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First characterization of the nuclear receptor superfamily in the Mediterranean mussel *Mytilus galloprovincialis*: developmental expression dynamics and potential susceptibility to environmental chemicals

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

The only documented invertebrate endocrine pathway involving NRs is that of ecdysteroids (12), and the ecdysteroid-responsive NRs of arthropods have been shown to be susceptible to a variety of different environmental contaminants, including pesticides, fertilizers, plasticizers, and organotins (13–15). Of note, previously thought to be exclusively present in the ecdysozoan clade of metazoan animals, ecdysone ligands, ecdysone-responsive nuclear receptors (EcR, E75, E78), and several components of the ecdysteroid metabolic machinery have recently been identified in several lophotrochozoan animals (10,16–18). However, the involvement of the ecdysteroid pathway in NR-mediated endocrine disruption in these animals has not yet been addressed (13–15).

The majority of invertebrate biodiversity is aquatic, and in particular marine, with many species inhabiting environmental niches that are known to be sinks for various chemicals with potential endocrine disrupting activity (19). For decades, their populations have suffered declines and mass mortalities with tangible impacts on environmental health and human food safety (8,20–22). This highlights the urgent need to expand our knowledge and understanding of NR signaling and endocrine disruption in invertebrates and to start addressing EDC toxicity as a taxon-specific phenomenon (2,6). Early development of the Mediterranean mussel *Mytilus galloprovincialis* is a standard model for ecotoxicology and ecophysiology research (23,24). The recent publication of a reference genome and of a developmental transcriptome (25,26) allowed us to characterize the NR superfamily complement in this model and to investigate NR expression during early *M. galloprovincialis* development, the life cycle stage with the highest susceptibility to environmental stressors (2,27). NRs were identified in a reliable assembly of the *M. galloprovincialis* genome and, their orthology was assessed by phylogenetic analyses. A detailed characterization of the expression dynamics of the *M. galloprovincialis* NR complement was performed during early development using a comprehensive developmental transcriptome dataset (26), and the spatiotemporal expression of a subset of NRs was evaluated by *in situ* hybridization using the hybridization chain reaction (HCR) approach. Our results suggest that NRs are pivotal regulators of different phases of early mussel development and indicate that the early larval stages of bivalve mollusks might be particularly susceptible to NR disruption by a much broader range of pollutants than previously thought.

Materials and Methods

Details of the materials and methods used in this study are available in Supplementary File 1. Briefly, members of the NR superfamily were isolated from the mg10 assembly of the *M. galloprovincialis* genome (25) by searching for proteins displaying the canonical domain architecture (28,29) and annotated in a phylogenetic context using the NRs of *Homo sapiens, Drosophila melanogaster*, *Crassostrea gigas*, *Mytilus coruscus, Mus musculus, Biomphalaria glabrata and Schistosoma mansoni* by Maximum likelihood (30) and Bayesian Inference (31). Developmental expression of *Mg*NRs was extrapolated from the genome-guided developmental transcriptome (accession: PRJNA996031 and ID: 996031), using libraries collected between 0 to 48 hours post fertilization (hpf) (26). *In situ* hybridization of a sub-set of NRs characterized either by conserved developmental functions or documented susceptibility to endocrine disruption (2) was performed by Hybridization Chain Reaction (HCR) in trochophore and D-veliger larvae as previously described (26). Larval samples for HCR analyses were obtained from larval cultures carried out in standard conditions at 16°C as previously described (32).

Results

The nuclear receptor superfamily of *Mytilus galloprovincialis*

Searches of the mg10 assembly of the *M. galloprovincialis* genome (25) resulted in 58 sequences with at least one NR DNA-binding domain (DBD) or one NR ligand-binding domain (LBD). Complete sequences were retrieved for 46 NRs, which included 43 canonical receptors (*i.e.*, with one DBD and one LBD), two 2DBD receptors, one

NR0, and 12 partial sequences (Supplementary Table 1). The *M. galloprovincialis* NRs were named based on phylogenetic analyses calculated by both Bayesian Inference (BI) and Maximum Likelihood (ML), and according to the current NR nomenclature (33). For the canonical NRs, the ML tree showing branch support values of both phylogenetic tree 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* members of the NR1 subfamily, 7 *M. galloprovincialis* members of the NR2 subfamily, two *M. galloprovincialis* members of the NR3 subfamily, and single *M. galloprovincialis* representatives of the NR4, 5, 6, and 7 subfamilies. Within the NR1 subfamily, each subgroup was characterized by the presence of at least one *M. galloprovincialis* NR. Interestingly, branch support values indicated that *M. galloprovincialis* has one canonical PPAR (NR1C) and two PPAR-like sequences, which were previously described as mollusk-specific duplicates within the NR1C subgroup (11,17). While the BI analysis supported an association of all three *M. galloprovincialis* sequences with the vertebrate PPARs (Supplementary Figure 1), the ML tree only recovered a branch with the canonical *M. galloprovincialis* PPAR and the vertebrate PPARs, at the exclusion of the two *M. galloprovincialis* PPAR-like sequences. We further found four *M. galloprovincialis* NR1J and 14 *M. galloprovincialis* NR1P sequences, which is similar to the NR1J and NR1P complements identified in the Pacific oyster (*Crassostrea gigas*) (10). Of note, we also identified three ecdysteroid NRs (EcR, E78, and E75) in *M. galloprovincialis*, which, in the tree, were associated with the human and/or fruit fly orthologs (10,17). Within the NR2 subfamily, we characterized single *M. galloprovincialis* orthologs of every vertebrate subgroup, and one additional *M. galloprovincialis* NR that was orthologous to the invertebrate HR83 receptors. For the NR3 subfamily, there were two *M. galloprovincialis* representatives, one orthologous to ER (NR3A) and one to ERR (NR3B). As expected, a *M. galloprovincialis* ortholog of the NR3C subgroup, which, in vertebrates, include AR, GR, MR, and PR was not identified. For NR4 and NR5, we found single *M. galloprovincialis* representatives for each of these two subfamilies. However, since the genomes of both *C. gigas* and *M. coruscus* encode a second member of the NR5 subfamily, HR39, we searched for this missing ortholog in a more recent assembly of the *M. galloprovincialis* genome (34) using the sequences from *C. gigas* and *M. coruscus* as assembly templates. This strategy allowed us to identify the HR39 ortholog of *M. galloprovincialis* (Supplementary Figure 2).

We further identified a representative of the NR6 subfamily in *M. galloprovincialis*, which was notable because bivalve mollusks were supposed to have lost all members of this subfamily in the course of evolution (10,17). The *M. galloprovincialis* NR complement also included one representative of the NR7 subfamily (35,36). In addition, separate phylogenetic analyses allowed us to define two *M. galloprovincialis* members of the 2DBD NR subfamily, which have previously been identified, for example, in *C. gigas* and the trematode worm *Schistosoma mansoni*, as well as a single *M. galloprovincialis* member of the NR0 subfamily, as previously documented, for example, in *C. gigas* (Supplementary Figure 3) (10,17).

Developmental gene expression patterns of *Mytilus galloprovincialis* nuclear receptors

The developmental expression dynamics of the *M. galloprovincialis* NR superfamily were studied using a developmental transcriptome (26) that included stages between 0 and 48 hours post fertilization (hpf) (*i.e.*, the unfertilized egg to the D-veliger stage) (Fig. 2). After fertilization, the *M. galloprovincialis* zygote undergoes spiral cleavage, resulting in embryos with larger cells at the vegetal pole and smaller, more numerous cells at the animal pole (Fig. 2A, 4 hpf). The embryos subsequently develop into gastrulae, forming the stomodeum (or presumptive mouth) and invaginating the shell field (Fig. 2A, 16 hpf). After gastrulation, the trochophore larva is formed. The stomodeum moves anteriorly, the prototroch and apical sensory organ appear, and the shell is starting to be secreted from the shell field (Fig. 2A, 28 hpf). The trochophore progressively expands its shell and develops into the D-veliger larva. The larval body is internalized into a D-shaped shell with a straight hinge and smooth D-borders. The prototroch differentiates into a ciliated epithelium (called the velum), the shell-secreting tissue becomes the mantle, and the larva has completed the formation of esophagus, gut, and anus (Fig. 2A, 44 hpf). Hierarchical clustering of NR expression levels during early development supported four main clusters (adjusted-unbiased p-values, au >95) (Fig. 2B). The clustering resulted in groups of subsequent developmental timepoints, indicating co-expression of subsets of NRs during specific periods of development. The first major cluster covered early embryonic development up to the late gastrula (8 to 20 hpf). The second cluster was divided into three clusters: 24 to 32 hpf, *i.e.*, trochophore stages; 0 to 4 hpf, *i.e.*, early cleavage stages; 36 to 48 hpf, *i.e.*, veliger stages. Clusters were named accordingly: Phase 1 (0 to 4 hpf), Phase 2 (8 to 20 hpf), Phase 3 (24

to 32 hpf), and Phase 4 (36 to 48 hpf). The correlation between developmental time and NR expression was further assessed by principal component analysis (PCA) (Fig. 2C). We found that principal component (PC) 1 and 2, respectively, covered 32.5% and 27.2% of the total variance of the dataset and spatially segregated each of the four developmental phases, thereby corroborating the results of the cluster analysis: NR expression in *M. galloprovincialis* embryos and larvae is correlated with developmental progression and falls into four distinct developmental phases. For each NR, the expression dynamics during each developmental phase was then visualized using a heatmap (Fig. 2D). Clustering of NRs by expression values reinforced the phase-specific patterns identified by hierarchical clustering. In addition, this analysis revealed an association of high NR expression levels with specific developmental phases. For example, while the two 2DBD NRs were highly expressed during early cleavage and D-veliger; PPAR, PPAR-like1, and PPAR-like2, respectively, defined early cleavage, trochophore, and D-veliger. Of the three NR1CDEFs, NR1CDEFα and NR1CDEFγ were highly expressed from the embryonic to the veliger phase, while NR1CDEFβ expression was highest at the veliger phase. For the ecdysteroid receptors, EcR and E75 were respectively characterized by expression peaks at the D-veliger and trochophore phases. In contrast, E78 expression was high throughout larval development. Of the NRs known to act as transcriptional regulators of animal development, we found HNF4 and HR38 expression to peak in embryos, that of TLX and RXR in D-veliger, and that of COUP-TF and RAR in trochophores. Dynamic expression also characterized the *M. galloprovincialis* orthologs of NRs targeted by EDCs in vertebrates (2). For example, THR expression peaked at the trochophore, ER expression in D-veligers, and ERR expression was conspicuous during both the embryo and D-veliger phases. Of note, we identified several NRs with high expression levels during both early cleavage and veliger phases (such as NR1P5 and FTZ-F1). These results are consistent with those obtained by hierarchical clustering and suggest that there are certain similarities in the transcriptional dynamics of *M. galloprovincialis* NRs at these temporally well-separated developmental phases.

Spatiotemporal expression patterns of *Mytilus galloprovincialis* nuclear receptors

The spatiotemporal expression patterns of a subset of *M. galloprovincialis* NRs were assessed by *in situ* hybridization using the hybridization chain reaction (HCR) approach (Fig. 3). We selected the *M. galloprovincialis* orthologs of NRs characterized by conserved developmental functions or known to be susceptibility to EDCs in invertebrates or vertebrates (2). For the former, we chose NR0B, HNF4, HR83, and TLX and for the latter, we selected ER, ERR, RXR, NR1Jβ, PPAR-like1, THR, EcR, and E75. Expression of these *M. galloprovincialis* NRs was then studied in trochophore (28 hpf or 32 hpf) and D-veliger (44 hpf) larvae. NR0B was expressed in groups of cells within the trochophore shell field at 28 hpf (Fig. 3A). At 44 hpf, NR0B was conspicuously expressed in two clusters at opposite sides of the shell hinge and much less conspicuous along the D-border (Fig. 3A). HNF4 expression was detectable in a group of anterior cells in proximity of the stomodeum at 28 hpf and in the presumptive gut at 44 hpf (Fig. 3A). HR83 was detectable in discrete islets of cells in the prototroch at 28 hpf and, at 44 hpf, HR83 expression was limited to a single anterior group of cells (Fig. 3A). TLX was expressed in the apical region as well as the posterior end of the trochophore at 28 hpf (Fig. 3A). In the D-veliger, at 44 hpf, TLX expression was observable in several islets of cells at the ventroposterior and dorsoanterior edges of the mantle as well as in groups of cells in the center of the larval body (Fig. 3A).

At 32 hpf, ER was expressed in cells beneath the larval shell outline and, at 44 hpf, at the extremities of the hinge and along the border of the D-veliger shell (Fig. 3B), in a pattern very similar to that of NR0B (Fig. 3A). At 28 hpf, ERR expression was detectable in the dorsal region as well as in islets of cells in the apical region of the trochophore (Fig. 3B), which was similar to the expression of HR83 (Fig. 3A). By 44 hpf, ERR expression was in a duct-like structure that, from a curl on the D-border, reached the presumptive gut in the dorsal hinge region (Fig. 3B). The early and late expression patterns of ERR were partially reminiscent of those described for HNF4 (Fig. 3A). RXR was expressed ubiquitously throughout the larva at both 28 hpf and 44 hpf (Fig. 3B). NR1Jβ was highly expressed in a group of apical cells and scattered dorsally at 32 hpf (Fig. 3B). By 44 hpf, expression was concentrated along the border of the D-veliger shell and in the dorsal portion of the larval body, in a pattern forming two central islets and expanding towards the hinge region (Fig. 3B). While PPAR-like1 expression was observable in the region of the shell field at 28 hpf, its expression was very inconspicuous at 44 hpf (Fig. 3B). THR was expressed in small islets of cells in the apical, stomodeal, and dorsal regions of the trochophore at 28 hpf (Fig. 3B). THR expression then became restricted to the dorsal side of the larva in the D-veliger at 44 hpf (Fig. 3B). EcR was expressed around the larval shell border at both 28 hpf and 44 hpf (Fig. 3B). E75 expression

was widespread dorsally and restricted apically at 32 hpf (Fig. 3B). At 44 hpf, E75 expression was restricted to a discrete cluster of anterior cells (Fig. 3B), resembling the cluster of cells expressing HR83 at the same stage of development (Fig. 3A).

Discussion

In vertebrates, members of the NR superfamily are pivotal regulators of development and endocrine functions, and their activities are highly susceptible to disruptive modulations by EDCs (3,37). Corroborated experimental evidence indicates that NRs also play important roles in the regulation of invertebrate development. However, the diversity of animal life makes it difficult to robustly infer conserved biological functions of orthologous NRs and to evaluate the overall susceptibility of invertebrate NRs to EDC-based disruption based solely on data obtained in vertebrates (2). For these reasons, it is necessary to characterize the expression and function of NR superfamily members in a wide variety of different taxa and to interpret the results obtained in a comparative context. These comparative developmental and toxicological analyses will not only shed light on conserved and divergent functions of NRs in vertebrates and invertebrates but will also be instrumental to evaluate the potential risks of NR-mediated endocrine disruption in different taxa. In this light, this work presents a first step in the characterization of NR superfamily members in the Mediterranean mussel *M. galloprovincialis*, a bivalve mollusk. Our results show that the NR complement of *M. galloprovincialis* includes at least 47 members. However, given the complex architecture of the *M. galloprovincialis* genome (25), it is likely that not all NR superfamily members could be identified in this study. For example, *M. galloprovincialis* NR orthologs were identified for every vertebrate, protostome, and bivalve NR subgroup, with the exception of HR39 (NR5B). This absence indeed represented a false negative, as a *M. galloprovincialis* HR39 ortholog was retrieved from a different genome assembly (34). In addition, we failed to complete the sequences of 12 NRs identified in the currently available genome assembly. Although additional work will be required to establish the complete NR complement of *M. galloprovincialis*, the present study already provides a first thorough characterization of the NR superfamily in this species. We thus unequivocally identified 47 NR superfamily members in *M. galloprovincialis*, including HR39, which is in line with previous descriptions of NR complements in bivalve mollusk, such as *C. gigas* (43 NRs), and gastropod mollusks, such as *Biomphalaria glabrata* (39 NRs) and *Lottia gigantea* (33 NRs) (10,17). When compared to other animals, mollusks are generally characterized by a relatively high total number of NRs. This can be explained by two phenomena: (1) mollusk NR complements also include receptors that have previously been thought to be specific to arthropods (such as EcR, E78 and NR2Es) and (2) several NR subfamilies have experienced lineage-specific expansions (such as the NR1Cs and NR1Js in all mollusks and the NR1Ps and NR1CDEFs in bivalve mollusks) (10,17). The identification of EcR homologs outside the ecdysozoan clade further supports the notion that at least one ortholog of protostome EcR and deuterostome FXR/LXR was already present in the last common ancestor of protostomes and deuterostomes, suggesting that chemicals targeting NR1H receptors could potentially affect all bilaterian animals (9).

For the NR1Cs, while two members have previously been described in gastropods and *C. gigas*, we found three representatives in mussels, suggesting that mytilids have experienced an additional NR1C duplication when compared to other mollusks. However, our phylogenetic analyses indicate that a robust association of two of the three mussel NR1Cs with the third mussel NR1C plus the vertebrate NR1Cs (*i.e.*, the PPARs) is method-dependent, suggesting that the phylogenetic status of this NR subgroup might need additional experimental corroboration. *M. galloprovincialis* also has three members of the NR1CDEF subgroup, which has previously been characterized in *C. gigas* (10). As in this previous analysis, we were unable to reliably resolve the phylogenetic signal within this subgroup of the tree, hence preventing an unambiguous assignment of the three *M. galloprovincialis* NRs to the NR1Cs, NR1Ds, NR1Es or NR1Fs (10). The lack of phylogenetic resolution within this subgroup might be due to a rapid evolutionary divergence following duplication (10), which for NR1 subgroups C, D, E, and F probably took place very early in bilaterian evolution (9). The NR complement of *M. galloprovincialis* also includes 14 members of the bivalve-specific NR1Ps, a subgroup that has already been described in *C. gigas* (10). The positioning of NR1Ps in close relationship to the NR1Cs, NR1Ds, NR1Es, and NR1Fs is consistent with previous observations and supports the notion that bivalves, and maybe all mollusks, have evolved a unique NR subgroup (10,38). Like in *C. gigas*, we also found four members of the protostome-specific NR1J subgroup in *M. galloprovincialis* (10). Furthermore, we identified a single *M. galloprovincialis*

representative for every other subgroup of the NR1 subfamily, including NRs that are potentially responsive ecdysone-like hormones, such as EcR, E75/Rev-Erb, E78, and HR3/ROR, which, in addition to arthropods, have previously been identified in other mollusks as well as in leeches and annelids (10,17,18).

The NR complement of *M. galloprovincialis* includes orthologs of all invertebrate and vertebrate NR subgroups that have been suggested to be implicated in endocrine disruption. For example, EcR and other ecdysone-responsive NRs of arthropods are currently the only invertebrate NRs known to be involved in hormonal control of the endocrine system, and these NRs are major targets of a broad range of EDCs, including steroids and pesticides (12,13,39). The transcriptional activity of members of the NR1C subgroup in vertebrates and gastropods has been shown to be disrupted by organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT) (3,11). The NR1Js, which include the arthropod HR96 and which form the outgroup of the vertebrate NR1Is, are susceptible to disruption by xenobiotics and biotoxins (40–42). Vertebrate THRs are major regulators of metamorphosis and notable targets of EDCs (3). Although a large body of evidence indicates that THR is also a regulator of metamorphosis in bivalves, its endogenous ligands (and potential disruptors) are yet to be discovered (2). RXR stands out due to its susceptibility to endocrine disruption by organotin, which has been documented in an astonishing diversity of metazoan animals (43). ER is a known target of EDCs in vertebrates, while its mollusk orthologs are devoid of any ligand-dependent transcriptional activity (2). Similarly, the transcriptional activity of vertebrate ERR is disrupted by bisphenol A (BPA) (44), but an involvement of ERR in endocrine disruption in mollusks still remains to be demonstrated. Given the presence of orthologs 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 (2,10,45). 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 (2,45). 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 (46), 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 (2,46). HR38 and TLX were expressed, presumably, in developing apical, pedal, and visceral ganglions (46,47), indicating that, as in other animals, NR2E receptors might function during development of the sensory nervous system in *M. galloprovincialis* (2,48,49). 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 was described previously in vertebrates (50). 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 (49). NR1Jβ 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 (46). In the fruit fly, the NR1J homolog HR96 is expressed in muscles and gut of the larva (49). 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 (47,49,51). 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 a 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 (20), 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 (55), 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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References

1. Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier MEA, et al. Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. PLoS Biol. 2010;8:e1000497.

2. Miglioli A, Canesi L, Gomes IDL, Schubert M, Dumollard R. Nuclear receptors and development of marine invertebrates. Genes. 2021;12:83.

3. Toporova L, Balaguer P. Nuclear receptors are the major targets of endocrine disrupting chemicals. Mol Cell Endocrinol. 2020;502:110665.

4. Tan H, Chen Q, Hong H, Benfenati E, Gini GC, Zhang X, et al. Structures of endocrine-disrupting chemicals correlate with the activation of 12 classic nuclear receptors. Environ Sci Technol. 2021;55:16552–16562.

5. La Merrill MA, Vandenberg LN, Smith MT, Goodson W, Browne P, Patisaul HB, et al. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. Nat Rev Endocrinol. 2020;16:45–57.

6. Cuvillier-Hot V, Lenoir A. Invertebrates facing environmental contamination by endocrine disruptors: novel evidences and recent insights. Mol Cell Endocrinol. 2020;504:110712.

7. Ford AT, Leblanc GA. Endocrine disruption in invertebrates: a survey of research progress. Environ Sci Technol. 2020;54:13365–13369.

8. Fernandez MA. Populations collapses in marine invertebrates due to endocrine disruption: a cause for concern? Front Endocrinol. 2019;10:721.

9. Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M. Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. Mol Biol Evol. 2004;21:1923–1937.

10. Vogeler S, Galloway TS, Lyons BP, Bean TP. The nuclear receptor gene family in the Pacific oyster, *Crassostrea gigas*, contains a novel subfamily group. BMC Genomics. 2014;15:369.

11. Capitão AMF, Lopes-Marques M, Páscoa I, Sainath SB, Hiromori Y, Matsumaru D, et al. An ancestral nuclear receptor couple, PPAR-RXR, is exploited by organotins. Sci Total Environ. 2021;797:149044.

12. Spindler KD, Hönl C, Tremmel C, Braun S, Ruff H, Spindler-Barth M. Ecdysteroid hormone action. Cell Mol Life Sci.

2009;66:3837–3850.

13. Pandey AK, Sharma V, Ravi Ram K. Drosophila ecdysone receptor activity-based *ex vivo* assay to assess the endocrine disruption potential of environmental chemicals. Environ Sci Pollut Res. 2022;29:56430–56441.

14. Jordaõ R, Campos B, Piña B, Tauler R, Soares AMVM, Barata C. Mechanisms of action of compounds that enhance storage lipid accumulation in daphnia magna. Environ Sci Technol. 2016;50:13565–13573.

15. Ruivo R, Sousa J, Neuparth T, Geffard O, Chaumot A, Castro LFC, et al. From extrapolation to precision chemical hazard assessment: the ecdysone receptor case study. Toxics. 2021;10:6.

16. Xiong X, Cao Y, Li Z, Huang R, Du X, Zheng Z. Ecdysone signal pathway participates in shell formation in pearl oysters *Pinctada fucata martensii*. J Steroid Biochem Mol Biol. 2022;217:106045.

17. Kaur S, Jobling S, Jones CS, Noble LR, Routledge EJ, Lockyer AE. The nuclear receptors of *Biomphalaria glabrata* and *Lottia gigantea*: implications for developing new model organisms. PLoS One. 2015;10:e0121259.

18. Laguerre M, Veenstra JA. Ecdysone receptor homologs from mollusks, leeches and a polychaete worm. FEBS Lett. 2010;584:4458–4462.

19. Pironti C, Ricciardi M, Proto A, Bianco PM, Montano L, Motta O. Endocrine-disrupting compounds: an overview on their occurrence in the aquatic environment and human exposure. Water. 2021;13:1347.

20. FAO. 2022. The state of world fisheries and aquaculture 2022. Towards blue transformation. Rome, Italy.

21. Yaghubi E, Carboni S, Snipe RMJ, Shaw CS, Fyfe JJ, Smith CM, et al. Farmed mussels: a nutritive protein source, rich in omega-3 fatty acids, with a low environmental footprint. Nutrients. 2021;13:1124.

22. Polsenaere P, Soletchnik P, Le Moine O, Gohin F, Robert S, Pépin JF, et al. Potential environmental drivers of a regional blue mussel mass mortality event (winter of 2014, Breton Sound, France). J Sea Res. 2017;123:39–50.

23. ISO 17244:2015. Water quality– Determination of the toxicity of water samples on the embryo-larval development of Japanese oyster (*Crassostrea gigas*) and mussel (*Mytilus edulis* or *Mytilus galloprovincialis*). International Standardization Organization (ISO). 2020.

24. ASTM E724-21. Standard guide for conducting static short-term chronic toxicity tests starting with embryos of four species of saltwater bivalve molluscs. American Society for Testing and Materials (ASTM) International. 2021.

25. Gerdol M, Moreira R, Cruz F, Gómez-Garrido J, Vlasova A, Rosani U, et al. Massive gene presence-absence variation shapes an open pan-genome in the Mediterranean mussel. Genome Biol. 2020;21:275.

26. Miglioli A, Tredez M, Boosten M, Sant C, Carvalho JE, Dru P, et al. The Mediterranean mussel, *Mytilus galloprovincialis*, a novel model for developmental studies of mollusks. BioRxiv. 2023;2023.07.27.550798.

27. Tyler J. Carrier, Adam M. Reitzel AH. Evolutionary ecology of marine invertebrate larvae. Oxford University Press; 2018. 16–33 p.

28. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: the protein families database. Nucleic Acids Res. 2014;42:D222–D230.

29. Finn RD, Clements J, Arndt W, Miller BL, Wheeler TJ, Schreiber F, et al. HMMER web server: 2015 update. Nucleic Acids Res. 2015;43:W30–W38.

30. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–1313.

31. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 2001;17:754–755.

32. Miglioli A, Balbi T, Montagna M, Dumollard R, Canesi L. Tetrabromobisphenol A acts a neurodevelopmental disruptor in early larval stages of *Mytilus galloprovincialis*. Sci Total Environ. 2021;793:148596.

33. Duarte J, Perrière G, Laudet V, Robinson-Rechavi M. NUREBASE: database of nuclear hormone receptors. Nucleic Acids Res. 2002;30:364–368.

34. Simon A. Three new genome assemblies of blue mussel lineages: North and South European *Mytilus edulis* and Mediterranean *Mytilus galloprovincialis*. BioRxiv. 2022;2022.09.02.506387.

35. Huang W, Xu F, Li J, Li L, Que H, Zhang G. Evolution of a novel nuclear receptor subfamily with emphasis on the member from the Pacific oyster *Crassostrea gigas*. Gene. 2015;567:164–172.

36. Beinsteiner B, Markov GV., Bourguet M, McEwen AG, Erb S, Patel AKM, et al. A novel nuclear receptor subfamily enlightens the origin of heterodimerization. BMC Biol. 2022;20:217.

37. Chung AC, Cooney AJ. The varied roles of nuclear receptors during vertebrate embryonic development. Nucl Recept Signal. 2003;1:e007.

38. Winnepenninckx BMH, Reid DG, Backeljau T. Performance of 18S rRNA in littorinid phylogeny (Gastropoda: Caenogastropoda). J Mol Evol. 1998;47:586–596.

39. LeBlanc GA. Crustacean

endocrine toxicology: a review. Ecotoxicology. 2007;16:61–81.

40. Karimullina E, Li Y, Ginjupalli GK, Baldwin WS. Daphnia HR96 is a promiscuous xenobiotic and endobiotic nuclear receptor. Aquat Toxicol. 2012;116–117:69–78.

41. Delfosse V, Huet T, Harrus D, Granell M, Bourguet M, Gardia-Parège C, et al. Mechanistic insights into the synergistic activation of the RXR–PXR heterodimer by endocrine disruptor mixtures. Proc Natl Acad Sci U S A. 2021;118:e2020551118.

42. Cruzeiro C, Lopes-Marques M, Ruivo R, Rodrigues-Oliveira N, Santos MM, Rocha MJ, et al. A mollusk VDR/PXR/CAR-like (NR1J) nuclear receptor provides insight into ancient detoxification mechanisms. Aquat Toxicol. 2016;174:61–69.

43. Fonseca E, Ruivo R, Borges D, Franco JN, Santos MM, Castro LFC. Of retinoids and organotins: the evolution of the retinoid x receptor in Metazoa. Biomolecules. 2020;10:594.

44. Tohmé M, Prud'Homme SM, Boulahtouf A, Samarut E, Brunet F, Bernard L, et al. Estrogen-related receptor γ is an *in vivo* receptor of bisphenol A. FASEB J. 2014;28:3124–3133.

45. Vogeler S, Bean TP, Lyons BP, Galloway TS. Dynamics of nuclear receptor gene expression during Pacific oyster development. BMC Dev Biol. 2016;16:33.

46. Dyachuk V, Odintsova N. Development of the larval muscle system in the mussel *Mytilus trossulus* (Mollusca, Bivalvia). Dev Growth Differ. 2009;51:69–79.

47. Voronezhskaya EE, Nezlin LP, Odintsova NA, Plummer JT, Croll RP. Neuronal development in larval mussel *Mytilus trossulus* (Mollusca: Bivalvia). Zoomorphology. 2008;127(2):97–110.

48. Kitambi SS, Hauptmann G. The zebrafish orphan nuclear receptor genes *nr2e1* and *nr2e3* are expressed in developing eye and forebrain. Gene Expr Patterns. 2007;7:521–528.

49. Wilk R, Hu J, Krause HM. Spatial profiling of nuclear receptor transcription patterns over the course of *Drosophila* development. G3. 2013;3:1177–1189.

50. Ehrlund A, Treuter E. Ligand-independent actions of the orphan receptors/corepressors DAX-1 and SHP in metabolism, reproduction and disease. J Steroid Biochem Mol Biol. 2012;130:169–179.

51. Kniazkina M, Dyachuk V. Neurogenesis of the scallop *Azumapecten farreri*: from the first larval sensory neurons to the definitive nervous system of juveniles. Front Zool. 2022;19:22.

52. Capelle JJ, Garcia AB, Kamermans P, Engelsma MY, Jansen HM. Observations on recent mass mortality events of marine mussels in the Oosterschelde, the Netherlands. Aquac Int. 2021;29:1737–1751.

53. Dégremont L, Maurouard E, Rabiller M, Glize P. Response to selection for increasing resistance to the spring mortality outbreaks in *Mytilus edulis* occurring in France since 2014. Aquaculture. 2019;511:734269.

54. Lupo C, Bougeard S, Le Bihan V, Blin JL, Allain G, Azéma P, et al. Mortality of marine mussels *Mytilus edulis* and *M. galloprovincialis*: systematic literature review of risk factors and recommendations for future research. Rev Aquac. 2021;13:504–536.

55. Avdelas L, Avdic-Mravlje E, Borges Marques AC, Cano S, Capelle JJ, Carvalho N, et al. The decline of mussel aquaculture in the European Union: causes, economic impacts and opportunities. Rev Aquac. 2021;13:91–118.

Figure and Table Captions

Figure 1: Phylogenetic relationship of nuclear receptors (NRs) from *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 μm. 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). NR expression levels are reported as scaled (by NR sequence) z-score values of the CPM (counts per million) gene count matrix.

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 to endocrine disrupting chemicals (EDCs) (2): ER, ERR, RXR, NR1Jβ, PPAR-like1, THR, EcR, and E75. Representative images of trochophore (28 hpf or 32 hpf) and D-veliger (44 hpf) stages. Maximum z-projections of Hoechst nuclear staining (grey) and fluorescent *in situ* hybridization signal (red), merged channels are shown. Shell outline is indicated with dotted white lines, orange arrowheads highlight presumptive outer and inner pairs of larval retractor muscles. aso: apical sensory organ; eso: esophagus; h: hinge; g: gut; sf: shell field; st: stomodeum; t: prototroch. Larval orientation as in Figure 2A. Scale bars: 20 μm.

Supplementary Material (https://doi.org/10.6084/m9.figshare.c.7007971.v1)

Supplementary Figure 1: Phylogenetic relationships of nuclear receptors (NRs) from *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 nuclear receptors (NRs) of the NR5 subfamily from *Mytilus galloprovincialis* (*Mg*), *Mytilus coruscus* (*Mco*), *Crassostrea gigas* (*Cg*), *Homo sapiens* (*Hs*), and *Drosophila melanogaster* (*Dm*). 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 Figure 3: Phylogenetic relationship of (A) 2DBD and (B) NR0 nuclear receptors (NRs) from *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).

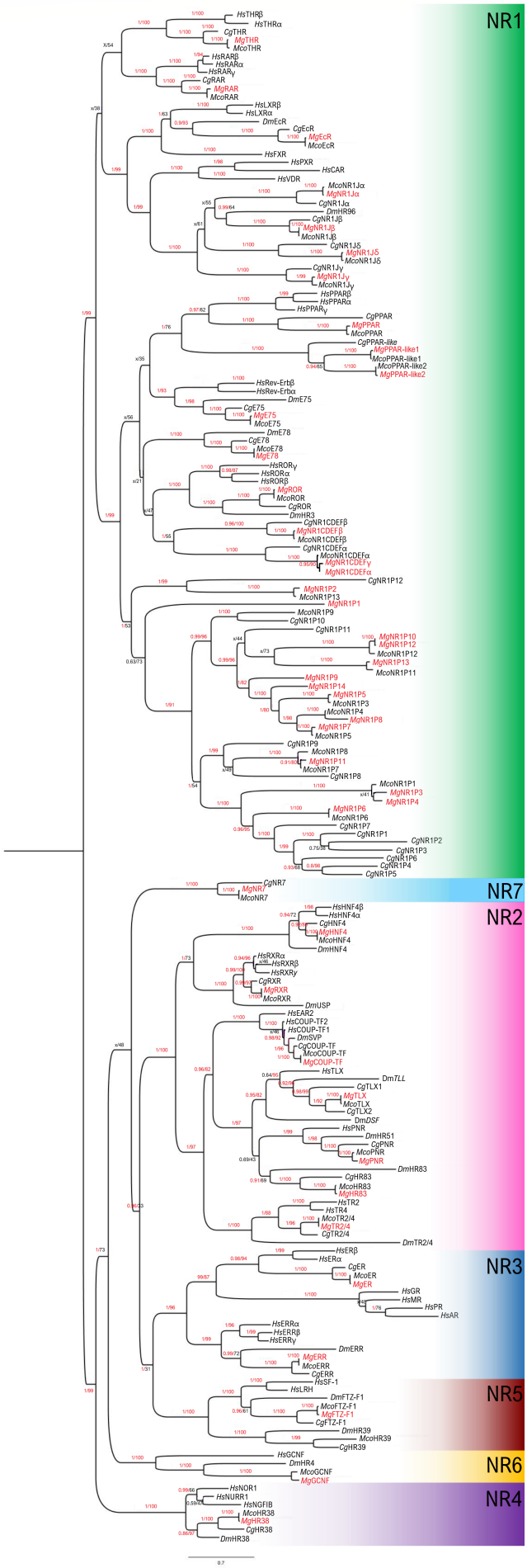
Additional Information

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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HsRARγ
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McoRAR
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MgEcR
McoEcR
HsFXR
HsPXR
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HsVDR
McoNR1Jα
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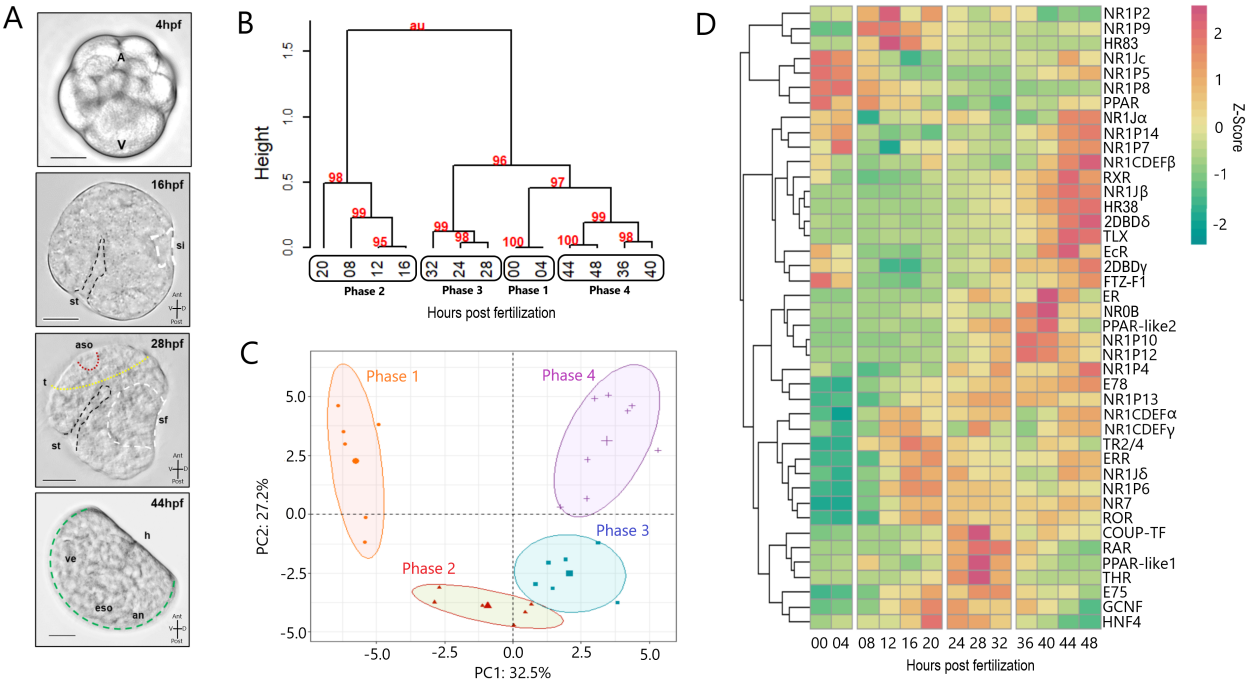
Figure 1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PC1: 32.5%
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HR83
NR1Jc
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PPAR
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NR1P7
NR1CDEFβ
RXR
NR1Jβ
HR38
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TLX
EcR
2DBDγ
FTZ-F1
ER
NR0B
PPAR-like2
NR1P10
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ERR
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NR1P6
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Figure 2

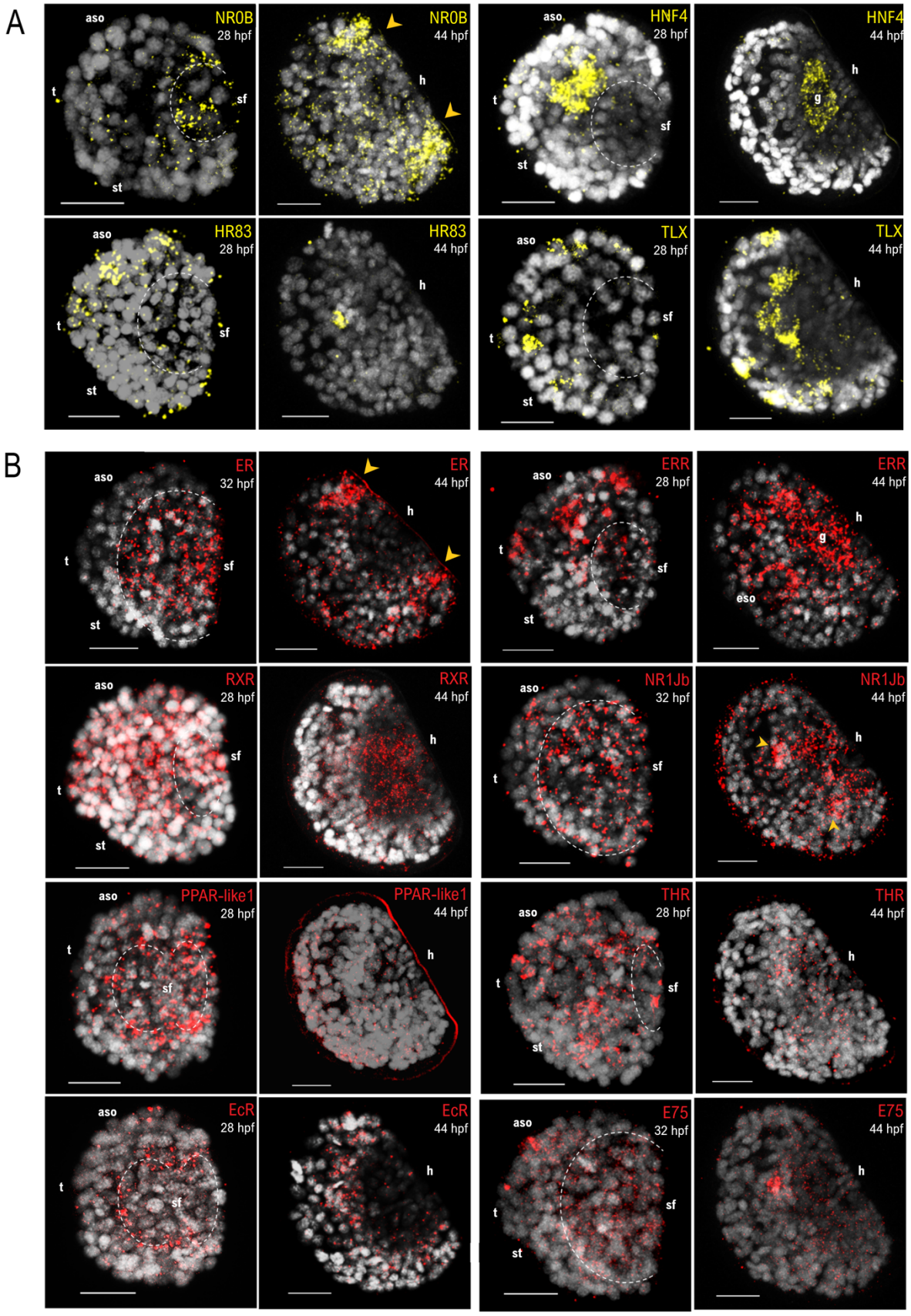
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Figure 3