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# 1 **Thyroid hormones regulate the formation and environmental** 2 **plasticity of white bars in clownfishes**

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21

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24

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26

## 27 **Authors contribution**

28 P.S., N.R., M.R. and N.R. performed research. B.P., P.M. designed and performed the  
29 inference approach. D.P. and D.H. performed CRISPR-Cas9 in *D. rerio*. O.S. and S.P.  
30 performed sampling of *A. percula*. A.M. and N.S. analyzed transcriptomic data. P.S., S.P.  
31 and V.L. designed research. P.S. and V.L. wrote the article.

## 32 **Abstract**

33 Determining how plasticity of developmental traits respond to environmental conditions is a  
34 challenge that must combine evolutionary sciences, ecology and developmental biology.  
35 During metamorphosis, fish alter their morphology and color pattern according to  
36 environmental cues. We observed that juvenile clownfish (*Amphiprion percula*) modulate the  
37 developmental timing of their adult white bar formation during metamorphosis, depending on  
38 the sea anemone species in which they are recruited. We observed an earlier formation of  
39 white bars when clownfish developed with *Stichodactyla gigantea* (*Sg*) than with *Heteractis*  
40 *magnifica* (*Hm*). As these bars, composed of iridophores, form during metamorphosis, we  
41 hypothesized that timing of their development may be thyroid hormone (TH) dependent. We  
42 treated clownfish larvae with TH and found that white bars developed earlier than in control  
43 fish. We further observed higher TH levels, associated with rapid white bar formation, in  
44 juveniles recruited in *Sg* than *Hm*, explaining the faster white bar formation. Transcriptomic  
45 analysis of *Sg* recruits revealed higher expression of *duox*, a dual oxidase implicated in TH  
46 production as compared to *Hm* recruits. Finally, we showed that *duox* is an essential  
47 regulator of iridophore pattern timing in zebrafish. Taken together our results suggest that TH  
48 control the timing of adult color pattern formation and that shifts in *duox* expression and TH  
49 levels are associated with ecological differences resulting in divergent ontogenetic  
50 trajectories in color pattern development.

## 51 **Significance statement**

52 Developmental plasticity is defined as the ability of an organism to adjust its development  
53 depending on environmental signals, thus producing alternative phenotypes precisely  
54 adjusted to the environment. Yet the mechanisms underlying developmental plasticity are not  
55 fully understood yet. We found that juvenile clownfish delay the development of their white  
56 bars during metamorphosis, depending on the sea anemone species in which they are  
57 recruited. To understand this developmental plasticity, we investigated roles for thyroid  
58 hormones, the main hormones triggering metamorphosis in vertebrates. We found that  
59 thyroid hormones regulate white bar formation and that a shift in hormone levels, associated  
60 with ecological differences, results in divergent color patterns in different sea anemone  
61 species in which the young fish is recruited.

62

63

## 64 **Introduction**

65 Understanding the origins of biodiversity is one of the major challenges of biology, but it  
66 should not be limited to the species level, which is however already a formidable task (1).  
67 Indeed, diversity is also present within species, as phenotypic variation between distinct  
68 populations, and also within populations, depending on individual genotype and the extent to  
69 which physiology, behavior, or development are influenced by the environment (1, 2). In  
70 some instances, this phenotypic variation can reflect adaptive developmental plasticity that is  
71 defined as the ability of organisms to change their developmental trajectories to generate  
72 phenotypes precisely adjusted to the environmental conditions (1–3). Remarkable examples  
73 of such plasticity are known in animals, giving rise to distinct color patterns and other  
74 morphological traits, as well as life-histories (4). For instance, different generations of  
75 butterfly can develop alternative color patterns on their wings, depending on the season in  
76 which they emerge (5). Water fleas can grow large helmets and spikes as a response  
77 induced by predator cues, such as the concentration of kairomones in the water (6).  
78 Spadefoot toad tadpoles living in semi-arid environments accelerate their metamorphosis in  
79 response to pond drying (7).

80

81 Determining how plastic developmental changes that occur in response to environmental  
82 conditions are coordinated at the physiological, cellular, and molecular levels is a challenge  
83 that must combine ecology with developmental biology (8, 9). The mechanisms that underlie  
84 the development of alternative phenotypes are still unclear for many systems and is one  
85 major goal of ecological developmental biology or Eco-Evo-Devo (1, 10).

86

87 Pigmentation is one of the conspicuous features of animals and often has clear ecological  
88 and behavioral significance. It is thus an outstanding model for understanding links between  
89 environment and developmental plasticity. There are several cases of teleost fishes  
90 exhibiting phenotypic plasticity in pigmentation (11). This is the case in cichlids, for which  
91 several species exhibit a conspicuous yellow-blue bright phenotype linked to social  
92 dominance (12), in the platyfish in which melanic spots phenotypes are polymorphic within  
93 and among populations of *Xiphophorus variatus* depending on stress status (13), in  
94 salmonids with various pigmentation phenotypes linked to stress and social dominance (14)  
95 and also in coral reef fishes such as the dottybacks depending on the presence of prey  
96 species (15).

97

98 One of the most extraordinary life history transitions in vertebrates is metamorphosis which is  
99 regulated by thyroid hormones (TH) (16). With the very large number of TH-regulated

100 morphological changes occurring during larval metamorphosis (17, 18), environmentally  
101 induced alterations to TH status during this developmental period have the potential to affect  
102 outcomes of the metamorphic process (19). TH is also required to shift the larval  
103 pigmentation toward adult pattern (20). In zebrafish, for instance, TH promotes the  
104 maturation of specific pigment cells, black melanophores and yellow xanthophores (21).  
105 Whereas TH drives the terminal differentiation and proliferative arrest of melanophores, thus  
106 limiting their final number, it promotes the accumulation of orange carotenoid pigments in  
107 xanthophores, making the cells more visible (21, 22).

108

109 Here, we investigate the potential role of TH in a case of developmental plasticity in color  
110 morphs of clownfishes and we tested the impact of two environments (*e.g.* sea anemone  
111 species) on that kinetic. Among these coral reef fishes, two closely related allopatric species,  
112 *Amphiprion ocellaris* and *Amphiprion percula*, live in mutualistic symbiosis with sea  
113 anemones in the tropical Indo-pacific (23, 24). We observed that *A. percula* young juveniles  
114 (referred to here as recruits) have a different rate of white bar formation depending on the  
115 sea anemone species, their obligate symbiotic partner, in which they are recruited: white  
116 bars develop more rapidly when fish are recruited in *Stichodactyla gigantea* than in  
117 *Heteractis magnifica*. Because *A. ocellaris* acquire their adult color pattern during  
118 metamorphosis (25, 26), we asked whether developmental plasticity in bar formation is  
119 associated with alteration in TH status. Using *A. ocellaris* we found that blocking TH  
120 production delayed white bar formation, whereas excess TH accelerated white bar formation,  
121 revealing a role for TH in determining the rate at which color pattern shifts from larva to  
122 juvenile form. To test the ecological significance of these findings, we assayed TH titers and  
123 gene expression in wild-caught *A. percula* and found that young recruits associated with *S.*  
124 *gigantea* exhibited a higher levels of TH and more abundant transcript of *duox*, a gene  
125 implicated in thyroid function and TH synthesis, as compared to recruits associated with *H.*  
126 *magnifica* (27). Further supporting a role for *duox* and TH in regulating the timing of  
127 iridophore patterning, we found that zebrafish deficient for *duox* activity were delayed in  
128 iridophore stripe formation relative to overall developmental progression. Taken together our  
129 results suggest that TH regulates color pattern formation in clownfish and that shifts in  
130 hormone levels are associated with ecological differences that result in divergent ontogenetic  
131 trajectories in color pattern formation.

132

## 133 Results

134

### 135 **Formation of white bars of *A. percula* new recruits is differentially influenced by age or** 136 **size depending on anemone species**

137 *Amphiprion* species acquire, in sequence, the head, body and finally peduncle white bars  
138 during post-embryonic development (26). In Kimbe bay, Papua New Guinea, *A. percula* is  
139 found in two different sea anemone hosts, *Stichodactyla gigantea* and *Heteractis magnifica*  
140 and the fish leaving in these two hosts belongs to the same population (28). We observed in  
141 the field that new *A. percula* recruits in *S. gigantea* have more white bars than new recruits in  
142 *H. magnifica* for juveniles of the same age and developmental stage (juvenile stage). In fact,  
143 33% of 148 new recruits (200–250 days old) in *S. gigantea* had 3 white bars, whereas only  
144 5% of 118 new recruits of the same age in *H. magnifica* had this pattern (Fig. 1 A and B, Test  
145  $\chi^2$  p-value=0.0011).

146

147 We tested by multiple regression whether sea anemone species affects the timing of white  
148 bar formation of *A. percula* new recruits from Kimbe bay, while accounting for ecological and  
149 social structure variables. These results confirm our observations that new recruits had  
150 consistently more bars in *S. gigantea* than in *H. magnifica* for a similar age or size (Fig. 1C  
151 and D, Supp Fig 1A and B and Supp Table 1-4).

152

153 As illustrated Fig. 1C and Supp. Fig. 1A, the speed at which bands were acquired varies with  
154 age (or with size) and how the acceleration and deceleration of band acquisition varied with  
155 age (or size) also depends of the anemone species. Thus, our results indicate that anemone  
156 species differentially modulate the dynamic to which bars were acquired with age (or size). In  
157 fact, available data allow us to detect differences between anemone species in the shape of  
158 the relationship between bars and age (or size), but more data would be needed to fully  
159 characterize the shape of these relationships.

160

### 161 **Adult color pattern formation is linked to a switch in pigment cell specific gene** 162 **expression**

163 Because we know that the sister species, *A. ocellaris* acquire their adult color pattern during  
164 metamorphosis (25, 26), we addressed whether TH is associated with developmental  
165 plasticity in color pattern using this species as a laboratory model (24, 25). *A. ocellaris*  
166 exhibits two pigmentation patterns during development: before stage 5 (around 9 dph; (25)),  
167 larvae have yellow larval xanthophores with a set of stellate larval melanophores forming two  
168 horizontal stripes covering the myotomes (Fig. 2A, B, C and D, red arrowheads). From stage

169 5, larvae acquire, in a rostro-caudal temporal gradient, three white vertical bars (Fig. 2E, F,  
170 G, white arrowheads), orange xanthophores outside of the future white bars (Fig. 2E, orange  
171 arrows) and melanophores dispersed all over the body (Fig. 2E and F, black arrows) (25,  
172 29). These melanophores are present over the body and are at higher density at the border  
173 of the white bars (Fig. 2F and G).

174 To better understand color pattern changes occurring around stage 4, we assessed the  
175 expression of pigmentation genes across post-embryonic stages. We extracted RNA from  
176 whole larvae at each of the seven *A. ocellaris* post-embryonic stages and performed  
177 transcriptomic analysis (29). We focused on pigmentation genes defined by (30, 31) (Fig. 2H,  
178 Supp Fig. 2A and Supp Table 5), and particularly on iridophore genes, as we showed  
179 previously that white bars are formed by iridophores (29) (Fig. 2I and J and Supp Table 5).  
180 We observed that stages 1 to 3 are clearly separated from stages 4 to 7 along principal  
181 component 2 (PC2) (Fig. 2 H and I). Among those genes, *fh12b*, *pnp4a* and *prkaca* have a  
182 highest fold difference at stages 5 to 7 compared to stages 1 to 3 whereas *gbx2*, *trim33*,  
183 *gmps* and *oca2* have a highest fold difference at stages 1 to 3 compared to stages 5 to 7  
184 (Fig. 2J). We also observed a clear separation across stages for all the functional categories  
185 described in (30) (Supp Fig. 2A): pigment cell specification (Supp Fig. 2B), xanthophore  
186 development (Supp Fig. 2C), pteridine pigment synthesis of xanthophores (Supp Fig. 2D), as  
187 well as melanophore development (Supp Fig. 2E), melanogenesis regulation (Supp Fig. 2F),  
188 and at a later stage, melanosome biogenesis (Supp Fig. 2G). These outcomes are consistent  
189 with changes across stages in pigmentation gene expression, complements of different  
190 pigment cell types, or likely both. They suggest that an important switch in the development  
191 of color pattern, involving each of the three pigment cells, occurs at stage 4.

192

### 193 **White bar formation is controlled by thyroid hormone signaling**

194 TH contributes to metamorphosis and the developmental program controlling pigmentation  
195 pattern in zebrafish and other teleosts (21, 32, 33). We hypothesized that TH regulates the  
196 timing of white bar formation during clownfish metamorphosis. To test this hypothesis, we  
197 exposed stage 3 larvae (5 dph) to different concentrations ( $10^{-6}$ ,  $10^{-7}$  and  $10^{-8}$  M) of the active  
198 thyroid hormone, T3. After 3 days of treatment with T3, we observed a more rapid  
199 appearance of white bars than in control larvae. This effect was dose-dependent with, at 3  
200 days post-treatment (dpt), 0 % of the fish exhibiting two bands in the control, 50% at  $10^{-8}$  M  
201 T3, 78% at  $10^{-7}$  M and 73% at  $10^{-6}$  M (Fig. 3A to D; Fig. 3E).

202 We then tested the effect of decreasing TH signaling by blocking TH production with a mix of  
203 goitrogens (34). Larvae treated from stage 3 (5 dph) had a delay in white bar development  
204 compared to controls at 9 dpt (Fig. 3H compared to the control Fig. 3G; Fig. 3F): whereas  
205 75% of controls had developed head and trunk white bars, only 15% of larvae treated with

206 MPI (Methimazol, Perchlorate Potassium and Iopanoic Acid) exhibited these bars and the  
207 remainder were devoid of any bars (Fig. 3F). It should be noted that after 25 days of  
208 treatment, white bars ultimately formed in MPI-treated fishes demonstrating that a delay,  
209 rather than blockade in bar formation is associated with TH inhibition (Fig. 3I).

210 Pigment cells other than iridophores were also affected by TH treatment, with melanophore  
211 numbers increasing significantly within 48 h of treatment with  $10^{-6}$  M T3 beginning at stage 3  
212 (5 dph) (Fig. 3J-  $p\text{-value}_{48\text{hpt}} = 0.0299$ ;  $p\text{-value}_{72\text{hpt}} = 0.0043$ ). In contrast, MPI treatments led  
213 only to a minor decrease (non-significant) in melanophore numbers at 48 or 72 hpt (Fig. 3J).  
214 We did not observe gross differences in xanthophore development, and it was not possible to  
215 identify individual xanthophores or to quantify their numbers.

216 Taken together, these results suggest that TH controls the timing of white bar formation  
217 relative to overall somatic development and may act on iridophores and melanophores.

218

### 219 **Expression of pigmentation genes is modified by T3 treatment**

220 To determine how TH affects iridophores, we assayed expression of iridophore genes (*fh12a*,  
221 *fh12b*, *apoda.1*, *saiyan* and *gpnmb*; 27) after treating larvae with exogenous TH. Stage 3  
222 larvae were treated with T3 at different concentrations ( $10^{-6}$ ,  $10^{-7}$  and  $10^{-8}$  M) for 12, 24, 48  
223 and 72 hours and expression of these genes was monitored by nanostring in RNA extracted  
224 from whole larvae. After T3 treatment, transcripts for all of these genes were significantly  
225 more abundant compared to levels in controls (Supp Fig. 3). In some cases (*apod1a* and  
226 *gpnmb*) this effect was evident by 12h and in others (*fh12a*, *fh12b* and *saiyan*) only after 24 or  
227 48h. This suggests that TH affects expression of genes known to be expressed in clownfish  
228 iridophores.

229

### 230 **Treatments with thyroid hormones or goitrogens lead respectively to ectopic 231 iridophores over the body and decrease in white hue in white bars**

232 To determine whether TH promotes iridophore differentiation, we treated stage 3 larvae with  
233 T3 at  $10^{-6}$  M for a longer period to compare juveniles at stage 6, when fish have developed  
234 both head and body bars. Interestingly, head and body bars were never fully formed in T3  
235 treated juveniles compared to controls (Supp Fig. 4D compared to Supp Fig. 4A) and close  
236 inspection of larvae revealed numerous ectopic iridophores across the flank of T3 treated fish  
237 (Supp Fig. 4F compared to Supp Fig. 4C- white arrowheads). Moreover, orange coloration  
238 was decreased in T3 treated juveniles compared to control (compare Supp Fig. 4B and Supp  
239 Fig. 4E). MPI treatment led to bars with normal shapes that were, nevertheless, more  
240 translucent presumably owing to deficiencies in the numbers of iridophores or the deposition  
241 of crystalline guanine within iridophores normally responsible for their white (or iridescent)  
242 appearance (N=2, Fig. 3I).



243

244 Together, these results indicate that exogenous TH leads to reduced orange coloration and  
245 defects in white bar formation accompanied by ectopic iridophores on the body, whereas  
246 blockade of TH production leads to a reduced number of white iridophores or reflective  
247 guanine within white bars.

248

249 **Ecological modulation in timing of white bar formation is linked to TH levels and *duox***  
250 **expression.**

251 As TH treatment accelerated white bar development in *A. ocellaris*, we asked whether the  
252 accelerated acquisition of bars in *A. percula* recruits in *S. gigantea* was linked to TH. We  
253 sampled a second set of new recruits of 12-27 mm (having one white bar either complete or  
254 being formed) living either in *S. gigantea* (N=6) or *H. magnifica* (N=6) and measured TH  
255 levels. Concentrations of T3 (in pg/ g of larvae) were significantly greater in new recruits  
256 sampled from *S. gigantea* compared to the those from *H. magnifica* (Fig. 4A).

257

258 To gain insight into mechanisms that explain these differences we compared gene  
259 expression between *A. percula* new recruits found in *H. magnifica* (n=3) or *S. gigantea* (n=3)  
260 by RNA-Seq of whole fish. Out of the 19063 analyzed genes, only 21 were significantly more  
261 expressed in new recruits from *S. gigantea* (adj. *P*-value < 0.05, Log<sub>2</sub>FC > 1), while 15 were  
262 significantly more expressed in new recruits from *H. magnifica* (adj. *P*-value < 0.05, Log<sub>2</sub>FC <  
263 1) (Fig. 4B, Supp. table 6). Within the differentially expressed genes, we observed *duox*,  
264 which encodes a dual oxidase implicated in TH production (27, 35). This gene was  
265 significantly overexpressed in new recruits from *S. gigantea* (adj. *P*-value = 0.038, Log<sub>2</sub>FC  
266 =2.53) compared to those from *H. magnifica*. Together, these results suggest that the rate of  
267 white bar formation in *A. percula* is linked to a differential level of T3, which is in turn linked to  
268 a differential expression of *duox*.

269

270 Last, we wanted to directly test whether *duox* is required for iridophore patterning. For this  
271 we used zebrafish *Danio rerio*, in which iridophores depend on TH for their maturation (22).  
272 *duox* requirements have been described for somatic development and melanophore  
273 numbers, but not iridophore pattern (27, 35). We therefore injected one-cell stage embryos of  
274 the iridophore reporter line Tg(*pnp4a:palm-mcherry*)<sup>wpr110Tg</sup> with highly efficient Alt-R CRISPR-  
275 Cas9 (36) targeting *duox*, resulting in phenotypes concordant with those for this locus (27,  
276 35) and other hypothyroid fish (21, 22). Figure 4C shows mCherry+ iridophores (dark cells;  
277 pixel values inverted) in representative uninjected (wild-type) and *duox*-deficient larvae of the  
278 same stage [10.6 mm standard length (SL)]. In wild-type, densely packed iridophores have  
279 formed one complete interstripe and a second interstripe has started to form ventrally; some

280 loosely arranged iridophores occur in between, where a melanophore stripe develops (37). In  
281 the *duox*-deficient larva only a single wider interstripe has developed and fewer stripe  
282 iridophores are visible (Fig. 4C) suggesting that iridophore development is slowed in *duox*-  
283 deficient animals. Consistent with this interpretation, most wild-type fish greater than 11.0  
284 mm SL had developed two complete interstripes (score=2.0), whereas equivalently staged  
285 *duox*-deficient fish had developed only one complete interstripe and were still developing a  
286 second interstripe (score=1.5) (Fig. 4D). Despite having fewer interstripes overall, *duox*-  
287 deficient animals had proportionally more of the flank covered by dense, interstripe  
288 iridophores, as compared to the wild type (Fig. 4E). These data show that *duox*, presumably  
289 acting through TH (27, 35), contributes to the timing of iridophore interstripe appearance and  
290 the patterning of interstripes in zebrafish.

291

292 To conclude, our findings suggest that reduced abundance of *duox* transcript in *A. percula*  
293 recruits within *H. magnifica* in comparison with those that are recruited in *S. gigantea* leads  
294 to a delay in the development of their white bars. This effect of *duox* in regulating the timing  
295 of iridophore development is conserved between the distantly related clownfish and  
296 zebrafish.

297

## 298 **Discussion**

299 During post-embryonic development, *A. ocellaris* lose their larval color pattern and acquire in  
300 a few days and in a rostro-caudal sequence the head, body and caudal peduncle white bars  
301 of their final adult color pattern. We showed here that during clownfish metamorphosis the  
302 formation of iridophore-containing white bars that are formed by iridophores is accelerated by  
303 TH, and that TH also underlie environmental (e.g. sea anemone species) plasticity in bar  
304 formation in wild populations. Interestingly a corresponding effect on iridophore patterning  
305 was also seen in zebrafish: *duox* mutants are hypothyroid (27, 35), and we found that  
306 iridophore patterning of *duox*-deficient animals was delayed. All these data converge toward  
307 the notion that variations in TH levels control a plastic pigmentation phenotype observed in  
308 clownfishes.

309

310 The observation that in both clownfish and zebrafish, TH affects white bar (clownfish) or  
311 interstripe (zebrafish) formation strongly suggests that these hormones directly or indirectly  
312 act on iridophores. Previous studies revealed that TH deficiency in zebrafish leads to an  
313 excess of melanophores and a loss of visible xanthophores (21). Further analyses showed  
314 that these hormones act differently on these two cell types, promoting maturation but via  
315 distinct mechanisms. TH promotes terminal differentiation and limits the final number of  
316 melanophores whereas it promotes accumulation of carotenoid pigments in xanthophores,  
317 making initially unpigmented precursors visible. A similar role for TH in promoting iridophore  
318 maturation was suggested by analyses of single cell transcriptomic states, though  
319 consequences for iridophore number and pattern were not assessed (22). In our analysis we  
320 observed that interstripe development is slowed in *duox*-deficient animals and that *duox*-  
321 deficient animals had proportionally more of the flank covered by dense, interstripe-  
322 iridophores, as compared to the wild type. Together these several observations support the  
323 idea that TH signaling has an evolutionarily conserved role in regulating the timing of  
324 iridophore development in two species having markedly different adult pigment patterns. TH  
325 receptors are expressed in iridophores of both species but analyses to date cannot indicate  
326 whether effects of TH are direct or mediated through other cell types (22).

327

328 We also observed an effect of TH on the shape of the trunk white bars in clownfish. Indeed,  
329 late in TH treated fishes, we observed abnormalities in this trunk white bar that is misshapen  
330 and incomplete (e.g. it does not cross the full body of the fish; see Supp Figure 4D). This is  
331 interesting as a similar phenotype is often observed in clownfish juveniles raised in the

332 laboratory and has been assumed to result from nutritional defects (38–40). In addition to  
333 abnormalities in the shape of white bars, we observed ectopic iridophores. We cannot  
334 exclude at this point that the defects in white bar shape could be linked to a role of TH on  
335 pigment cells migration.

336 We have observed that *A. percula* developing in association with *S. gigantea* acquire white  
337 bars and have higher levels of T3, than *A. percula* in *H. magnifica*. This difference can be  
338 explained by higher expression of *duox* by *A. percula* recruited in *S. gigantea* as compared to  
339 *A. percula* recruited in *H. magnifica*. Indeed, *duox*, encodes a dual oxidase that has been  
340 implicated in TH production both in mammals and zebrafish (27, 35). Beyond the effects of  
341 *duox* inactivation we observed on zebrafish iridophore patterning, *duox* mutants have growth  
342 retardation, ragged fins, thyroid hyperplasia and infertility and, a pigmentation phenotype with  
343 increased melanophore and reduced xanthophore (27, 35) typical of hypothyroid fish (21). As  
344 shown by Chopra *et al.*, some of these defects can be rescued with T4 treatment, even when  
345 initiated in adult fish (27). All these data allow us to suggest that in young juveniles which are  
346 recruited in *S. gigantea* there is an increased expression of *duox* that led to a higher TH level  
347 and a higher rate of white bar formation.

348 The results of our study leave two major questions unanswered: why is there an increased  
349 *duox* expression in *S. gigantea* recruits, and is there ecological significance to faster white  
350 bar formation in those fish. The regulation of *duox* gene expression in fish is still poorly  
351 known but it has been shown that *Duox1* and *Duox2* expression in mammals is tightly  
352 controlled and regulated by TSH, that is the hypothalamo-pituitary-thyroid axis (41). As *S.*  
353 *gigantea* has been shown to be a much more toxic sea anemone than *H. magnifica* by  
354 hemolytic and neurotoxicity assays (42), it is conceivable that clownfish recruited in this sea  
355 anemone perceive this harsher environment and hence activate their neuroendocrine axis to  
356 compensate. It is important to note in that respect that several anemonefish adults (*A.*  
357 *percula* but also *A. clarki*, *A. polymnus* or *A. chrysopterus*) exhibit a similar polymorphic  
358 melanistic morph when present in *Stichodactyla* vs. *Heteractis* (43). It is tempting to propose  
359 that these melanistic morphs are also linked to TH signaling in these species. The white bar  
360 phenotype we discussed here is therefore likely to be only one of a series of changes linked  
361 to the differential recruitment in various sea anemone species that allow the physiological  
362 adjustment of the fish in these distinct environments (44). However, the adaptative  
363 significance of this plastic phenotype is still only a hypothesis that remains to be tested  
364 experimentally in the field (44). It is interesting to note that *A. ocellaris* can also live in the  
365 same two sea anemone species but does not exhibit a melanistic morph when present in  
366 *Stichodactyla* (45). The rate of white bar appearance in young recruits of *A. ocellaris* living in  
367 the two sea anemone species is unknown. It will be interesting to study in the future the

368 differences in pigmentation plasticity between the two sister species, *A. ocellaris* and *A.*  
369 *percula*.

370 In conclusion, our study of white bar formation in clownfish highlight the interest of this new  
371 emerging system to investigate the cellular, molecular endocrine and developmental basis of  
372 alternative phenotypes that are detected in natural situation (24, 46). Combining analysis in  
373 the wild as well as in the lab, as we have done here using clownfish as model, offers great  
374 promises to understand the evolutionary and developmental basis of plastic phenotypes  
375 often observed in nature.

## 376 **Acknowledgments**

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380 Romans from the Aquariology Service at Observatoire Océanologique de Banyuls-Sur-Mer  
381 for expert technical help for clownfish husbandry. We also thank the Centre de Recherches  
382 en Cancérologie de Toulouse (CRCT UMR 1037 INSERM, Plateau Génomique et  
383 Transcriptomique) for the Nanostring experiments.

384

385

386

## 387 **Materials and methods**

388

389 See extended methods provided in *SI Appendix*.

390

391 **A. ocellaris larval rearing and ethics.** *A. ocellaris* were maintained as described in (25).  
392 We have approval for these experiments from the C2EA - 36 Ethics Committee for Animal  
393 Experiment Languedoc-Roussillon (CEEA-LR), number A6601601. The experimental  
394 protocols were following French regulation.

395

396 **RNA extraction and transcriptomic analysis.** Transcriptomic data of developmental stages  
397 of *A. ocellaris* larvae were taken from the transcriptomic analysis of *A. ocellaris* post-  
398 embryonic stages performed in (29). For more information see *SI Appendix*. Individuals of *A.*  
399 *percula* new recruits were sampled, euthanized in a MS222 solution (200 mg/l) and  
400 conserved in RNAlater. Total RNA of each individual was extracted using (Trizol Reagent  
401 15596-026 kit, Ambion) followed by DNase treatment (DNA-free AM1906 kit, Ambion) and  
402 then purified with 0.025 µm dialysis membranes. RNA-Seq libraries and sequencing were  
403 performed on a Illumina HiSeq4000 sequencer using a stranded protocol as Paired-end 50  
404 base reads. Transcriptomic analysis is described in *SI Appendix*.

405

406 **Drug treatment of *A. ocellaris* larvae.** T3 (3,3',5-Triiodo-L-thyronine) and IOP (Iopanoic  
407 Acid) were both diluted in dimethylsulphoxyde (T3: T2877, IOP: 14131, DMSO: D8418;  
408 Sigma-Aldrich Louis, MI, USA) to a final concentration 1 mM. To analyze the effect of a  
409 reduction of TH signaling we used a mix of goitrogens called MPI (Methimazole, Potassium  
410 Perchlorate and Iopanoic Acid) as in reference (47). Methimazole, Potassium Perchlorate  
411 and IOP (Methimazole: M8506, Potassium perchlorate 460494, Sigma-Aldrich Louis, MI,  
412 USA) were also diluted in DMSO to a respective final concentration of 100 mM, 10 mM and 1  
413 mM. Larvae were treated from 5 until 18 dph in 0.005% DMSO with T3+IOP at  $10^{-6}$ ,  $10^{-7}$  and  
414  $10^{-8}$  M (respective dilutions of 1/1000, 1/10000 or 1/100000) or MPI (dilution of 1/1000) or  
415 without (controls). For each condition, five larvae were treated in 500-mL fish medium in a  
416 beaker. One hundred milliliters of solution were changed every day.

417

418 **Nanostring gene expression analysis.** 400 ng of total RNA were analyzed using the  
419 Nanostring Counter. Each sample was analyzed in a separate multiplexed reaction including  
420 8 negative probes and 6 serial concentrations of positive control probes. Data were imported

421 into nSolver software (version 2.5) for quality checking and data normalization according to  
422 NanoString guidelines. Analysis was done using the R package TTCA1 (R version 3.5.1).

423 **Effect of ecological factors on the number of bars in new recruits of *A. percula*.** At the  
424 time of the sampling in Kimbe bay (5°12'22.56" S, 150°22'35.58" E), West New Britain Prov-  
425 ince, Papua New Guinea, we characterized the new recruit size, age (see SI appendix) eco-  
426 logical variables (geographic zone, primary host anemone species, depth), and the social  
427 structure of the new recruits within its sea anemone (total number of conspecifics inhabiting  
428 the sea anemone, size difference between the new recruit and the last subadult in the social  
429 hierarchy, female size) (28, 48). In the studied *A. percula* colonies located in Kimbe 43% are  
430 in *S. gigantea* and 57% in *H. magnifica*. To assess what factors affect the number of bars on  
431 new recruits, we modelled the number of bars as a response variable depending upon either  
432 size or age, their squared value, and ecological and social structure independent variables.  
433 We followed a multi-model inference approach (49, 50) to estimate predictors effect sizes  
434 and their 85% confidence interval (51). This approach was conducted independently in each  
435 anemone species to avoid confounding effects between anemone species and depth (see  
436 Supp. Methods for details of the statistical analysis). All analyses were performed with the  
437 MuMIn v1.43.6 package (52) in the statistical software R v3.6.3 (53).  
438

439 **Thyroid hormones extraction and dosage.** TH were extracted individuals from *A. percula*  
440 new recruits sampled in Kimbe Island, dry-frozen (previously euthanized in a 200 mg/l  
441 solution of MS-222) following the protocol described in (32). More details are described in *SI*  
442 *Appendix*.  
443

444 **Zebrafish *duox* CRISPR-Cas9.** Zebrafish *D. rerio* were reared under standard conditions  
445 (28 °C, 14L:10D) and staged according to (54). Embryos Tg(*pnp4a:palm-mcherry*)<sup>wprt10Tg</sup>  
446 expressing membrane-targeted mCherry (mem-Cherry) (55, 56) were injected at the one-cell  
447 stage with Alt-R CRISPR-Cas9 (36) targeting *duox*, and reared on a thyroid hormone free  
448 diet of brine shrimp and marine rotifers (21). Images of *duox* AltR-injected fish and uninjected  
449 controls were acquired on a Zeiss Axio Observer inverted microscope equipped with a  
450 Yokogawa CSU-X1M5000 laser spinning disk with Hamamatsu ORCA-Flash 4.0 camera.  
451 Regions of interest were defined by the anterior and posterior margin of the anal fin, and  
452 proportional coverage of dense interstripe iridophores relative to this region of interest were  
453 analyzed using ImageJ software. Numbers of completed or developing interstripes were  
454 scored qualitatively. Display levels were adjusted and inverted for visualization in Adobe  
455 Photoshop 2021.

457 **References**

- 458 1. M. J. West-Eberhard, Developmental plasticity and the origin of species differences.  
459 *Proc. Natl. Acad. Sci.* **102**, 6543–6549 (2005).
- 460 2. O. Leimar, Environmental and genetic cues in the evolution of phenotypic  
461 polymorphism. *Evol. Ecol.* **23**, 125–135 (2009).
- 462 3. B. Taborsky, *Developmental Plasticity: Preparing for life in a complex world* (Elsevier  
463 Ltd, 2017).
- 464 4. D. W. Pfennig, *et al.*, Phenotypic plasticity's impacts on diversification and speciation.  
465 *Trends Ecol. Evol.* **25**, 459–467 (2010).
- 466 5. H. F. Nijhout, Development and evolution of adaptive polyphenisms. *Evol. Dev.* **5**, 9–  
467 18 (2003).
- 468 6. E. Hammill, A. Rogers, A. Beckerman, Costs, benefits and the evolution of inducible  
469 defences: a case study with *Daphnia pulex*. *J. Evol. Biol.* **21**, 705–715 (2008).
- 470 7. S. S. Kulkarni, R. J. Denver, I. Gomez-Mestre, D. R. Buchholz, Genetic  
471 accommodation via modified endocrine signalling explains phenotypic divergence  
472 among spadefoot toad species. *Nat. Commun.* **8**, 993 (2017).
- 473 8. S. F. Gilbert, Mechanisms for the environmental regulation of gene expression:  
474 ecological aspects of animal development. *J. Biosci.* **30**, 65–74 (2005).
- 475 9. N. Aubin-Horth, S. Renn, Genomic reaction norms: using integrative biology to  
476 understand molecular mechanisms of phenotypic plasticity. *Mol. Ecol.* **18**, 3763–3780  
477 (2009).
- 478 10. S. F. Gilbert, D. Epel, *Ecological Developmental Biology: The Environmental  
479 Regulation of Development, Health and Evolution*. Sinauer Associates (2015) pp. 576.
- 480 11. A. C. Price, C. J. Weadick, J. Shim, F. H. Rodd, Pigments, patterns, and fish behavior.  
481 *Zebrafish* **5**, 297–307 (2008).
- 482 12. P. D. Dijkstra, *et al.*, The melanocortin system regulates body pigmentation and social  
483 behaviour in a colour polymorphic cichlid fish. *Proc. R. Soc. B Biol. Sci.* **284**,  
484 20162838 (2017).
- 485 13. Z. W. Culumber, Variation in the evolutionary integration of melanism with behavioral  
486 and physiological traits in *Xiphophorus variatus*. *Evol. Ecol.* **30**, 9–20 (2016).
- 487 14. L. Jacquin, *et al.*, Melanin in a changing world: brown trout coloration reflects  
488 alternative reproductive strategies in variable environments. *Behav. Ecol.* **28**, 1423–  
489 1434 (2017).
- 490 15. F. Cortesi, *et al.*, Phenotypic plasticity confers multiple fitness benefits to a mimic.  
491 *Curr. Biol.* **25**, 949–954 (2015).



- 492 16. V. Laudet, The origins and evolution of vertebrate metamorphosis. *Curr. Biol.* **21**,  
493 R726–R737 (2011).
- 494 17. S. K. McMenamin, D. M. Parichy, “Metamorphosis in Teleosts” in *Current Topics in*  
495 *Developmental Biology*, (2013), pp. 127–165.
- 496 18. M. A. Campinho, Teleost metamorphosis: the role of thyroid hormone. *Front.*  
497 *Endocrinol. (Lausanne)*. **10** (2019).
- 498 19. S. C. Lema, Hormones, developmental plasticity, and adaptive evolution: Endocrine  
499 flexibility as a catalyst for ‘plasticity-first’ phenotypic divergence. *Mol. Cell. Endocrinol.*  
500 **502**, 110678 (2020).
- 501 20. L. B. Patterson, D. M. Parichy, Zebrafish Pigment Pattern Formation: Insights into the  
502 Development and Evolution of Adult Form. *Annu. Rev. Genet.* **53**, 505–530 (2019).
- 503 21. S. K. McMenamin, *et al.*, Thyroid hormone-dependent adult pigment cell lineage and  
504 pattern in zebrafish. *Science (80-. )*. **345**, 1358–1361 (2014).
- 505 22. L. M. Saunders, *et al.*, Thyroid hormone regulates distinct paths to maturation in  
506 pigment cell lineages. *Elife* **8** (2019).
- 507 23. G. Litsios, *et al.*, Mutualism with sea anemones triggered the adaptive radiation of  
508 clownfishes. *BMC Evol. Biol.* **12**, 212 (2012).
- 509 24. N. Roux, P. Salis, S.-H. Lee, L. Besseau, V. Laudet, Anemonefish, a model for Eco-  
510 Evo-Devo. *Evodevo* **11**, 20 (2020).
- 511 25. N. Roux, *et al.*, Staging and normal table of postembryonic development of the  
512 clownfish ( *Amphiprion ocellaris* ). *Dev. Dyn.* **248**, 545–568 (2019).
- 513 26. P. Salis, *et al.*, Ontogenetic and phylogenetic simplification during white stripe  
514 evolution in clownfishes. *BMC Biol.* **16**, 90 (2018).
- 515 27. K. Chopra, S. Ishibashi, E. Amaya, Zebrafish *duox* mutations provide a model for  
516 human congenital hypothyroidism. *Biol. Open* **8**, bio037655 (2019).
- 517 28. O. C. Salles, *et al.*, First genealogy for a wild marine fish population reveals  
518 multigenerational philopatry. *Proc. Natl. Acad. Sci.* **113**, 13245–13250 (2016).
- 519 29. P. Salis, *et al.*, Developmental and comparative transcriptomic identification of  
520 iridophore contribution to white barring in clownfish. *Pigment Cell Melanoma Res.* **32**,  
521 391–402 (2019).
- 522 30. T. Lorin, F. G. Brunet, V. Laudet, J.-N. Volff, Teleost fish-specific preferential retention  
523 of pigmentation gene-containing families after whole genome duplications in  
524 Vertebrates. *G3; Genes|Genomes|Genetics* **8**, 1795–1806 (2018).
- 525 31. I. Braasch, F. Brunet, J.-N. Volff, M. Schartl, Pigmentation pathway evolution after  
526 whole-genome duplication in fish. *Genome Biol. Evol.* **1**, 479–493 (2009).
- 527 32. G. Holzer, *et al.*, Fish larval recruitment to reefs is a thyroid hormone-mediated  
528 metamorphosis sensitive to the pesticide chlorpyrifos. *Elife* **6** (2017).

- 529 33. Y. Inui, S. Miwa, Thyroid hormone induces metamorphosis of flounder larvae. *Gen. Comp. Endocrinol.* **60**, 450–454 (1985).
- 530
- 531 34. S. Remaud, *et al.*, Transient hypothyroidism favors oligodendrocyte generation  
532 providing functional remyelination in the adult mouse brain. *Elife* **6** (2017).
- 533 35. J.-S. Park, *et al.*, Targeted knockout of duox causes defects in zebrafish growth,  
534 thyroid development, and social interaction. *J. Genet. Genomics* **46**, 101–104 (2019).
- 535 36. K. Hoshijima, *et al.*, Highly efficient CRISPR-Cas9-based methods for generating  
536 deletion mutations and F0 embryos that lack gene function in zebrafish. *Dev. Cell* **51**,  
537 645-657.e4 (2019).
- 538 37. D. Gur, *et al.*, In situ differentiation of iridophore crystalotypes underlies zebrafish  
539 stripe patterning. *Nat. Commun.* **11**, 6391 (2020).
- 540 38. J. G. Eales, The influence of nutritional state on thyroid function in various vertebrates.  
541 *Am. Zool.* **28**, 351–362 (1988).
- 542 39. D. S. MacKenzie, C. M. VanPutte, K. A. Leiner, Nutrient regulation of endocrine  
543 function in fish. *Aquaculture* **161**, 3–25 (1998).
- 544 40. K. A. Leiner, D. S. Mackenzie, Central regulation of thyroidal status in a teleost fish:  
545 Nutrient stimulation of T4 secretion and negative feedback of T3. *J. Exp. Zool.* **298A**,  
546 32–43 (2003).
- 547 41. M. Milenkovic, *et al.*, Duox expression and related H2O2 measurement in mouse  
548 thyroid: onset in embryonic development and regulation by TSH in adult. *J. Endocrinol.*  
549 **192**, 615–626 (2007).
- 550 42. A. M. Nedosyko, J. E. Young, J. W. Edwards, K. Burke da Silva, Searching for a Toxic  
551 Key to Unlock the Mystery of Anemonefish and Anemone Symbiosis. *PLoS One* **9**,  
552 e98449 (2014).
- 553 43. T. A. Militz, M. I. McCormick, D. S. Schoeman, J. Kinch, P. C. Southgate, Frequency  
554 and distribution of melanistic morphs in coexisting population of nine clownfish species  
555 in Papua New Guinea. *Mar. Biol.* **163**, 200 (2016).
- 556 44. A. Ducrest, L. Keller, A. Roulin, Pleiotropy in the melanocortin system, coloration and  
557 behavioural syndromes. *Trends Ecol. Evol.* **23**, 502–510 (2008).
- 558 45. K. Hayashi, K. Tachihara, J. D. Reimer, Patterns of coexistence of six anemonefish  
559 species around subtropical Okinawa-jima Island, Japan. *Coral Reefs* **37**, 1027–1038  
560 (2018).
- 561 46. P. Salis, T. Lorin, V. Laudet, B. Frédérick, Magic traits in magic fish: understanding  
562 color pattern evolution using reef fish. *Trends Genet.* **35**, 265–278 (2019).
- 563 47. H. Dong, *et al.*, Transient Maternal Hypothyroxinemia Potentiates the Transcriptional  
564 Response to Exogenous Thyroid Hormone in the Fetal Cerebral Cortex Before the  
565 Onset of Fetal Thyroid Function: A Messenger and MicroRNA Profiling Study. *Cereb.*

566 *Cortex* **25**, 1735–1745 (2015).

567 48. M. L. Berumen, *et al.*, Otolith geochemistry does not reflect dispersal history of  
568 clownfish larvae. *Coral Reefs* **29**, 883–891 (2010).

569 49. K. P. Burnham, D. R. Anderson, *Model selection and multimodel inference* (Springer-  
570 Verlag New York, 2002).

571 50. M. R. E. Symonds, A. Moussalli, A brief guide to model selection, multimodel inference  
572 and model averaging in behavioural ecology using Akaike’s information criterion.  
573 *Behav. Ecol. Sociobiol.* **65**, 13–21 (2011).

574 51. H. Schielzeth, Simple means to improve the interpretability of regression coefficients.  
575 *Methods Ecol. Evol.* **1**, 103–113 (2010).

576 52. K. Bartoń, MuMIn: Multi-Model Inference, Version 1.43.6. *R Packag.* (2019).

577 53. R Core Team (2020), R: A language and environment for statistical computing. *R A*  
578 *Lang. Environ. Stat. Comput. R Found. Stat. Comput. Vienna, Austria* (2020).

579 54. D. M. Parichy, M. R. Elizondo, M. G. Mills, T. N. Gordon, R. E. Engeszer, Normal table  
580 of postembryonic zebrafish development: Staging by externally visible anatomy of the  
581 living fish. *Dev. Dyn.* **238**, 2975–3015 (2009).

582 55. D. S. Eom, E. J. Bain, L. B. Patterson, M. E. Grout, D. M. Parichy, Long-distance  
583 communication by specialized cellular projections during pigment pattern development  
584 and evolution. *Elife* **4** (2015).

585 56. J. E. Spiewak, *et al.*, Evolution of Endothelin signaling and diversification of adult  
586 pigment pattern in Danio fishes. *PLOS Genet.* **14**, e1007538 (2018).

587

588

## 589 **Figure legends**

590

### 591 **Figure 1. Formation of white bars of *Amphiprion percula* new recruits is differentially** 592 **influenced by age depending on the anemone species**

593 (A-B) Histograms representing percentage of new recruits having 1 (blue), 2 (green) or 3  
594 white bars (yellow) depending on their age, in new recruits living in *H. magnifica* (A) or *S.*  
595 *gigantea* (B). Statistical tests were done using  $\chi^2$  tests at each age between *H. magnifica* or  
596 *S. gigantea* and show statistical difference at 150-200 dph and 200-250 dph (respective p-  
597 value: 0.0032 and 0.0011). (C) Number of bars (85% CI) depending on age of individuals  
598 predicted from full averaging of the model candidates (D). Blue and orange represent  
599 respectively *A. percula* new recruits sampled in *H. magnifica* and in *S. gigantea*. Dots are  
600 observed data and are shifted around their number of bars for graphical representation.  
601 Predicted regressions of the number of bars are presented for the reference level “lagoon 0”.  
602 (D) Full model averaged estimates (85% CI) of linear regression parameters from models  
603 including age for each anemone species. Parameter estimates after model averaging of  
604 treatment were compared with “Lagoon 1” as reference for the geographic zone. A  
605 parameter estimate whose 85% CI includes zero is considered uncertain and parameter  
606 estimates whose 85% CI do not overlap are considered different.

607

### 608 **Figure 2. Adult color pattern formation in *Amphiprion ocellaris* is linked to a switch in** 609 **expression of pigment cells specific genes during post-embryonic development**

610 (A-B) Stereomicroscope images of entire larvae and the associated zoom of the trunk at  
611 stage 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F) and 7 (G) (Adapted from (25)). White and red  
612 arrowheads point white bars and black larval melanophores, black and orange arrows point  
613 respectively melanophores and xanthophores. (H and I) PCA analysis of the pigmentation  
614 genes (H) and iridophores genes (I) expression from transcriptomic analysis from entire  
615 larvae over post-embryonic stages. The two PCA exhibit a clear separation between stage 1  
616 to 3 and stage 4 to 7. The ellipses were arbitrarily drawn around arrays to help resolution:  
617 stages 1 to 3 (orange) and 4 to 7 (blue) arrays. All stages had 3 replicates. (J) Heatmap of  
618 the 7 iridophore genes having the highest fold change between stage 1-3 and stages 5-7.  
619 Color represents the intensity of the centered (but unscaled) signal that goes, for each gene,  
620 from low (blue) to medium (white) to high (red).

621

622

623

624 **Figure 3. White bars in *Amphiprion ocellaris* form earlier and later respectively after**  
625 **treatments with thyroid hormones or goitrogens**

626 (A-D) Stereomicroscope images of larvae treated at stage 3 during 3 days (dpt) in DMSO (A)  
627 or T3 at  $10^{-6}$  M (B),  $10^{-7}$  M (C) and  $10^{-8}$  M (D).

628 (E-F) Histogram showing the percentage of larvae having 0 (red), 1 (blue), 2 (green) or 3  
629 (yellow) white bars. (E) Larvae are treated at stage 3 for 3 days with DMSO, T3  $10^{-6}$  M,  $10^{-7}$   
630 M and  $10^{-8}$  M (nDMSO=16, nT3  $10^{-8}$  M=20, nT3  $10^{-7}$  M=18, nT3  $10^{-6}$  M=15 individuals). Chi2  
631 tests are significant between T3  $10^{-6}$  M and DMSO (p-value <0.0001). (F) Larvae are treated  
632 at stage 3 for 9 days with DMSO or MPI  $10^{-6}$  M (nDMSO=12, nMPI  $10^{-6}$  M= 13 individuals).  
633 Statistical test was done using  $\chi^2$  tests (p-value <0.0029).

634 (G-I) Stereomicroscope images of larvae treated at stage 3 during 9 days in DMSO (G) and  
635 MPI  $10^{-6}$  M (H) and MPI  $10^{-6}$  M stage 3 larvae treated for 25 days (I).

636 (J) Graphic showing the number of melanophores in a specific area of the trunk in DMSO  
637 (black), T3  $10^{-6}$  M (green) and MPI  $10^{-6}$  M (red) at 12, 24, 48 and 72 hpt (nDMSO>9, nT3>9,  
638 nMPI>9 individuals). Statistical tests were done using ANOVA between the T3 or MPI  
639 treatments and DMSO (control) at each time. Tests are significant between T3 and DMSO at  
640 48 hpt and 72 hpt (p-value are respectively equal to 0.0299 and 0.0043).

641 Bars correspond to the mean and crosses correspond to one experiment. hpt = hours post  
642 treatment. Scale bar = 1mm.

643

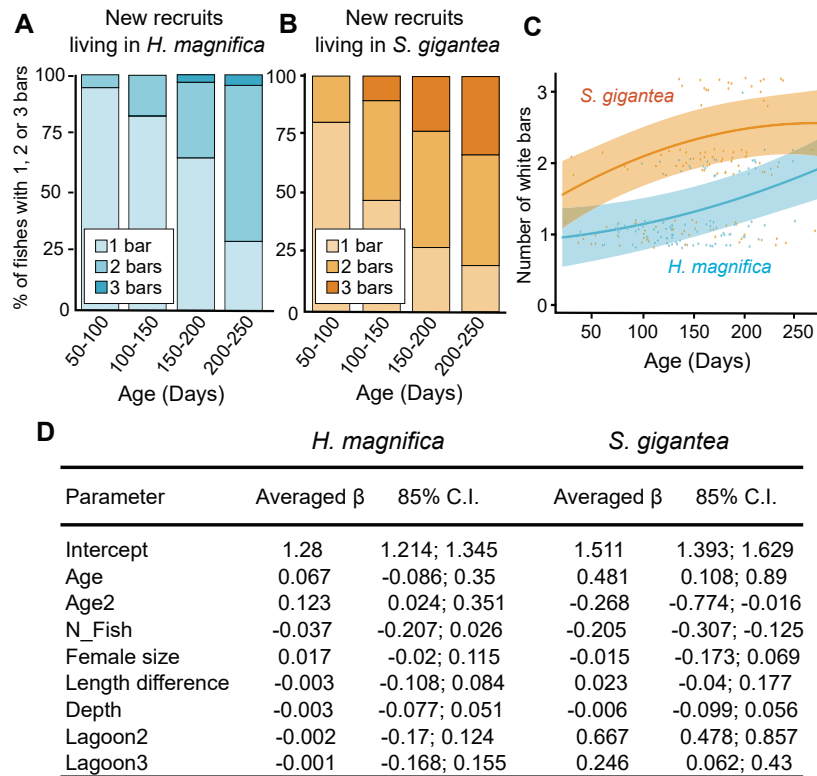
644 **Figure 4. *duox* requirement for the timing of color pattern formation in zebrafish**

645 (A) Graph representing T3 level (in pg.g<sup>-1</sup> of fish) in *A. percula* new recruits sampled in *H.*  
646 *magnifica* or *S. gigantea* (Non-parametric Mann Whitney Test, p-value=0.0022). (B) Volcano  
647 plot of differentially expressed genes between new recruits living in *H. magnifica* or *S.*  
648 *gigantea*. Positive Log2FC values correspond to an increased expression in recruits from *S.*  
649 *gigantea*, while negative Log2FC corresponds to increased expression in recruits from *H.*  
650 *magnifica*. Green and red points correspond to significantly differentially expressed genes.  
651 The vertical black lines delimit the Log2FC threshold of 1, while the horizontal line  
652 corresponds to the corrected p-value threshold. (C) Inverted fluorescence images show  
653 iridophores (dark cells) marked by *pnp4a:mem-mCherry* expression at 10.6 mm standard in  
654 wild-type (left) and *duox* CRISPR/Cas9 mutants (Right). (D) Numbers of interstripes were  
655 scored qualitatively over Standard Length (SL) in wild-type (blue, N=61) and *duox*  
656 CRISPR/Cas9 mutants (yellow, N=51). Complete interstripes received a score of 1 and  
657 developing interstripes received a score of 0.5. Each circle represents a single individual and  
658 points are jittered vertically for clarity, and equivalently smoothed splines are shown for ease  
659 of visualization. Differences in total numbers of interstripes and trajectories of interstripe  
660 addition resulted in significant effects of genotype (likelihood ratio test,  $\chi^2=91.7$ , p-

661 value<0.0001, d.f. = 1) and genotype x standard length interaction ( $\chi^2=21.9$ , p-value<0.0001,  
662 d.f. = 1). (E) Despite having fewer interstripes overall, *duox*-deficient animals had  
663 proportionally more of the flank covered by dense, interstripe-iridophores, as compared to  
664 the wild type ( $F_{1,43}=76.1$ ,  $P<0.0001$ ). Bars indicate means  $\pm$  95% confidence intervals. Scale  
665 bar in A = 200  $\mu\text{m}$ .

666

667

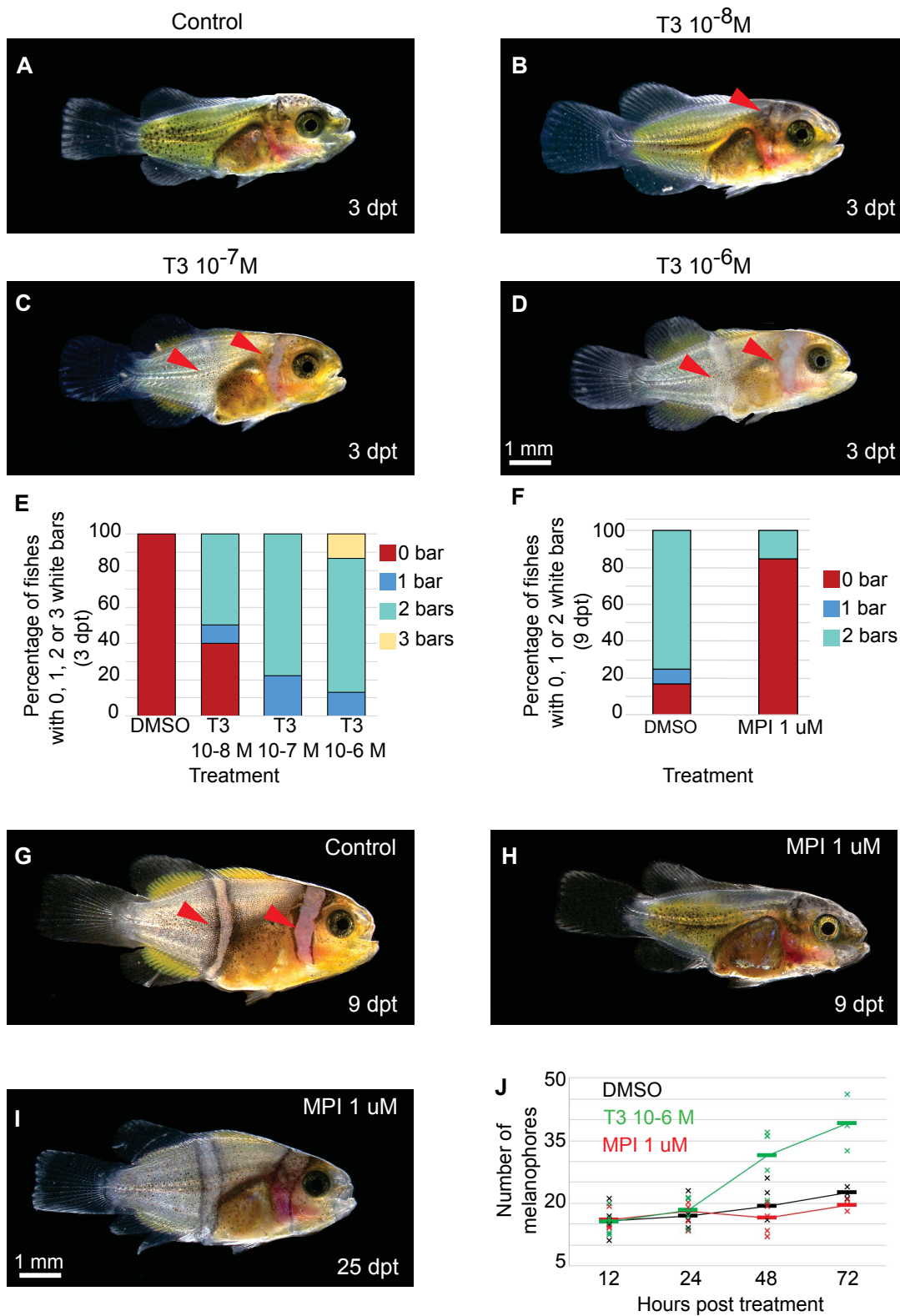


Salis et al. Fig 1





Salis et al.,  
Fig 3



Salis et al.,  
Fig 4

