Developmental Biology
Chromosomes and Gene Expression

The transcriptional landscape underlying larval development and metamorphosis in the Malabar grouper (*Epinephelus malabaricus*)

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Abstract

Teleost fishes typically have a bi-phasic life cycle with a transition between larval and juvenile phases called metamorphosis, which is known to be regulated by thyroid hormones (TH). However, other hormonal systems might be involved as it is the case in amphibians in which corticosteroids are interacting with TH pathways to trigger and regulate metamorphosis. Unfortunately, such interplay is poorly understood in teleost fishes. In order to investigate the potential involvement of these two hormonal pathways, we used the Malabar grouper (*Epinephelus malabaricus*) as a model system. We assembled a chromosome-scale genome and conducted a transcriptomic analysis of nine larval developmental stages. We studied the expression patterns of genes involved in TH and corticoid pathways, as well as four biological processes known to be regulated by TH in other teleost species: ossification, pigmentation, visual perception, and metabolism. Surprisingly, we observed an activation of many of the same pathways involved in metamorphosis at an earlier stage, suggesting an additional implication of these pathways in early larval development. Overall, our data reveal that on a common background (TH controlling metamorphosis) evolution is assembling species-specific peculiarities that allow to precisely align the molecular completion of metamorphosis with the ecological constraints.

eLife assessment

In this work, Huerlimann and colleagues suggest an intertalk between the thyroid and corticosteroid axis in regulating grouper metamorphosis. The work provides **valuable** genomic resources to address the endocrine control of a life cycle transition in the Malabar grouper fish. The evidence is still **incomplete** and it does not fully support an interaction between the thyroid and corticosteroid axis.

Introduction

Most teleost fishes have a stage-structured life cycle that includes a transition between larval and juvenile phases known as metamorphosis; this transition is regulated by thyroid hormones (TH)[1,2]. Of all teleost fishes, flatfishes experience one of the most extreme metamorphosis, with significant changes occurring in their body organization and appearance during this period, switching from a symmetrical to an asymmetrical body plan[3,4]. However, metamorphic changes are not always as pronounced in other fish species. For example, the metamorphosis of zebrafish is mainly marked by relatively discrete pigmentation changes that appear to be regulated by TH[5–8].

Metamorphosis in teleost fishes is not only marked by visible changes in the body, but also by a range of ecological, physiological, biochemical, and behavioral changes. These changes are thought to be initiated and coordinated by a surge of TH, which regulates various signaling pathways through the action of specific transcription factors known as thyroid hormone receptors (TRα, TRβ). For example, there is evidence that TH is associated with the transition between oceanic and coral reef environments in the convict surgeonfish[9], controls pigmentation changes in zebrafish, clownfish, and grouper[10,11], regulates ossification processes in zebrafish and flatfishes[12,13] and is involved in the shift of visual perception by controlling the expression of opsin genes in many species[14,15]. More recently, it has also been suggested that the metabolic changes that occur during larval development in teleosts may be regulated by TH, as demonstrated in clownfish[15].

Beside TH signaling pathway, other actors have been shown to be important in metamorphosis regulation. For example, studies have provided clear evidence that corticoids and TH are interacting together to regulate amphibian metamorphosis[16–18]. But as far as we know, there is limited information available regarding the interaction between corticoids and TH during fish metamorphosis. Although a synergistic effect of cortisol and TH has been observed in flatfish metamorphosis (advancement of morphological changes), there has been insufficient investigation into the communication between corticoids and TH pathways during teleost metamorphosis[19,20]. More research is needed, and the use of genomic analysis would be a good way to investigate if those pathways are associated with metamorphosis.

The use of high-throughput sequencing techniques, such as transcriptomics, has made it possible to study gene expression in greater detail, especially when combined with a high-quality annotated genome, which has enabled the identification of genes that may be involved in the key biological changes that occur during metamorphosis. These techniques have provided valuable insights into the underlying molecular mechanisms that drive metamorphosis in teleost fishes[21]. Most of the studies investigating the transcriptomic changes during marine fish larval development have been focused on commercial fish species used in aquaculture to: (*i*) gain insight into the key biological processes that occur, (*ii*) identify the genes involved in these processes, and (*iii*) find ways to improve rearing conditions to ensure high survival rates and harmonious

development[21]. However, these studies rarely mentioned metamorphosis to explain the onset of these processes, whereas it is likely that they correspond to the transition between the larval and the juvenile stages. This is another reason why studying the molecular changes occurring during the metamorphosis of the Malabar grouper *Epinephelus malabaricus* is very relevant. Indeed, this will allow for a better understanding of the biological processes at play and to understand the carry-over effect in the context of aquaculture. Indeed, it is well known that rearing conditions may impact welfare and growth at later stages and understanding the molecular changes occurring during the development of this species might be useful to enhance survival rates[22–24].

Grouper (Family Serranidae, Subfamily Epinephelinae) are a group of fish of both economic and ecological importance. Inhabiting temperate and tropical waters of eastern and southern regions Indo-Pacific region, East Atlantic, Mediterranean regions, and the intertropical American zone, they comprise 165 species in 16 genera[25,26]. Despite wide variations in growth rate, body size, and color, groupers share many biological traits and lifestyles, such as protogynous hermaphroditism and complex social structure[27]. Ecologically, groupers provide a wide variety of important functions as large top-level predators[28]. However, due to their high economic value on the food market, more than 40 species are at risk of extinction[29,30]. This has led to the widespread development of grouper aquaculture farms, which produced 155,000 tons per year according to the Food and Agriculture Organization of the United Nations in 2015, with 95% of global production occurring in Asia[31,32].

In order to gain insight into the molecular pathway involved during grouper metamorphosis, we assembled a chromosome-scale genome of *E. malabaricus* and conducted a transcriptomic analysis of nine developmental stages ranging from freshly hatched larvae to roughly two-month-old juveniles. We investigated the expression patterns of genes involved in the TH pathway and four biological processes known to be regulated by TH in other teleost species: ossification, pigmentation, visual perception, and metabolic transition. In addition, we used TH pathway and downstream regulated biological processes activation as indicators to look for the potential involvement of corticoids in metamorphosis. We observed the activation of the TH pathway during the regression of fin spines, which in other grouper species coincides with the surge of TH and marks the beginning of metamorphosis. Interestingly, the activation of the TH pathway at this stage was associated with the activation of corticoids pathways as well as the four biological processes we investigated. Very interestingly and unexpectedly, we observed an earlier activation of the two regulatory pathways (TH and corticoids) occurring before the formation of the elongated fin spines.

Results and Discussion

Genome assembly, phasing, scaffolding, and annotation

A total of 46 Gbp of PacBio HiFi reads (~43X coverage, Supp. Table S1) were assembled into a fully haplotype phased genome of the Malabar grouper (*Epinephelus malabaricus*) with the primary phase consisting of 298 contigs across 1.09 Gbp genome length, a contig N50 of 7.4 Mbp, and a genome level BUSCO completeness of 93.6% with 1.3% duplication (Table 1, Supp. Table S2). The raw assembly was further scaffolded by Phase Genomics using HiC data, resulting in a 1.03 Gbp assembly across 24 pseudo-chromosomes (Table 1). The scaffolded pseudo-chromosomes ranged from 22.5 Mbp to 50.6 Mbp in size and contained 90.5 % of the contigs and 92.8 % of the contig length (**Supp. Fig. S1**). The gene model annotation resulted in 26,140 protein coding genes, with a BUSCO completeness of 95.5% and a duplication level of 1.3%. The final GC content was 41.3 % and the assembly contained 56.4% repeat regions overall, which were mainly made up of DNA transposons (28.9%), followed by LINEs (5.3%), and LTR elements (2.2%) (**Table**

1, **Supp. Tables S2** and **S3**). The genome length, GC content, repeat content, number of gene models, and BUSCO values are similar to other published chromosome-level grouper genomes, for example *Epinephelus lanceolatus*[33], *E. akaara*[34], and *E. moara*[35].

General transcriptomic results

Transcriptomic analysis of *E. malabaricus* larval development was performed on grouper larvae raised in the Okinawa Prefectural Sea Farming Center. An average of 77.1 M reads were obtained per sample, which after quality control and mapping resulted in an average of 65.8 M uniquely mapped reads (85.6%) per sample for differential gene analysis. Sampled larvae from one day to two months old were sorted according to their morphology allowing us to sequence nine developmental stages (D01, D03, D06, D10, D13, D18, D32, D60, Juvenile) (**Table 1**). Principal component analysis (PCA) performed on all genes allowed to distinguish between three distinct groups: early developmental phase (composed of D01), intermediate developmental phase (composed of D03, D06, D10, D13 and D18) and late developmental phase (composed of D32, D60 and Juvenile) (**Fig. 1A**).

The analysis of upregulated genes during this post-embryonic development series revealed two major peaks of gene expression that underlies the clusters of regulated genes. Indeed, the cluster analysis shows 2,651 genes upregulated on D03 and to a lesser degree on D32 (clusters 1 and 2), 1,515 genes upregulated on D32 (cluster 3), and 785 genes upregulated on D32 and to a lesser degree on D03 (cluster 4) (**Fig. 1B**). Unsurprisingly, these two transitions, D01 to D03 and D18 to D32, also show the highest number of differentially expressed genes with 14,830 genes (7,151 up, 7,679 down) between D01 and D03, and 10,774 genes (5,320 up and 5,454 down) between D18 and D32 (**Supp. Table S4**). This suggests that there are two major events occurring in terms of gene expression: one early on, at day 3 and one later around day 32. This last event corresponds to the separation between the intermediate and late developmental phase and is concomitant with the regression of the elongated spines, an overall change of shape and progression of the pigmentation. In other grouper species, the regression of the elongated spines corresponds to the onset of metamorphosis and is associated with an increase of TH levels[36]. However, the very early event is more striking as such a global gene expression change very early on has, to our knowledge, never been reported in other teleost fish species.

Two periods of activation of the TH signaling pathway during grouper post-embryonic development

We investigated the expression patterns of key genes involved in the hypothalamo-pituitary-thyroid axis (*tshb, trhr1a, trhr1a-like, trhr1b, trhr2*) as well as in TH synthesis (*tg, tpo, nis*), TH metabolism (*dio1, dio2, dio3*), and finally the genes encoding thyroid hormone receptors (*tra, traβ, trβ*). These genes all play important roles in the regulation of TH levels and TH signaling in the body, and understanding their expression patterns during grouper metamorphosis can illuminate the underlying mechanisms that drive this process.

The gene encoding the pituitary thyroid stimulating hormone (*tshb*) is strongly expressed very early on during larval development at D01, decreases from D03, and then strongly increases again at D32, therefore following the two peaks mentioned earlier (**Fig. 2A**). Accordingly, we also observed two surges of expression for the hypothalamic factors *trhr1aa, trhr1a like, trhr1b*, and *trhr2*, at D03 and between D32 and D60, suggesting two distinct periods of stimulation of TH synthesis, one early on around D03 and one later at around D32. This pattern can also be seen in the expression of the corticotropin-releasing hormone (*crhb*) and receptors (*crhr1a, crhr1b*, and *crhr2*), which stimulates the synthesis of THS[37] (see section "**Possible involvement of corticoid pathways in metamorphosis**" and **Fig. 5** below). Interestingly, we also observed a peak of expression for *tg* at D03, the gene encoding for the TH precursor, and a strong increase of expression starting at D32 (**Fig. 2B**). A similar expression pattern was obtained for *tpo*, the gene

Contig assembly size	1,092,599,927 bp
Mean base-level coverage PacBio HiFi	43X
Contig length contained in scaffolds	92.8%
Scaffolded assembly size	1,027,628,325 bp
Number of scaffolds	24
Scaffold N50	43,313,630 bp
Largest Scaffold	50,623,973 bp
Smallest Scaffold	22,540,365 bp
GC contents	41.3 %
Repeat contents (DFAM)	56.4 %
Number of protein-coding genes	26,140
Gene annotation: BUSCO completeness	3476 (95.5%)
Gene annotation: Complete and single copy	3,429 (94.2%)
Gene annotation: Complete and duplicated	47 (1.3%)
Gene annotation: Fragmented	31 (0.9%)
Gene annotation: Missing	133 (3.6%)

Table 1

Statistics of the *Epinephelus malabaricus* chromosome-scale genome assembly, scaffolding and gene annotation.

Additional statistics can be found in **Supp. Table S2**.

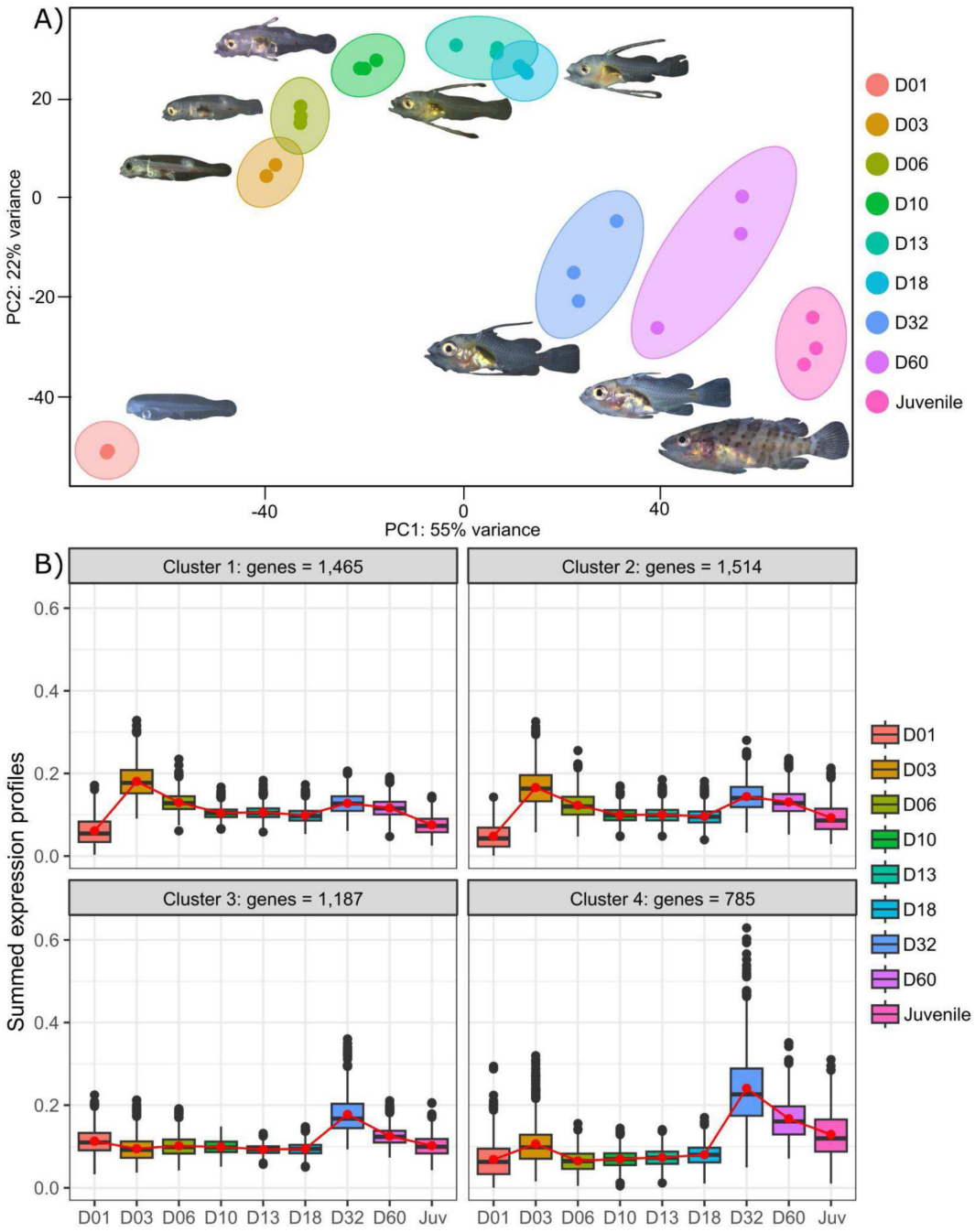

Fig. 1

Transcriptomic results of *E. malabaricus* larval development.

A) Principal component analysis of different larval stages using variance stabilizing transformed complete transcriptome. B) Cluster analysis using the coseq R package, focusing on genes that are upregulated on days 3 and/or 32. The number of genes in each cluster are shown above each graph. Adjusted p-values and functional annotations for the four gene clusters in this figure can be found in the **supplementary data** file.

encoding for the enzyme adding iodine to TH precursor, as well as for *sis*, gene encoding for the symporter involved in transferring iodide into thyrocytes. Once produced, TH, particularly T4, are transported into target cells where they convert them into the active form T3 mostly by *dio2* and *dio1* or degraded by *dio3* and *dio1*. As it has been observed for HPT factors, *tg, tpo*, and *sis*, we first noticed two peaks of expression of *dio2* with a very early one at hatching (D01) and a second one at D32 suggesting two distinct periods in which active TH (that is T3) is required. In accordance with this observation, we notice a minimal expression of the T3 degrading enzyme *dio3* at these two periods followed by a final late increase after D32. *dio1*, whose net function is unclear[38], shows a regular increase of expression that becomes maximal at the juvenile stages (J) (**Fig. 2C**). Finally, thyroid hormones receptors (TRs) expression levels increased throughout the entire larval development with a stronger increase of *trβ* at D60 (**Fig. 2D**). Taken together, these data reinforce the existence of two distinct periods of TH signaling activity, one early on at D3, and one late corresponding to the classical metamorphosis at D32 (**Fig. 2C**).

These results suggest the activation of the TH axis around D32, which coincides with the regression of the elongated spines and the appearance of the juvenile pigmentation pattern, indicating that metamorphosis in *E. malabaricus* occurs around D32 in our rearing conditions. These observations are consistent with what has been observed in *E. coioides*, in which TH levels peak around 40 dph when spines regression and pigmentation pattern formation are ongoing[36]. Interestingly, the high expression levels of *tshb, trhr, tg, tpo, sis, dio3*, and TRs at the very beginning of development (D1-D3) suggest a precocious activation of TH synthesis, which, to our knowledge, has not been observed in groupers nor in other teleost fishes so far (**Fig. 2**). Measurements of TH levels during these early development stages showed an early peak of T4 at D3, confirming the early activation of the TH pathway observed with gene expression patterns (**Fig. 2E**).

TH involvement in spine elongation and regression

In many marine fish larvae, there are several morphological features that help the larvae to improve its buoyancy during their pelagic phase[39]. This is what we observe in grouper with the formation of elongated spines of the dorsal and pelvic fins that have also an obvious defensive function[40–42]. These spines then regress during metamorphosis. It is well known that during fish larval development genes involved in ossification are under the control of TH. In zebrafish, TH control the proper morphogenesis and ossification in the majority of the bones, during post-embryonic development and metamorphosis[43]. This is why we investigated the expression changes of some of these genes in *E. malabaricus*. Interestingly, we observed, once again, two surges in the expression of the following genes: bone gamma-carboxyglutamate (*bglap*), periostin (*postnb*), and phosphate regulating endopeptidase (*phex*), three key genes implicated in the mineralization of tissues. The first at D13 following the early surge in thyroid hormone (TH) signaling genes, and the second starting at D60 (**Fig. 3A**). The first surge of gene expression coincides with the appearance and growth of the dorsal and pelvic elongated spines starting at D10 (**Fig. 3B**, shown by green arrowhead), while the second surge coincides with the regression of these spines, a process known to be regulated by TH in *E. coioides*[36]. The coincidence of both the growth and the regression of the elongated spines with the activation of the TH pathway in *E. malabaricus* suggests that in groupers TH may play a role not only in the regression of these spines but also in their formation.

Other TH regulated biological processes are also activated during grouper metamorphosis

Pigmentation changes are often the most visible changes in some teleost species such as clownfish[11]. In grouper, the pigmentation changes are accompanied by the regression of the dorsal and pelvic spines. The acquisition of an adult pigmentation pattern is characterized by the formation of brown and white vertical bars in *E. malabaricus* (**Fig. 3C**, juvenile stage). To reveal

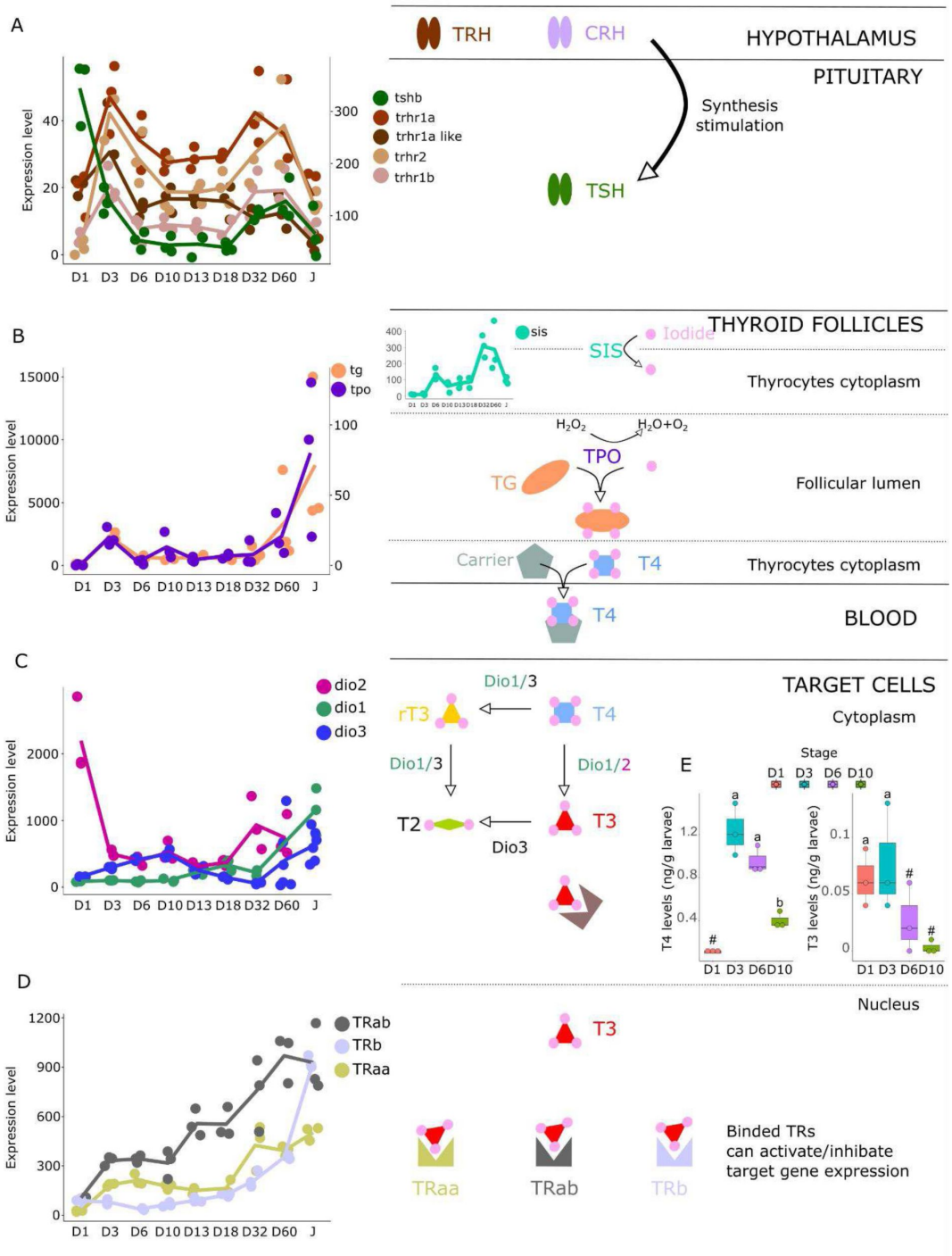

Fig. 2

Expression levels of TH signaling pathway genes in *E. malabaricus*.

A) TRH: thyroid releasing hormone, TSH: thyroid stimulating hormone. B), DUOX: dual oxidase, TG: thyroglobulin, TPO: thyroperoxidase, SIS: sodium iodine symporter. C) DIO: deiodinase. D) TR: thyroid hormone receptor. Colored lines join the average values of each stage. Biochemical pathways adapted from Roux *et al.*[15]. E) T4 and T3 levels (in ng/g of larvae) during early larval development. # indicates that the value is below the quantification limit, and different letters indicate significant differences (one-way ANOVA followed by a Tukey HSD test for T4 levels only as no significant differences were observed after ANOVA for T3 levels).

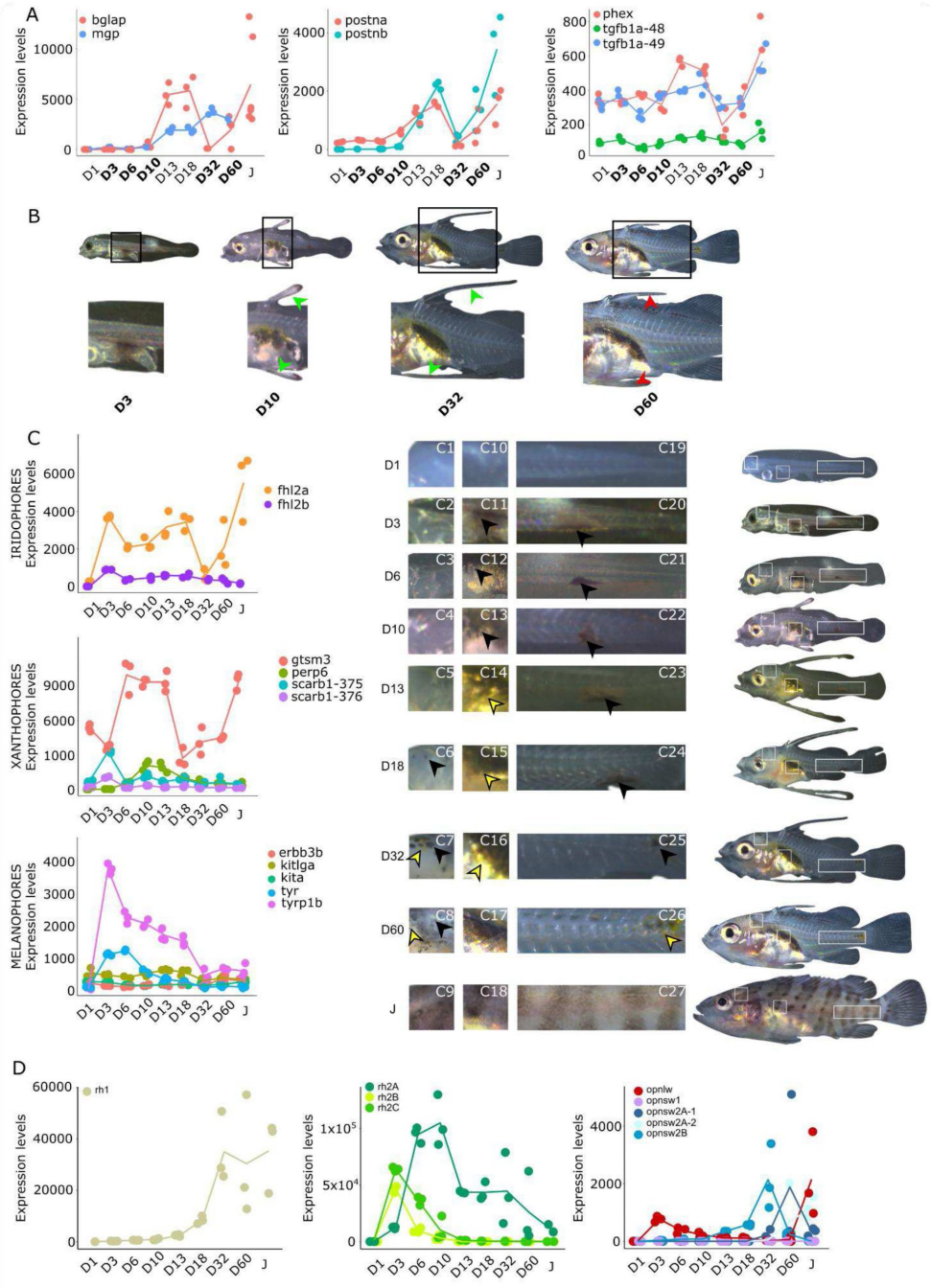

Fig. 3

Biological processes likely to be under TH control during *E. malabaricus* metamorphosis.

A) Expression patterns of key genes involved in ossification process and known to be regulated by TH in teleosts. *Bglap*: bone gamma carboxyglutamate protein, *mgp*: matrix gla protein, *postna*: periostin a, *postnb*: periostine b, *phex*: phosphate regulating endopeptidase homolog X linked. B) Pictures of *E. malabaricus* at D03, D10, D32, D60 illustrate the elongation of the dorsal and pelvic floating spines (green arrow heads at D10 and D32) and their regression (red arrow heads at D60). C) Expression patterns of genes involved in pigmentation. Three areas of interest were chosen to illustrate the appearance of melanophores (C6 to C8: at the top of the head, C11 to C13: above internal organs and, C20 to C25: close to the caudal peduncle,) and xanthophores (C7 to C8 at the top of the head, C14 to C16 above internal organs and C26: close to the caudal peduncle). D) Expression patterns of genes encoding for the rhodopsins (rh1) and the visual cone opsins (*rh2A, rh2B, rh2C, opnlw, opnsw1, opnsw2A-1, opnsw2A-2, opnsw2B*).

the molecular regulations driving these pigmentation changes, we assessed the expression of key pigmentation genes involved in white (iridophore genes), black (melanophore genes) and yellow (xanthophore genes) pigment cells known to be regulated by TH in zebrafish and clownfish[10,11]).

The expression level of the iridophore gene *flh2a* showed a strong increase from D03, followed by a decrease at D32 and a new surge at D60 (**Fig. 3C**). The first increase may correspond to the appearance of iridophores on the ventral cavity whereas the second may coincide with the formation of the white bars. In contrast, its paralogue *fhl2b* remained relatively stable throughout the development. Xanthophores start colonizing the larval body at D10, which may explain the increase of the expression level of two xanthophores markers, *gtsm3* and *perp6*, which play a role in concentrating and trafficking lipophilic pigments[44]. On the other hand, *scarb1*, which is involved in carotenoid deposition in zebrafish, increased slightly at D03 (**Fig. 3C**). Similarly, melanophore genes are displaying a strong increase of their expression level at D03 and D06 that may be related to the colonization of melanophores on larval body (*tyrp1b, tyr*, **Fig. 3C**).

During their metamorphosis in the wild, fish larvae also undergo ecological changes such as habitat transition (from ocean to coastal environment) and food habits. It is well known that in many fish species this ecological transition is accompanied by a change in color vision[45]. Since TH appeared critical in the regulation of genes involved in vision in salmonids, zebrafish and clownfish[14,15,46,47], we investigated the regulation of genes encoding for visual opsin. We expected to find at least eight visual cone opsin genes in *E. malabaricus* according to the phylogeny of opsin genes in teleosts[48] (*opnsw1, opnsw2Aa, opnsw2Ab, opnsw2B, rh2A, rh2B, rh2C, opnlw*) and one rhodopsin gene (*rh1*)[48,49]. These genes were indeed expressed in our transcriptomic data. We observed that the medium wavelength opsin (*rh2A, rh2B, rh2C*), and the long wavelength opsin (*opnlw*) were highly expressed at the beginning of the larval development (at D03 for *rh2B, rh2C and opnlw*, and at D10 for *rh2A*) (**Fig. 3D**). These surges of expression are followed by the increase of the expression levels of *opnsw2B, opnsw2Bb and opnlw* from D32. From D18 the rhodopsin involved in scotopic vision (*rh1*) increases. The expression level *opnsw1* remained low and stable during the entire development (**Fig. 3D**). It is again very interesting to note that these changes coincide with both TH signaling peaks. As these genes are regulated by TH in other species and according to the observed expression patterns, we may assume that this is also the case in *E. malabaricus*.

The timing of cone opsin (*opnsw2a1*, *opsnw2a2* and *opnlw*) expression in *E. malabaricus* is similar to *E. bruneus*[50], but different from *E. akaara* where *opnsw2* is strongly expressed early and then decreases[51]. However, the expression levels of mid wavelength opsins and *opnlw* are similar between *E. malabaricus* and *E. akaara*, suggesting their involvement in cone photoreceptor differentiation, while rod photoreceptors differentiate during metamorphosis in *E. akaara* and *E. malabaricus* larvae.

Metamorphosis is accompanied by a metabolic shift

Because metamorphosis is known to be energetically demanding and because the ecology of the planktonic larvae and the demersal juveniles are different, we investigated metabolic gene expression. **Fig. 4** shows the expression profile of the genes encoding for the rate limiting steps enzymes involved in glycolysis, (phosphofructokinase, *pfkma* and *pfkmb*), and citric acid cycle (citrate synthase, *cs*; isocitrate dehydrogenase, *idh3a*; oxoglutarate dehydrogenase complex, *ogdhl, dlst2*). The expression profile of all the genes associated with these pathways are shown in **Supp. Fig. 2**.

These profiles revealed a clear overall pattern: glycolysis genes are poorly expressed at the very beginning of the larval development while their expression increases throughout the development. This is particularly visible for *pfkma* which starts to increase from D10 and reaches its highest expression level at D32, likely coinciding with the onset of metamorphosis, and then

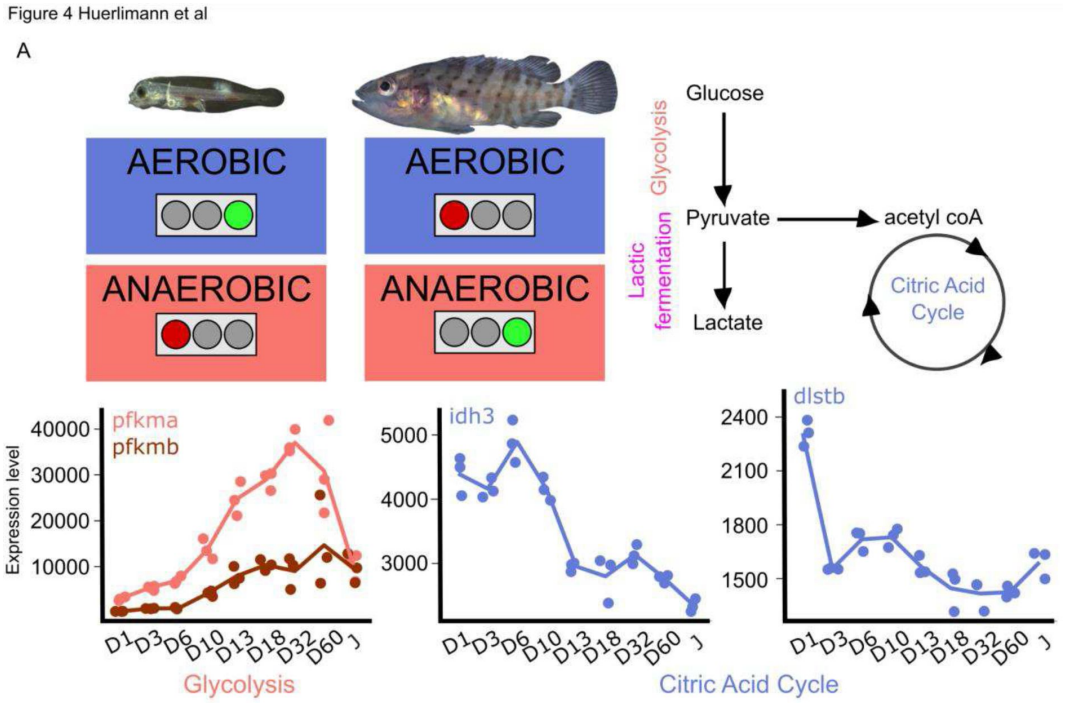

Fig. 4

Metabolic transition and corticoids expression levels of *E. malabaricus*.

A) Schematization of the metabolic transition occuring during *E. malabaricus* larval development showing that young larvae rely on aerobic metabolism whereas older larvae rely on anaerobic metabolism. Expression levels of genes involved in glycolysis (*pfkma, pfkmb*), krebs cycle (*idh3, dlstb*).

decreases until juvenile stage (J) (**Fig. 4A**). The genes involved in the rate limiting steps of the citric acid cycle (*cs, idh3, dlst*) are more expressed during early larval stages and then decrease progressively. It is also worth noting that several genes involved in both glycolysis and TCA cycle are encountering these two peaks of expression during the larval development (*gpi1b, aldoaa, gapdh1, pgam1a, pgam1b, pgam2, eno1b, pkma, dlsta, dldh, sdhb, mdh2*, **Supp. Fig. 2**). The lactic acid fermentation genes show an increase throughout the larval development with peaks of expression at D18 for *ldha* and at D03 for *ldhc* (**Supp. Fig. 2**). Taken together, these results reveal that at the very beginning of the development larval fish mainly rely on the citric acid cycle for aerobic energy production and then switch progressively to anaerobic energy production via glycolysis and lactic fermentation. This trend is similar to what has been observed in other fish species such as sea bass[21,52], but contrasts with the situation of other species such as the clownfish. Thyroid hormones are known to play a role in the regulation of metabolism in mammals[53], so it is likely that a similar regulatory process occurs during the development of *E. malabaricus* larvae, as it has been recently observed in the development of clownfish larvae[15]. Larval development and metamorphosis are very sensitive periods during which larvae must face a myriad of challenges: disperse into the open ocean, find food, escape from predators, locate and swim toward a suitable habitat, metamorphose and settle. All these challenges are highly demanding in terms of energy, it is thus very important for the larvae to properly allocate this energy to ensure the success of these various challenges. The regulation by TH of genes involved in processes such as glycolysis, lactic fermentation and citric acid cycle might be a way for larvae to tune their energetic source to enhance their survival and the success of metamorphosis.

Possible involvement of corticoid pathways in metamorphosis

Synergistic action of cortisol and THs has been encountered during flatfish metamorphosis, but crosstalk between corticoids and TH pathways has remained poorly investigated during fish metamorphosis[20]. For this reason, we decided to investigate eight key genes genes involved in the Hypothalamo-Pituitary-Interrenal axis: *crha, crhb, crhr1a, crhr1b, crhr2, pomc-a1, pomc-a2, pomc-b, mr, gr1, gr2* which encode respectively for the corticotropin releasing hormone (which stimulates the production of POMC and the stress hormone ACTH), the receptors of the CRH which are involved in the production of the stress-related hormone ACTH the pro-opiomelanocortin A1, A2 and B (precursors of several hormones such as ACTH) and corticoid receptors: mineralocorticoid receptor (MR) and glucocorticoid receptor (GR1&2) (**Fig. 5**) We also scrutinized the expression levels of genes encoding for key proteins involved in corticoid synthesis: *star, fdx1, fdx2, fdxr, cyp11a1, hsd3b1, cyp17a1, cyp21a2, cyp11c1, hsd11b1, hsd11b2*. Among these genes we were particularly interested in the expression of the steroidogenic acute regulatory protein (STAR) which is a rate-limited step in steroid hormone biosynthesis, the ferredoxin proteins (FDX1 & 2) and ferredoxin reductase involved in providing electrons to allow corticoid synthesis, the cytochrome p450 monooxygenase (or side cleavage enzyme) involved in the catalyzation of the cleavage of cholesterol into pregnenolone (CYP11A1), the 3 β hydroxysteroid dehydrogenase (HSD3B1) which catalyzes the transformation of pregnenolone into progesterone, or the 17-OH-pregnenolone into 17-OH progesterone, the cytochrome P450 17A1 enzyme catalyzes the transformation of Pregnenolone into 17-OH-pregnenolone and progesterone into 17-OH progesterone, the cytochrome P450 21A2 catalyzes the transformation of progesterone into 11 deoxycorticosterone and the transformation of 17-OH-Progesterone into 11 deoxycortisol, the cytochrome P450 11C1 catalyze the transformation of 11 deoxycortisol into cortisol and finally the two 11 β hydroxysteroid dehydrogenase (HSD11B1 & 2) catalyze the reaction to form either cortisol or cortisone.

Most of the genes of this pathway displayed a similar pattern as described previously above with a surge of expression between D03 and D10 and a second one between D32, D60 (*crhb, crhr1b, crhr2, pomc-a2, mr, gr1*) (**Fig. 5A**). The expression level of the gene encoding for CRHR2 started to increase after D01 and remained relatively stable all along whereas *crha* was lowly expressed (**Fig. 5A**). A surge of expression was observed for *pomc-a1* at D03 followed by a constant decrease until the juvenile stage. High expression of *pomc-b* was observed at D13, D18 and Juvenile stage.

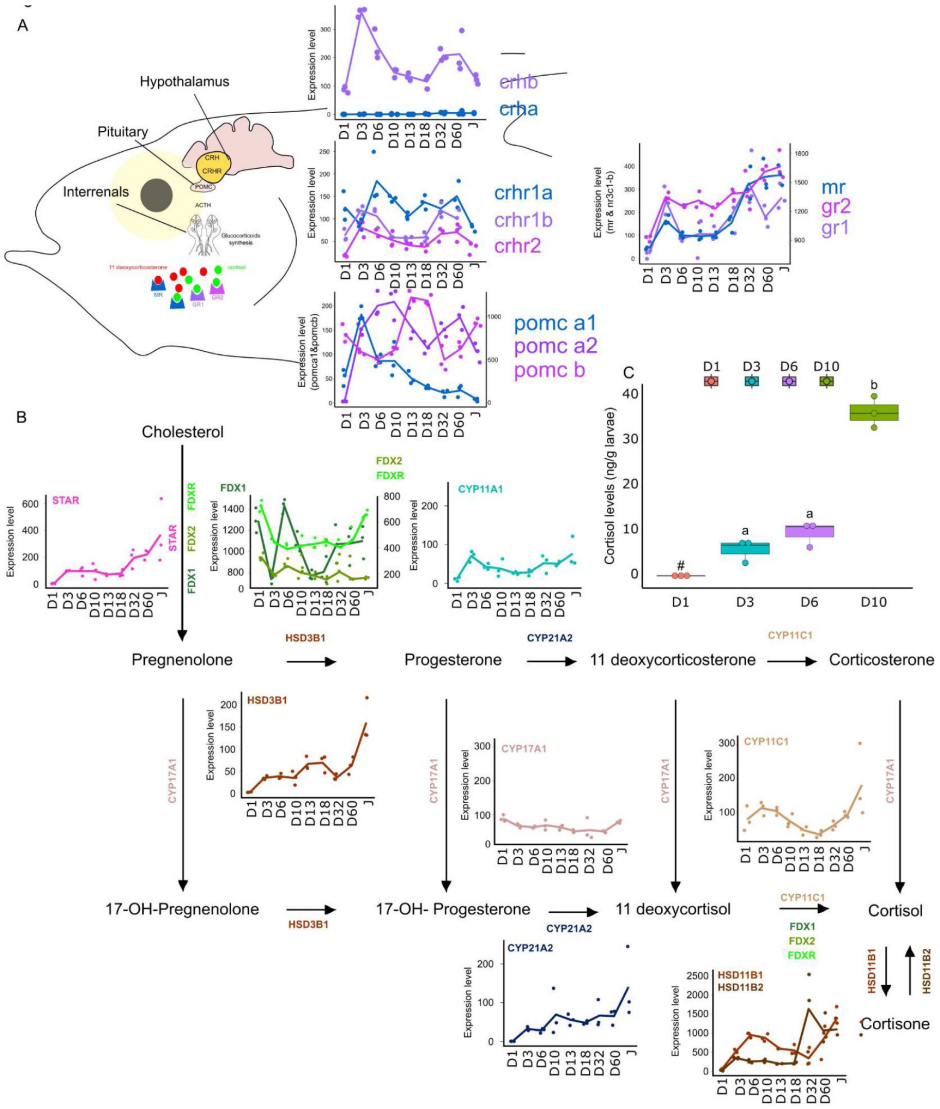

Fig. 5

Expression levels of genes involved in the Hypothalamo-Pituitary-Interranal axis (HPI) and corticoids synthesis.

A) Expression levels of genes involved in Hypothalamo-Pituitary-Interenal axis (HPI): *crha (corticotropin releasing hormone a), crhb (corticotropin releasing hormone b), crhr1a (corticotropin releasing hormone receptor 1a), crhr1b (corticotropin release hormone receptor 1b), crhr2 (cortico release hormone receptor 2), pomc-a1 (propiomelanocortin a1), pomc-a2 (propiomelanocortin a2), pomc-b (propiomelanocortin b), mr (mineralocorticoid receptor), gr1 (glucocorticoid receptor 1), gr2 (glucocorticoid receptor 2)*. B) Expression levels of genes involved in corticoids synthesis: *star* (steroidogenic acute regulatory protein), *fdx1* (ferredoxine 1), *fdx2* (ferredoxine 2), *fdxr* (ferredoxine reductase), *cyp11a1* (Cytochrome P450 Family 11 Subfamily A Member 1), *hsd3b1* (Hydroxysteroid dehydrogenases 3*β1*), *cyp17a1* (Cytochrome P450 Family 17 Subfamily A Member 1), *cyp21a2* (Cytochrome P450 Family 21 Subfamily A Member 2), *cyp11c1* (Cytochrome P450 Family 11 Subfamily C Member 1), *hsd11b1*, *hsd11b2* (Hydroxysteroid dehydrogenase 11 3β1&2). C) Cortisol levels (in ng/g of larvae) during early larval development. # indicates that the value is below the quantification limit, and different letters indicate significant differences (one-way ANOVA followed by a Tukey HSD test).

Finally, *gr2* expression level increased strongly at D03, then remained stable and increased again at D60. The relatively high expression of the *crhr* genes may suggest an increase in the sensitivity to CRH to mediate the production of POMC by the pituitary gland, a process that seems to occur twice during *E. malabaricus* larval development.

Concomitantly, a two-step increase in *star* is observed: first at D03 and a second at D32. This may suggest an increase in the production of cortisol following the high expression of *pomc-a2*. Indeed, POMC is the precursor of the Adreno Cortico Trophic Hormone (ACTH) which is the pituitary factor stimulating cortisol production by the inter-renal gland[54]. The expression levels of genes involved in cortisol production corroborate this hypothesis. Indeed, we observe an increase of expression around D03 and D06 for *fdx1, cyp11a1, hsd3b1, cyp11c1, hsd11b1, hsd11b2*, as well as a second increase of expression from D32 for *fdx1, fdxr, hsd3b1, cyp11c1, hsd11b1, hsd11b2*. Interestingly, measurements of cortisol levels during early larval development (between D1 and D10) showed that cortisol concentration starts to increase from D3, coinciding with the expression levels of star, and is followed by a stronger increase from D10. Those results first indicate that HPI axis and cortisol production are activated during metamorphosis suggesting a possible interaction between TH and corticoid pathways during metamorphosis. However, there is contrasting evidence of communication between these two pathways in teleost fish with some data suggesting a synergic and other an antagonistic relationship. In terms of synergy, an increase in cortisol level concomitantly with an increase in TH levels has been observed in flatfish[19], golden sea bream[100] and silver sea bream[101].

Cortisol was also shown to enhance in vitro the action of TH on fin ray resorption (phenomenon occurring during flatfish metamorphosis) in flounder[20]. TH exposure increases MR and GR genes expression in zebrafish embryo[55]. It has also been shown that cortisol regulates local T3 bioavailability in the juvenile sole via regulation of deiodinase 2 in an organ-specific manner[56]. On the antagonistic side, it has been shown that experimentally induced hyperthyroidism in common carp, decreasing cortisol levels[57], whereas cortisol exposure decreases TH levels in European eel[58]. Given this scattered evidence, the existence of a crosstalk active during teleost metamorphosis has never been formally demonstrated. The results we obtained in grouper are clearly indicating that HPI axis and cortisol synthesis are activated during early development and during metamorphosis and this may suggest that in some aspect cortisol synthesis can work in concert with TH, as has been shown in several different contexts in amphibians[17]. It is worth to note, however, that the increase of the gene encoding POMC-A2 may not only be linked to cortisol synthesis as POMC is also a precursor of other hormones and notably melanocytes-stimulating hormones[54]. Those hormones belong to the melanocortin system that is involved in body pigmentation, but also in social behavior, appetite, and stress physiology[59]. The increase in *pomc-a2* observed during *E. malabaricus* may thus also be involved in the onset of pigmentation pattern. Taken together, these results brought a first insight into the potential role of corticoids in the metamorphosis of *E. malabaricus* and call for functional experiments directly testing a possible synergy. Given the results obtained in our study, *E. malabaricus* could be a good model to investigate the role of corticoids in teleost metamorphosis. The interplay between corticoids and thyroid hormones could have relevant consequences in terms of aquaculture and claim for an examination of the role of stress in triggering metamorphosis.

Overall, the results obtained in this study revealed a very precocious surge of expression of genes involved not only in two connected key pathways (corticoids and TH) that are known to control ontogenetic transitions and to control many biological processes [60,61]. This indicates that the early post-embryonic period in grouper may correspond to such an ontogenetic transition that has been ignored until now. More generally, the fact that the outcome of metamorphosis is very variable from one species to another (e.g., differences in metamorphosis between clownfish and grouper) and that it also allows exquisite acclimation of the juveniles to their local environment[37] highlights the capacity of this transitional step, controlled by environmentally connected hormonal systems, to change rapidly in accordance with ecological needs. Finally,

considering that rearing conditions during larval metamorphosis in an aquaculture context may impact growth and welfare at later life stages, understanding the molecular changes occurring during the development of a species might prove useful to enhance survival rates.

Material and Methods

Larval husbandry

This study was conducted in partnership with the Okinawa Prefectural Sea Farming Center, Motobu-cho, Okinawa, Japan. *Epinephelus malabaricus* larvae and juveniles were obtained from various clutches obtained from natural spawning in 2020, 2021 and 2023. Larvae were reared under natural condition in 50,000 L of natural sea water in circular tanks. Light exposure duration followed natural daylight hours, salinity (approximately 33-34 ppm) and temperature (approximately 27°C on average) remained relatively stable as the tanks were constantly renewed with natural seawater. Microalgae (*Nannochloropsis* sp.) was added from hatching until 15 days post hatching (dph) to maintain the nutritional value of live-feed organisms and create a green-water environment. Rotifers *Brachionus* sp. (S type) were enriched with fish oil and distributed twice a day from 1 dph to maintain a concentration of 10 ind/mL until 13 dph. Artemia nauplii were added twice a day from 13 dph to 20 dph. Frozen copepods were given five times a day from 13 dph until 20 dph. Artificial food was given from 20 dph during daytime by automatic feeding (one distribution every hour).

Sample collection and tissue collection

Tissues for genome sequencing and transcriptome sequencing were collected on 08. September 2020 from two approximately four-month-old fish sourced from the Okinawa Prefectural Sea Farming Center. The fish were euthanized by cervical dislocation, and immediately dissected. Liver and muscle tissues of one fish were immediately frozen in liquid nitrogen for PacBio HiFi and Hi-C sequencing, respectively. Brain, gill, liver, heart, caudal fin, eye, spleen, stomach, intestine, muscle, skin spinal cord and spinal nerve tissues were taken from the second fish and stored in RNAlater™ (ThermoFisher Scientific) for tissue specific transcriptome sequencing.

Larvae and juveniles were sampled between 30. April 2021 and 02. June 2021 ranging from 1 dph to approximately 2 months juveniles. A total of four clutches spawned in early and late April were sampled during this period and larvae were collected and sorted according to their morphology allowing us to sequence 8 developmental stages. For the developmental transcriptome, whole individuals were sampled from the same farming center from 30. April 2021 to 02. June 2021, ranging from one day old larvae to roughly two-month-old juveniles (**Table 2**). Larvae and juveniles were euthanized in the afternoon (between 13:00 and 15:00) with MS222 solution (200 mg/L, Sigma-A5040) before being placed in RNAlater. Larger fish were cut open for improved RNAlater penetration and samples were kept at 4°C for two to eight days before being stored at -20°C until extraction. Larvae for TH and cortisol measurements were sampled in triplicates between 17. June 2023 and 26. June 2023 at D1 (n=120 per replicate), D3 (n=120 per replicate), D6 (n=60 per replicate) and D10 (n=40 per replicate). Larvae were collected as in Roux *et al.*[102] and kept at -80 until further analysis. TH and cortisol extraction and measurement were outsourced to ASKA Pharmaceutical Medical Co., Kanagawa, Japan. Detailed protocols can be found in **Supp. protocol S1** for TH and **Supp. protocol S2** for cortisol.

All sampling conducted in this study was done under the approval from the Animal Care and Use Committee at the Okinawa Institute of Science and Technology Graduate University (approval N°2021-328).

Age (dph)		Timpoint/ Stage	Morphological description
1	 2.5 mm NL/2.7 mm TL	D01	Newly hatched larva with a yolk sac; mouth unopened; eyes not pigmented; no pectoral fin
3	 2.7 mm NL/2.9 mm TL	D03	Yolk sac remains; mouth is opened; eyes are pigmented; pectoral fins are formed; large melanophores appear on the ventral cavity and on the second half of the body
6	 2.9 mm NL/3.1 mm TL	D06	Yolk sac has been resorbed; dorsal-fin spine starts to form within the finfold
10	 3.8 mm NL/4.0 mm TL	D10	Embryonic fin fold start differentiating in anal and dorsal fin while second spine of dorsal fin and spines of pelvic fins begin to extend with some melanophores colonizing the tips and xanthophores start covering the ventral cavity
13-15	 6.0 mm NL/6.4 mm TL	D13	Spines of dorsal and pelvic fins grow. First spine of dorsal fin appears, second spine of dorsal fin and spines of pelvic fins become serrated; head spines appear, caudal-fin rays start to form, tip of the notochord begins to flex; xanthophores continue their expansion
18-20	 6.8 mm SL/8.3 mm TL	D18	Notochord post-flexed; hypural bones are formed and in perpendicular position; caudal-fin rays are segmented; soft rays of dorsal and anal fins start to form and both fins start to form their final shape; fin rays are forming on upper part of the pectoral fin; soft rays of pelvic fins began to form; melanophores are appearing on top of the head and on the caudal peduncle
30-32	 10.0 mm SL/12.8 mm TL	D32	Second spine of dorsal fin and spines of pelvic fins start to regress; soft rays in dorsal, anal and pectoral fins are weakly segmented, caudal fin becomes truncated shape; melanophores are appearing at the basis of dorsal spines and along the notochord, melanophores ventrally on the caudal peduncle disappears; xanthophores start colonizing the caudal peduncle
60	 14.7 mm SL/19.1 mm TL	D60	Second spine of dorsal fin and spines of pelvic fins continue their regression. Soft rays in pectoral and pelvic fins segmented; caudal-fin rays branched; anterior two bands of melanophores start appearing in some individuals: xanthophores are disappearing from the ventral cavity
60	 27.6 mm SL/34.6 mm TL	J	Juvenile stage; scales covered the body surface; second spine of dorsal fin and spines of pelvic fins fully regressed and became plain without hooks; caudal fin reached its final round shape; adult pigmentation pattern is more visible with alternate light and brownish vertical bands making lateral line system fully visible

Table 2

Morphological description of the larval and juvenile stages sampled for the transcriptomic analysis.

D01: 1 day post hatching (dph), D03: 3 dph, D10: 10 dph, D:13 13-15 dph, D18: 18-20 dph, D32: 32-34 dph, D60: ca. 60 dph, J: ca. 60 dph with juvenile phenotype. NL is “notochord length” for preflexion and flexion larvae, SL is “standard length” for postflexion and older stages, and TL is “total length” for all stages.

DNA extraction and sequencing

Genomic DNA was extracted from liver tissue using the NucleoBond HMW DNA extraction kit (Machery-Nagel). Library preparation was carried out with the SMRTbell Express Template Prep Kit 2.0 and SMRTbell Enzyme Cleanup Kit, Sequencing primer v2, Sequel II Binding Kit 2.0, and Sequel II Sequencing Kit 2.0 (Pacific Biosciences). Sequencing was done on a Sequel II System, using three SMRT Cell 8M flow cells through diffusion loading of 60-100pM library. Hi-C library preparation and sequencing was carried out by Phase Genomics from muscle tissue using the Phase Genomics Proximo Animal Kit v3.0 and sequenced on a Illumina HiSeq 4000 with 150 bp PE.

RNA extraction and sequencing

For the tissue specific transcriptomics, samples were homogenized using a Kinematica Polytron PT1200E Homogenizer and RNA was extracted using the Maxwell RSC simplyRNA Tissue Kit (Promega: AS1340). Individually barcoded IsoSeq Express libraries of all 13 tissues were prepared by the OIST Sequencing Section using the SMRTbell Express Template Prep Kit 2.0. The libraries were sequenced on a PacBio Sequel 2 across two SMRT Cell 8M flow cells.

For the larval stage transcriptomics, samples from 1 to 32 dph were homogenized in thioglycerol using metal beads lysing matrix tubes (MPB) in an automated homogenizer (FastPrep-24 5G MPB). Bigger samples (60 dph and juveniles) were manually crushed in thioglycerol using 14 mL round bottom tubes and a tissue grinder (Tissue Ruptor II, Qiagen). Samples from 1 and 3 dph consisted of pools of three larvae in triplicates, while all remaining timepoints consisted of triplicates of single individuals. RNA Extraction was then carried out as for tissue samples using the Maxwell RSC simplyRNA Tissue Kit (Promega: AS1340). Library preparation was carried out at the OIST Sequencing Section using the NEBNext Ultra II Directional RNA Library Prep Kit. Sequencing was carried out with the pooled library split across two Illumina Nova Seq SP flowcells with 150 bp PE reads.

Genome assembly, scaffolding and phasing

The genome assembly was carried out using unprocessed PacBio HiFi reads with the diploid aware Improved Phased Assembler (IPA, V1.3.1, *https://github.com/PacificBiosciences/pbipa*), resulting in two phased genomes. The two phased genomes were assessed using purge_haplotigs[62] to generate a genome-wide read-depth histogram; however, no purging was necessary. Completeness of the final assembly was assessed using BUSCO[63] (V4.1.2) with the actinopterygii_odb10 database. Scaffolding and phasing were outsourced to Phase Genomics (See *Supp. Protocol S3* for detail).

Genome and functional annotation

Genome annotation was carried out as described Ryu et al.[64]. Briefly, repeat content analysis was done in RepeatModeler[65] (V2.0.1), RepeatMasker[66] (V4.1.1), the vertebrata library of Dfam[67] (V3.3), and GenomeTools[68] (V1.6.1). Annotation was done using BRAKER2[69] and associated programs[70–81]. For this, the ISO-seq data from the adult tissue and RNA-seq data from the larval samples (see below for quality control process) were used together with publicly available protein data (*Supp. Table S5*). Post-processing was carried out as described by Ryu et al.[64] using the Swiss-Prot protein database[82] (UniProt) with Diamond[74] (V2.0.9) and Pfam domains[83] identified by InterProScan[84] (V5.48.83.0). Gene model statistics were calculated using the get_general_stats.pl script from the eval package[85] (V2.2.8). Finally, functional annotation was carried out with the filtered gene models produced by BRAKER. The amino acid sequences were blasted against the non-redundant protein database (downloaded 15. November 2021) using blastp[86] (V2.10.0+; parameters: -show_gis -num_threads 10 -evalue 1e-5 -word_size 3 -num_alignments 20 -outfmt 14 -max_hsps 20). Additionally, protein domains were assigned using InterProScan[84] (V5.48.83.0; parameters: --disable-precalc -- goterms --pathways -f xml). The blast and interproscan results were then loaded into OmicsBox[87,88] for post-processing.

Differential gene expression analysis

Data from the two lanes per sample were merged before processing. Low quality bases and adaptor sequences were filtered using Trim Galore[89] (V0.6.5) and cutadapt[90] (V2.10). Kraken2[91] (V2.0.9-beta) was used to remove bacterial reads using the bacterial and archeal database (V4.08.20). Cleaned reads were mapped using STAR[92] (V2.7.9a) with "--quantMode GeneCounts", using the filtered gff file produced by the braker2 annotation outlined above for the genome indexing. The unstranded mapped reads were then loaded into Rstudio[93] (V2022.02.4) using R[94] (V3.6.3). DESeq2[95] (V1.36.0) was used for general data analysis, with coseq[96,97] (V1.20.0) being used for cluster analysis. In brief, the cluster analysis was carried out on differentially expressed genes only, as determined through likelihood ratio test (LRT) analysis (full model: design = ~ dph, reduced model: reduced = ~ 1, adjusted p-value threshold: 0.001) in DESeq2. Adjusted p-values and annotations for the group of genes represented in **Fig 1B** in this study can be found in the Suppl. Data File. Normalisation was done in DESeq2, while the following parameters were used for coseq: model = "Normal", transformation = "arcsin", seed = 1234, iter=10000. Specific genes belonging to clusters where D03 and/or Day32 showed upregulation were then re-clustered with the same parameter for visualization. A complete representation of all initial clusters found in this study can be found in **Supp. Fig. 3**. Pairwise analysis of differentially expressed genes between two time points was done using the Wald test in DESeq2 (design = ~ dph, adjusted p-value threshold: 0.01, log2FoldChange = ±0.58). Figures were plotted using ggplot2[98] (V3.4.1), and the analysis made general use of the tidyverse package[99] (V1.3.2).

Data Availability

All raw and assembled data used in this study has been deposited on GenBank under umbrella BioProject PRJNA798702, with the principal phased assembly in BioProject PRJNA798188, the alternate phased assembly in BioProject PRJNA798189, and the raw data in BioProject PRJNA794870. BioSamples can be found under SAMN24662200 (genome sequencing), SAMN24664212 (ISO-seq), and SAMN24664213 - SAMN24664234 / SAMN32359227 - SAMN32359229 (RNA-seq). PacBio and Illumina raw data can be found under SRR17639994 - SRR17640023 / SRR22859365 - SRR22859367. The final scaffolded assembly can be found under accession JANUFT000000000. The raw gene expression count data and hormone measurements can be found in the **supplementary data excel** file. Any additional processed data used in this publication (e.g., list of genes in each cluster) can be requested from the corresponding author.

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Author Contributions

R.H. carried out all bioinformatical analyses and contributed to the design of the work, the interpretation, and the writing of the manuscript. N.R. carried out the biological interpretation of the data and contributed to the writing of the manuscript. K.M. and P.P. designed and carried out the larval collection for transcriptomic analysis. S.M. and M.I. contributed to the lab work and data

collection for transcriptomic analysis and genome assembly. H.C. and S.M. sampled larvae for TH and cortisol measurements. V.L. contributed to the design of the work, the interpretation of the data, and writing the manuscript. T.R. contributed to the design of the work and writing of the manuscript.

Supplemental materials

Supp. protocol S1: T3 and T4 measurements

ASKA Pharmaceutical Medical Co., Ltd., 5-36-1 Shimosakunobe, Kawasaki Takatsu-ku, Kanagawa 213-8522, Japan

Before sample preparation, a whole fish body was homogenized in distilled water by a ball mill (ShakeMaster® NEO) with stainless beads. The homogenate sample was transferred to a polypropylene (PP) tube and spiked with isotope-labelled internal standards solution containing $^{13}C_6$-T4, $^{13}C_6$-T3, and $^{13}C_6$-rT3. The homogenate sample was denatured with acetonitrile and then, equilibrated for 30 min at room temperature on dark

After equilibration, the sample was centrifuged at 3000 rpm for 3 min, and then the supernatant was decanted into a new PP tube which was added distilled water. The sample was applied to an Oasis MCX cartridge which had been successively conditioned with methanol, distilled water, and 1% acetic acid solution. After the cartridge was washed with distilled water followed by methanol, the thyroid hormones were eluted with methanol/distilled water/ammonia solution (70:30:1,v/v/v). After the sample was evaporated to dryness, the residue was dissolved with methanol/distilled water pyridine solution (40:60:1, v/v/v). The sample was subjected to an LC-MS/MS system for determination of T4, T3 and rT3. The SRM transitions were *m/z* 777.8/731.6 for T4, 651.9/605.8 for T3 and 651.9/507.7 for rT3. The measurement ranges were 4-4000 pg/tube for T4 and 0.5-500 pg/tube for both T3 and rT3. The limits of quantification were 4 pg/tube for T4 and 0.5 pg/tube for both T3 and rT3..

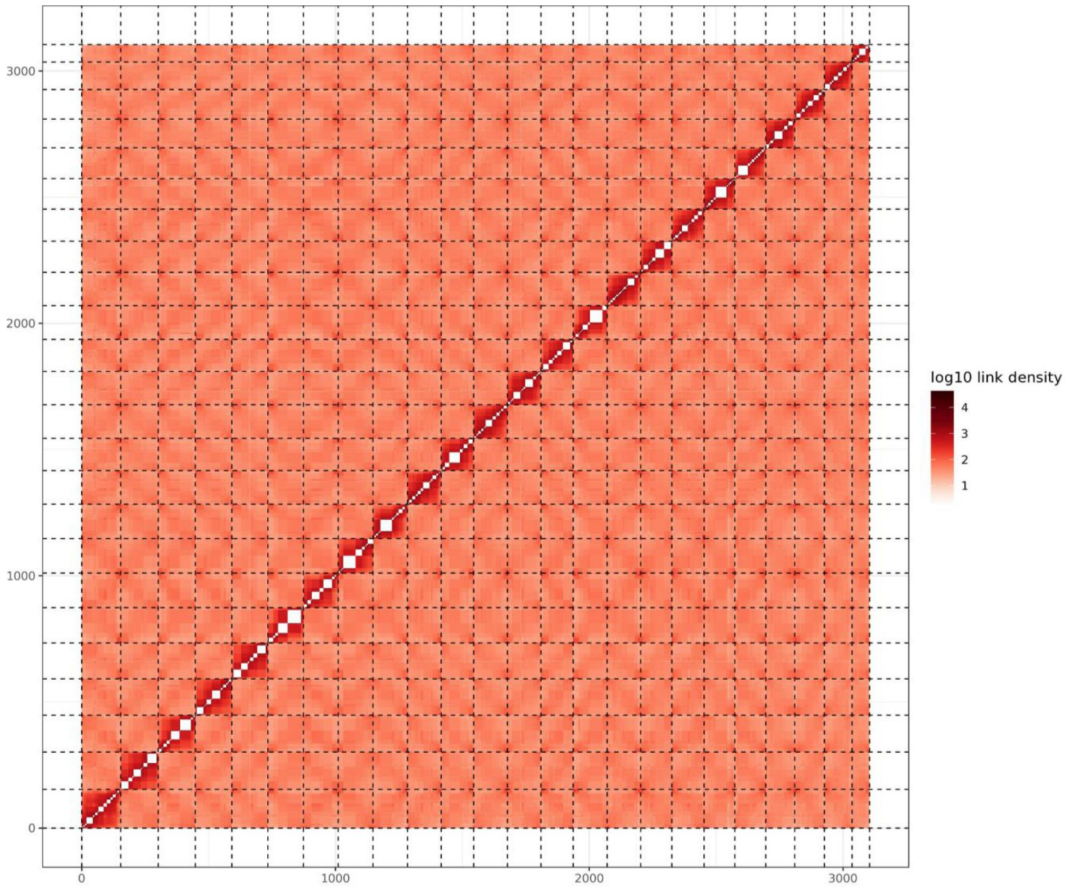
Supp. protocol S2: Cortisol measurements

ASKA Pharmaceutical Medical Co., Ltd., 5-36-1 Shimosakunobe, Kawasaki Takatsu-ku, Kanagawa 213-8522, Japan

Before sample preparation, a whole fish body was homogenized in distilled water by a ball mill (ShakeMaster® NEO) with stainless beads. The homogenate sample was transferred to a glass tube and spiked with isotope-labelled internal standard solution containing F-d4. F was extracted with 4 mL of methyl *tert*-butyl ether.

After the organic layer was evaporated to dryness, the extract was dissolved in 0.5 mL of methanol and diluted with 1 mL of distilled water. The sample was applied to OASIS MAX cartridge which had been successively conditioned with 3 mL of methanol and 3 mL of distilled water. After the cartridge was washed with 1 mL of distilled water, 1 mL of methanol/distilled water/acetic acid (45:55:1,v/v/v), and 1 mL of 1% pyridine solution, the F was eluted with 1 mL of methanol/pyridine (100:1,v/v).

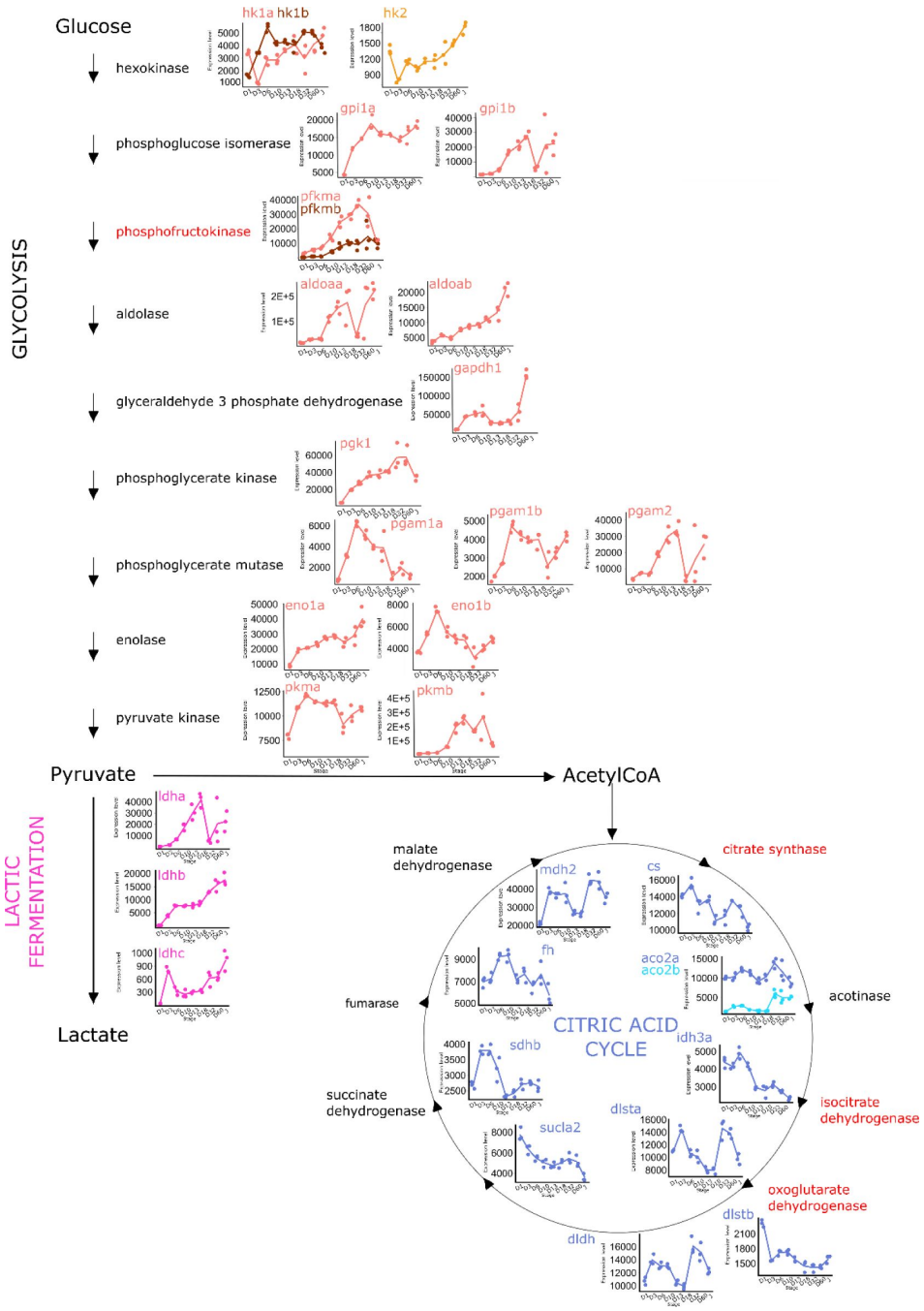
After evaporation, the residue was reacted with 50 µL of mixed solution (80 mg of 2-methyl-6-nitrobenzoic anhydride, 20 mg of 4-dimethylaminopyridine, 40 mg of picolinic acid and 10 µL of triethylamine in 1 mL of acetonitrile) for 30 min. at room temperature. After the reaction, the sample was dissolved in 0.5 mL of ethyl acetate/hexane/acetic acid (15:35:1, v/v) and the mixture was applied to HyperSep SI cartridge which had been successively conditioned with 3 mL of acetone and 3 mL of hexane. The cartridge was washed with 1 mL of hexane, and 2 mL of ethyl acetate/hexane (3:7, v/v). Steroids was eluted with 2.5 mL of acetone/hexane (7:3, v/v). After



Supp. Fig. S1

Hi-C contact map after scaffolding.

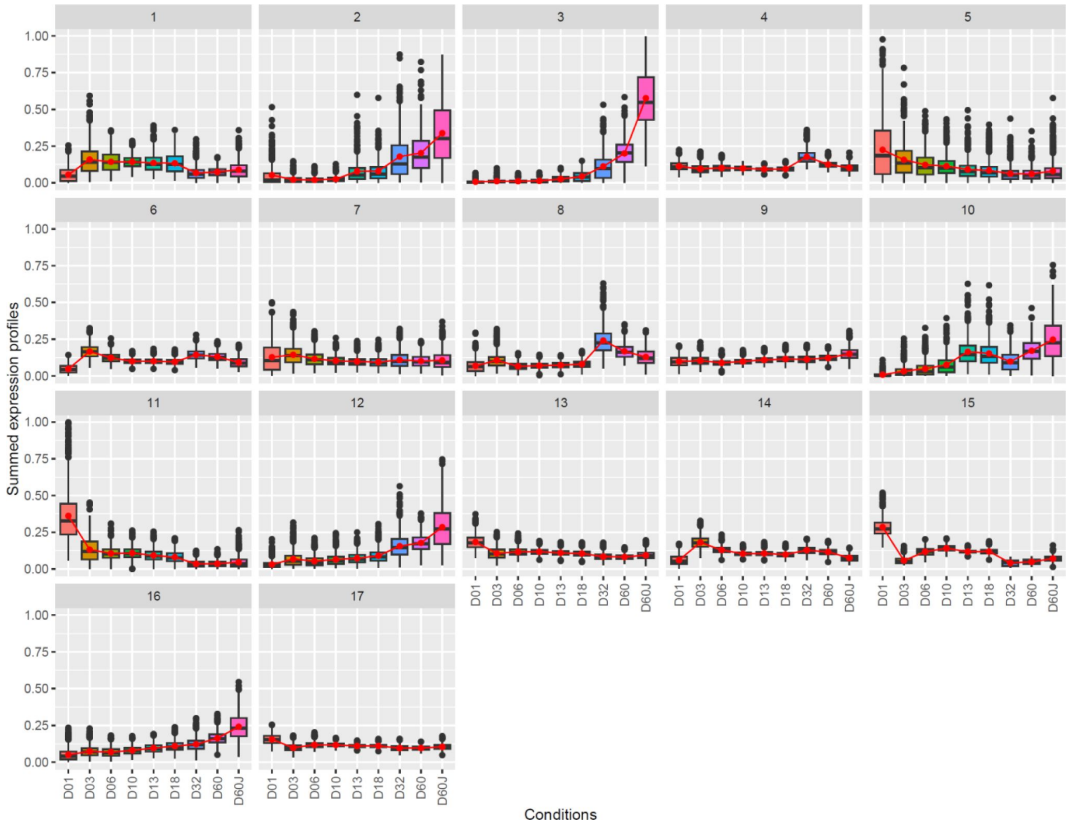
E. *malabaricus* genome contig contact matrix using Hi-C data. The color bar indicates contact density from dark red (**high**) to white (**low**).



Supp. Fig. S2

Metabolic shift during grouper metamorphosis.

Expression levels of genes involved in glycolysis, lactic fermentation, and citric acid cycle at each developmental stage (D01, D03, D06, D10, D13, D18, D60, J) extracted from transcriptomic data. Enzyme highlighted in red represents rate limiting steps for each metabolic pathway.



Supp. Fig. S3

Complete cluster analysis of differentially expressed genes.

Cluster analysis of differentially expressed genes (n = 22,135, LRT analysis (full model: design = ~ dph, reduced model: reduced = ~ 1, adjusted p-value threshold: 0.001). Genes contained in clusters: 1) 925, 2) 382, 3) 548, 4) 1,193, 5) 804, 6) 1,503, 7) 2,025, 8) 786, 9) 2,399, 10) 570, 11) 801, 12) 1,141, 13) 2,439, 14) 1,471, 15) 681, 16) 1,702, 17) 2,765.

	SMRT cell 1	SMRT cell 2	SMRT cell 3	Total
≥Q20 Reads	322,103	442,205	1,373,662	2,137,970
≥Q20 Yield (bp)	8,468,697,810	11,690,872,687	26,355,056,210	46,514,626,707
≥Q20 Read Length (mean, bp)	26,291	26,437	19,185	-

Supp. Table S1

PacBio HiFi data generated for *E. malabaricus* genome assembly based on three SMRT cells.

Contig assembly size	1,092,599,927 bp
Number of contigs	298
Contig N50	7,396,124 bp
Largest contig	26,202,351 bp
Contigs contained in scaffolds	90.5%
Non-ATGC characters	36,700 bp (0.003%)
Genome: BUSCO completeness	3,406 (93.6%)
Genome: Complete and single copy	3,359 (92.3%)
Genome: Complete and duplicated	47 (1.3%)
Genome: Fragmented	48 (1.3%)
Genome: Missing	186 (5.1%)
Number of protein-coding genes	26,140
Average gene length	20,718 bp
Average CDS length	1,750 bp
Average exons per gene	11.2

Supp. Table S2

Additional genome assembly, scaffolding and annotation statistics.

Total Genome length	1,027,628,325 bp		
Bases masked	579,515,295 bp (56.4 %)		
	number of elements	length occupied	percentage of sequence
Retroelements	660,880	123,046,856	11.97%
SINEs:	74,049	7,798,000	0.76%
Penelope	25,476	3,705,071	0.36%
LINEs:	422,597	86,199,668	8.39%
CRE/SLACS	1	100	0.00%
L2/CR1/Rex	266,320	53,565,106	5.21%
R1/LOA/Jockey	10,918	2,170,694	0.21%
R2/R4/NeSL	11,286	3,660,204	0.36%
RTE/Bov-B	47,451	10,563,888	1.03%
L1/CIN4	35,250	8,411,019	0.82%
LTR elements:	164,234	29,049,188	2.83%
BEL/Pao	10,186	2,183,697	0.21%
Ty1/Copia	4,658	823,228	0.08%
Gypsy/DIRS1	76,319	13,796,105	1.34%
Retroviral	32,317	5,441,530	0.53%
DNA transposons	1,604,009	296,558,272	28.86%
hobo-Activator	799,713	138,795,609	13.51%
Tc1-IS630-Pogo	138,975	24,805,298	2.41%
PiggyBac	22,605	3,909,912	0.38%
Tourist/Harbinger	161,216	38,134,353	3.71%
Other (Mirage, P-element, Transib)	52,802	10,665,183	1.04%
Rolling-circles	101,574	30,491,612	2.97%
Unclassified:	669,818	111,877,701	10.89%
Total interspersed repeats:		531,482,829	51.72%
Small RNA:	26,405	2,780,064	0.27%
Satellites:	11,580	2,523,082	0.25%
Simple repeats:	277,904	12,099,447	1.18%
Low complexity:	29,110	1,563,810	0.15%

Supp. Table S3

Detailed repeat annotation results using the DFAM repeat database.

Contrast	Differentially Expressed Gene	Upregulated genes	Downregulated Genes
D01 vs. D03	14,830	7,151	7,679
D03 vs. D06	8,381	4,464	3,917
D06 vs. D10	1,844	790	1,054
D10 vs. D13	1,976	671	1,305
D13 vs. D18	191	81	110
D18 vs. D32	10,774	5,320	5,454
D32 vs. D60	2,654	967	1,687
D60 vs. Juvenile	6,963	3,259	3,704

Supp. Table S4

Differentially expressed, upregulated and downregulated genes between consecutive time points.

Species	Common Name	Number of proteins	Source
Amphiprion ocellaris	Ocellaris clownfish	48,668	www.ncbi.nlm.nih.gov/protein
Danio rerio	zebrafish	88,631	www.ncbi.nlm.nih.gov/protein
Acanthochromis polyacanthus	spiny chromis damselfish	36,648	www.ncbi.nlm.nih.gov/protein
Oreochromis niloticus	Nile tilapia	36,648	www.ncbi.nlm.nih.gov/protein
Oryzias latipes	Japanese medaka	47,623	www.ncbi.nlm.nih.gov/protein
Poecilia reticulata	guppy	45,692	www.ncbi.nlm.nih.gov/protein
Salmo salar	Atlantic salmon	112,302	www.ncbi.nlm.nih.gov/protein
Stegastes partitus	bicolor damselfish	31,760	www.ncbi.nlm.nih.gov/protein
Takifugu rubripes	Japanese puffer	49,529	www.ncbi.nlm.nih.gov/protein
Epinephelus lanceolatus	Giant grouper	42,970	GCA_005281545.1, RefSeq
Epinephelus akaara	Red-spotted grouper	23,923	4398b9f, Dryad
Total aa sequences		**1,155,478**	

Supp. Table S5

Origin of protein sequences used for genome annotation in braker2.

evaporation, the residue was dissolved in 0.1 mL of acetonitrile/distilled water (2:3, v/v) and the solution was subjected to a LC-MS/MS. The SRM transitions was *m/z* 468.2/309.2 for F and 472.2/454.3 for F-d4. The measurement ranges was 10-100000 pg/tube. The limits of quantification of F was 10 pg/tube.

Supp. protocol S3: HiC scaffolding protocol used by Phase Genomics

Chromatin conformation capture data was generated using a Phase Genomics (Seattle, WA) Proximo Hi-C 4.0 Kit, which is a commercially available version of the Hi-C protocol [103]. Following the manufacturer's instructions for the kit, intact cells from two samples were crosslinked using a formaldehyde solution, digested using the DPNII restriction enzyme, and proximity ligated with biotinylated nucleotides to create chimeric molecules composed of fragments from different regions of the genome that were physically proximal in vivo, but not necessarily genomically proximal. Continuing with the manufacturer's protocol, molecules were pulled down with streptavidin beads and processed into an Illumina-compatible sequencing library. Sequencing was performed on an Illumina HiSeq 4000, generating a total of 159,606,973 read pairs. Reads were aligned to the draft assembly also following the manufacturer's recommendations [104]. Briefly, reads were aligned using BWA-MEM[105] with the -5SP and -t 8 options specified, and all other options default. SAMBLASTER[106] was used to flag PCR duplicates, which were later excluded from analysis. Alignments were then filtered with samtools[107] using the -F 2304 filtering flag to remove non-primary and secondary alignments. FALCON-Phase[108] was used to correct likely phase switching errors in the primary contigs and alternate haplotigs from FALCON-Unzip and output its results in pseudohap format, creating one complete set of contigs for each phase. Phase Genomics' Proximo Hi-C genome scaffolding platform was used to create chromosome-scale scaffolds from FALCON-Phase's phase 0 assembly, following the same single-phase scaffolding procedure described in Bickhart et al. [109]. As in the LACHESIS method [110], this process computes a contact frequency matrix from the aligned Hi-C read pairs, normalized by the number of DPNII restriction sites (GATC) on each contig, and constructs scaffolds in such a way as to optimize expected contact frequency and other statistical patterns in Hi-C data.

Juicebox[111,112] was then used to correct scaffolding errors, and FALCON-Phase was run a second time to detect and correct phase switching errors that were not detectable at the contig level, but which were detectable at the chromosome-scaffold level. Metadata generated by FALCON-Phase about scaffold phasing was used to generate matching .assembly files (a file format used by Juicebox) and subsequently used to produce a diploid, fully-phased, chromosome-scale set of scaffolds using a purpose-built script[113]. In these final scaffolds, phase 0 included 24 scaffolds spanning 1,027,591,625 bp (92.82% of input) with a scaffold N50 of 43,313,630 bp, and phase 1 included 24 scaffolds spanning 1,019,278,383 bp (91.98% of input) with a scaffold N50 of 43,164,609 bp.

3155248-data1.xlsx

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Editors

Reviewer #1 (Public Review):

Summary and strength:

The authors undertook to assemble and annotate the genome sequence of the Malabar grouper fish, with the aim of providing molecular resources for fundamental and applied research. Even though this is more mainstream, the task is still daunting and labor-intensive. Currently, high-quality and fully annotated genome sequences are of strategic importance in modern biology. The authors make use of the resource to address the endocrine control of an ecologically and developmentally relevant life cycle transition, metamorphosis. As opposed to amphibian and flat fish where body plan changes, fish metamorphosis is anatomically more subtle and much less known, although it is clear that thyroid hormone (TH) signaling is a key player. The authors thus provide a repertoire of TH-relevant gene expression changes during development and across metamorphosis and correlate developmental stages with changes in gene expression. Overall, this work has a strong potential to meet its target.

Weaknesses:

The manuscript needs proper editing and is not complete. Some wordings lack precision and make it difficult to follow (e.g. line 98 "we assembled a chromosome-scale genome of ..." should read instead "we assembled a chromsome-scla genome sequence of ...". Also, panel Figure 2E is missing.

The shortcomings of the manuscripts are not limited to the writing style, and important technical and technological information is missing or not clear enough, thereby preventing a proper evaluation of the resolution of the genomic resources provided:

- Several RNASeq libraries from different tissues have been built to help annotate the genome and identify transcribed regions. This is fine. But all along the manuscript, gene expression changes are summarized into a single panel where it is not clear at all which tissue this comes from (whole embryo or a specific tissue ?), or whether it is a cumulative expression level computed across several tissues (and how it was computed) etc. This is essential information needed for data interpretation.

- The bioinformatic processing, especially of the assemble and annotation, is very poorly described. This is also a sensitive topic, as illustrated by the numerous "assemblathon" and "annotathon" initiatives to evaluate tools and workflows. Importantly, providing configuration files and in-depth description of workflows and parameter settings is highly recommended. This can be made available through data store services and documents even benefit from DOIs. This provides others with more information to evaluate the resolution of this work. No doubt that it is well done,
but especially in the field of genome assembly and annotation, high resolution is VERY cost and time-intensive. Not surprisingly, most projects are conditioned by trade-offs between

cost, time, and labor. The authors should provide others with the information needed to evaluate this.

- Quantifications of T3 and T4 levels look fairly low and not so convincing. The work would clearly benefit from a discussion about why the signal is so low and what are the current technological limitations of these quantifications. This would really help (general) readers.

- Differential analysis highlights up to ~ 15,000 differentially expressed genes (DEG), out of a predicted 26k genes. This corresponds to more than half of all genes. ANOVA-based differential analysis relies on the simple fact that only a minority of genes are DEG. Having >50% DEG is well beyond the validity of the method. This should be addressed, or at least discussed.

https://doi.org/10.7554/eLife.94573.1.sa1

Reviewer #3 (Public Review):

Summary:

The manuscript by Huerlimann et al. entitled "The transcriptional landscape underlying metamorphosis in the Malabar grouper (Epinephelus malabaricus)." describes the transcriptional landscape of the Malabar grouper during selected metamorphic stages. The authors find evidence of dynamic regulation of HPT axis genes, TH signalling genes, and HPA and metabolic-related genes during post-natal development. Finally, the authors argue that the HPA is involved in grouper metamorphosis, given the related genes' dynamic expression during this developmental time.

Strengths:

The work is technically very good, and the methodology applied is solid.

Weaknesses:

However, the authors make substantial considerations that are not proven by experimental or functional data. In fact, this is a descriptive study that does not provide any functional evidence to support the claims made.

The consideration that cortisol is involved in metamorphosis in teleosts has never been shown, and the only example cited by the authors (REF 20) clearly states that cortisol alone does not induce flatfish metamorphosis. In that work, the authors clearly state that in vivo cortisol treatment had no synergistic effect with TH in inducing metamorphosis. Moreover, in Senegalensis, the sole pre-otic CRH neuron number decreases during metamorphosis, further arguing that, at least in flatfish, cortisol is not involved in flatfish metamorphosis (PMID: 25575457). Furthermore, the authors need to recognise that the transcriptomic analysis is whole-body and that HPA axis genes are upregulated, which does not mean they are involved in regulating the HPT axis. The authors do not show that in thyrotrophs, any CRH receptor is expressed or in any other HPT axis-relevant cells and that changes in these genes correlate with changes in TSH expression. An in-situ hybridisation experiment showing co-expression on thyrotrophs of HPA genes and TSH could be a good start. However, the best scenario would be conducting cortisol treatment experiments to see if this hormone affects grouper metamorphosis.

High TSH and Tg levels usually parallel whole-body TH levels during teleost metamorphosis. However, in this study, high Tg expression levels are only achieved at the juvenile stage, whereas high TSH is achieved at D32, and at the juvenile stage, they are already at their lowest levels.

It is very difficult to conclude anything with the TH and cortisol levels measurements. The authors only measured up until D10, whereas they argue that metamorphosis occurs at D32. In this way, these measurements could be more helpful if they focus on the correct developmental time. The data is irrelevant to their hypothesis.

Moreover, as stated in the previous review, a classical sign of teleost metamorphosis is the upregulation of TSHb and Tg, which does not occur at D32 therefore, it is very hard for me to accept that this is the metamorphic stage. With the lack of TH measurements, I cannot agree with the authors. I think this has to be toned down and made clear in the manuscript that D32 might be a putative metamorphic climax but that several aspects of biology work against it. Moreover, in D10, the authors show the highest cortisol level and lowest T4 and T3 levels. These observations are irreconcilable, with cortisol enhancing or participating in TH-driven metamorphosis.

Given this, the authors should quantify whole-body TH levels throughout the entire developmental window considered to determine where the peak is observed and how it correlates with the other hormonal genes/systems in the analysis.

Even though this is a solid technical paper and the data obtained is excellent, the conclusions drawn by the authors are not supported by their data, and at least hormonal levels should be present in parallel to the transcriptomic data. Furthermore, toning down some affirmations or even considering the different hypotheses available that are different from the ones suggested would be very positive.

https://doi.org/10.7554/eLife.94573.1.sa0

Author Response

> ***Reviewer #1 (Public Review):***
>
> *Weaknesses:*
>
> *The manuscript needs proper editing and is not complete. Some wordings lack precision and make it difficult to follow (e.g. line 98 "we assembled a chromosome-scale genome of ..." should read instead "we assembled a chromsome-scla genome sequence of ...". Also, panel Figure 2E is missing.*

We will make the suggested change of adding “sequence”. Concerning additional changes, we have carefully edited our manuscript and looked for any incomplete sections. Unfortunately, it is difficult to see what other issues are being raised here without any further information. And the example given is not helpful to ascertain what other changes may be necessary, since we cannot see any problem with the sentence “we assembled a chromosome-scale genome of” as this phrase is widely used in many similar publications.

As for panel E of figure 2, it is not missing. The panel located to the right, just below “Target Cells”.

> *The shortcomings of the manuscripts are not limited to the writing style, and important technical and technological information is missing or not clear enough, thereby preventing a proper evaluation of the resolution of the genomic resources provided:*
>
> - *Several RNASeq libraries from different tissues have been built to help annotate the genome and identify transcribed regions. This is fine. But all along the manuscript, gene expression changes are summarized into a single panel where*

> *it is not clear at all which tissue this comes from (whole embryo or a specific tissue ?), or whether it is a cumulative expression level computed across several tissues (and how it was computed) etc. This is essential information needed for data interpretation.*

No fertilised eggs or embryos have been sequenced, individual tissues derived from juvenile fish were used for the genome annotation and whole larval fish for the developmental analysis. We will specify in the figures and text that the results shown are from whole larvae, and add more detail to the material and methods section about which type of sample was analysed in which way.

> - *The bioinformatic processing, especially of the assemble and annotation, is very poorly described. This is also a sensitive topic, as illustrated by the numerous "assemblathon" and "annotathon" initiatives to evaluate tools and workflows. Importantly, providing configuration files and in-depth description of workflows and parameter settings is highly recommended. This can be made available through data store services and documents even benefit from DOIs. This provides others with more information to evaluate the resolution of this work. No doubt that it is well done,but especially in the field of genome assembly and annotation, high resolution is VERY cost and time-intensive. Not surprisingly, most projects are conditioned by trade-offs between cost, time, and labor. The authors should provide others with the information needed to evaluate this.*

We will upload the code used to assemble and annotate this genome to a public repository or add it to the supplementary material.

The genome assembly did not use a specific workflow (e.g., nextflow), but was done with a simple command and standard parameters in IPA. Scaffolding was carried out by Phase Genomics using their standardised proprietary workflow, of which a detailed description provided by Phase Genomics can be found in the supplementary material. The annotation workflow has been described in a previous publication already, but an in-depth description can also be found in the Material and methods section, including parameters used for specific steps. The RNA-seq mapping and analysis part has also been described in the Material and Methods section, including parameters and models for DESEq2.

> - *Quantifications of T3 and T4 levels look fairly low and not so convincing. The work would clearly benefit from a discussion about why the signal is so low and what are the current technological limitations of these quantifications. This would really help (general) readers.*

We will add a comment on this in the manuscript as suggested. Basically, the T3/T4 levels are consistent with other published work in fish. In the present manuscript for grouper we have a peak level of 1.2 ng/g (1,200 pg/g) of T4 and 0.06 ng/g (60 pg/g) of T3. This is a higher level of T4 and comparable level of T3 to what was found in convict tang (Holzer et al. 2017; Figure 2) with 30 pg/g of T4 and 100 pg/g of T3. Of course, there are also examples with higher levels, such as clownfish (Roux et al. 2023; Figure 1), with 10 ng/g (10,000 pg/g) of T4 and 2 ng/g (2,000 pg/g) of T3.

The differences could be due to different structure of fish tissues and therefore different hormone extraction efficiency, different hormone measurement protocols, different fish physiology, different fish size (e.g., the weighting of tiny grouper larvae is difficult and less precise than in convict tang). What is important is not the absolute level but the relative level, which shows the change within different larval stages of a species with identical extraction

and measurement protocols. Which means our data is internally consistent and coherent with what the grouper literature says.

Holzer, Guillaume, et al. "Fish larval recruitment to reefs is a thyroid hormone-mediated metamorphosis sensitive to the pesticide chlorpyrifos." Elife 6 (2017): e27595.

Roux, Natacha, et al. "The multi-level regulation of clownfish metamorphosis by thyroid hormones." Cell Reports 42.7 (2023).

> - *Differential analysis highlights up to ~ 15,000 differentially expressed genes (DEG), out of a predicted 26k genes. This corresponds to more than half of all genes. ANOVA-based differential analysis relies on the simple fact that only a minority of genes are DEG. Having >50% DEG is well beyond the validity of the method. This should be addressed, or at least discussed.*

As the reviewer notes, there are a large number of differentially expressed genes due to the fact that this is coming from a larval developmental transcriptome going from one day old larva to fully metamorphosed juveniles at around day 60.

While DESeq2 indeed works on an assumption that most genes are not differentially expressed, this affects normalization but not hypothesis testing (Wald-test, LRT tests or ANOVA). Normalisation in DESeq2 is fairly robust to this assumption. According to the author of DESeq2, Micheal Love, DESeq2 is using the median ratio for normalisation, and as long as the number of up and down regulated genes is relatively even, DESeq2 will be able to handle the data. As part of our general quality control for this project we consulted the MA plots, which do not show any overrepresented up or down expression patterns. Additionally see Michael Love comment on comparing different tissues, which is also applicable here when comparing vastly different larval stages (https://support.bioconductor.org/p/63630/): “For experiments where all genes increase in expression across conditions, the median ratio method will not be able to capture this difference, but this is typically not the case for a tissue comparison, as there are many "housekeeping" genes with relatively similar expression pattern across tissues.”

> ***Reviewer #3 (Public Review):***
>
> *Weaknesses:*
>
> *However, the authors make substantial considerations that are not proven by experimental or functional data. In fact, this is a descriptive study that does not provide any functional evidence to support the claims made.*

We agree with the reviewer that our paper lacks functional experiments but despite that, the transcriptomic data clearly show the activation of TH and corticoid pathways during two distinct periods; an early activation between D1 and D10, and a second one between D32 and juvenile stage. These data are interesting as they call for further examination of 1) the possible interaction of corticoids and TH during metamorphosis, a question that is certainly not settled yet in teleost fishes, and 2) the existence of an early larval developmental step also involving TH and corticosteroids.

Especially 2) is of interest and importance, since this early activation (unique to our knowledge in any teleost fish studied so far) raises a lot of new questions and once again will certainly be scrutinised by other groups in the years to come, therefore ensuring a good citation impact of our study. We hope that the reviewer, while disagreeing with some our statements, will recognize that our study will be stimulating at that level and that this is what scientific studies should do.

> *The consideration that cortisol is involved in metamorphosis in teleosts has never been shown, and the only example cited by the authors (REF 20) clearly states that cortisol alone does not induce flatfish metamorphosis. In that work, the authors clearly state that in vivo cortisol treatment had no synergistic effect with TH in inducing metamorphosis. Moreover, in Senegalensis, the sole pre-otic CRH neuron number decreases during metamorphosis, further arguing that, at least in flatfish, cortisol is not involved in flatfish metamorphosis (PMID: 25575457).*

We will do our best to improve the clarity of the revised manuscript to avoid any misunderstanding about our claims. However, we would like to point out the semantic shift in the reviewer first sentence: Indeed “being involved” is not the same as “cortisol alone does not induce”. In ref 20 the authors explicitly wrote that “Cortisol further enhanced the effects of both T4 and T3, but was ineffective in the absence of thyroid hormones” and in our view this indeed corresponds to ”being involved in metamorphosis”.

We are not claiming that cortisol alone is involved in metamorphosis as the reviewer suggests, but simply that there is a possible involvement of cortisol together with TH in metamorphosis. We stand on this claim as we indeed observed an activation of corticoid pathway genes around D32, which is sufficient to say it is involved. We do agree that functional experiments will be needed to properly demonstrate the involvement of corticoids in grouper metamorphosis, but this was not possible in the current study as it would imply to set up a full grouper life cycle in lab conditions which is impossible for the scope of this manuscript.

We also mentioned in the discussion that the role of corticoids in fish larval development is still debated, and we agree that this remain a contentious issue.

We wrote that “there is contrasting evidence of communication between these two pathways [TH and corticosteroids] in teleost fish with some data suggesting a synergic and other an antagonistic relationship. In terms of synergy, an increase in cortisol level concomitantly with an increase in TH levels has been observed in flatfish (ref 19), golden sea bream (ref 100) and silver sea bream (ref 101). Cortisol was also shown to enhance in vitro the action of TH on fin ray resorption (phenomenon occurring during flatfish metamorphosis) in flounder (ref 20). TH exposure increases MR and GR genes expression in zebrafish embryo (ref 55). It has also been shown that cortisol regulates local T3 bioavailability in the juvenile sole via regulation of deiodinase 2 in an organ-specific manner (ref 56) On the antagonistic side, it has been shown that experimentally induced hyperthyroidism in common carp, decreasing cortisol levels (ref 57), whereas cortisol exposure decreases TH levels in European eel (ref 58). Given this scattered evidence, the existence of a crosstalk active during teleost metamorphosis has never been formally demonstrated. The results we obtained in grouper are clearly indicating that HPI axis and cortisol synthesis are activated (i) during early development and (ii) during metamorphosis. This may suggest that in some aspect cortisol synthesis can work in concert with TH, as has been shown in several different contexts in amphibians (ref 17).” In the revised manuscript, we will also add the interesting case of the Senegal sole mentioned by the reviewer.

In the last revision, we had also added that our results “brought a first insight into the potential role of corticoids in the metamorphosis of E. malabaricus and call for functional experiments directly testing a possible synergy” meaning that we clearly acknowledge that we are only revealing a hypothesis that remains to be tested. We later follow up with a discussion about the most novel observation and focus of our study, the increase in THs and cortisol during early development, which was unexpected and very intriguing. Again, these results suggest that there might be a link between the two, as has been shown in amphibians. This is typically the kind of results that should encourage more investigations into other fish

species. Indeed, this has been pointed out by other authors and in particular by Bob Denver (probably the foremost expert on this topic) in Crespi and Denver 2012: “Elevation in HPA/I axis activity has been described prior to Metamorphosis in amphibians and fish, birth in mammals (reviewed in Crespi & Denver 2005a; Wada 2008)”. B. Denver also adds that: “Experiments in which GCs were elevated prior to metamorphosis or prior to hatching or birth (e.g. Weiss, Johnston & Moore 2007) or inhibited by treatments with GC synthesis blockers (e.g. metyrapone) or receptor antagonists (e.g. RU486, Glennemeir & Denver 2002) demonstrate that GCs play a causal role in precipitating these life-history transitions (also reviewed in Crespi & Denver 2005a; Wada 2008).” We believe the reviewer will be convinced by these elements coming from a colleague unanimously respected in the field.

> *Furthermore, the authors need to recognise that the transcriptomic analysis is whole-body and that HPA axis genes are upregulated, which does not mean they are involved in regulating the HPT axis. The authors do not show that in thyrotrophs, any CRH receptor is expressed or in any other HPT axis-relevant cells and that changes in these genes correlate with changes in TSH expression. An in-situ hybridisation experiment showing co-expression on thyrotrophs of HPA genes and TSH could be a good start. However, the best scenario would be conducting cortisol treatment experiments to see if this hormone affects grouper metamorphosis.*

We agree that functional experiments are needed to validate our hypothesis. As the early peaks of expression levels observed for many genes were very intriguing for us, we did carry out thyroid hormones and goitrogenic treatment on young grouper larvae to test their effect on the morphological changes. Unfortunately, such experiments, already tricky on metamorphosing larvae, are even more risky on such tiny individuals just after hatching and we encountered high mortality rates. We must add that because we cannot establish a full grouper life cycle under lab conditions, we have done these experiment in the context of a commercial husbandry system in Japan, which while excellent limits the scope of possible experiments. We were thus not able to provide functional validation of our hypothesis. Such experiments will be a full project in itself, requiring setting up a rearing system suitable for both larval survival and economical constraints related to drug treatments. We were further limited by the spawning times of the grouper in the operational aquaculture farm, which are limited to a short time during each year. So even if we strongly agree with the necessity of conducting such experiments, we think that this is not in the scope of the present paper, but something future research can explore.

> *High TSH and Tg levels usually parallel whole-body TH levels during teleost metamorphosis. However, in this study, high Tg expression levels are only achieved at the juvenile stage, whereas high TSH is achieved at D32, and at the juvenile stage, they are already at their lowest levels.*

This is exactly our point. We observe two peaks in TSH expression, one at D3 and one at D32. The peak at D3 coincides with high thyroid hormone levels on the same day, and while we have not measured TH at D32, existing literature shows that there is a peak in TH during that time (e.g., de Jesus et al., 1998). Similarly, there is a small peak of Tg at D3. Our manuscript focused more on the upregulation of these genes at D3, which has not been reported before in the literature and raised the question of the role of TH so early in the larval development, outside of the metamorphosis period.

Regarding the respective levels of TSH and Tg, we first would like to add that their respective order of appearance before metamorphosis (TSH at D32, Tg after) is consistent with what we would expect. We agree however that the strong increase of Tg and TPO expression is later than expected. We will make this clear in the revised manuscript.

> *It is very difficult to conclude anything with the TH and cortisol levels measurements. The authors only measured up until D10, whereas they argue that metamorphosis occurs at D32. In this way, these measurements could be more helpful if they focus on the correct developmental time. The data is irrelevant to their hypothesis.*

We respectfully disagree with the reviewer, considering that 1) TH levels have already been investigated in groupers coinciding with pigmentation changes and fin rays resorption, 2) that there is also evidence in numerous fish species that TH level increase is concomitant with increase of TH related genes, and 3) that we observed in our data an increase in the expression of TH related genes as well as pigmentation changes and fin rays resorption. Based on our experience in fish metamorphosis and the literature we can say confidently that those observations indicate that metamorphosis is occurring between D32 and the juvenile stage. To reinforce our point, we plan to add a figure to the revised manuscript, which puts our data in the context of earlier studies done in grouper. This will clearly show that our inference is correct. Additionally, we would like to point out that from our experience in several fish species transcriptomic data are more robust and precise than hormone measurements.

However, as we were surprised by the activation of TH and corticoid pathway genes very early in the larval development (at D3), which is clearly outside of the metamorphosis period, we decided to measure TH and cortisol levels during this period of time to determine if whether or not there this surprising early activation was indeed corresponding to an increase in both TH and cortisol. As such observation has never been made in other teleost species (to our knowledge), and as we were wondering if gene activation was accompanied by hormonal increase, the measurements we did for TH and cortisol between D1 and D10 are relevant. We will make sure to improve the clarity of the revised version of the manuscript to avoid any confusion between the two periods we are studying: early larval development (between D1 and D10) and metamorphosis (between D32 and juvenile stage).

> *Moreover, as stated in the previous review, a classical sign of teleost metamorphosis is the upregulation of TSHb and Tg, which does not occur at D32 therefore, it is very hard for me to accept that this is the metamorphic stage. With the lack of TH measurements, I cannot agree with the authors. I think this has to be toned down and made clear in the manuscript that D32 might be a putative metamorphic climax but that several aspects of biology work against it. Moreover, in D10, the authors show the highest cortisol level and lowest T4 and T3 levels. These observations are irreconcilable, with cortisol enhancing or participating in TH-driven metamorphosis.*

We thank the reviewer for this comment, but we think that there might be a misunderstanding here.

(1) We clearly observed an increase of TSHb (that occurs between D18 and juvenile stage) and an increase of tg from D32 which coincide with the activation of other genes involved in TH pathway (dio2, dio3, and also a strong increase of TRb). All this and put in the context of what we know from previous grouper studies, clearly supports our conclusion that TH-regulated metamorphosis is starting at around D32 in grouper. We also observed morphological changes such as fin rays resorption and pigmentation changes between D32 and juvenile stage. Such morphological changes have already been associated as corresponding to metamorphosis in groupers (De Jesus et al 1998) as they occur during TH level increase, and they also happen to be under the control of TH in grouper (De Jesus et al 1998). Based on this study but also on studies (conducted on many other teleost species) showing that the increase of TH levels is always associated with an activation of TH pathway genes and morphological and pigmentation changes we concluded that metamorphosis of E. malabaricus occurs between D32 and juvenile stage. We will improve the clarity of the manuscript to make sure

that our conclusion is based on our transcriptomic and morphological data plus the available literature.

(2) We clearly observed another activation of TH related gene earlier in the development (between D1 and D10, with a surge of trhrs, tg and tpo at D3. As this activation was very unexpected for us, we decided to focus the analysis of TH levels between D1 and D10 and very interestingly we observed high level of T4 at D3 indicating that THs are instrumental very precociously in the larval development of the malabar grouper which has never been shown before. We declared line 195 that our "data reinforce the existence of two distinct periods of TH signalling activity, one early on at D3 and one late corresponding to classic metamorphosis at D32". However, we agree that we could have been clearer and clearly explained that this early activation was very intriguing for us and that we wanted to investigate hormonal levels around that period. However, we never claimed anywhere in the manuscript that this early developmental period corresponds to metamorphosis. Something else is occurring and both TH and cortisol seem to be involved but further experiments need to be conducted to understand their role and their possible interaction.

(3) Finally, regarding the comment about cortisol enhancing or participating in TH driven metamorphosis, our data clearly showed an activation of the corticoid pathway genes around metamorphosis (between D32 and juvenile stage) suggesting a potential implication of corticoids in metamorphosis, but we agree with the reviewer that further experiment are needed to test that. We never claimed that cortisol was enhancing or participating in metamorphosis, on the contrary we are "suggesting a possible interaction between TH and corticoid pathway during metamorphosis". And we also say that our "results brought a first insight into the potential role of corticoids in the metamorphosis of E. malabaricus and call for functional experiments directly testing a possible synergy." Nonetheless, we agree that some parts of our manuscript can be confusing in regards of cortisol synthesis during metamorphosis as we did not measure cortisol levels between D32 and juvenile stage. We will correct this in the revised version.

> *Given this, the authors should quantify whole-body TH levels throughout the entire developmental window considered to determine where the peak is observed and how it correlates with the other hormonal genes/systems in the analysis.*

We did not measure TH levels at later stages as it has already been measured during Epinephelus coioides metamorphosis and the morphological changes observed in this species around the TH peak corresponds to what we observed in Epinephelus malabaricus around the peak of expression of TH pathway genes (see De Jesus et al., 1998 General and Comparative Endocrinology, 112:10-16). We are planning to add a figure reconciling all these data together. However, the main focus of this manuscript is the novel observation of the existence of an early activation period observed at D3, and for which we needed TH levels to determine if they were involved in another early developmental process (not related to metamorphosis). Our hypothesis is that this early activation might be related to the growth of fin rays necessary to enhance floatability during the oceanic larval dispersal. As we may have arrived at the explanation of this hypothesis too rapidly without setting up the context well enough, we will pay attention to improve that part too.

> *Even though this is a solid technical paper and the data obtained is excellent, the conclusions drawn by the authors are not supported by their data, and at least hormonal levels should be present in parallel to the transcriptomic data. Furthermore, toning down some affirmations or even considering the different hypotheses available that are different from the ones suggested would be very positive.*

We thank the reviewer for acknowledging the solidity of the method of our paper and the quality of the results. We agree that there were several parts where our message is unclear,

which we will address in the revised version of the manuscript to make sure there is no more confusion between the two distinct periods we studied in this paper (early larval development and metamorphosis). We will also make sure that our claims about TH/corticoids interaction during both periods remain hypothetical as we cannot yet, despite trials, sustain them with functional experiment.