The multi-level regulation of clownfish metamorphosis by thyroid hormones

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SUMMARY

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AUTHOR CONTRIBUTIONS

N.R. and V.L. wrote the manuscript with contributions from Y.G., K.G., D.L., and L.B. N.R., V.L., D.L., and L.B. designed the whole study with the help of Y.G. and K.G. for the metabolic aspects. Transcriptome assembly was performed by S.d.B., and analyses were performed by S.d.B. and N.R. N.R. and S.-h.L. performed all the pharmacological treatments with the help of S.M., M.D., Y.T., and M.R. Y.G. and K.G. brought their expertise on metabolic regulation and lipid metabolism. P.S. generated the transcriptomics dataset of *Amphiprion percula* recruits sampled in Kimbe, Papua New Guinea. A. Barua and N.R. analyzed the *A. percula* transcriptome. A. Boulahouf and P.B. performed the LXR antagonist activity experiment on zebrafish.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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Most marine organisms have a biphasic life cycle during which pelagic larvae transform into radically different juveniles. In vertebrates, the role of thyroid hormones (THs) in triggering this transition is well known, but how the morphological and physiological changes are integrated in a coherent way with the ecological transition remains poorly explored. To gain insight into this question, we performed an integrated analysis of metamorphosis of a marine teleost, the false clownfish (*Amphiprion ocellaris*). We show how THs coordinate a change in color vision as well as a major metabolic shift in energy production, highlighting how it orchestrates this transformation. By manipulating the activity of liver X regulator (LXR), a major regulator of metabolism, we also identify a tight link between metabolic changes and metamorphosis progression. Strikingly, we observed that these regulations are at play in the wild, explaining how hormones coordinate energy needs with available resources during the life cycle.

**In brief**

Roux et al. find that thyroid hormones coordinate multiple changes in anemonefish larvae, particularly color vision and metabolism. They also identify a link between metabolism and metamorphosis progression. Their research highlights the critical role of thyroid hormones in post-embryonic development of marine fish in captivity and in the wild.

**Graphical Abstract**

![Graphical Abstract](image-url)
INTRODUCTION

In vertebrates, metamorphosis is a common post-embryonic transition regulated by thyroid hormones (THs) during which a larva transforms into a juvenile. The role of THs as main triggers and coordinators of biological processes occurring during metamorphosis has been extensively studied, mostly in anurans and flatfish.\textsuperscript{1,2} These organisms exhibit a spectacular metamorphosis during which major morphological, physiological, and ecological transitions occur.\textsuperscript{3,4} THs not only trigger but also coordinate transformation of the larva into a juvenile at the tissue and cellular level. For example, in anuran tadpoles, cell-specific actions of THs are instrumental for transformation of several organs, illustrating the pleiotropic actions of these hormones.\textsuperscript{5} These actions are mostly mediated by the active TH T3 (3,3′,5-triiodothyrodine), whose precursor is T4 (thyroxine), through binding to specific transcription factors: the TH receptors (TRs).\textsuperscript{6} However, these flatfish and anuran models are more the exception than the rule because, in most vertebrate species, morphological changes are more subtle.\textsuperscript{3,7,8} All chordates go through such a post-embryonic transformative period even if it is not morphologically spectacular.\textsuperscript{7,9} In addition, several reports suggest that successful completion of metamorphosis is decisive for the quality and, therefore, the ecological success of the juvenile emanating from it.\textsuperscript{10} Therefore, understanding how THs orchestrate this transformation is critical to better understanding the pleiotropic action of these hormones and how they allow juveniles to adjust to their new environment.\textsuperscript{11}

A common challenge for all vertebrates is to align the transformation with the organism metabolic status and its environmental conditions.\textsuperscript{12,13} THs, with their known metabolic effects, are critical for this action.\textsuperscript{14,15} This has been shown in several species, like sticklebacks, whose freshwater populations, living in an energy-poor environment, control their metabolic rate.\textsuperscript{16} Similarly, studies of teleost fish have shown that THs impact the activity of lipid metabolism enzymes, therefore influencing energy availability.\textsuperscript{17} However, TH metabolic actions are mostly studied in juveniles or adults, and the mechanisms involved in metamorphosis remain unknown.\textsuperscript{17} Shedding light on how THs control metabolism during metamorphosis would be particularly desirable because metabolic pathways are heavily modified during larval transformation.\textsuperscript{18} Therefore, it remains to be determined (1) how THs regulate metabolism during metamorphosis and (2) how this action is integrated with the TH-regulated transformation of larvae into juveniles. To fill these gaps, using a marine fish model, we carried out an integrative study of metamorphosis to better understand the central role of THs in coordination with metabolic status. Understanding these processes is critical to better understand the ecological function of THs but also the constraints and biases affecting metamorphosis, a key process ensuring population replenishment. This is particularly important in the context of the global changes affecting natural populations worldwide.\textsuperscript{11}

We used the false clownfish, \textit{Amphiprion ocellaris}, as a model system to explore the hormonal basis of this metabolic integration. These fish live on coral reefs in symbiosis with sea anemones, in which they form colonies with one reproductive pair and a variable number of juveniles.\textsuperscript{19,20} Every 2–3 weeks, approximately 500 eggs are laid on a substrate near the host. Newly hatched larvae disperse in the ocean for 10–15 days. At the end of this pelagic phase, larvae metamorphose into juveniles, called recruits, which must locate...
a reef and actively look for a sea anemone using their sensory abilities, combining visual, chemical, and acoustic cues.\textsuperscript{21} During metamorphosis, larvae lose their larval characteristics (light pigmentation, elongated body shape) and transform into miniature adults with an ovoid body shape and a pigmentation pattern displaying white bars on a bright orange background.\textsuperscript{22} These pigmentation changes have been shown to be TH regulated, indicating that this metamorphosis is controlled by THs.\textsuperscript{23,24}

Here, we conducted an extensive study of clownfish metamorphosis by combining transcriptomics, TH levels measurements, behavioral observations, lipid analysis, \textit{in situ} observations, and functional experiments. By focusing on visual perception and metabolic changes, we uncover how THs control gene-regulatory programs as well as behavioral and physiological responses, highlighting their central role in regulating the whole metamorphosis. Our results demonstrated that THs link metabolic regulation, morphological transformation, and behavioral changes, ensuring full ecological transformation of the pelagic larvae into benthic reef-associated juveniles.

\section*{RESULTS}

\textbf{Three distinct post-embryonic phases}

The larval development period of \textit{A. ocellaris} has been divided into seven stages (S1–S7; Figure 1A).\textsuperscript{22} To obtain a global perspective on gene expression levels during post-embryonic development, we performed a transcriptomics analysis of these seven stages.

Principal-component analysis (PCA) and global hierarchical clustering were first applied to the 1,000 genes with the highest variance (Figures 1A and S1A). Both methods allowed distinguishing three groups: (1) early developmental stages, including S1, S2, and S3; (2) late developmental stages, including S5, S6, and S7; and (3) the pivotal S4. Of note, one S4 individual, 2S4, tends toward the late-stage cluster, indicating that there may be a gap between transcriptional regulation and the observed morphological changes (Figures 1A and S1A).

The same three groups can also be observed on the heatmap (Figure 1B). Interestingly, when we considered the number of differentially regulated genes between each consecutive stage, we observed that S4 coincides with a major transition of the regulatory program, with 177 genes significantly differentially expressed between S3 and S4 (Figure 1D; Table S1).

The two observed periods exhibit distinct morphological transformation. On a PCA performed with morphological measurements (body depth [BD]/standard length [SL], head length [HL]/SL, and snout vent length [SVL]/SL), we observed alignment of the first three stages along the main axis of variation (PC1), whereas after S4, there is an inflexion, and the metamorphic stages are varying along PC2 (Figure 1C). These changes can be explained by a change in body shape, with metamorphic stages becoming more ovoid (increase in BD). During the last stages, major changes in body pigmentation are observed.\textsuperscript{23} We conclude that S5–S7 correspond to the metamorphosis transforming the young larvae into miniature adults, whereas S4 is a pivotal stage assuring transition between the larval period and metamorphosis.
Interestingly, TH level measurements showed an increase in T4 from S4 with a peak at S5, followed by a slow decrease until S7 (Figure 1E). T3 levels also increase from S4 up to S7. This surge of T4 levels at S4 precedes appearance of the white bars at S5. Overall, these results reveal that *A. ocellaris* post-embryonic development is characterized by three distinct phases: (1) larval development, (2) the pivotal S4 marking metamorphosis onset with TH level increase, and (3) metamorphosis.

**The TH pathway is activated during metamorphosis**

To investigate THs’ role, we analyzed the expression levels of TH signaling genes. We selected key genes from the hypothalamic-pituitary-thyroid (HPT) axis and genes encoding TRs and followed their expression levels using transcriptomics analysis (Figure S2). We observed that these genes are effectively regulated during metamorphosis. Interestingly, a global PCA analysis of TH signaling gene expression levels separated the first three stages from S4 and S5–S7, indicating a clear activation of this pathway at S4 (Figure S1C). Taken together, TH signaling gene expression levels are in accordance with the increase in T4 that starts at S4 and surge at S5, confirming that clownfish metamorphosis starts at this stage.

These results suggest that several of these TH signaling genes may be regulated by THs, as observed in other species. We therefore investigated the effects of exogenous THs by treating S3 larvae for 2 days with T3 (10^{-7} M) and performed RNA sequencing (RNA-seq) analysis. A total of 66% of the 1,000 genes used for the PCA of the developmental stages were significantly regulated by T3, confirming that THs are the drivers of most of the changes observed during metamorphosis. We observed downregulation of expression of *tg* (thyroglobulin); *tpo* (thyroperoxidase), and *oatpc1c1* (organic anion-transporting polypeptide 1C1) as well as upregulation of *sis* (sodium/iodide cotransporter); *dio1*, *dio3a*, and *dio3b* (deiodinases); *trab*, and *trβ* (TRs) (Figure S3A). This is very similar to what has been observed in other species. We also inhibited TH synthesis by treating larvae for 19 days with a mix of goitrogens, called MPI (methimazole, potassium perchlorate, iopanoic acid), and proceeded to RNA-seq analysis. This timing allowed observation of a delay in metamorphosis compared with the DMSO control (Figure S3B). MPI effectively decreased TH production because lower levels of T3 and T4 were observed after treatment (Figure S3D). Interestingly, MPI treatment demonstrated contrasting regulation compared with T3 treatment. The expression levels of *tg* and *tpo* are upregulated, while *duox* and the three TRs are downregulated. Such results are in accordance with the delay in white bar formation observed in MPI-treated larvae (Figure S3B). Similar results were obtained in other fish species whose TH production, TRs, and deiodinase expression levels were altered, whether by methimazole or thiourea. It is also worth noting that fish may have the ability to compensate for the effects of goitrogenic compounds, which is why metamorphosis in most fish species (e.g., flatfish, fathead minnow) cannot be blocked but is only delayed, as observed in *A. ocellaris*. In summary, our analysis clearly demonstrates that TH signaling is activated during clownfish metamorphosis and that THs are the main driver of the complex transformation of a pelagic larva to a reef-associated juvenile.
**THs control molecular and behavioral shifts in vision**

During their transformation, clownfish larvae must switch from an oceanic environment to a colorful reef environment. It is well known that in many fish species this ecological transition is accompanied by a change in color vision.\(^{31}\) Because THs appeared to be critical for clownfish metamorphosis, we investigated the visual opsin genes.

Eight visual opsin genes (\textit{opnsw1-α}, \textit{opnsw1-β}, \textit{opnsw2B}, \textit{rh1}, \textit{Rh2A-1}, \textit{rh2A-2}, \textit{rh2B}, and \textit{opnlw}) have been identified in \textit{A. ocellaris}.\(^{32}\) We observed a reciprocal shift in their expression (Figure 2A); short-wavelength opsins (\textit{opnsw1-α}, \textit{opnsw1-β}, and \textit{opnsw2B}) and mid-wavelength opsins (\textit{rh2A} and \textit{rh2B}) are highly expressed at the beginning of larval development and downregulated after S4. Of note, we observed larval expression of both paralogs of \textit{opnsw1}, including \textit{opnsw1-α}, for which no clear expression has yet been detected.\(^{32}\) In contrast, the long-wavelength opsin (\textit{opnlw}) is poorly expressed in larval stages, and its expression strongly increases from stage 4 onward and remains high throughout metamorphosis (Figure 2A).

This reciprocal shift suggested that there could be a shift in visual perception from blue/green to yellow/red during metamorphosis. We therefore tested the visual preferences of \textit{A. ocellaris} before and after metamorphosis using a dual-choice chamber (Figure 2B). S2 larvae spent significantly more time in the blue compartment (50\% vs. 23\% in the orange compartment, \(p = 0.004\)), whereas S5 larvae prefer the orange one (41\% vs. 17\% in the blue compartment, \(p = 0.009\)). This indicates that opsin gene expression correlates with visual preference. This shift is also visible by \textit{in situ} hybridization. The \textit{opnsw2B} gene is strongly expressed in photoreceptors at S2 and slightly visible at S4 and S6 on the retina perimeter, except on the ventral side, whereas the \textit{opnlw} gene is not expressed at S2 and is detected in the photoreceptors at S6 (Figure 2C).

We next investigated whether THs could induce a shift in visual preferences. We treated S3 larvae for 72 h with either T3, MPI, or T3+MPI and assessed the color preference of larvae in a dual chamber, as described above. We observed that control larvae spent significantly more time in the blue compartment than T3-treated larvae (53\% vs. 1\%,
respectively; \( p < 0.05 \); Figure 2E), which can be correlated with the higher expression of \textit{opnsw2B} observed in control larvae (Figure 2D). Interestingly, MPI-treated larvae spent significantly more time in the blue compartment compared with T3-treated larvae (58\% vs. 1\%, respectively; \( p < 0.05 \)), and larval behavior was rescued when larvae were treated with T3+MPI and spent significantly less time in the blue compartment compared with DMSO (7\% vs. 53\%, respectively; \( p < 0.05 \)). Surprisingly, T3- and T3+MPI-treated larvae did not spend more time in the orange compartment compared with DMSO-treated larvae (11\% and 19\% vs. 16\%, respectively; \( p > 0.05 \)). Instead, most of the time they remained in the central compartment (88\% and 74\% vs. 31\% for the DMSO controls, \( p < 0.05 \)). Observation of T3− and T3+MPI-treated larvae after 72 h revealed that they effectively remained close to the bottom, barely swimming, whereas control larvae were actively exploring the blue compartment. The same behavior was observed when, 30 days post hatching, juveniles (15 days older than S7) were tested (Figure S4B). This indicates that T3 accelerates the appearance of juvenile-type behavior in accordance with the benthic lifestyle. Taken together, these results clearly demonstrate that THs control a shift in opsin gene expression that coincides with a color preference change occurring during metamorphosis. Even if the shift of opsin gene expression cannot solely explain the visual preference, our results indicate that THs coordinate a molecular, behavioral, and ecological transition essential for survival in the wild.

**A TH-regulated metabolic transition during metamorphosis**

Because THs are known to regulate metabolism in mammals, we investigated metabolic gene expression.\(^3^3\) Figure 3A shows the expression profile of the main genes involved in these pathways. And highlights in red the rate-limiting steps for glycolysis (phosphofructokinase, [\textit{pfkma} and \textit{pfkmb}]), the tricarboxylic acid (TCA) cycle (citrate synthase [\textit{cs}], isocitrate dehydrogenase [\textit{idh3A}], and the oxoglutarate dehydrogenase complex [\textit{ogdhl} and \textit{dlst2}]) and fatty acid \( \beta \)-oxidation (carnitine palmitoyltransferase [\textit{cpt1aa, cpt1b}, and \textit{cpt2}]).\(^3^4\) The expression profiles of all genes implicated in these pathways are shown in Figures S5 and S6.

These profiles revealed a clear overall pattern: glycolysis genes are highly expressed in larval stages (S1–S3), while their expression decreases during metamorphosis. This is particularly visible for rate-limiting enzymes (\textit{pfkma} and \textit{pfkmb}), which surge at S3 and strongly decrease up to S7. In sharp contrast, the expression of fatty acid \( \beta \)-oxidation genes showed an inverse profile with low expression at the larval stage and a sharp increase starting at S3 or S4. This is especially the case for \textit{acads}, which encodes acylcoenzyme A (CoA) dehydrogenase, an enzyme involved in the second step of \( \beta \)-oxidation. This trend is again encountered on the rate-limiting step (\textit{cpt1aa, cpt1b}, and \textit{cpt2}). Overall, the TCA cycle genes are expressed at lower levels during larval stages, and their expression tends to increase early during metamorphosis. This trend is more subtle and earlier than the one observed in glycolysis and fatty acid \( \beta \)-oxidation but is again observed in the three rate-limiting steps (\textit{cs, idh3A, ogdhl}, and \textit{dlst2}). The lactic acid fermentation genes (lactate dehydrogenase [\textit{ldha, ldhba, ldhbb}, and \textit{ldhd}]) show less homogeneous regulation, with \textit{ldha}
decreasing slowly, \textit{ldhba} showing a strong decrease, and finally \textit{ldhbb} increasing during metamorphosis.

Taken together, these results reveal that larvae mainly rely on glycolysis and lactic acid fermentation (anaerobic energy production), whereas during metamorphosis, juveniles rely more on fatty acids fueling \(\beta\)-oxidation and use the TCA cycle for aerobic energy production. Of note, this trend is the inverse to what is observed in other fish species, such as seabass.\textsuperscript{18,35} This difference may be due to contrasting life history traits, seabass being neritic fish and clownfish sedentary fish living inside sea anemones.

We then assessed whether THs were instrumental in controlling this transition in energy production by investigating their effects on metabolic gene expression levels. We scrutinized the effects of T3 (10\(^{-7}\)M, 2 days of treatment) and MPI (19 days of treatment) on genes involved in the four pathways mentioned above (Figure 3B). We observed that T3 down-regulated expression of glycolysis and lactic fermentation genes, whereas MPI upregulated them (Figure 3B). We observed, for the majority of the genes involved in lactic fermentation and the citric acid cycle, that T3 exerts regulation according to the expression pattern obtained during development. For example, \textit{aco2b}, \textit{ogdhl}, \textit{cpt1aa}, \textit{cpt2}, and \textit{acads}, which increase during metamorphosis, are upregulated by T3 (and downregulated by MPI). On the contrary \textit{cs}, \textit{dlstb}, \textit{suhb2}, \textit{sdha}, \textit{mdh2}, \textit{fh}, and \textit{cpt1b}, which decrease during metamorphosis, are downregulated by T3 (and upregulated by MPI). This clearly demonstrates that THs control a switch from glucose-based anaerobic toward a fatty acid-based aerobic energy production during metamorphosis.

**THs favor complexification of lipids during metamorphosis**

The previous data revealed a transition in energy source, with fatty acid \(\beta\)-oxidation being the main energy pathway during metamorphosis. In line with this observation, we noticed that the genes implicated in lipid biosynthesis and fat storage are not activated and sometimes even repressed during metamorphosis.

Lipid biosynthesis requires transport of citrate from the mitochondria to the cytosol by the tricarboxylate carrier protein SLC25A1 and then formation of acetyl-CoA by the ATP citrate lyase (\textit{acly}) (Figure 4A). This acetyl-CoA is then used by acetyl-CoA carboxylase (\textit{acaca} and \textit{acacb}), which form malonyl-CoA, the main starting substrate for fatty acid biosynthesis by fatty acid synthase (\textit{fasn}). In our transcriptomics data, we observed that \textit{slc25a1} expression decreased steadily from S1 until S6 with a final slight increase in S7 (Figure 4A). Other genes in the pathway were either slightly increasing (\textit{aclya} and \textit{acacb}) or constant (\textit{aclyb}, \textit{acaca}, and \textit{fasn}). All of this suggests that \textit{de novo} fatty acid biosynthesis is minimal during metamorphosis. In accordance with this notion, TH treatment decreased \textit{me2}, \textit{me3}, \textit{slc25a1}, \textit{acacb}, \textit{fasn}, and \textit{gpat2} expression, whereas MPI increased their expression (except \textit{acacb}), suggesting that TH does not favor \textit{de novo} fatty acid biosynthesis in this context (Figure 4C). These data also suggest that dietary fatty acids serve as the primary fuel source for \(\beta\)-oxidation, a situation already observed in other marine fish species because fatty acids are abundant in marine algae and zooplankton.\textsuperscript{36}
In contrast to these de novo fatty acid biosynthesis steps, we observed that the genes implicated in fatty acid desaturation and elongation are mostly upregulated during metamorphosis. This is important because long-chain fatty acids (polyunsaturated fatty acids [PUFAs] and highly unsaturated fatty acids [HUFAs]) participate in many biological processes and are precursors of key signaling molecules. PUFAs and HUFAs are generated by the action of front-end desaturases (FADS; SCD) and elongase (ELOVL). In contrast to mammals, clownfish, like most marine fish, only have one FADS encoded by the *fads2* gene. This gene is upregulated during metamorphosis (Figure 4A). The other desaturases (*scda* and *scdb*) are also activated with similar dynamics. The many elongase genes, including *elovl5* acting on C₁₈ and C₂₀ PUFAs, *elovl4* also acting on PUFAs, and *elovl6* acting on saturated fatty acids (FAs) and monounsaturated FAs (MUFAs), showed a wide variety of patterns suggesting an intricate level of regulation. *Elolv5* expression is upregulated, and so are the two *elovl6* genes, whereas *elovl4* expression decreases. We also observed that *chreb*, *srebp1*, *pparg*, and *lxr*, known transcription factors regulating *fads* and *elovl* genes in fish, are also upregulated during metamorphosis, suggesting that a major coordinated gene expression change related to lipid biochemistry is occurring (Figure 4A). These data reveal that fatty acids serve as an energy source during metamorphosis and are also metabolized to produce complex lipids, which act as precursors for signaling molecules. We therefore compared the lipid content between four post-embryonic stages: S3, S4, S5, and S6 (Figure 4B). In accordance with the increased expression of *fads2*, *elovl5*, and *elovl6*, we observed a statistically significant decrease in saturated lipids and a concomitant increase in MUFAs and PUFAs. In contrast to those two FA classes, HUFA content decreased steadily during metamorphosis. Figure S7A show the complex changes in individual lipid molecules we observed. Taken together, these results suggest that metamorphosis also coincides with a major change in FA biochemistry, and the complexification of the molecules that are present can be used as a cell membrane constituent, for energy storage and FA transport, and as a source of signaling molecules.

To determine whether these changes are under TH coordination, we analyzed the effects of T3 and MPI on the expression of genes involved in fatty acid desaturation and elongation. We observed that TH exposure effectively induces an increase in *fads2*, *elovl1a*, *elovl1b*, *elovl6l*, *elovl7a*, and *elovl8b* expression levels but also of *pparg*, *srebp*, and *lxr*, the major transcriptional regulators of desaturases and elongases. Most of these upregulations are in accordance with the developmental patterns described earlier, and opposite regulations are observed in MPI-treated larvae (except *elovl8b*) (Figure 4C). Surprisingly, TH repressed *elovl5* gene expression and MPI upregulated it, whereas it showed an increasing pattern during development (Figures 4A and 4C).

We also studied whether TH treatment (10⁻⁸ M, 10⁻⁷ M, 72 h post-treatment of S3 larvae) was effectively able to alter the amount of specific lipids. We observed a great variability from one fish to another, a situation probably linked to the fact that it is almost impossible to control the metabolic status and feeding time of such tiny aquatic organisms. This variability impacted the number of lipids for which we can get statistically significant differences between TH treatment and the control (DMSO). However, we observed that 37 lipids of...
the 1,323 studied show level differences after TH treatment (Figure S7), and we noticed a non-statistically significant trend for 197 others (data not shown). Overall, our data reveal a major implication of THs in lipid metabolism, with 144 lipids being increased after TH treatment and 137 being decreased.

Taken together, these data show that clownfish used free fatty acids (saturated and HUFAs) as a major energy source and dietary lipids as substrates to generate a myriad of molecules, many of which serve in FA transport (acyl carnitine) and energy storage (triacylglycerol) and as membrane constituents (ceramide, sphingomyelin, and phosphatidyl ethanolamine) or signaling molecules (phosphatidylglycerol sphingomyelin). As vision and energy metabolism, this transition in lipid biochemistry is also controlled by THs, as suggested by the T3/MPI experiment, which appear to be major conductors of the metamorphosis process.

Liver X regulator (LXR) modulation links metabolic transition and metamorphosis

Among the transcription factors regulating fatty acid biochemistry, LXR (also called NR1H3) is particularly interesting given its known pivotal role in lipid metabolism. This receptor regulates FA metabolism through, for example, control of srebp, elongase, and desaturase genes. Additionally, in the mouse, lxrβ controls TH signaling in the brain and adipose tissue, and its knockout results in upregulation of dio2, sis, and TH transport genes. We therefore tested whether inhibiting LXR action affected clownfish metamorphosis and its underlying gene-regulatory program.

We used SR9243, a selective LXR antagonist known to shut--down LXR activity. Importantly, we verified that this compound specifically inhibits zebrafish LXR activity and is inactive on other metabolic nuclear receptors such as PPARγ and PXR.

Larvae treated with SR9243 showed acceleration of metamorphosis compared with the control (Figure 5A). Indeed, using white bar presence as an indicator of metamorphosis progression, we observe a higher proportion of individuals with white bars on the trunk and head in T3- and SR9243-treated larvae compared with DMSO (Figure 5B: 36% for head and trunk bars in DMSO vs. 81% and 86% in SR9243 and 100% for head and trunk bars in T3, p < 0.05). The transcriptomics experiment reveals a clear separation between each treatment, as observed in the PCA of the 2,000 genes with highest variance (Figure 5C). Interestingly, the differential gene expression results showed similar regulation of SR9243 and T3 (Figure 5D) and are consistent with the observed metamorphosis acceleration because SR9243 is stimulating metamorphosis by upregulating expression of duox and trb and down-regulating expression of dio3a and dio3b to increase TH levels in target organs (Figure 5E). These results are consistent with what is observed in T3-treated larvae because duox and trb expression levels are also upregulated. However, the upregulation of dio3a and dio3b by T3 is solely due to the feedback mechanism operated by T3 to regulate its own level because DIO3 is involved in T3 degradation (Figure 5E). We also investigated the effects of SR9243 on genes involved in vision, glycolysis, the TCA cycle, and lipid metabolism and observed similar regulation as T3 on several of these genes. For example, SR9243 downregulated expression of opanw2b, pkma, pkmb, idh3a, and ldhba as T3 (Figure 5E). Most of the genes involved in phototransduction are significantly downregulated by both treatments.
compared with DMSO. It is well known that lipids are important for vision development in teleosts, but because T3 and SR9243 are acting on lipid metabolism regulation, one of the consequences might be downregulation of genes involved in vision (more specifically, in the phototransduction cascade; Figure 5D). Taken together, these results show that modulating lipid homeostasis by inhibiting LXR activity affects TH signaling and vision as well as glycolysis, lactic fermentation, the TCA cycle, and lipid metabolism, linking metabolic regulation with coordination of metamorphosis.

Natural TH regulation in an ecological context revealed metabolic and visual effects

We previously reported a natural situation where endogenous TH level regulation occurs. In Kimbe Bay (Papua New Guinea), Amphiprion percula can inhabit two different sea anemone hosts: the carpet sea anemone (Stichodactyla gigantea) and the magnificent sea anemone (Heteractis magnifica). In these two hosts, the new recruits exhibit differences in white bar formation that are linked to a higher TH level (Figure 6A) and higher duox expression in fish hosted by S. gigantea. Given the results described above, we measured the expression levels of genes implicated in vision and metabolic regulation. We compared gene expression between new recruits captured at similar depths in H. magnifica (n = 3) or S. gigantea (n = 3) by RNA-seq of whole fish. A PCA based on vision and metabolic genes showed a clear separation between both types of recruits (Figures 6B and 6C). We noticed that, in accordance with the higher TH level in S. gigantea recruits, the phototransduction gene guca1a is upregulated, whereas opnsw1 as well as rh1 and rh2a are downregulated (Figure 6D). We also detected a small downregulation of opnlw. The difference from what has been observed in A. ocellaris larvae treated with TH (Figure 2) is probably linked to the difference between the two sets of experiments: lab controlled with newly metamorphosed A. ocellaris on one side and wild-caught, month-old juveniles of A. percula on the other. It is clear that the differences observed in wild juveniles of A. percula could be partly due to ecological constraints. Future studies should focus on the ecological role of THs, and our study shows that anemonefish would be a relevant model to address such a question.

At the metabolic level, we also observed a clear separation of recruits living in S. gigantea from those living in H. magnifica, suggesting that, effectively, the metabolic status of both types is different (Figure 6C). When individual genes were studied, we observed an increase in expression of the glycolytic pfkmb gene, of the TCA cycle gene idh3, and of the β-oxidation genes acaa1 and acads (Figures 6E and 6F) in S. gigantea recruits (which contain higher TH levels). Similarly, we observed a decrease in the expression level of the fatty acid synthase gene fasn. These results are in accordance with the metabolic transition observed during metamorphosis and demonstrate the relevance of these effects in natural populations living in a pristine environment.

DISCUSSION

In this study, we show how THs control and coordinate a major ecological transition: metamorphosis of pelagic coral reef fish larvae into benthic reef-associated juveniles. Our data suggest a model, discussed below, according to which THs have a central function to ensure the ecological success of the metamorphosed juveniles by controlling and
coordinating the transition from larvae to juveniles, thus affecting various processes, such as white bar appearance, visual preference shift, and metabolic transition. In addition, blockage of lipid metabolism impacted metamorphosis triggering by interacting with TH pathway genes, showing a link between metabolic status and metamorphosis in teleost fish. Several lines of evidence suggest that what we observed during post-embryonic development in A. ocellaris is a development truly homologous to metamorphosis of other teleosts and not a developmental event specific to anemone fish. (1) We observe a clear peak of THs at S5 that occurs during morphological transformation of the larvae to juveniles. (2) We can accelerate or slow down the process by treatment with T3 or a mix of goitrogens that impair TH production, respectively. (3) We see coordinated change of TH signaling gene expression, demonstrating a higher activity of the pathway during metamorphosis. We recognize that some key genes (tg and tshb) are not clearly showing a statistically significant peak of expression. We believe this may be due to individual variation of the onset of metamorphosis between individuals.

Three main functions have been attributed to THs in vertebrates: (1) triggering of metamorphosis in amphibians and fish, (2) regulation of adult physiology and metabolism in mammals, and (3) adaptation to seasonality regulation in vertebrates. The extent to which these functions are shared across vertebrates remain unclear, but our data clearly suggest that the two first functions are active during clownfish metamorphosis; THs promote morphological and behavioral transformation of the larvae while also promoting a metabolic shift in energy source.

We studied in detail the action of THs on vision and metabolism, and in a recent report, we uncovered their function in adult pigmentation pattern formation. Interestingly, our transcriptomics data, obtained from whole larvae, reveal that several other important biological processes, such as bone mineralization and digestion, are also changing during metamorphosis. Additionally, a recent study of A. melanopus larvae also demonstrated that physiological changes related to hypoxia tolerance are occurring during the transition from oceanic larvae to reef juveniles. This, together with the detailed analysis of vision and metabolism we performed, clearly illustrates the pleiotropic TH action during metamorphosis.

A behavioral shift in vision controlled by TH

We observed a TH-induced shift between short- and long-wavelength opsin gene expression during clownfish metamorphosis. Interestingly, this shift occurs concomitant with a behavioral change; namely, a change in color preference. Larval fish prefer a blue background, in accordance with their natural pelagic environment, whereas juveniles prefer an orange background in accordance with the shallow, chromatically dynamic environment of the reef. In addition, we showed that TH exposure decreases the preference of treated fish for a blue background. In contrast, TH-treated larvae adopted a benthic behavior characterized by low swimming activity close to the bottom of the experimental tank, which is in accordance with the benthic lifestyle of anemonefish juveniles. Our data do not unequivocally demonstrate a direct effect of THs in color vision because many effects can also be elicited in neural circuit mechanisms for color vision. However, they
strongly suggest that THs globally control a visual shift that is ecologically critical for the recruitment success of juveniles.

Vision is one of the main sensory systems that has been studied by ecologists in the context of fish recruitment. A shift in opsin gene expression, and in particular an increase of opn1lw expression, has been observed in many fish species, such as flatfish, black bream, or cichlids. However, to date, there is only a partial understanding of the mechanistical control of this phenomenon and its integration with metamorphic transformation. In zebrafish, TH signaling has been shown to regulate opn1lw, and mutation of the TRβ gene results in an absence of red cones, but no link with an ecological transition was established. In surgeonfish, we have observed previously that shifts in retina structure and in the ability to visually recognize predators are controlled by THs, but no data on gene expression have been obtained so far, leaving the mechanism underpinning this recognition unclear.

The data we obtained here are in accordance with what has been observed in mammals. In mice, Eldred et al. have shown that THs control the temporal switch between S and M/L cones, which contain short- and medium/long-wavelength opsins, respectively. This suggests that the TH action on opsin gene expression and, more generally, visual function is a general phenomenon in vertebrates. By its ability to combine in vitro and field studies, the anemonefish model could allow integration of the actions of THs on retina formation and, more generally, on sensory system maturation in an ecologically relevant context.

The metabolic transition is instrumental for metamorphosis completion

We observed a major metabolic transition during metamorphosis and provided evidence that THs are implicated in this transition. Larvae preferentially use glycolysis and fermentation to produce energy, whereas they rely more on fatty acid β-oxidation and the TCA cycle during metamorphosis.

Our data show that there is a clear shift from glycolysis, used during early larval life (S1–S3), to β-oxidation starting at S4. To put this observation into perspective, we need to consider three parameters: (1) the changes in the organism’s growth, (2) the transition of the fish into a new ecological life, and (3) the energy produced by each pathway. In glycolysis, one molecule of glucose produces a net excess of only 2 ATP, 2 NADH, and 2 molecules of pyruvate, while oxidation of one molecule of palmitic acid (C16:0, one of the most common saturated fatty acid in vertebrates), produces a net gain of 129 ATP molecules by β-oxidation. Therefore, utilizing fatty acid as a fuel source generates much more energy than sugar. During clownfish post-embryonic development, we observe a massive increase in size soon after S4. Growth in vertebrates has a high demand for energy and, for larvae, has the ability to utilize a fuel source that produces a large number of ATP is certainly an advantage. Moreover, after S4, the transforming fish must actively swim to find its juvenile habitat and, when settled, will have to fight with congeners to be accepted into the colony. All of this will require high energy expenditure; hence, high ATP production explains the transition to a system producing more energy. In addition to this shift in energy production, we also show that THs stimulate production of complex lipids, suggesting that the biochemical landscape of the juvenile is more elaborate than the larval one.
Interestingly, this metabolic transition is deeply linked to the morphological and behavioral changes we describe in this study. Indeed, when we modified lipid metabolism by using an LXR antagonist, we observed an acceleration of white bar appearance as well as a shift in opsin gene expression, two of the most salient endpoints of metamorphosis. This suggests that LXR is active during this period, as demonstrated by the impact of its antagonist on the transcription of genes involved in phototransduction, as observed in zebrafish, but also by its impact on metabolic genes. These findings imply that LXR may play a role in regulating the pace of metamorphosis by ensuring that it occurs in tandem with the available energy production. In addition, by its action in regulating elongase and desaturase genes, LXR likely plays an important role in the complexification of fatty acids we observed.

The ecological function of THs

Collectively, our data suggest that THs are instrumental in integrating the complex remodeling that occurs during metamorphosis with the available environmental resources. This is highly relevant for marine fish larvae living in a very dynamic environment characterized by patchy food resources. It must be pointed out that the challenges faced by these tiny organisms are impressive; they must combine energy production from highly patchy food resources, mobilize their reserves in case of fasting, and, at the same time, mature sensory systems that can detect the future juvenile habitat—all of this while avoiding predators! Our study strongly suggests that THs play a major role in orchestrating this complex and challenging transformation.

Interestingly, the fact that metabolism must be coupled with developmental transition to fulfill the energy requirements during an organism’s life cycle have been pointed out previously in the context of insect metamorphosis. Nishimura has revealed that the planned regulation of metabolism by steroid hormones controls key steps during Drosophila metamorphosis. This is remarkably similar to the effects we observed here, with the major difference that, in contrast to insect pupae, the transforming fish larvae rely on external food sources. However, in both cases, coordination of the larval transformation must integrate environmental and internal conditions, adjusting metabolic regulation as a major tool for achieving this.

Our data are also relevant in light of previous work done on sticklebacks. These fish can live in a marine or freshwater environment, and the energy resources available in these two situations are drastically different. Interestingly, Kitano et al. have shown that freshwater sticklebacks exhibit fine-tuning of numerous physiological and metabolic traits that ensure reduced energy expenditure. These changes are driven by THs; a strong signature of divergent natural selection has been found at the TSHb2 genomic locus, which encodes a hypothalamic peptide controlling TH production. In freshwater sticklebacks, in which TH levels are low, there is also evidence of a shift in opsin gene expression linked to local adaptation to a different light environment. This suggests that, in sticklebacks as in anemonefish, there is a tight integration of metabolic and sensory changes and that THs are instrumental in this process. These cases provide a genuinely nice illustration of a still unappreciated potential for TH regulation to induce pleiotropic changes, allowing multi-level regulation of a developmental transition.
To conclude, it is worth noting that our model also has general significance regarding the effect of climate change and pollution in animal populations. The sensitivity of metamorphosis to environmental stressors has been emphasized recently.\textsuperscript{11} Metamorphosis, being a life history transition with abrupt ontogenetic changes tightly connected to environmental conditions, can make young life stages vulnerable to stressors. Indeed, we have demonstrated previously in convict surgeonfish that temperature and pollution synergistically disrupt TH signaling, affecting the ability of young juveniles to perform their ecological function.\textsuperscript{10} Our study therefore emphasizes the need to decipher how stressors affect not only metamorphosis endpoints but also the metabolic regulations that ensure its correct progression.

Limitations of the study

Although work on lab-reared animals allows us to have a better understanding of the biological processes occurring during metamorphosis, it does not tackle the role of the environment in such phenomena. Despite our efforts to conduct analyses on wild populations of anemonefish inhabiting two different species of sea anemone, much work remains to be done on individuals sampled before settlement in the reef. However, sampling pelagic larvae remains a challenge. Some experiments in this study were limited by the number of replicates, but we compensate for this with higher replicates for the functional transcriptomics experiments, in which we treated larvae with compounds that alter metamorphosis.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Vincent Laudet (vincent.laudet@oist.jp).

Material availability—This study did not generate new unique reagents.

Data and code availability—The RNA-seq datasets corresponding to the T3-treated, MPI-treated, and SR9243-treated larvae were generated in the purpose of this study. Single RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table as well as DOI for data already published in other articles.

This paper does not report original code.

Any additional information required to analyze the data reported in this paper is available from the lead contacts upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics approval—All experiments conducted in this study were done under the approval from the C2EA-36 Ethics Committee for Animal Experiment Languedoc-Roussillon (CEEALR - approval N°A6601601) as well as following the Animal Experiment Regulations at Okinawa Institute of Science and Technology Graduate University (approval
N°20052605) and Institutional Animal Care and Use Committee of Academia sinica (AS IACUC, approval N° 21–12-1769).

**Amphiprion ocellaris maintenance and rearing**—*Amphiprion ocellaris* larvae were obtained from ca 10 breeding pairs maintained as described in Roux et al.\(^65\) in rearing structures located in Banyuls sur mer (France), Okinawa (Japan) and Yilan (Taiwan). Reproductive pairs laid eggs every two weeks allowing to rear larvae regularly for the purpose of this study. Seven developmental stages were previously identified thanks to morphological criteria (such as notochord flexion, fin development, white bars appearance etc.) and were then used in this study.\(^22\) Even if it is difficult to liaise developmental stages and days post hatching because rate of development can be very different between one husbandry to another, the following relationship was often encountered in our rearing conditions: Stage 1: 1–3 dph, Stage 2: 2–8 dph, Stage 3: 3–10 dph, Stage 4: 5–14 dph, Stage 5: 9–15 dph, Stage 6: 10–20 dph, Stage 7: After 17 dph.

**Amphiprion percula sampling in the wild**—*Amphiprion percula* new recruits were sampled in Kimbe, Papua New Guinea in both *Heteractis magnifica* and *Stichodactyla gigantea*. A total of three new recruits per sea anemone were euthanized in MS222 solution and stored in RNAlater (ThermoFisher scientific, Waltham, USA) until RNA extraction and RNA sequencing of the whole fish individually (Salis et al.\(^23\)).

**Reporter cell lines**—The HG5LN Gal4-\(\text{zfPXR}\) and Gal4-\(\text{zfPPAR}_\gamma\) cell lines were previously described.\(^66,67\) Briefly, HeLa cells stably transfected with the GAL4RE5-\(\beta\)Globin-Luc-SVNeo plasmid (HG5LN cell line) were stably transfected with the pSG5-GAL4(DBD M1-S147)-\(\text{zfPXR}\) (LBD M111-T430)-puro or pSG5-GAL4(DBD M1-S147)-\(\text{zfPPAR}_\gamma\) (LBD K213-Y527)-puro plasmids. HG5LN Gal4-\(\text{zfPXR}\) and Gal4-\(\text{zfPPAR}_\gamma\) cell lines were selected for their inducibility in presence of clotrimazole 1 \(\mu\)M and GW3965 10 \(\mu\)M, respectively. The HG5LN Gal4-\(\text{zfLXR}\) was established in a similar manner. HG5LN cells were stably transfected with the pSG5-GAL4-(DBD M1-S147)-\(\text{zfLXR}\) (LBD A154-E412)-puro plasmid. This cell line was selected for its inducibility in presence of T091317 1 \(\mu\)M HG5LN GAL4-\(\text{zfPXR}\), GAL4-\(\text{zfPPAR}_\gamma\) and GAL4-\(\text{zfLXR}\) were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (D MEM/F-12) containing phenol red and 1 g/L glucose and supplemented with 5% fetal bovine serum, 100 units/mL of penicillin and 100 \(\mu\)g/mL of streptomycin supplemented with 1 mg/mL geneticin and 0.5 \(\mu\)g/mL puromycin in a 5% CO2 humidified atmosphere at 37°C.

**METHOD DETAILS**

**Transcriptomic analysis developmental stages**

**RNA extraction**: Three larvae per stage were sampled for a total of 21 samples. Larvae were euthanized in an MS222 solution (200 mg/L), photographed for stage identification, and kept in RNAlater (Thermofisher scientific, Waltham, USA) prior to RNA extraction. Total RNA was extracted from whole individual larval body using a Maxwell16 System (Promega, Madison, USA) and following the manufacturer’s instructions. RNA integrity and concentration was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA), with all samples having RNA Integrity Number values above 9.
RNA-seq libraries preparation and sequencing: RNA-Seq libraries were generated with TruSeq Stranded mRNA Sample Preparation Kit (Illumina Way, San Diego, CA) from 400 ng of total RNA according to manufacturer’s instructions. Surplus PCR primers were removed using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, USA). Final cDNA libraries were checked for quality and quantified using capillary electrophoresis. Libraries were loaded in the flow cell at 2 nM. Clusters were generated in the Cbot and sequenced on an Illumina HiSeq 4000 as paired-end 100 base reads. RNA-Seq library preparation and sequencing were performed by the IGBMC in Strasbourg, France (https://www.igbmc.fr/).

TH assay—To determine at which stage TH surge and mark the beginning of metamorphosis, 5 pools of clownfish larvae were sampled at each of the seven developmental stages identified in Roux et al. (2019) (15 larvae for S1, 12 for S2, 10 for S3, 4 for S4, 2 for S6 and 1 for S7). TH were extracted from dry-frozen larvae (previously euthanized in a 200 mg/L solution of MS-222) using the following method. Whole fish body was homogenized in distilled water by a ball mill (ShakeMaster NEO, Hirata, Japan) with stainless beads. The homogenate sample was transferred to a polypropylene (PP) tube and spiked with isotope-labelled internal standards solution containing 13C6-T4, 13C6-T3, and 13C6-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.

In situ hybridization—Digoxigenin RNA probes were synthesized using the T3/T7 Transcription Kit (Roche, Merk, Rahway, USA; Table S2). Larvae were collected, euthanized in MS222 at 200 mg/L and fixed 12 h in 4%. paraformaldehyde diluted in PBS (phosphate-buffered saline). Samples were subsequently dehydrated stepwise in PBS/ethanol, and then put three times 10 min in butanol 100% and finally in two bath of paraffin (respectively 1 and 4 h) before being embedded in blocks. Embedded larvae were sectioned transversally at 7 μm using a Leica Biosystems RM2245 Microtome the day before starting in situ hybridization (Figure 2C). The samples were then treated as in Thisse et al. 98

T3 and MPI treatments—To test the effects of TH on the metamorphosis of A. ocellaris, larvae were either exposed to T3+IOP (Sigma-Aldrich, Darmstadt, Germany) to trigger metamorphosis (iopanoic acid [IOP] used to block deiodinase activity), a mix of goitrogenous compounds called MPI (Methimazole, Potassium perchlorate, Sigma-
Aldrich, Darmstadt, Germany) to delay metamorphosis, or a mix of T3 + MPI to rescue the phenotype. These treatments were performed using the low-rearing volume protocol developed by Roux et al. Briefly larvae were divided in groups of 10 and placed in 800 mL beakers in a water bath to maintain the temperature at 27°C. Larvae were fed 3 times a day with rotifers at a final concentration of 10 rotifers/ml and once a day with nauplii of artemia. The algae *Nannochloropsis oculata* was added (500 μl/beaker) at each feeding to create a green environment and improve survival rates (except in the MPI treatment as we observed that MPI failed to delay metamorphosis in presence of algae likely due to the presence of iodine in the algae preventing MPI action). During the experiment, water changes of 100 ml (with addition of each treatment to maintain constant concentrations) were done daily to ensure water quality. Three sets of experiment were conducted: 1) larvae were treated for 24h and 72h hours with DMSO (control), T3 at 10–6M, MPI at 1/1000 dilution (see Salis et al., for the individual concentration of each compound) and T3+MPI (same concentrations as individual treatment). Larvae sampled at 24h were euthanized in a MS222 solution (200 mg/l) and photographed under a Zeiss stereomicroscope (V20 discovery Plan S) equipped with an Axiocam 105 camera for morphological analysis. Three replicates composed of pools of two larvae were then kept in RNA later at −20°C for gene analysis using nCounter technology from Nanostring (Figure 2D). Larvae sampled at 72h were used for visual perception behavioral analysis (Figure 2E, see detailed method below); 2) Larvae were treated for 48h with DMSO and T3 at 10–7M and sampled for transcriptomic analysis (Figures 3B, 4C, S3, and S4). 3) Larvae were treated for 19 days with DMSO and MPI (1/1000) and sampled for transcriptomic analysis (Figures 3B, 4C, S3, and S4). The concentrations used in this study were chosen based on previous experiments on fish metamorphosis.

**Behavioral test for visual perception**—Choice experiments were conducted on stages 2 and 5 to determine if there is a shift in visual perception before and after metamorphosis. Thus, a dual choice aquarium (measuring 25×10×10 cm) was built with white opaque Komassell, fitted with transparent plexiglass on the sides (Figure 2B). The aquarium was divided into three equal compartments (Figure 3B). The larvae were given the choice between a short wavelength color (blue) and a long wavelength color (orange). Each color was placed close to the plexiglass sides. Behavioral experiments were conducted in a dark room where the choice chamber was installed under a light ramp to ensure homogenous distribution of the light over the device. All the larvae were tested individually. The larvae were introduced carefully in the choice chamber without the colored panels and left for acclimation for 2 min. They were free to explore the three compartments. After acclimation and once the larvae were localized in the central compartment, blue and orange panels were installed, and the time spent by the larvae in each compartment was recorded during a 5 min period. We established that a larva preferred a color when it spent most of its time in the compartment of the given color (Figure 2B). Color panels were inverted after each tested larva to ensure that the choice was due to the color and not to the design of the experimental device. Results were expressed as the mean percentage of time spent in each compartment (Figure 2B).
**Gene expression using nCounter technology**

**Probe synthesis:** Probes of 100 nucleotides were designed by Integrated DNA Technology (https://sg.idtdna.com/pages) for all the genes investigated in this study (Table S2). However, no probes could be designed that specifically targeted TRαa, TRαb, rh2a-1 and rh2a-2 as sequences were too similar. For this reason, only one pair of probes has been designed and targeting both TRα and rh2a genes. Seven reference genes were chosen for normalization and expression analysis: Tuba1, PolD2, g6pd, eif4a3, tbp, rpl7 and rpl32.

**Sample processing:** Samples from each experiment were processed in multiplexed reaction including, in each case, six negative probes (to determine the background) and six positive control probes. Hybridization reactions lasted 16 hours at 67°C. Data were then imported into the nSolver analysis software (version 2.5) for quality checking and normalization of data according to NanoString analysis guidelines, using positive probes and 7 reference genes (Tuba1, PolD2, g6pd, eif4a3, tbp, rpl7 and rpl32).

**Lipid analysis**—Larvae (3 per stage) were sampled at stage 3, 4, 5 and 6 and starved for 1 h before being flash frozen in liquid nitrogen and stored at −80°C until analysis. Three individuals were collected for each condition and lipid extraction was done on each individual. Clownfish larvae were homogenized in 500μL of 10% methanol in water and 200μL of the homogenates were extracted by a modified BUME method. Extracts were dried and saponified using a 1:1 MeOH:KOH solution at 37°C for 30 min. Fatty acids were extracted by a biphasic solution of acidified methanol and isooctane, derivatized using PFBB, and analyzed using SIM by GC-MS on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 autosampler. Fatty acids were separated using a 15m ZB-1 column (Phenomenex, Le Pecq, France) and monitored using SIM identification. Similar analyses were performed on larvae (3 per condition) treated for 72 hours with 10⁻⁸ M and 10⁻⁷ M T3 (mixed with 10⁻⁷ M IOP) but with some variation in samples preparation following the method described in Hartler et al., Sampling was performed as explained previously.

**SR9243 experiments**—Specificity of a human LXR antagonist SR9243 was tested in reporter cell lines expressing the ligand binding domains of zebrafish receptors LXR, PPARγ and PXR. Briefly, SR9243 was tested alone for its agonistic activity in HG5LN-zfLXR, -zfPPARγ and zf-PXR cells. For its antagonistic activity on -zfLXR, -zfPPARγ and zf-PXR cells, SR9243 was tested in combination with T091317 30 nM, GW3965 3 μM and clotrimazole 0.1 μM respectively (Data not shown). (All antagonist and agonists were purchase on Sigma Aldrich, Darmstadt, Germany).

Larvae of *A. ocellaris* were sampled at stage 3 and treated for 5 days with DMSO (control condition), T3 at 10⁻⁷ M or LXR antagonist SR9243 at 10⁻⁷ M. Experiments were conducted following the protocol described in Roux et al., and detailed previously. Ten larvae per condition were sampled and photographed as previously and used for transcriptomic analysis (Figure 5).
Transcriptomic analysis T3/MPI and SR9243

RNA-seq libraries preparation and sequencing: RNA of each larva (n=10 per condition) used in these experiments was extracted using NucleoSpin RNA extraction kit (Macherey-Nagel, Düren, Germany). RNA-Seq libraries were generated from 2000 ng of total RNA using the Illumina Stranded mRNA Prep mRNA Sample Preparation Kit with UDI indices (Illumina, USA) according to the manufacturer’s instructions. Surplus PCR primers were removed using AMPure XP (Beckman Coulter Life Sciences, USA). Final cDNA libraries were checked for quality and quantified using Qubit (ThermoFisher Scientific, USA) and Fragment Analyzer for size profiling (Agilent, USA), and concentration normalized using KAPA Library Quantification Kit for Illumina Platforms (Roche, USA). Sequencing was performed on an Illumina NextSeq2000 for paired-end 150 base format. Libraries were loaded in the P2 flow cell at 645 nM. The fastQ files were generated and demultiplexed using bcl2fastq v2.20 pipeline. RNA-Seq library preparation and sequencing were performed by the High Throughput Genomics Core of Biodiversity Research Center in Academia Sinica in Taipei, Taiwan (http://ngs.biodiv.tw/NGSCore/contact-location/).

Transcriptomic analysis A. percula recruits

RNA-seq libraries preparation and sequencing: Total RNA of each individual (n=3 per sea anemone species) was extracted using TRizol Reagent kit (Thermofisher, Waltham, USA) followed by DNase treatment (Thermofisher, Waltham, USA) and then purified with 0.025-μm dialysis membranes. RNA-Seq libraries and sequencing were performed on an Illumina HiSeq 4000 sequencer using a stranded protocol as paired-end 50 base reads. Potential adapter contaminations were removed and trimmed to obtain raw reads with cutadapt (version 1.1382) and sickle (version 1.2983), respectively. The processed reads were mapped against A. percula reference genome (Ensembl ID: GCA_003047355.184 using HiSat2 version 2.1.085).

QUANTIFICATION AND STATISTICAL ANALYSIS

Transcriptomic analysis developmental stages

Pre-treatment (read quality, quantification, filtering, normalization): Quality of raw reads was assessed using FastQC quality control tool (version 0.11.886). Sample contamination was assessed using fastq_screen quality control tool (version 0.13.0, https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/). Amphiprion ocellaris transcript sequences and annotations (Ensembl release 97) were downloaded from the Ensembl website (http://www.ensembl.org/info/data/ftp/index.html) Some genes which had no official symbols in Ensembl were given the following symbols:

ENSAC0G00000023317 =tg, ENSAC0G0000008531 = mct8, ENSAC0G0000005709 = dio3a, ENSAC0G0000017526 = edn3b, and ENSAC0G0000019494 = bnc2.

Transcript expression quantification was performed from the raw read data using Salmon (version 1.1.087 with parameters –gcBias, –seqBias, –validateMappings, –discardOrphansQuasi, and –consistentHits. The gene counts were then loaded into RStudio using the tximport package (version 1.14.088 with parameters type="salmon" and countsFromAbundance="lengthScaledTPM"). Genes that did not have more than 0.502 counts per million counts in at least three samples were filtered out. To generate the
normalized signals, the effective library sizes are first computed using function estimateSizeFactors from package DESeq2 (1.26.0\textsuperscript{89}). The raw count values were then transformed using a variance stabilizing transformation (function VarianceStabilizingTransformation from package DESeq2 with parameter blind=TRUE).

**Hierarchical clustering:** Hierarchical clustering of samples was performed using the Wards agglomerative method, passing the euclidean distances between samples to function hclust from package stats (with parameter method=“ward.D2”). Cluster stability was estimated by multiscale bootstrap resampling using function pvclust from package pvclust (2.2–0\textsuperscript{90} with parameter nboot=10000). Hierarchical clusterings of genes was performed using the complete agglomerative method, passing the Euclidean distances between centered (for expression data) or uncentered (for fold change data) and scaled gene signals with function hclust from package stats (Figure 1B, Figure S1A).

**Principal component analysis:** Principal Component Analyses (PCA, Figure 1A) of sample expression levels were performed with gene signals centered but not scaled (using function prcomp from package stats). When displayed, gene coordinates correspond to these genes’ correlations with the presented components. Only the genes that contribute most to the components are displayed.

**Gene expression analysis:** Starting from the filtered, non-normalized counts, the effective library sizes were computed using function calcNormFactors from the edgeR package version 3.28.0\textsuperscript{91} Mean variance relationship was estimated using the voom function from the limma package version 3.42.0\textsuperscript{92} and injected into the statistical model as observational level weights. Linear regression models were applied using function lm from the stats package. Comparisons of interest were computed through statistical contrasts, using the emmeans package version 1.4.3.01\textsuperscript{93} (Figures 1D, S1B, and S2). The empirical Bayes method (function eBayes from the limma package) was used to compute moderated p-values. p-values were then corrected for multiple comparisons using the Benjamini and Hochberg’s false discovery rate (FDR) controlling procedure (function p.adjust from package stats with parameter method=“BH”).

**TH assay**—Relative TH value for both T3 and T4 were obtained by normalizing with sample weights and displayed in ng/g of larvae (Figure 1E). Statistical differences between each developmental stage were assessed for each hormone by performing a one way ANOVA followed by a pairwise t-test (using Bonferroni correction).

**Behavioral test for visual perception**—A Kruskall-Wallis test was performed to determine if the difference in the amount of time spent between larvae (stage 2 versus stage 5, Figure 2B) and a Dunn’s test was performed to determine the difference between the four treatment conditions (DMSO, T3, MPI, T3+MPI) was significantly different in each compartment (Figure 2E).

**Gene expression using nCounter technology**—After normalization using house keeping genes (see details above), analysis of the differential expression between treatments for each experiment was conducted using ANOVA (R software 3.2.3 version) and fold
change calculated on a log2 scale. A pairwise t-test for multiple comparison was used in R software (Figure 2D).

**Lipid measurements and analysis**—Statistical analysis on developmental stages was performed using one way ANOVA with R software for each lipid followed by a multiple pairwise comparison performed by pairwise t-test in case of significant differences (p-value<0.05, Figures 4B and S7A). Statistical analysis to compare each T3 concentration with DMSO control were performed using Student’s test with R software (Figure S7B).

**SR9243 experiments**—Effect of treatments (SR9243-LXR antagonist, T3) on white bars appearance was assessed by comparing the number of individuals displaying white bars on the head and trunk between control and each treatment using a Chi² test performed with R software (Figure S5B).

**Transcriptomic analysis T3/MPI and SR9243**—Analysis of these data sets were performed as described previously (Figures 3B, 4C, 5C–5E, S3, S4A, and S4C).

**Transcriptomic analysis A. percula recruits**—Raw counts for each gene were obtained with HTSeq (htseq-count, version 0.9.1, using the available gene annotation of the A. percula reference genome (Salis et al.)). Raw counts were then normalized as transcript per million to assess the difference in expression levels for gene involved in vision and metabolic processes using the test of Student (Figures 6D–6F). Principal component analysis was also performed on each biological process separately (Figures 6B and 6C).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

• A peak of thyroid hormones (THs) marks the onset of metamorphosis in *A. ocellaris*

• THs control opsin gene expression and color preference shift during metamorphosis

• THs promote a metabolic shift that is tightly linked with metamorphosis progression

• THs participate in aligning metabolism and environmental conditions in the wild
Figure 1. Three periods during clownfish post-embryonic development
(A) Principal-component analysis (PCA) according to the 1,000 genes with the highest variance, showing three groups in the transcriptomics data: early developmental stages, S4, and late developmental stages.
(B) Heatmap of the 100 genes with the highest variance, extracted from the transcriptomics data. Colors represent the intensity of the centered (but unscaled) signal that ranges from low (blue) to high (red).
(C) PCA of the morphological transformation according to body depth (BD), head length (HL), and snout vent length (SVL) over standard length (SL; red).
(D) Number of genes differentially expressed between contiguous developmental stages.
(E) Variation in TH levels (in nanograms per gram of larvae) during the post-embryonic stages of *A. ocellaris*.

See also Figures S1 and S2. Statistical differences (indicated by an asterisk) between each stage for each thyroid hormone are calculated by a one-way ANOVA followed by a pairwise t test (using Bonferroni correction).
Figure 2. THs control visual perception shift by regulating opsin gene expression

(A) Heatmap of the eight opsin genes involved in visual perception, organized from top to bottom according to short-wavelength \((opnsw2B, opnsw1a,\) and \(opnsw1b)\), medium-wavelength \((rh2B, rh2A-1, rh2A-2,\) and \(rh1)\), and long-wavelength \((opnlw)\) sensitivity.

(B) Mean percentage of time spent by S2 \((n=20)\) and S5 \((n=20)\) larvae in each compartment of a dual-choice chamber. Statistical differences (indicated by an asterisk) between each stage in each compartment were calculated by a chi-square test.

(C) In situ hybridization of sectioned S2, S4, and S6 larval retina using probes for \(opnsw2b\) and \(opnlw\). A black arrowhead indicates expression signals in the photoreceptors (external nuclear layer). Scale bar, 10 μm.
(D) Regulation of opnsw2B and opnfw expression after 24 h of treatment at $10^{-6}$ M T3+IOP, MPI, and T3+MPI (n = 3 pools of 2 larvae per condition). Expression levels, measured by nCounter technology, are expressed as fold change, and statistical differences between treatment and DMSO control (assessed by a pairwise t test following one-way ANOVA) are indicated by an asterisk.

(E) Effects of T3 at $10^{-6}$ M (n = 19) compared with MPI 1/1,000 diluted (n = 14), T3 $10^{-6}$ M+MPI 1/1,000 (n = 14), and DMSO control (n = 14) after 72-h treatment on the mean percentage of time spent by larvae in each compartment. Statistical differences (indicated by an asterisk) between each condition in each compartment were calculated by a chi-square test.

See also Figure S4.
Figure 3. THs induce a metabolic shift during metamorphosis

(A) Expression levels of genes involved in glycolysis, lactic fermentation, fatty acid β oxidation, and the tricarboxylic acid cycle at each developmental stage, extracted from transcriptomics data, clearly revealing a change of expression at S4 (indicated on each graph by a green rectangle) for each pathway. Black lines join the average values of each stage. Genes written in bold are significantly differentially expressed between S4 and metamorphosis stages (S5 and/or S6 and/or S7), and an asterisk indicates genes significantly differentially expressed between S1 and metamorphosis stages (S5 and/or S6 and/or S7).
(B) Effects of T3 (10^{-7} M, n = 10 larvae) and MPI (1/1,000 dilution, n = 10) after 2 and 19 days post-treatment (dpt), respectively, on the expression levels of the genes involved in glycolysis (hk2, gpih, pfkma, pfkmb, tpi1, gapdhBa, pgk1, pgam2, and eno3 pkma), the tricarboxylic acid cycle (cs, aco2b, idh3A, dlstb, ogdhl, sucla2, sdha, mdh2, and fhl), lactic fermentation (ldha, ldhba, and ldhbb), and β-oxidation (cpt1aa, cpt1b, cpt2, acads, and acaal). Expression levels obtained after transcriptomic analysis are expressed as log fold change, and statistical differences between treatment and DMSO control (differential gene expression analysis) are indicated by an asterisk. See also Figures S3, S5, and S6.
Figure 4. TH action on lipid metabolism regulation during metamorphosis
(A) Expression levels of genes involved in de novo lipogenesis and lipid desaturation and elongation at various stages. Black lines join the average values of each stage. Genes written in bold are significantly differentially expressed between S4 and metamorphosis stages (S5 and/or S6 and/or S7), and an asterisk indicates genes significantly differentially expressed between S1 and metamorphosis stages (S5 and/or S6 and/or S7).

(B) Fatty acid content of *A. ocellaris* at S3, S4, S5, and S6 (n = 3 per stage), illustrated with a heatmap (left) and boxplots (right). Significant differences are indicated by different
letters. Sat, saturated; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated FA; HUFA, highly unsaturated FA.

(C) Effects of T3 (10^{-7} M, n = 10) and MPI (1/1,000 dilution, n = 10) after 2 and 19 dpt, respectively, on the expression levels of representative genes for fatty acid synthesis (me1, me2, me3, slc25a1a, acaca, acacb, acly, aclyb, fasn, and gpat2), desaturation (scd, scdb, and fads2), and elongation (elovls) and for the transcription factors involved in lipid metabolism regulation (chreb, srebpl, srebp2, pparg, and lxr). Expression levels obtained after transcriptomics analysis are expressed as log fold change, and statistical differences between treatment and DMSO control (differential gene expression analysis) are indicated by an asterisk.

See also Figures S3 and S7.
Figure 5. Metabolic changes linked to metamorphosis revealed by LXR antagonist treatment

(A) Pictures of *A. ocellaris* larvae after 5 days of treatment (dpt) with DMSO control (top row) and LXR antagonist (SR9243) at $10^{-7}$ M (center row) and T3 at $10^{-7}$ M (bottom row).

(B) Quantitative analysis showing the percentage of individuals presenting a white bar on the head and trunk (DMSO, n = 19; SR9243, n = 22; T3, n = 29). Significant differences obtained after a chi-square test are indicated by an asterisk.

(C) PCA according to the 2,000 genes with the highest variance, showing a clear separation between each treatment condition (DMSO, SR9243, and T3; 10 larvae per treatment).
(D) Venn diagram illustrating the number of statistically significant differentially expressed genes in clownfish larvae treated with SR9243 and T3 for each signaling pathway (TH pathway, vision, glycolysis, TCA cycle, lactic fermentation, and lipid metabolism).

(E) Effect of SR9243 and T3 after 5 days of treatment on the expression levels of genes involved in TH signaling (tg, duox, trb, dio2, dio3a, and dio3b), vision (opnlw, opnsw2B, rh2a2, rh2b, guca1a, and arr3b), glycolysis (pfkma, pfkmb, eno3, pkma, pkmb, and pgk1), the citric acid cycle (TCA) (cs, idh3a, aco2b, and ogdhh), lactic fermentation (ldha and ldhba), and lipid metabolism (acads, elovl5, srebp1, lxr, abca1, and acaca1). Transcriptomic expression levels are displayed as normalized count, and significant differences between treatment conditions for each gene are displayed by different letters.
Figure 6. Natural variations in TH levels reveal metabolic and visual effects

(A) TH level (T3, expressed in picograms per gram of fish) of new recruits sampled in the sea anemone H. magnifica (blue, n = 5) versus S. gigantea (orange, n = 5) (from Salis et al.23).

(B and C) PCA of genes involved in vision and metabolism, showing the separation between A. percula recruits sampled in H. magnifica vs. S. gigantea.

(D–F) Expression levels (display as mean of transcript per million with standard error bars) of genes involved in vision (D) and metabolism (E and F), obtained after transcriptomics.
analysis of *A. percula* recruits sampled in both sea anemone species (n = 3 per condition). Significant differences (indicated by an asterisk) are calculated by a test of Student.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE                              | SOURCE                  | IDENTIFIER          |
|--------------------------------------------------|-------------------------|---------------------|
| Chemicals, peptides, and recombinant proteins    |                         |                     |
| MS222/Tricaine/ Ethyl 3 aminobenzoate methanesulfonate salt | Sigma-Aldrich           | A5040               |
| RNAlater                                         | ThermoFisher            | AM7021              |
| T3/T7 Transcription Kit                         | Roche                   | RPOLT3-RO / RPOLT7-RO |
| Formaldehyde                                     | Sigma-Aldrich           | F8775-500ml         |
| Butanol                                          | Sigma-Aldrich           | 33065               |
| T3 (or 3, 3', 5 Triiodo-L thyronine)             | Sigma-Aldrich           | T2877-250mg         |
| IOP (or Iopanoic Acid)                          | Sigma-Aldrich           | 14131-100mg         |
| Methimazole                                      | Sigma-Aldrich           | M8506-25g           |
| Potassium Perchlorate                            | Sigma-Aldrich           | 460494-25g          |
| SR9243 (LXR Antagonist)                         | Sigma-Aldrich           | SML1517             |
| T091317                                          | Sigma-Aldrich           | 575310              |
| GW3965                                           | Sigma-Aldrich           | G6295               |
| DNA-free kit                                     | AM1906                  | AM1906              |
| Critical commercial assays                       |                         |                     |
| Maxwell RSC SimplyRNA Tissue Kit                | Promega                 | AS1340              |
| Bioanalyser RNA Kit & Reagents                   | Agilent                 | 5067-1511           |
| TruSeq Stranded mRNA Sample Preparation Kit      | Illumina                | 20020595            |
| KAPA Library Quantification Kit                  | Illumina                | KK4824              |
| RNA Nucleospin extraction kit                    | Macherey-Nagel         | 740955.50           |
| Nanostring tagSet and Master Kit                 | Nanostring Technologies | N/A                 |
| Trizol Reagent RNA extraction kit                | Thermofisher            | 15596-026           |
| Deposited data                                   |                         |                     |
| Amphiprion ocellaris larval developmental stage  | Salis et al.24 (DOI: https://doi.org/10.1111/pcmr.12766) | NCBI BioProject PRJNA482393 |
| Amphiprion ocellaris T3 treated larvae           | This paper              | GEO accession GSE228441 |
| Amphiprion ocellaris MPI treated larvae          | This paper              | GEO accession GSE228438 |
| Amphiprion ocellaris SR9243 treated larvae       | This paper              | GEO Submission GSE232363 |
| Amphiprion percula wild recruit Kimbe (Papua New Guinea) | Salis et al.23 (DOI: https://doi.org/10.1111/pcmr.12766) | NCBI BioProject PRJNA828114 |
| Experimental models: Cell lines                  |                         |                     |
| Human : HG5LN Gal4-zfPXR                        | Laboratory of Patrick Balaguer-INSERM | N/A               |
| Human : HG5LN Gal4-zfPPARg                      | Laboratory of Patrick Balaguer-INSERM | N/A               |
| Human : HG5LN Gal4-zfLXR                        | Laboratory of Patrick Balaguer-INSERM | N/A               |
| Experimental models: Organisms/strains           |                         |                     |
| REAGENT or RESOURCE | SOURCE                                                                 | IDENTIFIER |
|---------------------|------------------------------------------------------------------------|------------|
| Amphiprion ocellaris larvae | Lab reared (Observatoire Océanologique de Banyuls sur mer France, Academia Sinica Taipei Taiwan, Okinawa Institute of Science and Technology Okinawa Japan) | N/A        |
| Amphiprion percula juveniles Kimbe bay (Papua New Guinea) | Sampled in the wild (Serge Planes-CRIOBE) | N/A        |
| Oligonucleotides    |                                                                        |            |
| nCounter Probes     | Integrated DNA Technology                                              | N/A        |
| Software and algorithms |                                                                  |            |
| R software          | R Core Team (2021)                                                    | https://www.r-project.org/ |