



The unpredictable effects of mixtures of androgenic and estrogenic chemicals on fish early life

M.P. Sárria^a, M.M. Santos^a, M.A. Reis-Henriques^a, N.M. Vieira^{a,b}, N.M. Monteiro^{c,d,*}

^a CIMAR/CIIMAR-Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 177, 4050-123 Porto, Portugal

^b Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

^c CIBIO, Research Centre in Biodiversity and Genetic Resources, Campus Agrário de Vairão, R. Padre Armando Quintas, 4485-661 Vairão, Portugal

^d CEBIMED, Faculdade de Ciências da Saúde da Universidade Fernando Pessoa, Rua Carlos da Maia, 296, 4200-150 Porto, Portugal

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ABSTRACT

Estuarine shallow areas and coastal lagoons are known to receive and concentrate multiple inputs, either from land, rivers or coastal areas, being intensively impacted by chemical contamination, namely endocrine disrupting chemicals (EDCs). Despite the ubiquitous coexistence of several classes of EDCs in most of these aquatic ecosystems, there is still limited information regarding their combined effects. Furthermore, given the immediate implications for population dynamics, the available laboratory studies almost invariably focus on very specific life history stages, such as embryonic development or reproduction, thus creating a gap on our knowledge of what happens in between. During this 'intermediate phase', the newborn larvae and juveniles face numerous challenges whose outcome may impair reproduction or even survival.

The black-striped pipefish, *Syngnathus abaster*, member of the Syngnathidae family (comprising pipefish, seahorses and seadragons), usually breeds in coastal areas such as estuaries, where its newborns are immediately exposed to EDCs. Given the ongoing decline of pipefish populations, together with the observed shrinkage and fragmentation of seagrass meadows, known to be impacted by EDCs, a first reasonable question to address is if pipefish newborns respond to environmentally relevant concentrations of ubiquitous EDCs, either single or in combination. Hence, a seven days exposure experiment to the estrogenic chemical ethinylestradiol (EE₂) and the androgenic chemical tributyltin (TBT), single and in binary mixtures, was conducted. Selected behavioural (e.g. predator avoidance) and developmental variables (e.g. growth) were monitored in pipefish juveniles after EDCs insult. The obtained results indicate that EE₂, TBT, or their combined exposure, do impact pipefish early life. However, the pattern of results emerging from the measured variables clearly indicates that mixtures significantly modulate newborn responses in distinct ways when compared to individual chemical's exposure. These findings further demonstrate the importance of addressing the issue of chemical mixtures of pollutants acting through dissimilar mode of action. Independently of all the observed response variations, an ultimate conclusion seems certain: EE₂ and TBT, single or in combination, induce disruption patterns able to imbalance pipefish survival. Since these (as well as other) contaminants are present in estuarine areas, profound implications in population structure could be expected, ranging from a decrease in recruitment to a disruption of sexual selection. Inexorably, these stressors simultaneously operate in already declining populations.

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1. Introduction

Endocrine disrupting chemicals (EDCs), a group of exogenous and endogenous compounds able to interfere with hormone-controlled physiological processes, are increasingly widespread in the environment. The adverse effects of EDCs upon the fauna and flora, together with the persistence in the natural ecosystems of several classes of EDCs (Trussell, 2001), fully justified the establishment of a growing public concern (COM(1999)706; WHO, 2002; EPA(2009)EDSP),

which was rapidly translated into a research increase in endocrine disruption mediated by contaminant exposure (e.g. crustaceans: Rodríguez et al., 2007; gastropods: Ellis and Pattisina, 1990; amphibians and reptiles: Crain and Guillette, 1998; Kloas, 2002; fish: Fent et al., 1998; Thibaut and Porte, 2004; Mills and Chichester, 2005; Rempel and Schlenk, 2008; Ankley et al., 2009; birds and mammals: Ottinger and Saal, 2002; humans: Murray et al., 2001; Waring and Harris, 2005; Caserta et al., 2008). From all the impacted ecosystems, the aquatic environments are amongst the most thoroughly studied, given the increasing concentration of chemicals either from point and non-point sources (Sumpter, 2005; Matthiessen et al., 2006).

* Corresponding author. Tel.: +35 1252660411; fax: +35 1252661780.
E-mail address: nmonteir@fc.up.pt (N.M. Monteiro).

The best-documented example of endocrine disruption in wildlife is still the masculinisation of neogastropod females (*imposex*) caused by the antifouling agent tributyltin (TBT) (Santos et al., 2005). The TBT masculinisation effect has also been recognized in fish (McAllister and Kime, 2003; Shimasaki et al., 2003; Santos et al., 2006). In fact, TBT is now known to affect several animal taxa (Moore et al., 1991; Fisher et al., 1999; Jensen et al., 2004; Zhang et al., 2008; Guo et al., 2010; Revathi and Munuswamy, 2010), an already anticipated outcome given the persistent nature of this xenobiotic together with its wide distribution, being easily detected both in freshwater, estuarine and coastal ecosystems (Fent, 1996). Besides TBT, other contaminant classes have also been thoroughly studied, with a particular incidence on estrogenic chemicals (Jaser et al., 2003; Vosges et al., 2008; Gyllenhammar et al., 2009; Soares et al., 2009; Saaristo et al., 2010). In natural ecosystems, EDCs occur in complex mixtures such as estrogenic, androgenic, anti-estrogenic or anti-androgenic. Hence, ecotoxicological evaluations of a single contaminant may not reflect the multitude of antagonistic or synergistic stimuli that wildlife species surely faces (Lopes et al., 2005). Recent studies have clearly demonstrated the importance of addressing the issue of mixture effects of EDCs if we want to improve risk assessment of this class of chemicals. Today, it is well established that mixtures of estrogenic chemicals (ECs), acting through a similar mode of action, operate in an additive manner. In fact, mixtures of ECs elicited biological responses for concentrations that were known to be ineffective when the selected compounds were administered alone (Silva et al., 2002; Brian et al., 2005; Correia et al., 2007). In contrast to the somewhat predictable effects of mixtures of ECs, the information available on mixture effects of EDCs that act through dissimilar modes of action seems to be a rather unexplored ground (Spurgeon et al., 2010). Interestingly, despite the ubiquitous coexistence of both estrogenic and androgenic contaminants in aquatic ecosystems, only a few published studies have addressed their combined effects in fish (Santos et al., 2006; Micael et al., 2007; Greco et al., 2007; Sun et al., 2009).

While the consequences of EDCs exposure, single or in combination, have been mainly addressed either during the embryonic development or reproductive phase, given the immediate implications for population dynamics, attention must be devoted also to the time period that lies in between. During this phase, the newborn larvae, or juvenile, face numerous challenges such as feeding or escaping predation. In fact, since predation is the main cause of early mortality (Cushing, 1974), any disturbance on the basic escape mechanisms, including startle responses or locomotion performance, potentially impact population structure more than imbalances occurring during reproduction (Houde, 1987). All of the above-cited behaviours can be severely impacted by EDCs (Little et al., 1990; Carlson et al., 1998; Zhou and Weis, 1998; McGee et al., 2009). However, data regarding effects on fish larvae movement and development under EDC exposure are still scarce, and even more so when xenobiotics are administered in mixtures, since the resulting interactions are vastly unknown.

Estuarine shallow areas and coastal lagoons are amongst the most productive aquatic habitats (Kneib, 1997). These particular environments perform a relevant ecological function, serving as spawning and nursery areas for numerous fish species (Franco et al., 2006). Interestingly, these areas are also intensively impacted by chemical contamination (Ridgway and Shimmield, 2002; Pojana et al., 2007). Specifically, *Zostera* sp. seagrass meadows have been recurrently reported as impacted by EDC exposure (Francois et al., 1989; Chesworth et al., 2004). This marine angiosperm, as well as related aquatic vegetation, has declined extensively worldwide (Martin et al., 2010), probably due to the ongoing anthropogenic pressure, which is causing increased fragmentation and degradation of estuarine habitats. This habitat loss is especially critical for some syngnathid species, already considered globally threatened given the declining

populations (IUCN, 2004). Even though the causes of syngnathid decline remain still partially unclear (Kendrick and Hyndes, 2003; Martin-Smith and Vincent, 2005), it remains to be addressed if EDC impacts not only the estuarine primary production but also the syngnathid populations. The family Syngnathidae (seahorses, seadragons and pipefishes) presents unique reproductive characteristics, namely male pregnancy which occurs in a specialized brooding area, the marsupium (Monteiro et al., 2005). Since some species, such as black-striped pipefish, *Syngnathus abaster* Risso (1826), migrate to estuarine areas during the breeding season, it can be predicted that these populations, comprising both reproductively mature individuals as well as their newborns, may be at risk. Even though there is virtually no information on endocrine disruption effects on syngnathids, this family, and especially *S. abaster*, can be viewed as an interesting model for the assessment of ecosystem health due to several factors: i) it is a species with a considerable life span, ii) it migrates to estuarine areas in order to breed, iii) male pregnancy and larvae release from the marsupium occurs within the estuarine zone, and iv) the first stages of development, from larvae to ready to migrate juveniles, occurs in the estuary, especially within seagrass meadows. If we take into consideration that the populations of *S. abaster* are also in decline (Pombo et al., 2002), a first reasonable question to address is if pipefish, especially newborns, are vulnerable to environmentally relevant concentrations of EDCs. Furthermore, since most of the current research is mainly focused on the action of EDCs on reproduction, it seems important to evaluate non-reproductive parameters, such as growth and activity patterns, that can act as rapid-measurable variables able to reflect the effects of EDCs at a much earlier stage of development. Any maladaptive alteration on larvae/juvenile morphology or behaviour can be translated into increased mortality rates, which can greatly contribute to the observed population declines. Thus, in the present study, special attention was devoted to the larvae response to the presence of a potential predator (the mosquitofish, *Gambusia affinis*), given that predation modulates early mortality, influencing fish larvae density that reach adulthood.

Finally, considering that newborn syngnathids immediately contact with TBT and the synthetic EC ethinylestradiol (EE₂), two molecules known to sometimes elicit antagonistic responses in marine organisms, it is important to clarify not only the hazard posed by each chemical independently, but also recognize the interactions created under mixture exposures. Furthermore, by simultaneously monitoring several distinct variables, either behavioural or morphological, it will be possible to determine if EDC mixtures globally induce any type of “predictable” alteration patterns in newborn pipefish.

2. Materials and methods

2.1. Chemicals

17- α -ethinylestradiol [C₂₀H₂₄O₂] (EE₂, purity \geq 98%, Sigma-Aldrich); tributyltin chloride [C₁₂H₂₇ClSn] (TBTCl, purity 97%, Sigma-Aldrich); acetone (pro-analysis, purity \geq 99.8%, Merck, Darmstadt).

2.2. Biological model

Syngnathus abaster (Pisces; Syngnathidae) is a small brown-green benthonic euryhaline pipefish with a restricted distribution that includes the Mediterranean, Black Sea and the Atlantic coast of southwest Europe up to southern Biscay (Dawson, 1986) (Fig. 1). This pipefish occurs either in coastal areas or in brackish and fresh waters habitats (Cakic et al., 2002), between 0.5 and 5 m, within a temperature range of 8 to 24 °C (Dawson, 1986). Males exhibit a brood pouch located ventrally on the tail (*Urophori*; Herald, 1959)—that consists of two skin folds that contact medially with their free edges. Females, usually larger than males deposit the oocytes in this specialized incubating area (marsupium), where the male fertilizes

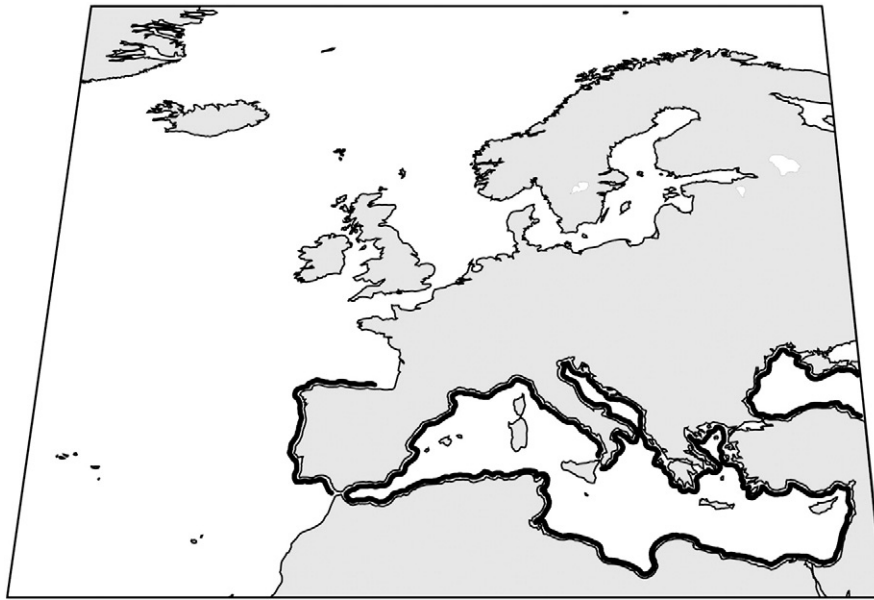


Fig. 1. Geographical distribution of *Syngnathus abaster* (as presented by Dawson, 1986).

them. Newborn pipefish abandon the marsupium, exhibiting a uniform dark colouration that is similar to that of the adults, and no further care is provided (Silva et al., 2006).

2.3. Normalization of prime conditions

88 pregnant males were captured, during the breeding season, at Ria de Aveiro, Portugal (40° 39' N; 8° 39' W) and initially maintained in a 150 L aquarium. Since parental quality or spawning investment may influence larvae morphology and behaviour (see Silva et al., 2009), special care was taken to ensure that the selected newborns were randomly selected from the hatchlings of a pool of 20 different males (all of which within the same stage of pregnancy, as determined by visual inspection). *Gambusia affinis* (Poeciliidae) adult females were also collected at Aveiro, using a hand net. The mosquitofish were maintained in separate aquaria, but in similar conditions as those described for *S. abaster*. Since the role of the mosquitofish was to be used as a potential predator (visual stimulus), the individuals were kept fasting, in order to avoid a complete lack of interest in *S. abaster* newborns.

2.4. EDCs exposure

Five days after exiting the marsupium, 180 newborns were randomly selected from a pool of approximately 1000 individuals. Juveniles, in groups of 10, were transferred to 5 L aquaria and exposed to EE₂ (nominal concentrations: 3 and 9 ng/L), TBTCl (nominal concentrations: 10 and 50 ngSn/L) as well as all possible combinations, during seven days. Acetone was used as organic solvent (0.000114%). Thus, a total of 9 treatments, including control, were replicated twice and maintained in closely controlled physicochemical conditions (25‰ artificial salt water; 14:10 h photoperiod; 21 ± 1 °C). 75% water renovation was conducted every other day. Newborns were fed daily with *Brachionus plicatilis* and 48 h *Artemia franciscana* nauplii.

2.5. Video recording and collected data

At the end of the exposure (day 7), the juveniles were randomly assigned to two 30 L aquaria. These aquaria were physically divided into two asymmetrical sections. A larger section (about two thirds) contained the pipefish juveniles while the smaller section contained

the potential predator (*G. affinis*). The glass division that separated the two sections allowed the juveniles to see the mosquitofish, but prevented any chemical communication, meaning that the pipefish were not receiving any chemical stimulus from the *G. affinis* females that, in turn, were not subject to any kind of EDC exposure. The stimulus fish were initially kept from sight by an opaque blind.

Given that newly born *S. abaster* tend to adopt a benthic distribution (Silva et al., 2006) while avoiding potential predators, it could be expected that newborns would tend to occupy the quadrant of the aquaria located near the bottom and further away from the glass division. Thus, this was the area selected for the registry of the selected variables: (1) number of movement bursts and (2) time spent on this more secluded area. Ten minutes after juvenile transference, the visual barrier was slowly removed and the juvenile *S. abaster* behaviour was digitally video-recorded (Sony DCR-SR36) for ten minutes.

Preliminary observations showed that juveniles individually placed in one aquarium tended to adopt abnormal behaviours (ranging from complete immobility to random patterns of locomotion). Thus, it was decided to mimic the conditions observed in the wild, where juveniles are present in groups, by placing a group of five individuals together, while only registering the behaviour of three. The response pattern obtained seemed to be consistent across treatments (see Results section), thus ruling out the effects of a hypothetical pseudo-replication, where the behaviour of one larvae would strongly influence the response patterns of the others.

At the end of the behavioural experiments, all 180 juveniles were photographed and measured (L_T) to the nearest millimetre. Measurements of the eye, pupil and dorsal fin (see Fig. 2a) were obtained using UTHSCSA IMAGE TOOL (v3.00). These variables were selected given their potential implications in vital processes such as swimming performance (predator escape or prey capture) or vision (predator and prey recognition).

2.6. Statistical analysis

All assumptions were met prior to data analysis. ANOVA model (two factors: EE₂ and TBT, each with three levels: zero, lower and higher EDC concentration) was conducted to determine if pipefish growth was affected by the selected contaminants, using body length as the dependent variable. To avoid biases associated with covariates, two ANCOVAs were conducted to determine the influence of the EDCs

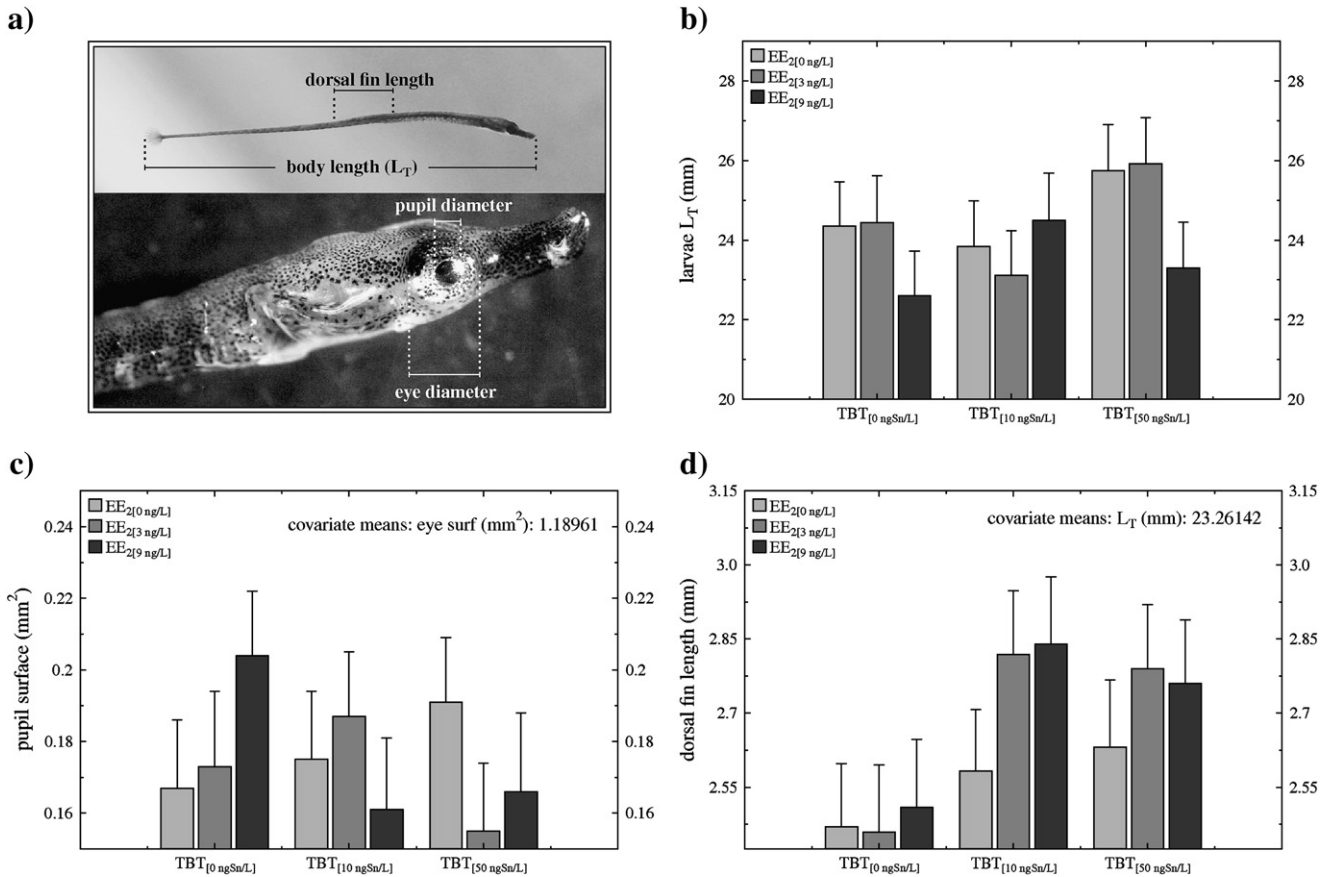


Fig. 2. The effects of EDCs exposure on *Syngnathus abaster* morphological development: a) a juvenile and a detail of the head showing the selected variables; b) effects of TBT and EE₂, single or in combination, on body length; c) pupil surface; and d) dorsal fin length.

on the pupil surface and dorsal fin length (eye surface and body length, respectively, were used as co-variables). The two behavioural variables considered (number of movement bursts and time spent on the secluded area) were used as dependent variables in a MANOVA design [two factors: EE₂ and TBT (each with three levels: zero, lower and higher EDC concentration)], to test for differences between treatments. Post hoc comparisons were conducted using Student–Newman–Keuls (SNK). A *P* value of 0.05 was used for significance testing. Analyses were performed in STATISTICA (v7).

3. Results

3.1. Larvae mortality

Given the team’s past experience in rearing *S. abaster* juveniles in aquaria, the juvenile mortality during the experimental period was considered reasonably negligible (7 out of 180 juveniles, scattered among 6 aquaria out of 18).

3.2. EDCs exposure and morphological development

When analysing the effects of TBT and EE₂, single or in combination, in juvenile growth, a significant interaction was observed between the selected EDCs (Table 1A). Even though the obtained pattern is of difficult interpretation, a general trend suggests that while EE₂ seems to depress growth, TBT produces the opposite results. Nevertheless, it can also be perceived that growth is diminished when juveniles are exposed to a mixture of the highest concentrations of both TBT and EE₂ (SNK post-hoc test, data not shown), suggesting that TBT effects in juvenile growth are counteracted when in presence of the highest EE₂ concentrations (Fig. 2b).

When considering the effects of the selected EDCs on the surface of the fish pupil, when adjusted for global eye surface (see Fig. 2c), the ANCOVA results show a significant interaction between the considered factors (Table 1B). An increase in EE₂ concentration is translated

Table 1

Syngnathus abaster morphometric analysis. (A) ANOVA results on the body length of juveniles exposed to EE₂ (nominal concentrations: 0, 3 and 9 ng/L) and TBT (nominal concentrations: 0, 10 and 50 ngSn/L), as well as all possible combinations. (B) ANCOVA results on the pupil surface of juveniles exposed to EE₂ and TBT, as well as all possible combinations (eye surface used as co-variable). (C) ANCOVA results on the dorsal fin length of juveniles exposed to EE₂ and TBT, as well as all possible combinations (body length used as co-variable).

Source of variation	DF	MS	F	P
A)				
EE ₂	2	24	3.672	<0.05
TBT	2	27	4.174	<0.05
EE ₂ *TBT	4	23	3.631	<0.01
Error	163	6		
B)				
Eye surface	1	0.027	40.768	<0.01
EE ₂	2	0.000	0.397	0.674
TBT	2	0.001	0.776	0.465
EE ₂ *TBT	4	0.003	5.092	<0.01
Error	62	0.001		
C)				
Body length (L_T)	1	3.067	73.134	<0.01
EE ₂	2	0.160	3.817	<0.05
TBT	2	0.648	15.461	<0.01
EE ₂ *TBT	4	0.051	1.221	0.309
Error	79	0.042		

into larger pupil surfaces. When TBT is administered alone, a similar pattern is also visible. Nevertheless, when both contaminants are combined, an opposite trend is observed with a decreasing pupil size.

The ANCOVA results on the effects of EDCs in dorsal fin length (adjusting for fish size), showed no significant interaction (Table 1C). Both considered factors, EE₂ and TBT, produced a similar (parallel) pattern of results, with the tested concentrations increasing the length of the dorsal fin (see Fig. 2d). SNK showed that both tested TBT concentrations were significantly different from the control. Although a similar pattern was visible for EE₂ (see Table 1C, where EE₂ showed significant effects), the post-hoc test was not able to detect differences between the concentration levels.

3.3. EDCs exposure and juvenile behaviour

When simultaneously considering the number of movement bursts and time spent on the aquarium's most secluded area, the MANOVA showed a significant interaction between the tested contaminants. Overall, similarly to what was observed for the pupil surface, EE₂ seems to increase both the number of bursts and time spent on the protected area (see Fig. 3). The highest EE₂ concentration produced a significantly higher number of movement bursts (SNK, data not shown). TBT alone favours an increase in time spent in the surveyed area, but not in the amount of bursts. When in combination, it seems that TBT is able to depress EE₂ effects on the number of bursts in the protected area, whereas EE₂ at 9 ng/L significantly decreased the time spent in the protected area at the highest TBT exposure level.

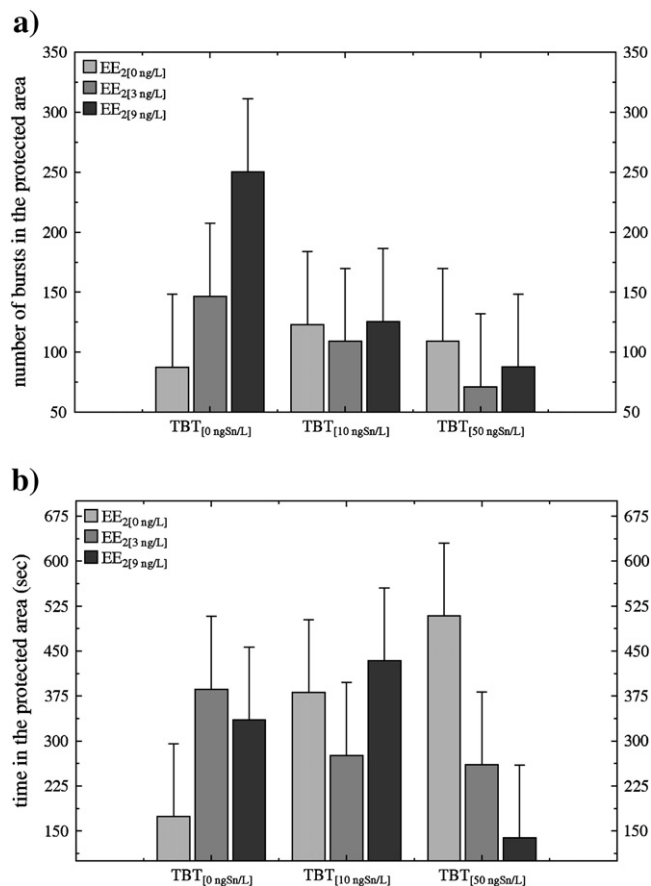


Fig. 3. The effects of EDCs exposure on *Syngnathus abaster* behaviour: a) effects of TBT and EE₂, single or in combination, in the number of movement bursts and b) time spent on the aquarium's most secluded area.

4. Discussion

Far from being considered a model organism for ecological risk assessment in aquatic environments, the present study has provided evidence that pipefish are sensitive to EDCs, even when exposed for a short time period during juvenile stages. This early life response is especially interesting if the long life span of this species is taken into account, meaning that imbalances in fish physiology or behaviour can be detected very early on, without the need of waiting for sexual maturity (where the majority of the endpoints traditionally addressed in endocrine disruption studies are concentrated). Variables related to basic survival mechanisms, such as predator recognition and avoidance, or phenotypic alterations, which ultimately relate to population structure and maintainability (if juveniles do not reach adulthood, no offspring will be produced), do have the potential to become valid early warning signals. In fact, recent investigations, using juvenile zebrafish (*Danio rerio*), unveiled specific behavioural fingerprints elicited by small neuroactive molecules (Kokel et al., 2010; Rihel et al., 2010). These results demonstrate the power of high-throughput locomotor responses in the discovery and characterization of psychotropic drugs. Relatively simple behavioral tests may, thus, realistically help estimate toxic effects that would only become apparent, when using more orthodox approaches, after longer time periods (Little et al., 1990). These rapid tests can act as reliable proxies for the determination of toxic effects even in species not usually considered for ecological risk assessment.

Fish larvae development is strictly dependent on a coordinated regulation of energy metabolism. Since lipids are viewed as the most energetic cost-effective biomolecules, any chemical able to disrupt lipid homeostasis, such as organotin compounds (Grun et al., 2006), can be viewed as having the potential to interfere with pipefish development. Alterations in size can have profound implications in pipefish reproductive dynamics since size does matter when it comes to selecting a partner. Both male and female *S. abaster* prefer larger partners (Silva et al., 2008, 2010).

As the generality of syngnathids, *S. abaster* mating choices are highly dependent on visual communication (Silva et al., 2007). Therefore, it can be predicted that any disruption on the sensory machinery responsible for picking up visual cues (e.g. size, coloration or behaviour) can severely impact sexual signaling. Recently, Sundin et al. (2010) stated that an environmental change, such as water turbidity, hampered the strength of mate choice in the sex role-reversed species *Syngnathus typhle*, due to vision impairment. Males of the broad-nosed pipefish did not seem to compensate for a reduction in visibility by using alternative sensory cues, such as olfaction, neither did females compensate by increasing their courtship activity. Even considering that there is limited information regarding the effects of toxicants on eye function and visual processing in fish species (Weis et al., 1987; Fent, 1992; Bentivegna and Piatkowski, 1998), the obtained results show that, when exposed to the mixture of TBT plus EE₂, pipefish juveniles developed smaller pupils and became more lethargic. Unlike most vertebrates, the iris of most teleosts does not present muscle cells and, thus, is unable to dilate and contract. The pupil diameter generally remains unchanged, even in response to a variation in ambient illumination (Douglas et al., 2002). Guthrie and Banks (unpublished data) demonstrated that several hours of light or dark adaptation produce no measurable change in the diameter of the pupil of the perch's eye. However, under the effect of chemicals, a reduction of the pupil's surface was observed within two hours. Even though it remains to be demonstrated that an alteration in pupil diameter is directly translated into poorer eyesight, juveniles exposed to the highest concentrations of TBT plus EE₂ greatly diminished the amount of time spent in the most secluded area. This 'kamikaze' behaviour (spending time near a starving mosquitofish) will surely decrease the chances of reaching sexual maturity, where visual communication is also crucial for reproduction.

The study of mixtures of EDCs in the environment is a major emerging issue in ecotoxicology and environmental risk assessment. Whereas most ecotoxicological studies have typically focused on single chemical exposures, the contact with complex mixtures, outside the laboratory, is the rule, not the exception (Hotchkiss et al., 2008). While the concept of concentration additive effects seems to accurately predict the toxicity of chemicals with a common mode of action, such as ECs, the established approaches have a limited use to predict the effects of mixtures of other classes of chemicals or EDCs acting through a dissimilar mode of action (Vijver et al., 2010; Spurgeon et al., 2010). In the present study the results from the measured variables clearly indicate that mixtures of TBT and EE₂ significantly modulate larvae responses in an unpredictable way when compared to single chemical exposure. Hence, the effects of the combined exposure of TBT and EE₂ are difficult to forecast, given that different patterns emerged when different variables and concentrations were considered. Nevertheless, although largely unpredictable, a common denominator can be withdrawn from all the experiments: mixtures of TBT and EE₂ have the potential to severely impact endpoints known to affect pipefish offspring survival.

5. Conclusion

All the presented results indicate that EE₂ and TBT influence morphological and behavioral development during pipefish early life. Nevertheless, the combined effects of the selected contaminants are far from producing a predictable outcome. Some of the measured variables showed clear interactions between the tested compounds. This unpredictability stresses the importance of considering mixture effects in environmental risk assessment of EDCs. Independently of all the observed response variation, an ultimate conclusion seems certain: EE₂ and TBT, single or in combination, induce disruption patterns able to imbalance pipefish early survival. Since these (as well as other) contaminants are indeed present in estuarine areas, profound implications in population structure could be expected ranging from a decrease in recruitment to a disruption of sexual selection, all simultaneously operating in already declining populations.

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