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Nuclear receptors and endocrine disruption in marine invertebrates: a case study with the Mediterranean mussel *Mytilus galloprovincialis***Angelica Miglioli^{1†}, Elza Fonseca², Lydia Besnardeau¹, Laura Canesi^{3*}, Michael Schubert^{1*} and Rémi Dumollard^{1*}**

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Keywords: bivalve mollusks, development, gene expression, hybridization chain reaction, phylogeny

Abstract

Endocrine disrupting chemicals (EDCs) represent a global threat to human health and the environment. In vertebrates, lipophilic EDCs primarily act by mimicking endogenous hormones, thus interfering with the transcriptional activity of nuclear receptors (NRs). The demonstration of the direct translation of these mechanisms into perturbation of NR-mediated physiological functions in invertebrates, however, has rarely proven successful, as the modes of action of EDCs in vertebrates and invertebrates seem to be distinct. In the present work, we investigated the members of the NR superfamily in a bivalve mollusk, the Mediterranean mussel *Mytilus galloprovincialis*. In addition to annotating the *M. galloprovincialis* NR complement, we assessed the potential developmental functions and susceptibility to EDC challenge during early development by gene expression analyses. Our results indicate that a majority of mussel NRs is dynamically expressed during early development, including receptors characterized by a potential susceptibility to EDCs. This study thus indicates that NRs are major regulators of early mussel development and that NR-mediated endocrine disruption in the mussel could be occurring at a larger scale and at earlier stages of the life cycle than previously anticipated. Altogether, these findings will have significant repercussions on our understanding of the stability of natural mussel populations.

Introduction

Nuclear receptors (NRs) are a superfamily of phylogenetically related transcription factors specific to metazoans, whose unique domain structure endows them with the ability to directly translate the presence/absence of signaling molecules and hormones into transcriptional responses (1,2). Although NRs are pivotal modulators of animal physiology, the complexity of their biological functions has mainly been studied and documented in vertebrates, where they act, for example, as receptors of lipophilic hormones in the endocrine system (1,3,4). Substances known to interfere with any aspect of hormone action are called endocrine disrupting chemicals (EDCs), and their introduction into the environment as pollutants can severely impact both human and animal health (3–5). Most chemicals catalogued as EDCs are molecules that mimic vertebrate hormones and primarily act as high affinity agonists or antagonists of NRs (3). In humans, 16 of the 48 NRs are involved, at least to a certain extent, in endocrine functions. These include the estrogen (ER), estrogen-related (ERR), androgen (AR), glucocorticoid (GR), mineralocorticoid (MR), and progesterone (PR) receptors of the NR3 subfamily, the thyroid hormone (THR), vitamin D (VDR), and peroxisome proliferator-activated (PPAR) receptors of the NR1 subfamily, and the retinoid X receptors (RXRs) of the NR2 subfamily (3,4).

However, a notable body of literature demonstrates that endocrine disruption also occurs in invertebrates, likely through modes of action and targets that are different from those known in vertebrates (6–8). Although important biological functions of NRs seem to be somewhat conserved between vertebrates and invertebrates,

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4 especially in early embryonic development, the ligands and gene regulatory networks involved in NR signaling
5 can differ significantly (2). For instance, invertebrate genomes are devoid of one-to-one AR, GR, MR, and PR
6 orthologs, and most invertebrate orthologs of ERs, THR, and PPARs do not transactivate in response to the
7 respective vertebrate hormone ligands (2,9–11). It is thus difficult, if not impossible, to infer NR-mediated
8 endocrine disruption in invertebrates from comparisons with published evidence on vertebrate models (2,6).

9 The only documented invertebrate endocrine pathway involving NRs is that of ecdysteroids (12), and the
10 ecdysteroid-responsive NRs of arthropods have been shown to be susceptible to a variety of different
11 environmental contaminants, including pesticides, fertilizers, plasticizers, and organotins (13–15). Of note,
12 previously thought to be exclusively present in the ecdysozoan clade of metazoan animals, ecdysone ligands,
13 ecdysone-responsive nuclear receptors (EcR, E75, E78), and several components of the ecdysteroid metabolic
14 machinery have recently been identified in several lophotrochozoan animals (10,16–18). However, the
15 involvement of the ecdysteroid pathway in NR-mediated endocrine disruption in these animals has not yet been
16 addressed (13–15). This highlights the urgent need to expand our knowledge and understanding of NR
17 signaling and endocrine disruption in invertebrates and to start addressing EDC toxicity as a taxon-specific
18 phenomenon (2,6).

19 The majority of invertebrate biodiversity is aquatic, and in particular marine, with many species inhabiting
20 environmental niches that are known to be sinks of EDCs (19). In the present work, we characterized the NR
21 superfamily complement of a marine bivalve, the Mediterranean mussel *Mytilus galloprovincialis* and
22 investigated NR expression during early development, the life cycle stage with the highest susceptibility to
23 environmental stressors (2,20). NRs were identified in the *M. galloprovincialis* genome and their orthology was
24 assessed by phylogenetic analyses. A detailed characterization of the expression dynamics of the *M.*
25 *galloprovincialis* NR complement was performed during early development using a comprehensive
26 developmental transcriptome dataset (21), and the spatiotemporal expression of a subset of NRs was evaluated
27 by *in situ* hybridization using the hybridization chain reaction (HCR) approach. Our results show that NRs are
28 pivotal regulators of different phases of early mussel development and indicate that the early larval stages of
29 bivalve mollusks might be particularly susceptible to NR disruption by a much broader range of pollutants than
30 previously thought.

36 Materials and Methods

37 Details of the materials and methods used in this study are available in Supplementary File 1. Briefly, members
38 of the NR superfamily were isolated from *M. galloprovincialis* published genome (22) by searching for proteins
39 displaying the canonical domain architecture (23,24) and annotated in a phylogenetic context using the NRs of
40 *Homo sapiens*, *Drosophila melanogaster*, *Crassostrea gigas*, *Mytilus coruscus*, *Mus musculus*, *Biomphalaria glabrata* and
41 *Schistosoma mansoni* by Maximum likelihood (25) and Bayesian Inference (26). Developmental expression of
42 MgNRs was extrapolated from the genome-guided developmental transcriptome (accession: PRJNA996031 and
43 ID: 996031), using libraries collected between 0 to 48 hours post fertilization (hpf) (21). *In situ* hybridization of a
44 sub-set of NRs characterized either by conserved developmental functions or documented susceptibility to
45 endocrine disruption (2) was performed by Hybridization Chain Reaction (HCR) in trochophore and D-Veliger
46 larvae as previously described (21). Larval samples for HCR analyses were obtained from larval cultures carried
47 out in standard conditions at 16°C as previously described (27).

51 Results

53 The nuclear receptor superfamily of *Mytilus galloprovincialis*

54 Searches of the *M. galloprovincialis* genome (22) resulted in 58 sequences with at least one NR DNA-binding
55 domain (DBD) or one NR ligand-binding domain (LBD). Complete sequences were retrieved for 46 NRs, which
56 included 43 canonical receptors (*i.e.*, with one DBD and one LBD), two 2DBD receptors, one NR0, and 12 partial
57 sequences (Supplementary Table 1). The *M. galloprovincialis* NRs were named based on phylogenetic analyses
58 calculated by both Bayesian Inference (BI) and Maximum Likelihood (ML), and according to the current NR
59 nomenclature (28). For the canonical NRs, the ML tree showing branch support values of both phylogenetic tree
60 reconstruction approaches is shown in Figure 1. Most *M. galloprovincialis* NRs branched with orthologs of known
NR superfamily members from other animals. The phylogenetic analyses thus identified 30 *M. galloprovincialis*

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4 members of the NR1 subfamily, 7 *M. galloprovincialis* members of the NR2 subfamily, two *M. galloprovincialis*
5 members of the NR3 subfamily, and single *M. galloprovincialis* representatives of the NR4, 5, 6, and 7 subfamilies.
6 Within the NR1 subfamily, each subgroup was characterized by the presence of at least one *M. galloprovincialis*
7 NR. Interestingly, branch support values indicated that *M. galloprovincialis* has one canonical PPAR (NR1C) and
8 two PPAR-like sequences, which were previously described as mollusk-specific duplicates within the NR1C
9 subgroup (11,17). While the BI analysis supported an association of all three *M. galloprovincialis* sequences with
10 the vertebrate PPARs (Supplementary Figure 1), the ML tree only recovered a branch with the canonical *M.*
11 *galloprovincialis* PPAR and the vertebrate PPARs, at the exclusion of the two *M. galloprovincialis* PPAR-like
12 sequences. We further found four *M. galloprovincialis* NR1J and 14 *M. galloprovincialis* NR1P sequences, which is
13 similar to the NR1J and NR1P complements identified in the Pacific oyster (*Crassostrea gigas*) (10). Of note, we
14 also identified three ecdysteroid NRs (EcR, E78, and E75) in *M. galloprovincialis*, which, in the tree, were
15 associated with the human and/or fruit orthologs (10,17). Within the NR2 subfamily, we characterized single *M.*
16 *galloprovincialis* orthologs of every vertebrate subgroup, and one additional *M. galloprovincialis* NR that was
17 orthologous to the invertebrate HR83 receptors. For the NR3 subfamily, there were two *M. galloprovincialis*
18 representatives, one orthologous to ER (NR3A) and one to ERR (NR3B). However, we failed to identify a *M.*
19 *galloprovincialis* ortholog of the NR3C subgroup, which, in vertebrates, include AR, GR, MR, and PR. For NR4
20 and NR5, we found single *M. galloprovincialis* representatives for each of these two subfamilies. We further
21 identified a representative of the NR6 subfamily in *M. galloprovincialis*, which was notable because bivalve
22 mollusks were supposed to have lost all members of this subfamily in the course of evolution (10,17). The *M.*
23 *galloprovincialis* NR complement also included one representative of the NR7 subfamily (29,30). In addition,
24 separate phylogenetic analyses allowed us to define two *M. galloprovincialis* members of the 2DBD NR
25 subfamily, which have previously been identified, for example, in *C. gigas* and the trematode worm *Schistosoma*
26 *mansoni*, as well as a single *M. galloprovincialis* member of the NR0 subfamily, as previously documented, for
27 example, in *C. gigas* (Supplementary Figure 2) (10,17).

31 32 **Developmental gene expression patterns of *Mytilus galloprovincialis* nuclear receptors**

33 The developmental expression dynamics of the *M. galloprovincialis* NR superfamily were studied using a
34 developmental transcriptome (21) that included stages between 0 and 48 hours post fertilization (hpf) (*i.e.*, the
35 unfertilized egg to the D-veliger stage) (Fig. 2). After fertilization, the *M. galloprovincialis* zygote undergoes spiral
36 cleavage, resulting in embryos with larger cells at the vegetal pole and smaller, more numerous cells at the
37 animal pole (Fig. 2A, 4 hpf). The embryos subsequently develop into gastrulae, forming the stomodeum (or
38 presumptive mouth) and invaginating the shell field (Fig. 2A, 16 hpf). After gastrulation, the trochophore larva
39 is formed. The stomodeum moves anteriorly, the prototroch and apical sensory organ appear, and the shell is
40 starting to be secreted from the shell field (Fig. 2A, 28 hpf). The trochophore progressively expands its shell and
41 develops into the D-veliger larva. The larval body is internalized into a D-shaped shell with a straight hinge and
42 smooth D-borders. The prototroch differentiates into a ciliated epithelium (called the velum), the shell-secreting
43 tissue becomes the mantle, and the larva has completed the formation of esophagus, gut, and anus (Fig. 2A, 44
44 hpf). Hierarchical clustering of NR expression levels during early development supported four main clusters
45 (adjusted-unbiased p-values, $au > 95$) (Fig. 2B). The clustering resulted in groups of subsequent developmental
46 timepoints, indicating co-expression of subsets of NRs during specific periods of development. The first major
47 cluster covered early embryonic development up to the late gastrula (8 to 20 hpf). The second cluster was
48 divided into three clusters: 24 to 32 hpf, *i.e.*, trochophore stages; 0 to 4 hpf, *i.e.*, early cleavage stages; 36 to 48
49 hpf, *i.e.*, veliger stages. Clusters were named accordingly: Phase 1 (0 to 4 hpf), Phase 2 (8 to 20 hpf), Phase 3 (24
50 to 32 hpf), and Phase 4 (36 to 48 hpf). The correlation between developmental time and NR expression was
51 further assessed by principal component analysis (PCA) (Fig. 2C). We found that principal component (PC) 1
52 and 2, respectively, covered 32.5% and 27.2% of the total variance of the dataset and spatially segregated each
53 of the four developmental phases, thereby corroborating the results of the cluster analysis: NR expression in *M.*
54 *galloprovincialis* embryos and larvae is correlated with developmental progression and falls into four distinct
55 developmental phases. For each NR, the expression dynamics during each developmental phase was then
56 visualized using a heatmap (Fig. 2D). Clustering of NRs by expression values reinforced the phase-specific
57 patterns identified by hierarchical clustering. In addition, this analysis revealed an association of high NR
58 expression levels with specific developmental phases. For example, while the two 2DBD NRs were highly
59 expressed during early cleavage and D-Veliger; PPAR, PPAR-like1, and PPAR-like2, respectively, defined early

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4 cleavage, trochophore, and D-Veliger. Of the three NR1CDEFs, NR1CDEF α and NR1CDEF γ were highly
5 expressed from the embryonic to the veliger phase, while NR1CDEF β expression was highest at the veliger
6 phase. For the ecdysteroid receptors, EcR and E75 were respectively characterized by expression peaks at the
7 D-Veliger and trochophore phases. In contrast, E78 expression was high throughout larval development. Of the
8 NRs known to act as transcriptional regulators of animal development, we found HNF4 and HR38 expression
9 to peak in embryos, that of TLX and RXR in D-Veliger, and that of COUP-TF and RAR in trochophores. Dynamic
10 expression also characterized the *M. galloprovincialis* orthologs of NRs targeted by EDCs in vertebrates (2). For
11 example, THR expression peaked at the trochophore, ER expression in D-Veligers, and ERR expression was
12 conspicuous during both the embryo and D-Veliger phases. Of note, we identified several NRs with high
13 expression levels during both early cleavage and veliger phases (such as NR1P5 and FTZ-F1). These results are
14 consistent with those obtained by hierarchical clustering and suggest that there are certain similarities in the
15 transcriptional dynamics of *M. galloprovincialis* NRs at these temporally well-separated developmental phases.
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18 **Spatiotemporal expression patterns of *Mytilus galloprovincialis* nuclear receptors**

19 The spatiotemporal expression patterns of a subset of *M. galloprovincialis* NRs were assessed by *in situ*
20 hybridization using the hybridization chain reaction (HCR) approach (Fig. 3). We selected the *M. galloprovincialis*
21 orthologs of NRs characterized by conserved developmental functions or known to be susceptibility to EDCs in
22 invertebrates or vertebrates (2). For the former, we chose NR0B, HNF4, HR83, and TLX and for the latter, we
23 selected ER, ERR, RXR, NR1J β , PPAR-like1, THR, EcR, and E75. Expression of these *M. galloprovincialis* NRs was
24 then studied in trochophore (28 hpf or 32 hpf) and D-veliger (44 hpf) larvae. NR0B was expressed in groups of
25 cells within the trochophore shell field at 28 hpf (Fig. 3A). At 44 hpf, NR0B was conspicuously expressed in two
26 clusters at opposite sides of the shell hinge and much less conspicuous along the D-border (Fig. 3A). HNF4
27 expression was detectable in a group of anterior cells in proximity of the stomodeum at 28 hpf and in the
28 presumptive gut at 44 hpf (Fig. 3A). HR83 was detectable in discrete islets of cells in the prototroch at 28 hpf
29 and, at 44 hpf, HR83 expression was limited to a single anterior group of cells (Fig. 3A). TLX was expressed in
30 the apical region as well as the posterior end of the trochophore at 28 hpf (Fig. 3A). In the D-veliger, at 44 hpf,
31 TLX expression was observable in several islets of cells at the ventroposterior and dorsoanterior edges of the
32 mantle as well as in groups of cells in the center of the larval body (Fig. 3A).
33

34 At 32 hpf, ER was expressed in cells beneath the larval shell outline and, at 44 hpf, at the extremities of the hinge
35 and along the border of the D-veliger shell (Fig. 3B), in a pattern very similar to that of NR0B (Fig. 3A). At 28
36 hpf, ERR expression was detectable in the dorsal region as well as in islets of cells in the apical region of the
37 trochophore (Fig. 3B), which was similar to the expression of HR83 (Fig. 3A). By 44 hpf, ERR expression was in
38 a duct-like structure that, from a curl on the D-border, reached the presumptive gut in the dorsal hinge region
39 (Fig. 3B). The early and late expression patterns of ERR were partially reminiscent of those described for HNF4
40 (Fig. 3A). RXR was expressed ubiquitously throughout the larva at both 28 hpf and 44 hpf (Fig. 3B). NR1J β was
41 highly expressed in a group of apical cells and scattered dorsally at 32 hpf (Fig. 3B). By 44 hpf, expression was
42 concentrated along the border of the D-veliger shell and in the dorsal portion of the larval body, in a pattern
43 forming two central islets and expanding towards the hinge region (Fig. 3B). While PPAR-like1 expression was
44 observable in the region of the shell field at 28 hpf, its expression was very inconspicuous at 44 hpf (Fig. 3B).
45 THR was expressed in small islets of cells in the apical, stomodeal, and dorsal regions of the trochophore at 28
46 hpf (Fig. 3B). THR expression then became restricted to the dorsal side of the larva in the D-veliger at 44 hpf
47 (Fig. 3B). EcR was expressed around the larval shell border at both 28 hpf and 44 hpf (Fig. 3B). E75 expression
48 was widespread dorsally and restricted apically at 32 hpf (Fig. 3B). At 44 hpf, E75 expression was restricted to
49 a discrete cluster of anterior cells (Fig. 3B), resembling the cluster of cells expressing HR83 at the same stage of
50 development (Fig. 3A).
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55 **Discussion**

56 In vertebrates, members of the NR superfamily are pivotal regulators of development and endocrine functions,
57 and their activities are highly susceptible to disruptive modulations by EDCs (3,31). Corroborated experimental
58 evidence indicates that NRs also play important roles in the regulation of invertebrate development. However,
59 the diversity of animal life makes it difficult to robustly infer conserved biological functions of orthologous NRs
60 and to evaluate the overall susceptibility of invertebrate NRs to EDC-based disruption based solely on data

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4 obtained in vertebrates (2). For these reasons, it is necessary to characterize the expression and function of NR
5 superfamily members in a wide variety of different taxa and to interpret the results obtained in a comparative
6 context. These comparative developmental and toxicological analyses will not only shed light on conserved and
7 divergent functions of NRs in vertebrates and invertebrates but will also be instrumental to evaluate the
8 potential risks of NR-mediated endocrine disruption in different taxa. In this light, this work presents a first
9 step in the characterization of NR superfamily members in the Mediterranean mussel *M. galloprovincialis*, a
10 bivalve mollusk. Our results show that the NR complement of *M. galloprovincialis* includes at least 46 members,
11 which is in line with previous work on NRs in the bivalve mollusk *C. gigas* (43 NRs) and the gastropod mollusks
12 *Biomphalaria glabrata* (39 NRs) and *Lottia gigantea* (33 NRs) (10,17). However, we failed to complete the sequences
13 of 12 NR fragments identified in the *Mg* genome, meaning that additional work will be required to establish the
14 complete NR complement once additional sequence resources are made available for this species.

15
16 *M. galloprovincialis* NR orthologs were identified for every vertebrate, protostome, and bivalve NR subgroup,
17 with the exception of HR39 (NR5B). The gene encoding HR39 was thus either lost in *M. galloprovincialis* or there
18 is currently not enough sequence information available to allow the unequivocal identification of the gene in
19 this mussel species (10,17). When compared to other animals, mollusks are generally characterized by a
20 relatively high total number of NRs. This can be explained by two phenomena: (1) mollusk NR complements
21 also include receptors that have previously been thought to be specific to arthropods (such as EcR, E78 and
22 NR2Es) and (2) several NR subfamilies have experienced lineage-specific expansions (such as the NR1Cs and
23 NR1Js in all mollusks and the NR1Ps and NR1CDEFs in bivalve mollusks) (10,17). For the NR1Cs, while two
24 members have previously been described in gastropods and *C. gigas*, we found three representatives in mussels,
25 suggesting that Mytilids have experienced an additional NR1C duplication when compared to other mollusks.
26 However, our phylogenetic analyses indicate that a robust association of two of the three mussel NR1Cs with
27 the third mussel NR1C plus the vertebrate NR1Cs (*i.e.*, the PPARs) is method-dependent, suggesting that the
28 phylogenetic status of this NR subgroup might need additional experimental corroboration. *M. galloprovincialis*
29 also has three members of the NR1CDEF subgroup, which has previously been characterized in *C. gigas* (10). As
30 in this previous analysis, we were unable to reliably resolve the phylogenetic signal within this subgroup of the
31 tree, hence preventing an unambiguous assignment of the three *M. galloprovincialis* NRs to the NR1Cs, NR1Ds,
32 NR1Es or NR1Fs (10). The lack of phylogenetic resolution within this subgroup might be due to a rapid
33 evolutionary divergence following duplication (10), which for NR1 subgroups C, D, E, and F probably took
34 place very early in bilaterian evolution (9). The NR complement of *M. galloprovincialis* also includes 14 members
35 of the bivalve-specific NR1Ps, a subgroup that has already been described in *C. gigas* (10). The positioning of
36 NR1Ps in close relationship to the NR1Cs, NR1Ds, NR1Es, and NR1Fs is consistent with previous observations
37 and supports the notion that bivalves, and maybe all mollusks, have evolved a unique NR subgroup (10,32).
38 Like in *C. gigas*, we also found four members of the protostome-specific NR1J subgroup in *M. galloprovincialis*
39 (10). Furthermore, we identified a single *M. galloprovincialis* representative for every other subgroup of the NR1
40 subfamily, including NRs that are potentially responsive ecdysone-like hormones, such as EcR, E75/Rev-Erb,
41 E78, and HR3/ROR, which, in addition to arthropods, have previously been identified in other mollusks, leeches,
42 and annelids (10,17,18).

43
44 The NR complement of *M. galloprovincialis* includes orthologs of all invertebrate and vertebrate NR subgroups
45 that have been suggested to be implicated in endocrine disruption. For example, EcR and other ecdysone-
46 responsive NRs of arthropods are currently the only known invertebrate NRs involved in hormonal control of
47 the endocrine system, and it is known that these NRs are major targets of a broad range of EDCs, including
48 steroids and pesticides (12,13,33). The transcriptional activity of members of the NR1C subgroup in vertebrates
49 and gastropods has been shown to be disrupted by organotin compounds, such as tributyltin (TBT) and
50 triphenyltin (TPT) (3,11). The NR1Js, which include the arthropod HR96 and which form the outgroup of the
51 vertebrate NR1Is, are susceptible to disruption by xenobiotics and biotoxins (34–36). Vertebrate THR is a major
52 regulator of metamorphosis and notable targets of EDCs (3). Although a large body of evidence indicates that
53 THR is also a regulator of metamorphosis in bivalves, its endogenous ligands (and potential disruptors) are yet
54 to be discovered (2). RXR stands out due to its susceptibility to endocrine disruption by organotin, which has
55 been documented in an astonishing diversity of metazoan animals (37). ER is a known target of EDCs in
56 vertebrates, while its mollusk orthologs are devoid of any ligand-dependent transcriptional activity (2).
57 Similarly, the transcriptional activity of vertebrate ERR is disrupted by Bisphenol A (BPA) (38), but an
58 involvement of ERR in endocrine disruption in mollusks still remains elusive. Given the presence of orthologs
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of all these NRs in the *M. galloprovincialis* genome, it seems possible that mussels, and probably bivalves in general, might be particularly sensitive to environmental chemicals, biotoxins, and EDCs.

Analysis of the developmental expression of *M. galloprovincialis* NRs yielded valuable information on the NR-dependent regulation of early development and larval morphogenesis in mollusks (2,10,39). For instance, three of the four developmental phases of NR expression we identified by hierarchical clustering in *M. galloprovincialis* have previously also been reported in *C. gigas*. It thus seems likely that NR expression, and potentially their functions, are largely conserved in bivalve mollusks (2,39). Furthermore, the homologs of NRs known to be targets of EDCs in both invertebrates and vertebrates (such as ER, ERR, THR, PPARs, EcR, and the NR1Js) are characterized by high expression levels at the trochophore and D-veliger stages in both *M. galloprovincialis* and *C. gigas*, suggesting that early bivalve larvae might be particularly susceptible to NR-mediated endocrine disruption. Expression analyses of NRs with potential developmental functions revealed that NR0B was detectable in the outer pair of larval retractor muscles (40), while HNF4 was expressed in the developing gut and hepatopancreas, which is line with the conserved role of HNF4 in development and differentiation of endodermal organs (2,40). HR38 and TLX were expressed, presumably, in developing apical, pedal, and visceral ganglions (40,41), indicating that, as in other animals, NR2E receptors might function during development of the sensory nervous system in *M. galloprovincialis* (2,42,43). Surprisingly, several NRs potentially susceptible to EDCs were characterized by expression patterns that were very similar to those of the developmental NRs. In D-Veligers, for example, the patterns of ER overlapped those of NR0B in the outer retractor muscles, which might be indicative of some level of functional interaction between the two NRs, as has previously been described in vertebrates (44). ERR expression partially overlapped that of HNF4 in the presumptive gut, while also outlining the larval esophagus. Similarly, ERR in the fruit fly is expressed in the larval midgut, indicating a potentially conserved function of ERR in metabolic functions (43). NR1J β was detectable in two groups of cells connected to the hinge region in D-Veligers, potentially labeling the inner pair of the larval retractor muscles (40). In the fruit fly, the NR1J homolog HR96 is expressed in muscles and gut of the larva (43). PPAR-like1 and EcR were expressed in the margins of the growing shell, and at least EcR has previously been suggested to support shell formation in the bivalve *Pinctada fucata martensii* (16). E75 was expressed in apical cells in both trochophores and D-Veligers, likely corresponding to cells destined to become peripheral sensory neurons associated with the apical organ, which is consistent with neural expression of E75 in the fruit fly (41,43,45). Of note, expression of THR was not restricted to a specific larval structure, suggesting that this NR might have several different roles during early development of *M. galloprovincialis*, and the organotin-binding NR RXR was ubiquitously expressed at both stages analyzed, indicating that EDCs targeting these NRs might represent a serious threat for mussel embryos and larvae. Together, these findings support the notion that larval shell formation, together with muscle and nervous system development, could be important targets of EDCs in bivalve mollusks.

Taken together, the identification and annotation of NR genes and the description of their early developmental expression in *M. galloprovincialis* is the first step to gain insights into the potential functions of these important transcription factors during development. Analysis of temporal and spatial expression profiles provides the foundation for identifying the physiological roles of specific NRs in this marine bivalve and for understanding how they may be affected by environmental pollutants and EDCs. Mass mortalities and population declines of marine bivalves are increasingly reported, including species of high ecological and economic values. For instance, one third of European aquaculture is represented by mussel production (46), and, although Mytilids, are generally considered more resilient to environmental stressors than other bivalves, a general decline in mussel production has been reported in the course of the last two decades (38–42). The causes for mortality episodes are multifactorial (51), but, as this report suggests, exposure to EDCs during early development may represent a serious threat to bivalve populations. The data presented here thus adds to our fundamental understanding of NR biology in bivalves and highlights possible functions and susceptibilities to endocrine disruption, which might have profound implications for protecting mussel populations from future mortality episodes.

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Figure and Table Captions

Figure 1: Phylogenetic relationship of nuclear receptors (NRs) in *Mytilus galloprovincialis* (Mg), *Mytilus coruscus* (Mco), *Crassostrea gigas* (Cg), *Homo sapiens* (Hs), and *Drosophila melanogaster* (Dm). The tree presents the phylogenetic branching pattern obtained from the Maximum Likelihood analysis. Posterior probabilities from Bayesian Inference and Bootstrap values from Maximum Likelihood are indicated on each branch. Red and black colors indicate, respectively, strong and weak branch support. NR subfamilies are highlighted in colors: NR1 in green, NR2 in pink, NR3 in blue, NR4 in violet, NR5 in red, NR6 in yellow, and NR7 in cyan.

Figure 2: Developmental expression dynamics of *Mytilus galloprovincialis* nuclear receptors (NRs). A) Representative images of the main stages of *M. galloprovincialis* early development. In the early embryo, at 4 hours post fertilization (hpf), the animal (A) and vegetal (V) poles are shown. In the gastrula, at 16 hpf, the stomodeum (st, black dotted line) and the shell field invagination (si, white dotted line) are indicated. In the trochophore, at 28 hpf, the prototroch (t, yellow dotted line), the region of the apical sensory organ (aso, red dotted line), the stomodeum (st, black dotted line), and the shell field (sf, white dotted line) are shown. In the D-veliger, at 44 hpf, the mantle edge is outlined by a green dotted line, and the anus (an), esophagus (eso), hinge (h), and velum (ve) are highlighted. Crosses indicate larval orientation: Ant-anterior; Post-posterior; D-dorsal; V-ventral. Scale bar: 20 μ m. B) Hierarchical clustering of NR expression from 0 to 48 hpf identifying four distinct clusters: Phase 1 (0 to 4 hpf), Phase 2 (8 to 20 hpf), Phase 3 (24 to 32 hpf), Phase 4 (36 to 48 hpf). Numbers in red represent approximate unbiased p-values. C) Principal component analysis biplot of NR expression in each sample: ellipses highlight the four developmental clusters (corresponding to Phases 1, 2, 3, and 4). D) Heatmap of NR expression dynamics during *M. galloprovincialis* early development clustered by rows, with column breaks separating the four developmental clusters (*i.e.*, Phases 1, 2, 3, and 4).

Figure 3: Developmental expression patterns of *Mytilus galloprovincialis* nuclear receptors (NRs). A) *In situ* hybridization-based expression of a selection of NRs with conserved roles during development (2): NR0B, HR83, HNF4, and TLX. Representative images of trochophore (28 hpf) and D-veliger (44 hpf) stages. Maximum z-projections of Hoechst nuclear staining (grey) and fluorescent *in situ* hybridization signal (yellow), merged channels are shown. B) *In situ* hybridization-based expression of a selection of NRs with known susceptibility

1 to endocrine disrupting chemicals (EDCs) (2): ER, ERR, RXR, NR1J β , PPAR-like1, THR, EcR, and E75.
2 Representative images of trochophore (28 hpf or 32 hpf) and D-veliger (44 hpf) stages. Maximum z-projections
3 of Hoechst nuclear staining (grey) and fluorescent *in situ* hybridization signal (red), merged channels are shown.
4 Shell outline is indicated with dotted white lines, orange arrowheads highlight presumptive outer and inner
5 pairs of larval retractor muscles. aso: apical sensory organ; eso: esophagus; h: hinge; g: gut; sf: shell field; st:
6 stomodeum; t: prototroch. Larval orientation as in Figure 2A. Scale bars: 20 μ m.
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Supplementary Material

Supplementary Figure 1: Phylogenetic relationships of in *Mytilus galloprovincialis* (Mg), *Mytilus coruscus* (Mco), *Crassostrea gigas* (Cg), *Homo sapiens* (Hs), and *Drosophila melanogaster* (Dm) obtained by Bayesian Inference. Posterior probabilities are indicated on each branch. Red and blue colors indicate, respectively, strong and weak branch support. Branch colors indicate NR subfamilies: NR1 in green, NR2 in pink, NR3 in blue, NR4 in violet, NR5 in brown, NR6 in orange, and NR7 in cyan.

Supplementary Figure 2: Phylogenetic relationship of 2DBD and NR0 nuclear receptors (NRs) in *Mytilus galloprovincialis* (Mg), *Mytilus coruscus* (Mco), *Crassostrea gigas* (Cg), *Homo sapiens* (Hs), *Schistosoma mansoni* (Sm), *Mus musculus* (Mm), and *Biomphalaria glabrata* (Bg). The tree represents the relationships derived from Maximum Likelihood analysis. Posterior probabilities from Bayesian Inference and Bootstrap values from Maximum Likelihood are indicated on each branch. Red and black colors indicate, respectively, strong and weak branch support.

Supplementary File 1: Extended Materials and methods.

Supplementary File 2: Supplementary Table 1: Protein domains of nuclear receptor (NR) proteins identified in the *Mytilus galloprovincialis* genome. **Supplementary Table 2:** Accession numbers and annotation of nuclear receptors (NRs) used for phylogenetic analyses from *Mytilus galloprovincialis* (Mg), *Mytilus coruscus* (Mco), *Crassostrea gigas* (Cg), *Homo sapiens* (Hs), *Schistosoma mansoni* (Sm), *Mus musculus* (Mm), and *Biomphalaria glabrata* (Bg). **Supplementary Table 3:** Table of *in situ* hybridization probe sequences using hybridization chain reaction (HCR).

Additional Information

Ethics

The authors have nothing to declare.

Data Accessibility

All further data is available upon request.

Authors' Contributions

AM, EF curated the datasets for phylogenetic analyses; AM performed phylogenetic and statistical analyses as well as gene expression assays, with the latter supported by LB; LC, MS, RD supervised analyses and experiments. AM, LC, MS, RD conceived the study and wrote the manuscript, with contributions from all authors.

Competing Interests

The authors declare that they have no competing interests.

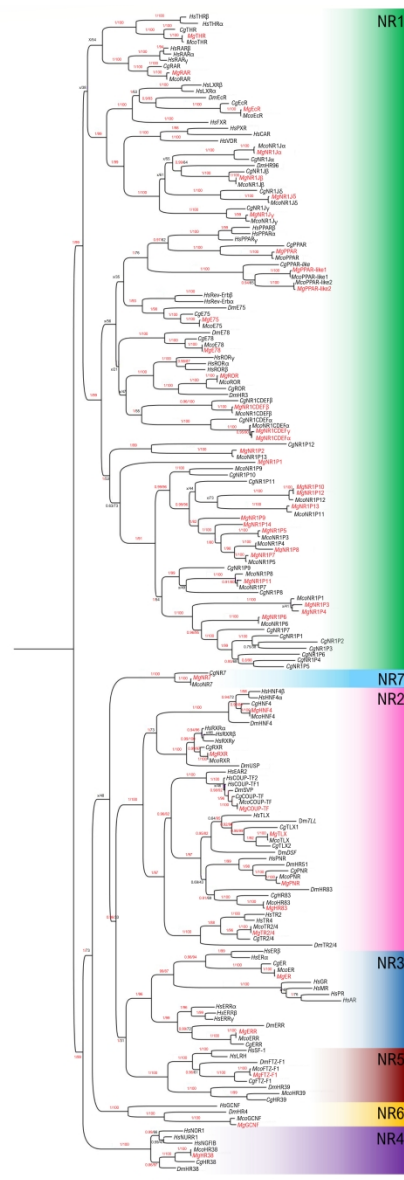


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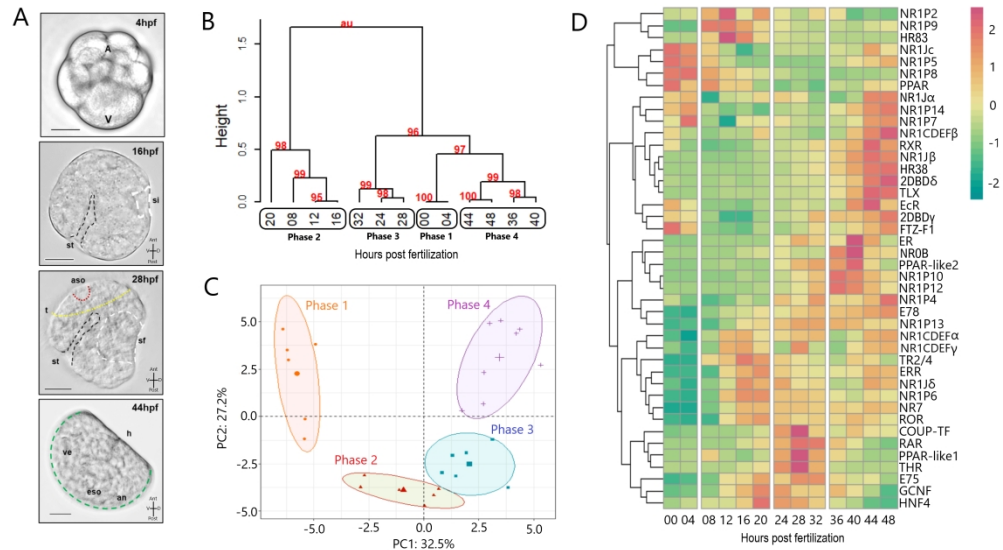


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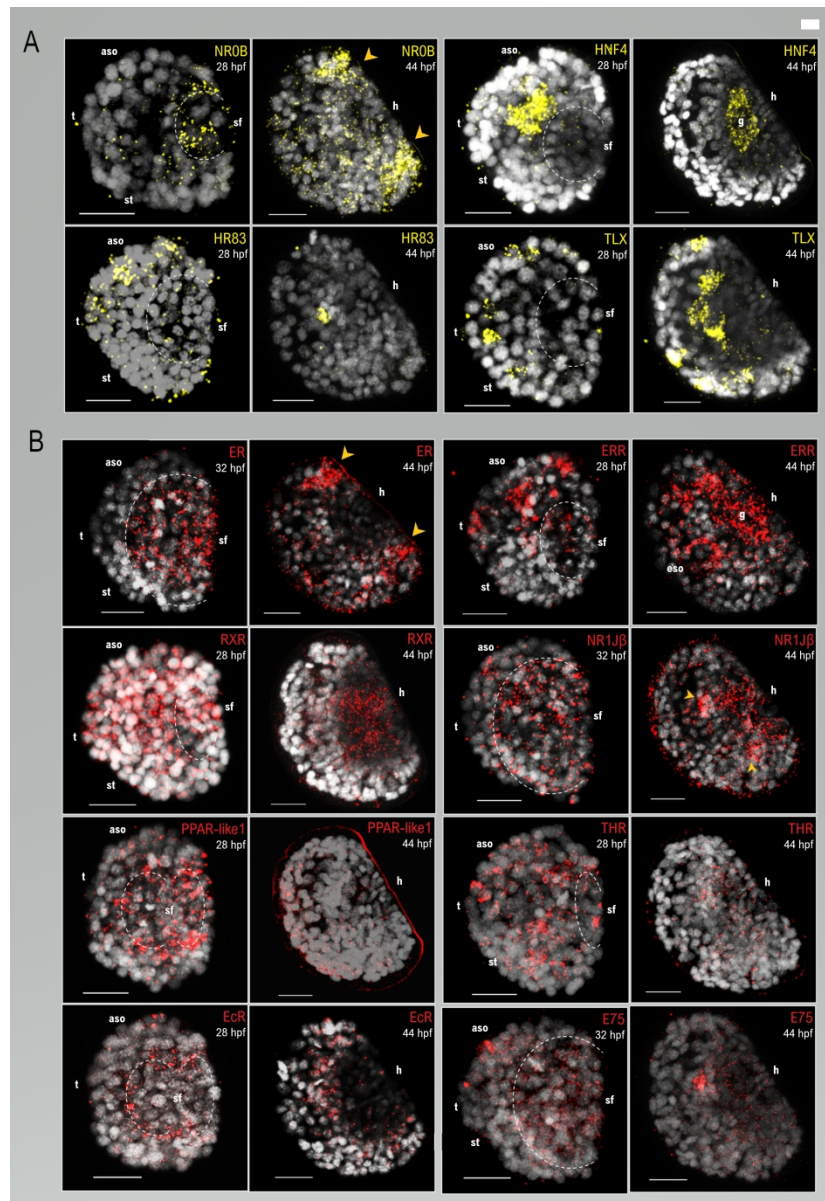


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