

Dose-response effects of a Chinese herbal extract mixture on masculinization and performance of *Oreochromis niloticus* (Linnaeus, 1758) across generations in earthen pond culture

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Abstract. Synthetic 17 α -methyltestosterone use for monosex tilapia production is increasingly restricted on environmental and food-safety grounds, creating demand for non-synthetic, hormone-free alternatives. This study evaluated four isonitrogenous, black soldier fly larvae (BSFL)-based diets containing a ten-herb Chinese herbal extract (CHE) mixture (*Sigesbeckia orientalis*, *Codonopsis pilosula*, *Citrus aurantium*, *Citrus sinensis*, *Aquilaria sinensis*, *Commiphora myrrha*, *Eclipta prostrata*, *Juglans nigra*, *Ligusticum chuanxiong* and *Cistanche deserticola*) at 0 (control), 0.5, 1.0 and 1.5% inclusion, fed to Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), broodstock and progeny across three generations (F0-F1, F1-F2, F2-F3) in earthen clay ponds in Selangor, Malaysia. All three CHE-supplemented diets progressively shifted the population from a near-balanced sex ratio at F0-F1 to a fully monosex, all-male population by F2-F3, whereas the unsupplemented control remained 42.6-43.9% male throughout. The 0.5% inclusion level produced the highest cumulative male output (538.7 fish; 75.3% of total growers), the best egg-to-grower survival (78.7% by F2-F3) and the most efficient feed conversion ratio (FCR) (1.24-1.27), and, among the CHE treatments, provided the most favourable overall combination of survival, feed efficiency, male output and water-quality parameters. We conclude that 0.5% dietary CHE inclusion is the most biologically effective and economically efficient dose for hormone-free, multigenerational, all-male *O. niloticus* production in earthen clay-pond systems typical of smallholder and commercial tropical aquaculture.

Keywords: monosex culture, Nile tilapia, phytogetic feed additive, sex reversal, soldier fly larvae, supermale.

Introduction. Tilapias, as a group of species (*Oreochromis* spp.), are among the most widely cultured freshwater finfish in the world, ranking second only to carps in global aquaculture production volume (Food and Agriculture Organization of the United Nations, 2022). Global production exceeded 7.2 million tonnes in 2021, with an estimated market value above USD 14.1 billion, and continued expansion is projected for the remainder of the decade (Roderick, 2025). Commercial culture is concentrated on three species, Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), Blue tilapia, *Oreochromis aureus* (Steindachner, 1864), and Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), of which *O. niloticus* dominates owing to its superior growth, disease resistance and environmental tolerance. *O. niloticus* is produced across a continuum of systems, from intensive recirculating and biofloc systems to extensive

and semi-intensive earthen ponds, and it is the earthen clay pond, fertilised by natural plankton production and supplemented with formulated feed, that remains the principal production unit for the majority of the world's *O. niloticus* farmers, particularly across tropical Asia, sub-Saharan Africa and Latin America.

A major biological constraint on commercial *O. niloticus* culture is the species' early sexual maturity and prolific reproduction. In mixed-sex grow-out systems, uncontrolled breeding generates large numbers of progeny that compete with the originally stocked cohort for feed, space and dissolved oxygen, producing stunted growth and a heterogeneous, low-value harvest (Pandian & Sheela, 1995). Because male *O. niloticus* outgrow females by 20–50% under equivalent rearing conditions as a result of higher circulating androgens and more efficient protein utilisation (Golan & Levavi-Sivan, 2014), the culture of monosex, all-male populations has become the standard commercial strategy, simultaneously removing the growth penalty of mixed-sex rearing and eliminating uncontrolled reproduction.

Several methods are used to generate monosex male *O. niloticus*, including manual sexing, interspecific hybridisation, hormonal sex reversal and the genetic production of YY supermales (Mair et al., 1991; Pandian and Kirankumar, 2003). Hormonal sex reversal with 17 α -methyltestosterone (MT) administered in medicated feed during early larval development remains the most widely applied method worldwide because of its efficacy, simplicity and low cost, typically achieving male ratios of 95–99% (Pandian & Sheela, 1995). However, regulatory restriction of MT use has intensified over the past two decades because of concerns over hatchery-worker exposure, environmental contamination from hormone-laden effluent and residues in food fish destined for human consumption (Phelps & Popma, 2000). This regulatory pressure has accelerated interest in the YY supermale technology as a chemical-free alternative: because *O. niloticus* sex determination follows an XY/XX system, a YY genotype produced through successive feminisation and back-crossing yields, when mated with normal XX females, progeny that are 98–100% genetically male without any further hormonal input (Scott et al., 1989; Mair et al., 1997). National programmes such as Indonesia's Genetically Supermale Indonesian Tilapia (GESIT) initiative (Aliah et al., 2010) and marker-assisted selection of YY lines from genetically improved farmed *O. niloticus* strains (Chen et al., 2019), together with farm-scale comparisons in which YY-derived genetically male progeny outperformed hormone-reversed fish (Hughes, 2004), have confirmed the commercial viability of this approach. The principal remaining technical bottleneck, however, is the establishment of the founding YY broodstock line itself, which still depends on an initial feminisation step using an estrogenic or otherwise bioactive compound to redirect gonadal differentiation in genetically male fish (Beardmore et al., 2001; Desprez et al., 2003), creating a continuing need for safe, non-hormonal agents capable of biasing sex differentiation in either direction.

Plant-derived phytochemicals have attracted growing interest as natural alternatives to synthetic steroids for influencing sex differentiation in fish (Gabriel et al., 2015; Gabriel, 2019). Three principal mechanisms have been proposed for masculinising or feminising activity of botanical extracts: direct androgen-receptor agonism by steroidal saponins and related compounds; inhibition of the aromatase enzyme (CYP19a1), which shifts the androgen:estrogen ratio toward androgens and has been demonstrated using both synthetic inhibitors (Kwon et al., 2000) and citrus-derived flavonoids such as naringenin (El-Kersh et al., 2021); and stimulation of the hypothalamic-pituitary-gonadal axis, increasing endogenous androgen output (Ganzera et al., 2001; Dabrowski et al., 2005). Saponin-rich extracts of *Tribulus terrestris* have produced significant masculinisation in *O. niloticus* larvae (Ganzera et al., 2001), and dietary flavonoid aromatase inhibitors have produced male ratios of 60–90% in *O. niloticus*, generally below the efficacy of MT but establishing clear proof of concept for phytochemical sex-reversal agents (Gabriel et al., 2015).

Traditional Chinese Medicine (TCM) represents one of the most extensively characterised botanical pharmacopoeias for endocrine-active compounds. Several TCM herbs are documented to possess androgenic, steroidogenic or receptor-modulating activity relevant

to reproductive endocrinology. *Cistanche deserticola*, known historically as "desert ginseng," has a centuries-long reputation as a male reproductive tonic and has been shown experimentally to upregulate testicular steroidogenic enzymes and elevate serum testosterone (Wang et al., 2012; Wang et al., 2020; Zhang et al., 2022). *Codonopsis pilosula* enhances androgen-receptor expression in experimental models (Zhang et al., 2020; Chu et al., 2024; Guo et al., 2024a). *Citrus aurantium* and *Citrus sinensis* peel and tissue are rich in the flavonoids naringenin, naringin and hesperidin, with naringenin acting as a potent aromatase inhibitor through the same mechanism exploited by synthetic compounds such as letrozole (Haggag et al., 1999; El-Kersh et al., 2021). *Sigesbeckia orientalis* contains saponins, alkaloids and sesquiterpene lactones associated with androgenic biosynthetic stimulation (Nwaogu et al., 2017), while *Aquilaria sinensis*, *Commiphora myrrha*, *Ligusticum chuanxiong*, *Juglans nigra* and *Eclipta prostrata* contribute sesquiterpenes, chromones, phthalides, naphthoquinones and phytosterols that have each been linked, individually, to endocrine-, vascular- or hepato-protective bioactivity in TCM pharmacology (Gabriel et al., 2015; Qin et al., 2022). Combining herbs with complementary, putative mechanisms across direct androgenic activity, aromatase inhibition and HPG-axis stimulation reflects the classical TCM principle of multi-component formulation; however, because the present mixture was not tested against its individual constituent herbs or sub-combinations, true pharmacological synergy among components cannot be demonstrated from this experiment.

Despite the accumulating evidence for individual TCM herbs, no published study has evaluated a formulated multi-herb Chinese extract mixture for its capacity to induce an all-male *O. niloticus* population under realistic, farm-relevant pond conditions. This is an important gap because the effective dose of a multi-herb formulation cannot be inferred from single-compound trials: botanical extracts commonly show non-monotonic, hormetic dose-response relationships in which intermediate doses outperform both lower and higher doses (Calabrese & Kozumbo, 2021), and the dilution, microbial degradation and clay-particle adsorption that bioactive compounds undergo in the semi-open, ecologically complex environment of an earthen pond cannot be replicated in laboratory aquaria. A second, independent driver of dietary formulation in the present trial was the use of black soldier fly larvae (BSFL, *Hermetia illucens*) meal as the sole protein source. BSFL meal is increasingly promoted as a sustainable, cost-efficient replacement for fishmeal in *O. niloticus* diets, capable of supporting comparable or superior growth performance, feed efficiency and economic return (Limbu et al., 2022), and its inclusion alongside the herbal mixture allowed the trial to evaluate the herbal dose-response on a feed platform that is itself representative of current sustainability priorities in aquafeed formulation. It should be noted that the present trial was designed to evaluate whole-mixture, farm-relevant production outcomes, sex ratio, survival, feed conversion, water quality and reproductive timing, rather than the molecular mechanisms outlined above; androgen-receptor expression, aromatase activity, circulating steroid concentrations and other endocrine endpoints were not measured, and mechanistic interpretation of the present results is therefore necessarily indirect.

The objective of the present study was therefore to determine the optimum dietary inclusion level, among 0.5, 1.0 and 1.5%, of a ten-herb Chinese herbal extract (CHE) mixture in a BSFL-based diet for the production of all-male *O. niloticus* in earthen clay ponds, evaluated across three successive generations in terms of water quality, reproductive performance and survival, the duration of the reproductive cycle, the progression of the population sex ratio, and feed conversion efficiency. By testing a multi-herb (multi-component) formulation under actual pond-farming conditions rather than in single-compound laboratory trials, this study extends the phytopharmacological sex-reversal literature toward direct, farm-applicable practice and provides clay-pond operators with an evidence-based dose recommendation for hormone-free, multigenerational monosex *O. niloticus* production.

Material and Method

Ethical approval and study site. The trial was conducted at VL Ocean Services, Semenyih, Selangor, Malaysia, following authorisation from the Animal Ethical Consent Committee of Universiti Malaysia Sarawak (UNIMAS) (Reference UNIMAS/AEC/R/F07/065). All procedures involving live fish, including handling, blood sampling and euthanasia, followed the approved animal-welfare protocol.

Experimental design. Four earthen clay ponds of equal dimensions (30 × 15 × 1.5 m) were used; each pond was assigned to a single dietary treatment, and three hapas (5 × 3 × 1 m) were installed within that pond as the three replicate units for the treatment, giving three replicates per treatment (12 hapas in total). Because the three replicate hapas within a pond shared the same pond water body and broader environment, they are not fully independent of one another; this pond-nested allocation is acknowledged as a limitation on the independence of replicates, and treatment comparisons should be interpreted with this restricted, pond-level form of replication in mind (see Statistical Analysis and Discussion). The trial was designed as a longitudinal, multigenerational dose-response study tracking the effect of graded dietary inclusion of a ten-herb CHE mixture on *O. niloticus* across three successive filial generations (F0-F1, F1-F2 and F2-F3). The overall research framework comprised collection and nutritional characterisation of the two principal raw dietary ingredients, formulation of the experimental diets, multigenerational production and observation, and continuous water-quality monitoring.

Collection and nutritional analysis of dietary raw materials. Ten Chinese herbal materials, *S. orientalis*, *C. pilosula*, *C. aurantium*, *C. sinensis*, *A. sinensis*, *C. myrrha*, *E. prostrata*, *J. nigra*, *L. chuanxiong* and *C. deserticola*, were sourced in raw form from commercial herbal suppliers in China and reduced to powder using a heavy-duty crusher. BSFL freeze-dried powder was obtained from BSF Borneo Sdn Bhd; according to the supplier, larvae were harvested at the pre-pupal stage to maximise protein and lipid content. Both ingredients were submitted to a commercial analytical laboratory (MY CO2 Group Sdn Bhd, Malaysia) for proximate analysis following Association of Official Analytical Chemists (AOAC) procedures (moisture, crude protein by Kjeldahl nitrogen, crude fat by ether extraction, crude fibre, ash and nitrogen-free extract), amino-acid profiling by high-performance liquid chromatography, and fatty-acid profiling by gas chromatography-mass spectrometry.

The ten herbal powders were found to be highly heterogeneous in composition: crude protein ranged from 2.6 to 32.0% and crude lipid reached 48.3% in the most lipid-dense herb, while ash content was more than fivefold higher in *C. deserticola* (26.6%) than in most of the remaining nine herbs (2.5-5.3%). This phytochemical and nutritional heterogeneity is consistent with a mixture deliberately assembled to combine complementary, rather than redundant, bioactive profiles. BSFL meal contained 490.3±0.19 g kg⁻¹ crude protein, 290.2±0.64 g kg⁻¹ crude lipid, 100.0±0.13 g kg⁻¹ crude fibre, 70.5±0.34 g kg⁻¹ ash and 49.1±1.30 g kg⁻¹ nitrogen-free extract (Table 1), confirming its suitability as a high-protein, high-energy alternative to fishmeal.

Table 1

Proximate composition (mean±SD) of black soldier fly larvae freeze-dried powder, the sole protein source of the basal experimental diet

<i>Component</i>	<i>Amount (g kg⁻¹)</i>
Crude protein	490.3±0.19
Crude lipid	290.19±0.64
Crude fibre	100.0±0.13
Ash	70.45±0.34
Nitrogen-free extract*	49.06±1.30

Note: * Nitrogen-free extract = 1000 – (moisture + crude protein + crude lipid + ash + crude fibre).

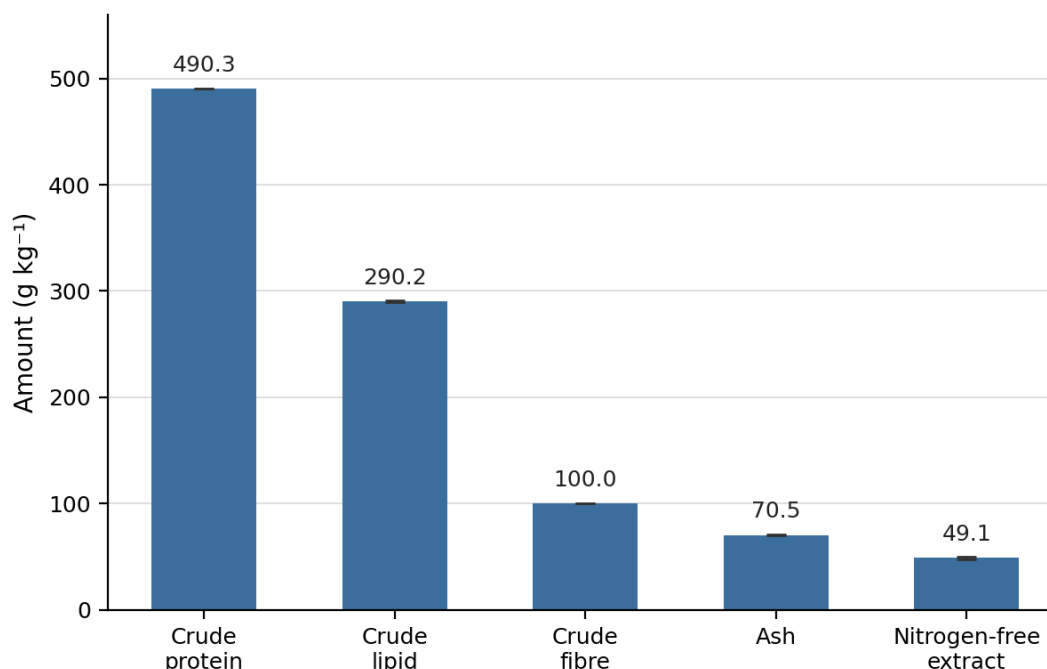


Figure 1. Proximate composition (mean±SD) of black soldier fly larvae freeze-dried powder, presented as a column chart of the data in Table 1.

Formulation of experimental diets. Four diets were formulated to provide comparable crude protein and lipid concentrations across CHE inclusion levels; however, because CHE replaced cellulose on a weight basis, increasing inclusion was accompanied by substantial changes in crude fibre, ash and nitrogen-free extract (Table 2), a confound discussed as a limitation below. The ten herbal powders were combined in equal proportions and blended to homogeneity, then coated onto a common BSFL-based basal mixture using a commercial coating/mixing machine. The control diet (T0) contained 0% CHE; treatment diets T1, T2 and T3 contained 0.5, 1.0 and 1.5% CHE by weight, respectively, with cellulose reduced proportionally to compensate for the added herbal mass and preserve a constant total batch weight (Table 2). All diets were steam-pelleted to preserve heat-sensitive bioactive constituents and stored at -20°C in vacuum-sealed packaging until use.

Table 2

Formulation and proximate composition (mean±SD) of the four experimental diets containing graded levels of the ten-herb Chinese herbal extract mixture

<i>Component</i>	<i>T0 (0%)</i>	<i>T1 (0.5%)</i>	<i>T2 (1.0%)</i>	<i>T3 (1.5%)</i>
BSFL freeze-dried powder (g)	825	825	825	825
Korean corn starch (g)	80	80	80	80
Fish oil (g)	11.5	11.5	11.5	11.5
Soybean oil (g)	30	30	30	30
Cellulose (g)	21	16	11	6
Carboxymethyl cellulose (g)	12.5	12.5	12.5	12.5
Vitamin premix (g)	10	10	10	10
Mineral premix (g)	10	10	10	10
CHE powder mix (g)	0	5	10	15
Crude protein (g kg ⁻¹)	400.9±0.22	401.3±0.18	402.0±0.49	400.2±0.11
Crude lipid (g kg ⁻¹)	41.64±0.16	40.67±0.29	42.11±0.24	40.85±0.32
Crude fibre (g kg ⁻¹)	18.05±0.09	32.79±0.14	71.2±1.17	96.5±1.59
Ash (g kg ⁻¹)	33.14±0.11	64.99±0.07	139.19±1.39	209.54±2.08
Nitrogen-free extract (g kg ⁻¹)	506.27±0.15	460.25±0.18	345.5±0.86	252.87±1.04
Gross energy (kcal g ⁻¹)	17.88±0.05	22.33±0.02	10.99±0.12	8.11±0.20

Note: Vitamin and mineral premix compositions, sourced from Rovimix and standard *O. niloticus* mineral-mix formulations, are available from the corresponding author on request.

Broodstock collection and husbandry. Wild *O. niloticus* broodstock (mean weight 179.4±2.6 g) was obtained from local fishermen at Sungai Batang Kali, Selangor. Forty pairs were stocked across four broodstock ponds and fed a commercial grower diet (3 mm pellet) until sexual maturity, identified by the presence of eggs in the buccal cavity of females. Mature broodstock was then allocated to the four dietary treatments to found the F0 generation.

Multigenerational production trial. Sex was determined manually by external genital papilla examination at the grower stage in every generation. The F0 founding broodstock and their F1 progeny were maintained on the four experimental diets (T0-T3) until egg production, with the F1 sex ratio expected to approximate the natural, near-50:50 Mendelian distribution in the absence of complete sex reversal, providing a baseline against which the masculinising potential of dietary treatment could be assessed. Fertilised eggs collected from the buccal cavity of brooding females were used to found the subsequent generation. From F1-F2 onward, only the basal (unsupplemented) feed was provided, so that any masculinisation observed in these later generations reflected the persistence of a male-biased phenotype in the parental broodstock lineage rather than ongoing dietary exposure of the offspring themselves; whether this persistence is heritable, parental or another form of carry-over effect cannot be established from the present design. The target production outcome was progressive masculinisation, reaching approximately 75% males in the F1-F2 generation and 100% males by F2-F3. The number of days required to collect a comparable batch of fertilised eggs was recorded at every generation as an index of reproductive cycle length.

Water quality monitoring. Water temperature, pH, un-ionised ammonia (NH₃), total ammonia nitrogen (TAN) and turbidity were monitored throughout the grow-out period of each generation using portable water-quality meters and recorded as treatment means (n = 3 ponds per treatment).

Survival, growth and feed-efficiency calculations. Survival rate (SR, %) was calculated as (final number of fish / initial number of fish) × 100, applied sequentially to the egg-to-hatching, hatching-to-fingerling and fingerling-to-grower intervals, and combined to give the overall egg-to-grower survival for each generation. The feed conversion ratio (FCR) was calculated as total feed consumed divided by total weight gain of the cohort over the same

interval. Both indices follow standard formulae widely applied in *O. niloticus* nutrition and production trials.

Statistical analysis. Data are presented as treatment mean±standard deviation. Differences among the four dietary treatments for each measured parameter and generation were tested by one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post-hoc test for pairwise comparisons where the overall F-ratio was significant. Because a separate one-way ANOVA was performed within each generation, this approach does not formally test for a CHE × generation interaction; testing such an interaction would require a two-way or mixed-effects model incorporating generation as a repeated factor, which was beyond the scope of the present analysis. Similarly, because the three replicate hapas within each treatment were nested within a single pond and therefore shared a common water body, hapa-level replicates are not fully independent; this pond-level pseudoreplication (Hurlbert, 1984) is a further limitation on the ANOVA reported here and is acknowledged accordingly. FCR and egg-collection time (Table 5) were calculated from pooled, treatment-level totals rather than from individual replicate hapas and are therefore reported descriptively rather than subjected to formal inferential statistics. Significance was accepted at $p < 0.05$ throughout.

Results

Water quality. Water temperature (27.9–28.7°C) did not differ among treatments or generations, indicating that thermal conditions did not confound the production results reported below. In contrast, TAN, un-ionised ammonia and turbidity rose significantly and progressively with increasing CHE inclusion in every generation ($p < 0.05$; Table 3). In the F0-F1 generation, ammonia increased from 0.011 mg L⁻¹ in the control to 0.037 mg L⁻¹ at 1.5% CHE, TAN rose from 0.19 to 0.71 mg L⁻¹, and turbidity increased more than tenfold, from 2.5 to 29.1 NTU, over the same dose range. The same dose-dependent pattern persisted in the F1-F2 and F2-F3 generations, although absolute ammonia, TAN and turbidity values were consistently lower in F2-F3 than in F1-F2 within each treatment.

Table 3
Water-quality parameters (mean±SD) in earthen clay ponds across three generations of *Oreochromis niloticus* fed graded levels of a ten-herb Chinese herbal extract mixture

Parameter	Generation	0% CHE	0.5% CHE	1.0% CHE	1.5% CHE
Temperature (°C)	F0-F1	28.4±0.6	28.5±0.5	28.3±0.4	28.4±0.5
	F1-F2	28.13±0.19	28.21±0.34	28.48±0.19	28.07±0.03
	F2-F3	28.00±0.24	28.39±0.71	28.73±0.55	27.93±0.34
pH	F0-F1	7.42±0.18	7.48±0.16	7.51±0.14	7.46±0.17
	F1-F2	7.44±0.08 ^a	7.55±0.01 ^b	7.83±0.02 ^c	7.61±0.07 ^{bc}
	F2-F3	7.23±0.09 ^a	7.32±0.15 ^{ab}	7.44±0.13 ^{bc}	7.59±0.06 ^c
Ammonia (mg L ⁻¹)	F0-F1	0.011±0.010 ^a	0.017±0.009 ^b	0.023±0.008 ^c	0.037±0.007 ^d
	F1-F2	0.009±0.003 ^a	0.011±0.08 ^{ab}	0.029±0.04 ^c	0.033±0.13 ^c
	F2-F3	0.002±0.001 ^a	0.004±0.02 ^{ab}	0.020±0.01 ^c	0.027±0.11 ^c
TAN (mg L ⁻¹)	F0-F1	0.19±0.09 ^a	0.31±0.08 ^b	0.43±0.07 ^c	0.71±0.06 ^d
	F1-F2	0.07±0.02 ^a	0.17±0.04 ^b	0.51±0.13 ^c	0.58±0.09 ^c
	F2-F3	0.04±0.01 ^a	0.06±0.04 ^{ab}	0.36±0.09 ^c	0.47±0.15 ^c
Turbidity (NTU)	F0-F1	2.5±3.8 ^a	8.08±2.5 ^b	19.6±3.2 ^c	29.1±1.1 ^d
	F1-F2	3.99±0.14 ^a	6.93±0.10 ^b	11.99±0.13 ^c	16.99±0.08 ^d
	F2-F3	2.91±0.13 ^a	3.11±0.01 ^b	8.14±0.04 ^c	12.04±0.19 ^d

Note: Different superscript letters within a row indicate significant differences among treatments ($p < 0.05$). F0-F1, F1-F2 and F2-F3 denote successive filial generations: n=3 ponds.

Reproductive performance and survival. The number of fertilised eggs collected per generation did not differ significantly among treatments (270.7-314.0 eggs across all generations and treatments). Hatching, fingerling and grower-stage survival, however, diverged sharply among treatments and generations (Table 4). In the F0-F1 generation, hatching percentage was broadly similar across treatments (87.2-92.0%), but fingerling and grower survival were already significantly higher in the 0.5% CHE group than in the 1.5% group. This divergence amplified in successive generations: by F2-F3, the 0.5% CHE group achieved 93.8% hatching and 90.0% grower survival, compared with 72.0% hatching and 71.5% grower survival in the 1.5% group, and 80.5% hatching and 65.4% grower survival in the unsupplemented control. Expressed as overall egg-to-grower survival, the 0.5% CHE diet achieved 74.6% in F0-F1, rising to 80.5% in F1-F2 and remaining at 78.7% in F2-F3, whereas the control fell from 59.0% to 49.8% and then to 37.2% across the same three generations, and the 1.5% group fell from 47.5% to 40.8% to 34.2%. The 1.0% group occupied an intermediate position throughout (57.9-60.1% across generations).

Table 4

Survival performance from fertilised egg to grower stage across three generations of *Oreochromis niloticus* fed graded levels of a ten-herb Chinese herbal extract mixture (mean±SD)

Parameter	Generation	0% CHE	0.5% CHE	1.0% CHE	1.5% CHE
Hatching (%)	F0-F1	87.2±1.6	92.0±1.2	90.0±1.9	89.7±2.5
	F1-F2	90.5±3.3 ^c	93.9±1.0 ^c	83.4±2.5 ^b	74.0±1.6 ^a
	F2-F3	80.5±0.8 ^b	93.8±0.6 ^c	81.9±0.8 ^b	72.0±0.9 ^a
Fingerling survival (%)	F0-F1	85.3±0.2 ^c	85.5±1.9 ^c	78.6±0.6 ^b	66.4±1.1 ^a
	F1-F2	79.0±2.9 ^a	92.8±1.4 ^c	84.9±0.2 ^b	80.7±0.6 ^{ab}
	F2-F3	70.9±0.9 ^a	93.3±0.7 ^c	83.5±1.1 ^b	66.7±3.9 ^a
Grower survival (%)	F0-F1	79.3±3.0 ^a	94.8±1.1 ^c	84.9±0.8 ^{bc}	79.7±1.4 ^a
	F1-F2	69.7±2.4 ^a	92.3±0.9 ^c	81.8±0.6 ^b	68.1±5.2 ^a
	F2-F3	65.4±4.6 ^a	90.0±1.0 ^c	77.7±3.7 ^b	71.5±1.6 ^{ab}
Overall egg-to-grower survival (%)	F0-F1	59.0	74.6	60.1	47.5
	F1-F2	49.8	80.5	57.9	40.8
	F2-F3	37.2	78.7	53.2	34.2

Note: Different superscript letters within a row indicate significant differences among treatments ($p < 0.05$). Overall egg-to-grower survival is the cumulative product of hatching, fingerling and grower survival for each generation.

Duration of fertilised-egg collection. The time required to collect a comparable batch of fertilised eggs differed significantly among treatments and changed systematically across generations (Table 5). The control group required a stable 125.7-126.7 days throughout the trial, with no significant difference among generations. All three CHE-supplemented groups required significantly longer in F0-F1 than the control (13.2% longer at 0.5%, 24.9% longer at 1.0%, and 43.1% longer at 1.5%; $p < 0.05$), but this delay contracted markedly with each successive generation. By F2-F3, the 0.5% group required exactly the same time as the control (126.7 days), while the 1.0% and 1.5% groups remained only 10.8% and 16.3% longer than the control, respectively, a substantial reduction from the 13.2-43.1% range recorded at F0-F1.

Sex-ratio progression and masculinization. The control group showed no evidence of progressive masculinisation, with the male proportion of the grower population remaining at 43.9, 43.3 and 42.6% in F0-F1, F1-F2 and F2-F3, respectively (Table 6). In contrast, all three CHE-supplemented diets produced a clear and progressive shift toward an all-male population. The male proportion in F0-F1 was already significantly elevated relative to the control (49.5, 49.9 and 51.5% at 0.5, 1.0 and 1.5% CHE, respectively), without significant differences among the three doses at this stage. By F1-F2, 73.8-74.6% of the grower population was male in all three CHE groups, and by F2-F3 the transition was complete: all three CHE-supplemented treatments yielded grower populations in which every individual

examined by external genital papilla inspection was phenotypically male (100%), with no females identified in any replicate; gonadal histological confirmation of sex was not performed, which is acknowledged as a limitation. Although the percentage male converged at 100% across all three CHE doses by F2-F3, the absolute and cumulative number of male fish produced differed substantially because of the divergent survival outcomes described above. Summed across all three generations, the 0.5% CHE group produced 538.7 male fish (75.3% of 715.7 total growers), compared with 370.0 males (73.3% of 504.7 growers) in the 1.0% group, 251.3 males (71.9% of 349.7 growers) in the 1.5% group, and only 186.0 males (43.3% of 429.4 growers) in the unsupplemented control.

Table 5
Duration of fertilised-egg collection and feed conversion ratio across three generations of *Oreochromis niloticus* fed graded levels of a ten-herb Chinese herbal extract mixture

Parameter	Generation	0% CHE	0.5% CHE	1.0% CHE	1.5% CHE
Egg-collection time (days)	F0-F1	126.0	142.7	157.3	180.3
	F1-F2	125.7	134.0	144.3	152.7
	F2-F3	126.7	126.7	140.3	147.3
Feed conversion ratio	F0-F1	1.89	1.27	1.49	1.62
	F1-F2	2.09	1.24	1.43	1.61
	F2-F3	2.06	1.26	1.44	1.67

Note: Feed conversion ratio and egg-collection time are pooled, treatment-level values (total feed consumed or total days per treatment); no replicate-level measure of variation (SD/SE) or formal statistical test is available for these two parameters, and they are therefore reported descriptively.

Table 6
Male:female sex-ratio progression of *Oreochromis niloticus* across three generations under graded dietary inclusion of a ten-herb Chinese herbal extract mixture (mean±SD)

Parameter	Generation	0% CHE	0.5% CHE	1.0% CHE	1.5% CHE
Grower population (n)	F0-F1	175.7±2.9	223.7±6.8	180.3±3.5	144.0±10.4
	F1-F2	145.7±9.0	252.7±7.6	169.7±4.9	113.0±16.1
	F2-F3	108.0±6.2	239.3±5.0	154.7±9.8	92.7±2.1
Male (%)	F0-F1	43.9±2.3 ^a	49.5±0.6 ^b	49.9±0.9 ^b	51.5±1.4 ^b
	F1-F2	43.3±3.5 ^a	74.6±0.6 ^b	73.8±1.4 ^b	74.6±0.8 ^b
	F2-F3	42.6±2.5 ^a	100.0 ^b	100.0 ^b	100.0 ^b
Female (%)	F0-F1	56.1±2.3	50.5±0.6	50.1±0.9	48.4±1.4
	F1-F2	56.6±3.4	25.3±0.6	26.1±1.4	25.4±0.8
	F2-F3	57.3±2.5	0.0	0.0	0.0

Note: Different superscript letters within a row indicate significant differences among treatments ($p < 0.05$).

Feed conversion ratio. The FCR was stable across generations within each treatment but differed substantially among treatments (Table 5). The 0.5% CHE diet produced the lowest (most efficient) FCR throughout the trial (1.27, 1.24 and 1.26 in F0-F1, F1-F2 and F2-F3, respectively), representing a 32.9-40.6% improvement over the unsupplemented control. The 1.0% CHE diet was intermediate (1.49, 1.43, 1.44) and the 1.5% diet less efficient still (1.62, 1.61, 1.67), although both remained more efficient than the control, which recorded the highest (least efficient) FCR of all treatments in every generation (1.89, 2.09 and 2.06). The ranking of treatments by feed efficiency ($0.5\% < 1.0\% < 1.5\% < 0\%$ CHE) was identical in all three generations.

Discussion

Diet formulation as a confound-control strategy. The compensatory reduction of cellulose against increasing CHE inclusion held crude protein (400.2–402.0 g kg⁻¹) and crude lipid (40.7–42.1 g kg⁻¹) essentially constant across all four diets (Table 2), which is the standard and necessary design for isolating the effect of a herbal additive from confounding variation in basal nutrient density (Citarasu, 2010; Abarike et al., 2019). However, because CHE replaced cellulose on a weight basis, increasing inclusion was accompanied by substantial increases in crude fibre and ash and a corresponding decline in nitrogen-free extract across the T0–T3 range (Table 2). The diets were therefore equalised for crude protein and lipid but not for fibre, ash or estimated digestible energy; consequently, the treatment differences reported here cannot be attributed to CHE bioactivity with certainty and may partly reflect these compositional differences among diets. This confound is acknowledged as a limitation of the present design and should be addressed in future work using diets equalised for digestible energy and fibre.

Water-quality dynamics in clay ponds. The progressive, dose-dependent rise in ammonia, TAN and turbidity with increasing CHE inclusion is readily explained by the proximate composition of the diets: as cellulose was replaced by the more fibrous and mineral-rich herbal powder mix, crude fibre rose from 18.1 to 96.5 g kg⁻¹ and ash rose more than sixfold, from 33.1 to 209.5 g kg⁻¹, across the T0–T3 range (Table 2). Earthen clay ponds are inherently more susceptible to particulate and dissolved organic loading than lined or concrete systems because suspended clay flocculates around organic and mineral colloids rather than settling cleanly, and pond water quality is governed largely by the balance between feed-derived loading and the assimilative capacity of the resident microbial and phytoplankton community (Boyd & Tucker, 1998). The decline in absolute ammonia, TAN and turbidity from F1–F2 to F2–F3 within each treatment suggests that this assimilative capacity strengthened as the pond ecosystem matured over the three-generation trial, although the underlying dose-dependent ranking among treatments was preserved throughout.

Survival and the non-monotonic dose-response. The finding that the lowest tested CHE dose (0.5%) produced the best, rather than the worst, survival outcome is consistent with a broad pattern in the phytogenic feed-additive literature, in which intermediate or low inclusion levels frequently outperform higher doses (Gabriel, 2019). Directly comparable non-monotonic dose-responses have been reported for *C. sinensis* peel extract in *Catla catla*, where a 6 g kg⁻¹ dose outperformed both 2 and 10 g kg⁻¹, the highest dose producing significantly poorer survival, growth and feed conversion together with biochemical evidence of liver stress (Shabana et al., 2019), and for a combined *C. pilosula*/*Astragalus membranaceus* extract in weaned piglets, where 1.5% inclusion increased the incidence of diarrhoea relative to 0.5 and 1.0% (Guo et al., 2024b). The present trial extends this well-established hormetic pattern (Calabrese & Kozumbo, 2021) to a ten-component CHE mixture used specifically for *O. niloticus* masculinisation, and identifies the underlying production cost of over-supplementation: at 1.0 and 1.5% CHE, the higher fibre and ash burden, together with the associated decline in water quality documented above, appears to impose a measurable physiological cost on hatching, fingerling and grower survival that more than offsets any additional masculinising benefit of the higher dose.

Egg-collection delay as an expected corollary of masculinization. The absence of a significant treatment effect on the number of eggs collected per generation indicates that CHE influenced downstream survival and sex differentiation rather than fecundity itself. The extended time required to collect a comparable egg batch in the CHE-supplemented groups, particularly evident at F0–F1, is a logical and even expected consequence of successful masculinisation rather than an unfavourable side-effect in its own right: as potential egg-

bearing females are progressively removed from the breeding population, fewer females remain available to spawn within a given period, extending the apparent reproductive cycle (Kwon et al., 2000). The practically important observation is that this delay contracted rapidly and specifically in the 0.5% group, reaching parity with the control by F2-F3, while the 1.0 and 1.5% groups still required 10.8 and 16.3% longer than the control at the same generation. For a commercial hatchery, in which each additional week of delay in egg collection reduces annual fingerling output per breeding female, the 0.5% dose therefore achieves full masculinisation with the smallest penalty to broodstock turnover and hatchery throughput.

Progressive multigenerational masculinization. The generation-by-generation shift from a near-balanced sex ratio at F0-F1 to a complete, 100% male population by F2-F3 in all three CHE-supplemented groups, against an essentially unchanged 42.6-43.9% male control, is the central biological result of this trial. Because dietary CHE supplementation was withdrawn after the F0-F1 generation and only the basal feed was provided thereafter, the persistence and progressive strengthening of the male-biased phenotype observed in F1-F2 and F2-F3 suggests a parental or transgenerational carry-over effect of the herbal treatment on the broodstock; however, genetic or epigenetic mechanisms cannot be established from the present experiment, and alternative explanations, including parental effects, selective breeder survival, sex-differential mortality, maternal effects, differences in reproductive behaviour, or sampling variation, cannot be excluded without further genetic or progeny-testing evidence. This multigenerational efficacy is of direct practical significance, since it implies that a single generation of dietary CHE exposure in the founding broodstock can seed a breeding line whose descendants progressively self-purify toward a fully monosex population, in a manner that may be broadly analogous to the feminisation step that underlies conventional YY supermale breeding programmes (Mair et al., 1997; Beardmore et al., 2001).

Feed conversion ratio and production economics. The feed-conversion-ratio data provide one of the most directly economically relevant measures in the dataset, because FCR translates immediately into feed cost per kilogram of fish produced, conventionally the single largest variable cost in intensive and semi-intensive finfish aquaculture. The 33-41% improvement in FCR delivered by the 0.5% CHE diet relative to the unsupplemented control, while still using the same BSFL-based basal formulation, indicates that the herbal mixture itself, rather than the protein source, was responsible for the gain in feed efficiency, plausibly reflecting improved nutrient digestibility or utilisation associated with the bioactive and prebiotic properties of several component herbs (Citarasu, 2010; Harikrishnan et al., 2011; Gruber et al., 2025). Dietary citrus-peel products, derived from two of the herbs included in the present mixture (*C. aurantium* and *C. sinensis*), have likewise been reported to improve growth performance, feed utilisation and disease resistance in *O. niloticus* and other cultured fish (Acar et al., 2015; Kesbiç et al., 2022). Because BSFL meal is itself promoted in the wider aquafeed literature principally on cost-efficiency grounds, as a lower-cost, more sustainable alternative to fishmeal capable of improving feed conversion and economic return (Limbu et al., 2022), it would be counter-productive from a whole-system cost perspective to erode this advantage by over-supplementing the basal diet with an unnecessarily large quantity of a comparatively expensive imported herbal additive.

Selecting the optimum CHE inclusion level. Bringing together the production endpoints measured in this trial (Table 7), every outcome that distinguishes among the three CHE-supplemented treatments, egg-to-grower survival, FCR and cumulative male output, places the 0.5% inclusion level at or above the 1.0 and 1.5% treatments, while water-quality loading and egg-collection delay place the 0.5% treatment at or below the higher doses. The single endpoint on which all three CHE doses are statistically indistinguishable is the percentage of males achieved by F2-F3 (100% in all three groups); even on this shared endpoint, however,

the 0.5% treatment delivers the result with the largest absolute and cumulative number of marketable male fish, the best feed efficiency and the smallest water-quality disturbance. There is, in short, no production outcome measured in this trial for which 1.0 or 1.5% CHE inclusion is superior to 0.5%, and several outcomes, survival, feed efficiency and water quality, for which the higher doses are measurably and consistently worse. Doubling or tripling the CHE inclusion rate therefore increases herbal raw-material cost per tonne of feed while simultaneously worsening feed conversion, egg-to-grower survival and reproductive cycle time, a pattern of diminishing-to-negative marginal returns that mirrors dose-response findings for other graded phytogetic feed additives in both aquaculture and terrestrial livestock production (Shabana et al., 2019; Guo et al., 2024b; Gabriel, 2019).

Table 7

Dose-response synthesis of key production and economic indicators across the four dietary treatments, drawn from the F2-F3 generation or cumulative three-generation totals as indicated

<i>Parameter</i>	<i>0% (Control)</i>	<i>0.5% CHE</i>	<i>1.0% CHE</i>	<i>1.5% CHE</i>
Grower output, F2-F3 (n)	108.0	239.3	154.7	92.7
Male proportion, F2-F3 (%)	42.6	100.0	100.0	100.0
Cumulative 3-generation male output (n)	186.0	538.7	370.0	251.3
Cumulative 3-generation male output (% of total growers)	43.3	75.3	73.3	71.9
Egg-to-grower survival, F2-F3 (%)	37.2	78.7	53.2	34.2
Feed conversion ratio, F2-F3	2.06	1.26	1.44	1.67
Egg-collection time, F2-F3 (days)	126.7	126.7	140.3	147.3
Total ammonia nitrogen, F2-F3 (mg L ⁻¹)	0.04	0.06	0.36	0.47
Turbidity, F2-F3 (NTU)	2.91	3.11	8.14	12.04

Conclusions. Dietary inclusion of a ten-herb CHE mixture in a BSFL-based diet induced a complete, multigenerational transition from a near-balanced to a fully monosex, all-male *O. niloticus* population by the third generation, in clear contrast to the unsupplemented control, which showed no progression toward masculinisation across the same period. Among the three doses tested, 0.5% CHE inclusion delivered the best egg-to-grower survival, the most efficient feed conversion ratio, the shortest reproductive cycle and the largest cumulative output of marketable male fish, while imposing the smallest burden on pond water quality and requiring the smallest quantity of the most expensive dietary component. No production outcome favoured the 1.0 or 1.5% inclusion levels over 0.5%. We therefore recommend 0.5% dietary inclusion of this ten-herb CHE mixture as the optimal, hormone-free strategy for multigenerational all-male *O. niloticus* production in earthen clay-pond systems, offering smallholder and commercial farmers a practical, low-cost and to synthetic androgen-based sex reversal. Comprehensive toxicological and residue screening of the herbal mixture were beyond the scope of the present trial and are recommended before broader environmental- or food-safety claims are made.

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Conflict of Interest. The authors declare that there is no conflict of interest.

Data Availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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