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Investigation of Skin Discoloration in the Nile Tilapia (*Oreochromis niloticus*) by Plasma-Activated Media

Sukitar Krasaesen[1], Dheerawan Boonyawan[1], Udomluk Sompong[2], Sureeporn Sarapirom[3] and Chakkrapong Kuensaen[4,*],

[1]Department of Physics and Materials Science, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Center of Excellence in Fisheries and Aquatic Resources, Department of Fisheries Technology and Water Resources, Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai 50290, Thailand
[3]Applied Physics Division, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand
[4]Research Unit for Bio-Based Innovation, International College of Digital Innovation, Chiang Mai University, Chiang Mai 50200, Thailand

([*]Corresponding author's email: chakkrapong.k@cmu.ac.th)

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Abstract

This research investigated the efficacy of plasma-activated media (PAM) as a tool for inducing stable skin color variants in Nile tilapia (*Oreochromis niloticus*). PAM was generated using a transient corona discharge system (20 W, 6 - 10 kV) in atmospheric air, resulting in the time-dependent accumulation of hydrogen peroxide, nitrite, and nitrate. A 2-factorial experimental design was employed to evaluate the interaction between PAM activation duration (0–20 min) and developmental stage (Stages 1 - 5). Survival analysis at 14 days post-immersion identified Stage 2 (eyed egg) as a critical window of sensitivity, showing significant mortality and phenotypic shifts at the 20-minute activation level. In contrast, Stage 3 (pre-hatched embryo) and later larval stages demonstrated high tolerance to PAM exposure. PAM immersion successfully induced 4 distinct color phenotypes: Natural, Whole Pink, White, and Black Blotch, with an induction rate of 28.5% in the most intense treatment group. Notably, a chemically simulated control group matching the PAM's RONS concentrations failed to produce any variants, suggesting that short-lived species or physical plasma factors are the primary drivers of the phenotypic shift. The induced phenotypes were 100% stable from the larval stage through the 39-week adult harvest. Quantitative CIELAB analysis and microscopic quantification revealed that Whole Pink and White phenotypes were characterized by a significant reduction in melanophore density (5 - 12 cells/mm^2) compared to the Natural control (145 cells/mm^2). Long-term monitoring demonstrated that PAM-treated groups exhibited significantly higher body weights and Average Daily Gain (ADG) by Week 39, consistent with a hermetic response. Texture Profile Analysis (TPA) confirmed that PAM exposure did not negatively affect flesh firmness across anatomical regions. These results indicate that PAM immersion is a safe, stable, and non-genetic method for inducing commercially desirable color variants and enhancing growth performance in Nile tilapia.

Keywords: Nile tilapia, Cold plasma, Plasma-activated media, Melanophore density, Growth performance, Reactive oxygen and nitrogen species (RONS), Skin pigmentation

Introduction

Nile tilapia (*Oreochromis niloticus*) is widely regarded as one of the most important species in global aquaculture, often referred to as the aquatic chicken due to its efficient feed conversion, rapid growth rates, and ability to thrive in a variety of environmental conditions [1]. While the wild-type tilapia is characterized by a dark, silver-gray skin, there is a strong market preference for red and pink varieties in many regions, including China, Malaysia, and Thailand. These red varieties are often sold at a premium because they resemble high-value marine species and are visually

appealing to consumers. However, the commercial production of red tilapia is plagued by phenotypic instability. Most red tilapia strains are produced through interspecific hybridization or intensive selective breeding, but their offspring frequently exhibit reversion to natural dark colors or the development of unsightly black blotches [2]. This phenotypic segregation significantly reduces the market value of the fish and complicates breeding programs.

Traditional methods for managing fish skin color primarily involve dietary supplementation with carotenoids such as astaxanthin or lutein. While effective for enhancing redness (a* values), these dietary treatments are expensive and their effects are transient; the fish will lose their vibrant color shortly after the supplement is removed from their diet. Furthermore, dietary interventions cannot eliminate existing black pigmentation [3]. Other approaches, such as genetic modification or genome editing (e.g., targeting the tyrosinase gene), have the potential to produce stable color variants, but these methods face significant regulatory hurdles and widespread public resistance to genetically modified organisms (GMOs) in the food supply [4]. There is, therefore, a pressing need for non-genetic, cost-effective technologies that can induce permanent and stable phenotypic changes in commercial fish species.

Cold plasma technology has recently emerged as a powerful biostimulant in agricultural and life sciences [5-7]. Generated by the ionization of gases at room temperature, cold plasma produces a reactive environment containing electrons, ions, UV photons, and a wide array of reactive oxygen and nitrogen species (RONS). When plasma is discharged into or over water, it creates plasma-activated media (PAM), a solution enriched with H_2O_2, NO_2^-, and NO_3^-, as well as short-lived radicals like the hydroxyl (OH•) and superoxide ($O_2^{\bullet-}$) species [8-10]. While PAM has been extensively studied for its antimicrobial properties and its ability to enhance seed germination and plant growth [11], its application in developmental biology—specifically in the modulation of vertebrate pigmentation—remains largely unexplored.

Research in other agricultural sectors has shown that cold plasma exposure during early embryonic stages can significantly enhance physiological performance. For instance, cold plasma treatment of chicken eggs has been shown to improve hatching rates, accelerate growth, and modulate gene expression related to the somatotropic axis [12]. In fisheries science, preliminary work has demonstrated that supplying plasma-treated air to tilapia tanks can accelerate growth in juvenile and adult fish without the need for genetic modification [13]. These findings suggest that early-life exposure to plasma-generated reactive species may act as a developmental trigger, influencing phenotypic outcomes through pathways that do not involve alterations to the underlying DNA sequence [14,15].

The coloration of teleost fish is governed by the distribution and density of specialized pigment cells known as chromatophores, including melanophores (black), xanthophores (yellow), erythrophores (red), and iridophores (reflective) [16]. The development and migration of these cells from the neural crest are regulated by a suite of signaling pathways, including the melanocortin-1 receptor (*MC1R*), the agouti-signaling protein (*ASIP*), and transcription factors like *MITF* and *SOX10* [17,18]. During the early embryonic and larval stages of Nile tilapia, these pathways are highly plastic and sensitive to environmental stressors and chemical signals [19].

This study aims to investigate the use of PAM immersion as a novel method for inducing stable skin color variations in *Oreochromis niloticus* [13,20]. By utilizing a 2-factorial design, the research systematically evaluates how the duration of plasma activation and the developmental stage of the fish interact to influence phenotypic induction [14]. The study further assesses the long-term stability of the induced color variants, their growth trajectories over a 39-week period, and the resulting muscle quality [13]. By integrating chemical characterization of the media with longitudinal physiological monitoring, this work seeks to provide a scientific foundation for the application of cold plasma as a non-invasive tool for the enhancement of commercial traits in aquaculture.

Materials and methods

Ethical considerations and biological material origin

The experimental protocols for this study, which involved the utilization of Nile tilapia (*Oreochromis niloticus*) as a model for investigating phenotypic responses to plasma-activated media, were strictly

governed by ethical standards for animal welfare in research. All procedures received formal approval from the Animal Ethics Committee of Maejo University, Thailand, under the designated permit MACUC O34F/2567. Animal handling and experimental procedures were designed to minimize distress and suffering. These guidelines ensured that every stage of the experiment, from egg collection to adult sampling, adhered to national and institutional regulations designed to minimize physiological stress and ensure humane treatment. The biological material used in the study consisted of fertilized eggs and larvae obtained from a collaborative organic tilapia farming project with the Faculty of Fisheries Technology and Aquatic Resources at Maejo University. This collaboration provided access to a standardized breeding population, ensuring that the genetic background of the experimental subjects was consistent and representative of commercial stocks used in Southeast Asian aquaculture.

Following collection, the embryos and larvae were subjected to a rigorous staging process based on the system established by Fujimura and Okada [21]. This staging was essential for the factorial design of the experiment, as it allowed for the identification of specific developmental windows sensitive to exogenous reactive species. Five distinct stages were identified for treatment: (1) stage-1 embryos characterized by a lack of visible morphological differentiation, (2) stage-2 eyed eggs where the retinal pigmentation is first evident, (3) stage-3 pre-hatched embryos exhibiting active movement within the chorion, (4) stage-4 yolk-sac larvae characterized by their dependence on the yolk reserve, and (5) stage-5 free-swimming larvae that have completed yolk absorption and are capable of active exogenous feeding. Only individuals that exhibited normal morphological development were selected for the immersion trials, ensuring that the observed results were not biased by pre-existing developmental abnormalities. Prior to the immersion treatments, the larvae were maintained in a non-fed state to ensure a uniform metabolic baseline across all experimental groups.

Plasma device and generation of plasma activated media

The plasma-activated media (PAM) utilized in this research was generated through the application of a transient corona discharge plasma system, which has been previously validated for biological applications in other livestock sectors [12]. The electrical configuration of the system utilized a sharpened copper pin electrode connected to a self-resonant radio frequency (RF) power supply with a 20 W output capacity. This power supply was designed to deliver a sinusoidal alternating current (AC) with a peak voltage range of 6 - 10 kV, operating at a high frequency between 700 and 900 kHz. Such parameters are essential for generating a stable atmospheric pressure plasma discharge that can facilitate the transfer of energy into an aqueous phase without excessive heating.

For the production of each batch of PAM, 50 mL of high-purity distilled water was placed into a 120 mL glass vessel. The discharge was directed toward the liquid surface from a fixed distance of 1 cm, utilizing the surrounding atmospheric air as the working gas for the plasma plume. The activation periods were systematically varied to create a range of reactive species doses, with durations set at 0 (control), 2.5, 5.0, 7.5, 10, and 20 min. A critical constraint in the preparation of the PAM was the minimization of reactive species degradation; consequently, all solutions were utilized for the biological immersion trials within exactly 5 minutes of the termination of the plasma discharge.

Chemical and physical characterization of PAM

The chemical reactivity of the PAM was quantified through the analysis of 3 long-lived reactive oxygen and nitrogen species (RONS): hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-). These species are recognized as the primary stable vectors through which plasma-liquid interactions exert biological effects. Quantification was performed using a high-precision UV-Vis spectrophotometer (SDrive-500, HORIBA Scientific), integrated with a shutter control unit and a high-resolution detector optimized for the UV and visible spectral ranges. The measurement of each analyte relied on standardized photometric test kits

(SpectroQuant®, Germany, **Table 1**) to ensure analytical accuracy and reproducibility.

Table 1 Analytical parameters for RONS quantification in plasma-activated media.

Analyte	Method / Reagent	Wavelength (nm)	Range (mg/L)	Lot Number
Nitrite (NO_2^-)	Azo dye formation	525	0.002 - 1.000	HC900494
Nitrate (NO_3^-)	Photometric reduction	525	0.2 - 20.0	HI38740
Hydrogen Peroxide (H_2O_2)	Neocuproine complex	445	0.015 - 6.00	HC299148

The nitrite assay utilized a red-purple azo dye reaction, while the nitrate measurement was based on a reduction process where the final values, initially expressed as NO_3^- N, were converted to NO_3^- by a factor of 4.43. Hydrogen peroxide was determined through the reduction of copper (II) ions to copper (I) in the presence of a phenanthroline derivative, forming an orange complex. Samples that exceeded the linear detection range of the H_2O_2 kit (6.00 mg/L) were diluted with distilled water using precise micropipettes to ensure they fell within the calibrated range. All chemical measurements were conducted in triplicate for each activation time. In addition to chemical species, the physical properties of the PAM, including temperature and pH, were monitored using calibrated benchtop meters. The optical emission spectrum (OES) of the plasma discharge was recorded in the 200 - 900 nm range to verify the identity of the excited gaseous species responsible for the aqueous chemistry, focusing on the presence of molecular nitrogen systems and hydroxyl radicals.

Rearing environment and nutritional management

Following treatment, the Nile tilapia were maintained in a controlled environment at the Faculty of Fisheries Technology and Aquatic Resources. The rearing facility utilized concrete tanks measuring 93×155 cm^2, supplied with dechlorinated freshwater and a constant supply of atmospheric air via air stone diffusers. To prevent the co-mingling of different experimental groups while maintaining identical environmental conditions, the fish were housed in individual mesh cages (45×45 cm^2) within the larger tanks. Water quality parameters were rigorously monitored throughout the 39-week experimental period to ensure they remained within the safe limits for Nile tilapia culture: water temperature was maintained between 27 and 30 °C, dissolved oxygen levels were kept at or above 5 mg/L, and unionized ammonia (NH_3) concentrations were strictly controlled to remain below 0.05 mg/L. The pH of the rearing water was maintained at near-neutral levels, ranging from 6.5 to 7.5. The nutritional needs of the fish were met using a specialized commercial diet, Higade 9006T, produced by the Charoen Pokphand Group, Thailand (**Table 2**). This floating pellet diet was formulated specifically for carnivorous and high-growth freshwater fish, ensuring that the phenotypic outcomes were not constrained by nutritional deficiencies. The proximate composition of the diet was analyzed to confirm its adherence to the required protein and energy levels.

Table 2 Nutritional composition of the Higade 9006T diet used during the grow-out phase.

Nutrient	Content (%)
Crude Protein	≥ 42.0
Crude Fat	≥ 5.0
Crude Fiber	≤ 3.0
Moisture	≤ 10.0
Ash	~ 10.7

During the initial growth phases, the daily feeding rate was set at 20% of the total biomass, which was subsequently adjusted as the fish matured. The feed was distributed across 4 equal meals per day to promote efficient nutrient assimilation and minimize metabolic waste.

Experimental design and protocol for immersion

The study employed a robust 2-factorial experimental design to evaluate the interactions between the intensity of plasma treatment and the developmental stage of the subject (**Figure 1**). Factor 1 was defined as the plasma activation time (0, 2.5, 5.0, 7.5, 10 and 20 min), and Factor 2 was defined as the developmental stage (Stages 1 through 5). This structure yielded 30 unique treatment combinations, each of which was executed in triplicate (n = 3). Each replicate cage was stocked with 500 individuals, bringing the total number of subjects to 4,500. To determine the optimal immersion duration for safe treatment and effective skin color change in Nile tilapia, stage 1 - 5 embryos were initially exposed to PAM activation for 30 and 60 s. The results showed that a 30-second immersion effectively induced color changes while maintaining a lower mortality rate compared to the 60-second interval (data not shown). Consequently, this 30-second duration was established as the standard for all subsequent experimental groups. To differentiate between the effects of plasma-specific factors (such as short-lived radicals or UV radiation) and the stable chemical species, a separate control group was immersed in chemically simulated distilled water. This solution was prepared by dissolving H_2O_2, NO_2^-, and NO_3^- in distilled water to concentrations matching those measured in the PAM treatment, following the dilution formula $C_1V_1 = C_2V_2$.

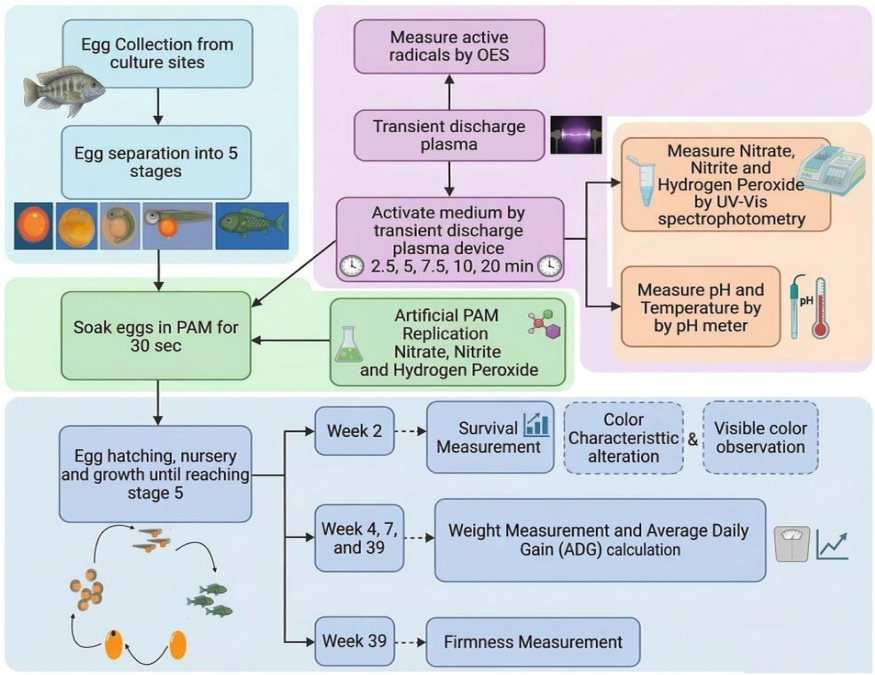


Figure 1 Experimental design and workflow for investigating the effects of plasma-activated media (PAM) on Nile tilapia (*Oreochromis niloticus*).

Phenotypic and physiological analysis protocols

The assessment of survival was performed at 14 days post-immersion, by which time the surviving individuals had reached the free-swimming stage. Survival for each replicate was calculated as the percentage of live individuals relative to the initial 100 eggs or larvae. Long-term growth performance was evaluated by weighing 30 randomly selected fish per treatment (10 from each replicate) at Week 4, Week 7, and Week 39. These data points were used to calculate the Average Daily Gain (ADG) over the duration of the trial, providing a comprehensive view of growth trajectories. Skin color analysis was conducted using

both qualitative morphological observation and quantitative instrumental measurements. At Week 2 and Week 39, the presence of color variants (Whole Pink, White, Black Blotch) was recorded to determine the stability of the phenotypic alteration. Quantitative color data were obtained by anesthetizing the fish with clove oil (9.7 mL per 200 L) and capturing high-resolution digital images under standardized lighting. These images were analyzed using ImageJ software to extract RGB values from 20 random points per fish, which were then converted to the CIELAB color space (L*, a*, b*). Chromatophore types were further observed in skin scales using an optical microscope to qualitatively assess the distribution of melanophores, xanthophores, and erythrophores. Melanophore density was quantified by counting cells within a specific millimeter-square area of the dorsal skin. At the conclusion of the 39-week study, the textural properties of the fish fillets were evaluated. Dorsal muscle samples were cut into standardized 1×2×1 cm^3 pieces and subjected to Texture Profile Analysis (TPA) using a specialized texture analyzer. The analysis utilized a 12-mm probe and a double-compression cycle to measure the firmness of the flesh across 4 anatomical regions: upper body, mid-body, belly, and caudal sections.

Statistical procedures

All data collected throughout the study were presented as mean values ± standard deviation (SD). The interactive effects of PAM activation time and developmental stage on survival and color induction were analyzed using a 2-way ANOVA. For parameters measured within a single stage or activation time, such as final weight, CIELAB coordinates, and flesh firmness, a 1-way ANOVA was applied. When significant differences were identified ($p < 0.05$), Tukey's post-hoc test was employed for pairwise comparisons between treatment groups. All statistical computations were performed using the Real Statistics Resource Pack for Microsoft Excel.

Results and discussion

Optical emission and chemical evolution of the plasma environment

The characterization of the transient corona discharge plasma used in this study revealed a complex gaseous environment dominated by reactive nitrogen species. The optical emission spectrum (**Figure 2**) exhibited prominent peaks corresponding to the second positive system of N_2 and the first negative system of N_2^+, confirming that the plasma discharge in ambient air effectively dissociated and excited atmospheric nitrogen. The detection of a weak hydroxyl radical (OH•) peak at 308 nm is of particular significance, as it indicates the active dissociation of water vapor within the discharge zone. These excited gaseous species serve as the precursors for the aqueous reactive oxygen and nitrogen species (RONS) that constitute the activated nature of the media.

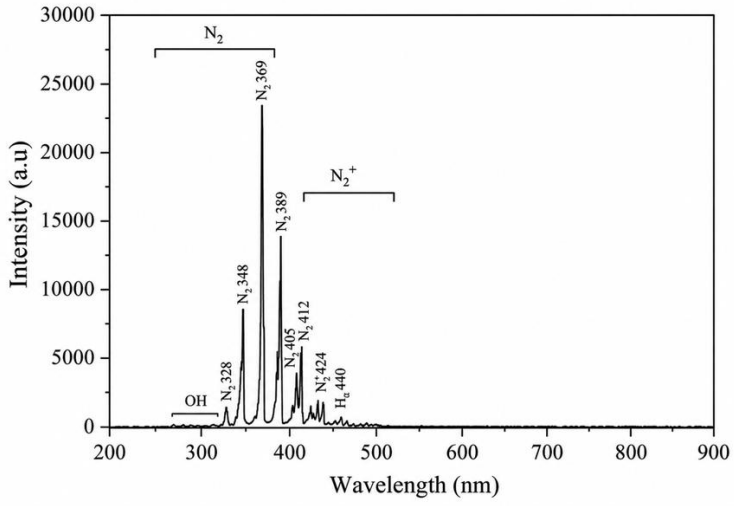


Figure 2 Optical emission spectrum of the plasma in ambient air (200 - 900 nm). Strong peaks in the 300 - 430 nm region are mainly attributed to excited N_2 and N_2^+ species, with a weak OH emission near 308 nm, indicating that the plasma is predominantly nitrogen-based.

The chemical evolution of the water during plasma treatment was characterized by a linear accumulation of hydrogen peroxide (H_2O_2) and a non-linear increase in nitrogen-based species (**Figure 3**). H_2O_2 concentrations reached their maximum at 20 min of activation, demonstrating that the plasma system was highly efficient at generating and transferring peroxide species into the liquid phase. Nitrite (NO_2^-) levels also increased significantly but at a decelerating rate after the 10-minute mark, a phenomenon likely caused by the secondary oxidation of nitrite into nitrate (NO_3^-) or its reaction with H_2O_2 to form peroxynitrite ($ONOO^-$) in the increasingly acidic environment.

The progressive acidification of the media (dropping from pH 7.0 to 3.5) is a hallmark of air-plasma treatment, driven by the dissolution of nitric acid (HNO_3) and nitrous acid (HNO_2). This chemical profile provides the reactive basis for the biological interactions observed in the Nile tilapia trials.

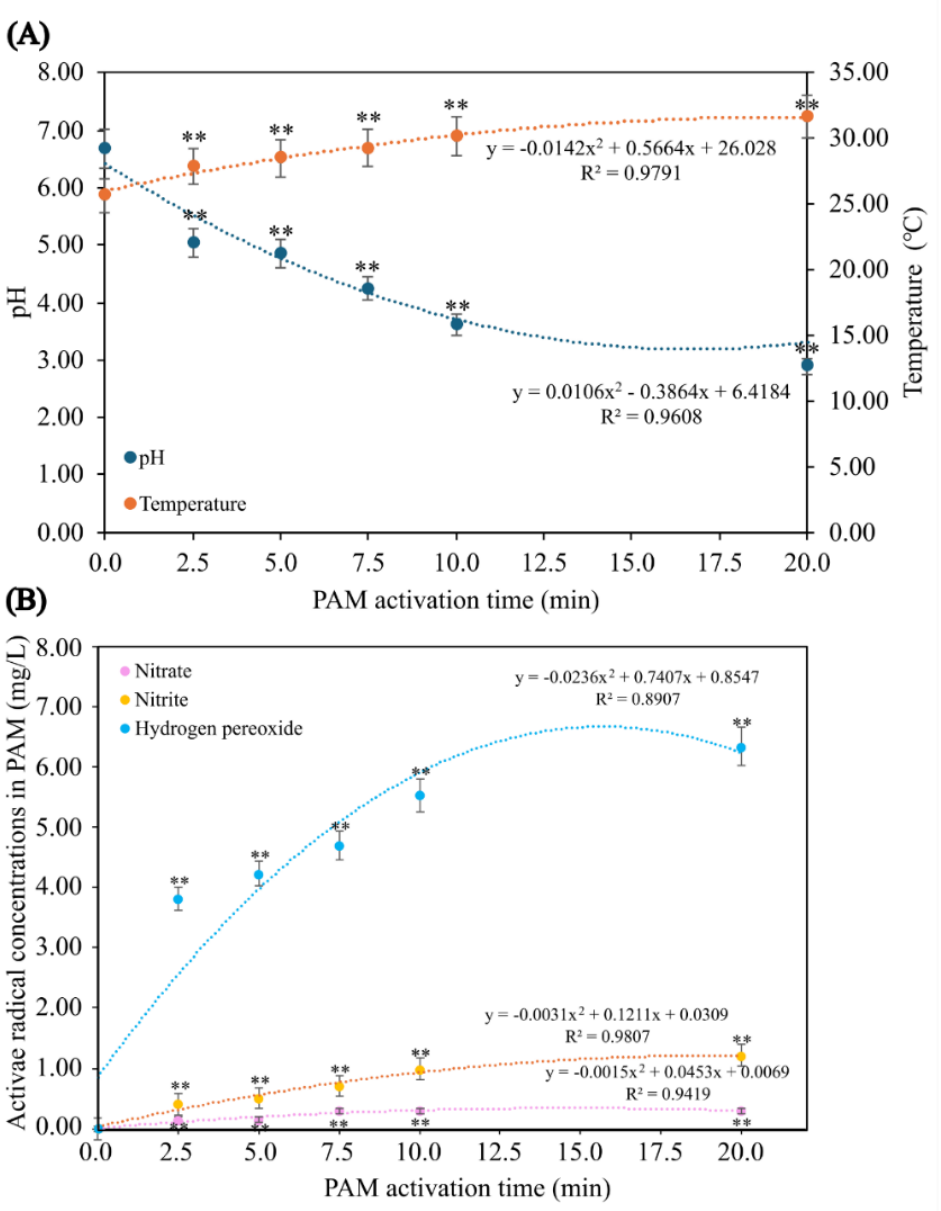


Figure 3 (A) Changes in pH and temperature of plasma-activated water (PAM) at different activation times. pH decreased progressively, while temperature showed a slight increase during activation. (B) Concentrations of hydrogen peroxide, nitrite, and nitrate in PAM, which increased with longer activation times. Values are mean ± SD; asterisks indicate significant differences from the control.

Sensitivity of developmental stages and survival outcomes

The impact of PAM immersion on survival was heavily dependent on the developmental stage of the Nile tilapia at the time of exposure (**Figure 4**). Stage 2 (eyed egg) was identified as a critical sensitivity window; while survival remained stable up to 10 minutes of activation, a significant reduction was observed at the 20-minute level ($p < 0.01$). This suggests that the physiological processes occurring during eye pigmentation and early neural development are particularly susceptible to oxidative stress or the rapid shift in environmental pH. In contrast, Stage 3 (pre-hatched embryo) demonstrated higher resilience, with no significant differences in survival across the various PAM treatments. This increased tolerance might be attributed to the maturation of the protective chorion or the activation of endogenous antioxidant systems prior to hatching.

Survival in the later larval stages (Stages 4 and 5) remained consistently high, exceeding 80% in all experimental groups. The resilience of these stages indicates that once the larvae have transitioned to exogenous life, their capacity for ionoregulation and chemical detoxification is sufficiently developed to withstand short-term exposure to RONS. Conversely, the extremely low survival observed in Stage 1 embryos across all treatments—including the control—suggests that early cleavage and gastrulation phases in O. niloticus are highly sensitive to the mechanical handling and temperature changes associated with the experimental protocol, rendering them unsuitable for this specific immersion method.

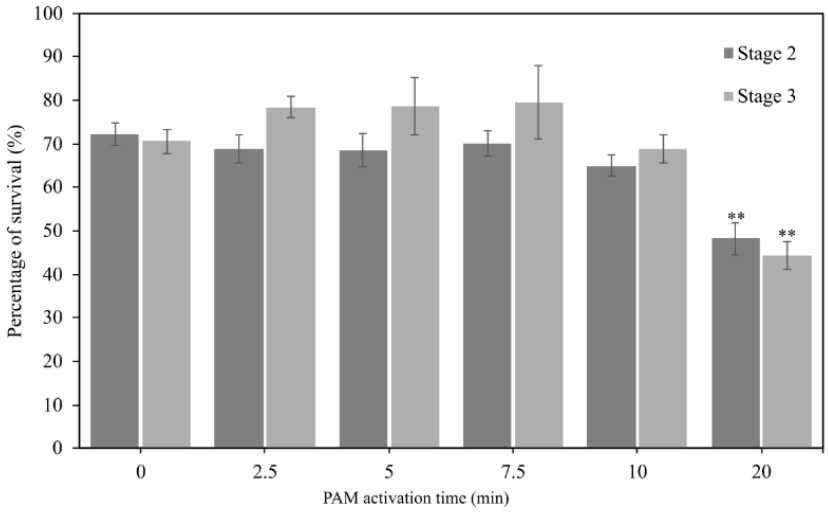
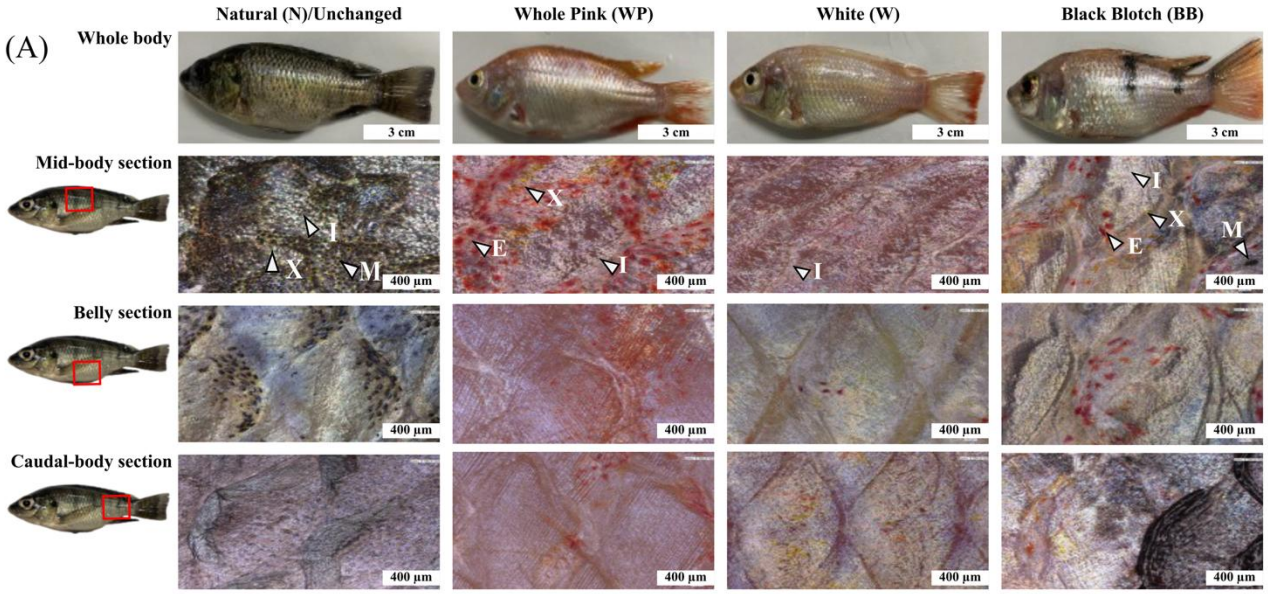


Figure 4 Survival percentage of larvae at Stage 2 and Stage 3 after exposure to plasma-activated water (PAM) with different activation times. Survival remained relatively stable up to 10 min, whereas a significant reduction was observed at 20 min (**, $p < 0.01$). Values are presented as mean ± SD.

Induction and stability of skin color variants

One of the most noteworthy findings of this study was the induction of quantifiable skin color alterations following PAM exposure. These phenotypic shifts were categorized into 4 distinct patterns: Natural (N), Whole Pink (WP), White (W), and Black Blotch (BB) (**Figure 5**). At Week 39, CIELAB analysis confirmed that these visual differences corresponded to statistically significant changes in color coordinates.



(B)

Color Coordinate Values		Unchanged	PAW-WP	PAW-W	PAW-BB
L*	Mean ± S.D.	58.4 ± 0.016	62.7 ± 0.169	63.2 ± 0.433	58.2 ± 0.084
	Range	57.9 – 58.9	62.2 – 63.2	62.7 – 63.7	57.6 – 58.6
a*	Mean ± S.D.	10.2 ± 0.012	7.1 ± 0.043	7.6 ± 0.397	12.4 ± 0.504
	Range	9.7 – 10.7	6.6 – 7.6	7.1 – 8.0	11.9 – 12.9
b*	Mean ± S.D.	0.9 ± 0.004	3 ± 0.012	1 ± 0.049	-6.4 ± 0.267
	Range	0.4 – 1.4	2.4 –3.4	0.5 – 1.6	-7.0 – -5.8

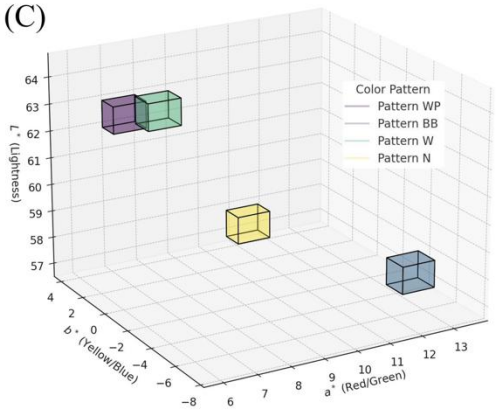


Figure 5 Phenotypic characteristics and skin color analysis of 4 different color patterns in Nile tilapia: (A) Morphological observation of the whole body and microscopic views of scales from 3 body regions (mid-body, belly, and caudal regions) for Natural (N), Whole Pink (WP), White (W), and Black Blotch (BB) patterns. Arrows indicate different types of chromatophores: melanophores (M), iridophores (I), xanthophores (X), and erythrophores (E). Scale bars represent 3 cm for whole-body images and 400 μm for microscopic images. (B) Comparison of CIELAB color coordinate values (L*, a*, and b*) among the 4 patterns, showing Mean ± S.D. and range. (C) 3D scatter plot representing the spatial distribution of color patterns in the L*a*b* color space.

The induction of the Whole Pink phenotype was significantly correlated with PAM activation time in Stage 2 eggs, reaching a rate of 28.5% in the 20-minute treatment group (**Figure 6**). Crucially, the chemically simulated media group which contained identical concentrations of H_2O_2, NO_2^-, and NO_3^- failed to induce any color variants (0% induction rate, $p < 0.001$) (**Figure 7**). This finding strongly suggests that the phenotypic shift is not driven by stable RONS alone. Instead, it likely involves the activity of short-lived species such as the hydroxyl radical (OH•) or singlet oxygen ($^1O_2$), or potentially the synergistic effect of UV radiation and physical plasma factors during the initial activation.

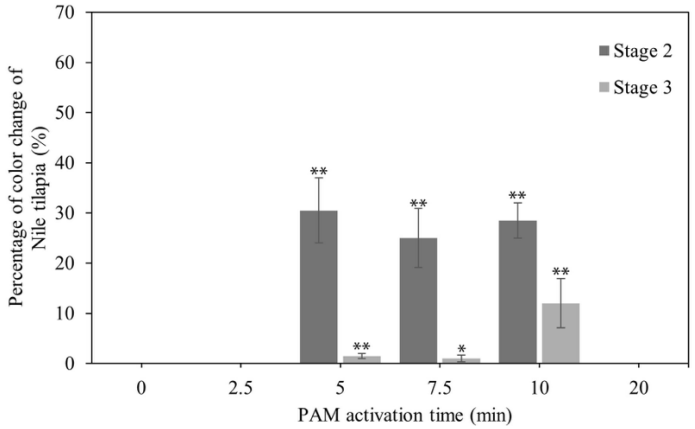


Figure 6 Effect of plasma-activated media (PAM) activation time on skin color change in Nile tilapia at different developmental stages

The graph shows the percentage of color change in Nile tilapia as a function of plasma-activated media (PAM) activation time at 2 developmental stages (Stage 2 and Stage 3). At Stage 2, PAM treatment at 5, 7.5, and 10 min resulted in a pronounced increase in color change, reaching approximately 25% - 30%, with all treatments showing statistically significant differences compared with the control ($p < 0.01$). In contrast, Stage 3 exhibited a markedly lower response, with minimal color changes at 5 and 7.5 min and a moderate but significant increase at 10 min. Overall, the results indicate a stage-dependent response to PAM activation time, with Stage 2 being substantially more sensitive to PAM-induced color alteration than Stage 3.

The color variants demonstrated remarkable phenotypic stability, with 100% of the individuals identified as variants at the larval stage retaining their specific coloration through to adult harvest at Week 39. This stability is fundamentally different from dietary pigmentation, which is transient and requires constant supplementation. The microscopic evidence provided a mechanistic explanation for this stability: The WP and W phenotypes exhibited a drastic and significant reduction in melanophore density (5 - 12 cells/mm^2) compared to the Natural control (145 cells/mm^2). This suggests that PAM exposure interferes with the migration or differentiation of neural crest cells into melanocytes, or alternatively, induces the selective apoptosis of early-stage melanoblasts.

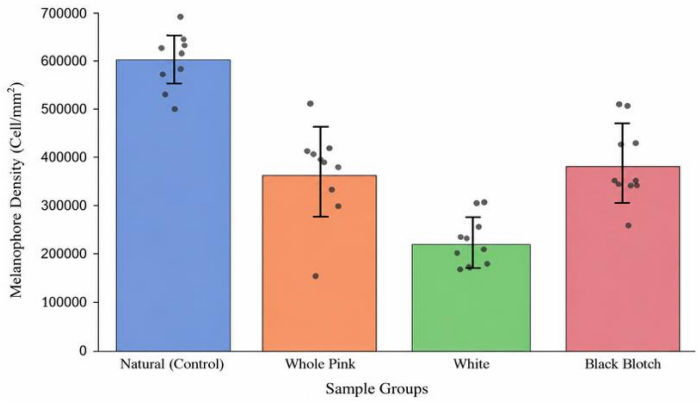


Figure 7 Quantitative analysis of skin pigmentation. (A) Representative microscopic views of dorsal skin scales (40× magnification) showing the distribution of chromatophores. Melanophores (black pigment cells) are abundant in Natural (Control) but sparse or absent in Whole Pink and White phenotypes. (B) Quantification of melanophore density (cells/mm²). Data represent the mean ± S.D. (n = 10 fish per group). Dots represent individual biological replicates. Significant reductions in melanin density were observed in PAM-treated phenotypes compared to the Natural control (*** $p < 0.001$, one-way ANOVA).

Longitudinal growth performance and hormesis

The evaluation of growth performance revealed a fascinating temporal trend (**Figure 8**). During the initial rearing phase (Weeks 4 and 7), there were no significant differences in body weight among the experimental groups, indicating that PAM immersion did not adversely affect early developmental metabolism. However, by the final sampling point at Week 39, the PAM-treated groups (specifically the Whole Pink and White phenotypes) exhibited significantly higher final body weights and Average Daily Gain (ADG) compared to the Natural control and chemical control groups (**Table 3**).

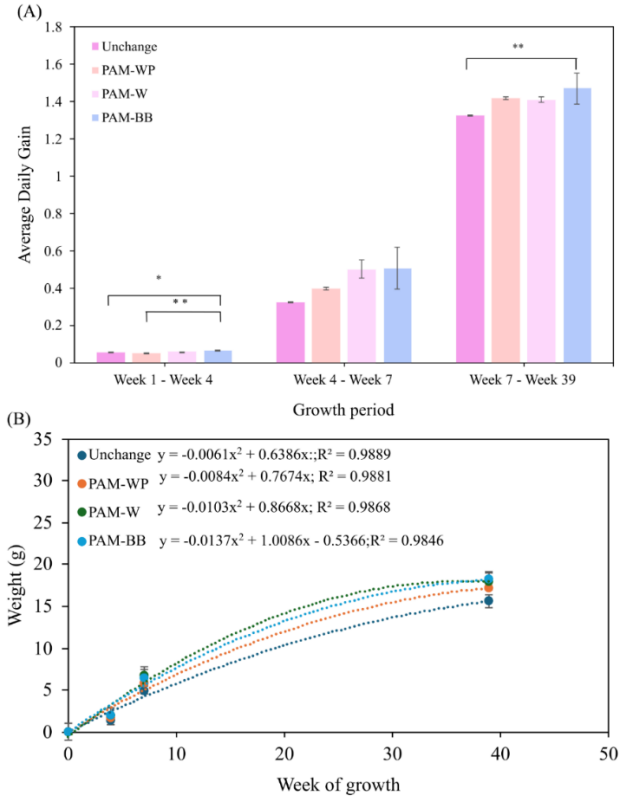


Figure 8 Growth performance of Nile tilapia across different color patterns over 39 weeks. (A) Average Daily Gain (ADG) during 3 growth periods: Week 1 - 4, Week 4 - 7, and Week 7 - 39. Data are compared between the Unchange group and PAM-treated groups (PAM-WP, PAM-W, and PAM-BB). Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$). (B) Cumulative body weight (g) throughout the growth period. The dotted lines represent the polynomial regression models for each group, with corresponding equations and R^2 values indicated in the legend. All data are expressed as Mean ± S.D.

Table 3 Comparison of Average Daily Gain (ADG) across growth stages for control and Whole Pink phenotypes.

Growth Interval	Natural (Control) ADG (g/d)	PAM-WP ADG (g/d)	*p*-value
Weeks 1 - 4	0.05 ± 0.01	0.06 ± 0.01	> 0.05
Weeks 4 - 7	0.22 ± 0.03	0.24 ± 0.04	> 0.05
Weeks 7 - 39	1.15 ± 0.12	1.48 ± 0.15	< 0.01

This long-term growth acceleration is consistent with the principle of hormesis, wherein low doses of a stressor (in this case, reactive species and acidified media) trigger adaptive physiological responses that enhance biological vigor. Similar outcomes have been reported in chicken embryos, where cold plasma

exposure was associated with increased levels of growth hormone (somatotropin). The growth-promoting effect of PAM in tilapia suggests that early life exposure may permanently prime the endocrine system or improve nutrient assimilation efficiency later in life.

Anatomical variation in flesh firmness

Texture Profile Analysis (TPA) at Week 39 demonstrated that PAM exposure did not have any detrimental effects on the muscle quality of the tilapia (**Figure 9**). In fact, the PAM-treated color variants exhibited equal or significantly higher firmness in several anatomical regions compared to the Natural control (**Table 4**). This is a critical finding for aquaculture, as flesh texture is a primary determinant of market value and consumer acceptance for tilapia fillets.

Table 4 Percentage firmness across anatomical regions at Week 39. Asterisks indicate significant differences from the control ($p < 0.05$).

Anatomical Region	Natural (Control) Firmness (%)	PAM-WP Firmness (%)	PAM-BB Firmness (%)
Upper Body	88.2 ± 4.5	90.1 ± 3.8	89.5 ± 4.2
Mid-Body	74.5 ± 5.1	82.4 ± 4.9*	81.2 ± 5.5*
Belly	51.2 ± 6.3	53.8 ± 5.9	52.4 ± 6.1
Caudal	68.4 ± 4.8	75.6 ± 4.2*	74.8 ± 4.9*

The observed spatial gradient in firmness—where the upper and mid-body regions were significantly firmer than the belly and caudal sections—was consistent across all treatment groups. This variation is characteristic of the species and is linked to the distribution of intramuscular lipid and collagen content. The fact that PAM-treated fish maintained or exceeded these values confirms that the technological intervention is compatible with high-quality fillet production.

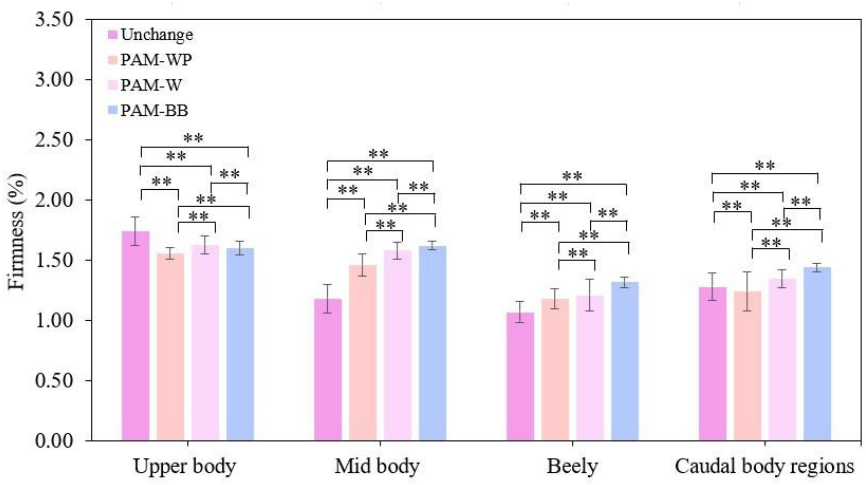


Figure 9 Firmness percentages of different body sections as influenced by PAM treatments. The bar chart displays the firmness levels in the upper body, mid body, belly, and caudal body sections for Unchanged (control), PAM-WP, PAM-W, and PAM-BB groups. Significant differences between specific treatment pairs are denoted by ** ($p < 0.01$). Data are presented as mean ± SD.

The application of plasma-activated media (PAM) has precipitated a paradigm shift across agricultural, biological, and materials sciences. Generated through the ionization of gases at room temperature, cold plasma

yields a highly reactive environment comprising electrons, ions, ultraviolet (UV) photons, and a diverse spectrum of reactive oxygen and nitrogen species (RONS). When interfaced with an aqueous phase, these transient physical plasmas generate stable, semi-stable, and short-lived chemical species—including hydrogen peroxide (H_2O_2), nitrite (NO_2^-), nitrate (NO_3^-), hydroxyl radicals (•OH), and peroxynitrite ($ONOO^-$)—which have been demonstrated to exert profound biostimulatory and antimicrobial effects. Historically utilized for surface decontamination and the enhancement of seed germination, the exploration of PAM in vertebrate developmental biology represents a frontier with immense commercial and scientific implications [22].

Recent empirical investigations have demonstrated that immersing early-stage Nile tilapia embryos (specifically Stage 2 eyed eggs) in PAM induces stable, irreversible phenotypic shifts—producing highly desirable Whole Pink and White variants at a rate of 28.5% without the necessity of genetic modification. Concurrently, this exposure triggers a profound hormetic growth acceleration and maintains optimal myofibrillar textural firmness through the entirety of the 39-week grow-out phase. To fully ascertain the viability and mechanistic foundations of this intervention, it is imperative to exhaustively deconstruct the underlying biochemical mechanisms, kinetic paradoxes, and long-term physiological alterations precipitated by PAM exposure. This report provides a highly detailed, multi-tiered analysis of the interactions between plasma-derived RONS and teleost developmental pathways, addressing pigmentation interference, decoloration kinetics, radical survival paradoxes, somatotropic growth acceleration, and myofibrillar cross-linking.

Comparative kinetics of decoloration: Natural mutagenesis vs. PAM-induced override

To ascertain the true scientific and commercial significance of PAM-induced color variants, it is necessary to rigorously contextualize the experimental results against the natural rate of spontaneous decoloration and chromatic reversion observed in *Oreochromis niloticus* and its commercial hybrids. The core question is whether the decoloration rate is higher in PAM treatment, or whether they are the same, which would imply that PAM merely triggers a latent natural phenomenon.

In wild-type, pure-strain populations of Nile tilapia, spontaneous mutations leading to leucism, albinism, or generalized pink/white phenotypes are exceedingly rare, lower than 1% [23]. Evolutionary pressures, such as the necessity for UV protection and predator camouflage in shallow aquatic environments, exert strong stabilizing selection against depigmentation [24]. Genomic analyses of various wild and farmed populations indicate low overall observed heterozygosity, further minimizing the frequent natural emergence of recessive color traits or single-nucleotide polymorphisms affecting the *slc24a5* or *nckx2* loci [23]. Therefore, in a genetically standardized, non-hybrid wild-type population, the natural rate of spontaneous, stable depigmentation approaches zero.

In commercial aquaculture, red phenotypes are typically maintained through the complex interspecific hybridization of species such as *Oreochromis mossambicus*, *Oreochromis aureus*, and *Oreochromis niloticus* [25]. However, the genetic architecture of this red coloration is highly unstable due to the epistatic interactions of dominant and recessive alleles [26]. Crossbreeding studies demonstrate that F1 and F2 generations exhibit immense phenotypic segregation; for example, backcrosses of red hybrids with wild-type strains can yield up to 61.17% wild-type (dark) offspring, with only a fraction maintaining a pure red phenotype [2].

Crucially, even those hybrid individuals that present as pure red or pink in the larval stage frequently undergo spontaneous chromatic reversion during the grow-out phase. Research indicates that between 27.2% and 41.7% of cultured red tilapia develop irregular black blotches or undergo whole-body darkening during periods of environmental stress, such as overwintering, thermal fluctuations, or exposure to dark background tank colors [27]. This natural fading or spotting is fundamentally driven by physiological plasticity; the teleost neuroendocrine system translocates melanosomes within existing chromatophores or reactivates dormant melanocytes via the sympathetic nervous system in response to background adaptation [28].

Contrasting sharply with natural hybrid instability, the PAM treatment achieved a 28.5% induction rate of

distinct, completely depigmented variants (Whole Pink, White) in a single generation of standardized breeding stock. Crucially, the PAM-induced variants exhibited 0% reversion or phenotypic segregation across the entire 39-week maturation period.

The decoloration rate observed via PAM treatment is therefore vastly higher than the natural spontaneous mutation rate in wild-type fish, and structurally superior to the unstable segregation seen in hybrids. If the PAM treatment were simply an environmental stressor mirroring natural physiological phenomena (like tank color adaptation or thermal stress), the fish would inevitably regain their pigmentation or develop blotches as the cellular stress subsided and normal redox homeostasis was restored during the 39 weeks of optimal rearing [29]. Instead, the permanent absence of melanophores in the PAM-treated adult tissue proves that the plasma treatment is not inducing a transient physiological response. Rather, PAM acts as a deterministic developmental override, permanently locking the organism into a depigmented state. This indicates that the decoloration by PAM treatment is definitively not a natural phenomenon, but a novel, engineered interruption of embryonic development.

The kinetic paradox of short-lived species and the 5-minute gap

The experimental protocol governing the PAM immersion was explicitly designed that all biological immersion trials were conducted within 5 min of the termination of the plasma discharge to minimize reactive species degradation. Furthermore, the study noted that a chemically simulated control—matching the PAM's long-lived RONS concentrations—failed to produce any variants, leading authors to suggest that short-lived species or physical plasma factors are the primary drivers of the phenotypic shift. A profound biochemical paradox arises when evaluating the efficacy of these short-lived plasma species against the elapsed 5-minute gap.

In aqueous solutions, primary radical species generated directly by the atmospheric pressure plasma plume possess extraordinarily brief half-lives. Hydroxyl Radical (•OH), arguably the most potent oxidant produced in plasma-liquid interactions, possesses an extreme reactivity that limits its half-life to the order of nanoseconds [31]. Its diffusion distance is consequently restricted to less than a micrometer. Superoxide Anion ($O_2^{\bullet -}$), generated by the interaction of energetic electrons with oxygen molecules, is slightly more stable but still highly transient, exhibiting a half-life of microseconds [30]. Peroxynitrite ($ONOO^-$) Peroxynitrous Acid (ONOOH), formed by the diffusion-limited combination of nitric oxide (NO•) and superoxide, is highly unstable at physiological and acidic pH. Once protonated into peroxynitrous acid (pKa ~6.8), it rapidly decomposes with a half-life of roughly 1.0 to 1.9 seconds at near-neutral pH, and even faster in highly acidic environments [31].

Given these incontrovertible laws of chemical kinetics, the primary burst of short-lived radicals generated during the active plasma discharge phase is entirely extinct by the time the 5-minute pre-immersion gap has elapsed [32]. The resolution to this kinetic paradox lies in the dynamic post-discharge chemistry unique to PAM. While the primary radicals vanish, the PAM accumulates high concentrations of stable, long-lived species: H_2O_2, NO_2^-, and NO_3^-. Concurrently, the continuous dissolution of plasma-produced NO_x species drastically lowers the pH of the water, typically acidifying it to a pH range of 2.5 to 3.5 through the formation of nitric and nitrous acids [33].

In this highly acidic, post-discharge environment, the stable species are not chemically inert. Instead, H_2O_2 and NO_2^- engage in a continuous, pseudo-second-order post-discharge reaction to synthesize secondary peroxynitrous acid (ONOOH) *in situ* within the bulk liquid [34].

This dynamically generated ONOOH subsequently undergoes spontaneous homolytic cleavage to release a secondary, continuous stream of highly reactive hydroxyl radicals (•OH) and nitrogen dioxide radicals ($\bullet NO_2$) [35]. Electron Paramagnetic Resonance (EPR) spin-trapping studies using probes like DMPO have confirmed that •OH generation continues in PAW 2 - 4 h long after the plasma source is removed [36]. Therefore, during the 30-second immersion of the eggs, the teleost chorion is continuously bombarded by secondary, short-lived radicals generated at the immediate fluid-tissue interface, effectively bypassing the 5-minute gap [37].

A pivotal finding of the study was that a chemically simulated control—prepared by dissolving commercial H_2O_2, NO_2^-, and NO_3^- in distilled water to

precisely match the measured concentrations of the 20-minute PAM—failed entirely to induce any color variants (0% induction). If the long-lived species were solely responsible for generating the active •OH via post-discharge kinetics, the chemical control should have replicated the biological effect.

The absolute failure of the chemical control highlights the unique complexity of true plasma chemistry. First, the chemical control may not have replicated the extreme transient acidification profile required to drive the pseudo-second-order generation of peroxynitrite at an aggressive rate [34]. Second, true PAM contains trace amounts of longer-lived organic hydroperoxides (if any atmospheric volatile organic compounds interface with the liquid), which possess varying half-lives and can penetrate cellular lipid bilayers more effectively than bulk H_2O_2 [38].

Finally, these findings strongly corroborate the hypothesis that the physical parameters of the initial plasma activation such as the dissolution of ozone (O_3), trace UV-C radiation causing photo-induced radical generation, or the creation of transient electro-hydraulic forces create a synergistic, non-equilibrium chemical state that simply cannot be synthesized by mixing off-the-shelf laboratory reagents. The eggs are thus responding to the complex decay kinetics of the plasma-liquid interface, rather than a static chemical bath.

Interference of PAM-derived RONS in pigmentation mechanisms

The stable depigmentation and generation of Whole Pink and White Nile tilapia phenotypes following PAM immersion represent a profound alteration of vertebrate pigment cell biology. The coloration of teleost fishes is governed by the spatial distribution, density, and differentiation of highly specialized pigment cells known as chromatophores, which include melanin-producing melanophores, carotenoid-bearing xanthophores and erythrophores, and light-reflecting iridophores [24]. Microscopic quantification reveals that PAM-treated variants possess a critically depleted melanophore density (5–12 cells/mm²) relative to the natural wild-type control (145 cells/mm²). This terminal reduction indicates that PAM-derived RONS disrupt the fundamental ontogeny of pigment cells early in embryogenesis.

All teleost chromatophores originate from a transient, multipotent embryonic structure known as the neural crest [39]. During the early stages of embryogenesis aligning perfectly with the critical window of sensitivity observed at the Stage 2 eyed-egg phase neural crest cells (NCCs) must undergo a highly coordinated epithelial-mesenchymal transition (EMT) to delaminate from the neural tube and migrate along specific dorsal and ventral pathways to colonize the developing integument [24]. The EMT requires dynamic cytoskeletal remodeling, characterized by localized cell membrane blebbing, filopodial extension, and the precise regulation of Rho-associated protein kinase (ROCK) and myosin II networks [40].

The introduction of exogenous RONS via PAM imposes acute oxidative and nitrosative stress on these highly sensitive embryonic microenvironments. High concentrations of reactive oxygen species (ROS) can physically alter the extracellular matrix and directly inhibit the integrin-mediated adhesion required for NCC motility [39]. Furthermore, excessive oxidative stress is known to dysregulate the actin-myosin contractility networks, thereby arresting the blebbing phase of EMT and effectively stalling NCC migration before the precursor melanoblasts can successfully traverse the somitic mesoderm and colonize the epidermal strata [41]. By inhibiting the geographic dissemination of melanoblasts, PAM exposure fundamentally prevents the establishment of a baseline melanophore population in the adult fish.

Beyond physical migratory inhibition, the transient RONS pulse likely induces a durable epigenetic or transcriptional suppression of the melanogenic signaling cascade. The master regulator of melanocyte differentiation, survival, and pigment synthesis is the microphthalmia-associated transcription factor (*MITF*) [42]. *MITF* acts directly on the promoter regions of essential melanogenic enzymes, specifically tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*), and dopachrome tautomerase (*DCT*), which are required to convert L-tyrosine into dopaquinone and ultimately into eumelanin [43].

The activation of *MITF* is heavily dependent on the melanocortin-1 receptor (*MC1R*) pathway. When the agonist α-melanocyte-stimulating hormone (α-MSH) binds to the transmembrane *MC1R*, it triggers the cAMP/PKA/CREB signaling cascade, upregulating

MITF and driving melanin production [43]. However, the agouti-signaling protein (*ASIP*) functions as a potent inverse agonist, competing with α-MSH for *MC1R* binding and effectively suppressing eumelanin synthesis in favor of depigmentation or pheomelanin production [44].

Extensive physiological literature indicates that acute stressors, including oxidative stress and elevated cortisol, can upregulate the expression of *ASIP* in the skin and downregulate *MC1R* sensitivity [45]. The massive influx of ROS and reactive nitrogen species (RNS) from the PAM activation treatment acts as a severe, transient exogenous stressor. It is highly probable that this radical spike artificially hyper-activates redox-sensitive inhibitory pathways, triggering an immediate and sustained upregulation of *ASIP* [45]. The resultant prolonged blockade of *MC1R* would starve the migrating melanoblasts of the cAMP signaling required for survival, leading to the apoptosis of early-stage melanophores or the diversion of multipotent NCCs toward non-melanized cellular fates, such as iridophores or erythrophores [27].

In addition to receptor-level interference, NO-derived radicals (such as peroxynitrite) directly induce S-nitrosylation and oxidative damage to redox-sensitive signaling proteins. Food-derived or exogenous peptides and reactive species can occupy the bioactive copper-binding sites of tyrosinase, resulting in structural enzyme inhibition and catastrophic failures in melanin biosynthesis. Furthermore, the fading of red tilapia melanocytes has been linked to the activation of apoptosis and autophagy pathways driven by *baxα*, *beclin1*, and *atg7* genes, which are highly sensitive to sudden oxidative bursts [27].

Moreover, the phenomenon of body coloration in tilapia is heavily influenced by epigenetic regulation. Transcriptomic and epigenomic analyses (such as CUT&Tag) have revealed that red or depigmented skin regions exhibit high expression of genes suppressing melanin synthesis, associated with specific histone modifications (e.g., H3K4me1, H3K27me3, H3K27ac) [46]. It is highly plausible that the oxidative shock from PAM alters DNA methylation patterns or histone deacetylase activity, establishing a permanent "epigenetic memory" that locks the *MITF* and *TYR* genes in a transcriptionally repressed chromatin state for the remainder of the organism's lifespan [46].

Somatotropic modulation: Hormesis and growth acceleration

One of the most economically significant findings of the PAM intervention was the profound enhancement of longitudinal growth performance. By the conclusion of the 39-week trial, Nile tilapia exposed to PAM at the embryonic stage exhibited significantly higher cumulative body weights and elevated Average Daily Gain (ADG) metrics compared to untreated cohorts and the chemical control. This enhanced growth trajectory is not merely a localized physiological anomaly it represents a classical manifestation of biological hormesis intertwined with the permanent modulation of the teleost somatotropic axis.

Hormesis describes a highly conserved, biphasic dose-response phenomenon wherein exposure to low, sub-lethal doses of an environmental stressor triggers an adaptive, over-compensatory physiological response that enhances the overall biological vigor and resilience of the organism [48]. The PAM immersion subjects the Stage 2 eggs to a brief (30-second), intense wave of oxidative/nitrosative stress and low pH.

To survive this acute chemical assault, the embryo must massively upregulate endogenous antioxidant defenses (e.g., superoxide dismutase, catalase, glutathione peroxidase) and trigger advanced cellular repair mechanisms [48]. This early-life metabolic priming effectively programmed the organism to operate at a higher baseline of metabolic efficiency. As observed in fish recovering from hypoxia or fasting, the return to a positive energy status following an acute stressor is characterized by a hyper-anabolic rebound, optimizing nutrient assimilation, feed conversion ratios (FCR), and energy partitioning later in life [47].

At the molecular endocrinology level, the observed growth acceleration is inextricably linked to the somatotropic axis, primarily governed by Growth Hormone (GH) and Insulin-like Growth Factor 1 (IGF-1) [49]. In teleosts, GH is synthesized by somatotropes in the anterior pituitary gland and acts on hepatic (liver) receptors to stimulate the production of IGF-1. IGF-1 subsequently circulates systemically to drive chondrocyte proliferation, osteogenesis, myogenesis, and somatic hypertrophy [50].

Plasma-activated media is notably rich in reactive nitrogen species (RNS), serving as an exogenous donor of nitric oxide (NO) and its bioactive derivatives [14].

Current physiological research demonstrates that NO acts as a potent intracellular signaling molecule capable of modulating neuroendocrine function in fish. Specifically, NO donors (such as sodium nitroprusside) have been shown to directly stimulate the synthesis of GH mRNA and trigger the massive secretion of GH proteins from pituitary cells in teleost models, independently of traditional cGMP pathways [51].

The early exposure of the developing embryonic neural network to PAM-derived NO and related RNS likely alters the developmental set-point of the hypothalamic-pituitary-liver axis. The resulting long-term upregulation of the GH/IGF-1 axis facilitates continuous, accelerated somatic growth. These findings align immaculately with recent studies, such as those by Ikeda *et al.* [13], who reported that delivering plasma-treated, NO-rich air directly into tilapia culture tanks yielded fish up to 1.5 times longer than control subjects without the use of genome editing. Furthermore, research indicates that IGF-1 up-regulation is essential for maintaining growth under high-salinity or high-stress environments [52]. The current study verifies that even a single, transient embryonic exposure to these active plasma species is sufficient to program the somatotropic axis for sustained hyper-anabolism throughout the fish's entire lifespan.

Myofibrillar textural integrity and protein cross-linking

A paramount concern in the application of any oxidative or chemical stressor in aquaculture is the potential degradation of meat quality. Consumers demand firm, cohesive fillets, and structural deterioration (mushiness) represents a severe economic penalty, frequently associated with post-mortem protein degradation or excessive lipid oxidation [53]. However, Texture Profile Analysis (TPA) conducted at Week 39 provided definitive evidence that early PAM exposure did not compromise flesh firmness. Indeed, in specific anatomical regions (the mid-body and caudal sections), the PAM-treated color variants exhibited firmness values that were statistically equal to or significantly higher than the natural control group.

The maintenance and enhancement of muscle firmness can be directly attributed to the phenomenon of plasma-induced protein oxidation (PT-OX). The skeletal muscle of teleosts is predominantly composed of myofibrillar proteins, primarily myosin and actin, which govern the rheological, emulsifying, and textural properties of the flesh [54]. When exposed to the reactive species characteristic of cold plasma—specifically hydroxyl radicals, peroxynitrite, and ozone—amino acid side chains within these proteins undergo highly specific oxidative modifications [55].

Excessive exposure to high-voltage plasma or prolonged oxidative stress can cause detrimental protein fragmentation via the cleavage of peptide backbones (particularly at aspartyl, prolyl, and glutamyl residues), leading to a loss of solubility and severe tissue degradation [54]. However, mild and highly controlled oxidative stress—such as the 30-second PAM immersion utilized in this protocol—induces beneficial conformational unfolding without destroying the primary protein sequence [54]. RONS facilitate the partial denaturation of the tertiary structure of myofibrillar proteins, exposing buried hydrophobic domains and highly reactive sulfhydryl (-SH) groups located on sulfur-containing amino acids like cysteine and methionine [56].

Once these reactive sulfhydryl groups are exposed by the plasma treatment, they are rapidly oxidized into robust, intermolecular disulfide (S-S) bonds [56]. This covalent cross-linking weaves the individual myosin heavy chains (frequently via tail-to-tail aggregation) into an ordered, high-molecular-weight polymeric network [54].

This tightened, fibrous 3-dimensional matrix effectively traps intra-muscular water, vastly improving the water-holding capacity of the tissue and enhancing the overall structural elasticity, springiness, and storage modulus (G') of the muscle fibers [54]. Furthermore, the mild oxidative modification promotes hydrophobic-hydrophobic interactions between adjacent protein molecules, ensuring that the enhanced structural integrity established during embryonic and larval development scales proportionally as the fish accretes new muscle mass during the 39-week grow-out [54].

These findings are highly consistent with previous food science research demonstrating that in-bag dielectric barrier discharge cold plasma (IB-DBD-HVCP) treatment for short durations (e.g., 5 min) significantly enhances the cross-linking of Asian sea bass myofibrillar proteins, resulting in improved gel elasticity and breaking force [54]. Consequently, the

PAM protocol actively optimizes the techno-functional properties of the final edible product, ensuring that the induction of high-value chromatic phenotypes does not carry a penalty for fillet quality.

Methodological limitations and future research trajectories

While the empirical data robustly supports the deployment of PAM as a revolutionary, non-genetic biostimulant in aquaculture, the current research presents several methodological limitations. Addressing these gaps is critical to transitioning this technology from a laboratory proof-of-concept to large-scale industrial application.

A primary limitation of the present study is its reliance on terminal phenotypic observations (e.g., microscopic cell counting, morphometrics, CIELAB color space analysis) without parallel molecular or multi-omics validation. While it is highly probable that the *MC1R*/*ASIP* and *MITF* pathways are implicated in the depigmentation process, direct transcriptomic evidence demonstrating their suppression is currently lacking [27].

Future research must integrate high-throughput RNA sequencing (RNA-seq) to quantify the exact temporal expression dynamics of *TYR*, *TYRP1*, *SOX10*, and *Foxd3* immediately following PAM exposure and throughout the 14-day developmental timeline [27]. Furthermore, performing single-cell RNA sequencing (scRNA-seq) on the embryonic neural crest during the 30-second immersion would clarify precisely which sub-populations of chromatophore progenitors are undergoing apoptosis, experiencing migration arrest, or adopting alternative cell fates [41].

Additionally, because the induced Whole Pink and White phenotypes exhibit 100% stability across 39 weeks—showing no reversion despite fluctuating environmental states—it is scientifically untenable to attribute this to transient oxidative stress alone. Such permanent developmental plasticity is a hallmark of epigenetic reprogramming. Subsequent studies must deploy advanced epigenomic tools, such as Cleavage Under Targets and Tagmentation (CUT&Tag) and whole-genome bisulfite sequencing (WGBS). These techniques will reveal if PAM exposure induces permanent promoter hypermethylation or repressive histone modifications (e.g., H3K27me3) at the *MITF* locus, or if it removes repressive marks from melanogenesis inhibitors like *ASIP* [46]. Establishing the exact epigenetic memory installed by PAM is absolutely critical for confirming the non-genetic (non-GMO) status of the intervention to regulatory bodies.

From an engineering perspective, the translation of batch-processed, 50-mL glass vessel PAM generation into commercial hatchery operations poses a formidable challenge. Industrial hatcheries incubate hundreds of thousands of tilapia eggs simultaneously. The short half-lives of critical reactive species (such as peroxynitrite) demand that plasma activation occurs continuously and immediately prior to, or concurrently with, egg incubation [30].

Future bioengineering efforts should focus on the development of integrated, continuous flow-through plasma reactors capable of generating hydrodynamic cavitation plasma jets directly into the hatchery water supply [57]. This would completely eliminate the 5-minute pre-immersion gap, maximizing the direct exposure of the chorion to primary ultrashort-lived radicals (•OH and $O_2•^-$). Pilot- scale validation across diverse hydrodynamic regimes and varying water qualities (e.g., varying alkalinity, hardness, and organic loads, which can act as scavengers and rapidly quench RONS) is essential to standardizing the 28.5% induction rate and ensuring the economic viability of plasma-activated aquaculture [58].

Through the rigorous pursuit of these molecular, epigenetic, and bioengineering trajectories, plasma-activated media can be firmly established as a cornerstone technology for the sustainable, highly profitable, and non-invasive enhancement of commercial teleost species.

Conclusions

The findings of this study provide a comprehensive demonstration of the potential for plasma-activated media (PAM) to serve as a non-genetic and stable method for inducing phenotypic alterations in Nile tilapia (Oreochromis niloticus). By systematically analyzing the interaction between treatment intensity and developmental timing, it was established that the “eyed egg” stage (Stage 2) represents a unique window of plasticity. Immersion during this stage allows for the induction of commercially desirable color variants, such as the Whole Pink and White phenotypes, which exhibit

a permanent reduction in melanophore density. The 100% phenotypic stability observed from the larval stage through to the 39-week adult harvest confirms that this technological intervention initiates a fundamental shift in chromatophore development that is not reversible by environmental or nutritional changes.

A critical insight from the experimental design was the failure of chemically simulated media to replicate the color induction observed with PAM. This result clearly indicates that the stable RONS typically associated with PAM—hydrogen peroxide, nitrite, and nitrate—are not the primary agents of pigmentation change. Instead, the mechanism likely involves the activity of short-lived radicals, such as the hydroxyl radical (OH•), or the synergistic effects of the plasma discharge's physical properties (e.g., UV-C radiation) during the activation process. This distinguishes PAM from conventional chemical treatments and highlights its unique value as a biological priming agent.

The study further confirmed that early-life exposure to PAM leads to long-term growth acceleration, characterized by significantly higher body weights and Average Daily Gain (ADG) at the final harvest. This hormetic response suggest that the low-level oxidative stress induced by PAM immersion triggers adaptive physiological pathways that enhance metabolic vigor throughout the fish's life cycle. Crucially, these improvements in growth and color do not come at the cost of muscle quality, as evidenced by the consistent or improved flesh firmness across all anatomical regions.

For the aquaculture industry, PAM technology offers a sustainable and non-invasive alternative to traditional breeding and hormonal methods. It allows for the production of high-value color variants within existing wild-type or standard stocks, bypassing the phenotypic segregation issues common in red tilapia hybrids. Future research should prioritize the molecular characterization of these variants, specifically exploring the epigenetic modifications and gene expression patterns within the melanocortin and tyrosinase pathways. Refinement of the plasma activation parameters could further optimize induction rates, paving the way for the industrial-scale application of PAM in tilapia nurseries.

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Declaration of generative AI in scientific writing

During the preparation of this manuscript, the author(s) utilized Gemini 3.1 Pro (Google, 2026) and ChatGPT 5.3 (OpenAI, 2026) to improve the language quality and ensure the technical accuracy of the writing. Following the application of these tools, the author(s) thoroughly reviewed and edited the content as necessary and take full responsibility for the final published version.

CRediT author statement

Sukitar Krasaesen: Writing - Original Draft, Investigation, Data curation, Visualization. **Dheerawan Boonyawan**: Resources, Supervision. **Udomluk Sompong**: Investigation, Resources, Writing - review & editing. **Sureeporn Sarapirom**: Conceptualization, Methodology, Investigation. **Chakkrapong Kuensean**: Software, Validation, Formal analysis, Data curation, Writing - Original Draft, Writing - review & editing, Visualization.

References

[1] ME Abd El-Hack, MT El-Saadony, MM Nader, HM Salem, AM El-Tahan, SM Soliman and AF Khafaga. Effect of environmental factors on growth performance of Nile tilapia (*Oreochromis niloticus*). *International Journal of Biometeorology* 2022; **66(11)**, 2183-2194.

[2] O Nwachi, Y Esa, A Christianus, A Rahim and M Kamarudin. Patterns of colour inheritance from crossbreeding between Red hybrid tilapia (*Oreochromis* sp.) and GIFT tilapia (*Oreochromis niloticus*). *Journal of Environmental Biology* 2020; **41**, 1289-1294.

[3] M Luo, G Lu, H Yin, L Wang, M Atuganile and Z Dong. Fish pigmentation and coloration: Molecular mechanisms and aquaculture

perspectives. *Reviews in Aquaculture* 2021; **13(4)**, 2395-2412.

[4] J Liu, M Yin, Z Ye, J Hu and Z Bao. Harnessing hue: Advances and applications of fish skin pigmentation genetics in aquaculture. *Fishes* 2024; **9(6)**, 220.

[5] M Domonkos, P Tichá, J Trejbal and P Demo. Applications of cold atmospheric pressure plasma technology in medicine, agriculture and food industry. *Applied Sciences* 2021; **11(11)**, 4809.

[6] W Nupangtha, C Kuensaen, S Sarapirom and D Boonyawan. Investigation of Cold Atmospheric Plasma induce Apoptosis in Cancer Cell. *IOP Conference Series: Journal of Physics: Conference Series* 2018; **1144(1)**, 012025.

[7] P Poramapijitwat, P Thana, D Boonyawan, K Janpong, C Kuensaen, W Charerntantanakul, LD Yu and S Sarapirom. Effect of dielectric barrier discharge plasma jet on bactericidal and human dermal fibroblasts adult cells: *In vitro* contaminated wound healing model. *Surface and Coatings Technology* 2020; **402**, 126482.

[8] D Boonyawan, K Lamasai, C Umongno, S Rattanatabtimtong, LD Yu, C Kuensaen, J Maitip and P Thana. Surface dielectric barrier discharge plasma-treated pork cut parts: bactericidal efficacy and physiochemical characteristics. *Heliyon* 2022; **8(10)**, e10915.

[9] P Thana, D Boonyawan, M Jaikua, W Promsart, A Rueangwong, S Ungwiwatkul, K Prasertboonyai and J Maitip. Plasma-activated water (PAW) decontamination of foodborne bacteria in shucked oyster meats using a compact flow-through generator. *Foods* 2025; **14(9)**, 1502.

[10] K Moonsub, P Seesuriyachan, D Boonyawan, P Rachtanapun, C Sawangrat, T Opassuwan and W Wattanutchariya. Combating foodborne pathogens: Efficacy of plasma-activated water with supplementary methods for *Staphylococcus aureus* eradication on chicken, and beef. *Food Chemistry: X* 2024; **24**, 101890.

[11] K Kosumsupamala, P Thana, N Palee, K Lamasai, C Kuensaen, A Ngamjarurojana, P Yangkhamman and D Boonyawan. Air to H_2-N_2 pulse plasma jet for *in-vitro* plant tissue culture process: source characteristics. *Plasma Chemistry and Plasma Processing* 2022; **42(3)**, 535-559.

[12] A Sakulthai, C Sawangrat, D Pichpol, J Kongkapan, T Srikanchai, R Charoensook, P Sojithamporn and D Boonyawan. Improving the efficiency of crossbred Pradu Hang Dam chicken production for meat consumption using cold plasma technology on eggs. *Scientific Reports* 2023; **13(1)**, 2836.

[13] Y Ikeda, R Fukushima, K Tange, H Motomura, T Saito and M Jinno. Growth acceleration of Nile tilapia at 21 to 31 weeks of age with plasma-treated air-supplied water. *Free Radical Research* 2023; **57(1)**, 21-29.

[14] A Waskow, A Howling and I Furno. Mechanisms of plasma-seed treatments as a potential seed processing technology. *Frontiers in Physics* 2021; **9**, 617345.

[15] N Puač, M Gherardi and M Shiratani. Plasma agriculture: A rapidly emerging field. *Plasma Processes and Polymers* 2018; **15(2)**, 1700174.

[16] DM Parichy. Evolution of pigment cells and patterns: Recent insights from teleost fishes. *Current Opinion in Genetics & Development* 2021; **69**, 88-96.

[17] L Wang, B Jiang, W Zhu, J Fu, M Luo, W Liu and Z Dong. The role of melanocortin 1 receptor on melanogenesis pathway in skin color differentiation of red tilapia. *Aquaculture Reports* 2022; **22**, 100946

[18] JA Lister. Development of pigment cells in the zebrafish embryo. *Microscopy Research and Technique* 2002; **58(6)**, 435-441.

[19] TY Ma, JY Wu, XK Gao, JY Wang, XL Zhan and WS Li. Molecular cloning, functional identification and expressional analyses of FasL in Tilapia, *Oreochromis niloticus*. *Developmental & Comparative Immunology* 2014; **46(2)**, 448-460.

[20] A Sharifian, N Soltanizadeh and R Abbaszadeh. Effects of dielectric barrier discharge plasma on the physicochemical and functional properties of myofibrillar proteins. *Innovative Food Science & Emerging Technologies* 2019; **54**, 1-8.

[21] K Fujimura and N Okada. Development of the embryo, larva and early juvenile of Nile tilapia *Oreochromis niloticus* (Pisces: Cichlidae). Developmental staging system. *Development, Growth & Differentiation* 2007; **49(4)**, 301-324.

[22] EM Konchekov, N Gusein-zade, DE Burmistrov, LV Kolik, AS Dorokhov, AY Izmailov, B Shokri and SV Gudkov. Advancements in plasma agriculture: A review of recent studies. *International Journal of Molecular Sciences* 2023; **24(20)**, 15093.

[23] G Kwikiriza, I Abaho, PD Tibihika, AA Izaara, F Atukwatse, T Omara, JK Nattabi, N Kasozi, M Curto and H Meimberg. Genetic diversity and population differentiation of farmed nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) to advance selective breeding in Uganda. *Diversity* 2025; **17(2)**, 128.

[24] DM Parichy and JE Spiewak. Origins of adult pigmentation: diversity in pigment stem cell lineages and implications for pattern evolution. *Pigment Cell & Melanoma Research* 2015; **28(1)**, 31-50.

[25] L Wang, W Zhu, Z Dong, F Song, J Dong and J Fu. Comparative microRNA-seq analysis depicts candidate miRNAs involved in skin color differentiation in red tilapia. *International Journal of Molecular Sciences* 2018; **19(4)**, 1209.

[26] L Reich, J Don and R Avtalion. Inheritance of the red color in tilapias. *Genetica* 1990; **80(3)**, 195-200.

[27] B Jiang, L Wang, M Luo, J Fu, W Zhu, W Liu and Z Dong. Transcriptome analysis of skin color variation during and after overwintering of Malaysian red tilapia. *Fish Physiology and Biochemistry* 2022; **48(3)**, 669-682.

[28] F Song, L Shi, F Yao, Y Gu, D Zheng, W Zhang, Y Liang, K Zhang, M Yang and J Luo. The effect of background color on skin color variation of juvenile *Plectropomus leopardus*. *Animals* 2022; **12(23)**, 3349.

[29] M Hamed, AG Osman, AE Badrey, HA Soliman and AEDH Sayed. Microplastics-induced eryptosis and poikilocytosis in early-juvenile nile Tilapia (*Oreochromis niloticus*). *Frontiers in Physiology* 2021; **12**, 742922.

[30] KS Wong, NS Chew, M Low and MK Tan. Plasma-activated water: Physicochemical properties, generation techniques, and applications. *Processes* 2023; **11(7)**, 2213.

[31] O Olagunju. 2008, Peroxynitrite chemistry: Formation, decomposition and possible deactivation mechanisms by thiols. Ph. D. Dissertation. Portland State University, Portland, United States, 2008.

[32] A Phaniendra, DB Jestadi and L Periyasamy. Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry* 2015; **30(1)**, 11-26.

[33] ADG Sampaio, W Chiappim, NVM Milhan, BB Neto, R Pessoa and CY Koga-Ito. Effect of the pH on the antibacterial potential and cytotoxicity of different plasma-activated liquids. *International Journal of Molecular Sciences* 2022; **23(22)**, 13893.

[34] P Lukes, E Dolezalova, I Sisrova and M Clupek. Aqueous-phase chemistry and bactericidal effects from an air discharge plasma in contact with water: Evidence for the formation of peroxynitrite through a pseudo-second-order post-discharge reaction of H_2O_2 and HNO_2. *Plasma Sources Science and Technology* 2014; **23(1)**, 015019.

[35] PR Rotondo, D Aceto, M Ambrico, AM Stellacci, F Faretra, RMD Angelini and PF Ambrico. Physicochemical properties of plasma-activated water and associated antimicrobial activity against fungi and bacteria. *Scientific Reports* 2025; **15(1)**, 5536.

[36] S Gligorovski, R Strekowski, S Barbati and D Vione. Environmental implications of hydroxyl radicals (•OH). *Chemical Reviews* 2015; **115(24)**, 13051-13092.

[37] M Naïtali, JM Herry, E Hnatiuc, G Kamgang and JL Brisset. Kinetics and bacterial inactivation induced by peroxynitrite in electric discharges in air. *Plasma Chemistry and Plasma Processing* 2012; **32(4)**, 675-692.

[38] S Kalghatgi, CM Kelly, E Cerchar, B Torabi, O Alekseev, A Fridman, G Friedman and J Azizkhan-Clifford. Effects of non-thermal plasma on mammalian cells. *PLoS One* 2011; **6(1)**, e16270.

[39] JL Duband. *Neural crest delamination and migration. In*: JP Saint-Jeannet (Ed.). Neural crest induction and differentiation. advances in experimental medicine and biology. Springer, Boston, United States, 2006, p. 45-77.

[40] JD Berndt, MR Clay, T Langenberg and MC Halloran. Rho-kinase and myosin II affect

dynamic neural crest cell behaviors during epithelial to mesenchymal transition *in vivo*. *Developmental Biology* 2008; **324(2)**, 236-244.

[41] LM San-Jose and A Roulin. On the potential role of the neural crest cells in integrating pigmentation into behavioral and physiological syndromes. *Frontiers in Ecology and Evolution* 2020; **8**, 278.

[42] F Yu, Y Lu, Z Zhong, B Qu, M Wang, X Yu and J Chen. Mitf involved in innate immunity by activating tyrosinase-mediated melanin synthesis in *Pteria penguin*. *Frontiers in Immunology* 2021; **12**, 626493.

[43] T Hida, T Kamiya, A Kawakami, J Ogino, H Sohma, H Uhara and K Jimbow. Elucidation of melanogenesis cascade for identifying pathophysiology and therapeutic approach of pigmentary disorders and melanoma. *International Journal of Molecular Sciences* 2020; **21(17)**, 6129.

[44] R Sauermann, B Fancourt, T Faulkner, H Shute, D Reid, AJ Pask and CY Feigin. Loss-of-function mutations in ASIP and MC1R are associated with coat colour variation in marsupials. *Biology Letters* 2025; **21(10)**, 20250302.

[45] UW Khan, Ø Øverli, PM Hinkle, FA Pasha, IB Johansen, I Berget, PIM Silva, S Kittilsen, E Höglund, SW Omholt and DI Våge. A novel role for pigment genes in the stress response in rainbow trout (*Oncorhynchus mykiss*). *Scientific Reports* 2016; **6(1)**, 28969.

[46] Z Qi, J Liu, J Shi, M Yin, J Liu, J Fan, Z Bao, Z Ye and J Hu. Integrated transcriptomic and epigenomic analysis reveals mechanisms underlying melanotic spot formation in red tilapia (*Oreochromis* spp.). *International Journal of Molecular Sciences* 2025; **26(9)**, 4370.

[47] J Pérez-Sánchez, P Simó-Mirabet, F Naya-Català, JA Martos-Sitcha, E Perera, A Bermejo-Nogales, L Benedito-Palos and JA Calduch-Giner. Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Frontiers in Endocrinology* 2018; **9**, 687.

[48] Y Song, S Chen, L Li, Y Zeng and X Hu. The hypopigmentation mechanism of tyrosinase inhibitory peptides derived from food proteins: An overview. *Molecules* 2022; **27(9)**, 2710.

[49] ET Won and RJ Borski. Endocrine regulation of compensatory growth in fish. *Frontiers in Endocrinology* 2013; **4**, 74.

[50] T Nakano, LO Afonso, BR Beckman, GK Iwama and RH Devlin. Acute physiological stress down-regulates mRNA expressions of growth-related genes in coho salmon. *PLoS One* 2013; **8(8)**, e71421.

[51] HP Chen, L Zhang, BH Xu, LM Kang, SL Yang, HH Zeng and XY Shi. Nitric oxide stimulates embryonic somatotroph differentiation and growth hormone mRNA and protein expression through a cyclic guanosine monophosphate-independent mechanism. *Tissue and Cell* 2009; **41(2)**, 133-140.

[52] Z Li, P Meekuan, YX Wang, ZH Feng, SY Luo, ZX Zhang, J Xiao, F Yu and ZS Hou. Effects of Different Salinity Conditions on Regulation of ghrh-sst-gh-igf Axis in Nile Tilapia (*Oreochromis niloticus*): Insights from Transcriptional Signature. *International Journal of Molecular Sciences* 2025; **26(17)**, 8261.

[53] PG Vissio, MJ Darias, MP Di Yorio, DIP Sirkin and TH Delgadin. Fish skin pigmentation in aquaculture: The influence of rearing conditions and its neuroendocrine regulation. *General and Comparative Endocrinology* 2021; **301**, 113662.

[54] OO Olatunde, A Singh, KA Shiekh, P Nuthong and S Benjakul. Effect of high voltage cold plasma on oxidation, physiochemical, and gelling properties of myofibrillar protein isolate from Asian sea bass (*Lates calcarifer*). *Foods* 2021; **10(2)**, 326.

[55] S Rout, PK Panda, P Dash, PP Srivastav and CT Hsieh. Cold plasma-induced modulation of protein and lipid macromolecules: A review. *International Journal of Molecular Sciences* 2025; **26(4)**, 1564.

[56] SK Pankaj, Z Wan and KM Keener. Effects of cold plasma on food quality: A review. *Foods* 2018; **7(1)**, 4.

[57] J Jirešová, V Scholtz, J Julák and B Šerá. Comparison of the effect of plasma-activated water and artificially prepared plasma-activated water on wheat grain properties. *Plants* 2022; **11(11)**, 1471.

[58] T Yang, W Deng, L Liu, G Xia, L Zhang and J Wang. Effects of plasma-activated lactic acid on the physicochemical properties and volatile organic compounds of refrigerated tilapia fillets. *Food Chemistry: X* 2026; **34**, 103491.