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Iodine indirect effect on thyroid gland: structure and hormone receptor (TRα) in common sole, *Solea solea* larvae

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Abstract
The effect of feeding *Solea solea* with *Artemia* enriched in iodine during metamorphosis was evaluated. In the present study, three treatment groups were used; i) control fish fed with untreated *Artemia*, ii) low dose (T_{Low}), in which sole were fed with instar I *Artemia* exposed to potassium iodate® (0.1g IKO3/L) for 3 hours and iii) high dose (T_{high}), in which sole were fed with instar I *Artemia* exposed to potassium iodate® (0.2 g IKO_3/L,). Growth performance, thyroid follicles (localization and morphometric analysis), and thyroid hormone receptor (TRα) were measured. A significant increase in the expression of thyroid hormone receptor type 1 (TRα) occurred in the iodine supplemented larvae and was 3.55±1.13 fold higher than the control. The thyroid gland was not significantly modified in the group fed on *Artemia* supplemented with iodine; indicating that iodine-supplementation for sole post-larvae is unnecessary in open circuit seawater systems.

Keywords: Sole larvae, gene expression, iodine, thyroid follicle, TRα

1. Introduction
Common sole (*Solea solea* L.) is considered one of the most important flatfish that spread along the Mediterranean coasts [1, 2]. The aquaculture potential of *S. solea* has been highlighted [3], and in Egypt, environmental conditions make it a good candidate for commercial production. However, more studies of the biology, nutrition and rearing of this species are mandatory; in order to facilitate its exploitation as a commercial species.

The common sole belongs to the Pleuronecti forms and like other flatfish species it undergoes metamorphosis, during which the symmetric pelagic larvae transforms into an asymmetric benthic juvenile. The process of metamorphosis includes eye migration, craniofacial remodeling, and also involves significant changes in feeding and digestive physiology [4, 5]. During this processes, it is common in aquaculture that flatfish post-larvae suffer from problems as; incomplete and/or delayed eye migration and malpigmentation, which decrease the chances for sustainable aquaculture of flatfish species [6, 8]. Nutrition insufficiency is one of the main difficulties during metamorphosis [9, 10]. During the period of metamorphosis when the eye is migrating, sole larvae are fed mainly on newly hatched *Artemia* followed by *metanauplii* enriched with commercial products. Live food (e.g. rotifers and *Artemia*) is mainly used as a marine source of essential nutrients to feed the larvae at the marine hatcheries [11, 12].Wild fish larvae depend mostly on the copepods as the main source of food, nutritionally different from the *Artemia*, which is suggested to be one of the factors explaining abnormal metamorphic development [13, 14].In the wild, the consumption of iodine-rich copepods and other zooplankton is considered to be adequate for the iodine necessities of larval flatfish. The deficient iodine contented of both rotifers and *Artemia* [12, 15, 16] has been proposed to explain the much higher iodine contents of larvae fed on copepods relative to those fed on *Artemia* [15]. However, it has been hypothesized that the iodine content of *Artemia* may be insufficient for production of the thyroid hormone (TH) concentrations that trigger metamorphosis, and that this might explain incomplete metamorphosis in flatfish [17]. Beside the nutrition deficient established at marine flatfish cultures, depleted iodide in the supplied water could cause thyroid hyperplasia and goiter [18].

Triiodothyronine (T_3) is considered the active ligand for the thyroid hormone receptors (TRs), formed by the removal of one iodine atom in enzymatic reaction from the thyroxin (T_4).

T_3 activates and increases the concentration of TR that consequently would improve the signal of thyroid hormone [19]. Thyroid hormones are conducted by their binding to TRs, to enhance or depress the gene expression that performs the cellular response to THs, which belong to nuclear steroid hormone receptor super-family [20, 21]. The role of bothT_4 and T_3 in triggering metamorphosis is widely investigated in flatfish [22, 24]. The THs are synthesized in the thyrocytes of the thyroid follicle epithelium by iodination of tyrosine residues [15] in thyroglobulin, the main constituent of the colloid in the follicle [25]. As well, larvae fed with exogenous iodine, enriched-*Artemia*) as; Atlantic halibut [16] and Senegalese sole [26] had an enhancing growth performance. Normal follicle histology occurred when larvae were fed extra iodine in both zebra fish and flatfish [27, 28].

In the present study, the importance of iodine for development and metamorphosis of common sole larvae was determined using iodine-enriched *Artemia* and by evaluating growth parameters, survival and thyroid status. Moreover, real-time PCR was used to quantify the thyroid hormone receptor α (TRα) in response to the changes of iodine levels.

2. Materials and Methods

2.1 Induced spawning

Common sole, brood stock were obtained from Damietta, north of Egypt, in January 2015. Fish was acclimated for 2 weeks to fiberglass raceway tanks containing sand and semi-covered with black sheets. Three females and six males (250-350 g for females and 200-250 g for males) were stocked in each tank. Eggs were collected and incubated in 70 L conical tanks until hatching, then transferred to cylindrical fiberglass rearing tanks until the experiment starts.

2.2 Larvae

Pro-metamorphic larvae, at 17 days post hatchery (dph), were collected from the cylindrical rearing tanks and randomly distributed between the experimental aquaria (30x20x20 cm). Replicate tanks (n=3/ treatment), containing 20 L of water were stocked with 120 larvae each. The water tank was substituted with freshly filtered seawater on daily basis and had a salinity of (38±2 ppt). The water temperature was16 - 18 °C, and photoperiod was adjusted to 8h light: 16h darkness. To evaluate the influence of the iodine, 3 experimental groups were established and included: a) larvae fed with instar I *Artemia* exposed to 0.1g potassium iodate® g IKO3/L (T_{low}) for 3 hours, b) larvae fed with instar I *Artemia* exposed to 0.2g IKO3/L (T_{high}) for 3 hours and c) the control group that received untreated *Artemia*. The feeding schedule is shown in Table (1).

Table 1: The feeding regime during the experiments

DPH	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47	49	51	53	55	57
Microalgae (*Nannochloropsisoculata*)	20,000 cell/ml																												
Rotifer, (*Brachionusplicatilus*)	10 ind./L																												
Artemia, (*Artemiaurmiana*)	*Artemia*																												
	Inster I, Untreated								Instar I; (Untreated, control; Treated, Low or High doses 0.1 and 0.2 g IKO3/L *Artemia*, respectively)												Metanuplii, untreated								
Weaning diet z (15% of total body weight)																					Artificial diet								

2.3 Sampling procedure

From each aquarium, 5 individuals were collected and fixed in 4% formalin for histological studies at 37 and 57 dph. To determine growth, 10 individuals were sampled at 37dph (after treatment) and 57 dph (the end of the experiment). Furthermore, 15 individuals were collected at 37 dph into RNA-Later and stored at -20°C until used to determine the TRα by quantitative PCR.

2.4 Histology and stereology of the thyroid follicles

Samples were decalcified with 0.5M EDTA pH 8 for about 10 days, changed daily and kept in the dark at 4°C. Larvae were dehydrated in a graded series of ethanol (50% to 100% ethanol) then xylene in an automatic tissue processing, embedded in paraffin using a tissue embedding center®. Serial sagittal sections (7µm) were cut with a rotary microtome and mounted on glass slides coated with poly-L-lysine. Sections were dried at 37°C for at least 24 h before use.

Hematoxylin and eosin (H&E) staining was performed after dew axing sections in xylene and rehydrating them through an ethanol series. Stained sections were examined for the presence of thyroid follicles (Tfo) using a light microscope and images captured using a digital camera. The number of follicles, the maximal diameter, length and width of all thyroid follicles on each section examined was determined using Image J software®.

2.5 Thyroid hormone receptor (TRα)

RNA-isolation and reverse transcription: Samples of sole larvae were collected at 37 dph from each of the control and treatment tanks. 15 larvae from each rearing tank were pooled, washed with distilled water and placed in DNA se /RNA se free tubes with RNA later (QIAGEN) and stored at -20ºC until RNA extraction. For total RNA extraction, pooled larvae were removed from RNA later, and homogenized in TRIZOL reagent (Ambien, life technologies) using a homogenizer and following the manufacturer's instructions.

For cDNA synthesis, 1µg of total RNA was reverse transcribed in a 20µl reaction volume containing 2µl of 10X RT Random primer, 50U of Multi Scribe TM Reverse Transcriptase, 2µl 10X buffer and 0.8µl 25xdNTP mix, 1µl RN ase inhibitor (High capacity cDNA reverse transcription kit, Applied Bio systems) and placed in a thermo cycler (Verity, Applied Bio systems) using thermal cycling conditions of 25 ºC for 10 min, 37 ºC for 120 min, 85 ºC for 5 min. Samples were then stored at -20 ºC until used for q PCR.

Real-time PCR: q-PCR for TRα was performed using SYBR green and an Applied Bio systems 7500 Real-Time PCR System. Duplicate reactions were carried out for each sample in a 20µl reaction volume containing; 1µl of diluted cDNA, 10µl of 2X SYBR Green real-time PCR Master Mix (Real MOD™ GH Green) and 1.5 µl (10µM) of the forward and reverse primers. For TRαrelative expression primers

were used as following (F 5´-GGA AAC AGA AGC GCA AGT TC- 3´ and R 5´-TCT TCA CAA GGC AGC TCT GA- 3´), and The housekeeping gene was acidic ribosomal protein (ARP) with primers (F5´-CTG AAC ATC TCG CCC TTC TC- 3´ and R 5´-TCT TCA CAA GGC AGC TCT GA- 3´), primers were selected and tested according to [28]. The thermo cycler used to amplify TRα and ARP was 3 min at 95°C, followed by 45 cycles of 15 s at 95ºC, 30 s at 58°C. A dissociation curve analysis was performed for each gene transcript amplified and revealed in all cases a single product reaction peak. Results were analyzed using the $2^{-\otimes\otimes CT}$ method [29] and the amplification efficiencies were 0.99 and 0.98 for TRα and ARP respectively. TRα mRNA expression was normalized using the reference gene ARP. Gene expression data are presented as the fold change relative to control untreated larvae.

2.6 Data analysis

The results are expressed as the mean ± standard error (± SE). Growth performance and TRα data were examined using both two-way and a one-way ANOVA, respectively, using the statistical software SPSS® V, (22). Follicles number, vacuole density, and diameter were examined using least square mean using the statistical software SAS®. All statistical analyses were followed by a Tukey test, significance was accepted at $p< 0.05$.

3. Results

3.1 Larval growth

There was no significant ($p>0.05$) increase among the groups. However, larvae from all treatments significantly ($p<0.05$) increased in length from 37 dph (the end of the treatment period) until 57 dph (the end of the experiment) (Fig. 1a).A slight but non-significant change in the larvae wet weight (TW) and weight gain (WG) occurred from 37 dph to 57 dph in all experimental groups (Fig. 1b).The specific growth rate (SGR%)/day significantly reduced ($p <0.05$) from 10.01±0.16 at 37 dph to 5.31±0.16 at 57 dph.

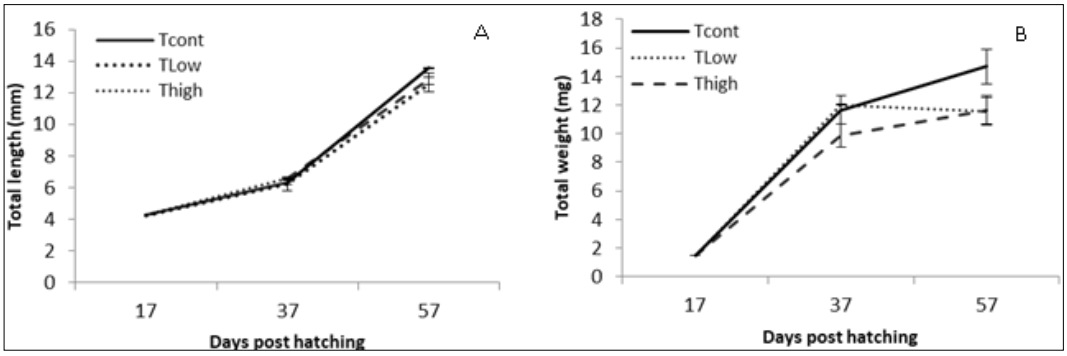

Fig 1: (a) Total length (mm), and (b) Total weight (mg), (mean±SE) for common sole larvae, fed T_{Cont}, T_{Low} and T_{High}from17dphuntil 57 dph.

3.2 Histological analysis of thyroid follicles

Data of thyroid follicles (Tfo) were analyzed (two-way ANOVA), to examine the differences between the groups above a portion of time (Fig.2 a & b).

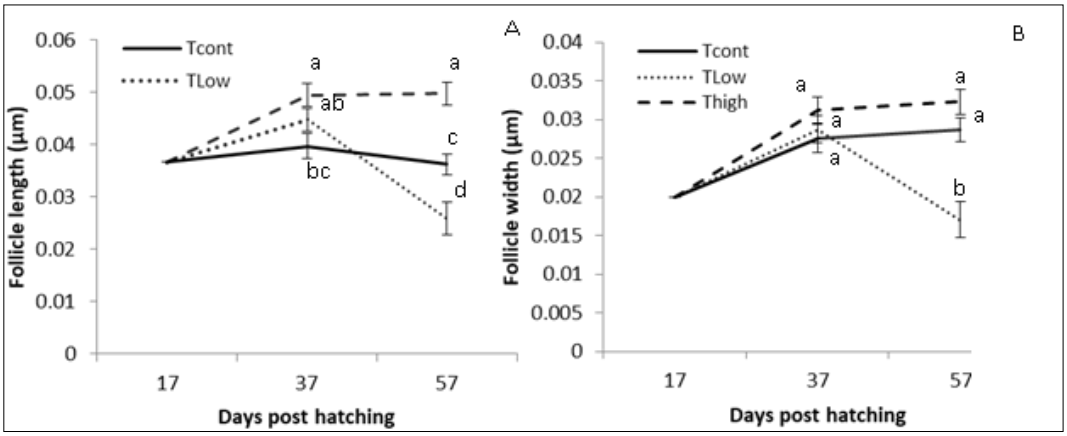

Fig 2: The thyroid follicle diameter; **(a)** length (µm), and **(b)** width (µm), (mean±SE) for Common sole larvae, fed T_{Cont}, T_{Low} and T_{High} from 17 dph until 57 dph. ($n=5^3$, 17 dph not included in the statistical analysis). [a-d] Mean values with different superscript letters are significantly different ($p<0.05$ two-way ANOVA)

Thyroid follicle diameters: thyroid follicle diameter significantly decreased with age (Tukey (HSD) test for unequal *N*, $p=0.05$). The thyroid follicle length significantly decreased ($p<0.05$) from 0.045 ± 0.0013 µm at 37 dph to 0.037 ± 0.0014µm at 57 dph. The thyroid follicle width didn't significantly change, being 0.029 ± 0.0010 µm in 37 dph larvae and 0.026 ± 0.0011 µm in 57 dph larvae.

The thyroid follicle diameter was also significantly ($p<0.05$) affected by the concentration of the iodine introduced in the larval feed. The thyroid follicle length was significantly ($p<0.05$) higher in the T_{high} (0.050 ± 0.0015 µm) relative to the T_{Low} (0.035 ± 0.0019 µm) and T_{Cont} (0.038±0.0016 µm). Likewise, the follicle width varied significantly ($p<0.05$) between the treatment groups and was highest in T_{high} (0.032 ± 0.0012 µm) and lowest in T_{Low} (0.023 ± 0.0015 µm) relative to the control group (0.028 ± 0.0012 µm).

The values of thyroid follicle diameter, length, and width, changed significantly ($p<0.05$) from 17 dph to 57 dph (Fig. 2a & b). Larvae fed on *Artemia* enriched in iodine (T_{high}) had significantly higher values ($p<0.05$) for the follicle diameter,

length, and width at 37 dph relative to 57 dph. In contrast, larvae fed on *Artemia* enriched in iodine (T_{Low}) had a significantly minor (p<0.05) follicle diameter at 57 dph (0.017 ± 0.0024 µm) relative to 37 dph (0.026 ± 0.0031 µm).

Thyroid follicle distribution and activity: In all studied larvae, Tfo were numerous and scattered in the tissue at the insertion of the branchial arches. The highest number of Tfo per histological section (n = 7) occurred in the T_{High} group at 57 dph (Fig. 3b).

The activity of the thyroid follicles was determined by assessing the abundance of vesicles in the periphery of the follicle lumen. The frequency of the vesicles was significantly (p<0.05) higher in the larvae fed on the T_{Low} iodine-enriched diet (relative to the control group at 37 dph and 57 dph (Fig. 4). No significant change in vesicle frequency occurred in the T_{High} relative to the control larvae. At 37 dph, a significant correlation (p<0.05) was found between the activity of the thyroid follicle and growth, vesicle density increased from 1.09±0.16, 1.55±0.17 to 1.85±0.18 that with increasing the iodine concentration as follow $T_{Cont.}$ T_{low}, and T_{high}, respectively. After the recovery period at 57 dph the thyroid follicle dense showed a significant (p<0.05) decay to reach 0.46±0.22 T_{high}, (Fig. 5 a, b & c).

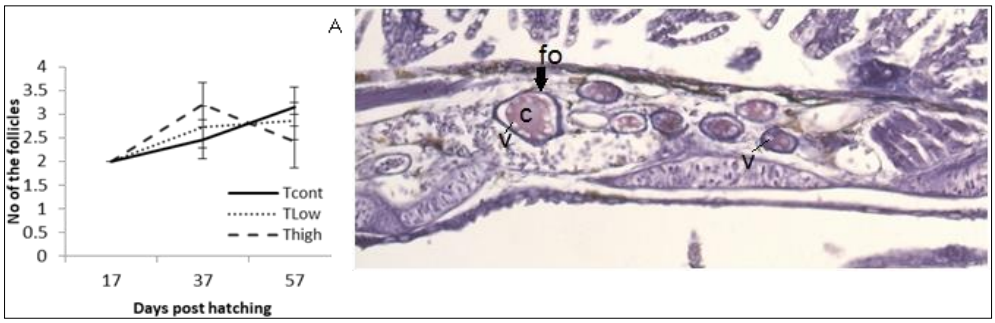

Fig 3: The thyroid follicle distribution in Common sole larvae; **(a)** number of follicles/section (mean±SE), fed T_{Cont}, T_{Low} and T_{High} from 17 dph until 57 dph. (n=5^3, 17 dph not included in the statistical analysis, p<0.05 two-way ANOVA), and (b) thyroid follicular cell (fo) from iodine treatment T_{High}at 37 dph, vesicles (v) are present at the periphery of the colloid (c), stained with H&E (magnification 200X)

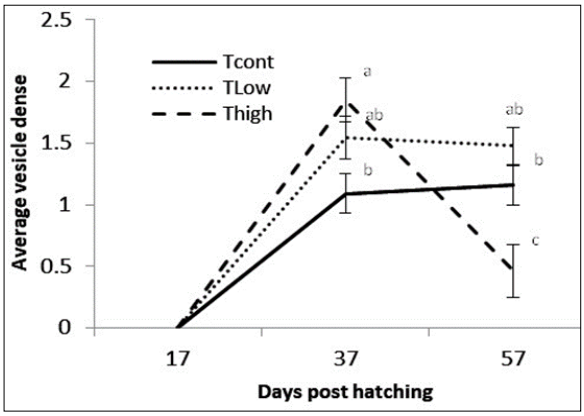

Fig 4: The thyroid follicle activity in Common sole larvae; Average vesicle frequency (mean±SE), fed T_{Cont}, T_{Low} and T_{High} from 17 dph until 57 dph. (n=5^3, 17 dph not included in the statistical analysis). $^{A-d}$ Mean values with different superscript letters are significantly different (p<0.05 two-way ANOVA)

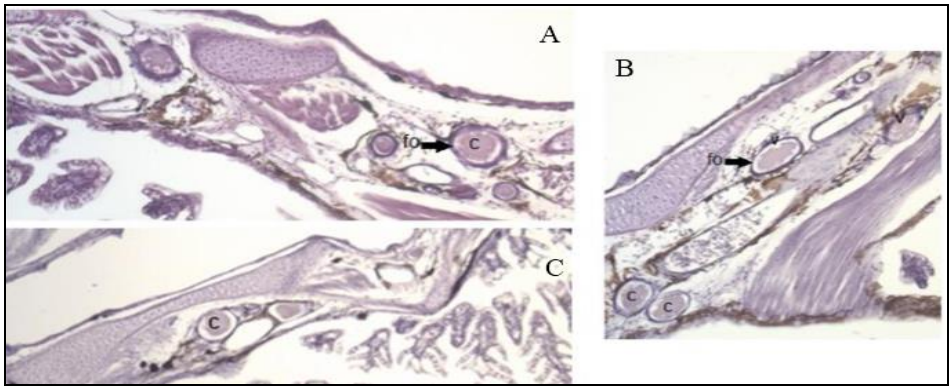

Fig 5: The thyroid follicle distribution and activity in Common sole larvae; fed (A)T_{Cont}, (B)$T_{Low}$and (C) T_{High} after recovery period at 57 dph. The thyroid follicular cell (fo) continued vesicles (v) that present at the periphery of the colloid (c) – stained with H&E (magnification 200X).

3.3 Thyroid hormone receptor (TRα)

Relative expression of TRα was determined at the end of the treatment period at 37 dph. Thyroid hormone receptor (TRα) expression increased significantly (p<0.05) in T_{Low} larvae group to be 3.55±1.13 fold in comparison to the control group. Meanwhile, no significant increase (p>0.05) was noticed at the T_{High} larvae, which record 1.13±0.16 fold from the control group (Fig. 6).

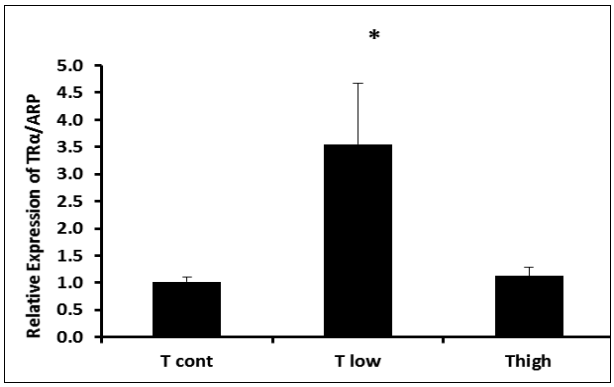

Fig 6: Relative TRα expression levels in the untreated control and potassium iodate treated groups after treatment period at 37 dph. Expression values were normalized to those of ARP. Data were expressed as the mean fold change (mean±SE, n = 3 in duplicate) from the calibrator (control). Values with ‘‘*’’ are significantly different (p<0.05) from the corresponding value for the control group

4. Discussion

Larvae growth

The result of this study revealed that iodine supplementation did not affect growth of *Solea solea*. Similar results were reported for Atlantic halibut larvae fed on iodine-enriched *Artemia*.[16] Moreover, exogenous iodine and selenium in marine larvae fish have been associated with decreased growth in Atlantic cod, *Gadus morhua*, larvae[12]; suggesting that iodine is present in an adequate quantity in larvae fed on rotifers. In contrast, *Solea senegalensis*, raised in a recirculation system [26, 28]. with ozone injection suffered from iodine deficiency and had improved growth when fed on iodine-enriched rotifers and *Artemia*. This suggests that the iodine from live organisms (*e.g.* Copepods containing 50-350 mg I kg^{-1} DW [15]. Promotes better marine larval growth performance. Exogenous iodine-enrichment, on the other hand, may cause iodine toxicity as observed in Atlantic cod fed on iodine-enriched rotifers and copepods (129 mg I kg^{-1}DW copepods [30]). Although they suggested that larvae fish could effectively and safely transfer iodine from copepods and that observed iodine toxicity was probably due to nutrient interactions (*e.g.* Bromine (Br^-)/ iodine (I^-) interaction).

Histological analysis of the thyroid follicles

The symptoms of severe iodine deficiency such as hyperplasia of the thyroid follicles (goiter) noticed at *Senegalese sole* larvae [26, 28]. Reared in a recirculating system or in Pacific threadfin, *Polydactylus sexfilis* [31]. Reared on well water is probably due to water parameters. This observation was also made in earlier studies of Atlantic halibut [15]. In recirculation systems, the observations of thyroid hyperplasia are in agreement with the present results on the common sole, in which the seawater in the system was frequently changed, no evidence of hyperplasia was observed in any of the experimental groups. Furthermore, colloid depletion and lumen diameter decrease were not observed in the present study, but have been associated with hyperplasia[32].However, at 37 dph, significant increase in thyroid follicle diameter and presence of vesicles in the periphery of the colloid and lack colloid were determined at larvae fed on iodine-enriched *Artemia*.

The iodine defiance and presence of high levels of goitrogens reported in *Solea senegalensis* are probably due to reared larvae in recirculation system [26, 28, 30] that decreased the accessibility of dissolved bioavailable iodine, iodide (I^-) to iodate (IO_3^-) in presence of ozone injection [33]. Furthermore, higher values of iodate versus iodide were considered cause of reduced growth and survival in well water used to rise pacific threadfin larvae [31]. In contrary, as sea water contains considerable amounts of iodine (64 μgl l^{-1}) [32]. Fish larvae reared with continuous water exchange, where iodide is continuously replaced didn't suffer from iodine defiance [30], as shown in the present investigation.

Concerning the Tfo morphology, Ribeiro *et al*. (2011 & 2012) [26, 28]. demonstrated that the shortage of iodine could result in the availability of T_4, according to their observations between iodine and thyroxin. This low production of T_4 could further lead to reduce the creation of the TSH from the pituitary. Hence, the thyroid follicles are continuously stimulated to grow and multiply to produce more T_4. This negative feedback could partly explain the increase in number of Tfo cell observed during this study. Whereas, at 37 dph, larvae fed on a high dose of iodine-enriched *Artemia* demonstrated a significant increase in thyroid follicles number and presence of vesicles at the colloid border that was noticed with a slight decrease at the growth.

Thyroid hormone receptors

Two types of thyroid hormone receptors, TRα and TRβ have been reported in teleost fish [34], and they are expressed in developing fish embryos even in the absence of thyroid tissue. TRα was evaluated in the present study as an indicator of TH activity [24]. Different expression patterns of TRs have been reported during larval development in several previous of flatfish, although in contrast to common sole, a significant decline in the TRα occurred during the metamorphic climax in Atlantic halibut[35] and Senegalese sole[36]. These data, taken together with the results of the present study, indicate that the significant variation in the TRα expression between sole larvae groups was probably due to the influence of the iodine-supplemented *Artemia*. For instance, at 37 dph, the TRα expressed 3.55-fold higher in larvae fed on a low dose of iodine-enriched *Artemia* versus the control group. While the larvae fed on a high dose of iodine-enriched *Artemia* present almost the same expression of the TRα that appeared in the control group.

5. Conclusion

In conclusion, experimental evidence has been demonstrated that it is not confidently approved that marine larval fish have their efficient amount of iodine from fresh supplementation of seawater. However, low doses of iodine-enriched *Artemia* showed a significant increase in the TRα expression, which can be considered as an indicator for increasing the thyroxin; leading to enhance the growth. Direct addition of iodine could be more efficient in enhancing the larval growth. Further studies on the mechanism of iodine-supplementation for marine larval fish are mandatory and highly recommended.

6. Acknowledgements

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