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Dietary inclusion of pine pollen alters sex ratio and promotes growth of Nile tilapia (Oreochromis niloticus, L. 1758)

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ABSTRACT

The potential of pine pollen (PP) to masculinize sexually undifferentiated Nile tilapia was evaluated by feeding graded levels of PP (0.08–3.20 g kg^{-1} basal diet) to triplicate groups of three-day-old Nile tilapia for 28 days. Masculinization and associated differences in growth were compared to fish individuals fed the same basal diet with no PP (CT; negative control) and with 0.06 g 17α-methyltestosterone (MT) kg^{-1} basal diet (positive control). Both PP and MT treatments significantly skewed the expected 50:50 (male: female) ratio towards more male individuals. Notably, MT and 1.28 g PP kg^{-1} of diet produced a significantly high proportion of males (89.2 ± 2.2% and 80.0 ± 2.9% respectively), compared to 50.8 ± 2.2% in the CT treatment ($P < 0.001$). Except for 0.08 g PP kg^{-1} of diet, the final body weight and specific growth rate of individuals fed PP and MT-supplemented feeds were significantly higher than fish from the CT group. Although 1.28 g PP kg^{-1} diet produced the highest masculinization, the fish fed 3.20 g PP kg^{-1} diet had the highest final weight (14.73 ± 0.54 g), suggesting the presence of growth enhancers in PP. Similarly, a superior feed conversion ratio was recorded in both PP and MT-treated groups compared to the CT treatment ($P = 0.024$). However, the condition factor and survival rate of fish in all groups did not differ significantly. Overall, 1.28 g PP kg^{-1} diet was potent in sex inversion and promoted the growth of Nile tilapia, making PP a promising alternative to MT in the production of all-male stock.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is the second most farmed fish worldwide, after carps. In 2018, an estimated 4.53 million tonnes of Nile tilapia were produced, representing 8.3% of the total farmed finfish production globally (FAO, 2020). Therefore, this fish contributes significantly to global human food and nutrition security and economic demands. Amongst the factors that have enabled its continued growth is the increased adoption of all-male culture technology in the production systems (Baroiller and D'Cotta, 2019; Nozu and Nakamura, 2020). All-male Nile tilapia production prevents uncontrolled reproduction, which is responsible for excessive recruitment of fingerlings, competition for food, stuntedness, and subsequently small-sized fish of low market value, characteristic of mixed-sex culture systems (Beardmore et al., 2001; Srisakultiew, 2013; Toguyeni et al., 2002). Besides, male individuals grow faster and bigger than females, resulting in a shortened production cycle and of uniform sized-fish at harvest that attract a good market price (Baroiller and D'Cotta, 2019; Chavez-Garcia et al., 2020; Snake et al., 2020). Consequently, the demand for all-male Nile tilapia seed is high.

Presently, most Nile tilapia hatcheries use exogenous synthetic hormones, mainly 17α-methyltestosterone (MT), to produce all-male individuals (Baroiller and D'Cotta, 2019; Celik et al., 2011; Snake et al., 2020). With this method, the sexually undifferentiated fish are fed diets mixed with MT hormone for three to four weeks post-hatching (Baroiller and D'Cotta, 2019; Celik et al., 2011; Mateen and Ahmed, 2007). However, long-term exposure to MT adversely affects the health of hatchery operators (Baroiller and D'Cotta, 2019; Golan and Levavi-Sivan, 2014; Megbowon and Mojekwu, 2014; Velazquez and Alter, 2004), including hepatotoxicity, menstrual irregularities, atrophy of breasts, impotence and prostatic hypertrophy conditions (Vick and Hayton, 2001; Yılmaz et al., 2013). Furthermore, the leakage of MT into the aquatic environments from uneaten or un-metabolized fish feed disrupts the endocrine systems of non-target organisms (Abo-Al-Ela, 2018; Abo-Al-Ela et al., 2017; Hulak et al., 2008; Ramirez-Godinez et al., 2013; Rivero-Wendt et al., 2013). Consequently, most countries have

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banned or instituted restrictions on using and selling synthetic hormones for aquaculture while advocating for organic fish production (Chakraborty et al., 2014; Leet et al., 2011; Mlalila et al., 2015). Research on potential alternatives to synthetic hormones has thus steadily progressed, and the utilization of organic plant-based products for control of unwanted reproduction in tilapia culture systems has received considerable attention (Ampofo-yeboah, 2013; Gabriel et al., 2017; Ghosal et al., 2021; Mukherjee et al., 2018; Nian et al., 2017).

Plant-based extracts contain phytochemicals with the potential to induce masculinization of tilapia in an attempt to control prolific breeding (Abaho et al., 2021; Ampofo-yeboah, 2013; Chakraborty et al., 2014; Gabriel et al., 2017; Ghosal et al., 2021; Mukherjee et al., 2018; Nian et al., 2017; Reverter et al., 2014). Currently, the seed, root, and leaf extracts of 20 plant species have been tested for masculinization potential to replace synthetic hormones with promising results (Abaho et al., 2021). Amongst the plant extracts, pollen, the male gamete of pine trees (pine pollen - PP) contains phytoandrogens; testosterone, epitestosterone, and androstenedione (Baluran et al., 2018; Tarkowska, 2019; Turan and Akyurt, 2005; Velasco et al., 2018), which have a potential to induce masculinization of Nile tilapia (Nian et al., 2017; Nieves, 2017). Besides sex masculinization, the bioactive compounds in PP boost the innate immune response in fish, subsequently accelerating fish growth (Baluran et al., 2018; Nian et al., 2017). However, inadequate information on the optimum concentration and application pathways of PP required to masculinize Nile tilapia, and the subsequent effects on fish growth, limit its commercial use. As such, this study explored the potential of oral administration of PP in feeds to induce sex masculinization in Nile tilapia and also examined the growth performance of treated fish. Specifically, the study determined the PP dose that would produce considerable masculinization of the fish, as well as its effects on growth, condition factor, and survival, to test the hypothesis that PP induces sex masculinization and enhances the growth of Nile tilapia.

2. Materials and methods

2.1. Ethical statement

The experimental procedures of the present study were conducted following the ethical guidelines for animal care and use in research. They were approved by the Animal Research Ethics Committee of Rhodes University (RU-AREC) under approval number: 2019-0792-973.

2.2. Phytochemical analysis of pine pollen

Qualitatively, the phytocompounds, including alkaloids, flavonoids, and steroids, were determined using visual observation of color changes or precipitate formation upon the addition of specific reagents to the PP sample (Ezeonu and Ejikeme, 2016; Sasidharan et al., 2011). The results were recorded as present (+, ++ and +++) or absent (-) depending on the outcome of the test and intensity of the color. The alkaloids, flavonoids, and steroids were extracted from PP using absolute ethanol and double distilled water. Since steroids are non-polar, i.e., they cannot be extracted by an aqueous solvent, screening was done with only ethanol. After qualitative confirmation of the target phytochemicals, quantification was performed following standard procedures (Gurupriya and Cathrine, 2021). Using a UV–vis spectrophotometer (752 UV-Vis, China), absorbance values of change in color intensities were measured at 510, 470, and 640 nm for flavonoids, alkaloids, and steroids, respectively. Atropine, quercetin, and cholesterol were used as standards for alkaloids, flavonoids, and steroids, respectively, to generate calibration curves of absorbance values against the concentrations of standards. The linear equation obtained from the standard curve plot was used to calculate the concentration of each phytochemical. The concentration of total alkaloids, flavonoids, and steroids in PP was expressed as μg g^{-1} of the dry powder. The steroids were further screened to identify specific phytosteroids: testosterone (T), androsterone (AN), androstenedione (AED), androstadienedione (ADD), epitestosterone (EPIA), and dehydroepiandrosterone (DHEA) present in PP using high-performance thin layer chromatography (HP-TLC) method.

2.3. Experimental facility and fish rearing

The experiments were conducted in the aquaculture facilities of Mukono Zonal Agricultural Research and Development Institute (MUZARDI) of the National Agricultural Research Organization, Uganda. Nile tilapia broodstock (60 females and 20 males) were mated at a ratio of three females to one male in one 60 m^3 circular concrete tank under a natural photoperiod (12-h light; 12-h dark) and water temperature (26 ± 2.1 ^{o}C). After two weeks, the mouths of female individuals were checked for eggs. The fertilized eggs from all brooding females were collected and transferred into an indoor hatchery and thereafter incubated at 27 ± 1.0 ^{o}C until hatching, using McDonald hatching jars. The newly hatched juveniles were left for three days to absorb the yolk sac. The three-day-old fish (3 dph; three days post-hatch) were randomly distributed into 21 experimental tanks (140 L), each filled with 120 L of water, at a density of two juveniles L^{-1}. The experimental tanks were set in a flow-through system with a water flow rate of 1.2 L min^{-1}. Continuous aeration was maintained in each tank using air stones connected to an air compressor (Hailea ACO-388D) to ensure a regular air supply throughout the experimental tanks. Water temperature, dissolved oxygen, and pH were monitored daily using a multi-parameter meter (In-Situ SmarTROLL™ MP, USA). Throughout the experiment, the water quality parameters were kept within the optimal range for tilapia culture (Mjoun et al., 2010). Water temperature was maintained at 27 ± 0.4 °C using thermostatic heating rods (Sera Aquarium heater thermostat, Germany). Dissolved oxygen and pH were also maintained at 5.4 ± 0.23 mg L^{-1} and 7.2 ± 0.42, respectively. Ammonia-nitrogen was monitored daily using a commercial freshwater aquaculture kit (LaMotte Company Ltd, USA) and kept below 0.2 mg L^{-1}. The experimental tanks were cleaned twice daily at 0700 h and 1900 h, before the first and after the last feeding, respectively, to remove uneaten food, solid wastes, and other debris by siphoning and replacing a third of the water volume with an equal volume of fresh water.

2.4. Experimental design

A total of 5040 three-day-old (three days post-hatch) juvenile Nile tilapia with mean body weight: 0.022 ± 0.001 g and mean total length: 11.51 ± 0.06 mm, of the same batch were randomly allocated to the experimental tanks (240 juveniles per tank). The treatments were grouped into: basal-diet with no PP nor MT treatment (CT- negative control); or the same basal diet supplemented with either graded levels of PP; or the same basal diet treated supplemented with MT (MT- positive control; Table 1).

2.5. Experimental diets

Commercially available pine pollen (PP; Jiangsu Qinshantang Health Industry Co., Ltd, Nanjing, China) harvested from *Pinus tabulaeformis,* and 17α-methyltestosterone hormone (purity ≥ 98%) (MT; Sigma Chemical Co., St Louis, MO, USA) were incorporated in the basal diet of juvenile Nile tilapia. Proximate analyses of PP and the other ingredients of the basal diet were determined prior to diet formulation. Thereafter, the experimental diets were formulated for a balanced composition of protein (46% protein), lipid (7.9% lipid), and energy (3865 kcal kg^{-1}). Pine pollen powder and MT were dissolved in 50 mL of 95% absolute ethanol, sprayed, and mixed well with the formulated diets, with respect to the treatment groups. The diets were treated with: 0.08, 0.16, 0.32, 0.64, 1.28, 1.92, 2.56 and 3.20 g kg^{-1} of PP and 0.06 g kg^{-1} of MT. The feed was completely dried at room temperature to prevent fungal contamination, vacuum-packaged, and stored at − 4 ℃ until use.

Table 1
Dietary ingredient formulation and proximate composition of the seven experimental diets with varying levels of pine pollen (PP): 0.08, 0.16, 0.32, 0.64, 1.28, 1.92, 2.56 and 3.20 g PP kg^{-1} diet for PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560 and PP3200 respectively; 17α-methyltestosterone (MT): 0.06 g kg^{-1} diet; and basal diet (CT): without PP and MT.

Ingredient (g kg^{-1})	**Treatment**									
	CT	**MT**	**PP80**	**PP160**	**PP320**	**PP640**	**PP1280**	**PP1920**	**PP2560**	**PP3200**
Fishmeal	625.20	625.20	625.20	625.20	625.20	625.20	625.20	625.20	625.20	625.20
Maize meal	245.60	245.60	245.60	245.60	245.60	245.60	245.60	245.60	245.60	245.60
Wheat flour	40.20	40.20	40.20	40.20	40.20	40.20	40.20	40.20	40.20	40.20
Sunflower meal	34.00	33.94	33.92	33.84	33.68	33.36	32.72	32.08	31.44	30.80
Fish oil	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00
Vitamin premix	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Mineral premix	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
PP†	0.00	0.00	0.08	0.16	0.32	0.64	1.28	1.92	2.56	3.20
MT††	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total (g)	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000.00**	**1000**	**1000**
Proximate analysis										
Total protein (%)	45.30	45.50	45.30	45.60	45.70	45.90	46.10	45.80	46.10	46.10
Total lipid (%)	7.70	7.50	7.60	7.40	7.50	7.30	7.20	7.40	7.60	7.50
Gross energy (Kcal kg^{-1})	3860	3861	3859	3860	3862	3861	3862	3861	3861	3862

†Jiangsu Qinshantang Health Industry Co., Ltd, Nanjing, China.
††Sigma Chemical Co., St Louis, MO, USA.

2.6. *Fish feeding*

The fish were fed the experimental diets from the 3rd dph to the 114th dph. From 3–30 dph, fish in the negative (CT) and positive control groups (MT) were only fed the basal diet and basal diet supplemented with MT, respectively. In contrast, those in the PP-treated groups were fed the basal diet supplemented with varying doses of PP (Table 1). From 30 dph, the fish in all treatments were fed the non-treated basal diet (CT) for 84 days. The experimental fish were fed six times a day (0800, 1000, 1200, 1400, 1600, and 1800 h) at a feeding rate of 20% of biomass during the first 28 days of the experiment. Subsequently, the fish were fed four times daily (0900, 1200, 1400, and 1700 h) at feeding rates of 15% and 10% of the biomass, from 31 to 90 dph and 91–114 dph, respectively. In cases where fish reached apparent satiation before the total ration was placed in the tank, feeding was stopped, and the weight of feed provided was recorded.

2.7. *Data collection*

Biometric measurements were performed at the start of the trial and bi-weekly, using a randomly selected sample of 30 individuals per replicate after the fish were purged for 12 h. The fish were anesthetized with a low dose of tricaine methanesulfonate (MS-222; Sigma-Aldrich) at 10 mg L^{-1} in aerated water, measurements taken, and immediately returned to the experimental tanks. The live wet body weight (g) and total length (mm) of the fish were measured with a digital Vernier caliper (Jpxvny Digital Vernier caliper; Resolution: 0.01 mm, Hanhe, China), and a digital scale (Philonext Digital Milligram Scale; 50 ×0.001 g and DBJB; 500 g * 0.01 g), respectively. Daily fish mortalities were recorded by visual counting during the experimental period. Growth performance was determined by considering the following parameters:

- Daily weight gain (DWG) = [final mean body weight (FBW) – initial mean body weight (IBW)] / number of days fed
- Specific growth rate (SGR) = [(ln FBW – ln IBW) / number of days fed] x 100

The feed conversion ratio (FCR) and survival were recorded as follows:

- FCR = total food fed / total wet weight gain by the fish
- Survival (%) = [final number of fish / initial number of fish] X 100

2.8. *Analysis of length-weight relationship and relative condition factor*

The relative condition factor (Kn), calculated using (Le Cren, 1951) formula: $Kn = W / aL^b$, where W is the observed individual fish weight, L is the observed individual fish total length, a is the intercept of the length-weight regression and b is the slope of the regression line, was used to determine the condition of fish in all treatments. The regression constants (a and b) in the length-weight relationship ($W = aL^b$) were generated from pooling data of the replicates for each treatment. Length and weight data were log-transformed and the resulting linear regression fitted by the least squares method using weight as the dependent variable.

2.9. *Sex ratio analysis*

The percentage of male and female individuals was determined at the end of the experiment. At 114 dph, 40 fish per replicate (i.e., 120 fish individuals per treatment) were randomly sampled and euthanized with 250 mg L^{-1} of MS-222 (Sigma-Aldrich). Gonadal tissues were harvested using surgical forceps, and sex was determined using the acetocarmine squash technique. Here, the gonad was mounted on a glass slide, crushed and phenotypic sex was determined microscopically at 100x magnification after acetocarmine coloration (Guerrero and Shelton, 1974).

To compare the level of masculinization, the squash method was followed by histological examination of gonads from MT and PP1280 (1.28 g PP kg^{-1} diet since it had the highest number of males) treated fish. The gonads were fixed in bouin solution for 24 h at room temperature. The samples were dehydrated in a series of ethanol with varying concentrations from 70%, 80%, 90%, 95%, and finally, absolute ethanol using an automated benchtop tissue processor (LEICA TP1020, USA). The dehydrated samples were cleared with xylene and thereafter wax-infiltrated with molten paraffin wax. Cross Section (4 µm) were performed using a microtome (LEICA RM2235, USA), stained using hematoxylin-eosin (H & E), examined and classified as ovary, testis or ovotestis under a light microscope (Nikon eclipse *ci*, USA). The photomicrographs were taken using a digital microscope-mounted camera (Nikon Digital Sight, USA).

2.10. *Data analysis*

Data were analyzed using SPSS statistical package (IBM SPSS Statistics, Version 21.0, USA). The data were expressed as mean ± standard error (SE) and checked for homogeneity of variance and normality of residuals using the Levene's and Shapiro-Wilk tests, respectively.

Treatment means were compared using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. *Concentrations of alkaloids, flavonoids, and steroids in pine pollen*

Qualitative phytochemical analysis showed the presence of steroids, alkaloids, and flavonoids (in descending order) in PP for both aqueous and ethanolic extracts, where applicable (Table 2). Quantitatively, the concentration of phytocompound, in descending order, was steroids > alkaloids > flavonoids. Meanwhile, testosterone (T) and androsterone (AN) were the only steroids present in PP.

3.2. *Gender determination*

At the end of the experiment, fish gonads were microscopically classified into male and female using the Aceto-carmine squash method (Fig. 1). The female gonads were characterized by the presence of oocytes (Fig. 1a), while male gonads had thin thread-like structures typical of testicular tubules (Fig. 1b).

Histological examination of the gonads from the fish treated with 1.28 g PP kg^{-1} of diet and MT revealed no discernable differences in the ovaries of non-masculinized individuals in both treatments. Ovaries with oocytes, typical female phenotypical attributes, were observed in non-masculinized females (Fig. 2a and b). Meanwhile, most of the fish specimens from both 1.28 g PP kg^{-1} of diet and MT groups showed phenotypical male characteristics, including spermatocyte (SP), spermatid (ST), and spermatozoa (SZ) (Fig. 2c and d). Numerous SP were observed in the testes of PP-treated fish (Fig. 2c) compared to ST and SZ in the MT group (Fig. 2d). Overall, no noticeable damages were observed in the testicular and ovarian structures.

3.3. *Sex ratio*

At the end of the experimental period, microscopic examination of the fish gonads demonstrated a sex ratio of 1:1 (male: female) in the control group and predominantly male progeny in the MT and PP treatment groups (Table 3). Both PP and MT induced a significant shift from females to males, hence deviating from the expected 1:1 sex ratio (ANOVA, $F_{(9,\ 20)} = 7.537$, $P < 0.001$). A dose-dependent increase in male individuals was observed in juvenile Nile tilapia fed diets supplemented with PP. The dose of 1.28 g PP kg^{-1} diet significantly (80.0 ± 2.9%) altered the sex ratio from the expected 50:50 pattern in favor of males as compared to 50.8 ± 2.2% on the non-treated (negative control) diet (ANOVA, $P < 0.001$). With regard to the proportion of masculinization in PP treatments, the male individuals from tanks fed 1.28 g PP kg^{-1} diet was higher than fish fed low and high concentrations (Table 3). Notably, the percentage of males produced by MT treatment (89.2 ± 2.2%) was not significantly different from 80.0 ± 2.9% produced in the group fed diets supplemented with 1.28 g PP kg^{-1} of diet (ANOVA, $P = 0.087$).

Table 2
Screening and quantification results for steroids, flavonoids, and alkaloids in pine pollen.

Extract	*Qualitative analysis*		*Quantitative analysis*
	Phytochemical	**Deduction**	**Mean concentration ± SD ($\mu g\ g^{-1}$ dry weight)**
Ethanolic	Steroids	+++	433.14 ± 8.58
	Alkaloids	++	172.25 ± 0.64
	Flavonoids	+	3.47 ± 0.26
Aqueous	Steroids	N/A	N/A
	Alkaloids	++	118.29 ± 12.67
	Flavonoids	+	1.66 ± 0.06

+, ++, and +++: low, moderate, and high concentrations of the phytochemical, respectively; N/A: Not applicable since steroids are non-polar and thus cannot be extracted by an aqueous solvent.

3.4. *Growth performance*

Dietary inclusion of PP significantly augmented the growth of Nile tilapia than fish fed basal diet only (ANOVA, $F_{(9,\ 20)} = 9.303$, $P < 0.001$; Table 4). The results revealed a corresponding increase in fish weight with an increase in the concentration of PP. Generally, the growth pattern showed two phases of weight gain. An exponential increase in fish weight with time in the first 12 weeks of the experiment and a gradual increase between the 12th and 14th weeks in all treatments (Fig. 3). The mean final weights of the fish ranged from 8.63 ± 0.34 g to 14.73 ± 0.54 g across the treatments. The final mean body weight (14.73 ± 0.53 g) observed in the 3.20 g PP kg^{-1} of diet treatment, was significantly higher than 8.63 ± 0.34 g. and 12.62 ± 0.78 g in control (ANOVA, $P < 0.001$; Table 4) and MT- treatments respectively (ANOVA, $P = 0.010$; Table 4). Notably, fish individuals fed diets treated with 1.28 g PP kg^{-1} (with comparable levels of masculinization to MT) were significantly heavier than fish fed diets supplemented with MT (Table 4). Likewise, the daily weight gain (DWG) and specific growth rate (SGR) of fish fed PP-treated feeds were significantly higher than individuals from CT and MT groups (ANOVA, $F_{(9,\ 20)}$, $= 9.303$, $P < 0.001$ for DWG and ANOVA, $F_{(9,\ 20)} = 14.196$, $P < 0.001$ for SGR; Table 4). In particular, fish fed diets supplemented with 1.28, 1.92, 2.56, and 3.20 g PP kg^{-1} of diet displayed significantly higher weight gain and specific growth rate than those fed either CT or MT (ANOVA, $P < 0.05$).

3.5. *Feed conversion ratio*

The mean (± SE) FCR of fish fed PP (1.23 ± 0.15) and MT (1.55 ± 0.34) diets were significantly lower than one recorded among individuals fed only basal diet (2.27 ± 0.26; ANOVA, $F_{(9,\ 20)} = 2.869$, $P = 0.024$; Table 4). In PP-treated groups, FCR ranged from 1.03 ± 0.33–1.42 ± 0.06. .

3.6. *Survival*

The mean percentage survival (% ± SE) of Nile tilapia did not vary significantly among the treatments. The survival rate of the fish ranged between 72.50 ± 5.21% and 75.69 ± 0.61% (ANOVA, $F_{(9,\ 20)} = 0.106$, $P = 0.999$; Table 4).

3.7. *Relative condition factor and length-weight relationship*

The relative condition of Nile tilapia did not differ significantly amongst the treatments (ANOVA, $F_{(9,\ 20)} = 0.493$, $P = 0.923$; Table 4) and ranged from 1.01 ± 0.01–1.03 ± 0.01. The power curve equations showed a strong degree of association between the total length and weight of the fish among all treatments, with the coefficient of determination (R^2) ranging from 0.991 to 0.995 (Fig. 4). Positive allometric growth was observed across treatments, and the regression coefficients ("b" values) were above 3 (3.066–3.110) in all groups. Further, MT, PP640, PP1280, PP1920, PP2560, and PP3200 treatments had larger individuals compared to the other treatments. However, the length-weight relationship of these individuals was consistent with one observed in smaller ones in these and other treatments (Fig. 4).

4. Discussion

All-male Nile tilapia culture eliminates uncontrolled breeding and allows for the production of market-sized fish in a shorter period compared to all-female or mixed-sex populations (Beardmore et al., 2001; El-Greisy and El-Gamal, 2012). Recently, the application of plant

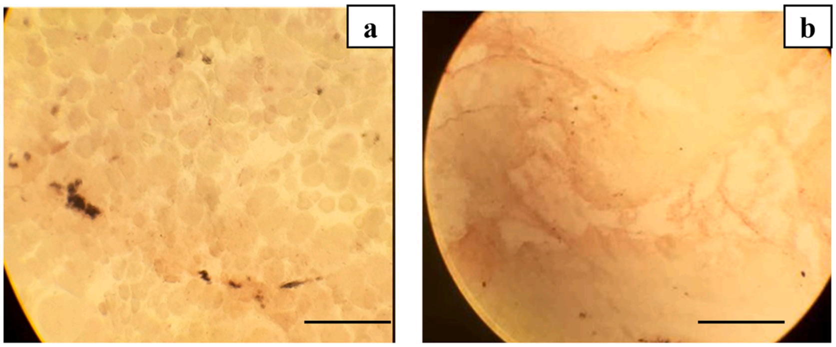

Fig. 1. Nile tilapia reproduction organs at 114 day post hatching (dph), stained with aceto-carmine (100x): a) female ovary with oocytes and b) male testis with testicular tissues. Scale bar (a – b): 50 µm.

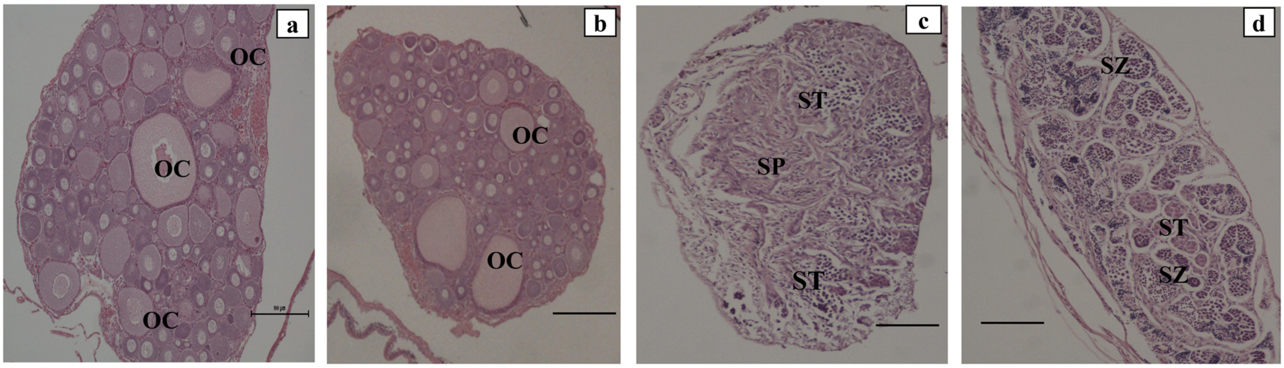

Fig. 2. The histological features of Nile tilapia gonads at 114 day post hatching (dph): a) and b) ovarian cavity (OC) in female individuals obtained from 1.28 g PP kg^{-1} of diet and MT treatments, respectively, c) spermatocyte (SP) and spermatid (ST) observed in male fish obtained from 1.28 g PP kg^{-1} of diet treatment, and d) testis containing spermatid (ST) and spermatozoa (SZ) in specimens from MT treatment. Scale bar (a – d): 100 µm.

Table 3
Fish sex ratios observed across the different treatments of the experiment. Means in a column with a different superscript are significantly different (ANOVA, $F_{(9, 20)} = 7.537$, $P < 0.001$).

Treatment		male	female	% of males
	n	n	n	
CT	120	61	59	50.8 ± 2.2^{a}
MT	120	107	13	89.2 ± 2.2^{b}
PP80	120	83	37	69.2 ± 5.1^{c}
PP160	120	86	34	71.7 ± 3.6 ^{cd}
PP320	120	92	28	76.7 ± 3.6 ^{cd}
PP640	120	93	27	77.5 ± 5.2 ^{cd}
PP1280	120	96	24	80.0 ± 2.9^{bd}
PP1920	120	94	26	78.3 ± 1.7 ^{cd}
PP2560	120	91	29	75.8 ± 2.2 ^{cd}
PP3200	120	93	27	77.5 ± 5.0 ^{cd}

Pine pollen (PP) concentrations: 0.08, 0.16, 0.32, 0.64, 1.28, 1.92, 2.56 and 3.20 g kg^{-1} diet for PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560 and PP3200 respectively; 0.06 g 17α-methyltestosterone kg^{-1} diet (MT) and basal diet (CT).

extracts as alternatives to synthetic chemicals to produce only male stocks has been considered a safe and eco-friendly approach (Bilen et al., 2019; Hasan et al., 2021). For instance, the use of pine pollen (PP) is viewed as a potential substitute to MT in the production of all-male tilapia populations (Nian et al., 2017; Nieves, 2017). Pollen is a yellowish powder that is harvested from the male reproductive parts of pine trees (*Pinus* spp.) such as *P. kesiya, P. nigra, P. silvestris* and *P. tabulaeformis*, which belong to Pinales order and Pinaceae family (Christenhusz et al., 2011). It contains testosterone (T) as one of its major bio-active compounds (Janeczko and Skoczowski, 2005; Saden-Krehula et al., 1979, 1971; Velasco et al., 2018), which is an androgenic steroid that promotes the development of male reproductive characteristics in vertebrates including fish (Celik et al., 2011; Leet et al., 2011). Therefore, the inclusion of PP in fish diets prior to gonadal differentiation is expected to alter the process of sex development in favor of male individuals. In the present study, PP was incorporated in the diets of sexually undifferentiated Nile tilapia, and its potential to induce masculinization was examined. The study utilized the early stages of sexual development, where the gonads are bi-potential and can be modified to follow a differentiation pathway oriented to either ovary or testis by exogenous hormonal treatment (Baroiller et al., 2009; Budd et al., 2015; Ijiri et al., 2008). Exposure to PP and MT was done from the onset of first feeding, i.e., 3 dph, up to 30 dph, to match the labile period for sex inversion (Kobayashi et al., 2008; Nivelle et al., 2019). Considerable sex inversion to a gender of choice, male individuals, was observed in addition to increased feed utilization with corresponding superior growth performance.

In the present study, sex masculinization of Nile tilapia increased with the incorporation of PP in diets in a dose-dependent manner, up to 1.28 g PP kg^{-1} of diet. Compared to the control group, the dietary inclusion of PP in the basal diet shifted the sex ratio from the expected 1:1 (male: female), producing a higher percentage of males. Comparable findings were obtained from previous studies that used PP from *P. tabulaeformis* to produce all-male Nile tilapia populations (Nian et al., 2017; Nieves, 2017). Similarly, feeding juvenile African catfish (*Clarias gariepinus*) on PP-treated diets resulted in a higher proportion of male individuals (Adenigba et al., 2017). These results are attributed to the presence of T in PP powder (Adenigba et al., 2017; Saden-Krehula et al., 1979, 1971; Tarkowska, 2019; Velasco et al., 2018), which induces androgenic activity in animals (Gharaei et al., 2020; Guiguen et al., 2010; Wang et al., 2017). This study confirmed the presence of steroids,

Table 4

Initial body weight (IBW), final body weight (FBW), specific growth rate (SGR), daily weight gain (DWG), feed conversion ratio (FCR), and relative condition factor (Kn) of Nile tilapia across different treatments (values are mean ± SE). Means with a different superscript in a column are significantly different (SPSS, ANOVA, $P < 0.05$).

Treatment	**Parameter**						
	IBW (g)	**FBW (g)**	**SGR (% day^{-1})**	**DWG (g)**	**FCR**	**Survival (%)**	**Kn**
CT	0.022 ± 0.001	8.63 ± 0.34^{a}	5.30 ± 0.04^{a}	0.08 ± 0.00^{a}	2.27 ± 0.26^{a}	74.58 ± 7.56^{a}	1.02 ± 0.01^{a}
MT	0.022 ± 0.001	12.62 ± 0.78^{b}	5.56 ± 0.06^{b}	0.11 ± 0.01^{b}	1.55 ± 0.34^{b}	72.50 ± 5.21^{a}	1.02 ± 0.01^{a}
PP80	0.022 ± 0.001	9.63 ± 0.48^{a}	5.38 ± 0.05^{ac}	0.09 ± 0.00^{a}	1.42 ± 0.06^{b}	74.31 ± 1.64^{a}	1.02 ± 0.01^{a}
PP160	0.022 ± 0.001	11.51 ± 0.74^{b}	5.46 ± 0.07^{bc}	0.10 ± 0.01^{b}	1.20 ± 0.06^{b}	75.00 ± 1.88^{a}	1.02 ± 0.01^{a}
PP320	0.022 ± 0.001	11.58 ± 0.61^{b}	5.53 ± 0.06^{b}	0.10 ± 0.01^{b}	1.21 ± 0.08^{b}	74.17 ± 4.59^{a}	1.02 ± 0.01^{a}
PP640	0.022 ± 0.001	11.60 ± 0.71^{b}	5.52 ± 0.06^{b}	0.10 ± 0.01^{b}	1.36 ± 0.09^{b}	75.28 ± 1.21^{a}	1.03 ± 0.01^{a}
PP1280	0.022 ± 0.001	14.21 ± 0.54^{c}	5.76 ± 0.05^{d}	0.13 ± 0.00^{c}	1.30 ± 0.14^{b}	75.56 ± 2.18^{a}	1.02 ± 0.01^{a}
PP1920	0.022 ± 0.001	14.28 ± 0.39^{c}	5.79 ± 0.04^{d}	0.13 ± 0.00^{c}	1.21 ± 0.27^{b}	72.92 ± 2.71^{a}	1.02 ± 0.01^{a}
PP2560	0.022 ± 0.001	14.46 ± 0.52^{c}	5.78 ± 0.04^{d}	0.13 ± 0.00^{c}	1.10 ± 0.15^{b}	72.78 ± 2.41^{a}	1.01 ± 0.01^{a}
PP3200	0.022 ± 0.001	14.73 ± 0.54^{c}	5.80 ± 0.04^{d}	0.13 ± 0.00^{c}	1.03 ± 0.33^{b}	75.69 ± 0.61^{a}	1.02 ± 0.01^{a}

Pine pollen (PP) concentrations: 0.08, 0.16, 0.32, 0.64, 1.28, 1.92, 2.56 and 3.20 g kg^{-1} diet for PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560 and PP3200 respectively; 0.06 g 17α-methyltestosterone kg^{-1} diet (MT) and basal diet (CT).

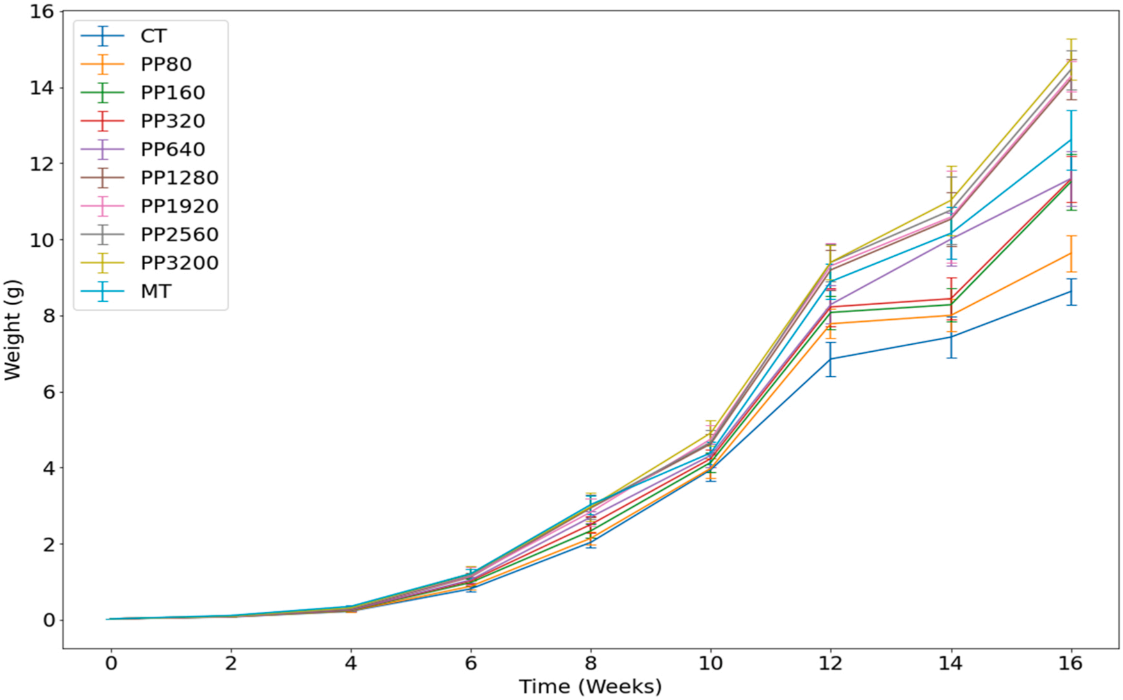

Fig. 3. Wet weight of Nile tilapia raised on pine pollen (PP) based-diets at concentrations of 0.08, 0.16, 0.32, 0.64 1.28, 1.92, 2.56 or 3.20 g PP kg^{-1} diet for PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560 or PP3200 respectively; 0.06 g 17α-methyltestosterone kg^{-1} diet (MT); or a basal diet only (CT). Error bars represent the standard errors of three replicates for each treatment.

mainly T, as the major phytochemicals in the used PP. Like MT, the T in PP could have augmented the endogenous T levels in the fish serum, thereby shifting the balance of the androgen: estrogen ratio in favor of androgens. (Baroiller et al., 1999; Golan and Levavi-Sivan, 2014; Leet et al., 2011). This, in turn, may have stimulated the development of male characteristics while inhibiting feminization (Golan and Levavi-Sivan, 2014; Velasco et al., 2018). In line with this hypothesis, sex inversion of juvenile Nile tilapia to male gender using puncture vine (*Tribulus terrestris)* seed and shatavari (*Asparagus racemosus)* root extracts, coincided with elevated levels of 11-ketotestosterone (11-KT) levels in the gonads of the treated fish (Ghosal et al., 2021). Therefore, future studies should explore the changes in the levels of endogenous T in the PP-treated fish to understand its role in the masculinization process (Gennotte et al., 2014; Shi et al., 2017).

Although MT and PP significantly increased the proportion of male individuals, both treatments produced a low number of all-male fish compared to previous studies. For example, 0.06 g MT kg^{-1} diet yielded 95 – 100% male individuals (El-Greisy and El-Gamal, 2012; Mehrim et al., 2019), while 0.32 g PP kg^{-1} diet produced 89% masculinization rate of Nile tilapia (Nian et al., 2017). This discrepancy could be attributed to variability in the amount of the sex steroids available to the fish, arising from the non-uniform distribution of MT and PP during feed mixing, as well as competition for feed amongst the experimental fish (Budd et al., 2015; Silva et al., 2021). Owing to feed competition by fish, access to food by some individuals is limited due to the dominance hierarchy experienced in the culture system (Fortes, 2005; Obirikorang et al., 2020). Furthermore, oral administration of androgens exposes them to hepatic metabolism; hence, very low levels reach systemic circulation, reducing their androgenic activity. This situation could have been more pronounced in the PP treatments since the T is more susceptable to metabolism, unlike MT (Phelps and Popma, 2000) The structural modification of T to include an alpha-methyl group at 17 carbon position attenuates catabolism in the gut, making it more bioavailable (Gao et al., 2005; Phelps and Popma, 2000). Besides,

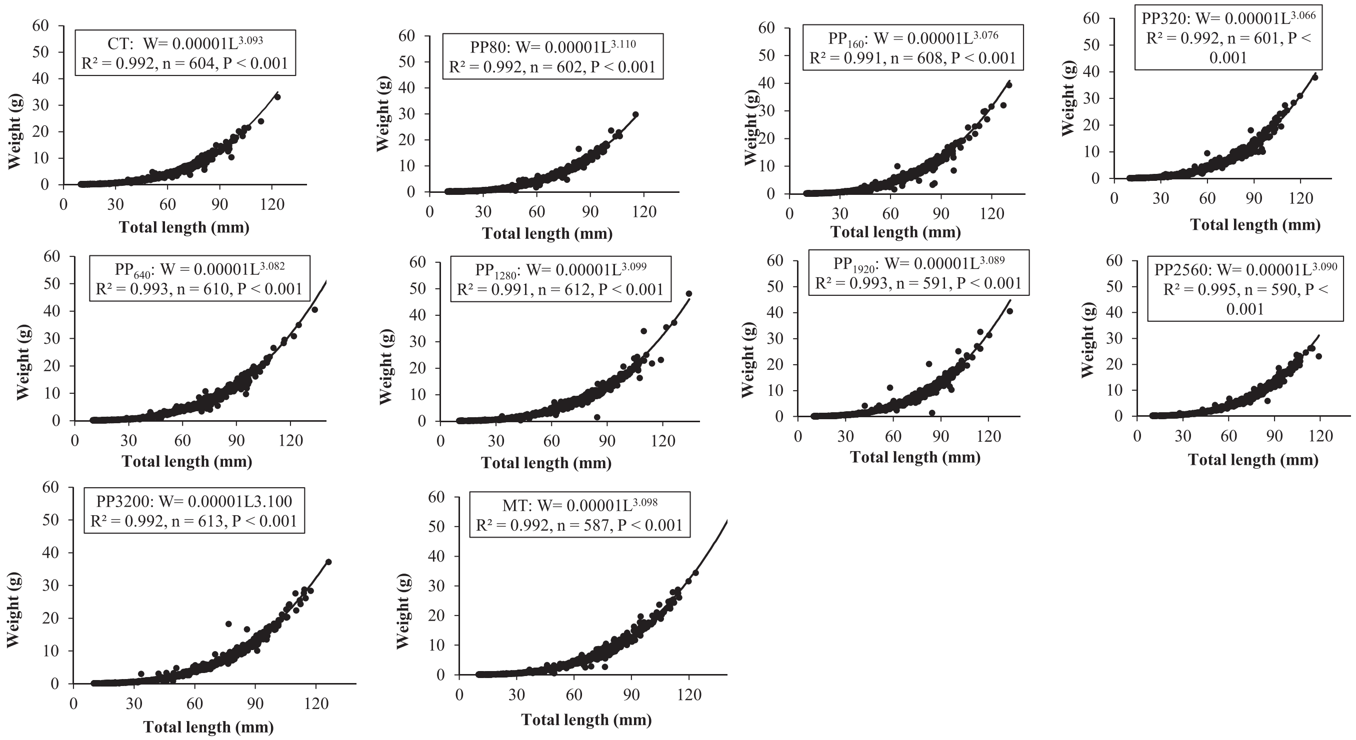

Fig. 4. Relationship between weight (g) and total length (mm) of Nile tilapia fed diets supplemented with varying levels of pine pollen (PP): 0.08, 0.16, 0.32, 0.64, 1.28, 1.92, 2.56 and 3.20 g PP kg^{-1} diet for PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560 and PP3200 respectively; 0.06 g 17α-methyltestosterone kg^{-1} diet (MT); or a basal diet only (CT), for 28 days, and thereafter only basal diet for 84 days.

α-alkylation minimizes the aromatization of the MT to estrogens (Attardi et al., 2008; Fragkaki et al., 2009; Mor et al., 2001). These scenarios could explain the observed differences in the proportions of male individuals between the MT and PP-treated fish. Notably, while higher doses of PP were expected to increase serum T levels in fish and consequently stimulate considerable female-to-male sex change, opposite results were obtained. Related results were obtained while masculinizing Zebra fish (*Danio rerio*) with extracts from *T. terrestris* extract (Gharaei et al., 2020). Testosterone is more prone to aromatization, which results in estrogens that bind to estrogen receptors hence eliciting feminization effects. In the present study, higher doses of PP could have produced more T, which catalyzed aromatization as a substrate (Attardi et al., 2008; Pawlowski et al., 2004).

Regarding the effectiveness of PP in masculinizing Nile tilapia, the gonads of fish treated with 1.28 g PP kg^{-1} of diet were histologically examined since a maximum proportion of males was obtained based on the acetocarmine squash approach. To compare the level of masculinization, the gonads of fish from MT treatment were also analyzed. The testes were fully differentiated with various stages of spermatogenesis, i. e., spermatocytes, spermatids, and spermatozoa. No ovarian tissues were observed in both treatments, which suggests complete masculinization. A fully differentiated testis of male Nile tilapia contains spermatogenic cysts at all stages of spermatogenesis, including spermatogonia, spermatocytes, spermatids, and finally, spermatozoa (Tokalov and Gutzeit, 2005). Therefore, the testicular characteristics observed in both MT and PP1280 indicate successful masculinization at a mature stage of spermatogenesis (Martinez Chavez et al., 2021), which is evidence that PP masculinizes Nile tilapia. Since sex differentiation in Nile tilapia occurs between 21 and 35 dph (Ijiri et al., 2008; Kobayashi et al., 2013; Melo et al., 2019; Nakamura et al., 1998), it can be concluded that by 114 dph, all the sex inversed germ cells were fully differentiated, and the direction of gonadal development determined.

A suitable natural alternative to MT for the production of all-male fish population should also provide desirable growth effects. In this regard, the present study analyzed the effects of PP on the growth performance of Nile tilapia. Overall, PP demonstrated a positive effect on the growth performance of this fish in a dose-dependent manner. The study was cognizant of the effects of water quality on fish growth and thus maintained the critical water parameters within the optimal range (Mjoun et al., 2010) for all treatment groups during the experimental period. The obtained growth-promoting effect of PP was consistent with the findings of (Nian et al., 2017) and (Nieves, 2017), that observed improved weight gain, SGR, and FCR of Nile tilapia fed PP-treated diets. Similar findings were obtained in African catfish (Adenigba et al., 2017) and milkfish (*Chanos chanos*) (Baldove et al., 2019) fed diets supplemented with PP, and Common carp (*Cyprinus carpio*) fed diets supplemented with peony pollen (Ren et al., 2021). A possible hypothesis that explains the ability of PP to augment the growth performance of fish is the presence of growth-promoting compounds, i.e., testosterone and androsterone in PP, which are anabolic-androgenic steroids, and hence stimulate growth by increasing muscle mass (Albano et al., 2021; Turan and Akyurt, 2005). Besides, the bioactive compounds in plant extracts are vital in promoting stress tolerance and boosting the immunity of the fish (Elabd et al., 2022; van Doan et al., 2019, 2018). For PP, the presence of anti-oxidants and polyphenolics could also have enhanced the growth promotion of fish in the present study (Baldove et al., 2019; Baluran et al., 2018; Velasco et al., 2018). Nile tilapia also exhibits sexual growth dimorphism in favor of male individuals (Yue et al., 2018). The male tilapia individuals exhibit greater growth potential, with less metabolic energy allocated towards reproduction (Beardmore et al., 2001; Chavez-Garcia et al., 2020). In addition, unlike females, males also benefit from the androgens in PP and MT, which enhance anabolism (Turan and Akyurt, 2005; Yue et al., 2018). As such, the superior weight gain and specific growth rate in both PP and MT-treated groups could also be partly attributed to the highest proportion of male individuals in these treatments. Notably, the higher growth performance in both MT and PP treatments coincided with the lower FCR values. Since FCR is an indicator of enhanced feed utilization, with the fish effectively converting the consumed feed into body growth (Abaho et al., 2020), lower FCR values are desired in aquaculture.

Condition factor assesses the robustness and the well-being of the fish. As such, the length-weight relationships were used in the present study to understand the condition of the experimental fish. Fish is considered to be in good and poor growth condition when the relative condition factor (K_n) is ≥ 1 and < 1, respectively (Abaho et al., 2020; Froese, 2006; Ighwela et al., 2011). In the present study, the relative condition of all the experimental fish was above one, suggesting good growth. The condition factor of cultured fish depicts the environmental parameters of the rearing system, as well as the level of management (Araneda et al., 2008). Therefore, the ideal water quality parameters and diet maintained during the experiment could also have been responsible for the good condition of the fish. This fish condition conforms to the positive allometric growth pattern ($b > 3$) observed among all treatment groups, with a positive relationship between weight and length of fish as evidenced by higher correlation coefficient values ($R^2 = 0.99$) from the LWR regression equations (Alhassan et al., 2015). The higher b values suggest that fish increase in weight at a faster rate in relation to body length, hence becoming heavier as they increase in length (Ahmed et al., 2011; Datta et al., 2013), an attribute ideal for aquaculture.

Comparable survival rates were observed across the treatment groups in the present study. Notably, no adverse effects were observed in Nile tilapia whose diets were supplemented with PP, contrary to the fact that some plant extracts can result in deleterious effects on the physiological processes in the animals, including fish (Ayotunde and Ofem, 2008). As such, the better survival rates observed in PP-treated groups could be associated with the immunostimulatory effect of PP on fish (Baldove et al., 2019; Baluran et al., 2018). Besides, during this study, optimal water parameters for the culture of Nile tilapia were maintained across the treatments, further promoting better survival of the fish. Otherwise, non-ideal water parameters are very detrimental to fish and often result in high larval and juvenile mortalities in hatcheries and, subsequently, significant economic losses (Abu-Elala et al., 2021).

5. Conclusion

Dietary inclusion of PP has a masculinization effect on Nile tilapia. This implies that PP is a potential alternative to MT hormone in the sex inversion of Nile tilapia towards controlling unwanted reproduction in tilapia culture systems. Besides, PP offers better growth performance, survival rate, and feed utilization, which are vital for the success of an aquaculture enterprise. However, further studies with all-female Nile tilapia progeny are required to precisely describe its effectiveness in inducing masculinization. Additionally, analysis of the functional mechanisms responsible for the androgenic potency of PP in Nile tilapia is paramount. Also, to get detailed information on the effect of PP on the growth of fish, comparing the PP-treated individuals with non-treated ones based on sex is necessary. Finally, hematological and biochemical studies to further understand the effect of PP on fish health are necessary before recommending the product for large-scale commercial application.

CRediT authorship contribution statement

Ivan Abaho: Formal analysis, Writing – original draft, Writing – review & editing, **Peter Akoll:** Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition, **Clifford L.W. Jones:** Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition, **Charles Masembe:** Methodology, Investigation, Writing – review & editing, Supervision, Funding acquisition. All authors read and contributed to the manuscript. The authors have approved the manuscript and agree with the submission to Aquaculture Reports journal.

Declaration of Competing Interest

The authors of this article declare that they have no known competing financial interests or personal relationships that could have influenced any work reported in this paper.

Data availability

The data that support the findings of this study are accessible from the corresponding author upon request.

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