor™ AF1, pH 10, Agar scientific). Samples were imaged with a Leica Sp8 CLSM. Hoechst, 5-HT immunoreactivity and Brightfield signals were sequentially scanned with a 0.3 µm Z-stack interval. Using ImageJ software, channels were merged and subsequent Z-stacks were assembled in order to assess the number of 5-HT-ir (immuno-reactive) cells as previously described (Miglioli et al., 2021a). Larvae stained with GABA Abs were entirely imaged in 0.6 µm Z-stacks for a total of 100 z planes, with Z-projections made with 10 sequential z planes (from z10 to z100). At least 50 individuals were analyzed for each sample and experimental condition (N=5).

204

2.8 Statistics

Data obtained from 4 or 5 independent parental pairs, depending on the experiment (mean ± SD) were analysed by one-way nonparametric ANOVA (Kruskal-Wallis test) followed by the Tukey's test (p<0.05) using GraphPad Prism 5 software (GraphPad Inc.).

209

3. Results

3.1 Phenotypic changes induced by TBBPA in mussel larvae at 48 hpf

The phenotypic effects of TBBPA were first evaluated in 48 hpf larvae and the results are reported in Fig. 1. TBBPA induced a concentration-dependent decrease in the percentage of normal D-veligers as previously described (Fabbri et al., 2014) (Fig. 1A). At all concentrations, immature larvae (pre-veligers), as well as larvae withheld at the trocophora stage were observed, together with shell malformations. These effects could be better appreciated by Calcofluor/Calcein double staining, that shows deposition of each shell component, the organic matrix (blue) and CaCO₃ (green) (Fig. 1B). The shell of normal D-veligers showed extensive calcification, accretion rings and straight hinge. In TBBPA-exposed samples (10 µg/L), malformed larvae were characterized by irregular calcification

220 and hinge malformations; immature D-veligers were identified by incomplete calcification and
221 protrusion of the velum, the larval feeding organ; other larvae showed the arrested trocophora
222 phenotype (Fig. 1B).

223 The effects of TBBPA (10 and 1 $\mu\text{g/L}$) on larval shells were investigated in more detail by SEM, and
224 representative images are reported in Fig. 2 and 3, respectively. As previously described (Balbi et al.,
225 2016, 2017, 2018) normal D-veliger shells were characterized by a straight hinge, symmetric valvae
226 and a uniform, smooth surface (Fig. 2A, Fig. 3A). In TBBPA-exposed larvae (10 $\mu\text{g/L}$) shells showed
227 irregular surfaces, holes, convex hinges, and fractures (Fig. 2B-E) often perpendicular to the hinge
228 axis (Fig. 2F). At lower concentrations (1 $\mu\text{g/L}$) in addition to shell fractures (Fig. 3B), immature
229 shells, characterized by polygonal shapes, asymmetric valvae (Fig. 3C, D) and protruding velum (Fig.
230 3E) were observed. Irregular calcification at the center of shell was also detected (Fig. 3F).

231

232 *3.2 Effects of TBBPA on shell formation at earlier larval stages*

233 The effects of TBBPA were further investigated by Calcofluor/Calcein staining at shorter times post-
234 fertilization (24, 28, and 32 hpf), and the results are shown in Fig. 4. In Fig. 5, quantification of areas
235 of occupied by the organic matrix and calcified shell in different experimental conditions are reported.
236 In control larvae, at 24 hpf (the trocophora stage) the initial shell was only made of organic matrix
237 (blue) and characterized by a saddle shape (the shell field); calcification (green) was detectable from
238 28 hpf, starting from the center of each valva, and increased at 32 hpf, with evident shell accretion
239 rings, whereas the hinge region was not calcified yet. Complete calcification, including the hinge,
240 was observed at 48 hpf (Fig. 4). Exposure to TBBPA (10 $\mu\text{g/L}$) induced a significant decrease in the
241 calcified shell area from 28 hpf, and in matrix deposition at 32 hpf (Fig. 4 and Fig. 5B, C).
242 Interestingly, in TBBPA-exposed larvae, absence of calcification was observed in the center of the
243 valvae from 28 to 48 hpf (Fig. 4), corresponding to the irregular shell calcification observed by SEM
244 in some individuals at 48 hpf. Overall, TBBPA (10 $\mu\text{g/L}$) induced a significant increase in the ratio
245 organic matrix/calcified shell at 28 hpf (Fig. 5D). Stronger effects were observed at higher

concentrations (100 µg/L) (Fig. 4 and Fig. 5A-D). All the following experiments were performed at 10 µg/L TBBPA.

3.3 Effects of TBBPA on transcription of genes involved in early shell formation

Transcription of selected genes involved in first shell formation (Miglioli et al., 2019) was evaluated by qPCR in larvae exposed to TBBPA (10 µg/L) at different times pf. In Fig. 6 data are reported on changes in mRNA level for genes involved in matrix deposition and remodeling (chitin synthase-CS; tyrosinase-TYR) and calcification (carbonic anhydrase-CA; extrapallial protein-EP) in control and TBBPA-exposed samples across different stages with respect to eggs. Transcription of CS was upregulated at 32 and 48 hpf in control samples, and TBBPA induced a further small but significant increase at 48 hpf (+20% with respect to controls) (Fig. 6A). Although basal expression of TYR in control larvae is very high with respect to eggs at all times pf (Miglioli et al., 2019, 2021a), TBBPA induced a further general upregulation at all stages, that was highest at both 28 and 48 hpf (+85% vs controls, $p<0.05$) (Fig. 6B). Transcription of CA was unaffected by TBBPA (Fig. 6C); however, TBBPA induced a general increase in mRNA levels for EP, with highest upregulation at 28 and 48 hpf (+105 and +135% vs controls, respectively, $p<0.05$) (Fig. 6D).

The expression pattern of tyrosinase and of chitinase, the enzyme involved in degradation of chitin, the main component of the organic matrix, was also investigated by ISH, and the results are reported in Fig. 7. As previously demonstrated (Miglioli et al., 2021a), tyrosinase was strongly expressed at all time pf in the areas corresponding to the organic matrix of the growing shell. TBBPA exposure resulted in a general upregulation of tyrosinase, in line with the results obtained by PCR, without significantly affecting its localization. Chitinase showed expression in the central part of the shell in larvae from 24 to 32 hpf, followed by a sharp decrease in expression in D-veligers. TBBPA did not affect chitinase localization at 24 and 28 hpf, whereas it induced some downregulation at 32 hpf and some upregulation at 48 hpf (Fig. 7).

272 *3.4 Role of thyroid and dopamine signaling in mussel shell morphogenesis as possible targets of*
273 *TBBPA*

274 Since in vertebrate developmental models, including the zebrafish, TBBPA mainly affects thyroid
275 hormone signalling (Zhu et al., 2018 and refs. therein; Yu et al., 2021) the role of this pathway was
276 first investigated by exposure of fertilized eggs to the thyroid Receptor-THR agonists Triiodotyronine
277 (T₃) and Tetraiodotyronine (T₄), the THR antagonist 1-850, and the antithyroid drug thiourea (TU).
278 None of these compounds significantly affected larval development at 48 hpf, in terms of changes in
279 proportion of larval phenotypes (Fig. S1). Only the THR antagonist 1-850 induced a slight decrease
280 in the percentage of normal D-veligers; however, the values were above the acceptability of the test
281 results (>75% normal larvae) (ASTM, 2012). The results indicate that thyroid hormone signalling
282 does not play a key role in shell morphogenesis in mussel early larval stages.

283 In analogy with data obtained in oysters (Liu et al., 2018, 2020), the possible involvement of the
284 dopaminergic system in mussel larval development was investigated. Exposure to the DR1 antagonist
285 SCH 23390 resulted in a complete disruption of shell formation. In particular, no calcification was
286 observed up to 32 hpf (Fig. S2) and, from 24 to 32 hpf, the area of the shell field occupied by the
287 organic matrix was reduced to less than 20% of controls (Fig. S2B). At 48 hpf, a severe impairment
288 of calcification was observed (Fig. S2B), the overall effects resulting in about an 80% decrease in the
289 percentage of normal D-larvae (Fig. S2C).

290
291 *3.5 TBBPA affects the expression pattern of dopaminergic components*

292 The effects of TBBPA (10 µg/L) on key components of the dopaminergic system were then
293 investigated. To this aim, expression pattern of key enzymes involved in DOPA synthesis (Tyrosine
294 Hydroxylase) and metabolism (Dopamine-β-Hydroxylase) and of the DOPA receptor DR1 were
295 evaluated by ISH, and the results are shown in Fig. 8. Exposure to TBBPA induced changes in the
296 expression pattern of all genes from 24 hpf. In control larvae, the dopaminergic marker TH,
297 responsible for the synthesis of DOPA from the amino acid tyrosine, showed a diffuse localization at

298 24 hpf, followed by progressive concentration in two clusters dopaminergic neurons at 28 hpf and 32
299 hpf; at 48 hpf, a clear localization of TH in a single cluster was observed. TBBPA-exposed larvae
300 showed a stronger and more diffuse signal at earlier stages, with two observable clusters remaining
301 at 48 hpf (suggesting a delay in the differentiation of dopaminergic cell ganglion). Expression of D β H
302 (the enzyme that catalyzes the conversion of DOPA to noradrenaline) was detectable in control
303 samples from 28 hpf at the center of the larval body and showed a distinct localization only at 48 hpf.
304 TBBPA resulted in a general upregulation of D β H at all times pf, except for at 28 hpf. DR1 in control
305 larvae showed a weak expression in the trocophorae at 24 hpf and subsequently increased during
306 transition to the D-veliger, showing a sharp localization along the margins of the growing shell. In
307 TBBPA-exposed larvae, DR1 was not expressed at 24 hpf; a weaker signal and less distinct
308 localization were observed from 28 to 32 hpf, followed by strong upregulation at 48 hpf.

309

310 *3.6 TBBPA affects serotonin and GABA immunoreactivity*

311 Development of serotonin immunopositive cells (5-HT-*ir*), a key step in early mussel larvae
312 neurodevelopment, was evaluated by immunocytochemistry as previously described (Miglioli et al.,
313 2021a), and the results are reported in Fig. 9 and Fig. 10. In control larvae, the number of 5-HT-*ir*
314 cells progressively increased from 2 to 7 from 24 to 48 hpf (Fig. 9A and Fig. 10A) as previously
315 reported (Miglioli et al. 2021a). In samples exposed to TBBPA (10 μ g/L), the 5-HT positive ganglion
316 was still present: however, the number of 5-HT-*ir* neurons was generally lower at all times pf (Fig.
317 9B and Fig. 10B). In particular, at 48 hpf, such a decrease, was clearly evident in immature veligers
318 and arrested trocophorae (Fig. 10B), and statistically significant in all treated larvae with respect to
319 controls (Fig. 11). However, TBBPA did not affect mRNA levels for the serotonin receptor 5-HTR
320 at any time pf (not shown).

321 Development of GABA-*ir* cells was also evaluated from 24 to 48 hpf (Fig. 12 and Fig. 13). Due to
322 strong GABA signal observed at all times pf, larvae were entirely imaged in 100 z-stacks, with Z-
323 projections made every 10 sequential stacks (from z10 to z100) to better identify the localization of

GABA-*ir* cells (Fig S3 and S4). In control larvae, several GABA-*ir* cells were detected at 24 and 28 hpf, along the shell field and at the center of the body (Fig. 12A and Fig S3). From 32 hpf, immunoreactivity was concentrated along the external margins of the larvae. In TBBPA-exposed samples (10 µg/L), a clear decrease in the GABA signal was observed from 24 to 32 hpf (Fig. 12B). At 48 hpf, GABA immunoreactivity was concentrated along the external margin of the body in control samples (Fig. 13A and Fig. S4). At this stage, in samples exposed to 10 µg/L TBBPA, GABA-*ir* cells were localized in the protruding velum of immature veligers (pre-veligers), and arrested trocophores showed the strong immunoreactivity observed in control 24-28 hpf trocophores (Fig. 13B).

4. Discussion

The results here obtained extend previous observations indicating that TBBPA interferes with early larval development of *M. galloprovincialis* (Fabbri et al., 2014) at concentrations encompassing those detectable in contaminated coastal areas (Gong et al., 2017 and refs. therein). Moreover, we provide first information on the mechanisms of action of TBBPA on mussel shell biogenesis and neurodevelopment from earlier larval stages. The effects of TBBPA on all endpoints measured from molecular to individual level were observed at 10 µg/L, a concentration of the same order of magnitude as those detected in polluted environments (low µg/L) (Liu et al., 2016; Gong et al., 2017 and refs. therein; Li et al., 2020).

TBBPA has been reported as a potential thyroid endocrine disruptor and neurotoxicant in mammalian and fish (i.e. the zebrafish) models through multiple mechanisms, indicating that particular attention should be paid to the effects of this compound on early developmental stages (reviewed in Zhou et al., 2020). In marine organisms, the effects of TBBPA have been investigated in a range of species across different trophic levels, indicating that some fish and molluscs are particularly sensitive to TBBPA (Pittinger and Pecquet, 2018; Nos et al., 2020). With regards to marine bivalves, acute exposure of the Eastern oyster *Crassostrea virginica* to TBBPA (18 µg/L, 96 h) significantly reduced

350 shell growth (Springborn Life Sciences, 1989). In *Mytilus edulis*, chronic exposure (70 days) to
351 TBBPA affected growth, with NOEC and LOEC values of 17 and 32 µg/L, respectively
352 (AstraZeneca, 2005a,b). In juveniles of the clam *Ruditapes phillipinarum* exposed to TBBPA (62.5 -
353 1000 µg/L, 28 days) inhibition of shell growth and filtration rate were observed (Jiang et al., 2019).
354 Overall, these data indicates that shell growth represents a target for TBBPA in adult and juvenile
355 bivalves, although at higher concentrations than those detected in the environment.

356 In *M. galloprovincialis*, TBBPA in a wide concentration range, encompassing environmental
357 exposure levels (from ng to µg/L), affected early larval development, increasing the percentage of
358 trocophorae/immature D-veligers (Fabbri et al., 2014). However, the possible mechanisms of action
359 were not investigated, due to little information available on the molecular pathways regulating shell
360 biogenesis in mussels. Subsequent studies shed some light on the expression pattern of genes involved
361 in shell formation, and in neuroendocrine pathways in early mussel larvae grown both in
362 physiological conditions and in the presence of different emerging contaminants (Balbi et al., 2016,
363 2017, 2018; Franzellitti et al., 2019; Miglioli et al., 2019, 2021a).

364 The results here presented provide information on the mechanisms of action of TBBPA in developing
365 larvae across different stages, focusing on shell morphogenesis and neurogenesis. For a mechanistic
366 study, all experiments were carried out at 10 µg/L, a concentration about two-fold higher than both
367 the EC₅₀ obtained in the 48 h standard larval assay and TBBPA levels detected in polluted coastal
368 areas. The utilization of calcein/calcofluor staining to visualize and quantify the deposition of each
369 shell component, clearly demonstrated that TBBPA significantly increased the ratio organic
370 matrix/calcified shell at 28 hpf, when the first calcification of the growing shell occurs (Miglioli et
371 al., 2019), indicating a perturbation of the firsts steps of shell biogenesis. Moreover, TBBPA affected
372 the pattern of calcification; in particular, no CaCO₃ deposition at the center of the valvae was
373 observed, resulting in a ‘key hole’ phenotype similar to that previously observed in mussel larvae
374 exposed to acidification (Kapsenberg et al., 2018). These data indicate that TBBPA affects the
375 mechanisms involved in deposition of the organic matrix, which represents the blueprint for

376 biomineralization and normal shell biogenesis. The effects of TBBPA on shell formation could be
377 appreciated by SEM imaging at 48 hpf, at concentrations as low as 1 µg/L, confirming delayed
378 development and irregular calcification.

379 Data on gene expression offer a first glimpse into the underlying mechanisms of TBBPA on shell
380 formation, indicating a general dysregulation of the expression pattern of genes involved in matrix
381 deposition and calcification across different developmental stages, with tyrosinase representing the
382 most upregulated gene from 28 hpf. Tyrosinase has been identified as a key enzyme in organic matrix
383 remodeling in early mussel larvae grown in physiological conditions (Miglioli et al., 2019), as well
384 as a target of BPA (Miglioli et al., 2021a). The results here obtained indicate that TBBPA induced a
385 stronger upregulation of tyrosinase with respect to BPA at similar concentrations (10 *vs* 11.4 µg/L)
386 at all stages pf (Miglioli et al., 2021a); the effects were also stronger when compared on a molar basis
387 (18 nM TBBPA *vs* 50 nM BPA). TBBPA also resulted in upregulation of EP, that plays a key role in
388 calcification, with a similar trend across different stages. Only at 48 hpf, increased expression of
389 chitin synthase and decreased that of chitinase were observed, as shown by both qPCR and ISH.
390 These results indicate that increased synthesis of chitin, the main component of the shell organic
391 matrix, typical of the initial stages of shell deposition, is upregulated by TBBPA at later stages, further
392 supporting a delay in development. Overall, these data partly contribute to explain the phenotypic
393 effects of TBBPA on shell formation.

394 BPA has been previously shown to affect expression of genes involved in detoxification response in
395 mussel larvae (Balbi et al., 2016). However, TBBPA (up to 10 µg/L) did not induce changes in mRNA
396 levels for glutathione-s-transferase (GST π) and the ABC transporter p-glycoprotein (ABCB) with
397 respect to controls at any developmental stage (not shown), suggesting that mussel early larvae have
398 little capacity for TBBPA biotransformation/detoxification.

399 The possible endocrine mechanisms of action of TBBPA were then investigated. In vertebrate
400 systems TBBPA, structurally resembling thyroid hormones, can act as an endocrine disruptor
401 affecting TH signaling and metabolism (Zhang et al., 2014; Zhou et al., 2020). These mechanisms

402 have been shown to be associated with the neurodevelopmental toxicity of TBBPA in the zebrafish
403 embryo (Zhu et al., 2018; Pang et al., 2020; Yu et al., 2021). The presence of THs and related
404 signaling components has been demonstrated in developing and adult bivalves (Huang et al., 2015;
405 Taylor and Heyland, 2017; Miglioli et al., 2021b). In clam juveniles, prolonged exposure to TBBPA
406 caused alterations in tissue content of THs and in expression of TH synthesis-related enzymes (Jiang
407 et al., 2019). In the oyster *C. gigas*, TH signaling was shown to be involved in embryogenesis and
408 metamorphosis; however, from the trocophora to the first D-veliger stage, lowest values of T₃ and T₄
409 and absence of THR expression were observed (Huang et al., 2015). Accordingly, the results here
410 obtained with *M. galloprovincialis* show that exposure to different TH agonists and antagonists did
411 not affect larval development at 48 hpf. These observations support the hypothesis that TH signaling,
412 which is considered a common target for the endocrine effects of TBBPA also in aquatic species,
413 does not represent a key pathway in the transition from the trocophora and the first D-veliger of
414 bivalve larvae. Therefore, other mechanisms may be involved in the action of TBBPA as a potential
415 developmental disruptor in early developmental stages of mussel larvae.

416 Neuroendocrine mechanisms are known to play a vital role in larval development of invertebrates
417 (Cann-Moisan et al., 2002). Molecular components of the neuroendocrine system have been
418 characterized in marine bivalves (catecholaminergic, cholinergic, enkephalinergic, serotonergic,
419 gamma-aminobutyric acid-ergic, and neuropeptide systems), and they share a similar molecular basis
420 with their vertebrate counterparts (Wang et al., 2018; Liu et al., 2018; Joyce and Vogeler, 2018). In
421 larvae of the oyster *C. gigas*, expression of a DOPA receptor Cg-DR-1 peaked during the transition
422 from the trocophora to the D-veliger stage, indicating a role in shell biogenesis (Liu et al., 2018). In
423 a subsequent study, exposure of trocophorae to DR1 and 5-HTR inhibitors was found to prevent shell
424 formation (Liu et al., 2020). Metabolomic and transcriptomic analyses suggested that the synthesis
425 of DOPA and 5-HT may modulate initial shell formation through the TGF- β smad pathway, by
426 activating the expression of tyrosinase and chitinase, that are essential for initial shell biogenesis (Liu
427 et al., 2020).

428 The results here presented extend the findings in oysters by showing that in *M. galloprovincialis*,
429 exposure of fertilized eggs to the selective DR1 inhibitor SCH 23390 disrupted shell formation at a
430 concentration 20 times lower than those utilized in oyster trocophorae (Liu et al., 2020). Therefore,
431 DOPA signaling has a key role in mussel shell morphogenesis. TBBPA induced alterations in the
432 expression pattern of enzymes involved in DOPA synthesis and metabolism and of the DOPA
433 receptor DR1 from 24 hpf, indicating a delay in the differentiation of dopaminergic neurons and DR1
434 expression, as well as an increased metabolism of DOPA. The effects on the dopaminergic system
435 were detectable before the changes in expression of shell related genes observed from 28 hpf. We
436 hypothesize that impairment of dopamine system interferes with the initial processes of shell
437 deposition, resulting in altered shell phenotypes and delayed development. Only at later stages (48
438 hpf), TBBPA-induced upregulation of DR1 was accompanied by increased expression of tyrosinase,
439 chitin synthase, EP, whereas chitinase was downregulated. Another link between dopaminergic
440 signaling and shell formation is suggested by the results obtained on expression of mussel EP protein,
441 that is involved in controlling the deposition of CaCO₃ polymorphs, including vaterite, the most
442 unstable crystalline phase formed during initial biomineralization (Ji et al., 2010). In the pearl mussel
443 *Hyriopsis cumingii* DOPA has been involved in stability of vaterite (Kim and Park, 2010).

444 Serotonin represents a key navigational cue during bivalve neurodevelopment (Yurchenko et al.,
445 2019) and shell formation (Liu et al., 2020). In *M. galloprovincialis*, BPA affected expression of
446 tyrosinase, larval patterning and 5-HT signalling (Miglioli et al., 2021a). The results here presented
447 show that also exposure to TBBPA impairs the development of serotonergic neurons from 24 hpf,
448 whose number was decreased in 48 hpf larvae, characterized by delayed development and arrested
449 phenotypes. Overall, the results indicate that tyrosinase, that represents a link between monoamine
450 neurotransmitter signaling and shell biogenesis in both oysters and mussels (Liu et al., 2020; Miglioli
451 et al., 2019, 2021a), is a potential target for endocrine disruptors in early bivalve development.

452 Finally, TBBPA showed dramatic effects on the development of GABA-*ir* cells in mussel larvae at
453 early stages (24 hpf). In mammalian cells, TBBPA and other brominated flame retardants inhibited

uptake of GABA and 5-HT (Mariussen and Fonnum, 2003). GABA is a conserved neurotransmitter in different phyla (Miller, 2019; Smart and Stephenson, 2019): in molluscs, GABA has been shown to affect larval settlement and metamorphosis, with possible downstream effects on serotonin pathways (Biscocho et al., 2018; Joyce and Vogeler, 2018, and refs therein). The high abundance of GABA-ir cells observed in control mussel trocophorae (at 24 hpf) is in line with the role of this neurotransmitter in the early developing mammalian brain, where the GABAergic system plays an important trophic role, even prior to synaptogenesis, in the proliferation of neuronal progenitor cells (reviewed in Wu and Sun, 2016). Overall, on the basis of our first observations, the dramatic decrease in GABA immunoreactivity induced by TBBPA further suggests that, in mussel larvae, TBBPA may act upstream of the first stages of neurodevelopment, to affect subsequent differentiation of serotonergic or other neurons. Interference with GABA signalling has been involved in TBBPA neurotoxicity in *Xenopus* and zebrafish embryos (Hendriks et al., 2012; Ye et al., 2016). However, irrespective of the mechanisms of action, in vertebrate developmental models significant effects of TBBPA were observed from concentrations generally higher than those utilized in the present study; this further emphasize the sensitivity of mussel early larval stages towards TBBPA.

469

470 **5. Conclusions**

The results demonstrate that TBBPA affected components of DOPA, 5-HT and GABA systems in mussel larvae from 24 hpf; from 28 hpf, these effects were accompanied by changes in deposition of organic matrix and calcification of the growing shell, and altered transcription pattern of genes crucial for shell formation. The overall effects of TBBPA resulted in larval malformations and delayed/arrested development at 48 hpf. Although for a mechanistic study all the endpoints were evaluated at a concentration two-fold higher than those detected in polluted coastal areas, to our knowledge, these results represent the demonstration of the effects of TBBPA at the lowest concentrations tested so far in bivalves.

479 The signaling pathways linking development of neurotransmitters with shell formation still need to
480 be elucidated; however, the data obtained so far (this work, Miglioli et al., 2021a) indicate that EDs
481 such as TBBPA and BPA can affect the complex processes involved in early neurodevelopment and
482 shell biogenesis in the model marine bivalve *Mytilus*.

483 The identification of the mode of action (MOA) of EDs in marine invertebrates can contribute
484 building Adverse outcome pathways (AOPs) (Ankley et al., 2010; Ford and Le Blanc, 2020),
485 encompassing relationships from molecular initiating events (MIE) caused by the initial exposure, to
486 a series of key events (KE), that lead to identification of effects on individuals (i.e. developmental
487 effects) and potential loss of population sustainability. Although the possible consequences of
488 TBBPA at the population level cannot at present be demonstrated, the results of this work provide
489 data on possible progression of the effects of this compound from the molecular to the individual
490 level.

491 A better understanding of the neuroendocrine function during development in model marine
492 invertebrates will greatly help understanding the impact of emerging pollutants, in particular EDs, as
493 well as of other environmental stressors, on more susceptible life stages of ecologically important
494 species in coastal environments.

495

496 **CRedit authorship contribution statement**

497 Angelica Miglioli: Investigation, Methodology, Data curation, Writing. Teresa Balbi: Investigation,
498 Data curation, methodology, Writing, review & editing, funding acquisition. Michele Montagna: data
499 curation, methodology. Remi Dumollard: Conceptualization, Supervision, Writing, funding
500 acquisition. Laura Canesi: Conceptualization, Supervision, Writing.

501

502 **Declaration of Competing Interest**

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

505

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Figure Legends

Figure 1 - Effect of TBBPA (1, 10 and 100 µg/L) on larval phenotypes at 48 hpf.

A) percentage of different phenotypes: normally developed D-Veligers (white), malformed larvae, (light gray), immature larvae with protruding mantle (dark grey), arrested trochophorae (black). Measurements were made on at least 50 larvae for each experimental condition obtained from 5 parental pairs (N=5).

B) representative images of the main phenotypes obtained with calcofluor/calcein staining in larvae exposed to 10 µg/L TBBPA, showing organic matrix (blue) and calcified areas (green) in the growing shell (scale bar: 20 µm).

Figure 2 - Effects of TBBPA (10 µg/L) on shell morphology of *Mytilus* larvae at 48 hpf, evaluated by SEM.

A) control samples with symmetric valves, straight hinge, uniform surfaces. B-E) TBBPA-exposed samples, showing shells with irregular surfaces, holes (arrowhead in B), fractures (C-E), convex hinges (C, D). Fractures perpendicular to the hinge were often observed (D, square in E enlarged in F).

717 **Figure 3 - Effects of TBBPA (1 µg/L) on shell morphology of *Mytilus* larvae at 48 hpf, evaluated**
718 **by SEM.**

719 A) control D-veligers, with symmetric valvae and uniform surface. B-D) TBBPA-exposed samples,
720 showing malformed shell with fractures perpendicular to the hinge region (B), immature shells,
721 characterized by polygonal shapes and asymmetric valvae (C, D). E-F) details of D (squares),
722 showing protruding mantle (E), and irregular calcification at the center of the valvae (F).

723

724 **Figure 4 - Effect of TBBPA (10 and 100 µg/L) on the time course of early shell formation in *M.***
725 ***galloprovincialis* from the trochophora to the first D-veliger stage at different times post-**
726 **fertilization (from top to bottom: at 24, 28, 32, 48 hpf).**

727 Columns correspond to experimental conditions (from left to right: Control, 10 and 100 µg/L
728 TBBPA). Representative images (lateral view) show merged fluorescent signals of calcofluor (blue)
729 and calcein (green) staining, respectively, the organic matrix and CaCO₃ deposition. White
730 arrowheads indicate missing calcification in the centre of each valva (scale bars: 10 µm).

731

732 **Figure 5 - Effect of TBBPA (10 and 100 µg/L) on the growth of organic matrix (blue) and**
733 **calcified shell (green) in mussel larvae.**

734 Areas of each shell component (µm²) measured in a single valva of TBBPA-exposed larvae are
735 reported as % values of control (unexposed) larvae at 24 (A), 28 (B) and 32 hpf (C). In D) ratios of
736 areas matrix/calcified shell in different experimental conditions are reported. Measurements were
737 made on at least 50 larvae for each condition obtained from 4 parental pairs (N=4). * p≤0.05, TBBPA-
738 exposed vs Controls; # Control 28 hpf vs 32 hpf. Oneway ANOVA Kruskal-Wallis followed by
739 Tukey's test.

740

741 **Figure 6 - Expression of genes related to shell formation in control and TBBPA-exposed larvae**
742 **of *M. galloprovincialis* evaluated by qPCR at different times pf.**

743 Controls (white bars); TBBPA (10 µg/L) (grey bars).

744 A) Chitin synthase; B) tyrosinase; C) carbonic anhydrase; D) Extrapallial Protein.

745 Data, reported as fold changes with respect to eggs, represent the mean $\pm$ SD (N=4). Data were
746 analysed by one way ANOVA Kruskal-Wallis followed by Tukey's test and statistical differences
747 between control and TBBPA-exposed samples at each developmental stage are reported (* = $p < 0.01$).
748 As previously described (Miglioli et al., 2019), expression of all transcripts was significantly different
749 at all times pf with respect to eggs ($p < 0.01$, not shown).

750

751 **Figure 7 - Expression pattern of Tyrosinase and Chitinase in control and TBBPA-exposed**
752 **larvae of *M. galloprovincialis* evaluated by ISH at different times pf.**

753 C: Control; TBBPA (10 µg/L); rows (from top to bottom: 24, 28, 32, 48 hpf). Scale bar: 20 µm.

754

755 **Figure 8 - Expression pattern of dopaminergic components control and TBBPA-exposed larvae**
756 **of *M. galloprovincialis* evaluated by ISH at different times pf.**

757 C: Control; TBBPA (10 µg/L); rows (from top to bottom: 24, 28, 32, 48 hpf). Tyrosine Hydroxylase
758 (TH), Dopamine- β -Hydroxylase (D β H), Dopamine Receptor 1 (DR1). Scale bar: 20 µm.

759

760 **Figure 9 - Confocal images of serotonin immunoreactive cells (5-HT-ir) in early larval stages of**
761 ***M. galloprovincialis* from 24 to 32 hpf.**

762 5-HT-ir cells are shown in Red/Pink (Ex/Em: 590/617 nm), Hoechst stained nuclei are shown in blue
763 (Ex/Em: 358/461 nm). A) control; B) TBBPA (10 µg/L).

764 Rows: 24, 28, 32 hours post fertilization-hpf.

765 Columns:

766 a) brightfield images; scale bar: 20 µm.

767 b) 5-HT and Hoechst merged channels; scale bar: 20 µm.

768 c) 4-5 X magnified images of column; white asterisks indicate the nuclei of 5-HT-*ir* cells (*) and
769 white arrowheads emerging neurites, respectively; scale bar: 5 µm.

770

771 **Figure 10 - Confocal images of serotonin immunoreactive cells (5-HT-*ir*) in early larval stages**
772 **of *M. galloprovincialis* at 48 hpf.**

773 5-HT-*ir* cells are shown in Red/Pink (Ex/Em: 590/617 nm), Hoechst stained nuclei are shown in blue
774 (Ex/Em: 358/461 nm). A) control; B) TBBPA (10 µg/L). In B) 5-HTR immunoreactivity is reported
775 in representative TBBPA-induced phenotypes.

776 Columns:

777 a) brightfield images; scale bar: 20 µm.

778 b) 5-HT and Hoechst merged channels; scale bar: 20 µm.

779 c) 4-5 X magnified images of column; white asterisks indicate the nuclei of 5-HT-*ir* cells, showing
780 that TBBPA-induced phenotypic alterations are associated with a reduction in the number of 5-HT-
781 *ir* cells (*); arrowheads indicate emerging neurites; scale bar: 5 µm.

782

783 **Figure 11 - Effect of TBBPA (10 µg/L) on development of serotonin immunoreactive cells (5-**
784 **HT-*ir*) in mussel larvae at 48 hpf.**

785 Box plot reporting the number of 5-HT-*ir* cells quantified in at least 12 larvae from 4 different parental
786 pairs (N=4). Data are reported as mean ± SD, indicating significant differences between control and
787 TBBPA-exposed samples * = p<0.01 (Mann-Whitney U test).

788

789 **Figure 12 - Confocal images of GABA immunoreactive cells (GABA-*ir*) in early larval stages of**
790 ***M. galloprovincialis* from 24 to 32 hpf.**

791 GABA-*ir* cells are shown in Red/Pink (Ex/Em: 590/617 nm), Hoechst stained nuclei are shown in
792 blue (Ex/Em: 358/461 nm). A) control; B) TBBPA (10 µg/L).

793 Rows: 24, 28, 32 hours post fertilization-hpf.

794 Columns:

795 a) brightfield images; scale bar: 20 μ m.

796 b) GABA and Hoechst merged channels; the z-stack acquisition is indicated in white (see Fig. S3).

797 Red arrowheads indicate the shell field region, and the perimeter of the larvae is indicated by a white
798 dotted line; scale bar: 20 μ m.

799

800 **Figure 13 - Confocal images of GABA immunoreactive cells (GABA-*ir*) in early larval stages of**
801 ***M. galloprovincialis* at 48 hpf.**

802 GABA-*ir* cells are shown in Red/Pink (Ex/Em: 590/617 nm), Hoechst stained nuclei are shown in
803 blue (Ex/Em: 358/461 nm). A) control; B) TBBPA (10 μ g/L). In B) GABA immunoreactivity is
804 reported in representative TBBPA-induced phenotypes.

805 a) brightfield images; scale bar: 20 μ m.

806 b) GABA and Hoechst merged channels, shown in 3 different z-stack acquisitions indicated in white
807 (see Fig. S4). Red arrowheads indicate the hinge region, and the perimeter of the larvae is indicated
808 by a white dotted line; scale bar: 20 μ m.

Figure 2

Figure 3

Figure 6

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Supplementary material for on-line publication only
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CRedit authorship contribution statement

Angelica Miglioli: Investigation, Methodology, Data curation, Writing. Teresa Balbi: Investigation, Data curation, methodology, Writing, review & editing, funding acquisition. Michele Montagna: data curation, methodology. Remi Dumollard: Conceptualization, Supervision, Writing, funding acquisition. Laura Canesi: Conceptualization, Supervision, Writing.

SUPPLEMENTARY INFORMATION to Miglioli et al.,

Tetrabromobisphenol A acts as a neuro-developmental disruptor in early larval stages of *Mytilus galloprovincialis*

qPCR

Primers pairs employed for qPCR analysis and their accession numbers in GenBank are reported in **Table S1** (Balbi et al., 2016, 2017, 2018; Miglioli et al., 2019).

Table S1

Gene	Primers	Amplicon size bp	Amplification efficiency (%)	Accession number
<i>EF1</i>	5'- CGTTTTGCTGTCCGAGACATG -3' 5'- CCACGCCTCACATCATTCTTG -3'	135	99	AB162021
<i>Hel</i>	5'- GCACTCATCAGAAGAAGGTGTC -3' 5'- GCTCTCACTTGTGAAGGGTGAC -3'	129	132	DQ158075
<i>CS</i>	5'- AACAGAAGCCAGGCACTATATC -3' 5'- GTCAGAACCAGCACAGTAGTC -3'	106	104	EF535882
<i>CA</i>	5'- ACCAGATGGTCTTGCAGTTT -3' 5'- TCATCTCTGACTGCTGCTAATG -3'	102	95	LK934681
<i>EP</i>	5'- TAAACTCTGGACACGCATACC -3' 5'- GAGTCCCTCTTGGTGCATATT -3'	100	100	AY364453
<i>TYR</i>	5'-CGATTCTTTATACATGAAATCTGTG -3' 5'-AAACCGTTATAACAACGTGCTAA -3'	113	111	KV583276.1
<i>5-HTR</i>	5'- CAGCTGCAAGATCGAGGATT -3' 5'- TGAAGCCATCTTGACTGACG -3'	144	117	AB526218

EF1 = *M. galloprovincialis* elongation factor-1 α ; HEL = *M. galloprovincialis* helicase; CS = *M. galloprovincialis* chitin synthase; CA = *M. edulis* carbonic anhydrase; EP = *M. edulis* extrapallial protein; TYR = *M. galloprovincialis* tyrosinase; 5-HTR = *M. edulis* Serotonin receptor.

Effect of Thyroid receptor agonists/antagonists on early larval development

Fertilized eggs were exposed to thyroid hormones [3,3,5,5-triiodothyronine-T₄ (T2376, Sigma Aldrich) and 3,3,5-triiodothyronine-T₃ (T6397, Sigma Aldrich)], the selective antagonist of human THR (1-850; 17248, Cayman Chemical Company) and Thiourea-TU (T8656, Sigma Aldrich). Stock

solutions of T₃ (0.3 M) and T₄ (0.4 M) were prepared in 0.2 M Sodium Hydroxide (NaOH). For TU and 1-850, 6 and 0.2 M stock solutions, respectively, were prepared in DMSO. Upon 10X serial dilution, fertilized eggs were exposed to final concentrations of 3, 4, 6 and 2 µM of T₃, T₄, TU and 1-850, respectively. The effect on development was evaluated in 48 hpf larvae by phenotype scoring.

Effect of the dopamine antagonist SCH 23390 on early larval development

The role dopaminergic signalling in shell formation was first evaluated utilizing the selective antagonist of DR1, SCH 23390 (Liu et al., 2018). Fertilized eggs were exposed to R(+)-SCH-23390 hydrochloride (D054, Sigma Aldrich). A stock solution of SCH (0.5 M in DMSO) was diluted in MFSW at 500 µM and tenfold diluted to obtain the final concentration 0.5 µM. Samples were analyzed by calcofulor/calcein staining at different times pf (24, 28, 32, 48 h) and phenotypes evaluated at 48 hpf as described above.

Results

Figure S1 - Effect of THR agonists and antagonists on larval development at 48 hpf.

Effects of T_3 (3,3,5-triiodothyronine), T_4 (3,3,5,5-triiodothyronine), 1-850 the selective antagonist of human THR, and Thiourea-TU on larval phenotypes: normally developed D-Veligers (white), with hinge malformations (light grey), pre-veligers (dark grey) and arrested trocophorae stage (black). Measurements were made on at least 50 larvae for each experimental condition obtained from 5 parental pairs (N=5).

Figure S2 - Effects of the DOPA Receptor 1 (DR1) inhibitor SCH 23390 (0.5 μ M) on *M. galloprovincialis* larval development.

- A) Calcofluor/calcein staining, showing organic matrix (blue) and calcified areas (green) in the growing shell at 24, 28, 32 and 48 hpf as in Fig. 1B and Fig. 4. Due to the weakness of both signals, images of SCH samples were overexposed to show the shell field from 24 to 32 hpf (arrowheads) and calcified areas at 48 hpf.
- B) Areas of the organic matrix (μm^2) measured in a single valva of TBBPA-exposed larvae. Data are reported as % values of control larvae at 24, 28 and 32 hpf. Details as in Fig. 5.
- C) Percentage of larval phenotypes: normally developed D-Veligers (white), malformed larvae (light gray), immature larvae with protruding mantle (dark grey), arrested trochophorae (black). All details as in Fig. 1A.

Figure S3 - GABA immunoreactivity and Hoechst staining in control (A) and TBBPA (10 µg/L) (B) exposed *Mytilus* larvae at 24, 28 and 32 hpf.

Larvae stained with GABA Ab were entirely imaged in 100 z-stacks, with Z-projections made every 10 sequential stacks (from z10 to z100). GABA-ir cells are shown in Red/Pink (Ex/Em: 590/617 nm), Hoechst stained nuclei are shown in blue (Ex/Em: 358/461 nm); the z-stack is indicated in white; the white dotted line indicates the perimeter of the larva. Scale bar: 20 µm.

Figure S4 - GABA-*ir* and Hoechst staining at 48 hpf in control (A) and TBBPA-exposed larvae (10 µg/L) (B-D).

Larvae stained with GABA Ab were entirely imaged in 100 z-stacks, with Z-projections made every 10 sequential stacks (from z10 to z100). GABA-*ir* cells are shown in Red/Pink (Ex/Em: 590/617 nm), Hoechst stained nuclei are shown in blue (Ex/Em: 358/461 nm); the z-stack is indicated in white; the white dotted line indicates the perimeter of the larva. Scale bar: 20 µm.

A) Normal D-veliger; B-D) altered phenotypes observed in TBBPA-exposed larvae; B) Indented hinge; C) Pre-veliger; D) Arrested trochophora.