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Research Article

Bromelain-Supplemented Diet Improves Morphology and Spawning Performance in *Clarias macrocephalus*

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ABSTRACT

This study aimed to investigate the effects of a bromelain-supplemented diet on the external morphological and reproductive characteristics of *Clarias macrocephalus*, and to assess how these changes influence spawning performance. Functional food enzymes are attracting increasing interest in aquaculture due to their potential to improve digestion and physiological performance. Bromelain, a proteolytic enzyme derived from pineapple, was evaluated for its effects on external morphology and reproductive characteristics. The experiment comprised two dietary treatments: a control diet and a bromelain-supplemented diet. External morphology was assessed by examining skin pigmentation, tail curvature, mucus secretion, muscle tone and reproductive traits such as genital papillae development and abdominal distension. External morphologies were scored using a standardised scale of 1–5, and statistical analyses were performed using independent t-tests and chi-square tests. Fish fed the bromelain diet showed significantly greater skin pigmentation (3.65 ± 0.49 vs. 1.15 ± 0.37), increased tail curvature (3.40 ± 0.60 vs. 1.60 ± 0.50), higher mucus secretion (3.80 ± 0.89 vs. 1.50 ± 0.51) and reduced muscle tone (3.40 ± 0.60 vs. 1.25 ± 0.55) compared to the control group (p<0.001). In contrast, reproductive characteristics including genital papilla structure and abdominal distension showed no significant differences (p>0.05), although successful spawning and a high fertilisation rate (87.4 ± 5.2%) were observed in the bromelain-fed pairs. The findings suggest that bromelain supplementation has a marked effect on the external morphology and physiological condition of catfish, which could enhance spawning readiness despite limited visible changes in reproductive anatomy. This indicates that the effects of bromelain have an internal or metabolic potential. The study highlights the potential of bromelain as a feed additive in aquaculture and recommends further research to investigate its effects on reproductive hormones, gonadal histology and synergistic interactions with bioactive compounds to optimise reproductive outcomes in *C. macrocephalus* culture.

Key words: Bromelain, *Clarias macrocephalus*, external morphology, functional feed, reproductive characteristics

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INTRODUCTION

Aquaculture plays a central role in global food security and contributes significantly to the production of protein-rich food for human consumption. As demand for sustainable fish farming increases, optimising fish growth, reproductive characteristics and overall physiological health has become a major focus of aquaculture research. According to the Agriculture Census 2024, catfish is among the top five species commercially cultured in Malaysia (DOSM, 2025). Currently, the catfish species most commonly reared by Malaysian fish farmers are either *Clarias gariepinus* or hybrids of *C. macrocephalus x C. gariepinus*. This preference is due to the slower growth rate, poorer feed conversion ratio (FCR) and lower nutrient uptake efficiency of *C. macrocephalus* compared to its hybrid or African catfish counterparts, making it less economically viable under intensive rearing systems (Na-Nakorn & Brummett, 2009; Bautista *et al.*, 2022; Duong *et al.*, 2022). Despite its high market value and desirable meat quality, the aquaculture of *C. macrocephalus* faces several constraints. These include difficulties in broodstock management, low seed availability due to poor spawning performance and high larval mortality under artificial rearing systems (Duong *et al.*, 2022). Moreover, environmental sensitivity, particularly to poor water quality and fluctuating temperatures, results in increased susceptibility to stress and disease outbreaks (Thaiso *et al.*, 2024; Tahiluddin *et al.*, 2025). As aquaculture depends on artificial reproduction techniques to maintain consistent and continuous fish production, spawning performance is a critical factor, in which *C. macrocephalus* performs poorly. This limitation affects not only its potential in Malaysia's aquaculture sector but also its conservation efforts.

One promising approach to achieving optimal results is the inclusion of bioactive compounds, such as enzymes, probiotics, or phytogenic additives, in fish diets as functional aquaculture nutrition. Incorporating dietary enzymes such as bromelain, a cysteine protease extracted from pineapple (*Ananas comosus*), offers several unique advantages over conventional enzymes used in aquaculture (e.g., trypsin, papain, or microbial proteases) (Chakraborty *et al.*, 2021). The proteolytic activity of bromelain includes both endo- and exopeptidase functions, enabling it to break down complex proteins into peptides and bioavailable amino acids more efficiently (Wiszniewski *et al.*, 2019; Yaseen *et al.*, 2023). Its use as a functional feed has been associated with improved feed conversion efficiency, accelerated growth performance, and optimal nutrient absorption, especially under intensive rearing conditions (Saleh *et al.*, 2022; Thaiso *et al.*, 2024). Bromelain has also been shown to influence muscle fibre development, epithelial tissue remodelling, and immune modulation, making it a multifunctional additive in aquaculture systems (Rehman *et al.*, 2021; Chen *et al.*, 2024).

In natural conditions, bromelain is not typically abundant in aquatic ecosystems. However, fish living in tropical agro-linked waters, such as rice fields or floodplains, may be exposed to bromelain indirectly through pineapple plant waste or water systems near agricultural land. Pineapple stem and fruit residues, which are rich in bromelain, can enter aquatic systems during heavy rains or agricultural drainage, providing intermittent natural exposure to the enzyme (Wiboonsirikul *et al.*, 2024). This leads to a natural presence of bromelain at low levels where *C. macrocephalus* is often found, in contrast to the higher levels used in formulated aquafeeds.

Bromelain maintains its activity from pH 5.5 to 8.0, making it suitable for a wide range of digestive conditions in fish (Chakraborty *et al.*, 2021). The anti-inflammatory and immunomodulatory properties of this enzyme can reduce oxidative stress, enhance mucosal immunity, and promote gut health by modulating inflammatory markers, which is uncommon among standard proteases (Chakraborty *et al.*, 2021). In addition to aiding digestion, bromelain has been shown to influence pigmentation, muscle tone, and reproductive physiology in several species, offering greater benefits than conventional digestive enzymes (Chen *et al.*, 2024; Thaiso *et al.*, 2024). Bromelain can also affect external morphological characteristics such as pigmentation, skin condition, and reproductive organ morphology by modulating protein turnover and enzymatic pathways involved in tissue maintenance (Zhao *et al.*, 2015; Badriyya *et al.*, 2020). However, the full extent of these effects, particularly in freshwater catfish species such as *C. macrocephalus*, remains underexplored. Bromelain, which is of natural origin and derived from pineapple, is considered safe, biodegradable, and environmentally friendly, making it advantageous for sustainable aquaculture practices (Wiszniewski *et al.*, 2019; Saleh *et al.*, 2022). Given its multifunctional effects, bromelain is a promising candidate in functional aquaculture nutrition aimed at improving physiological and reproductive health in cultured fish species.

External morphological characteristics, including skin pigmentation, mucus secretion, tail curvature, fin structure and reproductive organ development, serve as important indicators of fish physiological health, stress levels and reproductive status (Taft *et al.*, 2018; Zheng *et al.*, 2019; Wu *et al.*, 2024). In fish, external reproductive traits such as genital papilla size and abdominal expansion are often used to assess sexual maturity and reproductive capacity (Marin, 2016; Bond & Forsgren, 2021; Gopalraaj *et al.*, 2024). Changes in external morphology can indicate nutritional imbalance, metabolic stress or hormonal fluctuations, which can directly affect breeding efficiency and species survival in aquaculture (Wiszniewski *et al.*, 2019).

Diet plays a fundamental role in regulating the reproductive physiology of fish. Previous studies have examined high-protein and enzyme-treated diets to enhance gonadal development and reproductive characteristics (Torrecillas *et al.*, 2019; Saleh *et al.*, 2022; Wu *et al.*, 2024). However, few studies have assessed the effects of bromelain supplementation on external morphological changes and reproductive traits in *C. macrocephalus*. Although enzymatic feeds have been shown to improve growth and protein metabolism, their potential effects on skin integrity, mucus secretion and reproductive organ morphology remain largely unexplored (Zheng *et al.*, 2019).

The proteolytic properties of bromelain may influence external morphological characteristics by affecting protein turnover in epithelial tissues, muscle structure and metabolic processes (Badriyya *et al.*, 2020; Yaseen *et al.*, 2023). The role of the enzyme in reducing muscle stiffness, altering pigmentation patterns and modifying external reproductive traits (Zhao *et al.*, 2015; Wiszniewski *et al.*, 2019) has yet to be systematically investigated in *C. macrocephalus*. Therefore, it is crucial to understand how bromelain supplementation affects external morphology and reproductive traits, as this information could help optimise feed formulations to improve fish health and reproductive efficiency in aquaculture.

Despite existing studies on the effects of bromelain on muscle integrity and digestive efficiency, little is known about its role in shaping external reproductive traits (Wiszniewski *et al.*, 2019; Bottega *et al.*, 2021), particularly in freshwater catfish species. This study aims to evaluate the effects of a bromelain-supplemented diet on the external morphological and reproductive characteristics of *C. macrocephalus*, and to determine how these effects contribute to spawning performance. In particular, we analyse skin pigmentation, mucus secretion, fin and tail morphology, as well as external reproductive characteristics such as the structure of the genital papillae and abdomen expansion. The findings will clarify the possible effects of enzymatic supplementation on external reproductive morphology and general physiology in this species. The effects on external morphology and reproductive traits were also analysed and validated to determine whether bromelain supplementation produced significant changes. By achieving these objectives, this study will provide valuable insights in the field of fish nutrition and reproductive physiology and pave the way for more efficient nutritional strategies in aquaculture.

MATERIALS AND METHODS

Fish collection and experimental setup

Wild adult *C. macrocephalus* specimens were collected from a rice field in Gurun, Kedah, Malaysia. The fish were weighed and selected within a target weight range of 100–200 g (Serrano *et al.*, 2022) to ensure uniformity across experimental groups. Upon arrival, the fish were acclimatised under laboratory conditions for two weeks to adapt to the captive environment (Mugwanya *et al.*, 2022). During acclimation, they were housed in aerated fibreglass tanks (360 L capacity each) and fed a standard basal diet without bromelain to standardise physiological conditions. The basal diet used in this study consisted of 50% fishmeal, 28% cornmeal, 16% wheat flour, 2% vitamin-mineral premix and 4% fish oil, according to common formulations for catfish and other freshwater carnivorous species (El-Sayed, 2006; Ng & Romano, 2013; Zheng *et al.*, 2023).

After acclimation, the fish were randomly assigned to treatment groups using a completely randomised design (CRD). Each treatment group was replicated in three tanks (triplicates), with 15 fish per tank. The experiment was conducted for four months, with a total of 90 healthy fish used in the feeding trial. The initial average weight was 182.37 ± 31.27 g, and the average total length was 27.06 ± 3.10 cm. Fish were of mixed sex and were not separated before treatment to avoid stress from invasive methods and to reflect standard aquaculture rearing conditions. Any sex-specific reproductive responses were later analysed based on external and internal morphological characteristics following the feeding trial. The feeding trial was conducted in a controlled aquaculture facility using six fibreglass tanks (360 L capacity each). Tank positions were randomly arranged to minimise positional bias and environmental variation. The tanks were equipped with mechanical and biological filtration units to maintain optimal water quality. During the experiment, all fish were fed to satiation twice daily with their respective experimental diets. Uneaten feed was collected and removed to prevent the water from becoming cloudy. Water quality parameters were closely monitored and maintained within optimal ranges throughout the 16-week trial: an average temperature of 29 ± 2℃, a pH of 8.23–8.70, and a dissolved oxygen content of 6.5 mg/mL. Total ammonia concentration (average 0.28 ± 0.13 mg/L), nitrite levels (average 0.28 ± 0.11 mg/L), alkalinity (average 64.5 ± 13.3 mg $CaCO_3$/L), and water hardness (average 115 ± 5.3 ppm) were all held consistently throughout the study period. Partial water exchange (15-20%) was conducted twice weekly to ensure stability. Water quality was measured using standard portable meters (YSI, USA) and test kits (API, USA). Tanks were cleaned regularly to prevent the accumulation of organic matter and uneaten feed, and faeces were siphoned daily after feeding.

Source of bromelain enzyme

The bromelain used in this study was commercial pineapple enzyme powder (Bromelain powder; CAS No. 9001-00-7; EINECS No. 253-387-5) obtained from a certified supplier. This enzyme is derived from the stem of *Ananas comosus* (pineapple) and is classified as food grade with bromelain as the active ingredient. The product has an activity range of 300,000–1,250,000 IU/g and a mesh size with 90% passing through 100 mesh, ensuring fine uniformity for dietary incorporation. The enzyme was stored at 4℃ in a closed container until use to maintain its proteolytic activity.

Preparation of experimental diets

Experimental fish feed was prepared in two diets: a control diet without bromelain and a treatment diet with bromelain. Bromelain was included at 200 mg/kg of feed (0.02%), a concentration previously reported to be safe and effective in enhancing nutrient digestibility, improving feed conversion ratio (FCR), and promoting growth performance in aquaculture species (Sharma *et al.*, 2021). All dry ingredients were weighed and thoroughly mixed using a meat mixer to ensure uniform distribution. The mixture was then moistened with water (approximately 30-35% moisture content) and formed into pellets using a meat grinder with a 4 mm diameter. The pellets were dried at 40℃ in a forced-air dryer for 6-8 hr to reduce moisture content below 10% and prevent enzyme damage. Table 1 presents the formulation and proximate analysis (AOAC, 2003) of the experimental diet.

To preserve the enzymatic activity of bromelain, the pellets were dried at a low temperature (40℃) to prevent thermal inactivation. After drying, the pellets were cooled to room temperature and stored in airtight containers at 4℃ to prevent moisture absorption, protease oxidation, and photodegradation, all of which can reduce enzyme function over time (Chakraborty *et al.*, 2021; Sharma *et al.*, 2021). A concentration of 0.02% has been shown in previous studies to maintain high enzyme stability while significantly improving nutrient digestibility and fish growth performance (Sharma *et al.*, 2021; Saleh *et al.*, 2022). At this low dose, bromelain is less susceptible to autolysis and retains its structural conformation during mixing and short-term storage, making it a suitable and efficient dietary additive under aquaculture conditions (Chakraborty *et al.*, 2021).

Dietary treatments

A control group (basal diet) and bromelain-supplemented diet groups were fed twice daily, at 09:00 and 17:00, for four months. The daily feed ration was set at 3% of total body weight, a practice commonly used in catfish feeding trials (Saleh *et al.*, 2022; Yaseen *et al.*, 2023). Feed amounts were adjusted every two weeks based on average body weight measurements to maintain accuracy throughout the experiment.

Morphological and reproductive assessments

Before the feeding trial, all fish were examined for baseline external morphological and reproductive characteristics. Four traits were selected and scored using a standardised numerical rating system based on visual assessment. External morphological assessment focused on tail curvature, skin pigmentation, mucus secretion and

Table 1. The composition of ingredients and proximate analysis of the experimental diets.

Ingredients (1 kg)	Control group	Bromelain group
Bromelain (%)	0	0.02
Fishmeal (%)	50	50
Cornmeal (%)	28	28
Wheat flour (%)	16	16
Vitamin-mineral premix (%)	2	2
Fish oil (%)	4	4
Proximate composition (%)		
Moisture	5.31 ± 1.04	7.79 ± 0.93
Protein	34.47 ± 0.62	34.93 ± 1.03
Fat	12.09 ± 3.82	20.92 ± 2.34
Ash	5.21 ± 0.23	5.66 ± 0.19
Carbohydrate	38.55 ± 0.41	30.46 ± 1.08
Fiber	8.76 ± 0.51	6.87 ± 0.34

Data expressed as mean ± standard deviation (SD).

muscle tone (Öngün *et al.*, 2021). Observations were recorded on a numerical scale (1 – 5), with 1 representing no change (normal condition) and 5 representing extreme morphological changes (Table 2). Initial mean scores across all traits showed no significant differences between the two groups (p>0.05), confirming homogeneity at baseline. These numerical ratings were used as a reference for comparison with post-treatment assessments, ensuring consistency in morphological and reproductive evaluation. Furthermore, the characteristics of the gonads were examined externally based on the size of the genital papillae, abdominal expansion and reproductive behaviour (Serrano *et al.*, 2022). The morphological and reproductive examinations were carried out after four months of feeding. For validation, feeding was continued for one year until the breeding season in February 2025 (Đa *et al.*, 2024).

Breeding procedures

Artificial breeding methods were used to assess the reproductive characteristics of *C. macrocephalus* fed a bromelain-supplemented diet. Sexually mature males and females were selected based on external morphological criteria and confirmed by gentle abdominal palpation. Females were induced to spawn by intramuscular injection of Ovaprim (0.5 mL/kg body weight) to stimulate final oocyte maturation and ovulation. Males were sacrificed to extract milt, as *C. macrocephalus* sperm are not easily stripped. Eggs were manually stripped from females into clean, dry trays using gentle abdominal pressure to prevent injury and ensure the collection of mature ova. After mixing, fertilised eggs were incubated in a flow-through system with gentle aeration. Fertilisation and hatching rates were recorded at 12 and 24 hr after fertilisation, respectively. This breeding protocol followed standard hatching techniques for *C. macrocephalus*, as described by Na-Nakorn and Brummett (2009) and Duong *et al.* (2022) and adhered to best practices in Southeast Asian catfish hatcheries.

Observation of egg, embryonic and larval development

Fertilisation success was assessed by observing three random samples of 100 eggs from each treatment under a stereomicroscope (Leica M205 A) at 30-min intervals during the first three hr post-fertilisation (hpf). Translucent eggs were recorded as fertilised, while opaque or whitened eggs were considered unfertilised or non-viable, following the criteria of Liu *et al.* (2023) and Péter *et al.* (2023). The fertilisation rate (%) was calculated using the formula:

$$\text{Fertilisation rate (\%)} = \frac{N_f}{N_t} \times 100$$

Equation 1

Where N_f is the number of fertilised eggs and N_t is the total number of eggs examined.

Egg morphology (size, shape, and colour) and adhesiveness were documented using a digital photomicrography system (Leica Flexacam-C1 4K HD Digital Camera) at 40× magnification. Embryogenesis was observed hourly until hatching under a stereomicroscope. The embryonic stages, including gastrulation, blastoderm formation, yolk sac segmentation, tail elongation, pigmentation, and ocular rudiment formation, were identified according to Morioka *et al.* (2013) and Geladakis *et al.* (2022). Hatched larvae were transferred to 50 L rearing tanks and observed daily for morphological and behavioural changes. Growth was monitored from hatching to day 50 post-fertilisation. Observations included pigmentation, barbel development, fin elongation, muscle tone, swimming behaviour, yolk sac absorption, and onset of exogenous feeding. Larval and post-larval photographs were taken using a digital stereomicroscope to document developmental milestones. These observations were compared with standard developmental benchmarks for *C. macrocephalus* and related species to confirm normal growth and ontogenetic progression (Aripin *et al.*, 2015; Hung *et al.*, 2022).

Statistical analysis

The control and bromelain treatment groups were compared quantitatively using a numerical scale and analysed with an independent samples t-test, as only two dietary treatments were included in this study. This test determined whether significant differences existed in external morphological parameters (e.g. pigmentation, tail curvature, mucus secretion, muscle tone) between groups. In addition, a chi-square test of independence assessed the association between categorical reproductive traits and dietary treatment. Statistical significance was set at P<0.05. All analyses were conducted using SPSS v20.0 (IBM Corp., Armonk, NY, USA), and results are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Baseline morphological and reproductive characteristics before dietary treatment

External morphological and reproductive traits of *C. macrocephalus* were assessed before the feeding trial commenced to ensure that

Table 2. Numerical rating scale (1–5) for external morphological characteristics of *Clarias macrocephalus*.

Scale (1-5)	Tail curvature (skeletal deformation)	Skin pigmentation (discolouration/lesions)	Mucus secretion (protective layer)	Muscle tone (firmness)
1 (None/Normal)	Tail completely straight; no visible curvature or deformity	Even, natural pigmentation; no discolouration or lesions	Evenly distributed, normal mucus layer	Very firm muscle mass; no soft spots or indentations
2 (Mild)	Slight deviation or curvature of tail; minimal skeletal irregularity	Slight fading of colour; small pale areas or dullness	Slightly increased or decreased mucus production; even overall	Slight decrease in firmness; slight softening compared to control
3 (Moderate)	Noticeable tail curvature; slight skeletal misalignment or stiffness	Irregular pigmentation with visible patches or bluish sheen	Uneven secretion; some dry areas or localised overproduction	Moderate loss of firmness; slight indentations; less muscle tone
4 (Severe)	Marked tail curvature; rigid or partially twisted posture	Extensive discolouration; presence of rough texture or lesions	Thick mucus accumulation or significant patchy mucus loss	Soft muscle tissue; visibly reduced mass and tone
5 (Extreme)	Extreme tail curvature; curled tail affecting mobility or swimming	Widespread damage; ulcers, necrotic areas, or signs of oxidative injury	Excessive mucus coating or complete absence over large areas	Severely degraded muscle; structurally compromised, very soft

any differences observed after treatment could be attributed to the effects of the diet. Initial assessments confirmed that the two groups, control and bromelain-treated, were statistically and visually comparable in tail curvature, skin pigmentation, mucus secretion, muscle tone, genital papilla morphology and gonadal status. As shown in Table 3, there were no significant differences (p>0.05) between the two groups in any of the observed traits. Mean muscle tone scores were similar (1.25 ± 0.250) in the control group and 1.23 ± 0.398 in the bromelain group, as were the levels of pigmentation and genital papilla morphology. Both groups had a balanced sex ratio of sexually mature males and gravid females, confirmed by external abdominal appearance and internal examination of the gonads in subsamples. These consistent pre-treatment characteristics validated the experimental design, ensuring that any morphological or reproductive differences observed after the intervention resulted from the bromelain-supplemented diet.

External morphology of *Clarias macrocephalus* when fed bromelain-supplemented feed and control feed

Bromelain, a plant-derived proteolytic enzyme, has been widely studied for its role in improving protein digestion by hydrolysing dietary proteins into smaller peptides and amino acids, thereby enhancing bioavailability, nutrient absorption, feed efficiency and metabolic utilisation (Sharma *et al.*, 2021; Yaseen *et al.*, 2023). In the present study, dietary supplementation with bromelain resulted in marked changes in the external morphology of *C. macrocephalus*, specifically in four traits: tail curvature, skin pigmentation, mucus secretion and muscle tone (Table 4). Independent t-tests showed significant differences in all four traits between the bromelain and control groups (p<0.001), supporting the enzyme's systemic physiological influence.

Tail curvature

Tail curvature is a sensitive morphological indicator of musculoskeletal system development, often influenced by dietary composition, enzymatic activity and environmental stress (Kerniske *et al.*, 2021). In this study, bromelain-fed fish exhibited higher tail curvature scores (3.40 ± 0.60) than the control group (1.60 ± 0.50), with a t-statistic of 9.00 and a p-value of 1.48×10^{-12}, indicating a highly significant difference (Table 4; Figure 1a–b). These findings suggest that prolonged exposure to bromelain may disrupt the synthesis or structural integrity of collagen and myofibrillar proteins essential for skeletal stability (Saleh *et al.*, 2022). An overactive proteolytic process could degrade connective tissues prematurely, leading to axial deformities, particularly in the caudal region. These morphological deformities not only impair swimming performance and predator evasion but also compromise growth and feed conversion efficiency due to increased energy expenditure required to maintain posture (Butler *et al.*, 2017; Luis *et al.*, 2021). Enzymatic modulation of the neuromuscular axis, possibly through acetylcholinesterase (AChE) inhibition as documented in zebrafish by Luis *et al.* (2021), could further explain aberrant locomotor patterns and postural abnormalities.

Although bromelain improves digestive efficiency and is associated with specific growth rate (SGR) and feed conversion ratio (FCR) (Wiszniewski *et al.*, 2019), these benefits can be offset by morphological compromises if dosage and feeding duration are not carefully optimised. Tail deformities in aquaculture are more than cosmetic; they reduce competitive fitness, increase susceptibility to predation and disease and diminish market value (Hassaan *et al.*, 2019). Therefore, these results underscore the importance of optimising bromelain inclusion levels and exploring co-supplementation strategies, such as structural protein stabilisers (e.g. gelatin, hydroxyproline) or antioxidant support (e.g. vitamin E, selenium), to mitigate potential skeletal deformities while harnessing bromelain's digestive benefits.

Skin pigmentation

Fish fed a bromelain-supplemented diet showed marked changes in skin pigmentation, with a significantly higher mean pigmentation score (3.65 ± 0.49) compared to the control group (1.15 ± 0.37) (Table 4; Figure 1c–d). This difference was statistically robust, with a t-statistic of 16.25 and a p-value of 1.97×10^{-20}, indicating a highly significant reduction in pigmentation intensity. The visual evidence supports this statistical outcome, as shown in Figure 1c-d, where bromelain-fed fish display faded, irregular pigmentation with reduced melanin concentration compared to the uniformly dark skin tone of control fish. These external changes are closely associated with physiological disturbances in the activity of pigment cells, melanophores, which are responsible for melanin synthesis. Mechanistically, bromelain, as a proteolytic enzyme, can interfere with pigment biosynthetic pathways either by disrupting tyrosinase enzyme activity (essential for melanin production) or by modulating neuroendocrine signals such as melanocyte-stimulating hormone (α-MSH). Furthermore, bromelain-induced oxidative stress has been reported to damage chromatophores, leading to pigment cell apoptosis or functional suppression (Gopalraaj *et al.*, 2024). These findings are consistent with previous reports. Wu *et al.* (2024) observed depigmentation in fish experiencing protein metabolic stress, while Dong *et al.* (2011) demonstrated the sensitivity of chromatophores to environmental and dietary stressors. Barua *et al.* (2022) emphasised the evolutionary roles of pigmentation in camouflage, UV protection and sexual selection, functions that are compromised when pigmentation is lost.

Importantly, pigmentation loss also correlates with reduced mucus production, as both functions are associated with skin integrity and immune barrier function. Zheng *et al.* (2021) found that stress-induced pigment loss coincided with increased susceptibility to skin infections due to compromised mucus quality. In summary, while bromelain supplementation can improve digestion, its unintended effects on skin pigmentation highlight the importance of dosage control. Co-supplementation with antioxidant compounds or micronutrients such as selenium or vitamin C could be explored to mitigate oxidative damage. Additionally, pigmentation scoring

Table 3. Baseline morphological and reproductive characteristics of *C. macrocephalus* before treatment (n = 20 per group).

Morphological characteristic	Bromelain group (mean ± SD)	Control group (mean ± SD)	*p*-value
Tail curvature	1.42 ± 0.498	1.45 ± 0.303	0.913
Skin pigmentation	1.33 ± 0.489	1.35 ± 0.321	0.803
Mucus secretion	1.48 ± 0.894	1.5 ± 0.513	0.904
Muscle tone	1.23 ± 0.398	1.25 ± 0.250	0.863
Genital papilla morphology	Normal (100%)	Normal (100%)	–
Sex ratio (male:female)	10:10	10:10	–
Gonadal status	Mature in both sexes	Mature in both sexes	–

can serve as a sensitive, non-invasive biomarker of metabolic health in aquaculture systems.

Mucus secretion

Mucus secretion was significantly increased in *C. macrocephalus* fed a bromelain-supplemented diet (3.80 ± 0.89) compared with the control group (1.50 ± 0.51), as shown in Table 4 and Figure 1e–f. This difference was highly significant (t=9.88, p=3.65 × 10^{-12}), indicating a clear physiological change induced by dietary bromelain. Bromelain is known to modulate mucosal responses in vertebrates by triggering inflammatory pathways, increasing permeability, and altering secretory cell activity (Chakraborty *et al.*, 2021). In fish, excessive proteolytic stimulation can provoke stress responses and epithelial irritation, leading to hypersecretion of mucus as a protective adaptation (Badriyya *et al.*, 2020). The uneven distribution of mucus observed in bromelain-fed fish likely reflects dysregulated mucous cell activity, possibly exacerbated by metabolic or oxidative stress associated with enzyme-rich diets (Barua *et al.*, 2021; Alves & Agustí, 2022).

Mucus in fish serves not only as a mechanical barrier against pathogens but is also rich in antimicrobial peptides, lysozymes, and immune cells that are sensitive to dietary changes (Díaz-Puertas *et al.*, 2023). Increased secretion can provide short-term benefits by enhancing immune protection, but chronic overproduction can disrupt skin integrity, osmotic balance, and respiration. Studies in other species show that dietary enzymes such as bromelain can modify mucin composition and glycoprotein structures, potentially altering mucus viscosity and antimicrobial efficiency (Saleh *et al.*, 2022; Wainwright *et al.*, 2024). Moreover, pigmentation disturbances and mucus alterations often co-occur, possibly due to shared metabolic or hormonal pathways. Pigment cells and mucous cells originate from the same ectodermal layer and may respond similarly to enzymatic or oxidative stress, reinforcing the observed co-pattern (Zheng *et al.*, 2021). Given the importance of mucus as a non-specific immune defence, these findings suggest that bromelain supplementation, while beneficial for digestion, requires cautious dosing. Long-term exposure can compromise mucosal integrity unless balanced with dietary antioxidants (e.g. spirulina, vitamin E) to buffer stress responses (Gopalraaj *et al.*, 2024). Monitoring mucus characteristics, consistency, and antimicrobial properties should be standard practice in nutritional testing with exogenous enzymes.

Muscle tone

Bromelain supplementation significantly altered the muscle tone of *C. macrocephalus*, with bromelain-fed fish scoring 3.40 ± 0.60, compared to 1.25 ± 0.55 in the control group (Table 4; Figure 1g–h). Statistical analysis yielded a t-statistic of 10.70 and a p-value of 2.61 × 10^{-14}, confirming a strong effect of dietary bromelain on somatic muscular traits. This marked reduction in muscle tone suggests potential degradation of structural proteins, particularly myofibrillar components such as actin and myosin, which are critical for muscle contraction and elasticity (Bao *et al.*, 2020; Qin *et al.*, 2020). Proteolytic enzymes like bromelain can excessively cleave these structural proteins when used without dosage control, compromising muscle fibre integrity. Sharma *et al.* (2021) noted that while bromelain improved protein digestibility in fish diets, unregulated proteolytic activity can cause undesirable side effects, including textural changes in flesh. The weakened muscle tone observed could manifest as sluggish swimming, poor postural control and increased vulnerability to environmental stressors or predators. This aligns with findings by Perelló-Amorós *et al.* (2021), who reported that muscle quality directly affects locomotor performance and feeding efficiency in aquaculture species.

Saleh *et al.* (2022) and Chen *et al.* (2024) reported similar findings, showing that enzymatic diets can degrade muscle proteins and impair locomotor function. Furthermore, chronic stress or inflammation potentially induced by enzymatic irritation could trigger catabolism of muscle proteins, compounding the problem (Ordóñez-Grande *et al.* 2020). In aquaculture, these outcomes result in lower market value, higher mortality and reduced feed conversion efficiency, undermining production sustainability. Shoemaker and LaFrentz (2015) emphasised the importance of balanced muscle tone for posture and energy-efficient movement in water, highlighting how structural impairments can reduce fitness. Therefore, while bromelain's benefits for nutrient bioavailability and digestion are evident, this study underscores the importance of controlled enzyme dosing. Future studies should explore histological analysis of muscle fibres, enzymatic thresholds and antioxidant co-supplementation strategies to mitigate muscle degradation while preserving digestive benefits (Wiszniewski *et al.*, 2019; Qin *et al.*, 2020; Mugwanya *et al.*, 2023). This is also supported by Byrnes *et al.* (2022), who emphasised that morphological changes in widgeon grass affect fish habitat. Similarly, bromelain could positively influence fish morphology and reproductive traits by contributing to better nutrition and health. More focused research is needed to explicitly evaluate the effects of bromelain on fish morphology and reproductive traits.

Morphological changes associated with reproductive characteristics

External reproductive morphology provides essential indicators of sexual maturity and physiological readiness in *C. macrocephalus*. In this study, prolonged exposure to a bromelain-supplemented diet for 12 months enabled reliable differentiation between sexes based on visual and behavioural traits, particularly in the context of reproductive development.

Characteristics of gravid females

Gravid females in the bromelain-fed group showed notably distended abdomens, indicating ovarian development and advanced vitellogenesis, consistent with late-stage oocyte maturation and reproductive readiness (Figure 2a). This physical change was accompanied by asymmetrical body contours, likely due to uneven gonadal expansion (Chen *et al.*, 2024; Gopalraaj *et al.*, 2024). These females also demonstrated reduced swimming activity, a behaviour interpreted as energy conservation before spawning events (Wiszniewski *et al.*, 2019). An important marker of reproductive readiness was the genital papilla, which in gravid females appeared blunt, protruding and slightly swollen (Figure 2a), traits consistent with the onset of oviposition in catfish species (Das Neves *et al.*, 2019; Wu *et al.*, 2024). This was further supported by the observation of distinct orange to yellow ovarian tissue upon inspection (Figure 2c), confirming the advanced reproductive stage. Pigmentation patterns are well-documented indicators of late oocyte development (Saleh *et al.*, 2022; Gopalraaj *et al.*, 2024). Although bromelain primarily acts on digestive efficiency, its secondary systemic effects can enhance nutrient availability for gonadal development. Previous studies (Sharma *et al.*, 2021; Thaiso *et al.*, 2024) suggest that dietary proteases can improve reproductive condition by supporting hormonal balance and enhancing amino acid uptake, essential for oocyte formation and ovarian hydration. However, caution is warranted as over-supplementation can also affect protein reserves required for muscle function or immune regulation, potentially influencing reproductive trade-offs (Rehman *et al.*, 2021; Yaseen *et al.*, 2023).

Morphology of the males

Male *C. macrocephalus* displayed distinct external and internal morphological traits indicative of reproductive maturity, particularly after prolonged exposure to the bromelain-supplemented diet. Externally, males exhibited a flat, non-distended ventral profile with symmetrical body conformation, in contrast to the abdominal asymmetry observed in gravid females. This symmetrical profile indicates an absence of internal pressure from gonadal mass, characteristic of sexually mature males in a non-gravid state. Their

Table 4. Comparative analysis of bromelain-supplemented feed and control feed on the external morphology of *Clarias macrocephalus.*

Morphological characteristic	Bromelain group (mean ± SD)	Control group (mean ± SD)	*t*-statistic	*p*-value	Significance
Tail curvature	3.4 ± 0.598	1.6 ± 0.503	9.00	<0.001	***
Skin pigmentation	3.65 ± 0.489	1.15 ± 0.366	16.25	<0.001	***
Mucus secretion	3.8 ± 0.894	1.5 ± 0.513	9.88	<0.001	***
Muscle tone	3.4 ± 0.598	1.25 ± 0.550	10.70	<0.001	***

***Significance at $p<0.001$.

behaviour was consistently active and responsive, with increased lateral swimming, traits commonly associated with mate-seeking and spawning readiness in catfish (Wiszniewski *et al.*, 2019; Sharma *et al.*, 2021). Bromelain, by improving digestive efficiency, can support energy metabolism and muscular endurance, contributing to increased male mobility and reproductive activity (Rehman *et al.*, 2021; Thaiso *et al.*, 2024).

Fig. 1. External morphological differences in adult *Clarias macrocephalus* after four months of feeding a bromelain-supplemented diet and a control diet. (a) Pronounced tail curvature and rigid posture in fish fed bromelain-supplemented diet; (b) straight tail with no visible deformities in the control group; (c) discolouration spots, skin lesions and rough texture in bromelain-fed fish; (d) intact skin with uniform pigmentation in control fish; (e) irregular mucus secretion with localised dryness and excess in bromelain-fed fish; (f) normal and evenly distributed mucus secretion in control fish; (g) soft muscle tone with reduced mass and visible muscle atrophy in bromelain-fed fish; (h) firm, well-developed musculature in control fish. Scale bar = 3.5 cm.

The genital papilla in males was elongated, pointed and rigidly protruding (Figure 2b), consistent with well-documented external indicators of androgen-driven reproductive development (Zheng *et al.*, 2019; Wu *et al.*, 2024). This elongation suggests active testicular stimulation and androgenic regulation, potentially influenced by improved nutrient assimilation through enzyme-aided digestion. Internal gonadal inspection further confirmed maturity, as milky white testes and motile sperm were consistently observed (Figure 2d). These characteristics confirm active spermatogenesis and reproductive viability, commonly observed during the peak spawning phase in male catfish (Saleh *et al.*, 2022; Chen *et al.*, 2024). Although bromelain's primary role is proteolytic, it can also indirectly influence male gonadal development by improving amino acid bioavailability and endocrine balance, both crucial for hormone synthesis and sperm production. Studies by Yaseen *et al.* (2023) and Gopalraaj *et al.* (2024) support the view that dietary enzymes can enhance reproductive organ function by modulating nutrient-hormone interactions. However, the long-term physiological trade-offs of enzymatic feeding, such as potential oxidative stress or impacts on sperm quality, should be explored in future research.

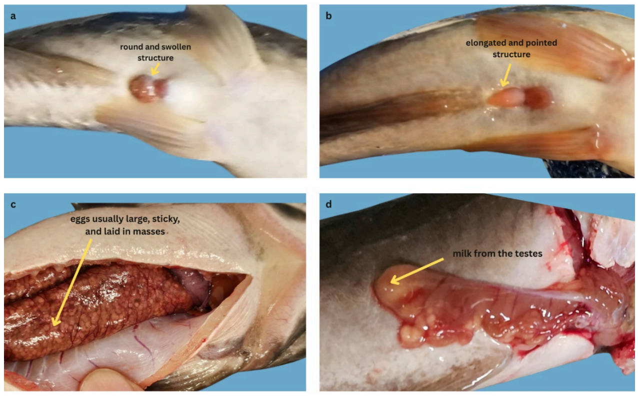

Fig. 2. External and internal reproductive characteristics of *Clarias macrocephalus*; (a) External genital papilla of a gravid female showing a blunt and swollen structure; (b) External genital papilla of a mature male showing an elongated and pointed structure; (c) Internal gonads of a gravid female showing orange-yellow mature oocytes; (d) Internal gonads of a mature male showing paired whitish testes.

Comparative analysis of reproductive morphology

A comprehensive and in-depth comparative analysis of reproductive morphology between gravid females and reproductively mature males of *C. macrocephalus* revealed clear external traits useful for non-invasive reproductive assessment. Table 5 and Figure 3 present detailed distinctions in abdominal shape, genital papilla morphology, symmetry, behaviour and internal gonadal condition. Gravid females consistently exhibited distended, rounded abdomens, a traditional indicator of ovarian development (Figure 2a), likely caused by asymmetrical gonadal mass accumulation, as also noted by Gopalraaj *et al.* (2024) and Chen *et al.* (2024). In contrast, males showed flat, symmetrical abdomens (Figure 2b), indicating no egg mass development (Wiszniewski *et al.*, 2019; Thaiso *et al.*, 2024). These differences were clearly observable even before dissection, supporting external sex identification practices. The genital papilla also differed significantly; females presented short, blunt and slightly swollen papillae, indicating readiness for spawning (Figure 2a), while males had elongated and pointed structures, associated with active sperm production (Figure 2b). These observations align with Zheng *et al.* (2019) and Wu *et al.* (2024).

Behaviourally, females exhibited reduced swimming activity and longer resting periods, which are pre-spawning energy conservation mechanisms (Wiszniewski *et al.*, 2019; Thaiso *et al.*, 2024). In contrast, males showed active, agile movements, supporting their role in territorial or courtship behaviours. Upon dissection, female gonads appeared as large, orange-yellow egg masses (Figure 2c), while males had milky, lobulated testes with visible sperm (Figure 2d), confirming active gametogenesis in both sexes. Recent studies by Ordóñez-Grande *et al.* (2020) and Byrnes *et al.* (2022) indicate that reproductive morphology is also influenced by dietary interventions such as bromelain, which can accelerate ovarian maturation and testicular development by enhancing nutrient absorption and proteolytic processing.

These clearly defined morphological differences, supported by the qualitative and quantitative data in Figure 3, highlight the value of external reproductive traits as reliable indicators for sex determination. This approach reduces the need for invasive procedures and is therefore particularly useful for large-scale broodstock selection and breeding programs in commercial aquaculture (Fragkoulis *et al.*, 2022; Oyedokun *et al.*, 2023). In conclusion, the findings reinforce the use of external reproductive traits as reliable indicators for sex identification, reducing reliance on invasive methods. This is especially valuable in broodstock selection, reproductive staging and aquaculture breeding programs. However, future research should include hormonal profiling and histological validation, particularly under bromelain-supplemented diets, to clarify mechanistic links between nutrition and reproductive development.

Statistical analysis of reproductive characteristics

The relationship between external morphological characteristics and reproductive status of *C. macrocephalus* was analysed using the chi-square test of independence. This approach enabled assessment of the association between categorical morphological features and confirmed sex (gravid female versus mature male). As shown in Table 6, none of the eight traits evaluated, including abdominal shape, genital papilla structure, body symmetry, swimming behaviour, gonadal colouration, and presence of gametes, showed statistically significant differences ($p>0.05$) between the sexes. In particular, characteristics typically considered reliable for sex identification, such as abdominal expansion (χ^2= 3.100, p=0.876), presence of sperm (χ^2 = 3.875, p=0.794) and presence of eggs (χ^2 = 3.100, p=0.876), showed no significant correlations. Similarly, visually observable features such as genital papilla morphology, symmetry and behavioural differences did not statistically differentiate the sexes. This finding contradicts the common assumption that external morphology alone is sufficient for reliable sex differentiation. Instead, it is consistent with the findings of Zheng *et al.* (2019) and Wu *et al.* (2024), who demonstrated that morphological indicators can be affected by environmental variables, stress levels and individual physiological variability. Saleh *et al.* (2022) likewise reported that abdominal and genital features often reflect nutritional or hormonal states rather than reproductive maturity itself.

Importantly, the lack of statistical significance should not be interpreted as an absence of biological meaning. Rather, it suggests that subtle sexual dimorphism in *C. macrocephalus* limits the effectiveness of gross external morphology as the sole diagnostic tool. Catfish species, including *C. macrocephalus*, are known to exhibit low external sexual differentiation, especially outside the active spawning period (Thaiso *et al.*, 2024). Additionally, individual variation, such as differences in fat deposition or gonadal regression in older fish, can obscure visual markers (Gopalraaj *et al.*, 2024).

One underexplored factor is the effect of dietary proteases such as bromelain on these traits. Emerging evidence suggests that

Table 5. Morphological changes in *Clarias macrocephalus* fed a bromelain-supplemented diet in relation to reproductive characteristics.

Trait	Observation	Possible implication	Supporting references
Gravid female			
Abdominal region	Slightly distended, rounded abdomen	Possible sign of egg development in a gravid female	Saleh *et al.*, 2022; Thaiso *et al.*, 2024
Genital papilla	Slightly swollen, protruding	A common gravid characteristic in catfish species preparing to spawn	Zheng *et al.*, 2019; Wu *et al.*, 2024
Body symmetry	Slight lateral asymmetry (more pronounced on one side)	Probably due to the development of the ovaries and increased egg mass	Chen *et al.*, 2024; Gopalraaj *et al.*, 2024
Swimming behaviour	Slower movements, frequent resting periods	Gravid females reduce their activity to save energy for spawning	Wiszniewski *et al.*, 2019
Eggs (if present)	Orange/yellow material observed inside	Confirms female reproductive organs (mature ovaries)	Saleh *et al.*, 2022; Gopalraaj *et al.*, 2024
Males			
Abdomen shape	Flat, not inflated	Indicates the absence of eggs, consistent with a male fish	Wiszniewski *et al.*, 2019; Thaiso *et al.*, 2024
Genital papilla	Protruding	A common characteristic in mature males approaching reproduction	Zheng *et al.*, 2019; Wu *et al.*, 2024
Gonad colouration	Not visible externally	Typically, males have whitish-milky testes, while females have orange-yellow ovaries	Saleh *et al.*, 2022; Gopalraaj *et al.*, 2024
Presence of sperm	Whitish milky substance observed	Confirms male reproductive organs (mature testes)	Zheng *et al.*, 2019; Chen *et al.*, 2024

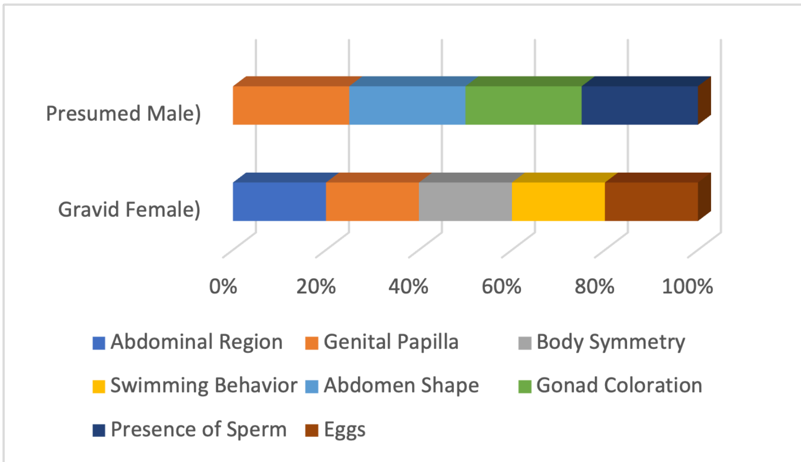

Fig. 3. Comparison of the gravid features of presumably male and gravid female *Clarias macrocephalus*. Stacked bar chart showing the distribution of reproductive morphological traits in presumed male and female *Clarias macrocephalus* (n=12). Parameters include abdominal region, genital papillae, abdominal shape, swimming behaviour, body symmetry, gonadal colouration and presence of eggs or sperm. The results reflect external indicators of reproductive status used for non-invasive sex determination.

bromelain supplementation can accelerate gonadal development or alter the hormonal pathways that modulate reproductive morphology, thereby masking or exaggerating certain external traits (Perelló-Amorós *et al.*, 2021; Fragkoulis *et al.*, 2022). For instance, bromelain's role in increasing bioavailable amino acids and stimulating steroidogenic pathways can disrupt the usual correlation between morphology and sex, particularly in long-term fed populations (Byrnes *et al.*, 2022). Given these limitations, it is recommended that multimodal sexing approaches, combining external morphology, ultrasonography, and hormone profiling, are used for accurate broodstock management (Saleh *et al.*, 2022; Wu *et al.*, 2024). These integrative methods are particularly important in hatchery settings, where sex misclassification can lead to reproductive inefficiency or poor selection outcomes. In conclusion, while external morphological traits offer a rapid, non-invasive method for preliminary sex assessment, their lack of statistical association with confirmed reproductive status highlights the need for a more robust, multimodal framework. Future research should further investigate the influence of nutritional interventions such as bromelain on reproductive phenotype, including endocrine and molecular changes, to better tailor sexing protocols in aquaculture operations.

Validation of spawning in *Clarias macrocephalus* fed a bromelain-supplemented diet

The spawning success of *C. macrocephalus* broodstock fed a bromelain-supplemented diet was clearly demonstrated through comprehensive observations of reproductive behaviour, egg quality and embryonic development, providing both visual and quantitative confirmation of reproductive viability. Bromelain, a plant protease, enhances nutrient absorption, intestinal morphology and hormonal modulation, thereby supporting reproductive health in aquaculture species (Rehman *et al.*, 2021; Yaseen *et al.*, 2023). The observed spawning behaviour and egg release in bromelain-fed females occurred after hormonal induction with Ovaprim, consistent with the artificial breeding protocol described earlier. The manually stripped eggs were uniform in shape and orange-yellow in colour, confirming successful oocyte maturation and ovulation induced by hormonal stimulation. Similar reproductive responses to Ovaprim-induced spawning have been reported in *C. gariepinus* and *C. macrocephalus*, where exogenous hormones effectively synchronise final oocyte maturation and promote ovulation (Na-Nakorn & Brummett, 2009; Duong *et al.*, 2022). Egg morphology was consistent with viable development; small, spherical, dark-coloured eggs adhered well to the substrate. The fertilisation rate averaged 87.4 ± 5.2%, which is high compared to other enzyme-based interventions (Geladakis *et al.*, 2022). Translucent eggs indicated successful fertilisation, while opaque or whitened eggs were identified as non-viable (Figure 4a). These visual cues correspond well to standard fertilisation diagnostics in tropical catfish culture (Liu *et al.*, 2023; Péter *et al.*, 2023).

Post-fertilisation, embryogenesis proceeded normally, with gastrulation visible at six hr post-fertilisation, and blastoderm formation, yolk sac segmentation and primitive streak structure evident (Figure 4b) (Morioka *et al.*, 2013). At 23 hr after fertilisation, the larvae developed pigmentation, ocular rudiments and tail elongation, which are hallmarks of robust embryonic progression (Figure

Table 6. Chi-square analysis of external morphological traits in *Clarias macrocephalus*.

Morphological trait	Chi-square value	p-value	Significance (p<0.05)
Abdominal region	3.100	0.876	Not significant
Genital papilla	3.875	0.794	Not significant
Body symmetry	3.100	0.876	Not significant
Swimming behaviour	3.100	0.876	Not significant
Abdomen shape	3.875	0.794	Not significant
Gonad colouration	3.875	0.794	Not significant
Presence of sperm	3.875	0.794	Not significant
Presence of eggs	3.100	0.876	Not significant

4c) (Geladakis *et al.*, 2022; Kwan *et al.*, 2024). At 48-72 hr, larvae remained in the yolk sac stage, demonstrating low mobility and endogenous feeding behaviour. This stage marks the transition from protected in-egg development to environmental interaction, requiring sufficient energy reserves and immune readiness. The bromelain-fed broodstock showed no deformities, indicating that early nutritional programming supported normal larval architecture. By the fourth day, post-larval features emerged, such as functioning fins, initiation of exogenous feeding and organ development (Aripin *et al.*, 2015; Molinari *et al.*, 2020). These developmental milestones, achieved within standard timelines, signal successful early life-stage viability, even under enzyme-modulated dietary protocols.

From weeks two to eight, larvae gradually transitioned into juveniles. Notable morphological changes included fin elongation and segmentation, barbel development, muscle tone consolidation, increased pigmentation and enhanced swimming activity. By days 16–18, larvae exhibited distinct suprabranchial organ formation, facilitating aerial respiration, a significant adaptation in *C. macrocephalus* (Morioka *et al.*, 2013). At day 50, juveniles reached 5 cm in length, had developed strong swimming responses and were fully adapted to weaning diets (Figure 4d). These traits indicate stable metabolic programming and robust physiological condition, likely supported by the digestibility-enhancing and hormone-modulating effects of bromelain (Chen *et al.*, 2024; Thaiso *et al.*, 2024). This aligns with established timelines for *Clarias* fry development (Aripin *et al.*, 2015; Hung *et al.*, 2022). The successful transition to the juvenile phase indicates that early development was effectively supported by the nutritional intervention.

The successful spawning, fertilisation, embryonic development and larval survival of *C. macrocephalus* in this study confirm that bromelain-supplemented diets do not impair reproductive success; rather, they can enhance it. The enzymatic action of bromelain likely improved protein digestibility, stimulated endocrine function and promoted nutrient bioavailability, all of which are critical during gametogenesis and early development (Perelló-Amorós *et al.*, 2021; Fragkoulis *et al.*, 2022). This supports findings from Yaseen *et al.* (2023), who reported increased egg quality and hatchability in fish fed enzyme-rich diets. Additional mechanisms may include enhanced gonadotropin release through improved gut-brain signalling, reduced oxidative stress during spawning and increased availability of arginine, glutamate and lysine. These amino acids are involved in reproductive hormone biosynthesis. While this study confirms the efficacy of bromelain in reproductive support, further work should determine the profile of reproductive hormones (e.g. estradiol, testosterone, GnRH), explore epigenetic modifications triggered by enzymatic diets and assess synergistic supplementation with antioxidants or immune-enhancing additives. Given the ecological vulnerability and commercial importance of *C. macrocephalus*, nutritional strategies such as bromelain supplementation could become vital tools in sustainable hatchery protocols, improving seed quality, reproductive efficiency and larval survival under controlled and variable environmental conditions.

CONCLUSION

Dietary supplementation with bromelain resulted in significant changes in the external morphology of *C. macrocephalus*, including increased tail curvature (3.40 ± 0.60), increased skin pigmentation (3.65 ± 0.49), increased mucus secretion (3.80 ± 0.89) and decreased muscle tone (3.40 ± 0.60) (p<0.001). However, external reproductive traits such as genital papilla size and abdominal distension did not differ significantly (p>0.05). Notably, the high fertilisation rate of 87.4 ± 5.2% confirmed reproductive capacity. These findings suggest that bromelain improves external physiology and supports successful spawning, although reproductive changes may be internal. Further studies are recommended to evaluate the hormonal, histological and synergistic nutritional effects to improve broodstock performance, immunity and overall aquaculture sustainability. This study provides valuable insights into enzyme-based nutritional strategies to improve fish health, reproductive characteristics and the success of *C. macrocephalus* in the early life stage in aquaculture systems.

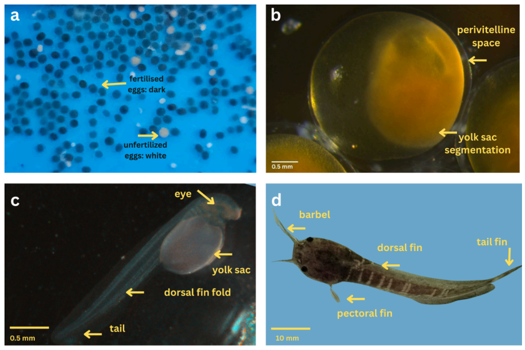

Fig. 4. Development of embryos and larvae from *Clarias macrocephalus* broodstock fed a bromelain-supplemented diet. (a) Fertilised eggs appear dark, while unfertilised eggs appear white; (b) Embryo at mid-gastrula stage, 6 hr after fertilisation, showing blastoderm expansion and yolk sac segmentation; (c) Hatched larva, showing tail elongation, eye pigmentation, and advanced organogenesis at 23 hr post-fertilisation; (d) Juvenile (day 50), showing developed fins, pigmentation, and complete weaning adaptation.

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ETHICAL STATEMENT

This study was approved by the ethical committee of Universiti Selangor, approval number UNISEL/CRIL/EC/0033.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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