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► To cite this version:

Pauline Salis, Natacha Roux, Delai Huang, Anna Marcionetti, Pierick Mouginot, et al.. Thyroid hormones regulate the formation and environmental plasticity of white bars in clownfishes. *Proceedings of the National Academy of Sciences of the United States of America*, 2021, 118 (23), pp.e2101634118. 10.1073/pnas.2101634118 . hal-03368778

HAL Id: hal-03368778

<https://hal.science/hal-03368778v1>

Submitted on 5 Nov 2021

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1 **Thyroid hormones regulate the formation and environmental**

2 **plasticity of white bars in clownfishes**

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21

22 Keywords: Pigmentation, Developmental plasticity, Clownfishes, Thyroid Hormones,
23 Metamorphosis

24

25 Classifications: Biological sciences- Developmental Biology

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 χ^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, Log₂FC > 1), while 15 were
262 significantly more expressed in new recruits from *H. magnifica* (adj. *P*-value < 0.05, Log₂FC <
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, Log₂FC
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*)^{wpr110Tg} 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**

377 This study was supported by Agence Nationale de la Recherche (ANR-19-CE34-
378 0006-Manini and ANR-19-CE14-0010-SENSO) as well as by National Institute of Science
379 (NIH R35 GM122471). We thank Valentin Logeux, Remi Pillot, Nancy Trouillard and Pascal
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*)^{wprt10Tg}
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.

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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 χ^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 χ^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

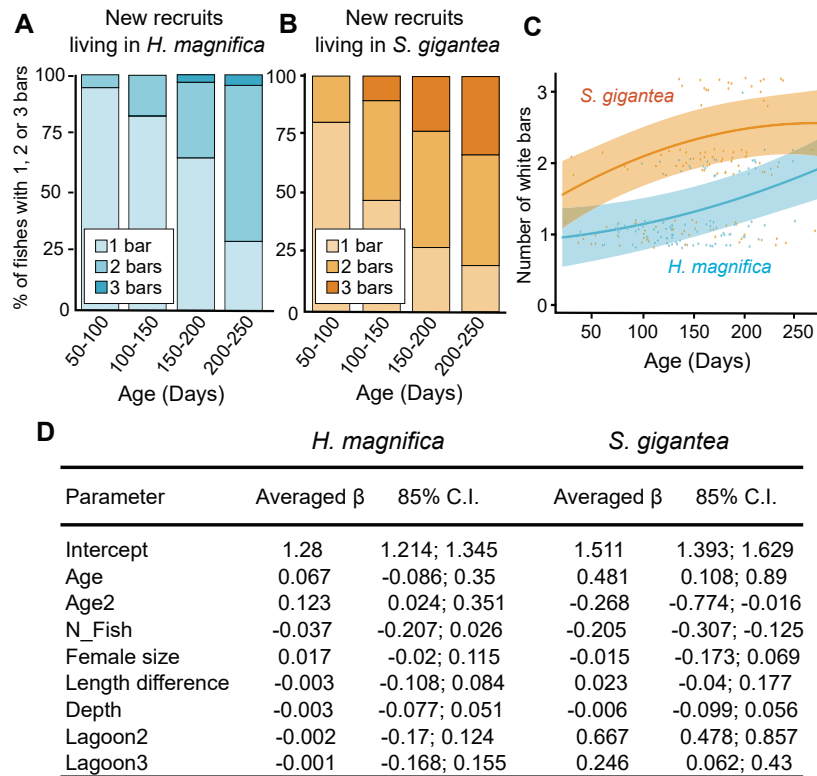
644 **Figure 4. *duox* requirement for the timing of color pattern formation in zebrafish**

645 (A) Graph representing T3 level (in pg.g⁻¹ 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 μm .

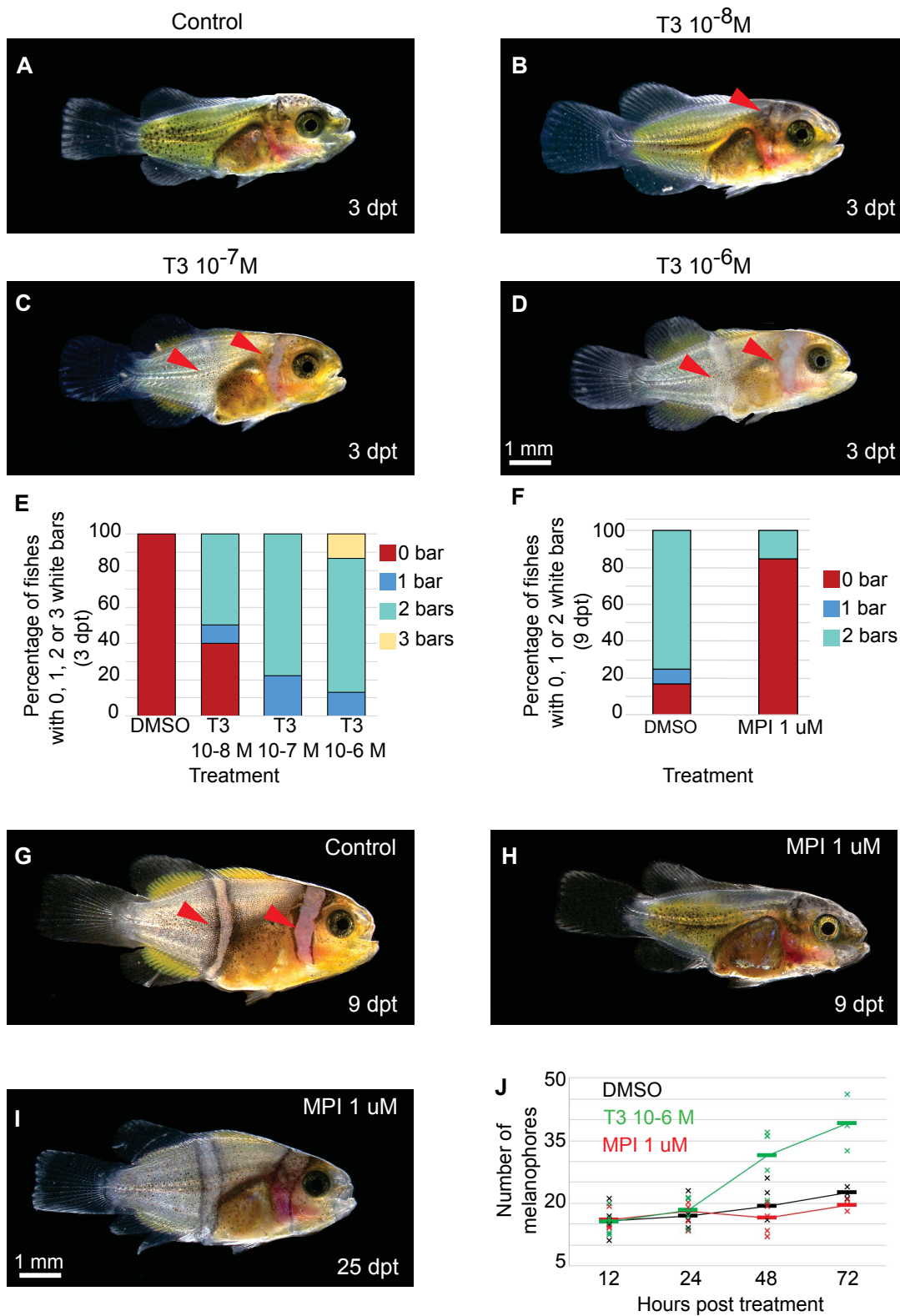
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Salis et al. Fig 1

Salis et al.,
Fig 3



Salis et al.,
Fig 4

