Invertebrate Biology

Effects of Bisphenol A on pigmented organ development in the ascidian Phallusia mammillata

Journal:	*Invertebrate Biology*
Manuscript ID	Draft
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Messinetti, Silvia; Università degli Studi di Milano, Department of Environmental Science and Policy MERCURIO, SILVIA; Università degli Studi di Milano, Department of Environmental Science and Policy Pennati, Roberta; Università degli Studi di Milano, Department of Environmental Science and Policy
Keywords:	otolith, ocellus, rescue of function, tunicate, nervous system

Effects of Bisphenol A on pigmented organ development in the ascidian *Phallusia mammillata*

Messinetti Silvia, Mercurio Silvia*, Pennati Roberta

Department of Environmental Science and Policy, University of Milan, Via Celoria 2, 20133 Milan, Italy

*corresponding author sil.mercurio@gmail.com

Abstract

Bisphenol A (BPA) is an organic compound used in the manufacture of polycarbonate plastic and epoxy resins that is released into the environment from sewage treatment effluents, landfill leachate and degradation of plastic products. BPA can act both as teratogenic substance and as endocrine disruptor. Recently, alterations of sensory organs in zebrafish have been described after exposure to BPA. It was proposed that these alterations were due to the binding of BPA to Estrogen Related Receptor γ (ERR). The phylogenetic position of tunicates as sister group of vertebrates and their cosmopolitan distribution in marine ecosystems coupled with their ecology and easy manipulability make them reliable model organisms for ecotoxicology bioassays. Here we evaluated the effects of different concentration of BPA on ontogenetic processes in the ascidian *Phallusia mammillata*. BPA did not influence sperm fertilization capability but it impaired the development causing a phenotype characterized by short and kinked tail. Larvae developed from BPA exposed embryos presented also malformations to pigmented organs such as altered pigment deposition, absence of one or both pigmented organs and extranumerary organs. The co-exposure with 4-OHT, an ERR inverse agonist, produced a rescue of the normal phenotype of pigmented organs, supporting the hypothesis that BPA exerts its teratogenic effects binding to ERR as in vertebrate models.

Keyworlds: otolith, ocellus, rescue of function, tunicate, nervous system

Running title: Bisphenol A effects on *Phallusia mammillata*

Bisphenol A (BPA; 2,2-bis-(4-hydroxyphenyl)-propane; CAS Registry No. 80-05-7) is an organic compound originally developed as a synthetic hormone and then employed as a monomer for production of polycarbonate plastics and epoxy resins (Flint et al 2012). Nowadays, BPA production is continuously rising (Lin et al 2017) leading to a significant increase of its release in the environment (Flint et al 2012). This is particularly worrying as BPA is a well known teratogen and endocrine disruptor exerting its action through different nuclear and cytoplasmic receptors (Rubin 2011, Tohmé et al 2014). Initially, it was believed that BPA acts by binding to Estrogen Receptor and Androgen Receptor, even if its affinity for these receptors is extremely weak, 1000–10,000 times lower than those of natural hormones (Kuiper et al 1998, Rubin 2011). Thus, it was hard to explain how BPA could cause the severe adverse effects reported even after low doses exposure. Recently, the high specificity of BPA to the nuclear orphan Estrogen Related Receptor (ERR) γ was demonstrated in human cell lines, in which a competitive receptor binding assay revealed that BPA binding affinity to ERR γ (IC50) is extremely high, being of 13.1±2.34 nM (Takayanagi et al 2006, Okada et al 2008). ERRs get their name from sequence homology with estrogen receptors, but they do not bind estrogens or other steroid hormones. They possess their own Estrogen Related Receptor response Elements (ERREs), but they can also bind and activate transcription through both the Estrogen Responsive Elements (ERE) and the palindromic thyroid hormone response element (TREpal) in absence of a ligand (Xie et al 1999). In humans, three ERR are present, α, β, and γ. The homologs of these receptors are also present in zebrafish where they are segmentally expressed in the hindbrain. Similarly, the single ERR gene present in amphioxus genome is expressed in a segmented manner in a region considered homolog to the vertebrate hindbrain (Bardet et al 2005).

ERRs have a very high level of spontaneous constitutive activity that is increased by the binding of BPA through one of its phenol-hydroxyl groups (Okada et al 2008, Tohmé et al 2014). In zebrafish (*Danio rerio*), ERRγ is expressed in the otic vesicle (Bardet et al 2005, Tohmé et al 2014) and it was demonstrated that the binding of BPA to ERRγ induces alterations in otic vesicle and otolith formation during the first stages of embryonic development, from 5 to 48 hours post fertilization (Tohmé et al 2014). Moreover, these alterations were recovered by the co-exposure of zebrafish embryos to BPA and 4-Hydroxytamoxifen (4-OHT) (Tohmé et al 2014). 4-OHT is an inverse agonist of ERRγ that is known to decrease the constitutive activity of the receptor (Coward et al 2001). Alterations of sensory organs induced by the presence of BPA were also reported in the ascidians *Ciona robusta* (Messinetti et al unpublished data) and *Phallusia mammilata* (Dumollard et al 2017). In addition, in *C. robusta* BPA caused trunk and tail malformations and alteration of GABAergic and dopaminergic cells of the central nervous system (Messinetti et al unpublished data).

Ascidians, or sea squirts, are marine invertebrates, characterized by a filter feeding sessile adults and a swimming lecitotrophic larva. They have been confirmed as reliable model organisms for ecotoxicological bioassay, thanks to their easy manipulability, rapid embryonic development and high production of gametes (Zega et al 2009, Gallo & Tosti 2015). Moreover, they offer valuable advantages for exploring the molecular mechanism of pollutants due to their phylogenetic position, as members of the sister group of vertebrates, and their smaller genome compared to vertebrate ones (Passamaneck & Di Gregorio 2005). The solitary ascidian *Phallusia mammillata* develops through a tadpole larva, composed of a trunk and a tail, which shows the typical chordate body plan. The trunk comprises three adhesive papillae at its anterior end and the first portions of central nervous system, namely the sensory vesicle with the two pigmented sensory organs, the otolith and the ocellus, the

neck and the visceral ganglion. The tail contains the caudal nerve cord, the notochord and the muscles.

The aim of this work was to study the effects of BPA exposure on different ontogenetic processes in the ascidian *P. mammillata* in order to evaluate if different ascidian species display different sensitiveness to this molecule. Then, we co-exposed *P. mammillata* embryos to BPA and 4-OHT in order to gain insight into the molecular mechanism involved in the teratogenic effects of BPA, testing if the binding of this pollutant to ERR is responsible for the developmental impairment induced by BPA in these animals.

Methods

Animals and chemicals

Adults of the ascidian *P. mammillata* were collected from natural populations in Lerici (La Spezia, Italy), and maintained in aquaria filled with artificial sea water (ASW, Instant Ocean®, Aquarium System) and equipped with mechanical, chemical and biological filters. Salinity was set at 36 ‰ and temperature fixed at 16±1 °C. Constant light promoted the production of gametes and avoided their precocious release (Lambert & Brandt 1967). Eggs and sperm were obtained by the dissection of gonoducts of three hermaphrodite adults for each experiment. Since the use of self-sperm decreases fertilization success, cross fertilization was always performed. All procedures were carried out at 18±1°C in a thermostatic chamber. All the exposure experiments were conducted in glass Petri dishes (Ø 4 cm) filled with 10 ml of the tested solutions. Bisphenol A (BPA, PM=228.29) and 4-Hydroxytamoxifen (4-OHT, PM=387.51) were purchased from Sigma, Italy. Stock solution of 100 mM BPA was made dissolving 11.4 mg in 500 µl of dimethyl sulfoxide (DMSO, Sigma) and then diluted in filtered artificial sea water with 0.005 M Hepes pH 8.0 (ASWH) to reach the final test concentrations (0.1, 0.5, 1, 5, 10, and 20 µM). 4-OHT stock solution of 50 mM was obtained

dissolving 10 mg in 500 µl of DMSO and then diluted in ASWH to get the test solutions (1 and 2.5 µM). As a control and a solvent control, a solution of ASWH and 0.02% DMSO in ASWH was used respectively in each experiment. Fresh solutions were prepared every time.

Effects on sperm viability

To analyze BPA effects on sperm viability, 20 µl of dry sperm were diluted in 2.4 ml of ASWH and 300 µl of this solution were exposed to 5 ml of BPA at the different concentrations for 30 minutes. 200 µl of BPA-sperm solution were used to fertilized 60 eggs in 10 ml of ASWH in glass Petri dishes. 1 hour post fertilization (hpf), embryos were fixed in 4% Paraformaldehyde (PFA) dissolved in phosphate butter saline (PBS), and the fertilization rate (FR, percentage of fertilized eggs on total eggs) was calculated. The experiment was performed in triplicate and results were considered reliable only when FR of control samples was ≥ 80%.

Effects on fertilization

To test the BPA effects on fertilization, 60 unfertilized eggs and 15 µl of sperm dilution (20 µl of dry sperm in 2.4 ml of ASWH) were added to Petri dishes containing 10 ml of a solution of BPA at different concentrations (0.1, 0.5, 1, 5, 10, and 20 µM) in ASWH. After 1 hour, embryos were fixed in 4% PFA and the percentage of samples that completed the first cell division was calculated. The experiment was performed in triplicate and results were considered reliable only when FR of control samples was ≥ 80%.

Effects on embryogenesis

To analyze the effects of BPA, 4-OHT and their co-exposure on embryogenesis, cross-fertilization was performed in Petri glass dishes containing ASWH and maintained at 18±1 °C until, after 1 hour, the first division occurs. Then, about 100 embryos at two-cell stage were

exposed to the different concentrations of BPA (0.1, 0.5, 1, 5, 10, 20 µM), to two concentrations of 4-OHT (1 and 2.5 µM) and, based on observed incidence of malformations, to combinations of the two molecules (5/10/20 µM BPA plus 1/2.5µM 4-OHT). Each experiment was performed in triplicate and for each exposure 100 embryos (n:~300) were used. When controls reached the larva stage (~19 hpf), all specimens were fixed in 4% PFA and examined under a dissection microscope. For each treatment, the percentages of normal larvae, larvae with malformations and dead embryos were calculated.

Effects on pigmented organs

50 larvae exposed to BPA or to the mixtures of BPA and 4-OHT were observed under a dissection microscope to analyze malformations at the pigmented organs. These organs were scored as normal (one otolith and one ocellus completely formed), mildly affected (one otolith and one ocellus with abnormal deposition of pigments) or severely affected (absence of one or both pigmented organs or extranumerary pigmented organs).

Immunohistochemistry

Immunostaining of the nervous system was performed, as described in (Pennati et al 2003), on fixed larvae treated with different concentration of BPA. Monoclonal anti β-tubulin (clone 2-28-33, Sigma, Italy) antibody developed in mouse was used to label nervous system fibers, while polyclonal anti GABA antibodies made in rabbit (Sigma, Italy) was specific for GABAergic neurons. In brief, specimens were rinsed in 0.1% Tween-20 in Phosphate Buffer Saline (PBT), permeabilized with three washes for 10 minutes in a solution of 0.25% Triton X-100 in PBT and incubated for 2 hours in 50% PBT /50% Normal Goat Serum (NGS), previously deactivated at 55°C for 30 min. Then, samples were incubated overnight at 4°C with primary antibody diluted 1:500 in 10% NGS in PBT. After several washes in PBT, the

samples were incubated in 1% bovine serum albumine (BSA) in PBT for 2 h at room temperature and then incubated at 4°C overnight in PBT, in which Alexa Fluor 488 anti mouse IgG or anti rabbit IgG antibody, diluted 1:800, was added. Next, the specimens were washed multiple times in PBT and mounted in 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma, Italy) on microscope slides. Samples were examined using a confocal laser scanning microscope Leica SP2 (Leica Microsystems, Heidelberg, Germany), equipped with argon/krypton laser.

Statistical analysis

We used the analysis of variance (ANOVA) followed by HSD Tukey's post hoc test, performed with R software (R-Core-Team 2013) and 'agricolae' package (de Mendiburu 2015) to evaluate the statistical significance of the differences in fertilization rate and in larval development of samples treated with different concentrations of BPA or/and 4-OHT and controls. A Cochran test was performed to test the homogeneity and normality of the variances and percentage data were transformed when they did not meet the assumptions of the analysis (normality and homoscedasticity). The differences in pigmented organs development were statistically analyzed with χ^2 test.

Results

Effects on sperm viability and fertilization

The fertilization capability of sperm was not affected by the 30-minute pre-exposure to the different concentrations of BPA (from 0.1 µM to 20 µM): the differences in fertilization rate (FR, percentage of fertilized eggs on total eggs) were not statistically significant (F=0.7665; p=0.623; Fig. 1A).

FR was not altered even when the fertilization occurred in presence of the tested concentration of BPA (Fig. 1B). However, some of the fertilized eggs exposed at concentrations higher than 5 µM did not complete the first cell division and the number of these aberrant embryos increase with BPA concentration (Fig. 1C). The percentage of malformed two-cell embryos resulting from exposure to BPA was significantly different from that of control embryos starting from the 5µM BPA ($F=262.77$; $p<0.0001$; Tukey's post hoc test control versus 5µM $p<0.0002$; control versus 10µM $p<0.0001$; control versus 20µM $p<0.0001$).

Effects on embryogenesis

Exposure to BPA at concentrations lower than 10 µM did not significantly affect the general anatomy of *P. mammillata*. The body shape of the larvae appeared comparable to that of control larvae, at least by light microscopy (Fig. 2A), although at 10 µM BPA a slight increase in the percentage of body malformations was registered. On the contrary, exposure to 20 µM BPA resulted in a high percentage (94.3%) of severely malformed embryos; this incidence was significantly higher than that of controls ($F=98.338$ $p<0.0001$, Fig. 2A). The anatomical malformations consisted in shorted and kinked tail and malformed trunk with a reduction of papillae.

Immuno-labeling of the nervous fibers by anti β-tubulin antibody highlighted the impairment of the nervous system induced by the highest concentration of BPA. In control larvae, the antibody labeled the neurons of the papillae, the papillary nerves that connect the sensory cells of the papillae to the sensory vesicle, the complex of nervous fibers around the sensory vesicle and in the posterior dorsal neural tube (Fig. 3A and D). Larvae exposed to 20 µM BPA showed an altered nervous system, with disorganized fibers around the sensory vesicle and reduced or absent papillary sensory neurons (Fig. 3B-C and E-F).

Effects on pigmented organs

In addition to the previously described body malformation, abnormal development of pigmented organs was observed in larvae exposed to 5, 10 and 20 µM BPA. Indeed, a statistically significant increase of affected pigmented organs was recorded in BPA treated larvae compared to controls ($p<0.001$; Fig. 4A). We identified mild malformations consisting in abnormal deposition of pigment, and severe malformations consisting in the presence of extranumerary pigmented organs or absence of one or both organs (Fig. 5). Larvae with normal pigmented organs were almost absent in samples exposed to the 3 highest concentrations, while percentage of larvae with severe phenotypes increased with BPA concentration (Fig. 4A-C).

To better characterize these malformations, we performed an immunlocalization of GABA. GABA immunoreactivity in *P. mammillata* control larvae is associated with pigmented organs. In particular, GABA is localized in a fan of positive cells dorsal to the ocellus and in few cells of the stalk of the otolith, forming a cup-shaped structure (Fig. 6A and D). In larvae exposed to low concentrations of BPA, the localization of GABA was similar to that of control larvae and it was always recognizable a signal associated with the ocellus and one associated with the otolith (Fig. 6B-C and E-F). In larvae exposed to 20 µM BPA, where a higher percentage of severe phenotypes was recorded, we observed different conditions: 1) GABA was localized in a dorsal fan of cells in correspondence to the single pigmented organ that was for this reason identified as an ocellus (Fig. 6G an J); 2) the peculiar cup shaped signal of GABA allowed us to identify the presence of extranumerary otoliths (Fig. 6H and K); 3) it was not possible to discern which pigmented organ was differentiated as the signal was distorted in correspondence to extruded pigment (Fig. 6I and L).

Effects of 4-OHT co-exposure on pigmented organs

Malformations to sensory organs were similar to those observed in BPA treated zebrafish in which abnormal otoliths were reported. Thus, we considered that these alterations were due to a specific action of the molecule possibly by binding to EERs. To verify this hypothesis, we co-exposed embryos to BPA and 4-OHT, an inverse agonist of EER. Before testing the effects of the co-exposure of BPA and 4-OHT, we verified that the chosen 4-OHT concentrations did not affect *P. mammillata* development: the exposure to 1 and 2.5 µM 4-OHT did not alter the percentage of larvae that developed normally compared to controls ($F=1.776$, $p=0.2293$; Figure 2B). Due to the absence of any malformations to the larval anatomy and pigmented organs at the lowest BPA doses, co-exposure experiments were made combining 5, 10 and 20 µM BPA with the two 4-OHT concentrations. The co-exposure to 5 µM BPA and 4-OHT produced a partial rescue of the normal phenotype at the pigmented organ level. In fact, the percentage of normal phenotype increased from an average of 2.17% in 5 µM BPA exposed larvae to 24% in larvae co-exposed to 1 µM 4-OHT and 12.73% in larvae co-exposed to 2.5 µM 4-OHT. The differences in the incidence of normal phenotype between co-exposed larvae and BPA treated larvae were statistically significant ($p<0.001$ and $p=0.0126$ respectively) (Fig. 4A). The efficiency of 4-OHT to rescue the phenotype decreased with the increment of BPA concentrations. After co-exposure to 10 µM BPA and 1 µM 4-OHT, 11.32% of larvae showed a normal phenotype, a percentage significantly different from that observed after exposure to 10 µM BPA alone ($p=0.0218$; Fig. 4B). The reduction of severely affected phenotypes and the increment of mildly affected phenotypes after co-exposure to 20 µM BPA and 4-OHT was not statistically significant ($p>0.1150$; Fig. 4C).

Discussion

There is a high concern about the effects induced by the organic pollutant Bisphenol A on human and wildlife health (Crain et al., 2007; Rochester, 2013; Rubin, 2011). To understand

the possible consequences of an increase of this pollutant in aquatic environment, ecotoxicological bioassays have been performed on numerous species, reporting different sensitiveness to BPA (Flint et al 2012). For example, low doses as 0.1 µg/l and 0.08 µg/l caused developmental inhibition and delayed larval emergency in the crustacean *Tigriopus japonicas* and in chironimids respectively (Marcial et al 2003, Watts et al 2003), while concentrations ranging from 0.83 and 100 µg/l administrated for 90 days, did not produce any effects in the amphibian *Xenopus laevis* (Pickford et al 2003). These differences of effects at similar concentration can be due to the distant phylogenetic position of the aforementioned taxa. However, the dose of 250 mg/kg/day produced an increase in testosterone levels in the mammal *Microtus agrestis* (Nieminen et al 2002b), while it did not produce any effects in the mammal *Mustela putorius* (Nieminen et al 2002a), highlighting that BPA is differently tolerated also in closely related species. On the other hand, different studies performed on *X. laevis* found really variable results based on the concentration tested, the length and the developmental stages of exposure (Kloas et al 1999, Iwamuro et al 2003, Oka et al 2003, Levy et al 2004, Sone et al 2004), underling that differences can be observed also in the same species. As regarding ascidians, previous studies have been conducted in *C. intestinalis* and *C. robusta* (Cangialosi et al 2013, Matsushima et al 2013, Messinetti et al unpublished data), reporting a variety of effects on developmental processes. In the present work, we explore the ontogenetic impairments induced by BPA in another ascidian species, *P. mammillata*. Comparably to what observed in *C. robusta* (Messinetti et al unpublished data) the exposure of sperm to BPA did not alter the fertilization rate in *P. mammillata* and the co-exposure of sperm and eggs compromised the first cell division at BPA concentrations higher than 5 µM. If the exposure occurs after the first cell division, embryos of *P. mammillata* could reach the larval stage also in presence of BPA, even if at the highest tested concentration, 20 µM BPA, all the larvae were malformed. On the contrary, in *C. robusta,* 20 µM BPA was lethal

(Messinetti et al unpublished data), suggesting that differences in the tolerance to BPA action are also common in these marine invertebrates. Immunostaining with anti-β tubulin showed an alteration of neural fiber pattern in *P. mammillata*, comparable to that observed in *C. robusta*. These malformations can be ascribed to a pleiotropic toxic action of the molecule (Wetherill et al 2007). Moreover, we observed that in *P. mammillata* the sensory organs, otolith and ocellus, showed a wide range of alterations at concentrations of BPA that did not produce external morphological malformations. Mild alterations, as the abnormal deposition of pigmentation, and severe alterations, as the presence of supernumerary pigmented organs, the presence of a single organs or the absence of pigmented organs were observed. These highly reproducible malformations were considered to be due to a specific action of BPA. In fact, alterations of the sensory organs were reported also in zebrafish after exposure to BPA (Gibert et al 2011, Tohmé et al 2014). In this animal, it was demonstrated that induced malformations were due to the interaction between BPA and an Estrogen Related Receptor (Tohmé et al 2014). The *C. intestinalis* genome revealed the presence of 17 nuclear receptors that comprise a single ERR. A ERR sequence is also present in *P. mammillata* transcriptome and it is available on the databank ANISEED (https://www.aniseed.cnrs.fr). The partial rescue of the phenotype that we observed after co-exposure of embryos to BPA and 4-OHT suggests that also in ascidians the teratogenic mechanism of BPA may be triggered by binding to this nuclear receptor. The rescue was observed in larvae treated with 5 µM BPA and 1µM 4-OHT. Higher concentrations of BPA were toxic and the presence of 4-OHT did not manage to reverse the effects of the pollutant. Interestingly, as revealed also by GABA immunolabelling, we observed both the presence of extranumerary otoliths and ocelli and the absence of one or both pigmented organs. Since ascidians develop with a fixed cell lineage, it has been possible to trace the embryological origin of the pigmented organs. In *Ciona intestinalis*, they derived from a symmetrical pair of cell from the a-line, particularly from the a8.25 pair (Nishida

1987, Nicol & Meinertzhagen 1988b, Nicol & Meinertzhagen 1988a). Origin and differentiation of pigmented organs have been well studied in the ascidian *Halocynthia roretzi* (Nishida & Satoh 1989, Darras & Nishida 2001), whom development is similar and comparable to that of *Ciona intestinalis* and *Ciona robusta*. The fate of the two pigmented cells is induced by A-line nerve cord precursors (Nishida 1991), but the left-right position is not correlated to the final structure (otolith or ocellus; (Nishida 1987). After neural tube closure, the two a10.97 cells line up along the dorsal midline, the anterior one becomes the otolith and the posterior one the ocellus (Darras & Nishida 2001), and this differentiation is probably due to a gradient in expression of *bone morphogenetic protein* (*BMP*) and *chordin* (Darras & Nishida 2001). Different experiments on ablation of pigment cells precursors and overexpression of *BMP* or *chordin* produces phenotypes without pigmented organs or with one or more than 2 pigmented organs (Nishida & Satoh 1989, Darras & Nishida 2001).

It is to be established which step of the pathway that lead to the correct differentiation of the two organ is altered by the action of the BPA. The mechanism is finely tuned by extracellular and intracellular environment, and it is possible to suppose that according to which step is altered by the action of the BPA, it can be produced an extra otolith and extra ocellus or none of them. The findings of this work suggested that the effects of BPA on *P. mammillata* are comparable to the ones obtained in *C. robusta,* even though *P. mammillata* tolerated higher concentrations of BPA during embryogenesis. These considerations are of particular importance for future ecotoxicological studies confirming the great variability of sensibility to pollutants even among strictly related species. The reasons of this different sensibility are not known but can be associated to different detoxificant capability or to different permeability of the egg coat that can partially block the diffusion of the pollutants through the chorion membrane. Moreover, the co-exposure with an inverse agonist of ERR partially rescued the normal phenotype, suggesting that in ascidians the mechanism of action of this pollutant is the

same reported in zebrafish. Finally, this study confirmed the emerging role of ascidians as model organisms to predict the effects and define the mode of action of pollutants on vertebrates and humans.

Aknowledgements/Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors have no conflict of interest to declare.

References

Bardet PL, Schubert M, Horard B, Holland LZ, Laudet V, et al. 2005. Expression of estrogen-receptor related receptors in amphioxus and zebrafish: implications for the evolution of posterior brain segmentation at the invertebrate-to-vertebrate transition. *Evolution and Development* 7: 223-33

Cangialosi MV, Mansueto V, Faqi AS. 2013. Bisphenol A (BPA) and atrazine inhibits the embryonic development of *Ciona intestinalis* (ascidiacea, urochordata). *Caryologia* 66: 97-102

Coward P, Lee D, Hull MV, Lehmann JM. 2001. 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor γ. *Proceedings of the National Academy of Sciences* 98: 8880-84

Darras S, Nishida H. 2001. The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. *Developmental biology* 236: 271-88

de Mendiburu F. 2015. agricolae: Statistical Procedures for Agricultural Research.

Dumollard R, Gazo I, Gomes IDL, Besnardeau L, McDougall A. 2017. Ascidians: An Emerging Marine Model for Drug Discovery and Screening. *Current Topics in Medicinal Chemistry* 17: 2056-66

Flint S, Markle T, Thompson S, Wallace E. 2012. Bisphenol A exposure , effects , and policy : A wildlife perspective. *Journal of Environmental Management* 104: 19-34

Gallo A, Tosti E. 2015. The Ascidian *Ciona Intestinalis* as model organism for ecotoxicological bioassays. *Journal of Marine Science: Research & Development* 5: e138

Gibert Y, Sassi-Messai S, Fini J-B, Bernard L, Zalko D, et al. 2011. Bisphenol A induces otolith malformations during vertebrate embryogenesis. *BMC developmental biology* 11: 1471-213.

Iwamuro S, Sakakibara M, Terao M, Ozawa A, Kurobe C, et al. 2003. Teratogenic and anti-metamorphic effects of bisphenol A on embryonic and larval *Xenopus laevis*. *General and comparative endocrinology* 133: 189-98

Kloas W, Lutz I, Einspanier R. 1999. Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. *Science of the Total Environment* 225: 59-68

Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, et al. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* 139: 4252-63

Lambert CC, Brandt CL. 1967. The effect of light on the spawning of *Ciona intestinalis*. *Biological Bulletin* 132: 222-28

Levy G, Lutz I, Kruger A, Kloas W. 2004. Bisphenol A induces feminization in *Xenopus laevis* tadpoles. *Environmental Research* 94: 102-11

Lin Z, Wang L, Jia Y, Zhang Y, Dong Q, Huang C. 2017. A Study on Environmental Bisphenol A Pollution in Plastics Industry Areas. *Water, Air, and Soil Pollution* 228: 98

Marcial H, Hagiwara A, Snell TW. 2003. Estrogenic compounds affect development of harpacticoid copepod *Tigriopus japonicus*. *Environmental toxicology and chemistry* 22: 3025-30

Matsushima A, Ryan K, Shimohigashi Y, Meinertzhagen Ia. 2013. An endocrine disruptor, bisphenol A, affects development in the protochordate *Ciona intestinalis*: Hatching rates and swimming behavior alter in a dose-dependent manner. *Environmental Pollution* 173: 257-63

Messinetti S, Mercurio S, Pennati R. unpublished data. Bisphenol A affects neural development of the ascidian *Ciona robusta.*

Nicol D, Meinertzhagen IA. 1988a. Development of the central nervous system of the larva of the ascidian, *Ciona intestinalis* L: I. The early lineages of the neural plate. *Developmental biology* 130: 721-36

Nicol D, Meinertzhagen IA. 1988b. Development of the central nervous system of the larva of the ascidian, *Ciona intestinalis* L: II. Neural plate morphogenesis and cell lineages during neurulation. *Developmental biology* 130: 737-66

Nieminen P, Lindstrom-Seppa P, Juntunen M, Asikainen J, Mustonen AM, et al. 2002a. In vivo effects of bisphenol A on the polecat (*Mustela putorius*). *Journal of Toxicology and Environmental Health, Part A* 65: 933-45

Nieminen P, Lindstrom-Seppa P, Mustonen A, Mussalo-Rauhamaa H, Kukkonen JVK. 2002b. Bisphenol A affects endocrine physiology and biotransformation enzyme activities of the field vole (*Microtus agrestis*). *General and Comparative Endocrinology* 126: 183-89

Nishida H. 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. . *Developmental biology* 121: 526-41

Nishida H. 1991. Induction of brain and sensory pigment cells in the ascidian embryo analyzed by experiments with isolated blastomeres. *Development* 112: 389-95

Nishida H, Satoh N. 1989. Determination and regulation in the pigment cell lineage of the ascidian embryo. *Developmental Biology* 132: 355-67

Oka T, Adati N, Shinkai T, Sakuma K, Nishimura T, Kurose K. 2003. Bisphenol A induces apoptosis in central neural cells during early development of *Xenopus laevis*. *Biochemical and biophysical research communications* 312: 877-82

Okada H, Tokunaga T, Liu X, Takayanagi S, Matsushima A, Shimohigashi Y. 2008. Direct evidence revealing structural elements essential for the high binding ability of bisphenol a to human estrogen-related receptor-γ. *Environmental Health Perspectives* 116: 32-38

Passamaneck YJ, Di Gregorio A. 2005. *Ciona intestinalis*: Chordate development made simple. *Developmental Dynamics* 233: 1-19

Pennati R, Groppelli S, Sotgia C, Zega G, Pestarino Ma. 2003. WAY-100635, an antagonist of 5-HT(1A) receptor, causes malformations of the CNS in ascidian embryos. *Development genes and evolution* 213: 187-92

Pickford DB, Hetheridge MJ, Caunter JE, Hall AT, Hutchinson TH. 2003. Assessing chronic toxicity of bisphenol A to larvae of the African clawed frog (*Xenopus laevis*) in a flow-through exposure system. *Chemosphere* 53: 233-35

R-Core-Team. 2013. R: A language and environment for statistical computing.

Rubin BS. 2011. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. *The Journal of Steroid Biochemistry and Molecular Biology* 127: 27-34

Sone K, Hinago M, Kitayama A, Morokuma J, Ueno N, et al. 2004. Effects of 17β-estradiol, nonylphenol, and bisphenol-A on developing *Xenopus laevis* embryos. *General and comparative endocrinology* 138: 228-36

Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y. 2006. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. *Toxicology Letters* 167: 95-105

Tohmé M, Prud'homme SM, Boulahtouf A, Samarut E, Brunet F, et al. 2014. Estrogen-related receptor γ is an in vivo receptor of bisphenol A. *FASEB Journal* 28: 3124-33

Watts MM, Pascoe D, Carroll K. 2003. Exposure to 17α-ethinylestradiol and bisphenol A e effects on larval moulting and mouthpart structure of *Chironomus riparius*. *Ecotoxicology and Environmental Safety* 54: 07e215

Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, et al. 2007. In vitro molecular mechanisms of bisphenol A action. *Reproductive Toxicology* 24: 178-98

Xie W, Hong H, Yang NN, Lin RJ, Simon CM, et al. 1999. Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. *Molecular endocrinology,* 13: 2151-62

Zega G, Pennati R, Candiani S, Pestarino M, De Bernardi F. 2009. Solitary ascidians embryos (Chordata , Tunicata) as model organisms for testing coastal pollutant toxicity. *Invertebrate survival journal* 6: S29-S34

Figures and tables

Figures

Figure 1: Effects on sperm viability (A), on fertilization rate (B) and proportion of fertilized eggs that reach correctly the two cell stage (C) at different concentrations of BPA in *P. mammilata*. Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from Control. The number of each symbol indicate the level of significance according to R significance codes: $p<0.001$ '***' ; $p<0.01$ '**'; $p<0.05$ '*'.

Figure 2:Effects of BPA (A) and 4-OHT (B) on embryogenesis in *P. mammillata*. Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from Control. The number of each symbol indicate the level of significance according to R significance codes: $p<0.001$ '***' ; $p<0.01$ '**'; $p<0.05$ '*'.

Figure 3: Immunostaining of the nervous system with anti β-tubulin antibody in *P. mammillata*. Bright field images and confocal microscope. (A,D) Control larvae, (B,C,E,F)

Larvae developed from embryos treated with 20 µM BPA. psn=Papillary sensory neurons; pn= papillary nerves; sv= sensory vesicle; cnc= caudal nerve corde.

Figure 4: Effects of co-exposure to BPA and 4-OHT on pigmented organs at 5 µM BPA (A), 10 µM BPA (B) and 20 µM BPA (C). Legend of symbols: *= differences from Control; °=differences from 5 µM BPA; +=differences from 10 µM BPA. The number of each symbol indicate the level of significance according to R significance codes: $p<0.001$ '***' ; $p<0.01$ '**'; $p<0.05$ '*'

Figure 5: Malformation of pigmented organs. (A) normal phenotype, (B) mildly affected phenotype, (C-F) severlely affected phenotype, particularly (C-D) extranumerary pigmented organs, (E) one pigmented organ, (F) absence of pigmented organs.

Figure 6: Immunostaining of the nervous system with anti GABA antibody in *P. mammillata*. Bright field images and confocal microscope. (A,D) Control larvae, (B,E,C,F) Larvae with mild malformation of pigmented organs, (G-L) Larvae with severe malformation of pigmented organs.

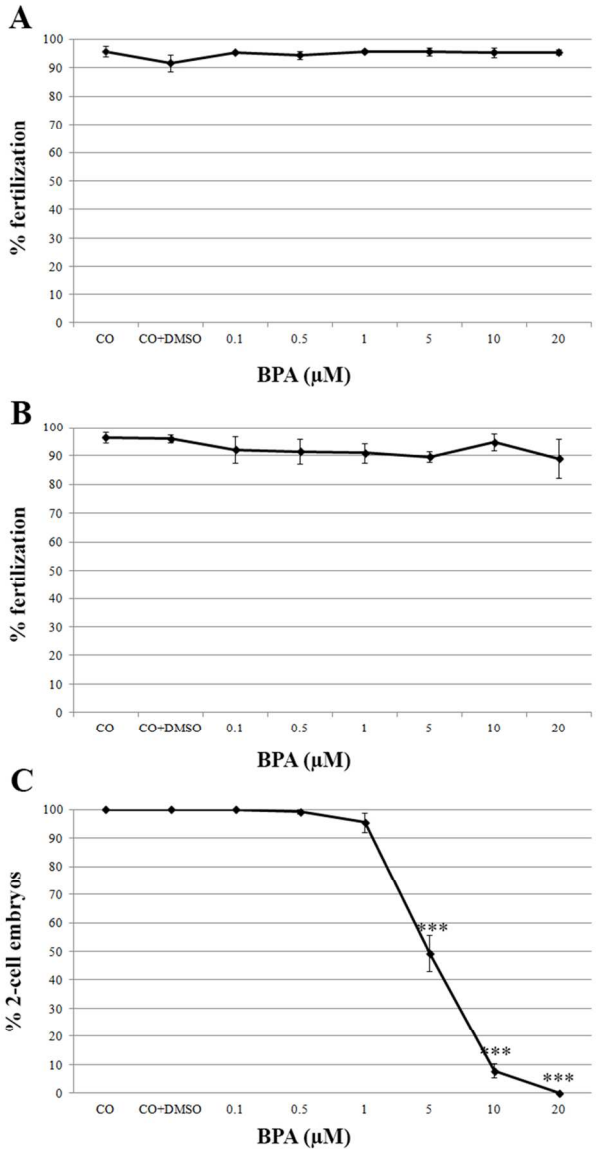

Figure 1: Effects on sperm viability (A), on fertilization rate (B) and proportion of fertilized eggs that reach correctly the two cell stage (C) at different concentrations of BPA in P. mammilata. Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from Control. The number of each symbol indicate the level of significance according to R significance codes: p<0.001 '***' ; p<0.01 '**'; p<0.05 '*'.

87x155mm (300 x 300 DPI)

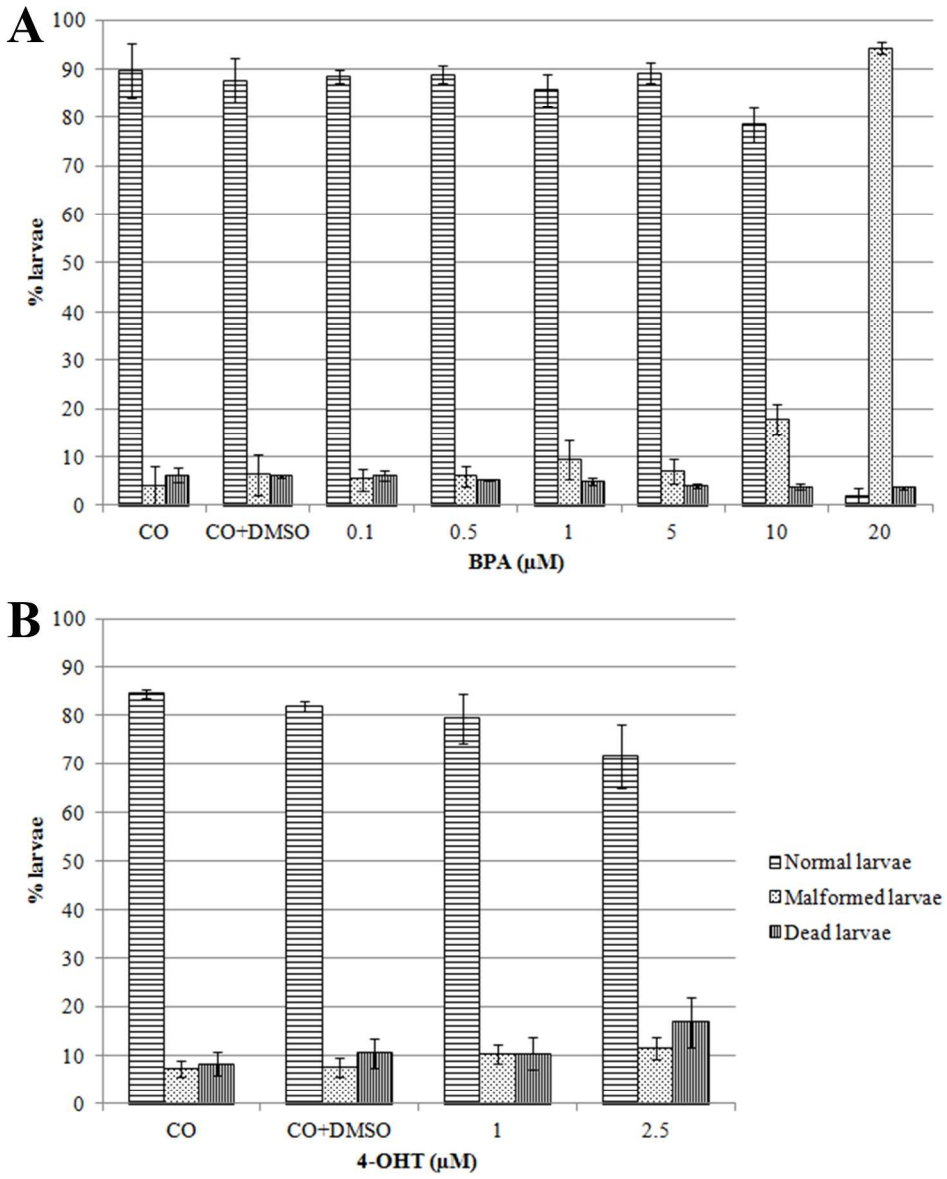

Figure 2: Effects of BPA (A) and 4-OHT (B) on embryogenesis in P. mammillata. Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from Control. The number of each symbol indicate the level of significance according to R significance codes: p<0.001 '***' ; p<0.01 '**'; p<0.05 '*'.

232x291mm (300 x 300 DPI)

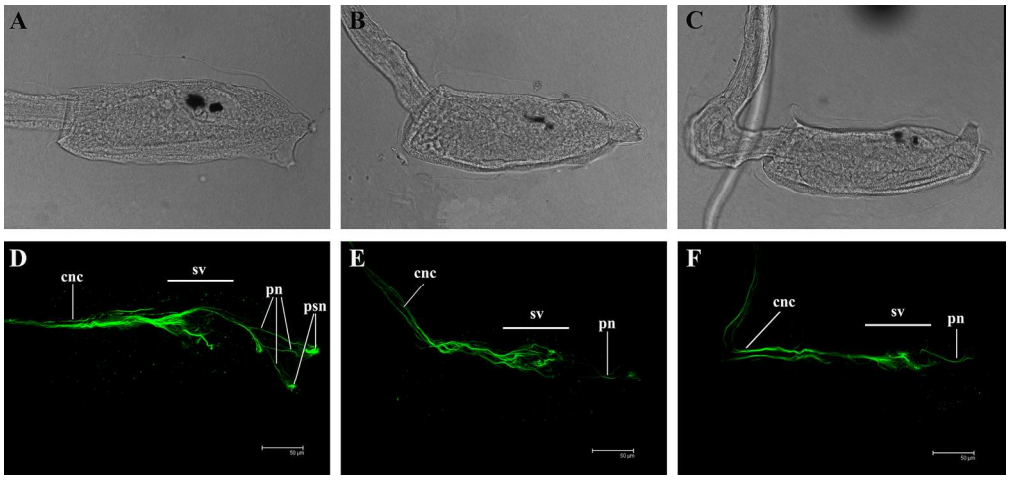

Figure 3: Immunostaining of the nervous system with anti β-tubulin antibody in P. mammillata. Bright field images and confocal microscope. (A,D) Control larvae, (B,C,E,F) Larvae developed from embryos treated with 20 µM BPA. psn=Papillary sensory neurons; pn= papillary nerves; sv= sensory vesicle; cnc= caudal nerve corde.

175x81mm (300 x 300 DPI)

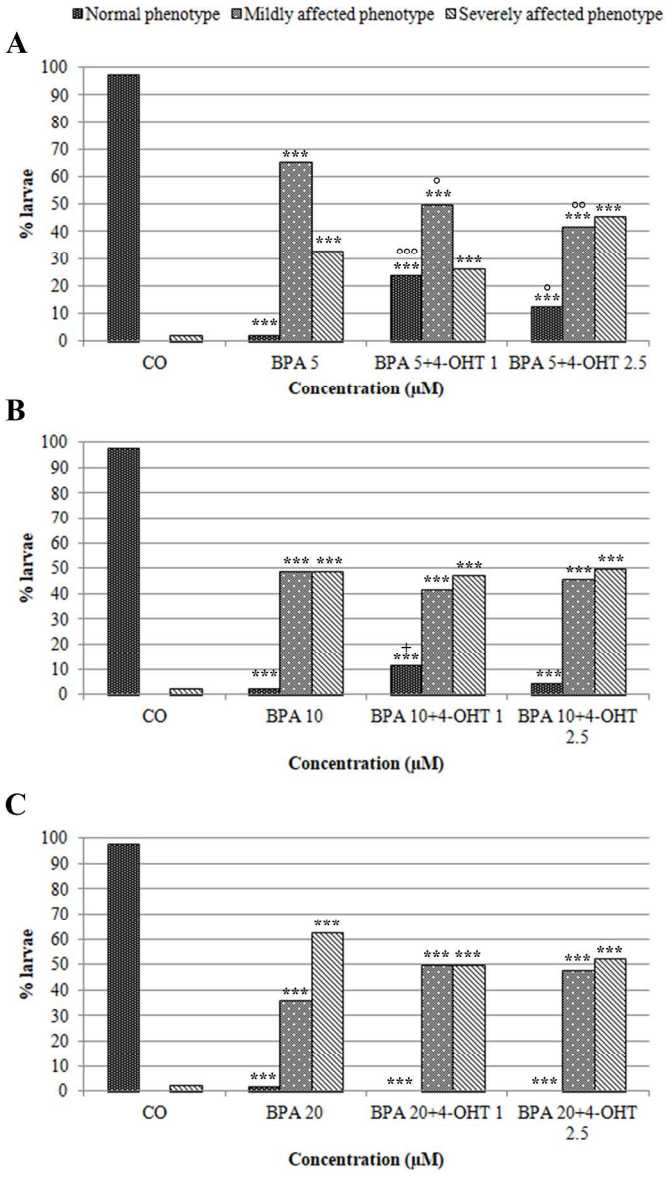

Figure 4: Effects of co-exposure to BPA and 4-OHT on pigmented organs at 5 µM BPA (A), 10 µM BPA (B) and 20 µM BPA (C). Legend of symbols: *= differences from Control; °=differences from 5 µM BPA; +=differences from 10 µM BPA. The number of each symbol indicate the level of significance according to R significance codes: $p<0.001$ '***' ; $p<0.01$ '**'; $p<0.05$ '*'

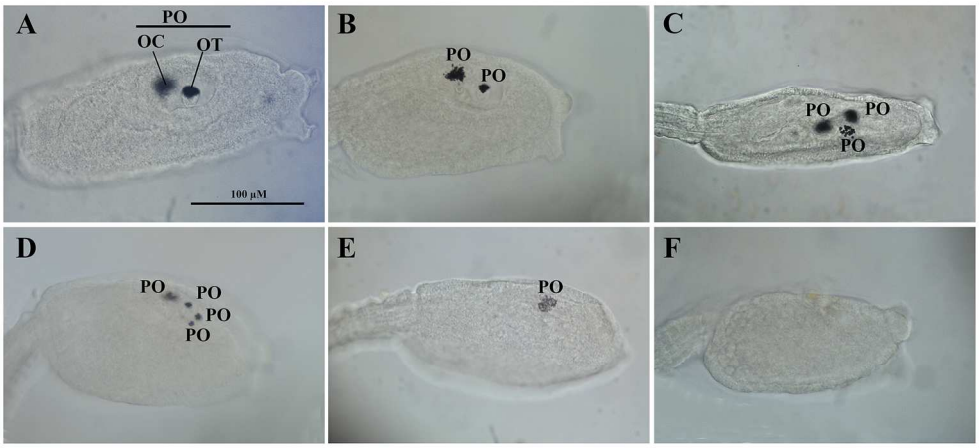

Figure 5: Malformation of pigmented organs. (A) normal phenotype, (B) mildly affected phenotype, (C-F) severlely affected phenotype, particularly (C-D) extranumerary pigmented organs, (E) one pigmented organ, (F) absence of pigmented organs.

175x80mm (300 x 300 DPI)

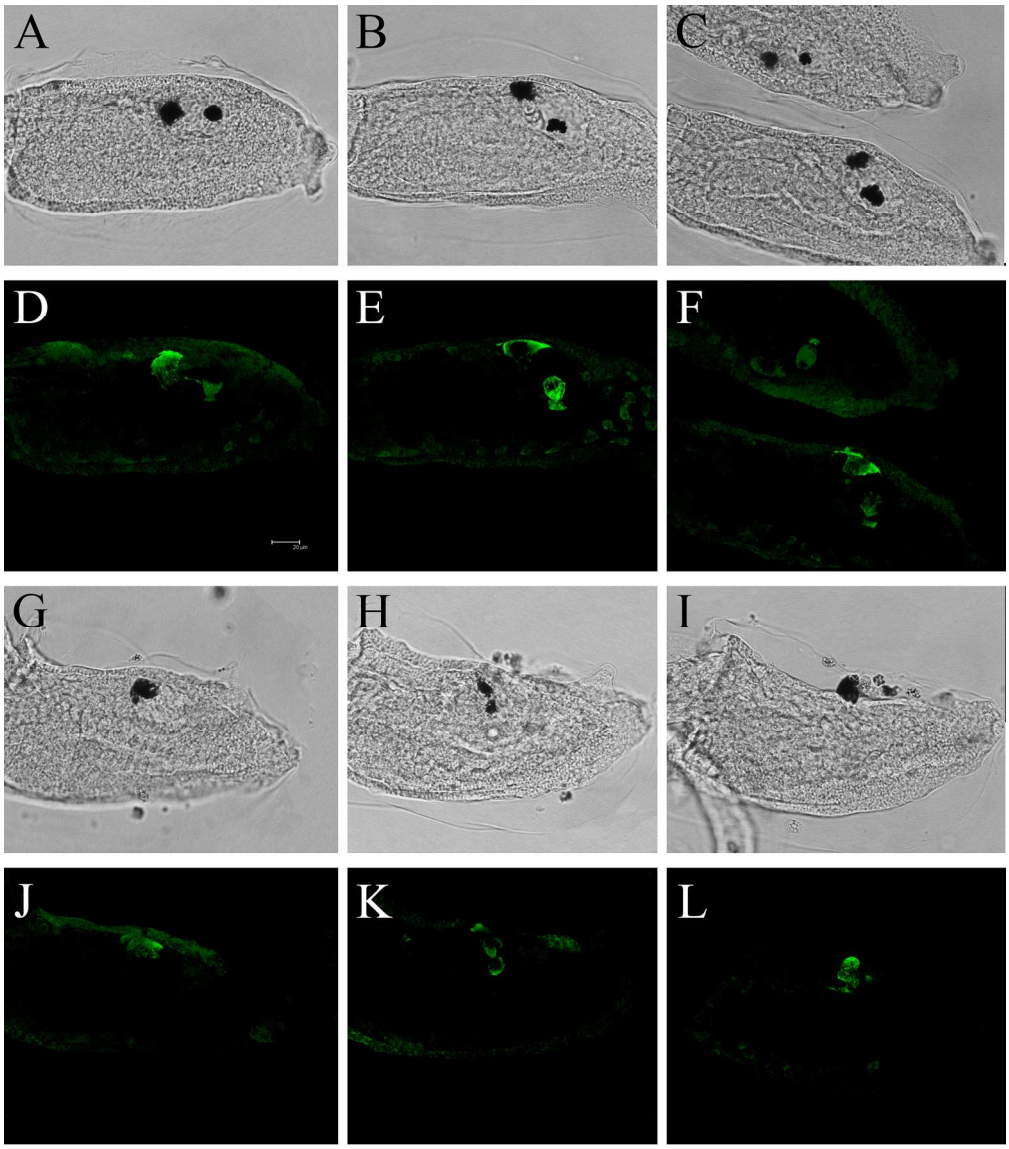

Figure 6: Immunostaining of the nervous system with anti GABA antibody in P. mammillata. Bright field images and confocal microscope. (A,D) Control larvae, (B,E,C,F) Larvae with mild malformation of pigmented organs, (G-L) Larvae with severe malformation of pigmented organs.

175x198mm (300 x 300 DPI)