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Original Article

Effect of human chorionic gonadotropin (hCG) and salinity on growth, reproductive indexes, and hormonal profiles of male short-finned eel (*Anguilla bicolor*)

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Abstract: *Anguilla bicolor* is a catadromous teleost with high aquaculture potential; however, captive reproduction is constrained by males failing to mature spontaneously in captivity. This study evaluated the factorial effects of salinity manipulation (0 or 30 ppt) and with and without human chorionic gonadotropin (hCG) induction on growth performance, reproductive indices, and hormonal profiles of male *A. bicolor*. The experiment was conducted for 7 weeks. Findings reveal that survival was 100% across all treatment groups, while seawater at 30 ppt combined with hCG significantly enhanced the gonadosomatic index (GSI: 0.47%), hepatosomatic index (HSI: 1.54%), and plasma testosterone (66.91 ng/mL) compared with freshwater groups. Histological analysis confirmed more advanced spermatogenic progression in males reared in 30 ppt with hCG, including the presence of spermatocytes and early spermatozoa. Eye index, body coloration, and condition factor remained relatively similar across all groups (P>0.05), indicating that external silvering indicators were not initiated under the experimental conditions. These findings demonstrate a synergistic interaction between salinity and hCG in captive male *A. bicolor*, providing a scientific basis for broodstock conditioning in eel aquaculture.

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Introduction

The short-finned eel, *Anguilla bicolor*, is a catadromous teleost of high economic importance in Southeast Asian aquaculture (SEAFDEC, 2020). Despite its commercial potential and the abundance of wild juveniles in estuarine areas of Java, western Sumatra, and Sulawesi, uncontrolled harvesting threatens long-term sustainability (Raganata et al., 2024; Samuel et al., 2024). Captive production remains limited because commercially viable closed-cycle artificial propagation has not yet been successfully established for this species (Sugeha and Ganesa, 2015).

A major challenge is the failure of male *A. bicolor* to mature spontaneously in captivity. Male reproductive biology in *A. bicolor* remains poorly understood, and the limited availability of mature male broodstock constrains sperm production and reduces fertilization success (Wahyudi et al., 2025). Hormonal induction using human chorionic gonadotropin (hCG) is widely used to stimulate gonadal development in eel species, as it mimics the role of luteinizing hormone (LH) by activating the hypothalamic-pituitary-gonadal (HPG) axis (Ijiri et al., 2011; Benini et al., 2022). However, most protocols were developed for the European eel (*A. anguilla*) and the Japanese eel (*A. japonica*), with limited applicability to tropical species such as *A. bicolor*.

In addition to hormonal induction, salinity manipulation is a potentially important environmental trigger for reproductive activation in euryhaline species. *Anguilla bicolor* naturally migrates from freshwater to marine environments for spawning, with salinity serving as a primary cue activating the HPG axis (Zydlewski et al., 2013). Positive steroidogenic effects of elevated salinity have been reported in striped mullet (*Mugil cephalus*), Asian seabass (*Lates*

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calcarifer), and common carp (*Cyprinus carpio*) (Mohanty et al., 2020); However, its combined effect with hCG on male *A. bicolor* has not yet been evaluated under controlled aquaculture conditions. This study, therefore, investigated the factorial effects of salinity (0 vs. 30 ppt) and hCG (with and without) induction on growth performance, reproductive indexes, and hormonal profiles of male *A. bicolor*, with the aim of identifying an effective baseline protocol for broodstock reproductive conditioning.

Materials and Methods

Broodstock procurement and management: Wild *A. bicolor* were collected from Pelabuhan Ratu, West Java, and reared for two years in a freshwater recirculating aquaculture system (RAS) until reaching the silver eel stage (170-208 g). One hundred individuals were selected for an 18-month broodstock conditioning program in 3-ton RAS tanks, with water quality monitored twice daily: dissolved oxygen at 6.5-6.8 ppm, pH at 7.8-8.2, and temperature at 27.7-28.1°C. Fish were fed three times per week at 1% body weight using high-protein pellets (JAPFA Comfeed®; 52% crude protein).

Experiment design and treatments: Forty adult male *A. bicolor* (142-248 g) were equally distributed into four treatment groups (n = 10; duplicate tanks, 5 fish per tank): 30 ppt with hCG (30 T); 30 ppt without hCG (30 NT); 0 ppt with hCG (0 T); and 0 ppt without hCG (0 NT). Fish assigned to the 30 ppt treatment were gradually acclimated at a rate of 10 ppt per day until reaching 30 ppt, and were subsequently individually tagged with Passive Integrated Transponder (PIT) tags. Fish injected with hCG received weekly intraperitoneal injections at 2000 IU/kg body weight under phenoxyethanol anesthesia (0.4 mg/L; 5 min). The experiment lasted 7 weeks.

Growth and morphological measurements: Growth metrics (body weight, total length, and body girth) and eye index (EI) were recorded at 5-day intervals. Condition factor was calculated as $K = (W/L^3) \times 100$. Eye index was calculated as $EI = [(EL + EH)/HL] \times 100$, where EL = eye length, EH = eye height, and HL = head length. Body coloration was quantified by grayscale analysis in ImageJ (ver. 1.54, NIH, USA; Schneider et al., 2012). Pearson correlation coefficients were computed among growth traits, coloration, and eye index.

Reproductive indices: At the end of the experimental period, all 40 fish were humanely euthanized with tricaine methanesulfonate (MS-222; 100-150 mg/L). Testes and livers were dissected and weighed to calculate gonadosomatic index (GSI = gonad weight/body weight × 100) and hepatosomatic index (HSI = liver weight/body weight × 100) according to Flores et al. (2019). Testes were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-7 μm, and stained with H&E for histological staging. Plasma testosterone was quantified by competitive ELISA (MyBioSource®, USA; absorbance at 450 nm).

Statistical analysis: All analyses were conducted in SPSS v. 26.0 (IBM Corp.). Data were presented as mean ± SE; significance was set at $P<0.05$. Shapiro-Wilk and Levene tests verified normality and homogeneity of variance; data were $\log_{10}$-transformed where assumptions were violated. A 2×2 factorial ANOVA was used to evaluate main effects and interactions of salinity and hCG; Tukey's HSD post hoc test was applied for multiple comparisons. The Mann–Whitney U test was used when parametric assumptions could not be met.

Results

Survival and growth performance: Survival was 100% across all treatment groups throughout the 7-week experimental period. After 7 weeks, the 30 NT group reached a significantly higher final body weight (187.0±11.3 g) compared to the other groups ($P<0.05$; Table 1). However, final total length (41.8-43.4 cm) and condition factor K (0.21-0.25) did not differ significantly among treatments ($P>0.05$). Body weight and body girth showed slight fluctuations over the 7-week period, but no significant differences were detected (Fig. 1).

Body coloration: Findings on body coloration did not differ significantly among treatments ($P>0.05$) (Fig. 2). The 0 T group exhibited the highest grayscale

Table 1. Growth performance of male *Anguilla bicolor* subjected to the factorial effects of salinity and hCG induction (n = 5/treatment; mean±SE). Different superscript letters indicate significant differences (P<0.05). BW = body weight; TL = total length; K = condition factor.

Treatment	Survival (%)	Final BW (g)	Final TL (cm)	K
30 T	100.0±0.0[a]	176.0±10.2[a]	43.4±2.1[a]	0.25±0.04[a]
30 NT	100.0±0.0[a]	187.0±11.3[b]	41.8±1.2[a]	0.21±0.06[a]
0 T	100.0±0.0[a]	179.0±7.8[a]	43.2±1.6[a]	0.22±0.07[a]
0 NT	100.0±0.0[a]	169.0±8.8[a]	41.8±1.5[a]	0.23±0.04[a]

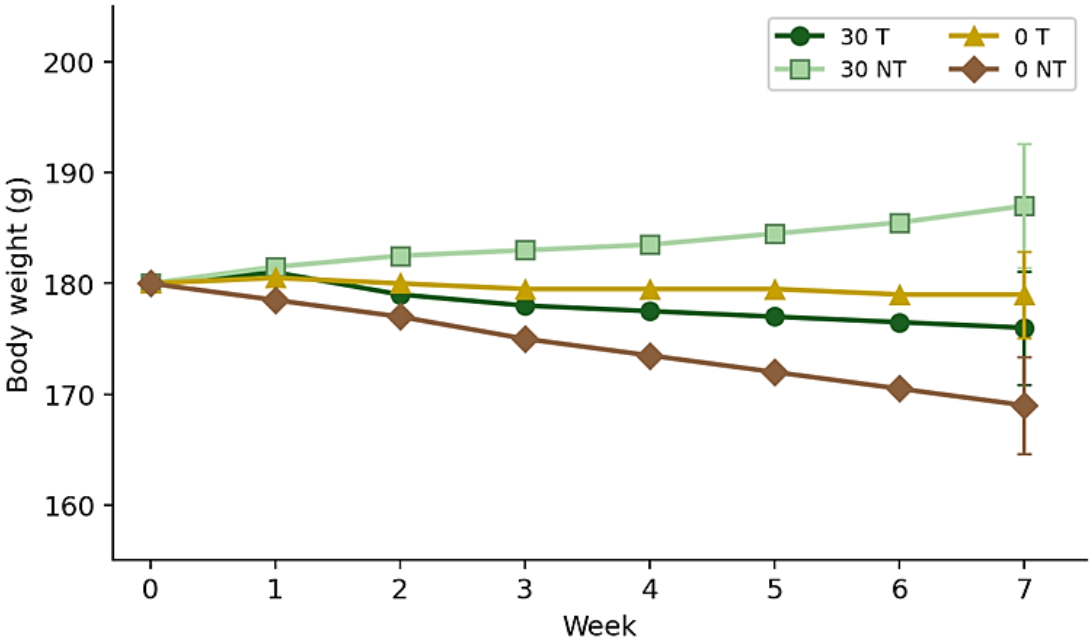

Figure 1. Changes in body weight (g) of male *Anguilla bicolor* over 7 weeks under different salinity and hormonal treatments (30 T, 30 NT, 0 T, and 0 NT). Values are mean±SE. No significant differences were detected among treatments (P>0.05).

Parameters	30 T	30 NT	0 T	0 NT
Final Body Coloration				
Image Convert to Greyscale				
Scale	86.7 ± 13.5	93.6 ± 13.4	112.8 ± 9.7	88.5 ± 9.7
Final Coloration (%)	44.2 ± 1.5	36.7 ± 3.0	34.7 ± 2.3	34.0 ± 2.5

Figure 2. Body coloration of *Anguilla bicolor* reared under different salinity levels (0 and 30 ppt) with and without hormone treatment. Upper row: color photographs of representative fish; lower row: corresponding grayscale conversions for ImageJ analysis. Final coloration values are expressed as mean±SE.

value (112.8±9.7), indicating a lighter body tone, whereas the 30 T group showed the lowest (86.7±13.5). Percentage coloration was highest in the 30 T group (44.2±1.5%), followed by 30 NT (36.7±3.0%), 0 T (34.7±2.3%), and 0 NT (34.0±2.5%).

Eye index and correlation analysis: The eye index showed no significant differences among treatment groups (P>0.05) (Fig. 3), ranging from 0.67% (0 NT) to approximately 1.0% (30 NT). Neither salinity nor hCG induction had a measurable effect on eye index during the 7-week experimental period. Pearson correlation analysis revealed a strong positive relationship between eye index and final body weight

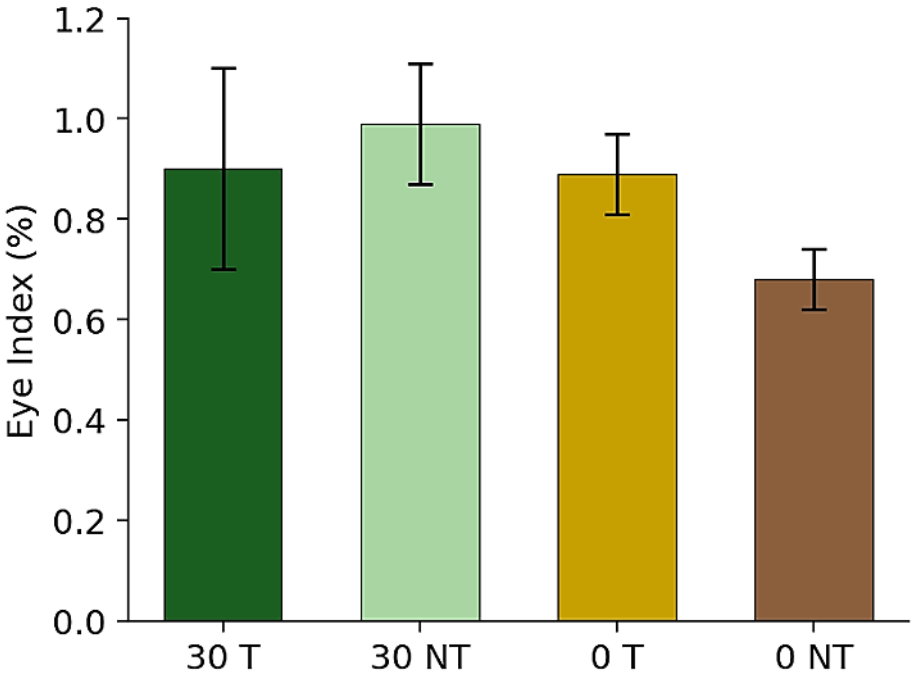

Figure 3. Eye index (%) of male *Anguilla bicolor* over 7 weeks under different salinity and hormonal treatments (30 T, 30 NT, 0 T, and 0 NT). Values are mean ± SE. No significant differences were detected among treatments (P>0.05).

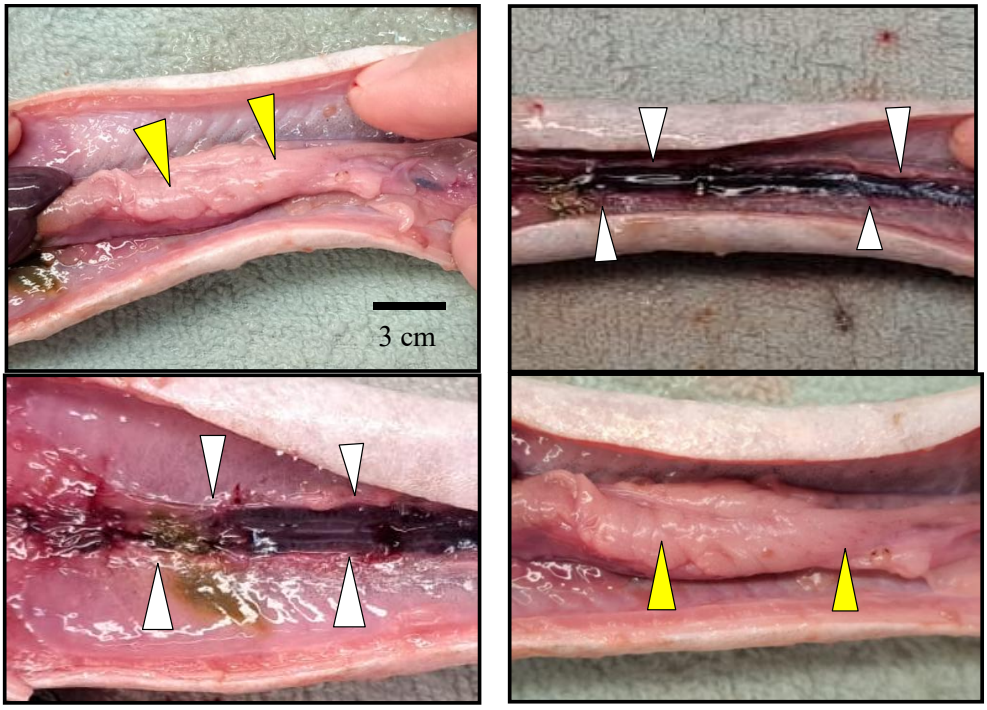

Figure 4. Testicular condition of male *Anguilla bicolor* subjected to different salinity and hormonal treatments. (a) 30 ppt + hCG (30 T): enlarged testes; (b) 30 ppt without hCG (30 NT): thin testes; (c) 0 ppt + hCG (0 T): narrow testes; (d) 0 ppt without hCG (0 NT): fat deposits with minimal testicular development. White arrows = testis; yellow arrows = fat deposits (Scale bar = 3 cm).

(r = 0.9) and the strongest positive correlation between condition factor and body coloration (r = 0.7; Table 2).

Gonadal morphology: Macroscopic examination at the end of week 7 revealed clear treatment-dependent differences in testicular appearance (Fig. 4). Fish in the 30 T group (30 ppt + hCG) displayed the most advanced testicular development, with enlarged, whitish, and elongated gonads. Meanwhile, fish in 30 NT showed thin but visible testes. Fish in the 0 T group were found with narrow and underdeveloped

Table 2. Pearson correlation matrix of eye index, final body weight, final total length, condition factor (K), and body coloration in male *Anguila bicolor*. Strength interpretation: |r| < 0.3 = weak; 0.3-0.7 = moderate; |r| ≥ 0.7 = strong.

	Eye Index (%)	Final Body Weight (g)	Final Total Length (cm)	Condition Factor (K)	Final Coloration (%)
Eye Index (%)	-	0.9	0.3	-0.3	0.4
Final Body Weight (g)	0.9	-	-0.1	-0.6	0.1
Final Total Length (cm)	0.3	-0.1	-	0.6	0.6
Condition Factor (K)	-0.3	-0.6	0.6	-	0.7
Final Coloration (%)	0.4	0.1	0.6	0.7	-

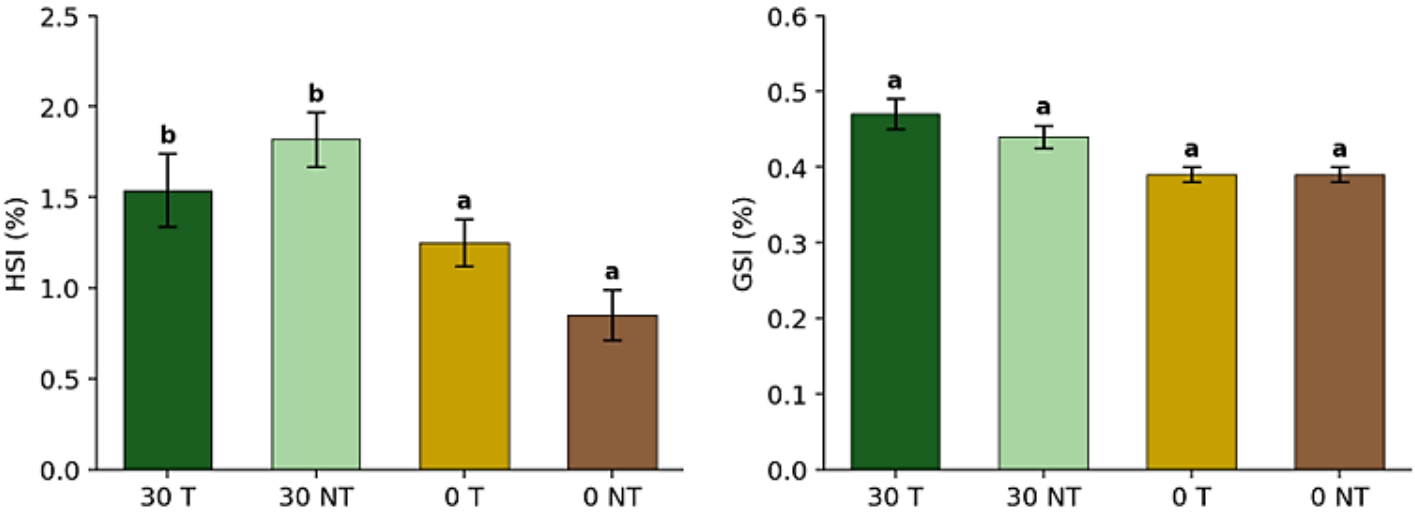

Figure 5. Hepatosomatic index (HSI, left) and gonadosomatic index (GSI, right) of male *Anguilla bicolor* reared under different salinity and hormonal treatments (30 T, 30 NT, 0 T, and 0 NT). Values are mean ± SE. Different letters indicate significant differences (P<0.05).

testes, while those in the 0 NT group exhibited predominantly fat deposits along the gonadal region with minimal testicular tissue, indicating energy diversion toward fat accumulation rather than reproductive development.

Hepatosomatic index (HSI) and gonadosomatic index (GSI): HSI was significantly higher in 30 ppt groups (30 NT: 1.82±0.15%; 30 T: 1.54±0.12%) compared with 0 ppt groups (P<0.05). GSI values trended upward in 30 T (0.47±0.05%) and 30 NT (0.44±0.04%) compared with freshwater groups (0.39±0.02%), but differences were not statistically significant (P>0.05) (Fig. 5).

Testicular histology: Histological analysis confirmed treatment-dependent differences in spermatogenic progression (Figure 6). In 30 T fish, seminiferous tubules were well organized and contained germ cells at multiple developmental stages, including early and late B-type spermatogonia (SG-B), spermatocytes (SC), and spermatozoa (SZ), indicating partial maturation. The 30 NT group showed reduced spermatogenic activity, with spermatogonia and spermatocytes present but fewer spermatozoa and more prominent connective tissue. Both freshwater groups (0 T and 0 NT) were dominated by early spermatogonia with no detectable spermatozoa, reflecting limited or regressed gonadal development (Fig. 6).

Plasma testosterone concentration: Plasma testosterone was significantly higher in the 30 T group (66.91±2.1 ng/mL) than in 30 NT (21.98±3.1 ng/mL), 0 T (19.31±2.4 ng/mL), and 0 NT (9.81±4.2 ng/mL) (P<0.05; Fig. 7), showing a strong synergistic stimulatory effect of seawater salinity and hCG on androgen synthesis in male *A. bicolor*.

Discussions

The 100% survival rate confirms that male *A. bicolor* tolerate both freshwater and seawater rearing, as well as repeated hCG injections, without mortality,

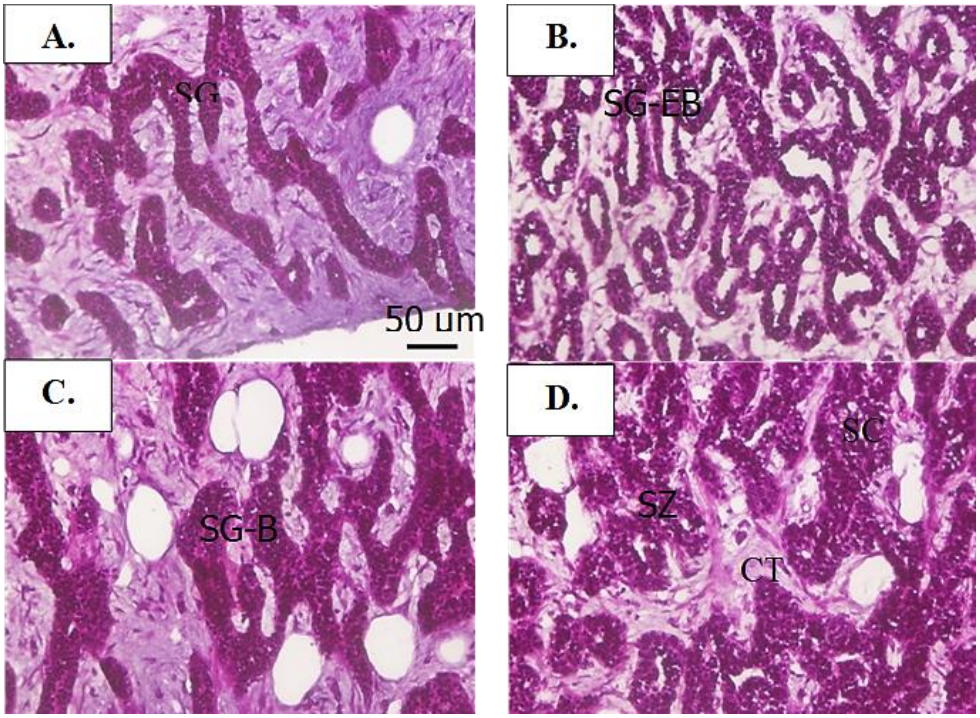

Figure 6. Gonad histology of male *Anguilla bicolor* after 7 weeks of treatment (4000× magnification). (a) 30 T; (b) 30 NT; (c) 0 T; (d) 0 NT. SG = spermatogonium; SG-EB = early B spermatogonium; SG-B = late B spermatogonium; SC = spermatocyte; SZ = spermatozoa; CT = connective tissue; R = regression (Scale bar = 50 µm).

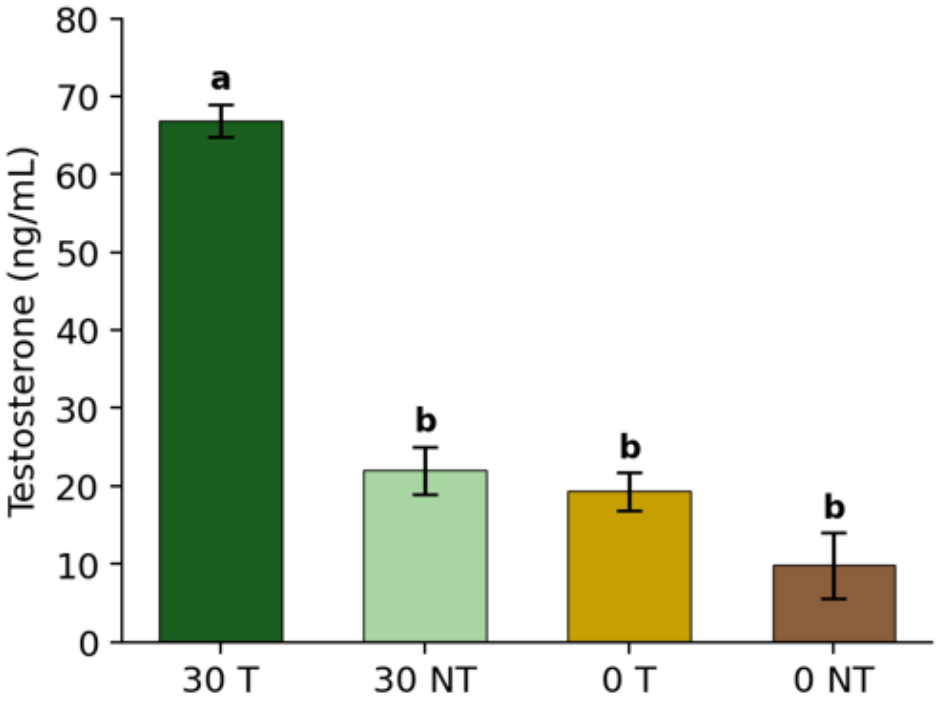

Figure 7. Plasma testosterone concentration (ng/mL) in male *Anguilla bicolor* reared under different salinity and hormonal induction treatments (30 T, 30 NT, 0 T, 0 NT). Values are mean ± SE. Different letters indicate significant differences (P<0.05).

consistent with the catadromous and euryhaline nature of the species (Arai, 2016; Raganata et al., 2024). The significantly higher body weight in the 30 NT group (187.0±11.3 g) versus the hCG-treated 30 T group (176.0±10.2 g) is consistent with hormonal stimulation redirecting energy from somatic growth toward gonadal development. Similar energetic trade-offs have been documented in European eel (Mordenti

et al., 2014) and *A. japonica* (Okamura et al., 2007; Ijiri et al., 2011), confirming that this is a common feature in anguillid eels during hormonal induction.

Eye index remained stable across all groups, indicating that 7 weeks of saline exposure and/or hormonal stimulation were insufficient to initiate the silvering cascade. In anguillid eels, eye enlargement is a hallmark of silvering, typically triggered by declining photoperiod and rising salinity (Durif et al., 2009). The dissociation observed here suggests that gonadal activation via exogenous hCG can precede external silvering indicators in *A. bicolor*, meaning that internal measures (GSI, histology, hormone levels) are more reliable indices of reproductive readiness (Flores et al., 2019; Gentile et al., 2022).

Seawater (30 ppt) combined with hCG produced the most advanced testicular development. Freshwater-reared fish showed limited germ cell progression regardless of hormonal treatment, confirming that environmental salinity acts as an upstream regulator of reproductive processes. The results suggest that the osmoregulatory and neuroendocrine adjustments triggered by seawater exposure create a permissive physiological environment for hCG to act on the gonads, potentially through upregulation of gonadotropin receptor expression or enhanced intratesticular steroidogenesis. This is consistent with observations in *A. japonica* and *A. anguilla*, where seawater conditioning was required for full testicular maturation under artificial induction (Ohta et al., 1997; Lokman et al., 2016).

The significantly elevated plasma testosterone in the 30 T group (66.91±2.1 ng/mL) compared with freshwater groups (9.81-19.31 ng/mL) provides strong evidence that salinity and hCG act synergistically to stimulate androgen biosynthesis in male *A. bicolor*. Seawater exposure may enhance gonadotropin receptor sensitivity or Leydig cell steroidogenic capacity, as proposed by Kuo et al. (1974) in grey mullet and supported by more recent observations in other euryhaline teleosts (Mohanty et al., 2020). The concurrent elevation in HSI reflects intensified hepatic involvement in energy mobilization and steroidogenesis to support gonadal maturation (Takeuchi et al., 2021).

Conclusion

This study demonstrates that the combined effects of salinity and hCG significantly influence the reproductive physiology of captive male A. bicolor. Rearing in seawater (30 ppt) coupled with weekly hCG injection (2000 IU/kg) markedly enhanced GSI, HSI, and plasma testosterone levels, and resulted in more advanced spermatogenesis based on histological assessment compared with freshwater treatments. In contrast, external morphological indicators (eye index, body coloration, and condition factor) were not responsive to the treatments, indicating that internal physiological parameters are more reliable indicators of reproductive readiness. Therefore, seawater rearing combined with weekly hCG administration is recommended as a baseline strategy for reproductive induction in male *A. bicolor*. Further studies incorporating longer induction periods and optimized dosing regimes are necessary to achieve complete gonadal maturation and consistent spermiation.

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