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Involvement of Retinoid X Receptor in Imposex Development in *Nucella lapillus* and *Nassarius reticulatus*—Preliminary Results

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Abstract—The imposex phenomenon provides one of the best examples of endocrine disruption in wildlife and has been studied for about four decades. However, the exact biochemical mechanism by which it develops is still unclear. Several pathways have been proposed: neuroendocrine, steroid and retinoic. In the present work the possible role of retinoid X receptor (RXR) in the development of imposex in *Nucella lapillus* and *Nassarius reticulatus* was investigated: snails of both species were injected with ethanol containing tributyltin (TBT) or with fetal bovine serum (FBS) containing 9-*cis*-retinoic acid (9CRA: the natural ligand for humans RXRs) and maintained in a flow-through system with artificial seawater for 30 days (temperature: 18.4 ± 0.44°C; salinity: 33.0 ± 1.06‰).

Both TBT and 9CRA induced imposex development in *N. lapillus* and *N. reticulatus*. Significant ($p < 0.05$) increases in the *vas deferens* sequence index (VDSI) and female penis length (FPL) were registered between the ethanol control and the TBT treatment and between the FBS control and the 9CRA treatment. The obtained results provide further evidence of the involvement of RXR signalling pathway on imposex development in both species.

Keywords: imposex, *Nassarius reticulatus*, *Nucella lapillus*, retinoid X receptor (RXR), tributyltin (TBT), 9-*cis*-retinoic acid (9CRA)

INTRODUCTION

Imposex is considered the best documented example of endocrine disruption in wildlife (Matthiessen and Gibbs, 1998). About 200 gastropod species worldwide are affected by this phenomenon (Shi *et al*., 2005) and some are routinely used as bioindicators of TBT pollution. Although the causal relationship between TBT and imposex is more than consensual in the scientific literature, the exact mechanism of TBT action on imposex induction still remains under debate. Several hypotheses have been postulated in order to explain the mechanisms underlying imposex induction and three possible pathways have been identified:

the neuroendocrine, the steroid and the retinoic. Succinctly, the neuroendocrine pathway suggests that the homeostasis of certain neuroendocrine factors can be disrupted by TBT, leading to the formation of accessory sex organs in females (Féral and Le Gall, 1983; Oberdörster and McClellan-Green, 2000, 2002). The steroid pathway postulates that TBT causes an imbalance in steroid hormones characterized by higher testosterone levels in females which leads to the imposex development; this disruption in steroid homeostasis can be promoted either through interferences with the steroid biosynthesis (see Spooner *et al*., 1991; Bettin *et al*., 1996) or with steroid excretion (see Ronis and Mason, 1996; Gooding *et al*., 2003). The retinoic pathway proposes that TBT and triphenyltin (TPT) mimic the endogenous ligand of retinoid X receptor (RXR) and thus activate the signaling cascades which are retinoic acid dependent (Nishikawa *et al*., 2004; Castro *et al*., 2007; Horiguchi *et al*., 2007a, 2007b, 2010, in press). So far, both neuroendocrine and steroidal theories could not provide unequivocal experimental results as they were unable to promote the increase in female penis length to the same extent as TBT whilst in the RXR theory a single injection of 9-*cis*-retinoic acid (9CRA), the natural ligand for human RXRs, induced the development of imposex (percentage of affected females) as well as the substantial growth in female penises to the same extent as TBT or TPT, when administrated at similar concentrations (Horiguchi, 2006).

The present work aims to understand if 9CRA is able to induce imposex in *N. lapillus* and in *N. reticulatus* and therefore to provide insights on imposex mechanism in these species.

MATERIALS AND METHODS

Sampling and animal's selection

N. lapillus and *N. reticulatus* collection was performed in March 2008, at two locations in the North Western Portuguese coast. The dogwhelks were collected by hand at the open coast near Aveiro (Poço da Cruz, 40°29′22.91″ N/ 8°47′37.06″ W, Portugal) whereas the netted whelk specimens were captured with baited hoop nets inside the Ria de Aveiro estuarine system (Magalhães Mira, 40°38′34.65″ N/8°44′06.80″ W, Portugal). Once in the laboratory, the animals were narcotized with magnesium chloride so that females could be selected: for *N. lapillus* only the ones without penis were chosen whereas for *N. reticulatus* only those with small penis (<1 mm) were considered since imposex-free snails could not be found.

Specimens maintenance, transportation and acclimatization

The selected females were maintained in the laboratory with artificial seawater (Crystal Sea® Bioassay Formulation, Marine Enterprises International; salinity of 33‰) for two weeks and then transported to the National Institute for Environmental Studies (NIES, Japan) in refrigerated conditions. Once arrived at NIES, the animals were maintained in an aquarium with artificial seawater

(Tomita Pharmaceutical Co., Tokushima, Japan) under the same salinity and allowed to acclimate for ten days before the experiments started. During the acclimatization period, animals were fed with mussels (*Septifer virgatus*), which were collected at Hiraiso, Japan, a less contaminated site by organotins and therefore usually used with this purpose (Nishikawa *et al*., 2004; Horiguchi *et al*., 2007a).

In vivo experiments

Females of both species were narcotized in 72 g/L magnesium chloride hexahydrate (Nacalai Tesque, Japan) and then injected with the test solutions in the foot using a 10 uL Hamilton micro syringe (Hamilton 80300 - 10 uL syringe 701 N). For each species four groups of 20 animals each were injected with: fetal bovine serum (designated as "FBS control"); 9-*cis*-retinoic acid ("9CRA"); ethanol ("EtOH control") and tributyltin chloride ("TBTCl"). The 9CRA (Wako Pure Chemicals Industries, Japan) solution was prepared in FBS (Flow Laboratories, USA) whilst the TBTCl (95% pure, Tokyo Kasei Kogyo Co., Japan) solution was prepared in EtOH (Wako Pure Chemicals Industries, Japan). Each animal in solvent control (EtOH and FBS) was injected with 1 uL per gram of soft tissue (in a wet weight (ww) basis). The same volume was injected in the other treatments: 9CRA in FBS was injected at a concentration of 1 ug/g ww in *N. lapillus* and *N. reticulatus* whereas TBTCl was injected in EtOH at a concentration of 1 ug/g ww in *N. lapillus* and 2 ug/g ww in *N. reticulatus*.

The animals of each experimental group where kept separately in 2-L beakers immersed in a temperature controlled water bath (TK 60; Takara Japan). Each beaker was provided with oxygen saturated artificial seawater and was connected to a flow-through system (Eyela Mp, Tokyo Rikakikai Co., Japan) at a constant rate (20 L/beaker/day). *N. lapillus* containing beakers were covered with plastic film to prevent animals to escape. Water parameters were monitored daily and presented mean values (±St. Dev) of: 8.0 ± 0.10 for pH; 33.0 ± 1.06 for salinity and 18.4 ± 0.44°C for temperature. Animals where fed *ad libitum* three times a week. For *N. lapillus* live mussels (*Septifer virgatus*) were provided whereas for *N. reticulatus* open dead mussels were offered.

Imposex analysis

At the end of the experimental period animals were morphologically examined for imposex development. Imposex parameters were assessed after narcotisation: percentage of females affected by imposex (%I), *vas deferens* sequence index (VDSI), and mean female penis length (FPL). The penis length was measured with a digital vernier calliper to the nearest 0.01 mm. The *vas deferens* sequence in *N. lapillus* was classified according to the scoring system proposed by Gibbs *et al*. (1987) and in *N. reticulatus* according to the system proposed by Stroben *et al*. (1992a). Soft tissues of each animal were preserved for future histological studies and for chemical analysis, except the 9CRA group where no specimens for chemical analysis were reserved.

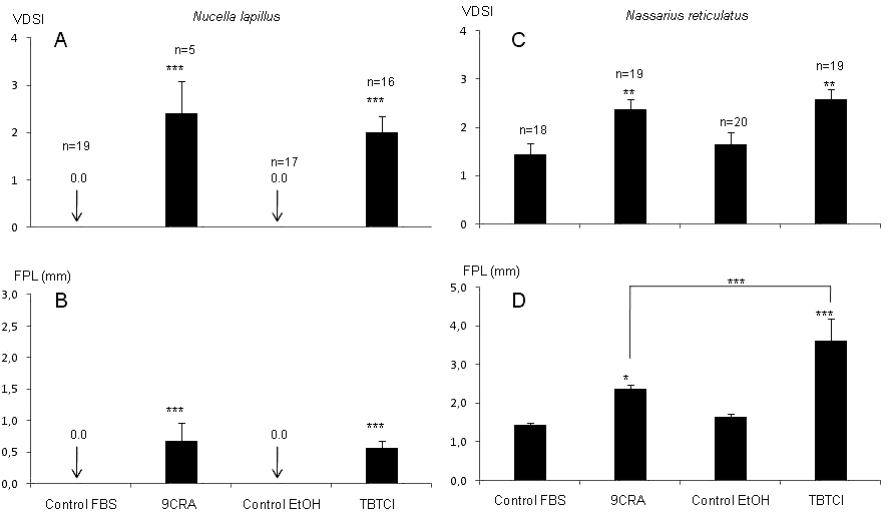

Fig. 1. Variation of *vas deferens* sequence index (VDSI) and female penis length (FPL) in *N. lapillus* (A and B) and *N. reticulatus* females (C and D) after 30 days of exposure. Error bars denote standard error. Significant differences in relation to control groups (FBS for 9CRA and EtOH for TBTCl) are marked: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Significant differences between 9CRA and TBTCl are also indicated.

Statistical analysis

SigmaStat software v3.5 was used to perform all statistical analysis. Differences in VDSI between groups were tested through the non-parametric Kruskal Wallis test (Anova on ranks) followed by the post-hoc Dunn's test whereas differences in FPL between groups were tested through one way ANOVA after confirming data normality and homocedasticity. The adopted critical significance level was 5%.

RESULTS AND DISCUSSION

Imposex induction in Nucella lapillus

N. lapillus mortality rates varied between 5% in EtOH control and 75% in 9CRA. In FBS control and TBTCl group mortality rates were 15 and 20%, respectively. Highly significant VDSI increases ($p < 0.001$) were observed between the EtOH control (VDSI = 0.0) and the TBTCl treatment (VDSI = 2.0) and between the FBS control (VDSI = 0.0) and the 9CRA treatment (VDSI = 2.4), (Fig. 1A). Female penises also increased significantly from the EtOH control (0.0 mm) to the TBTCl (0.6 mm) and from the FBS control (0.0 mm) to the 9CRA (0.7 mm), (Fig. 1B). No significant differences in VDSI or FPL were observed between both controls or between the TBTCl and the 9CRA groups (Figs. 1A and B).

Our results indicate that 9CRA induces imposex in *N. lapillus* with the same severity as the positive control TBTCl, suggesting that both compounds may act through the same signalling pathway. To the author's best knowledge, only two studies on RXR and imposex in *N. lapillus* were performed (Castro *et al*., 2007; Oehlmann *et al*., 2007) and they disclose contradictory results. Oehlmann and co-workers injected 9CRA in *N. lapillus* females and reported that injection of 9CRA did not cause any effect on the development of imposex (i.e., percentage of affected females, increase in penis length and the development of *vas deferens* in females) after a 56 days period, even exposed at the highest concentration tested (2.5 ug/g). Castro *et al*. (2007) injected *N. lapillus* females with the 1 ug/g of 9CRA and demonstrated that it induces imposex to the same degree as TBT, when administered at similar doses (1 ug/g). These authors suggested that the use of different carriers (FBS in both Castro *et al*. (2007) and this study and vitamin A-free peanut oil in Oehlmann *et al*. (2007)) might explain the observed differences since dosing 9CRA in peanut oil can affect its biodisponibility during the experiment course, rendering it less bioactive. It should also be noticed that 9CRA is easily photo decomposed. Even knowing that the number of females in the 9CRA group at the end of the experiment was extremely low ($n = 5$), our results corroborate the findings of Castro *et al*. (2007) reinforcing the hypothesis that imposex in *N. lapillus* is mediated trough the RXR pathway.

Imposex development in Nassarius reticulatus

N. reticulatus mortality rates varied between 0% and 10% (0% in EtOH, 5% in TBTCl and 9CRA; 10% in FBS). As already referred, *N. reticulatus* females used in the present study were slightly affected by imposex (mean VDSI and FPL of 1.6 and 0.4 mm, respectively) in the beginning of the experiment.

VDSI levels in *N. reticulatus* females increased significantly ($p < 0.01$) from the EtOH control (VDSI = 1.7) to the TBTCl (VDSI = 2.6) and from the FBS control (VDSI = 1.4) to the 9CRA (VDSI = 2.4), (Fig. 1C). Female penis length increased in a highly significant manner ($p < 0.001$) between the EtOH control (0.6 mm) and the TBTCl (3.6 mm) whereas between FBS control (0.3 mm) and 9CRA (0.9 mm) the increase was not so substantial, despite being significant ($p < 0.05$), (Fig. 1D). Females injected with TBT developed longer penises than those injected with 9CRA ($p < 0.001$) and, besides, no significant differences in FPL between EtOH and FBS controls were found (Fig. 1D). Such results might suggest that 9CRA does not promote imposex in the same way as TBT does, however it should be stressed that this difference is most probably a consequence of the different concentrations used: 1 ug/g ww for 9CRA and 2 ug/g ww for TBTCl. According to Stroben *et al*. (1992b) *N. reticulatus* is less sensitive than *N. lapillus* and for this reason a higher TBT concentration was used. For 9CRA the selected concentration was the same as the one used in other studies (Nishikawa *et al*., 2004; Castro *et al*., 2007; Oehlmann *et al*., 2007) so that comparisons could be performed.

As far as we are aware, the only study with *N. reticulatus* is the one by Oehlmann *et al*. (2007) and, as for *N. lapillus*, these authors did not detect any

differences in the development of imposex between the control and the 9CRA groups. Our results point in the opposite direction, with significant differences in both female penis length and VDSI between the control and the 9CRA groups. The use of different carriers (FBS and vitamin A-free peanut oil) can be a possible reason to explain such differences, as suggested by Castro *et al.* (2007). Our results also demonstrate that RXR plays an important role in imposex development in *N. reticulatus* and reinforce the retinoid theory originally proposed by Nishikawa *et al.* (2004) for *T. clavigera* and already confirmed for *N. lapillus* (Castro *et al.*, 2007).

The role of RXR in imposex development

Several theories have been proposed to explain the imposex phenomenon, but so far the exact mechanism by which imposex develops is still elusive. Until recently the aromatase inhibition and the neuroendocrine theories were the most consistent ones but recently a new pathway was proposed—the retinoic pathway. The retinoid X receptor (RXR) is a member of the nuclear receptor super-family which is highly conserved throughout metazoans (Nishikawa, 2006; Castro *et al.*, 2007; Sternberg *et al.*, 2008). Retinoid signaling is involved in the regulation of male reproductive differentiation and development (Sternberg *et al.*, 2008). Recently, it was proposed that imposex in gastropods is mediated trough the RXR signaling pathway (Nishikawa *et al.*, 2004). Organotins (TBT and TPT) apparently mimic the role of the natural ligand, binding RXR with high affinity (Nishikawa *et al.*, 2004). This theory was initially proposed for the rock shell *T. clavigera* (Nishikawa *et al.*, 2004), and was confirmed for the same species through a series of laboratory experiments (Horiguchi *et al.*, 2007a, 2010) and also through the assessment of RXR gene expression and protein content in wild animals (Horiguchi *et al.*, 2007b, in press). The involvement of RXR in imposex induction was further investigated by Oehlmann *et al.* (2007) in *N. lapillus* and *N. reticulatus*. However, these authors showed that for both species 9CRA had no significant effects on imposex parameters after almost two months following injection. Such contradictory results lead Castro *et al.* (2007) to perform a series of experiments with *N. lapillus* in order to test if the RXR is or not involved in the imposex induction and the obtained results indicate that RXR is the primary target for TBT in *N. lapillus*.

In order to provide further evidences of the involvement of RXR in imposex mechanism we injected 9CRA in *N. lapillus* and in *N. reticulatus*. Our results confirm the findings of Castro *et al.* (2007) for *N. lapillus* and provide evidences that imposex is also mediated through the RXR signaling pathway in *N. reticulatus*. Recent studies also seem to provide further evidences of RXR involvement, with the cloning of RXR in imposex susceptible gastropods as the mud-snail *Ilyanassa obsoleta* (Sternberg *et al.*, 2008) and the above mentioned *N. lapillus* (Castro *et al.*, 2007) and *T. clavigera* (Nishikawa *et al.*, 2004). Additional reports confirmed that TBT activates the RXR-PPAR heterodimer through a covalent interaction with Cys432 residue in the RXR-α (le Maire *et al.*, 2009). Despite the increasing amount of evidences linking imposex and the RXR signaling pathway many

questions remain unsolved (as for example the interplay between pathways) and further research is mandatory.

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