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Dynamics of nuclear receptor gene expression during Pacific oyster development

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Abstract

Background: Nuclear receptors are a highly conserved set of ligand binding transcription factors, with essential roles regulating aspects of vertebrate and invertebrate biology alike. Current understanding of nuclear receptor regulated gene expression in invertebrates remains sparse, limiting our ability to elucidate gene function and the conservation of developmental processes across phyla. Here, we studied nuclear receptor expression in the early life stages of the Pacific oyster, *Crassostrea gigas*, to identify at which specific key stages nuclear receptors are expressed

Results: We used quantitative RT-PCR to determine the expression profiles of 34 nuclear receptors, revealing three developmental key stages, during which nuclear receptor expression is dynamically regulated: embryogenesis, mid development from gastrulation to trochophore larva, and late larval development prior to metamorphosis. Clustering of nuclear receptor expression patterns demonstrated that transcriptional regulation was not directly related to gene phylogeny, suggesting closely related genes may have distinct functions. Expression of gene homologs of vertebrate retinoid receptors suggests participation in organogenesis and shell-formation, as they are highly expressed at the gastrulation and trochophore larval initial shell formation stages. The ecdysone receptor homolog showed high expression just before larval settlement, suggesting a potential role in metamorphosis.

Conclusion: Throughout early oyster development nuclear receptors exhibited highly dynamic expression profiles, which were not confined by gene phylogeny. These results provide fundamental information on the presence of nuclear receptors during key developmental stages, which aids elucidation of their function in the developmental process. This understanding is essential as ligand sensing nuclear receptors can be disrupted by xenobiotics, a mode of action through which anthropogenic environmental pollutants have been found to mediate effects.

Keywords: Nuclear receptors, Gene expression, Development, *Crassostrea gigas*, Invertebrates

Background

Nuclear receptors (NRs) are one of the largest classes of transcription factors in metazoan species and regulate many cellular functions through manipulation of gene expression. Although NRs are present even in the simplest animals in low numbers (demosponge: 2 NRs [1]), extensive diversification of NR families has occurred in Bilateria through gene duplications, gene loss and diversification [1–4]. In vertebrate species, NRs are essential for regulating gene expression during complex

processes, in particular during development, which is one of the most dynamic periods of NR activity [5, 6]. For invertebrates, however, information on NR regulated gene expression is limited. Nevertheless, many developmental processes in metazoans are highly conserved and similar features are shared among diverse phyla. Understanding the presence and participation of NRs in phases of tightly controlled gene expression, particularly during developmental stages, is highly desirable. Not only is this a requirement for understanding the intrinsic biology, but also because alteration of NR function is one of the key routes through which normal biology can be disrupted by external factors, often resulting in abnormal phenotypes.

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Typical NRs consists of five different domains, which include the highly conserved C domain, also denoted as the DNA-binding domain (DBD), and the moderately conserved ligand binding (E-) domain (LBD). The high sequence conservation of the DBD and LBD in particular, allows for phylogenetic classification of NR subfamilies (NR0-NR6) and their constituent subgroups [7, 8]. NRs regulate gene expression by attaching the DBD to specific response elements in the promoter of target genes, with the whole protein structure functioning as monomer, homodimer or heterodimer [9, 10].

One of the most interesting characteristics of NRs is their capability to interact with endogenous or exogenous compounds through ligand binding, a feature which has been described for a subset of NRs, such as for hormonal (steroid and thyroid hormones) and retinoid regulated receptors. In addition, other NRs do not require any ligand binding and function in a constitutively activated manner [9]. Ligand-binding NRs are able to bind to exogenous compounds. When present in high doses or as mimics of natural ligands, these xenobiotics can lead to disruption of normal NR function [11]. Xenobiotics in the environment are commonly introduced by anthropogenic pollution and can affect various developmental and physiological processes in humans and wildlife [12, 13]. In many fish species exposure to xenoestrogens such as bisphenol A (BPA), 17 α -ethinylestradiol and dichlorodiphenyltrichloroethane, have caused developmental malformations and had negative effects on reproduction [12, 14, 15]. Many of the effects of environmental estrogens are known to be mediated through interaction with NRs [15–18]. Tributyltin (TBT), an organotin and an additive in antifouling paints for boats, has been shown to cause imposex, (superimposition of male organs on females) in >20 gastropod species [19] as well as developmental failure and reproduction impairment in bivalves [20–23]. TBT has been identified as a xenobiotic ligand for vertebrate and gastropod NRs [24–26] and a link between NRs and TBT-mediated disruption has been proposed [26–28]. As the expression of NRs varies between different life stages, the response of an animal to a xenobiotic can vary according to the life stage.

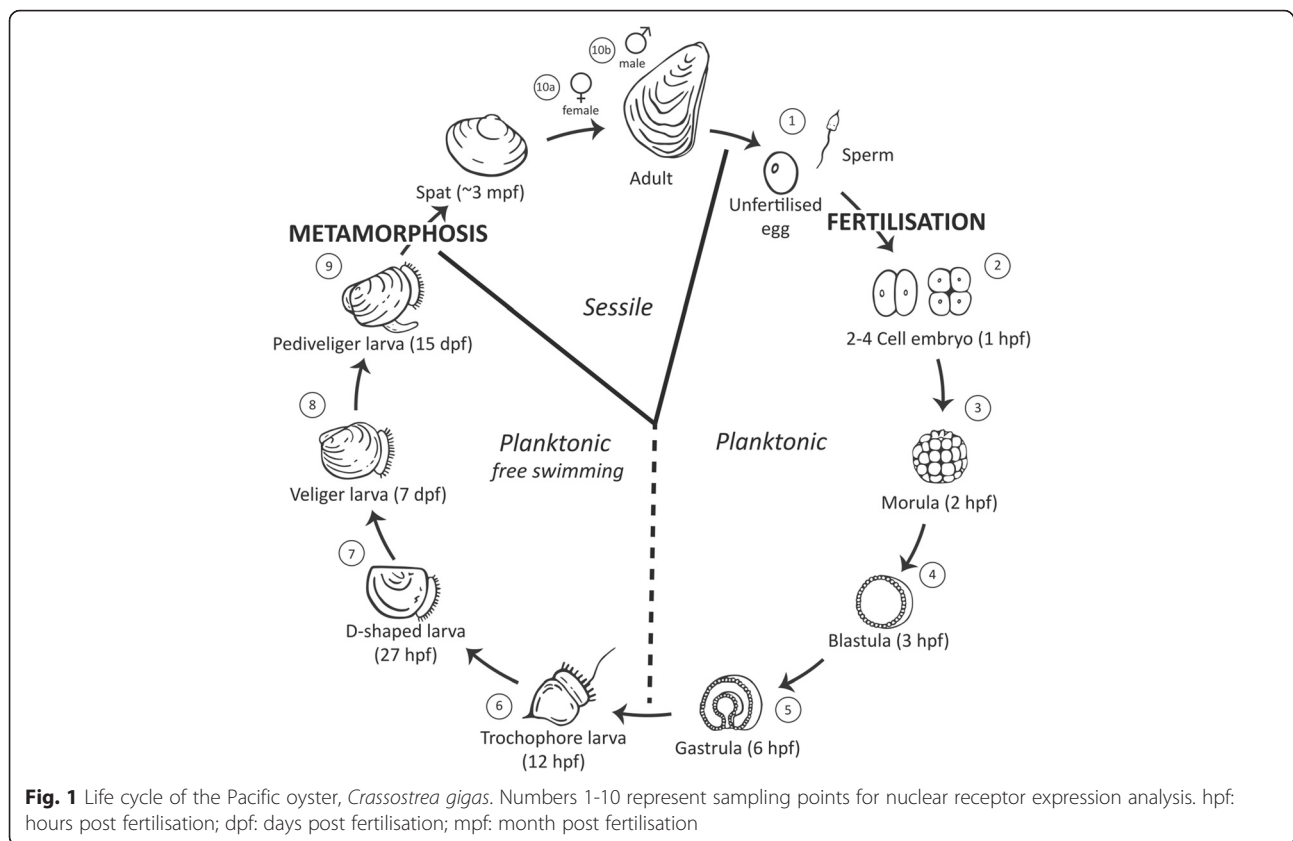
The function and presence of NRs in development, reproduction and homeostasis in vertebrate species is well studied, but knowledge of receptor participation in invertebrate systems has been less well investigated. Previously, we [29] reported the presence of 43 NRs in the Pacific oyster, *Crassostrea gigas*, and described their phylogenetic relationship to other known NR homologs in human and *Drosophila*. The Pacific oyster is a bivalve species (Clade: Lophotrochozoa, Phylum: Mollusca) and as a sessile filter feeder, it is a commonly used organism for biological monitoring [30–32]. Oysters live along

coasts and estuaries worldwide and are under constant anthropogenic pressure including from industrial, agriculture and sewage pollution. Although oyster development has been well studied due to the high economic interests in aquaculture for food, the underlying molecular mechanisms of gene regulation during development remain mostly unknown. Here, we provided an overview of the presence of NRs in Pacific oyster life stages, including the early embryo and larva. We studied 34 of the 43 NRs genes, for which expression could be verified by quantitative RT-PCR (qPCR), and assessed the expression patterns for phylogenetically related groups of genes across early life stages, also including a comparison with adults of both sexes. We discuss the results in the context of the three identified developmental stages (early, mid and late development) and the potential participation of NR genes in embryonic development and sexual differentiation. In particular we focused on those receptors which have previously shown a potential for xenobiotic disruption in other species; the retinoid X receptor (RXR), retinoic acid receptor (RAR), thyroid receptor (THR), estrogen receptor (ER), estrogen-related receptor (ERR), peroxisome proliferator-activated receptor (PPAR), ecdysone receptor (EcR), and xenobiotic-sensing receptor subfamily group NR1].

Results

Quantitative RT-PCR was used to measure the expression of 34 NRs in ten different life stages of the Pacific oyster, including nine developmental stages, and male and female adult individuals (Fig. 1). In general, NR expression was measured during all life points and showed variation between developmental stages (Fig. 2). A few NR transcripts (CgNR2E2, CgNR2E3 and CgNR2F), were below the limit of detection (N/A) at some life stages. Expression of each receptor was calculated relative to a normalisation factor derived from three reference genes. Elongation factor-1 α (EF-1), ribosomal protein S18 (RS18) and ribosomal protein L7 (RL7) were verified as the most suitable reference genes among other commonly used housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, glutathione S-transferase, α -tubulin) by the programme geNorm v3 (Additional file 1).

Principal component analysis (PCA) for developmental stages (excluding adult stages) (Fig. 3) was conducted for 31 of 34 NRs, excluding those receptors which showed an expression below detection limit. Principal component 1 (PC1) accounted for 42 % of the overall variance in gene expression among developmental stages and principal component 2 (PC2) accounted for 25 % of the overall variance. Based on the observed variance in the expression levels of the NRs, four distinct life events are clearly distinguishable (Fig. 3a): early embryo



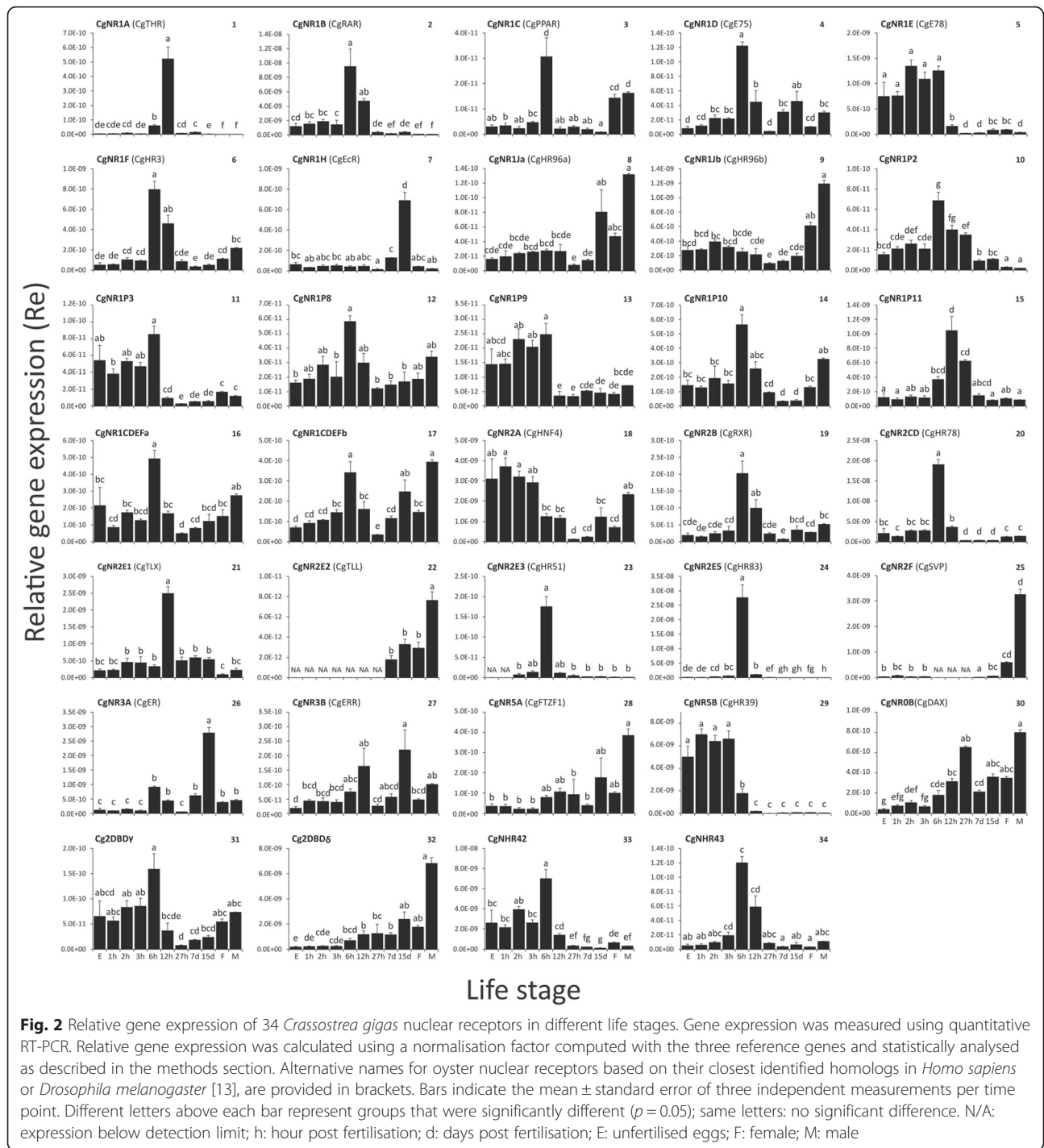
development (unfertilized egg – 3 hpf), mid development, divided into gastrulation (6 hpf) and trochophore larval stage (12 hpf), and late larval development (27 hpf – 15 dpf).

Similar clustering was detected by separating the NRs based on the observed variance in different life stages (Fig. 3b). Three clusters of NRs emerged and could be categorised in three of the four previously detected key stages: early, mid and late development. Within the mid development group, the gastrulation (6 hpf) and trochophore larval (12 hpf) stages were not as clearly distinguished as they had been during the previous observation of the variance in the expression levels of NRs. Most of the NRs in the detected early and mid development stages contribute to the observed principle components (Fig. 3c): early development: CgNR1P3, CgNR1E, CgNHR42, CgNR5B, Cg2DBD γ ; CgNR1P9; mid-development: CgNR2CD, CgNHR43, CgNR1B, CgNR1F, CgNR2B, CgNR2E5, CgNR1CDEFa, CgNR1P10, CgNR1P8. Only two NRs (Cg2DBD δ , CgNR0B) display a contribution higher than the expected average contribution for the late development stage. CgNR1H and CgNR1Ja could not be clearly assigned to one of the three key developmental stages by the PCA (Fig. 3) and did not contribute towards the observed variance in different developmental life stages (Fig. 3c). The expression profile

of CgNR1H (Fig. 2) showed an increase at 15 dpf compared to all other developmental and adult stages. CgNR1Ja, showed a comparable expression pattern to its closest oyster receptor paralog CgNR1Jb, which assigned with the NR group expressed during early development. The moderate expressions in early and mid development of both receptors are replaced by low expression during later development and high expression in adult life stages. In addition, CgNR1Ja showed a high expression peak at 15 dpf.

Previously we identified a novel subfamily group, NR1P, in the pacific oyster comprising 11 NRs [29]. The expressions of six of these receptors (NR1P2, NR1P3, CgNR1P8, CgNR1P9, CgNR1P10, CgNR1P11) were analysed and they all display differences in their expression profiles among different life stages (Fig. 3b): two receptors (NR1P3(11), NR1P9(13)) are mainly expressed at early development; three at mid-development (NR1P2(10), NR1P8(12), NR1P10(14)); and one at late development (NR1P11(15)).

The sex of adults was determined by visually observing the presence of developed oocytes (eggs) or spermatozoa in the gonads. Gene expression data (Fig. 2) shows a difference in expression patterns between unfertilized eggs and adult females. Therefore, we assume that the expression measured in female



adults, which were at the beginning of the gametogenesis, was not entirely due to the presence of eggs. The same is assumed for males as RNA concentration in sperm is very low [33].

Individual analyses of adults (Fig. 2) show equal levels of expression for most males and females, with only five NRs showing significant differences between sexes. An additional PCA was conducted (Additional file 2) to

identify general expression patterns between males and females among developmental and adult stages. Males and females vary in their NR expression and each sex also separates from most of the developmental stages (likenesses to pediveliger stage (15 dpf)). The cladogram of a heat map analysis of all NRs (Fig. 4) shows a similar separation of adults and developmental stages, in particular to early and mid developmental stages.

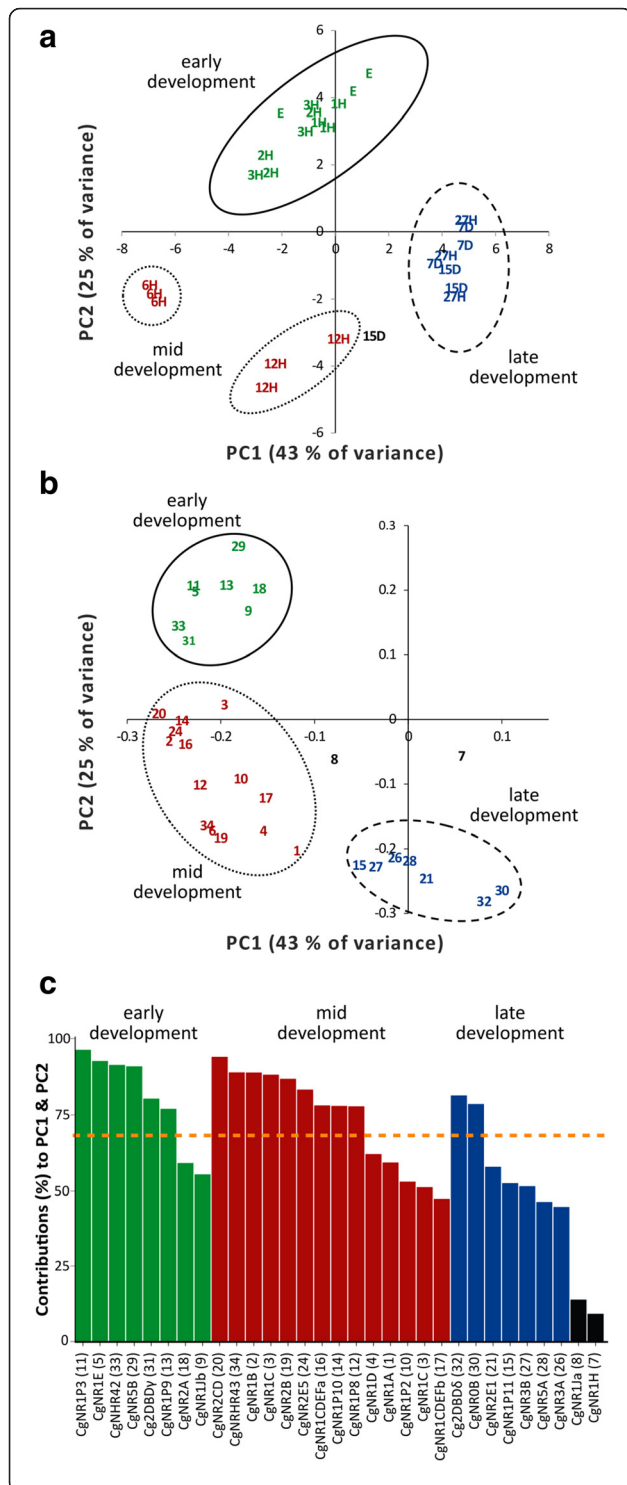
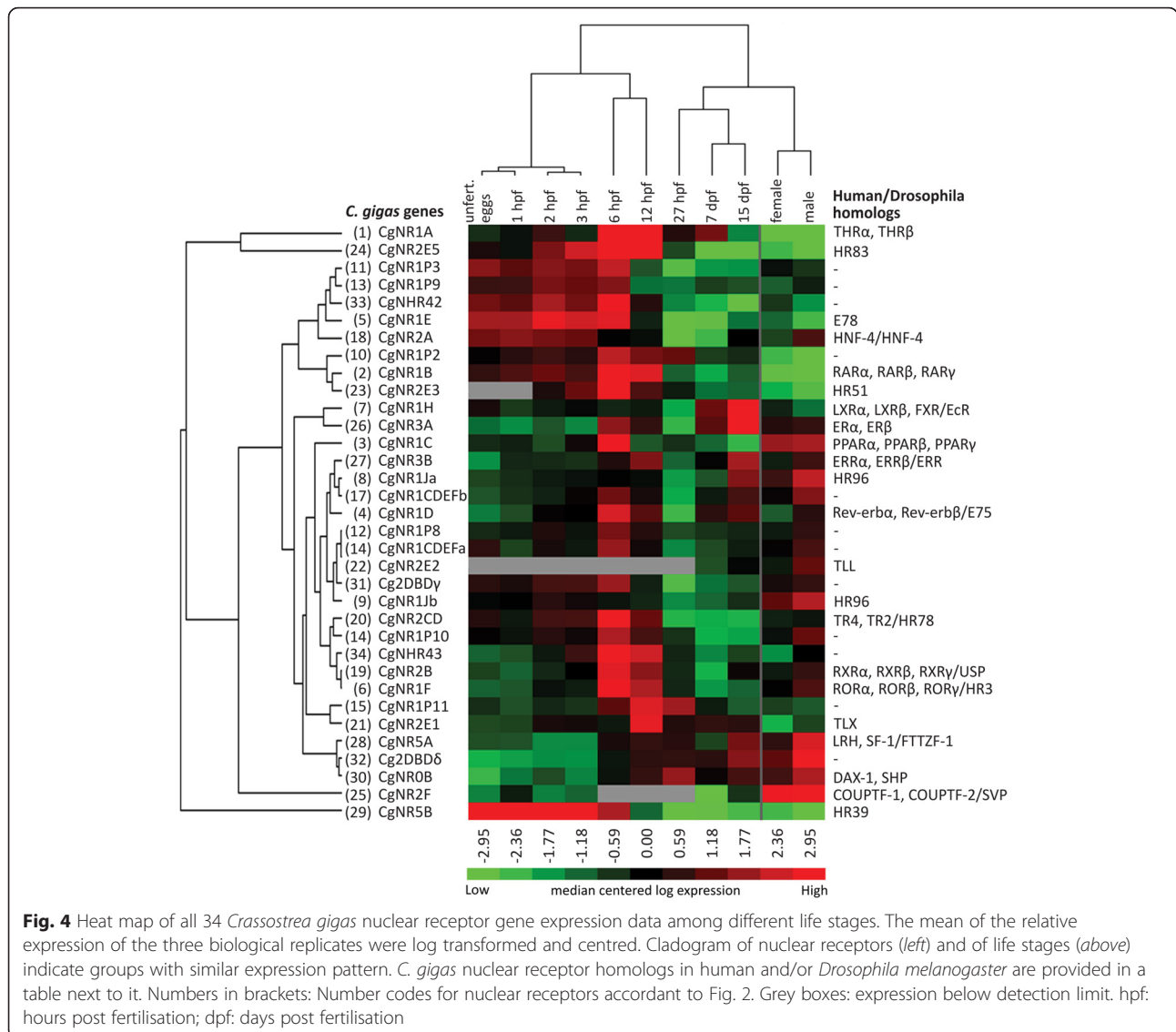


Fig. 3 Principle component analysis (PCA) of 31 *Crassostrea gigas* nuclear receptor gene expression data. **a** Scatterplot of the first two PCA components of developmental stages indicating separation based upon the variance observed in the expression levels of 31 of the 34 nuclear receptor genes. Principal component 1 (PC1) and 2 (PC2) explain 43 and 25 % of variance, respectively. **b** Scatterplot of PC1 and PC2 scores indicating the separation of 31 nuclear receptors based on expression across life stages. Circles around measurements represent distinct clustering for developmental stages or nuclear receptors: early development (solid line), mid development (dotted lines), late development (dashed line). Number codes for nuclear receptors can be found in (c). **c** Bar chart showing the contribution (in percentage) of each nuclear receptor towards the variability of PC1 and PC2. The orange dashed line: expected average contribution. Letters/numbers/bars: early development (green), mid development (red), late development (blue), receptors not assigned (black). h: hour post fertilisation; d: days post fertilisation; E: unfertilised eggs

The clustering of the gene expression analysis does not correspond with the phylogenetic clustering of the NR subfamily groups, which also shows divergent temporal expression patterns during development. This becomes particularly apparent in the heat map analysis of all NR expressions (Fig. 4), in which all developmental stages and both adult life stages have been included. The heat map shows comparable results to the PCA; three main developmental stages, early, mid and late development, plus a further adult life stage were identified with hierarchical clustering of the life stages. Clustering of NRs based on their expression profile does not concur with the phylogenetic relationship of these NRs. The dendrogram of the NR hierarchical clustering shows no coherence between phylogenetically related NRs. For example, during early development (unfertilised eggs – 3hpf), receptors belonging to subfamily 1 (e.g., 11, 13, 5, 10, 2) group together with receptors member of subfamily 2 (e.g., 18, 23) or non-assigned receptors (e.g., 33) (Fig. 4).

Discussion

We show here that the Pacific oyster, like many other complex multicellular metazoans, differentially expresses a variety of NRs during its lifetime. The expression pattern analysis of 34 NRs throughout nine developmental time points clusters into three distinct life stages, showing dynamic changes in receptor expression: an early, a mid and a late developmental stage. Observationally, the mid developmental stage can be further divided into two separate developmental processes: gastrulation and trochophore larval stages, which include organ differentiation and shell development. Male and female adult life stages show an overall separation from the other developmental stages as well as from each other. However, the individual analysis of male and female expression patterns demonstrates only a few cases for which NRs are differentially expressed. The observed clustering of



NR expression does not correlate with each receptor’s phylogenetic relationship, and belonging to the same subfamily does not result in similar patterns of expression, indicating differential or redundant functions. The members of the novel subfamily group NR1P also show differences in their expression among each life stage, which suggests that these receptors fulfil different functions, irrespective of their close phylogenetic relationship.

Early development: embryogenesis

Embryogenesis, defines the first few steps in the life of a freshly fertilised egg in metazoans. During this event, RNA gene transcripts are thought to be maternal or zygotic in origin: maternally synthesized RNAs and proteins are stocked in oocytes during female gametogenesis [34]. A Pacific oyster zygote undergoes its first 2–4 cell division within an hour of fertilisation and is shortly

after followed by the morula (2 hpf), blastula (3 hpf) and gastrula (6 hpf) stages (Fig. 1).. The expression of NRs at these early life stages did not significantly differ from the expression in unfertilised eggs for any tested NR. This suggests that the first NRs expressed during early development in oysters are provided by the mother. Nevertheless, maternal RNA is not universally stable and will degrade over time. It is replaced or diluted by zygotic gene expression, a process that is called maternal-to-zygotic transition (MZT) [34, 35]. This RNA destabilisation varies spatially and temporally, depending on gene, degradation mechanism and species, but the event of maternal RNA decay appears to be evolutionarily conserved among metazoans [36, 37]. Embryonic transcriptional activation through zygotic genome activation (ZGA) for a specific gene usually results in an increase in expression during early

embryogenesis [35]. In this study, the PCA of gene expression for 34 oyster NRs did not distinguish a sub-grouping within the early developmental group (Fig. 3a), which would indicate a collective ZGA onset for the majority of the NRs. The data suggests that the MTZ time schedule is not concordant for most NRs. It is unclear if and which NRs are actually regulated by ZGA in invertebrates. Although research on *Drosophila* suggests that most of the transcription factors are strictly zygotic [38], other data indicates that some transcription factors are of maternal origin and are required for ZGA onset and maternal RNA destabilisation [38–40]. Nevertheless, the resolution of this study for distinguishing between maternal/zygotic RNA is low and additional research including a higher temporal resolution is required to comprehensively identify the origin of early NR expression or proteins.

Compared to their expression level during other life stages, a few NRs showed a high expression level during early embryogenesis: CgNR2A, CgNR5B, CgNR1P9 mostly decrease in expression after 3 hpf, and CgNR1E, CgNR1P3, Cg2DBD, CgNHR42 mostly decrease after 6 hpf. Assumptions of their putative function can only be made based on their closest homologs present in other species (this is a possibility for CgNR2A, CgNR1E and CgNR5B). Early development is shaped by mitotic division, germ cell layer formation and initiation of organogenesis. CgNR2A is a homolog to the hepatocyte nuclear factor 4 (HNF4) [29], a maternally transferred NR [41–43]. CgNR2A expression also suggests a maternal origin for this transcript and its high expression indicates an important role during early development. In the frog species *Xenopus laevis* HNF4 is thought to contribute to zygotic activation of a transcription factor regulating tissue specification [43–45]. Mouse HNF4 participates in regulation processes for primary endoderm development [46] and gastrulation [47], and in *Drosophila* it plays a role in gut formation [41]. Knockout experiments in insect species indicated that E78, a CgNR1E ortholog, and HR39, a CgNR5B ortholog, are required for successful early embryogenesis, beside their main functions in female reproduction [48, 49].

Mid development: gastrulation and shell development

The mid development stage combines two of the most decisive developmental processes in the bivalve life cycle. Gastrulation (~6 hpf) is defined by three germ layers, basic body structure development and organogenesis. At the trochophore larval stage (~12 hpf), shell development is initiated and larvae become free living, using their circular ciliary bands for locomotion [50, 51]. Differentiation and development of organs and shell begins, and, in conjunction with axial patterning, requires intensive gene regulation. Many of the oyster NRs are

highly expressed during gastrulation and the trochophore larval stage, indicating a participation during these pivotal developmental stages.

CgNR2B, an ortholog to vertebrate and invertebrate retinoid X receptors (RXR), is highly expressed at 6 hpf and moderately expressed at 12 hpf. Research on different molluscan species suggests participation of RXR in organogenesis and shell development. In embryos of fresh-water snails of three different gastropod species (sister-class to bivalves), exposure to 9-*cis* retinoic acid and all-*trans* retinoic acid, natural metabolites of vitamin A, caused significant eye and shell deformation. Some embryos were even developmentally arrested at the trochophore larval stage [52–54]. 9-*cis* retinoic acid was identified as a ligand of RXR in a fresh-water and a marine gastropod snail species [25, 55], which in combination with reported exposure effects in other gastropods, indicates a biological function of RXR during optic development and shell formation in snails. Additionally, exposure of developing molluscs, including oyster species, to the organotin TBT caused serious disruption of development including shell deformities, delayed growth and larval development to the point of high death rates even at low TBT concentrations [56–58]. TBT has been identified as a xenobiotic ligand for gastropod [25] and deuterostome RXRs [24, 26].

Heterodimerisation is a common feature of NRs and RXR is the preferred heterodimer partner for many species studied so far [59, 60]. CgNR1B, an ortholog to the retinoic acid receptor RAR, is mainly expressed between 6 hpf and 12 hpf and shows a similar expression pattern as CgNR2B. This provides room for speculation about a possible interaction between these receptors, a hypothesis which is supported by the findings in two gastropod species, for which gastropod RAR orthologs were observed to heterodimerise with their RXR homologs and are able to regulate gene expression in vitro [61, 62]. To what extent the oyster RAR is sensitive to xenobiotic disruption is currently unknown. In contrast to vertebrate RARs, which are highly responsive to natural and synthetic retinoic acids, molluscan RARs seem to have no ability to bind to ligands [61, 62]. More research on oyster RXR and RAR homologs could reveal links between their prominent mid developmental expression, chemical exposure and putative binding, which has been reported for their molluscan relatives. Nevertheless, retinoid metabolism in invertebrates is proposed to be partially conserved among bilaterians [63]. In vertebrates the RAR/RXR heterodimers are involved in the regulation of a diverse variety of genes contributing to organogenesis, axial patterning and neuronal differentiation [64].

Two additional oyster NRs, which are highly expressed during mid development, should also be mentioned: The first is the peroxisome proliferator-activated receptor

(PPAR) homolog CgNR1C. Protostome homologs to PPARs have only been identified in one bivalve species, *C. gigas* [29], and two gastropod species, *Lottia gigantea* and *Biomphalaria glabrata*, across all of the currently studied protostomes [65], but information on their putative function or mode of action is sparse. The high expression at 6 hpf could potentially point towards a participation of CgNR1C in gastrulation supported by research on vertebrate species. In vertebrates PPARs fulfil different functions during embryo/larval development, especially during gastrulation, ranging from fat metabolism and adipocyte development [66] to cell, neural and muscular differentiation [66, 67]. TBT has also been proposed as a xenobiotic modulator of the RXR/PPAR γ heterodimer in vertebrates [24, 26, 68, 69].

The second NR is CgNR1A, an ortholog to human thyroid receptors (THR) [29]. Our data demonstrates high levels of CgNR1A expression during the trochophore larval stage and lower but significant expression during gastrulation. The vertebrate THR reacts to stimuli of thyroid hormones and is a common ligand-activated NR. The thyroid, and THRs in particular, play important roles in vertebrates during organogenesis and neural development [70] and it is suggested that the thyroid signalling pathway is conserved in invertebrate protostomes [8, 71]. Exposure to BPA causes disruption of developmental, reproduction and physical processes in vertebrates and invertebrates [15]. The xenoestrogen BPA is also a known antagonist to THR in rats in vitro [72, 73].

Late development: pre-metamorphosis

The later stages of development in bivalves and gastropods are mainly defined by growth and shell expansion as free swimming planktonic larvae. Some of the fundamental organs and the central nervous system are further defined [74], and new features appear, so called larval organs (velum for swimming and feeding, a foot for crawling, an eyespot as a light sensing organ). The duration of the free swimming stage varies depending on species and environmental cues and terminates in metamorphosis at which larvae transform to their juvenile form (spat). After a substantial re-organisation of body parts and disappearance of the larval organs, the individual attaches to a substrate and becomes sessile [50, 75]. Some NRs are expressed just before this life changing event. CgNR1H, a ortholog to the ecdysone receptor (EcR) in Ecdysozoans [29], the sister clade of Lophotrochozoans, is highly expressed at 15 dpf. In conjunction with an RXR homolog, EcR initiates and regulates life changing events such as moulting and metamorphosis in Ecdysozoans, binding to ecdysteroids [76]. After receiving an ecdysone signal the EcR/RXR homolog heterodimer initiates a transcriptional cascade of NRs, which are

responsible for further gene regulation [77]. EcR is a common target for pesticides, which results in a disturbance of this cascade, leading to disruption in insect moulting [78]. The presence of an EcR ortholog in oyster pediveliger larvae raises the question whether a similar cascade in oyster individuals is activated to initiate metamorphosis and settlement. *In silico* modelling of EcR orthologs in other lophotrochozoans already suggested the possibility for the binding of EcR to ecdysone or ecdysone-like compounds outside the ecdysozoan lineage [79]. Further research on oyster EcR will be needed to investigate its putative participation in metamorphosis and their potential for xenobiotic disruption through pesticides.

The estrogen receptor (ER) and estrogen-related receptor (ERR) homologs CgNR3A and CgNR3B [29], both highly expressed at the end of the larvae stages (15 hpf), are also worth mentioning. The ERR, also present in ecdysozoans, is a proposed precursor gene of metamorphosis in insects along with many other functions in animal development [80–82]. The ER is not present in insects, but has been widely studied in fish, regulating brain development and sexual differentiation in larvae [83, 84]. The relatively high expressions of ER and ERR homologs during the mid development stage in oysters, although unresponsive to any estrogen or estrogen-like ligands [85–87], suggest these genes might be fulfilling similar functions, in a constitutive, rather than ligand dependant, manner.

Adults: males and females

The adult life style of the Pacific oyster differs distinctly from its free-living planktonic developmental stages and requires the regulation of different genes. Being sessile, adult individuals depend completely on their surrounding environment and do not undergo any further fundamental reconstruction of their body plan. In general, our data suggests a separation of NR expression between most of the developmental stages and adult male/female individuals, indicating that different sets of NRs are switched on during the high dynamic developmental and the stationary adult stages.

Our data also indicates a difference in NR expression for male and female adults. The Pacific oyster has separate sexes and has an annual reproductive cycle, which includes the development of oocytes and spermatozoa. As a protandric species, most Pacific oyster individuals first develop as males and may change sex to female after a few annual cycles [50, 88]. In the nematode roundworm *Caenorhabditis elegans* [89], as in vertebrates [90], sex change and reproduction is directly or indirectly regulated by NRs. The oysters used in this study were at an early stage of gametogenesis for sex identification. However, the data for individual NR comparison

showed only five receptors with significant differences (CgNR1E, CgNR1CDEFb, CgNR2A, CgNR2E2, CgNR2E5), of which most have not been linked to sex dimorphism in other species. Only homologs to CgNR1E in insects have been connected to female reproduction and oogenesis [49]. CgNR1E, although expressed highly in early development and less in late and adult life stages, shows a higher expression in females than in males. Expression of the ER and ERR homologs (CgNR3A and CgNR3B respectively) was at similar levels. Previous research in gastropod species showed differences in expression for male and female reproductive tissues and no differences for other tissues [85, 86, 91]. Nevertheless, gene expression was measured in whole individuals (not separated by tissue) and sex-dependent expression could still be neutralized by pooling of tissues (e.g., high expression in gonads, but low expression in mantle and vice versa).

CgNR1C, the PPAR ortholog, is highly expressed during gastrulation, but also moderately expressed in adult life stages. TBT, a known gastropod and vertebrate RXR ligand [25, 26] causes shell thickening in adult bivalve [92, 93], as well as the development of male sex organs in females (imposex) in many gastropod species [19]. There have been previous hypotheses that this is triggered through disruption of the RXR/PPAR heterodimer, since exposure to rosiglitazone, a strong PPAR γ agonist for vertebrates [94], also causes imposex in gastropods [95]. Additionally, the vertebrate RXR/PPAR γ heterodimer has been identified as a target for TBT, which binds to either RXR alone or both receptors [24, 26, 68, 69]. Expression of a PPAR ortholog in combination with CgNR2B in adult individuals in the Pacific oyster supports the conceivable theories of interaction between RXR and PPAR, and further adds to the theory that this may be the primary mechanism of TBT-based endocrine disruption in molluscs.

CgNR1Jb, originally assembled with the early development group, displayed its highest expression in the adult life stages (Figs. 2 and 4), but also showed measurable expression during most of the other life stages. CgNR1Jb and CgNR1Ja, which showed a similar expression profile to CgNR1Jb, are members of the protostomes subfamily group NR1J, a homologous group to the deuterostome subfamily group NR1I. Representatives of the NR1I and NR1J subfamilies have been linked to xenobiotic-sensing [96–101], a mechanism of defence against natural and anthropogenic environmental stressors through which expression of genes involved in detoxification is induced [100, 101]. Research in the bivalve *Scorbcularia plana* demonstrated that the bivalve homolog NR1J β is also able to interact with such compounds, suggesting a conserved xenobiotic-sensing mechanism in bivalves [96].

Conclusion

This study provides a detailed overview of the NR expression dynamics in the Pacific oyster. We have demonstrated that a large variety of NRs are expressed at different respective stages throughout oyster lifetime, ranging from fertilisation, through embryo and larval development, to the point of adulthood. Different NRs cluster together into groups in a non-phylogenetic manner, representing different life events such as early, mid and late development. Differences between sexes were also recognized.

NRs are known to interact with ligands, which makes them vulnerable to exogenous xenobiotic compounds. Therefore, detecting the expression dynamics in different life stages is important in predicting putative functions of NRs and helping to uncover at which life points the Pacific oyster is vulnerable to xenobiotics. Our study on NRs in a molluscan species is therefore an important step towards understanding invertebrate development and for the study of anthropogenic impacts on the environment.

Methods

Oyster husbandry

Development studies were adapted from the oyster embryo-larval bioassay protocol [102]. Three independent fertilisations were performed using four female and five male conditioned adult individuals (Guernsey Sea Farm, Guernsey, UK). Artificial seawater (ASW) was prepared several days prior at 34 psu and pH 8.25. Approximately 3,000–4,000 eggs/ml in a total volume of 1 L were fertilized per biological replicate 3 hours post fertilization (hpf) each replicate was diluted 1:20 (to ~150–200 embryos/ml) in ASW to prevent oxygen depletion. Life stages were microscopically assessed and samples were taken of unfertilized eggs (~100,000 eggs/sample) and at 1 hpf, 2 hpf, 3 hpf (~100,000 embryos/sample), 6 hpf, 12 hpf and 27 hpf (3000–6000 embryos/sample) (Fig. 1: 1–7) from the three independent developmental experiments. The experiments were validated by assessing the percentage of dead/abnormally developed D-shelled larvae (ranging from 0 to 15 % abnormal development), which did not exceed the critical rejecting value of 30 %.

Veliger (7 days post fertilisation (dpf)) and pediveliger (15 dpf) larvae stages, as well as adult individuals were also obtained from Guernsey Sea Farm. The larvae were re-suspended in 12 °C artificial seawater for one hour and sampled (Fig. 1: 8–9). Three samples of veliger (1200–2000 larvae/sample) and pediveliger (500–1000 larvae/samples), respectively, were taken for further analysis. Three male and three female adult individuals at the beginning of the gametogenesis were shucked, and tissues homogenised and pooled separately by sex (Fig. 1: 10a–10b). Three homogenized pooled tissue samples

(~100 mg) for each sex were taken for further analysis. The sex of the individuals was determined through examination of the gonads to verify whether sperm or eggs were present. Only individuals in the early stages of gametogenesis were used.

RNA extraction and reverse transcription

The RNA was extracted from three biological replicates at each time point (developmental stages: unfertilized eggs, 1 hpf – 27 hpf; veliger larvae (7 dpf); pediveliger (15 dpf; pooled female and adult individuals). Total RNA was extracted using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich), following the manufacturer's protocol, and genomic DNA was removed with RQ1 RNase-Free DNase (Promega). RNA purity and quantity were determined by ND-1000 spectrophotometer (Nanodrop). For each sample, 900 ng of total RNA, divided into two independent 20 µl reactions (each 450 ng RNA), was converted to cDNA with the ThermoScript RT/PCR System (Invitrogen), using oligo (dT) primers, and then pooled together and further diluted (1:1) with nuclease-free water.

Primer design and optimisation

Forward and reverse oligonucleotide primers were designed with Primer-Blast at the National Centre for Biotechnology Information (NCBI) [103] to amplify each of the 34 *C. gigas* NRs. Primers were 18–23 nt, with a GC content of 40–60 % and produced predicted amplicons of 100–200 bp (Additional file 3). The primer pairs were optimized by changing final primer concentration, temperature and/or final MgCl₂ concentration to reach a primer pair efficiency between 90 and 115 %. The efficiency was tested by a dilution series resulting in a standard curve with a slope between 3.0 and 3.55. The efficiency was calculated as follows [104]: Efficiency (E) = 10^(-1/slope).

Each primer pair amplification product was verified by sequencing, using a common polymerase chain reaction (PCR) with the GoTaq system (Promega) for amplification and the products were purified with the QIAquick PCR Purification Kit (Qiagen, UK). Sequencing was conducted by Eurofins MWG Operon (Ebersberg, Germany).

Quantitative RT-PCR

Quantitative RT PCR was performed using the SsoFast EvaGreen Supermix (Bio-Rad), and the reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad). For each gene, each of the biological replicates per time point was run in duplicate (technical replicates) on a single plate. 0.5 µl of cDNA dilution was used per 10 µl reaction. The MgCl₂ concentration of the SsoFast EvaGreen supermix and the primer concentration were adjusted for primer optimisation (Additional file 3). qPCR conditions

were as follows: 95 °C for 2 min, 45 cycles of 95 °C for 15 s, 60–63.1 °C for 30 s (primer dependent) and 72 °C for 1 min. A melt curve was run after each PCR (65–95 °C at a temperature transition rate of 0.05 °C/s). For each reaction the melt curves were analysed to verify the specificity of the amplified product, and to confirm that a single PCR product had been amplified. A non-template control was analysed in parallel for each gene as well as a positive control.

Data analysis

The calculation of the relative expression (RE) for gene transcripts (mRNA copies) of each NR was based on the modified comparative Ct method [104, 105], using the average Ct (avCt_{target}) of each biological replicate per time point, corrected for efficiency (E) and compared to the normalisation factor of combined reference genes (NF_{refs}). Three housekeeping genes (elongation factor-1 α (EF-1), ribosomal protein S18 (RS18), ribosomal protein L7 (RL7)) were chosen as reference genes (Additional file 1). The normalisation factor of the combined reference gene (NF_{refs}) has been determined by the programme geNorm v3 [106], using the Ct values of the reference genes corrected for their efficiency:

Normalisation Factor (NF_{refn}) = $\sqrt[n]{E_{ref1}^{avCtref1} * E_{ref2}^{avCtref2} * \dots * E_{refn}^{avCtrefn}}$. Thus, the relative expression was calculated as follows [105]: Relative expression (RE) = $\frac{NF_{refn}}{E_{target}^{avCt_{target}}}$.

Relative expressions of all NRs were statistically analysed using RStudio v0.98.1091 (RStudio, Inc.). The data was transformed (log or sqrt) to normal distributions, tested using a Shapiro-Wilk test, and the expression patterns were analysed with a one-way ANOVA followed by multiple pairwise comparisons with Tukey's Honestly Significant Difference Test. Cluster analysis was performed to demarcate the expression patterns during all stages using Cluster 3.0 v1.52 [107]. Hierarchical and *k*-means clusters were obtained by logarithmically transformed centred data, by gene, using the Euclidean similarity metric. The hierarchical cluster was visualized using Java TreeView [108]. In addition, to examining gene expression patterns along NR subfamilies and developmental stages excluding and including adult stages, principal component analyses (PCA) were conducted in R version 3.2.4 [109], using `prcomp()`, a built-in function in the R stats package, and the packages `FactorMineR` [110] and `factoextra` [111].

Additional files

Additional file 1: Gene expression of selected reference genes in *Crassostrea gigas* among different life stages. Three housekeeping genes selected as reference genes (centred Ct values): elongation factor-1α: white bars; ribosomal protein S18: light grey bars; ribosomal protein L7:

dark grey bars; mean of all reference genes including significantly different groups; black bars and letters. hpf: hour post fertilisation. dpf: days post fertilisation. E: unfertilised eggs. F: female. M: male. (DOC 43 kb)

Additional file 2: Principle component analysis (PCA) of 31 *Crassostrea gigas* nuclear receptor gene expression data across developmental and adult life stages. Scatterplot of the first two PCA components of developmental stages including adult life stages indicating separation of all life stages based upon the variance observed in the expression levels of 31 of the 34 nuclear receptor genes. Principal component 1 (PC1) and 2 (PC2) explain 36 and 25 % of variance, respectively. Circles around measurements and colours of measurements representing distinct clustering for all life stages or nuclear receptors: early development (green numbers + letters, black solid line), mid development (red numbers + letters, black dotted lines), late development (blue numbers + letters, black dashed line), adult life stages (yellow letters, grey dashed lines). h: hour post fertilisation; d: days post fertilisation; E: unfertilised eggs; F: female; M: male. (DOC 196 kb)

Additional file 3: Primer information used in qPCR analysis. Table of forward and reverse primer sequences for 34 *Crassostrea gigas* nuclear receptors and three reference genes including amplicon length (bp), annealing temperature, final primer concentration and MgCl₂ concentration, primer efficiency, and accession number of receptor gene. (XLS 41 kb)

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Authors' contributions

SV, TB, BL and TG contributed to study concept and design. Interpretation of data and manuscript preparation were conducted by all authors. The laboratory studies for quantitative PCR for gene expression analysis and the statistically analyses were carried out by SV. All authors take responsibility for the data, its interpretation and presentation. All authors read and approved the final manuscript.

Competing interests

Authors declare that they have no competing interests.

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