The androgenic and anabolic effects of pine pollen on Nile tilapia (*Oreochromis niloticus*)

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BY

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ABSTRACT

All-male tilapia aquaculture is desirable to control unwanted breeding. Besides, male tilapia individuals grow faster and bigger than females. Presently, most farmers use 17α-methyltestosterone (MT) to produce an all-male stock, although the hormone is associated with human health and environmental risks. Recently, plant-based products have been reported to induce masculinisation in fish and are considered safe nature-based alternatives to MT. The present study utilised pine pollen (PP) to induce female-to-male sex change in Nile tilapia (*Oreochromis niloticus*). Prior to the start of the research, there was insufficient information on the use of PP for sex inversion, with no published data on the sex change mechanism, hence limiting the progress in the application of the product from experimental to hatchery levels.

In this study, the optimal dietary inclusion of PP for maximum masculinisation of Nile tilapia was investigated by feeding three-day-old fish graded PP levels (80, 160, 320, 640, 1,280, 1,920, 2,560 and 3,200 mg kg^{-1} basal diet) from 3 to 30 days post-hatch (dph). This was compared with fish of the same batch fed the same basal diet with no PP (CT; negative control) or the same basal diet supplemented with 60 mg MT kg^{-1} (MT; positive control). To confirm whether the sex change was complete, fish in all treatments were fed only a basal diet for an additional 84 days. The associated differences in the growth of the fish were also determined. Pine pollen and MT significantly skewed the expected 50:50 (male: female) ratio towards more male individuals (Chi-square: $X^2 = 54.396$, df = 9, $P < 0.001$). The 1,280 mg PP kg^{-1} of diet equally induced masculinisation (80.0 ± 2.9 % males) as MT (89.2 ± 2.2 %), and both were significantly higher than 50.8 ± 2.2 % in the CT treatment. In addition to masculinization, dietary inclusion of 1,280 mg PP kg^{-1} improved fish growth, with the specific growth rate significantly higher than fish from the MT and CT treatments (One-way ANOVA:

$F_{(9, 20)}$ =14.196, $P < 0.001$). An increment in the dietary levels of PP from 1,280 to 3,200 mg kg^{-1} further promoted the growth of the fish but did not affect masculinisation.

The mechanism underlying PP-induced sex masculinisation was investigated using all-female Nile tilapia fed a basal diet supplemented with 1,280 mg PP kg^{-1} for 28 days from 3 dph, in comparison with fish fed a basal diet incorporated with 60 mg MT kg^{-1} (MT treatment) or only a basal diet (CT treatment). The expression of sex-related genes (*dmrt1*, *amh*, *cyp19a1a*, and *foxl2*), changes in sex steroid profiles (T: testosterone, 11-KT: 11-ketotestosterone, and E2: 17β-estradiol), and gonadal histology were analysed. Gene expression and sex steroid concentrations were significantly influenced by the interaction between dietary treatment and time, with the expression changing differently over time among the treatments (RM-ANOVA: $P < 0.001$). Pine pollen significantly up-regulated the expression of *dmrt1* and *amh*, while *cyp19a1a* and *foxl2* were down-regulated. Corresponding to male sex gene up-regulation, male-based steroids (11-KT and T) levels were also significantly amplified in both PP and MT-treated fish. The gene expression pattern and changes in sex steroids corresponded to a higher proportion of male individuals obtained in the MT and PP treatments (MT: 97.8 ± 1.1 % and PP: 77.8 ± 2.9 % males), implying female-to-male sex change induction. Subsequently, spermatogonia and spermatocytes were the dominant germ cells in the histological sections of the gonads obtained from the PP-treated fish. At the same time, the individuals from the MT treatment exhibited mainly spermatids and spermatozoa. In contrast, all the fish from the CT treatment remained females, having only ovarian tissues.

This thesis confirmed that PP induces female-to-male sex change in Nile tilapia and enhances fish growth. The research contributed novel information on the mechanism underlying PP-

induced sex change, which included disrupting the expression of sex genes and the androgen-to-estrogen balance, ultimately determining the sexual fate of the fish. The findings provide a foundation for understanding the role of PP in masculinisation, with broad potential application in the aquaculture industry.

DECLARATION

I, **Ivan Abaho,** hereby declare that this thesis for the degree of Doctor of Philosophy of Rhodes University is my original work and that it has not been, previously in its entirety or in part, submitted for a degree in any other academic institution. All information therein from other sources has been appropriately acknowledged.

Date: **01st August 2023**

Signed: ……

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DEDICATION

To

My dearest parents, Tushabe Colonel and Kyomugisha Provia; thank you for your support towards achieving my grandest dream.

And

My lovely spouse Leticia Komugisha Abaho, our sons: Travis and Taylor, and daughters: Theresa and Tricia; I salute you for being my continuous motivation in life.

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ETHICS APPROVAL STATEMENT

All experimental procedures presented in this thesis were conducted in accordance with the ethical guidelines for animal care and use in research. They were approved by the Animal Research Ethics Committee of Rhodes University (RU-AREC), South Africa, under approval number: 2019-0792-973. All efforts were made to minimise stressing the fish during handling.

LIST OF PUBLICATIONS DERIVED FROM THIS THESIS

This thesis is partly based on published articles and proceedings from conferences described below:

Peer-reviewed journal articles

Chapter 1 of this thesis was published as: Abaho I, Masembe C, Akoll P, Jones CLW (2022). The use of plant extracts to control tilapia reproduction: Current status and future perspectives. *Journal of the World Aquaculture Society* 53: 593–619.

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Conference presentations

Abaho I, Jones CLW, Masembe C, Akoll P (2022). Phytoandrogens in pine pollen – Nature's way of altering sex ratio in Nile tilapia (*Oreochromis niloticus*). Conference organised by Aquaculture association of Southern Africa from 11 – 15 July 2022, Stellenbosch, South Africa (Oral presentation).

Abaho I, Akoll P, Jones CLW, Masembe C (2022). Harnessing the androgenic potential of pine pollen to produce all-male Nile tilapia (*Oreochromis niloticus*). Conference organised by the centre for postgraduate studies from 01 – 02 September 2022, Rhodes University, South Africa (Oral presentation).

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ABBREVIATIONS AND ACRONYMS

11-KT	11-Ketotestosterone
amh	Anti-mullerian hormone
ANOVA	Analysis of variance
Bp	Base pair
cDNA	complementary Deoxyribonucleic Acid
CF	Condition factor
Ct	Cycle threshold
cyp19a1a	Cytochrome P450, family 19, subfamily A, polypeptide 1a
Df	Degree of freedom
dmrt1	Doublesex and mab-3 related transcription factor 1
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
Dph	Day post-hatch
E2	17β-estradiol
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation
FCR	Feed conversion ratio
foxl2	Forkhead box protein L2
FBW	Final mean body weight

IBW	Initial mean body weight
RM-ANOVA	Repeated measures analysis of variance
mRNA	messenger ribonucleic acid
MT	17α-methyltestosterone hormone
MS-222	Tricaine methanesulfonate
MUZARDI	Mukono Zonal Agricultural Research and Development Institute
NARO	National Agricultural Research Organisation
PCR	Polymerase chain reaction
pH	Molar concentration of hydrogen ions
PP	Pine pollen
RNA	Ribonucleic acid
SD	Standard deviation
SE	Standard error
SGR	Specific growth rate
T	Testosterone
Tm	Melting temperature
Tukey's HSD	Tukey's honest significant difference
UK	United Kingdom
USA	United States of America

CHAPTER 1[1]

General introduction and literature review

There are increasing concerns over using chemicals in aquaculture and a shift in preference for organically produced fish products. For instance, the utilisation of a synthetic chemical, "17α-methyltestosterone hormone (MT)", to produce all-male progeny as a control technique for unwanted spawning in Nile tilapia (*Oreochromis niloticus*) aquaculture has adverse effects on human health and the aquatic environment (Velazquez and Alter 2004, Leet *et al.* 2011, Rivero-Wendt *et al.* 2013, Megbowon and Mojekwu 2014, Rivero-Wendt *et al.* 2020). The all-male populations are preferred because male phenotypes exhibit faster growth than the female and mixed-sex individuals (Beardmore *et al.* 2001, Toguyeni *et al.* 2002, Srisakultiew and Kamonrat 2013, Yue *et al.* 2018, Baroiller and D'Cotta 2019, Chavez-Garcia *et al.* 2020). As a result, research efforts have shifted focus to producing all-male stock using naturally occurring plant compounds since they are cheap, safe, and environmentally sustainable. The phytocompounds have proven capacity to induce sex masculinisation in fish (Gabriel *et al.* 2017, Gabriel 2019, Hasan *et al.* 2021, Abaho *et al.* 2022b, Aziz *et al.* 2022, Mansour *et al.* 2022). Among the plant-based products with the potential to masculinise Nile tilapia is pine pollen (PP) powder (Nian *et al.* 2017; Aziz et al. 2022). However, inadequate information is available on the optimal quantities of PP required for permanent female-to-male sex change as well as the molecular and endocrine regulations responsible for the induction of masculinisation. Therefore, the present study assessed the effect of PP on the sex ratio and growth of Nile tilapia. In addition, the changes in the expression of sex-specific genes, sex

[1]Sections 1.2 and 1.3 of the chapter were published as a review paper: Abaho I, Masembe C, Akoll P, Jones CLW 2022. The use of plant extracts to control tilapia reproduction: Current status and future perspectives. *Journal of the World Aquaculture Society* 53: 593–619.

steroids, and gonadal histology were examined to understand the mechanism underlying PP- induced sex inversion of Nile tilapia. The information generated during the study contributes to fine-tuning the utilisation of PP as a potential alternative to MT in producing all-male tilapia individuals towards availing safe and ecologically sustainable fish products.

1.1 Overview of global aquaculture

Aquaculture involves farming fish, crustaceans, bivalves, and seaweeds (Cai and Leung 2017). It started as an Asian freshwater fish production system but was later adopted in other continents (Subasinghe *et al.* 2009). In 2020, aquaculture contributed 57.3 % (approximately 122.6 million tonnes) to the global production of aquatic products, comprising 87.5 and 35.1 million tonnes of all farmed animals and plants, respectively (FAO 2022). The fish farming practice has continued to grow faster than other food-producing and income-generating sectors (Naylor *et al.* 2021, FAO 2022, Mapfumo 2022). The rapid growth in the aquaculture industry is accelerated by: 1) improvement in culture technologies; 2) continuous expansion in the production areas; and 3) development and implementation of aquaculture-centred policies (Basha *et al.* 2013, Adeleke *et al.* 2021, Naylor *et al.* 2021). Therefore, the sector presents a viable option to meet the increasing gap between the demand and supply of fish arising from the stagnation of capture fishery production and the rapidly growing human population. With the global human population projected to reach 9.8 billion in 2050, production must increase by 20 to 70 % by 2050 to allow adequate food supply (Hunter *et al.* 2017, United Nations 2017). Aquaculture is envisioned as one of the critical sectors that will significantly contribute to meeting the populace's food, nutrition, and income demands(Ahmed *et al.* 2019, FAO 2022).

Although fish aquaculture production has increased over time, the expansion is skewed on a regional basis, with Asia as the major producer of farmed fish. In 2020, Asia met 88.43 % of the total global fish production, followed by the Americas at 5.00 %, while Africa contributed 2.57 % (Figure 1.1). The aquaculture output from the African continent is still meagre, with Egypt accounting for 70.74 % of the total fish production, followed by Nigeria at 11.63 %, and the remaining 17.63 % produced from the rest of the countries (FAO 2022). Despite the availability of immense land, water, genetic, and feed resources, aquaculture growth in Africa is hampered by: 1) limited improved fish strains; 2) limited access to quality feed due to high prices; 3) limited expertise and infrastructure for research and market; and 4) weak governance and regulations (Adeleke *et al.* 2021, Shikuku *et al.* 2021, Mapfumo 2022).

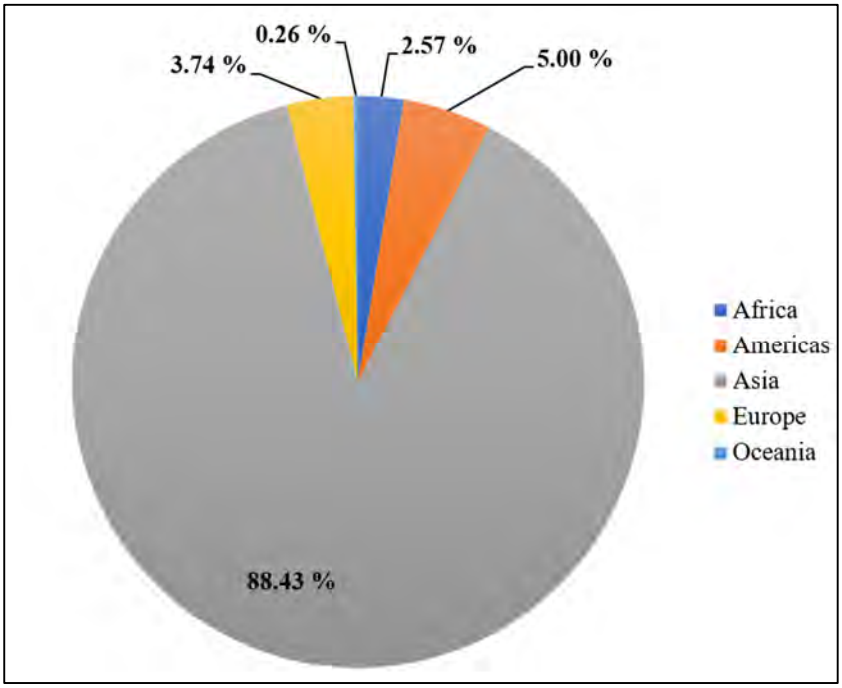

Figure 1.1: Global contribution to aquaculture fish production by region (FAO 2022).

The upward growth trajectory of aquaculture has adverse impacts on the ecosystem, including habitat destruction, water pollution, eutrophication, disease outbreaks, and biotic depletion

(Naylor *et al.* 2000, 2021, Hall *et al.* 2011, Waite *et al.* 2014), that threaten the sustainability of the industry. Therefore, environmentally sustainable and socially sound management practices focusing on preserving aquatic ecosystem health, protecting biodiversity, and preventing pollution must be adopted to ensure long-term aquaculture growth (Ahmed and Thompson 2019, Naylor *et al.* 2021). Such practices comprise the investment in: a) organic aquaculture to reduce the use of chemicals and drugs; b) increasing feed digestibility and bioavailability to minimise wastage; c) recirculating aquaculture systems and aquaponics to conserve water; and d) integrated multi-trophic aquaculture (Ahmed *et al.* 2019, Marta Correia *et al.* 2020, FAO 2022).

1.2 Tilapia aquaculture

Tilapia are freshwater cichlids native to Africa (Tibihika *et al.* 2018, Lind *et al.* 2019) but have been introduced into several parts of the world and are reared in over 120 countries (Cai *et al.* 2018). In 2020, the species contributed 11.2 % of the total freshwater-farmed fish production, rising from 1.13 in 2000 to 5.48 million tonnes in 2020. As such, the tilapia species are among the most important aquaculture species of the 21st century (FAO 2022). In developing countries, tilapia is one of the primary animal protein sources, contributing to food, nutrition, and income security (Fitzsimmons 2017, FAO 2020, Mapfumo 2022).

The tilapia species exhibit desirable attributes such as: 1) ease to breed in captivity; 2) short production cycle due to fast growth rate; 3) tolerance to wide environmental conditions (such as temperature, salinity and high density); 4) feed on low trophic levels, with very versatile food habits and acceptability of artificial feeds after yolk-sac absorption; 5) adaptability to various culture systems and practices (extensive to intensive systems and monoculture to

polyculture); and 6) marketable (Watanabe *et al.* 2002, Gupta and Acosta 2004, El-Sayed 2006, 2019, Baroiller and D'Cotta 2019, Prabu *et al.* 2019). Further, technological advances associated with the intensification of cultural practices, including the development of new tilapia strains and hybrids, all-male culture, quality fish-formulated feeds, adoption of semi-intensive and intensive culture systems (ponds, cages, tanks, and raceways), and advanced water treatment methods, have accelerated adoption and growth of tilapia aquaculture (El-Sayed 2019, Prabu *et al.* 2019). The ideal biological, economic and technological characteristics have contributed to the exponential increase in global tilapia production (Figure 1.2; Yue *et al.* 2016; Fitzsimmons 2016).

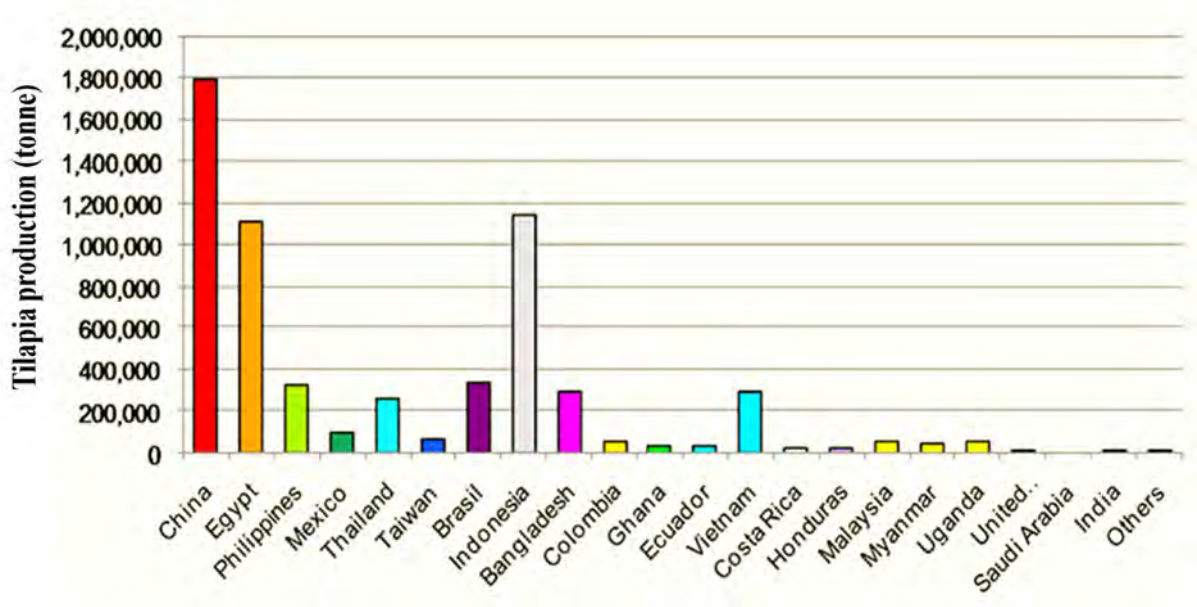

Figure 1.2: Global tilapia production in 2016 by country (Fitzsimmons 2016).

1.3 Culture of Nile tilapia

Amongst the tilapines, Nile tilapia has superior aquaculture attributes and is thus the most widely farmed on all continents except Antarctica (Zambrano *et al.* 2006). Nile tilapia is produced from extensive backyard ponds to large-scale commercial culture facilities (Gupta

and Acosta 2004, Wang and Lu 2016, Yue *et al.* 2016, FAO 2020). In this fish, reproduction involves maternal mouth brooding of fertilised eggs that hatch within 70 - 90 h of incubation. Thereafter, the female mouth broods the larvae and gives parental care until the swim-up fry stage, which lasts between 6 and 10 days. The fry can now ingest natural or artificial feeds, move easily in the surrounding waters, and do not need parental care (de Graaf *et al.* 1999, Hussain 2004, El-Sayed 2019). During incubation, the female individuals suspend feeding, resulting in a reduced growth rate (Budd *et al.* 2015, Baroiller and D'Cotta 2019). As such, males are preferred for aquaculture. Besides, under farming conditions, Nile tilapia attains sexual maturity within 30 - 50 g before reaching harvestable size (de Graaf *et al.* 1999, Baroiller and Toguyeni 2004 Abdel-Tawwab 2005). In addition, the female individuals produce fry continuously, in monthly batches, resulting in overpopulation of the culture units. Therefore, the precocious maturation and prolific spawning under farming conditions lead to competition for resources and crowding, which induces stress, consequently retarding growth (Teichert-Coddington *et al.* 2000, Toguyeni *et al.* 2002, Ahmed *et al.* 2007, Srisakultiew and Kamonrat 2013, Baroiller and D'Cotta 2019, Opiyo *et al.* 2021). Therefore, controlling prolific spawning is essential to improving economic returns from tilapia production enterprises.

1.3.1 Reproduction control in Nile tilapia culture systems

Numerous interventions including: 1) periodic harvesting of fry and fingerlings; 2) high-density culture; 3) cage culture through disruption of the breeding cycle as fish are unable to access the natural breeding grounds; 4) polyculture with the predator fish; 5) sterilisation by application of heat shock; and 6) adoption of all-male culture, are applied to minimise the unwanted reproduction in the production systems (Mair and Little 1991, Phelps and Popma 2000, Fortes 2005). Currently, all-male tilapia culture is desirable because male individuals have minimal energy loss to reproduction and gain more weight than females. Thus, the all-

male populations result in a shortened production cycle, with reduced size variation at harvest and are hence more productive than female and mixed-sex individuals (Baroiller and Toguyeni 2004, Angienda *et al.* 2010, El-Greisy and El-Gamal 2012, Baroiller and D'Cotta 2019, Chavez-Garcia *et al.* 2020, Snake *et al.* 2020).

1.3.2 All-male tilapia production

The techniques utilised to produce Nile tilapia progeny with a high proportion of male individuals include: 1) sex-sorting by hand, 2) hybridisation, 3) environmental manipulation (such as temperature treatment), 3) genetic/chromosomal manipulation, and 4) hormonal sex change using androgens (Mair and Little 1991, Vera Cruz and Mair 1994, Mair *et al.* 1997, Abucay *et al.* 1999, Gale *et al.* 1999, Beardmore *et al.* 2001, Desprez *et al.* 2003, Abad *et al.* 2007, Dauda *et al.* 2014, Linda 2019, Snake *et al.* 2020, Abaho *et al.* 2022b).

Sex-sorting by hand

Sex sorting by hand involves separating males from females manually before sexual maturation and spawning. The technique is based on a visual examination of the urogenital papillae of each fish. The male genital papilla is protruding and larger with two openings: the urogenital opening (excreting urine and releasing milt) and the anus. The females' genital papilla is flat and smaller with three openings; the anus, the urethra, and the oviduct (Guerrero and Shelton 1974, Hussain 2004, Budd *et al.* 2015, Rahma *et al.* 2015, Baroiller and D'Cotta 2019). Sex-sorting by hand requires sufficient expertise to distinguish between the male and female urogenital papillae, hence the high chances of misidentification. As such, human errors committed during fish sexing process result in mixed-sex populations in the production system. Besides, the method is time-consuming and leads to the wastage of female individuals as they

are discarded, while the males are selected because they grow faster and bigger (Pandian and Varadaraj 1987, Beardmore *et al.* 2001, Fortes 2005, Linda 2019).

Hybridisation

Hybridisation involves mating genetically different individuals, with crossing either within a species or between species (Bartley *et al.* 2000, Beardmore *et al.* 2001, Budd *et al.* 2015, Rahman *et al.* 2019, Snake *et al.* 2020). For example, the crossing of a homogametic XX female Nile tilapia and a homogametic ZZ male blue tilapia (*Oreochromis aureus*) yields mainly male progeny. Also, crossing of female male Nile tilapia and Wami tilapia (*Oreochromis urolepis hornorum*) results in predominately male offspring (Cnaani *et al.* 2008, El-Zaeem and Salam 2013, Baroiller and D'cotta 2019). Besides being utilised for production of all-male offspring, the technique can result in hybrids with superior growth, quality flesh and disease resistance (Bartley *et al.* 2000, Gupta and Acosta 2004, Lozano *et al.* 2014, Rahman *et al.* 2019). As such, hybridisation has led to the adoption of some tilapia hybrids such as blue tilapia x Nile tilapia (Pruginin *et al.* 1975, Chapman 1992) and red tilapia strains from crosses between Mozambique tilapia and Nile tilapia (McAndrew *et al.* 1988). However, the technique is associated with various limitations, such as: a) inability to maintain a parent stock that consistently produces 100% male offspring due to misidentifications and subsequent mixing between hybrids and parental species, resulting in reappearance of females in the progeny; b) limited fecundity of parent fish, which restricts fry production; and c) difficulty in producing a sufficient number of hybrid fry due to spawning incompatibility between parent species (Pruginin *et al.* 1975, Varadaraj and Pandian 1989, Wohlfarth 1994, Baroiller and D'Cotta 2019, Rahman *et al.* 2019).

Environmental manipulation through heat treatment

Nile tilapia is a thermo-sensitive species, and during the critical period of sexual differentiation, the species can shift sex orientation in response to changes in temperature (Poonlaphdecha *et al.* 2011, Nivelle *et al.* 2019, Wang *et al.* 2022). Increasing culture temperature (heat shock) before and during gonadal differentiation results in the masculinisation of the fish, whereas reduced temperature (cold shock) leads to feminisation. An increase in rearing temperatures from 32 to 37 °C, applied from 10 days post-fertilization for 28 days, skewed sex ratios towards males (Baroiller *et al.* 1995, Baras *et al.* 2001, D'Cotta *et al.* 2001, Tessema *et al.* 2006, Rahma *et al.* 2015, Nivelle *et al.* 2019, Habibah *et al.* 2021, Wang *et al.* 2022). Elevated temperatures alter the expression of sex-specific genes, with the up-regulation and down-regulation of male and female-sex genes, respectively, promoting testicular differentiation (Poonlaphdecha *et al.* 2011, 2013, Rahma *et al.* 2015, Zhao *et al.* 2020, Lu *et al.* 2022). The method is environmentally sustainable and does not pose a health hazard to humans. However, the temperature approach for masculinisation in tilapia culture does not result in complete sex change and is capital intensive, limiting commercialisation (Fuentes-silva *et al.* 2013).

Genetic manipulation / YY super male

The production of all-male tilapia using the YY super male technique involves crossing the YY genotypic males with the normal XX females, resulting in all XY male progeny (Mair *et al.* 1997, Baroiller and D'Cotta 2019). The technique combines genetic breeding programme and hormones for feminisation (Beardmore *et al.* 2001, Rowell *et al.* 2002, Ezaz *et al.* 2004). The production of YY super males first involves the production of XY females by sex reversal treatment. The normal XY males are feminized using synthetic estrogens to produce the neo-females (XY). During feminization, a limited number of individuals are needed, and hence low

quantities of hormones are used. The neo-females are mated with normal male genotypes (XY), producing YY genetically male tilapia (Beardmore *et al.* 2001, Ezaz *et al.* 2004, Baroiller and D'cotta 2019). The approach is relatively environmentally sustainable since low quantities of the synthetic hormone in the treatment of broodstock are used (Mair *et al.* 1997). The YY super male technology requires highly skilled personnel and involves a lengthy procedure of producing and identifying putative YY male individuals (Mair and Little 1991, Tessema *et al.* 2006, Baroiller and D'Cotta 2019). Consequently, this technology has not been embraced, especially in developing countries.

Endocrine / hormonal manipulation

Endocrine manipulation involves treating fish with exogenous sex steroids to produce the desired fish sex. In Nile tilapia, the embryonic gonad is bi-potential and can differentiate into either the ovary or testis during the labile period of sex differentiation (Yamamoto 1969, Hunter and Donaldson 1983, Fitzpatrick *et al.* 1993, Budd *et al.* 2015). Besides, the fish exhibits sexual plasticity that allows for the alteration of gonadal differentiation to either the female or male sex, even in the presence of the genetic material responsible for sex determination (Kobayashi *et al.* 2013, Li *et al.* 2022). As such, exposure to exogenous steroids does not alter the genotypic sex of the fish but re-directs the differentiation of phenotype sex. This knowledge led to the development of synthetic androgens to produce all-male individuals in tilapia aquaculture (Yamazaki 1983, Mair and Little 1991, Gale *et al.* 1999, Pandian and Kirankumar 2003, Biswas *et al.* 2005, Budd *et al.* 2015, Baroiller and D'Cotta 2019, El-Sayed 2019, Snake *et al.* 2020). The common androgens used for sex masculinisation of Nile tilapia include 17α-methyltestosterone (MT), 17α-methyldihydrotestosterone, 17β-trenbolone, mibolerone and mesterolone (Yamazaki 1983, Mair and Little 1991, Pandian and Sheela 1995, Biswas *et al.* 2005, Srisakultiew and Kamonrat 2013, Rahma *et al.* 2015, El-Sayed 2019). The exogenous

steroids are easy to apply and often yield consistent results, and hence are commonly utilised for the commercial production of all-male tilapia fry (Phelps and Popma 2000, Phelps 2006, Budd *et al.* 2015, Baroiller and D'Cotta 2019). The androgenic steroids are preferred for female-to-male sex inversion because they also promote anabolic activity; hence fish grow faster, yielding higher weight at harvest (Rothbard *et al.* 1990, Yue *et al.* 2018). The growth-promoting effect of the androgens is attributed to the ability to elevate growth hormone metabolism and insulin-like growth factors (Yue *et al.* 2018a). The MT steroid is widely used in the commercial production of tilapia, with masculinisation success ranging from 90 to 95%, at a dose of 60 mg kg^{-1} feed (Phelps and Popma 2000, Beardmore *et al.* 2001, Homklin *et al.* 2011, El-Greisy and El-Gamal 2012, Vinarukwong *et al.* 2018, Baroiller and D'Cotta 2019, El-Sayed 2019, Karaket *et al.* 2023).

The androgens are administered during the first 30 days post-hatch (dph) (Mateen and Ahmed 2007, Kobayashi *et al.* 2008, Nivelle *et al.* 2019) to trigger testicular differentiation and stimulate precocious spermatogenesis along with phenotypic and behavioural masculinisation (Nakamura and Nagahama 1985, Nakamura 2010, Zhao *et al.* 2019). The routes of administration of androgens include a) incorporation in fish feeds (oral method), or b) immersion or c) intraperitoneal injection (Devlin and Nagahama 2002, Phelps 2006, El-Greisy and El-Gamal 2012, Gabriel 2019). Oral administration involving the dissolution of steroids in alcohol and thereafter mixing with the diet is the predominant method (Phelps and Popma 2000). The method is preferred because of the low cost of application, potential to treat large numbers of fish, causes no stress or reduced stress, and yields at least 95 % masculinised individuals (Devlin and Nagahama 2002, Phelps 2006, El-Greisy and El-Gamal 2012, Budd *et al.* 2015, Gabriel 2019, Abaho *et al.* 2022b). Nonetheless, the non-uniform distribution of the

hormone in the diets results in variations in the concentration of sex steroids available to the fish.

The immersion technique of steroid application is crucial in fish species whose gonadal labile period occurs before the first feeding (Devlin and Nagahama 2002). In Nile tilapia, fry ingests exogenous diets during the gonadal differentiation period (Mateen and Ahmed 2007, Kobayashi *et al.* 2008, Nivelle *et al.* 2019). As such, the masculinisation by immersion technique has been utilised, yielding up to 90% male individuals (Gale *et al.* 1999, Karaket *et al.* 2023). However, the method requires mixing the steroid with culture water at each exposure time, which results in the wastage of the masculinisation agent and a high rate of release into the environment.

Intraperitoneal injection of fish is efficient and rapid, necessitated by quick absorption of the steroids (Reverter *et al.* 2014). However, the process of injecting fish is expensive, laborious, stressful to fish, and impractical for small fish, i.e., less than 15 g $fish^{-1}$ (Sakai 1999, Beardmore *et al.* 2001, Reverter *et al.* 2014). Besides, the method requires technical skills to avoid inflicting damage on the fish (Hoga *et al.* 2018). As such, the injection method is rarely utilised to administer sex inversion agents in tilapia.

1.3.3 Sex genes and steroids in sex determination and differentiation of Nile tilapia

Sexual determination involves initial events ascertaining the ability of the vertebrates' gonads to develop either as testes or ovaries (Capel 2017). In fish, the genetic sex determination with sex chromosomes XX/XY or ZW/ZZ and environmental sex determination systems exist (Devlin and Nagahama 2002, Schartl 2004, Capel 2017, Nagahama *et al.* 2021, Rajendiran *et*

al. 2021). In either sex determination system, genes are the primary factors which control the initial decision for sex differentiation. Therefore, sex determination is a master switch to the bipotential gonad, which triggers the sex differentiation cascade to take over the process of gonadal development, ultimately producing either the male or female sex phenotype (Rajendiran *et al.* 2021). The sex differentiation process is modulated by various genes, which produce hormones, enzymes, and transcription factors responsible for either male or female sex differentiation. The sex-determining genes act as upstream factors and control the downstream sex-differentiating genes to promote the appropriate sexual phenotype (Figure 1.3).

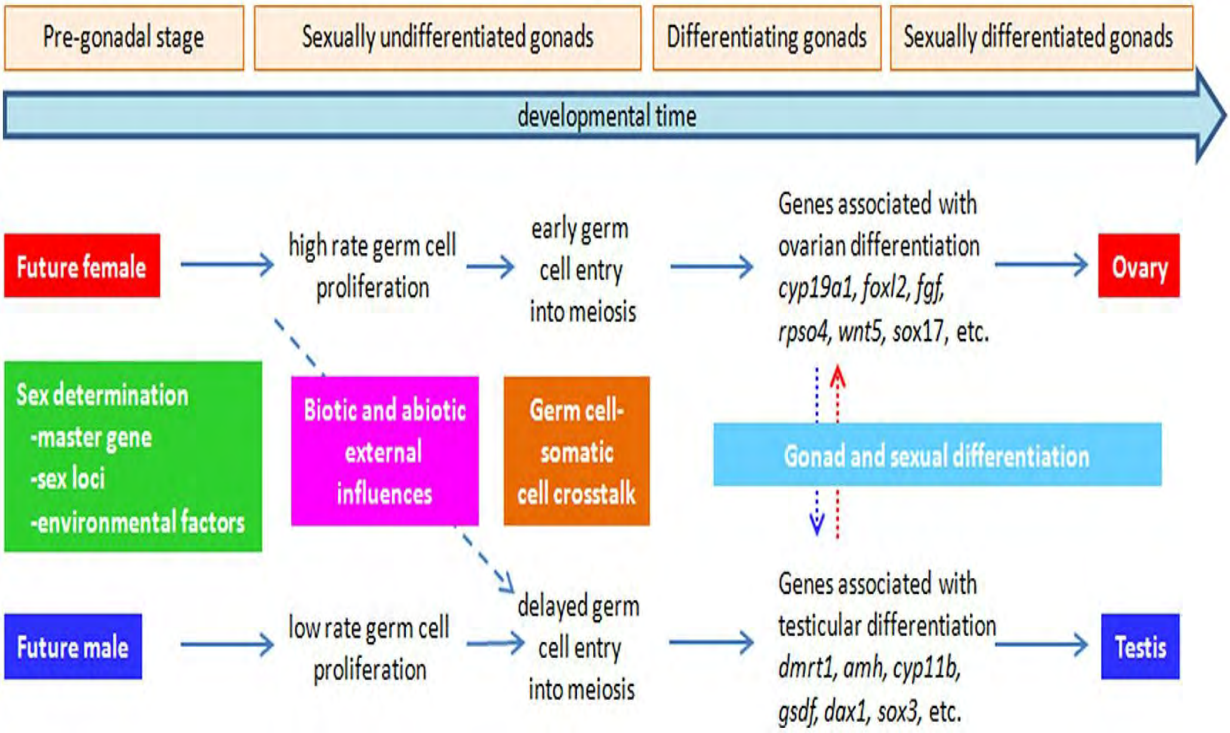

Figure 1.3: Major events leading to ovarian and testicular differentiation (Martinez *et al.* 2014)[2].

[2] *cyp19a1*: cytochrome P450, family 19, subfamily A; *foxl2*: forkhead transcriptional factor 2; *fgf*: fibroblast growth factor; *rpso4*: R-spondin 4; *wnt5*: Wnt family member 5; *sox17*: SRY-box transcription factor 17; *dmrt1*: doublesex/mab-3 related transcription factor 1; *amh*: anti-müllerian hormone; *cyp11b*: cytochrome P450 family 11 subfamily B; *gsdf*: gonadal soma-derived factor; *dax1*: dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; and *sox3*: SRY-box transcription factor 3

Nile tilapia has a XX/XY sex-determining system, and its gonads develop either into ovaries or testes depending on the prevailing intrinsic and extrinsic factors, and sexual orientation is maintained throughout the lifespan (Ijiri *et al.* 2008). During the gonadal development process, the germinal ridge is initially formed, followed by the appearance of the gonads in the dorsal mesentery (Devlin and Nagahama 2002). Within three days post-hatch primordial germ cells are formed. Sexual dimorphism is established at nine days post-hatch with the formation of either intra-testicular efferent ducts in males or the ovarian cavity in females. Finally, the first meiosis occurs at 35 dph in females and at least 50 dph in males (Kobayashi 2010). Overall, sex differentiation in Nile tilapia occurs within 20 dph in females with ovarian cavity formation and within 25 dph after testicular differentiation, indicated by the development of intra-testicular efferent ducts in males (Kobayashi *et al.* 2013).

Like in other teleosts, sex differentiation in Nile tilapia involves differential expression of sex-specific genes in the gonad, namely, *dmrt1*, *amh*, and *gdsf* in the male-dominant sex, and *cyp19a1a* and *foxl2* in females (Ijiri *et al.* 2008, Li *et al.* 2014, Kaneko *et al.* 2015, Teng *et al.* 2020a, Nagahama *et al.* 2021, Lu *et al.* 2022). The expression of the sex-biased genes regulates the sex steroid balance and ultimately determines the fate of the gonad (Kitano *et al.* 2000, Bhandari *et al.* 2006, Kobayashi *et al.* 2008, Golan and Levavi-Sivan 2014, Gennotte *et al.* 2015, Banh *et al.* 2017, Rajakumar and Senthilkumaran 2020, Bhat *et al.* 2021). The primary steroids in Nile tilapia are 17β-estradiol (E2) and 11-ketotestosterone (11-KT), as estrogen and androgen, respectively, which are produced from testosterone (T). The production balance of these steroids controls the orientation of gonadal differentiation and development of the fish (Wang and Orban 2007, Guiguen *et al.* 2010, Wang *et al.* 2017a, Aleksandr and Antonina 2019).

The sex differentiating genes are susceptible to extrinsic factors and act antagonistically, with the prevailing network determining the direction of sex development (Graves and Peichel 2010, Mcclelland *et al.* 2012, Lau *et al.* 2016, Todd *et al.* 2016, Ortega-Recalde *et al.* 2020). Therefore, the ultimate phenotypic sex is a result of the intrinsic factors (genetic sex) coupled with extrinsic factors (environment) influence (Hayes 1998, Devlin and Nagahama 2002, Brown *et al.* 2014, Sun *et al.* 2018a, Hayasaka *et al.* 2019, Li *et al.* 2019, Imiuwa 2020, Yan *et al.* 2021, Zhou *et al.* 2021, Sharma *et al.* 2022). For example, once the fish is exposed to sex steroids, the initially genetically pre-programmed sex determination mechanism is overridden, hence re-directing the phenotypic sex differentiation (Kitano *et al.* 2000, Kobayashi *et al.* 2008, Palaiokostas *et al.* 2013, Wessels *et al.* 2014, Gennotte *et al.* 2015, Banh *et al.* 2017, Nivelle *et al.* 2019, Habibah *et al.* 2021, Lu *et al.* 2022). Successful sex change has been reported once the steroids are administered during the sensitive period (3 - 30 dph) in which the ovarian cavity or the intratesticular efferent ducts are formed (Devlin and Nagahama 2002, Bhandari *et al.* 2006, Kobayashi *et al.* 2008, Kobayashi *et al.* 2013, Habibah *et al.* 2021, Li *et al.* 2022). Therefore, the development of functional gonads specific to the desired sex can be accomplished even in the presence of the genetic material which determines sex (El-Sayed *et al.* 2012, Gennotte *et al.* 2014, Golan and Levavi-Sivan 2014, Chen *et al.* 2016, Sun *et al.* 2018a).

The exogenous steroids mimic the endogenous sex hormones (E2, T, and 11-KT) to override the natural process of sex differentiation by acting on mechanisms of gonad morphogenesis. For example, androgen treatment suppresses the female sex genes, notably *cyp19a1a*, *foxl2*, and *wnt5*, while male-biased genes, including *sox9*, *dmrt1*, *amh* and *gsdf* are up-regulated (Mei and Gui 2015, Chen *et al.* 2016). In Nile tilapia, MT exposure resulted in upregulation of *dmrt1* and *amh* directing testicular development (Kobayashi *et al.* 2003, 2008, Tao *et al.* 2013, Chen

et al. 2016). In contrast, the administration of estrogen 17α-ethynylestradiol (EE2) to tilapia fry induced ovarian differentiation after down and up-regulating *dmrt1* and *cyp19a1a*, respectively (Kobayashi *et al.* 2003, 2008). The alteration in gene expression disrupts the endocrine system triggering the development of the targeted sex (Denslow and Sepulveda 2007, Guiguen *et al.* 2010, Leet *et al.* 2011, Segner *et al.* 2013, Golan and Levavi-Sivan 2014). The exposure of fish to androgens leads to the conversion of T to 11-KT, resulting in testicular differentiation, while estrogen treatment results in the conversion of T to E2, promoting ovarian differentiation (Guiguen *et al.* 1999, Devlin and Nagahama 2002, Uchida *et al.* 2004). Upon sex differentiation, the exogenous hormones continue regulating the secondary characteristics, including sexual behaviour in fish and maintenance of sexual phenotype (Melo *et al.* 2015, Rajakumar and Senthilkumaran 2015, Shi *et al.* 2017).

1.3.4 Human health and environmental hazards associated with the use of synthetic steroids in tilapia aquaculture

Aquaculture contributes to the elevation of synthetic hormone levels in the aquatic environment through uneaten treated feeds or excretion of partially metabolised steroids (Kolodziej *et al.* 2004, Scott and Ellis 2007, Macintosh 2008, Rivero-Wendt *et al.* 2013, Czarny *et al.* 2017) or leaching into the production system from the treated diets (Beardmore *et al.* 2001, Vick and Hayton 2001, Ramirez-Godinez *et al.* 2013, Neto *et al.* 2021). Synthetic hormones have been detected in water, suspended particles, sediment, feed, and fish faeces from different aquaculture farms (Liu *et al.* 2011, 2017, Wang *et al.* 2012). The release of untreated wastewater, with synthetic steroids, directly into the aquatic environment increases the spread as well as the transformation into toxic products, subsequently compromising the integrity of the ecosystems (Kim *et al.* 2007, Farre *et al.* 2008, Homklin *et al.* 2011, Khalil *et al.* 2011).

Notwithstanding MT, 17β-estradiol (E2), 17α-ethynylestradiol (EE2), glucocorticoids and progestogens pose direct risk to humans through dietary exposure due to low metabolism and persistence in fish tissues after use (Liu *et al.* 2012, 2015, Yang *et al.* 2014). As such, the use of MT in tilapia aquaculture has attracted increasing public criticism due to environmental and human health (Leet *et al.* 2011, Ramirez-Godinez *et al.* 2013, Rivero-Wendt *et al.* 2013, 2016, Chakraborty *et al.* 2014, Mlalila *et al.* 2015, Abo-Al-Ela 2018, Leticia *et al.* 2020, Ghosal *et al.* 2021, Zhang *et al.* 2022). The androgenic MT is carcinogenic; thus, prolonged exposure to the hormone during the application process can cause hepatotoxicity and fetotoxicity (Wilkins 1960, Schardein 1980, Hartleb and Nowak 1990, Vick and Hayton 2001, Velazquez and Alter 2004). In addition, MT can result in menstrual irregularities and atrophy of breasts in women, while impotence and prostatic hypertrophy have been reported in men (Ferner 1994). As such, the hormone poses a health risk to hatchery personnel involved in tilapia seed production (Megbowon and Mojekwu 2014). Besides, 30% of the administered hormone-treated diet is unavailable to the fish during feeding (Vick and Hayton 2001, Ramirez-Godinez *et al.* 2013). Meanwhile, only 10% of the hormone consumed in the diet is utilised for sex inversion (Ziegler and Fanchin 2000, Ong *et al.* 2012). As a result, 90% of the remaining hormone build up in the environments as either active metabolites excreted by the treated fish or leachates from uneaten food (Abucay and Mair 1997, Thanasupsin *et al.* 2021, Zhang *et al.* 2022). In the environment, MT rapidly adsorbs onto sediments due to its hydrophobic nature, resulting in residual accumulation (Mlalila *et al.* 2015).

Apart from human health, leakages of MT and the metabolites in the aquatic environment disrupt the endocrine and reproductive systems of non-target aquatic organisms (Gomelsky *et al.* 1994, Abucay and Mair 1997, Hulak *et al.* 2008, Ramirez-Godinez *et al.* 2013, Rivero-Wendt *et al.* 2013, Wang *et al.* 2020). For example, the exposure of Common carp (*Cyprinus*

carpio L.) progeny to residual water from feeding trials with MT treatment caused masculinisation in the species (Gomelsky *et al.* 1994, Hulak *et al.* 2008). In addition, small quantities of MT up to 0.004 mg L^{-1} induced vitellogenin (Vtg) in aquatic organisms (Rivero-Wendt *et al.* 2016). Likewise, androgen in wastewater induced cytogenetic toxicity, embryo malformations and hatching delay in the non-target species (Rivero-Wendt *et al.* 2013).

With the global expansion in tilapia aquaculture operations, the use of MT is projected to increase, and consequently, the magnitude of the adverse effects. Concerns regarding the use of the hormone in fish culture operations have recently been reflected in the low acceptability of fish treated with synthetic chemicals by consumers (Biswas *et al.* 2005, Reverter *et al.* 2014). Several countries, especially in Europe and the United States of America, have also instituted stringent measures, including strict licensing and prohibiting the use of hormones in food fish production (Penman and McAndrew 2000, Bhandari *et al.* 2004, Leet *et al.* 2011, Chakraborty *et al.* 2014, Mlalila *et al.* 2015, Ribeiro *et al.* 2015, Baroiller and D'Cotta 2019). Although aquaculture is still in the infancy stage in developing countries of Sub-Sahara Africa, the use of steroids in fish hatcheries is a fast-growing practice, albeit without guidelines. The accreditation and certification of fish hatcheries are also not common practices, with no or limited monitoring and surveillance of hormone use. As such, the farmers use different synthetic hormones indiscriminately. The situation is worsened by poor infrastructure, lack or poor protective equipment, and limited expertise in the hatchery facilities (Fortes 2005, Gabriel *et al.* 2017). Therefore, using MT poses a significant threat to the sustainability of aquatic ecosystems and human health in developing countries. As such, the demand for economically viable, environmentally sustainable, and socially acceptable plant-based alternative products for producing all-male tilapia populations is increasing (Ampofo-yeboah 2013, Gabriel *et al.*

2015, 2017, Nian *et al.* 2017, Mukherjee *et al.* 2018, Gabriel 2019, Ghosal *et al.* 2021, Abaho *et al.* 2022b).

1.3.5 Use of plant extracts to control tilapia reproduction

Plant extracts are becoming an integral part of fish culture, as alternatives to chemicals, drugs, and hormones, in response to the increasing pressure to reduce adverse impacts associated with aquaculture on human and environmental health. Organic plant products are relatively safe, inexpensive, easy to prepare, and easily accessible, and are thus capable of contributing to achieving sustainable fish production (Logambal *et al.* 2000, Makkar *et al.* 2007, Olusola *et al.* 2013, Chakraborty *et al.* 2014, Reverter *et al.* 2014, Hoseini *et al.* 2019). Moreover, consumers are increasingly demanding good quality and safe fish products which are free of pollutants (Leet *et al.* 2011, Chakraborty *et al.* 2014). Therefore, adopting safe and environmentally clean fish production practices will facilitate meeting the requirements of new markets. Accordingly, efforts are underway to identify and develop novel plant-based products for use in aquaculture to replace synthetic hormones and chemicals (Turan and Akyurt 2005, Citarasu 2010, Mehrim *et al.* 2019, Abaho *et al.* 2022b).

To date, several plant extracts are predominantly used to improve fish growth, enhance innate immune responses, and control disease in aquaculture, as compared to reproduction control (Logambal *et al.* 2000, Olusola *et al.* 2013, Reverter *et al.* 2014, Baluran *et al.* 2018). However, the bioactive compounds, i.e., phytochemicals (phytoestrogens or phytoandrogens), such as flavonoids, tannins, terpenoids, alkaloids, and steroids, obtained from the extracts of different plant parts have been reported to induce masculinisation or fertility impairment in fish (Citarasu 2010, Chakraborty and Hancz 2011, Chakraborty *et al.* 2014, Emeka *et al.* 2014, Gabriel *et al.*

2015, 2017, Ghosal *et al.* 2021). The phytochemicals are structurally similar to animal hormones and hence act as endogenous steroids in regulating fish reproduction (Glazier and Bowman 2001). As such, numerous research efforts are exploring the possibility of utilising plant extracts to control indiscriminate spawning in Nile tilapia culture systems through oral in diets and immersion techniques (Table 1.1). The preference for oral in diets and immersion methods to administer plant extracts to the fish is attributed to the ease of application and the non-invasiveness of the methods (Yoshida *et al.* 1995, Bulfon *et al.* 2015).

Table 1.1: Summary of results obtained by previous studies from the utilisation of plant extracts to control reproduction in Nile tilapia. The major bioactive compounds, usable parts and products, dose, exposure period, mode of administration and the androgenic and fertility impairment attributes of the plant extracts are described (Abaho *et al.* 2022b).

Plant species	Bioactive compound	Treatment	Dose	Duration (days)	Delivery	Study	Best dose	Summary of results	Reference
True aloe, *Aloe vera*	Saponins and flavonoids	Crude powder extract	0, 1.0, 2.0 and 4.0 % of feed	30	Oral	Sex ratio	4.0% of feed	Induced 67.62 ± 4.37 % of male individuals after the treatment period	(Gabriel *et al.* 2017)
		Aloe vera latex	0, 0.5, 1.0, 1.5- and 2.0 mL kg^{-1} feed	60	Oral	Gonadal histology	2.0 mL kg^{-1}	Disintegrated spermatids in males, ruptured follicles, and gonadal necrosis in females.	(Jegede 2011, Kushwaha 2013)
Shatavari, *Asparagus racemosus*	Saponins	Root methanol extract	0.1, 0.15 and 0.2 g kg^{-1} of feed	30	Oral	Sex ratio	0.2 g kg-1 of feed	Yielded 92.24 ± 0.13 % of male individuals	(Mukherjee *et al.* 2018)
		Root aqueous extract	0.01, 0.015 and 0.02 g L^{-1}	30	Immersion	Sex ratio	0.015 g L^{-1}	Sex inversion results produced 90.60 ± 1.56 % of males	(Mukherjee *et al.* 2015a)
Aspilia plant, *Aspilia mossambicensis*	Saponins and flavonoids	Leaf powder	1.0, 2.0, 4.0 and 8.0 g kg^{-1} feed	90	Oral	Hatchlings production	8 g kg^{-1}	Low hatchlings number (101.9 ± 19.7)	(Kapinga *et al.* 2018)
		Leaf powder	1.0, 2.0, 4.0 and 8.0 g kg^{-1} of diet	90	Oral	Gonadal histology	2.0 g kg-^{1}	Degenerated of seminiferous tubules and atretic follicles	(Kapinga *et al.* 2019)
Neem tree,		Crude ethanol extract	0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 g kg^{-1} diet	56	Oral	Hatchlings number	1.0 g kg^{-1}	No breeding from the 5th week of the treatment	(Obaroh and Achionye-Nzeh 2011)

Azadirachta indica	Saponins and flavonoids	Leaf crude extract (Saponin)	0.0, 0.5, 2.0, 4.0 and 8.0 g kg^{-1} feed	56	Oral	Hatchlings number	4.0 and 8.0 g kg^{-1}	No spawning was observed	(Obaro *et al.* 2012)
		Leaf Powder	0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 g kg^{-1} feed	56	Oral	Hatchlings number	1.0, 2.0, 4.0 and 8.0 g kg^{-1}	No spawning observed	(Obaroh and Nzeh 2013)
		Leaf powder	1.0, 2.0, 4.0 and 8.0 g kg^{-1} feed	90	Oral	Hatchlings production	8 g kg^{-1}	Low hatchlings number (62.5 ± 6.2)	(Kapinga *et al.* 2018)
Indian spinach, *Basella alba*	Steroids	Leaf powder of aqueous, methanol, ethanol, dichloromethane, hexane and successive methanol extracts	0.5, 1.0 and 1.5 g kg^{-1} of feed	30	Oral	Sex ratio	1.0 g of ethanol extract kg^{-1} of feed	Sex inversion with 83.2 ± 0.7 % of males was observed	(Ghosal *et al.* 2015)
		Leaf aqueous extract	0.00, 0.05, 0.10 and 0.15 g L^{-1}	30	Immersion	Sex ratio	0.1 g L^{-1}	A skewed sex ratio with 70.3 ± 1.9 % males obtained	(Ghosal and Chakraborty 2014a)
		Leaf powder	0.0, 5.0, 10.0 and 15.0 g kg^{-1} of feed	30	Oral	Sex ratio	10.0 g kg^{-1} of feed	Triggered sex inversion to 70.3 ± 1.2 % of males	(Ghosal *et al.* 2016)
		Leaf aqueous extract	0.05, 0.10 and 0.15 g L^{-1}	30	Immersion	Sex ratio	0.1 g L^{-1}	Sex masculinisation producing 71.9 ± 1.9 % of males observed	(Ghosal *et al.* 2016)

Red kwao krua, *Butea superba*	Flavonol and flavonoid glycoside	Root powder	100, 200, and 300 g kg^{-1} of feed	30	Oral	Sex ratio	200 g kg^{-1} of feed	Male individuals increased to 72.2 ± 25.5 % from the normal 1:1 sex ratio	(Mengumphan *et al.* 2006)
		Root powder ethanol extract	0.00, 0.04, 0.08, 0.12, 0.16 and 0.20 g kg^{-1} of feed	21	Oral	Sex ratio	0.20 g kg^{-1} of feed	Induced 100 % testicular development	(Kiriyakit 2014)
Pawpaw, *Carica papaya*)	Saponin (oleanolic acid 3-glucoside)	Seed powder	6 g kg^{-1} of feed	45	Oral	Sex ratio	6 g kg^{-1} of feed	68 % of male individuals observed at the end of the study	(Mehrim *et al.* 2019)
		Seed powder	0, 4, 8, and 12g kg^{-1} feed	60	oral	Gonadal histology	8 g kg^{-1}	Atretic follicles in ovaries and degenerated spermatozoa in the testes	(Waweru *et al.* 2019)
		Seed powder	3 and 6 g kg^{-1} of diet	30	Oral	Gonadal histology	6 g kg^{-1}	Permanent sterility in mature male tilapia	(Abbas and Abbas 2011)
		Seed powder	0, 2, 4, 6 and 8 g kg^{-1} of feed	28	Oral	Gonadal histology	4 g kg^{-1}	Gonadal deformity resulting in sterility of female individuals	(Solomon *et al.* 2017)
		Seed powder	4.9, 9.8 g kg^{-1} of feed per day	30	Oral	Gonadal histology	9.8 g kg^{-1} per day	Permanent sterility	(Ekanem and Okoronkwo 2003)
		Seed powder	0, 0.5, 1.0, 1.5 and 2.0 g kg^{-1} diet	60	Oral	Gonadal histology	2.0 g kg^{-1}	The disintegration of gonadal cells, rendering the testes and ovaries devoid of spermatids and oocytes, respectively	(Jegede and Fagbenro 2008)

		Seed powder	60 ,90 and120 g kg^{-1} of feed	60	Oral	Gonadal histology	120 g kg^{-1}	Permanent sterility	(Abdelhak *et al.* 2013)
Jack plant, *Eurycoma longifolia*	Phytosterol (stigmastero l)	Root methanol extract	0.00, 0.03, 0.06 and 0.09 g kg^{-1} of feed	30	Oral	Sex ratio	0.06 g kg^{-1} of feed	82.10 % male individuals were obtained	(Yusuf *et al.* 2019)
		Root powder ethanol extract	0.00, 0.02, 0.04, and 0.06 g L^{-1}	60	Immersi on	Sex ratio	0.06 g L^{-1}	Altered gonadal differentiation to 67.44 % of males	(Rinaldi *et al.* 2017)
Bitter kola, *Garcinia kola*	Flavonoids (apigenin)	Seed powder	0, 6 and 10 % feed	70	Oral	Gonadal histology	6%	Impaired gonadal development in female individuals	(Nyadjeu *et al.* 2019)
		Seed powder	0, 1, 3 and 6% diet	44	Oral	Number of eggs spawned	83.45 %	Significantly reduced fecundity	(Sulem-Yong *et al.* 2018)
		Seed powder	0, 10, 20 and 30 g kg^{-1} of feed	28	Oral	Sex ratio	30 g kg-1 of feed	Treatment resulted in 65.75 ± 4.19 % of male individuals	(Tigoli *et al.* 2018)
Cotton, *Gossypium Herbaceum)*	Polyphenol (gossypol)	Root bark powder	0, 5, 10, 15 and 20 gkg^{-1} diet	70	Oral	Gonadal histology	20 g kg^{-1}	Disintegration in seminiferous lobule and oocytes damaged plus connective tissue	(Akin-Obasola and Jegede 2016)
		Cotton seed meal	0, 25, 50, 75 and 100 % soybean meal protein substituted	90	Oral	Gonadal histology	25, 50, 75 and 100 %	Destroyed spermatocytes and distorted vitellogenic stages	(Tope-Jegede *et al.* 2019)
Red hibiscus, *Hibiscus rosa-sinensis*	Flavonoids	Leaf powder	0, 1.0, 2.0, 3.0 and 4.0 g kg^{-1} feed	60	Oral	Gonadal histology	3.0 and 4.0 g kg^{-1}	Induced sterility by destructing testes and ovary tissues	(Jegede 2010)

Mango, *Mangifera indica*	Saponins	Leaf Powder	0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 g kg^{-1} feed	56	Oral	Hatchlings number	2.0, 4.0 and 8.0 g kg^{-1}	No spawning was observed during the experiment	(Obaroh and Nzeh 2013)
Moringa, *Moringa oleifera* Lam.	Triterpenoids (oleanolic acid-3-glucoside and β-sitosterol)	Leaf powder	0%, 5%, 10% and 15% of total dietary protein	90	Oral	Gonadal histology	5% of total dietary protein	Severe oocyte cytoplasm degeneration with normal hepatocytes	(Nwankpa 2017)
Velvet bean, *Mucuna pruriens*	Steroids	Seed methanol extract	0.1, 0.15 and 0.2 g kg^{-1} of feed	30	Oral	Sex ratio	0.2 g kg^{-1} of feed	Masculinisation producing 93.79 ± 0.95 % of males observed	(Mukherjee *et al.* 2018)
		Seed powder	0.0, 2.0, 3.5 and 5.0 g kg^{-1} of feed	30	Oral	Sex ratio	5.0 g kg^{-1} of feed	73.33 ± 0.67 % of males were obtained	(Mukherjee *et al.* 2015b)
		Seed aqueous extract	0.02, 0.035 and 0.05 g L^{-1}	30	Immersion	Sex ratio	0.05 g L^{-1}	Induced sex masculinisation producing 74.67 ± 0.33 % of males	(Mukherjee *et al.* 2015b)
Pine tree, *Pinus tabulaeformis*	Steroids (test osterone, epitestosterone, and Androstenedione)	Pollen powder	0.00, 0.08, 0.16, 0.32 and 0.64 g kg^{-1} of feed	60	Oral	Sex ratio	0.32 g kg^{-1} of feed	Increased male individuals to 89.1 ± 3.6 % from the expected 50%	(Nian *et al.* 2017)
Pine tree, *Pinus kesiya*		Pollen powder	(0.5g of pine pollen +0.5g MT) kg^{-1} of feed and 1.0 g of pine pollen kg-1 of feed	28	Oral	Sex ratio	1.0 g of pine pollen kg^{-1} of feed	Sex inversion with 88 % of males obtained	(Nieves 2017)

Guava, *Psidium guajava*	Saponins and flavonoids	Crude Leaf extract	0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 g kg^{-1} diet	56	Oral	Gonadal histology	4 .0, 8.0 g kg^{-1}	Atrophy of the testicular tissues and ripe oocytes	(Obaroh *et al.* 2018)
Soapbark tree, *Quillaja Saponaria*	Triterpenoidal saponins (triterpene glycoside)	Saponin (QS; Sigma, St. Louis, MO, USA)	0.05, 0.15, 0.30, 0.50 and 0.70 g kg^{-1} of feed	60	Oral	Sex ratio	0.70 g kg-1 of feed	69 % of male individuals obtained	(Francis *et al.* 2002)
		Saponin methanol extract	40, 60 and 80% QS (0.15 and 1.00 g kg^{-1} of feed)	120	Oral	Sex ratio	80% QS (0.15 g kg-1 of feed)	Sex masculinisation with 73 % of male individuals obtained	(Stadtlander *et al.* 2008)
Puncture vine, *Tribulus terrestris*	Steroidal saponin (protodioscin)	Seed powder aqueous, methanol, ethanol, dichloromethane, hexane and successive methanol extracts	0.5, 1.0 and1.5 g kg^{-1} of feed	30	Oral	Sex ratio	1.5 g of ethanol extract kg^{-1} of feed	Altered the normal 1:1 sex ratio producing 88.9 ± 1.1 % male individuals	(Ghosal *et al.* 2015, Ghosal and Chakraborty 2020)
		Trib 60 Extract (Tonvara Premium Natural Supplements, UK).	0.0, 1.0, 1.5, 2.0 and 2.5 g kg^{-1} of feed	42	Oral	Sex ratio	2.5 g kg^{-1} of feed	Induced female-to-males ex inversion with 83.67 ± 4.04 % of males	(Omitoyin *et al.* 2013)
		Seed powder	0.0, 5.0, 10.0 and 15.0 g kg^{-1} of feed	30	Oral	Sex ratio	15.0 g kg^{-1} of feed	76.6 ± 0.5 % male fish were obtained	(Ghosal *et al.* 2016)

		Seed aqueous extract	0.05, 0.1 and 0.15 g L^{-1}	30	Immersion	Sex ratio	0.15 g L^{-1}	Sex inversion producing 81.4 ± 0.5 % of males	(Ghosal *et al.* 2016)
		Seed aqueous extract	0.00, 0.05, 0.10 and 0.15 g L^{-1}	30	Immersion	Sex ratio	0.15 g L^{-1}	81.4 ± 0.5 % of male individuals obtained	(Ghosal and Chakraborty 2014b)
		Seed powder ethanol extract	0.0, 2.0, 2.5 and 3.0 g kg^{-1} of feed	30	Oral	Sex ratio	2.0 g kg^{-1} of feed	Sex masculinisation resulting in 91.85 ± 0.38 % of males	(Ghosal *et al.* 2021)
		Root, flower, and aqueous leaf extract	0.0, 1.0, 2.0, and 3.0	28	Oral	Sex ratio	2.0 g kg^{-1} feed	Sex inversion was effective, producing 97.43 ± 0.13 % of males	(El Deen *et al.* 2020)
Fenugreek, *Trigonella foenum-graecum*	Steroidal saponins (Diosgenin)	Saponin methanol extract	0.15 and 0.30 g kg^{-1} of feed	30	Oral	Sex ratio	0.30 g kg^{-1} of feed	Low levels of masculinisation with 56 ± 12.6 % of males	(Stadtlander *et al.* 2013)
		Saponin methanol extract	40, 60 and 80% TS (0.15 and 1.00 g kg^{-1} of feed)	120	Oral	Sex ratio	80% TS (0.15 g kg^{-1} of feed)	Induced sex change with 73 % of males at the end of the experiment	(Stadtlander *et al.* 2008)
Honeysuckle tree, *Turraea heterophylla*	Steroids	Root powder	0, 10, 20 and 30 g kg^{-1} of feed	28	Oral	Sex ratio	30 g kg^{-1} of feed	76.82 ± 3.34 % of male individuals were obtained	(Tigoli *et al.* 2018)

MT, 17α-methyltestosterone; TS, *Trigonella foenum-graecum* saponin; QS, *Quillaja saponaria* saponin.

1.3.6 Mechanism of action of plant extracts during sex control in fishes

Plant extracts modulate sex inversion in fish by disrupting the biosynthesis, distribution, and functions of steroid hormones, subsequently interfering with the reproductive physiology of fish (Omeje 2016). The synthesis of sex steroids is interrupted through: a) inhibiting aromatase enzyme; and b) antagonising estrogen nuclear receptors, subsequently interfering with the process of gonadal differentiation (Francis *et al.* 2002, Cek *et al.* 2007a, 2007b, Cheshenko *et al.* 2008, Chakraborty *et al.* 2014).

The phyto-compounds can inhibit aromatase enzyme cytochrome P450, which catalyses the conversion of androgens to estrogens, thus favouring the development of female characteristics (Eng *et al.* 2001). The enzyme is inhibited through either competitive inhibition of natural substrates for the enzyme, decreasing the expression of cyclic adenosine monophosphate (cAMP) responsive element binding (CREB) protein or inhibiting the generation of cAMP. Consequently, the pathway regulating aromatase expression is altered, which in turn augments the production of androgens, modifying the sex ratio in favour of male individuals (Cheshenko *et al.* 2008). Phytochemicals such as flavonoids (Miyahara *et al.* 2003, Tarigan *et al.* 2016) and steroidal saponins (Golan *et al.* 2008) attenuate estrogen production by inhibiting aromatase enzyme activity hence increasing the production of testosterone (T), a process that induces masculinisation. Previous studies confirmed increased T levels and a significant number of male individuals after feeding Nile tilapia diets supplemented with saponin extracts from the soapbark tree (*Quillaja saponaria*) and *Tribulus terrestris* were (Omitoyin *et al.* 2013, Stadtlander *et al.* 2013).

The phyto-extracts may also antagonise endogenous estrogens by interacting with estrogen nuclear receptors. The antagonistic nature of phyto-compounds is facilitated by the structural similarity to estrogens and the high affinity for estrogen receptors. Besides, the phytochemicals have stable structures and low molecular weights, which permit passage through the cell membranes (Ososki and Kennelly 2003). Within the cytoplasm, the phyto-compounds compete with endogenous estrogens for binding sites of estrogen receptors, hence acting as estrogen antagonists (anti-estrogens). The anti-estrogens block or alter estrogen receptors, preventing estrogenic activity and consequently reversing the estrogenic effects (Eng *et al.* 2001, Miyahara *et al.* 2003, Ososki and Kennelly 2003, Golan *et al.* 2008, Matozzo *et al.* 2008). The structures of anti-estrogenic compounds such as flavonoids mimic estrogens, enhancing the affinity for estrogen receptors (Bennetau-Pelissero *et al.* 2001, Miyahara *et al.* 2003, Chen and Chang 2007, Green and Kelly 2009, Tarigan *et al.* 2016). Therefore, the compounds can easily bind to the estrogen receptors, subsequently disrupting the effect of estrogens. Consequently, the phytochemicals act as "phytoandrogens," with functional effects similar to testosterone in animals, involving the induction of the development of male reproductive characteristics (Turan and Akyurt 2005).

Histologically, the phyto-compounds may impair fertility by inducing histological changes in the gonads of fish: a) delay gonadal maturation and b) obstruct reproductive functions. The histological changes in fish testes and ovaries include the disintegration of gonad cells, rupture of seminiferous lobule and follicles, and gonadal necrosis. As a consequence of gonadal damage, testes and ovaries become devoid of spermatids and oocytes, respectively (Jegede and Fagbenro 2008, Jegede 2010, Abdelhak *et al.* 2013, Ampofo-yeboah 2013). For instance, the saponins from *C. papaya* seed powder rendered the testes and ovaries of Nile tilapia and Mozambique tilapia

devoid of spermatids and oocytes (Jegede and Fagbenro 2008, Abdelhak *et al.* 2013, Ampofo-yeboah 2013, Ugonna *et al.* 2018). Similarly, feeding Nile tilapia on feeds treated with Red kwao krua root extracts resulted in sterility (Kiriyakit 2014), confirming the presence of reproduction inhibition factors.

1.3.7 Limitations to the utilisation of plant extracts

The use of plant extracts to control prolific spawning in tilapia culture systems is an emerging innovation. As such, several drawbacks continue to constrain the commercial application of phytoextracts from experimental to field levels (Chakraborty *et al.* 2014, Mukherjee *et al.* 2018, Kapinga *et al.* 2019, Ghosal and Chakraborty 2020). Inadequate knowledge on: a) ideal extraction methods of phytochemicals; b) effective bioactive compounds; c) the effect of seasons, environmental parameters and stage of plant growth on the yield and biological activity of extracts; d) optimal doses; e) mechanism of action; and f) long-term effects of plant extracts on quality of fish's physiological processes such as growth and immunity, continue to limit the use of extracts (Stadtlander *et al.* 2008, Chakraborty *et al.* 2014, Mukherjee *et al.* 2015a, Gabriel *et al.* 2017, Ghosal *et al.* 2021, Abaho *et al.* 2022b).

Various studies have reported different extraction methods with varying levels of sex masculinisation (Table 1.1). Notably, phytochemicals' yield and functional properties depend on extraction techniques (Altemimi *et al.* 2017, Dhanani *et al.* 2017). Besides, the bioactive compounds from different plant extracts have varying solubility properties in different solvents such as aqueous, ethanol and methanol (Truong *et al.* 2019). Therefore, selecting an ideal

extraction solvent is vital, especially of appropriate polarity, to maximise the yield of the target bioactive compounds with no disruption of the functional activities (Dhanani *et al.* 2017). Little is also known about the effect of the growth stage of the plant (Akula and Ravishankar 2011), seasons and environmental parameters (Isah 2019), and geographical origin (Dinchev *et al.* 2008) on the concentration and composition of phytochemicals that are believed to control tilapia reproduction. Regarding the effectiveness of plant extracts, variable optimal doses are reported by different studies, even for extracts from the same plant (Table 1.1). In addition, the precise mechanism modulating tilapia sex inversion or fertility impairment is not understood, further hampering the harmonisation of the optimal doses and treatment regimen.

Research on developing efficient methods for isolating and quantifying bioactive compounds from plant extracts, including determining appropriate extraction solvents for the target phytochemicals, is vital. Furthermore, the mechanism or mechanisms of action necessitates comprehensive analysis, either by searching for sex gene expression profiles or changes in the sex steroid levels (Ross and Capel 2005, Capel and Tanaka 2013), to validate the specific role of plant extracts in the sex differentiation process of tilapia. The research findings will be instrumental in designing a standard utilisation protocol, including the: 1) extraction procedure for bioactive ingredients of interest; 2) effective extraction solvents; and 3) best administration technique and dose of the extracts (Dhanani *et al.* 2017, Mukherjee *et al.* 2019).

Besides reproduction control, plant extracts can also enhance the immune system and, subsequently growth performance of fish. However, the extracts may also negatively modulate the physiological functioning of fish, depending on plant type and the administered dose. As such,

a specific dosage of the extracts, which is effective in controlling unwanted spawning, may trigger a negative growth response in the same fish. For example, some plant extracts contain anti-nutritional factors such as gossypol in cotton, which interfere with feed utilisation, hence adversely affecting the health and growth of fish (Ayotunde and Ofem 2008, Prasad and Mukthiraj 2011, Chakraborty *et al.* 2014, Gabriel *et al.* 2015, Kapinga *et al.* 2018). Thus, in-depth explorations focusing on determining optimal doses of plant extracts for sex manipulation, with no undesirable effects on the fish's physiological processes, are needed.

In summary, although some positive results have been registered while using plant extracts to control unwanted reproduction in tilapia, further research to address the above-described limitations is still required. The research results will improve the effectiveness of the phyto-extracts and consequently foster their adoption as environmentally sustainable and socially acceptable alternatives to synthetic hormones in tilapia aquaculture.

1.3.8 The use of pine pollen as a masculinising agent in Nile tilapia aquaculture

Pine pollen (PP) is a male reproductive part of pine trees. The trees belong to the Pinales order and Pinaceae family (Christenhusz *et al.* 2011). The purplish male cones of pine trees are a source of pollen, which has traditionally been utilised as a medicinal product and food for years, especially in China. Pine pollen is used to treat colds, prostate disease, anaemia, diabetes, hypertension, asthma, and rhinitis (Lee *et al.* 2009) and attenuate ageing-related diseases (Mao *et al.* 2012). The pollen powder is referred to as a seedbed of androgens, mainly testosterone, epitestosterone and androstenedione (Saden-Krehula *et al.* 1971, 1979, Jones and Roddick 1988, Zhong-han *et al.*

1994, Turan and Akyurt 2005, Adenigba *et al.* 2017, Baluran *et al.* 2018, Velasco *et al.* 2018, Tarkowska 2019). For example, *Pinus bungeana* and *P. tabulaeformis* were reported to contain 11 ng g^{-1} and 27 ng g^{-1} dry weight of pollen, respectively, of testosterone (Zhong-han *et al.* 1994, Janeczko and Skoczowski 2005, Tarkowska 2019). Other pine trees, such as *Pinus silvestris Pinus nigra*, *P. bungeana*, and *P. kesiya,* have also been described as stores of phytoandrogens (Saden-Krehula *et al.* 1971, 1979, Jones and Roddick 1988, Zhong-han *et al.* 1994, Velasco *et al.* 2018). The phytoandrogens in PP, including testosterone, androstenedione, and dehydroepiandrosterone, have been implicated in the sex masculinisation of fish (Godwin *et al.* 2003, Turan and Akyurt 2005). The pollen also contains antioxidants, which are essential for boosting the immunity of the fish (Nian *et al.* 2017, Baluran *et al.* 2018, Rahman *et al.* 2019). In addition, the powder is rich in amino acids, minerals, vitamins, enzymes, and flavonoids (Saden-Krehula and Tajic 1987, Wang *et al.* 2005). The presence of natural inducers for female-to-male sex change and the anabolic compounds has consequently opened the door for using PP in aquaculture, particularly in producing all-male fish populations (Adenigba *et al.* 2017, Nian *et al.* 2017).

The present thesis used PP from *P. tabulaeformis* Carr as a potential alternative to MT in the production of all-male Nile tilapia. To our knowledge, only two studies had utilised PP to masculinise Nile tilapia (Nian *et al.* 2017, Nieves 2017), and one on African catfish (*Clarias gariepinus*) for male sex development (Adenigba *et al.* 2017), at the inception of the study. Therefore, insufficient information on the PP-induced masculinisation of tilapia was available, with the underlying mechanism modulating PP-induced sex inversion unknown. As such, the present study set out to determine the effect of PP on masculinisation and growth of Nile tilapia. Furthermore, the mechanism governing PP-induced sex differentiation of Nile tilapia was

investigated. The changes in sex gene expression profiles of four main sex differentiating genes: doublesex and mab-3 related transcription factor 1 (*dmrt1*); anti-mullerian hormone (*amh*); cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*); and forkhead box L2 (*foxl2*), in the gonads of PP-treated Nile tilapia were determined. Also, sex steroids (T: testosterone, 11-KT: 11-ketotestosterone, and E2: 17β-estradiol) profiles were examined, as well as changes in gonadal morphological differentiation. The approach was premised on the sexual plasticity attribute in Nile tilapia, where sexually undifferentiated individuals can be converted to male individuals after exposure to androgens. The androgenic treatments modify the expression of sex differentiation genes in teleosts, where the male-specific gene expression is up-regulated while suppressing female-promoting genes, consequently shifting the fate of undifferentiated gonads (Kobayashi *et al.* 2008, Golan and Levavi-Sivan 2014, Chen *et al.* 2016, Shi *et al.* 2017, Wang *et al.* 2022). Additionally, alterations in sex gene expressions disrupt the levels of sex steroids, which consequently directs sex differentiation without affecting genotype (Bhandari *et al.* 2004, Sun *et al.* 2014, Chen *et al.* 2016, Shi *et al.* 2017, Wang *et al.* 2018, 2022, Nagahama *et al.* 2021). Besides, the female-to-male sex change involves the degeneration of female germ cells followed by the appearance of testicular tissue after androgen treatment (Bhandari *et al.* 2006, Kobayashi *et al.* 2008, Wang *et al.* 2022). Therefore, sequential changes in the gonadal histology were examined to further clarify the role of PP in the female-to-male sex inversion of Nile tilapia.

1.4 Aim and objectives of the study

The present study aimed to investigate the androgenic and anabolic effects of PP on Nile tilapia towards providing a safe and environmentally sustainable masculinisation agent as an alternative to synthetic hormones. Specifically, the objectives of the study were to:

1. determine the optimal dietary inclusion level of PP for maximum masculinisation of Nile tilapia;
2. explore how PP affects the growth performance and survival of Nile tilapia;
3. examine the effects of PP on the expression of sex genes (*dmrt1*, *amh*, *cyp19a1a,* and *amh*) during and after sex differentiation of Nile tilapia; and
4. to assess the effects of PP on sex steroid profiles (T, 11-KT, and E2) and gonadal histology during and after sex differentiation of Nile tilapia.

1.5 Thesis structure

The thesis consists of five chapters. Chapter 1 includes the general introduction, while Chapters 2, 3, and 4 address specified research objectives. Finally, Chapter 5 presents a general discussion of the study results. Chapters 1 and 2 have been published in peer-reviewed journals and were incorporated in the thesis with minor modifications. Notably, "sex inversion" and "sex reversal" are sometimes used interchangeably in fish reproduction. Sex inversion involves directing an undifferentiated gonad to the desired sex, unlike sex reversal, where the already differentiated, gonad is induced to become an opposite sex (Green *et al.* 1997). In the present thesis, both sex inversion and sex reversal were adopted since they all occur during the 30 dph of PP or MT treatment of Nile tilapia.

Chapter 1 reviewed the available literature, focusing on global tilapia production trends and reproduction aspects of Nile tilapia, with a discussion of the various approaches used to control unwanted spawning in tilapia production systems. In addition, the challenges associated with

using synthetic hormones in producing all-male Nile tilapia are highlighted. The chapter gives an account of the use of plant extracts as potential alternatives to synthetic hormones in the sex inversion of Nile tilapia. Finally, PP is proposed as a safe and eco-friendly alternative to MT in producing all-male Nile tilapia individuals. Sections 1.2 and 1.3 of the chapter are based on the review publication (Abaho *et al.* 2022b) with modifications.

In Chapter 2, the potential of PP to masculinise Nile tilapia and the effects on the growth of the fish were determined. The changes in the sex ratio, growth and survival of PP-fed Nile tilapia were examined. The results were used to design the subsequent chapters of the thesis. The chapter has been published (Abaho *et al.* 2022a) and is included here with minor changes to the materials and methods section.

Chapter 3 elucidated the molecular mechanism underlying the PP-induced sex inversion of Nile tilapia. The chapter addressed objective three of the thesis. A candidate sex gene approach was adopted to investigate the mechanism underlying PP-induced sex inversion. The expression of four main sex-biased genes (*dmrt1*, *amh*, *cyp19a1a*, and *foxl2*), which are markers within the sex differentiation network of Nile tilapia, were analysed during and after gonadal differentiation.

The effect of PP on the sex steroids (T, 11-KT, and E2) and the associated histological changes in the gonads of Nile tilapia were examined in Chapter 4. Since sex hormones play a vital role in the sex differentiation process of fish, it was imperative to understand how PP interferes with the sex steroid profiles. Besides, a shift in sex-specific gene expression alters the sex steroid profiles,

consequently directing the sex differentiation process in fish. The changes in the concentration of sex steroidal profiles were related to the histological changes in the gonads of the fish.

Finally, Chapter 5 includes a general discussion that presents novel aspects of the research and suggests future research avenues for using PP in tilapia aquaculture.

CHAPTER 2[3]

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

2.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is the second most farmed fish worldwide, after carps. In 2018, an estimated 4.53 million tonnes of the species were produced, representing 8.3 % of the total farmed finfish production globally (FAO 2020). Therefore, Nile tilapia contributes significantly to global human food and nutrition security and economic demands. Amongst the factors contributing to the continued production growth of this fish is the increased adoption of all-male culture technology (Baroiller and D'Cotta 2019, Nozu and Nakamura 2020). The culture of all-male individuals 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, Toguyeni *et al.* 2002, Srisakultiew and Kamonrat 2013). Besides, males grow faster and bigger than females, resulting in a shortened production cycle and uniform-sized fish at harvest, which attracts good market prices (Baroiller and D'Cotta 2019, Chavez-Garcia *et al.* 2020, Snake *et al.* 2020). Consequently, the demand for all-male seed continues to increase.

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Presently, most Nile tilapia hatcheries use the synthetic 17α-methyltestosterone hormone (MT) mainly to produce all-male individuals (Celik *et al.* 2011, Baroiller and D'Cotta 2019, Snake *et al.* 2020). While using MT, sexually undifferentiated fish are fed diets mixed with the hormone for three to four weeks post-hatch (Mateen and Ahmed 2007, Celik *et al.* 2011, Baroiller and D'Cotta 2019). However, as described in Chapter 1, the synthetic hormone is carcinogenic. Hence, long-term exposure to MT adversely affects the health of hatchery operators (Velazquez and Alter 2004, Yılmaz *et al.* 2013, Golan and Levavi-Sivan 2014, Megbowon and Mojekwu 2014, Baroiller and D'Cotta 2019, Zhang *et al.* 2022). Furthermore, MT leakages from uneaten or un-metabolised diets pollute the aquatic ecosystems and disrupt the endocrine systems of non-target organisms (Hulak *et al.* 2008, Ramirez-Godinez *et al.* 2013, Rivero-Wendt *et al.* 2013, Abo-Al-Ela *et al.* 2017, Abo-Al-Ela 2018). Consequently, most countries have banned or instituted restrictions on using and selling synthetic hormones for aquacultural use while advocating for organic fish production (Leet *et al.* 2011, Chakraborty *et al.* 2014, Mlalila *et al.* 2015, Ribeiro *et al.* 2015, Zhang *et al.* 2022). Research on potential alternatives to synthetic hormones has thus steadily progressed, and the utilisation 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, Nian *et al.* 2017, Mukherjee *et al.* 2018, Ghosal *et al.* 2021).

Plant-based extracts contain phytochemicals with the potential to induce the masculinisation of tilapia in an attempt to control prolific breeding (Ampofo-yeboah 2013, Chakraborty *et al.* 2014, Reverter *et al.* 2014, Gabriel *et al.* 2017, Nian *et al.* 2017, Mukherjee *et al.* 2018, Ghosal *et al.* 2021, Abaho *et al.* 2022b). Currently, the seed, root, and leaf extracts of 20 plant species have been tested for masculinisation potential to replace synthetic hormones with promising results

(Abaho *et al.* 2022b). Amongst the plant extracts, pollen, the male gamete of pine trees (pine pollen - PP) contains phytoandrogens (Turan and Akyurt 2005, Baluran *et al.* 2018, Velasco *et al.* 2018, Tarkowska 2019), which have the potential to induce masculinisation of Nile tilapia (Nian *et al.* 2017, Nieves 2017). Besides sex masculinisation, the bioactive compounds in PP also boost the immunity of the fish, subsequently stimulating fish growth (Nian *et al.* 2017, Baluran *et al.* 2018). However, inadequate information on the optimum concentration of PP required to masculinise Nile tilapia and the subsequent effects on fish growth limit the use of the extract in hatcheries. As such, the present study explored the potential of oral administration of PP in feeds to induce sex masculinisation in Nile tilapia and enhance the growth performance of the fish. Specifically, the study determined the PP dose, which results in maximum masculinisation and investigated the effects on growth, condition factor, and survival of the fish.

2.2 Materials and methods

2.2.1 Phytochemical analysis of pine pollen

Alkaloids, flavonoids, and steroids were the target phytochemicals from PP powder. The extraction process was conducted from the analytical biosciences laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Uganda. Absolute ethanol and double distilled water were used as the extraction solvents. The plant powder and the solvent were mixed in a ratio of 1:2, and thereafter, the soaked samples were kept in the dark for seven days while being shaken three times daily. The filtrates were stored at -20 °C in amber glass bottles until further analysis.

Qualitatively, the phyto-compounds: alkaloids, flavonoids, and steroids were screened following the laboratory manual, adapted from the standard protocols for analysis of phytochemicals in plant extracts (Harborne 1973, Sasidharan *et al.* 2011, Ezeonu and Ejikeme 2016, Usman *et al.* 2020, Jeevitha *et al*. 2021). Each test was conducted in triplicate, and the results were recorded as present (+, ++ and +++) or absent (-) depending on colour changes or precipitate formation upon adding specific reagents. Briefly, to confirm the presence of flavonoids in ethanolic and aqueous filtrates, 3 mL of 3 % Sodium hydroxide (NaOH) was added to 6 mL of each extract. The formation of an intense yellow colour that turned colourless upon adding three drops of diluted Hydrochloric acid (HCl) confirmed the presence of flavonoids in the sample. The alkaloids were screened by adding Mayer's reagent (1mL) to 2 mL of ethanolic and aqueous filtrates. The formation of the brownish precipitate confirmed the presence of alkaloids in the sample. Finally, the steroids were qualitatively confirmed by evaporating 3 mL each of ethanolic filtrate to dryness using a water bath. The dried residue was dissolved in 1 mL of chloroform; thereafter, 1.5 mL of concentrated Sulphuric acid (H_2SO_4) was added. The formation of brown colour with green effervescence confirmed the presence of the steroids.

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 colour 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 equations obtained from the standard curve plot were 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.

Once the presence of steroids was confirmed, screening was done, in triplicate, to identify specific phytosteroids in the powder. Specifically, 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. The plate was developed in chloroform: methanol mixture (2:1), and the presence of the steroids was confirmed under an ultra-violet (UV) lamp (264 nm). Each steroid was observed based on its physical properties, i.e., colour and the rate of displacement on TLC, which is dependent on weight. The amount of T in PP was quantified using an enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical Co., USA).

2.2.2 Experimental facility and fish rearing

The experiments were conducted from the aquaculture facilities of Mukono Zonal Agricultural Research and Development Institute (MUZARDI) of the National Agricultural Research Organization (NARO), Uganda. Nile tilapia broodstock, comprising 60 females and 20 males with mean body weight of 152.74 ± 6.21 g and 188.02 ± 8.34 g, respectively, 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 ºC). After two weeks, the mouths of female individuals were checked for eggs. The fertilised eggs from all brooding females were collected and transferred into an indoor hatchery and thereafter incubated at 27 ± 1.0 ºC 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 30 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}. Water temperature, dissolved oxygen, and pH were monitored daily using a multi-parameter meter (In-Situ SmarTROLL^{TM} MP, USA), while ammonia-nitrogen was monitored using a commercial freshwater aquaculture kit (LaMotte Company Ltd, USA). Throughout the experiment, the water quality parameters were kept within the optimal range for tilapia culture (Mjoun *et al.* 2010). Dissolved oxygen was maintained at 5.4 ± 0.23 mg L^{-1} by continuous aeration of 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 was maintained at 27 ± 0.4 ºC using thermostatic heating rods (Sera Aquarium heater thermostat, Germany). The pH and ammonia- nitrogen were maintained at 7.2 ± 0.42 and below 0.2 mg L^{-1}, respectively. 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.

2.2.3 Experimental design

A total of 7,200 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 only basal diet with no PP and MT (CT treatment) or the same basal diet supplemented with either graded levels of PP (80, 160, 320, 640, 1,280, 1,920, 2,560 and 3,200 mg PP kg^{-1} for treatments

PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560, and PP3200, respectively) or the same basal diet supplemented with 60 mg MT kg^{-1} (MT treatment; Figure 2.1).

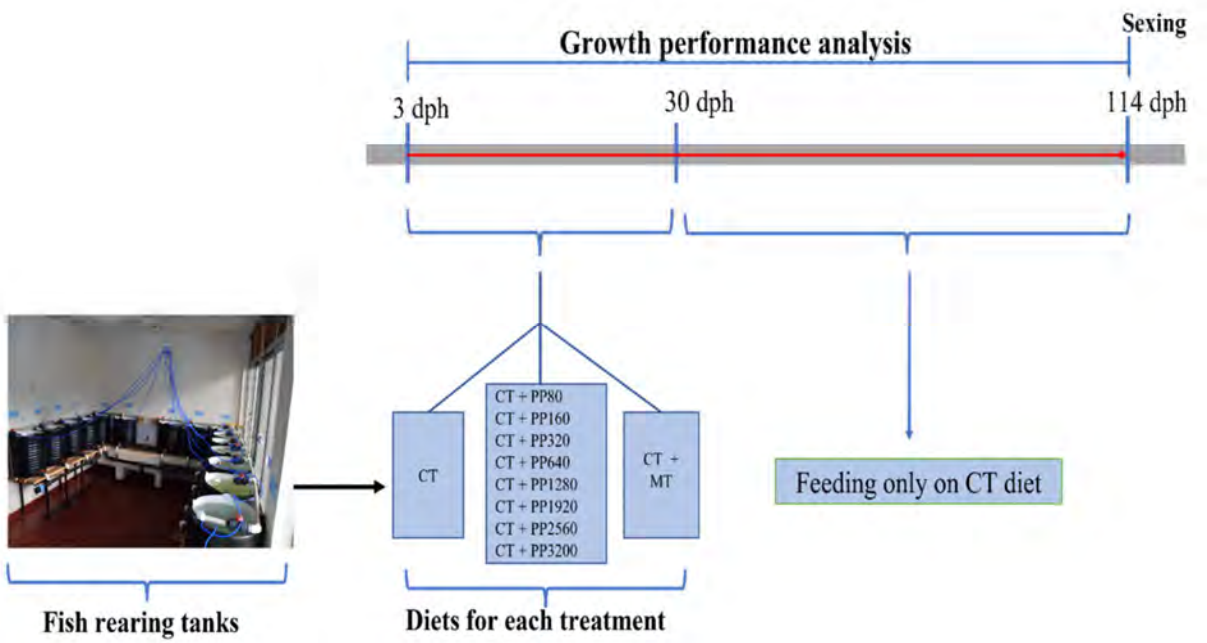


Figure 2.1: The experimental design illustrating dietary pine pollen (PP) and 17α-methyltestosterone hormone (MT) treatments used in the study and the control diet (CT) from three to 114 days post-hatch (dph).

2.2.4 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 (3,865 kcal kg^{-1}) (Table 2.1).

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 (Adenigba *et al.* 2017, Nian *et al.* 2017). The diets were treated with: 80, 160, 320, 640, 1,280, 1,920, 2,560, and 3,200 mg kg^{-1} of PP and 60 mg kg^{-1} of MT. The feed was completely dried at room temperature to prevent fungal contamination, vacuum-packaged, and stored at -4˚C until use.

2.2.5 Fish feeding

The experimental fish were reared from 3 to 114 dph. From 3 to 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 2.1). From 31 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 to 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.

Table 2.1: Dietary ingredient formulation and proximate composition of the ten experimental diets with varying levels of pine pollen (PP): 80 to 3,200 mg PP kg^{-1} (diet PP80 to PP3200, respectively) and 17α-methyltestosterone (MT; 60 mg kg^{-1}) and basal diet (CT).

Ingredient	**Treatment**									
(g kg^{-1})	**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)	**1000.00**	**1000.00**	**1000.00**	**1000.00**	**1000.00**	**1000.00**	**1000.00**	**1000.00**	**1000.00**	**1000.00**
Target formulation										
Total protein (%)	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00	46.00
Total lipid (%)	7.90	7.90	7.90	7.90	7.90	7.90	7.90	7.90	7.90	7.90
Gross energy (Kcal kg^{-1})	3,865.00	3,865.00	3,865.00	3,865.00	3,865.00	3,865.00	3,865.00	3,865.00	3,865.00	3,865.00
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})	3,860.00	3,861.00	3,859.00	3,860.00	3,862.00	3,861.00	3,862.00	3,861.00	3,861.00	3,862.00

*Nutritive value of raw ingredients is proprietary information of Aquaculture Research and Development Center, Uganda

†Jiangsu Qinshantang Health Industry Co., Ltd, Nanjing, China. dry matter, 91.49 %; crude protein, 15.96 %; crude lipid,7.02 %; and gross energy, 5,245.39 Kcal g^{-1}

††Sigma Chemical Co., St Louis, MO, USA

Vitamin premix (mg kg^{-1}): Vitamin A, 10; vitamin D, 0.05; vitamin E, 400; vitamin K, 40; vitamin B1, 50; vitamin B2, 200; vitamin B3, 500; vitamin B6, 50; vitamin B7, 5; vitamin B11, 15; vitamin B12, 11; vitamin C, 1,000; inositol, 2,000; and choline, 5,000

Mineral premix (mg kg^{-1}): Sodium 1500; iron, 3000; copper,90; zinc, 1500; manganese, 800; selenium, 4.3; iodine, 21; and cobalt, 3

2.2.6 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 anaesthetised with a low dose of tricaine methanesulfonate (MS-222; Sigma-Aldrich, USA) 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 using a digital Vernier calliper (Jpxvny Digital Vernier calliper; Resolution: 0.01 mm, Hanhe, China), and a digital scale (Philonext Digital Milligram Scale; 50 X 0.001 g and DBJB; 500g* 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 apparent feed conversion ratio (AFCR) and survival were recorded as follows:

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

2.2.7 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.

2.2.8 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 euthanised with 250 mg L^{-1} of MS-222 (Sigma-Aldrich). Gonadal tissues were harvested using surgical forceps, and sex was determined using the aceto-carmine squash technique. Here, the gonad was mounted on a glass slide, crushed, and phenotypic sex was determined microscopically at 100x magnification after acetocarmine colouration (Guerrero and Shelton 1974).

To compare the level of masculinisation, the squash method was followed by a histological examination of gonads from MT and PP1280 (1,280 mg PP kg^{-1} diet since it had the highest number of males) treated fish (Sarker *et al.* 2022). 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 sections (4.0 μm) were performed using a microtome (LEICA RM2235, USA), stained using haematoxylin-eosin (H &

E), examined, and classified as ovary, testis or ovotestis under a light microscope (Nikon eclipse c*i,* USA). The photomicrographs were taken using a digital microscope-mounted camera (Nikon Digital Sight, USA).

2.2.9 Testosterone leaching rate

A leaching trial was conducted to determine the concentration of testosterone (T) in the PP-supplemented diet after exposure to water. In the experiment, 1.28 g PP kg^{-1} was used since the dose produced the highest proportion of male individuals. Before the investigation, the average time the feed remains in the water before consumption by tilapia juveniles was determined as 49 s. Ten grams of the PP-supplemented diet was placed onto the water surface of a 10 L bucket. Once every 10 s, the water and the feed were mixed using a spatula to trigger disturbance. The diet was then removed from the water after 49 s and dried at room temperature for 24 h. The trial was repeated four times following the same procedure. The concentration of T in the experimental diet exposed to water was analysed using an ELISA kit (Cayman Chemical Co., USA). In addition, the amount of the steroid in the dietary samples before leaching was determined.

2.2.10 Data analysis

Data were analysed using SPSS statistical package (IBM SPSS Statistics, Version 21.0, USA). The differences in the percentages of male individuals among the treatments were analysed using a comparison of proportions by Chi-square statistical test (X^2). For the FBW, SGR, DWG, AFCR, Kn, and survival, the values from the fish in the same replicate tank were averaged before analysis to avoid bias from pseudoreplication. 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 (One-way ANOVA), followed by Tukey's post hoc test for multiple comparisons. The differences in the concentration of testosterone in the diet before and after exposure to water were tested using an independent t-test. Differences were considered significant at $P < 0.05$.

2.3 Results

2.3.1 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 2.2). Quantitatively, the concentration of phyto-compounds, in descending order, was steroids > alkaloids > flavonoids. Meanwhile, testosterone (T) and androstenedione (AED) were the only steroids present in PP. Quantification results showed T levels of 0.028 ± 0.005 µg g^{-1} dry weight of PP. There was no evidence of T leaching after the diet was placed in the water. The concentration of T in the diet exposed to water for 49 s was 0.026 ± 0.002 µg g^{-1}, which was similar to 0.026 ± 0.001 µg g^{-1} in the same diet prior to water exposure (Independent t-test: $t_{(6)} = 0.264$, $P = 0.257$).

Table 2.2: Screening and quantification results for steroids, flavonoids, and alkaloids in pine pollen.

Extract	*Qualitative analysis*		*Quantitative analysis*
	Phytochemical	**Deduction**	**Mean concentration ± SD (µg 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 less soluble in aqueous solvents.

2.3.2 Gender determination

At the end of the experiment, fish gonads were microscopically classified into male and female using the aceto-carmine squash method (Figure 2.2). The female gonads were characterised by the presence of oocytes (Figure 2.2a), while male gonads had thin thread-like structures typical of testicular tubules (Figure 2.2b).

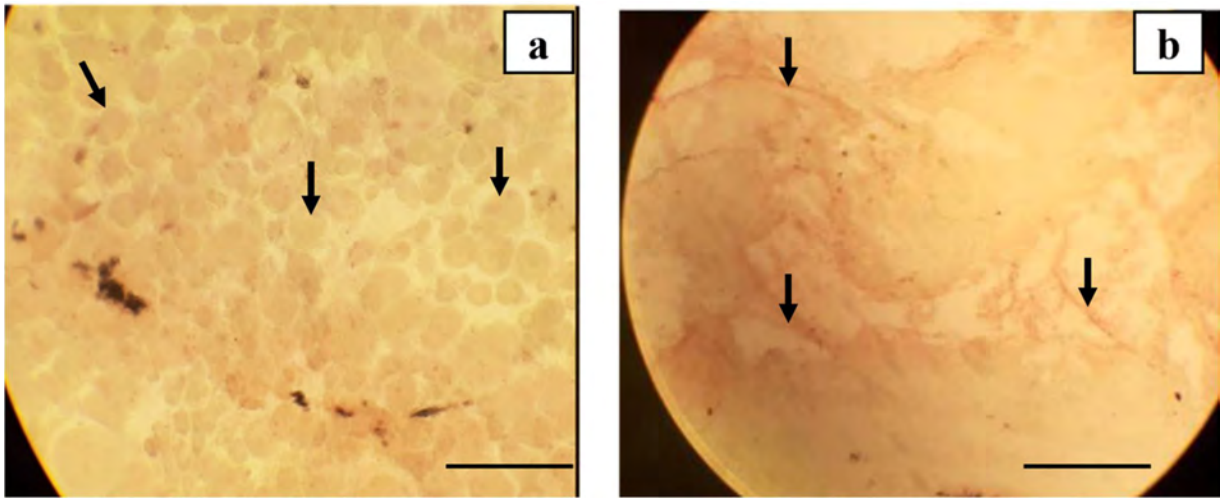


Figure 2.2: Nile tilapia reproduction organs at 114 days post hatching (dph), stained with aceto-carmine (100x): a) female ovary with oocytes (represented by arrows) and b) male testis with testicular tissues (represented by arrows). Scale bar (a – b): 50 µm.

Histological examination of the gonads from the fish treated with 1,280 mg PP kg^{-1} of diet and MT revealed no discernible differences in the ovaries of non-masculinised individuals in both treatments. Ovaries with oocytes, typical female phenotypical attributes, were observed in non-masculinised females (Figures 2.3a and b). Meanwhile, most of the fish specimens from both 1,280 mg PP kg^{-1} of diet and MT groups showed phenotypical male characteristics, including spermatocyte (SP), spermatid (ST), and spermatozoa (SZ) (Figures 2.3c and d). Numerous SP were observed in the testes of PP-treated fish (Figure 2.3c) compared to ST and SZ in the MT group (Figure 2.3d). Overall, no noticeable damages were observed in the testicular and ovarian structures.

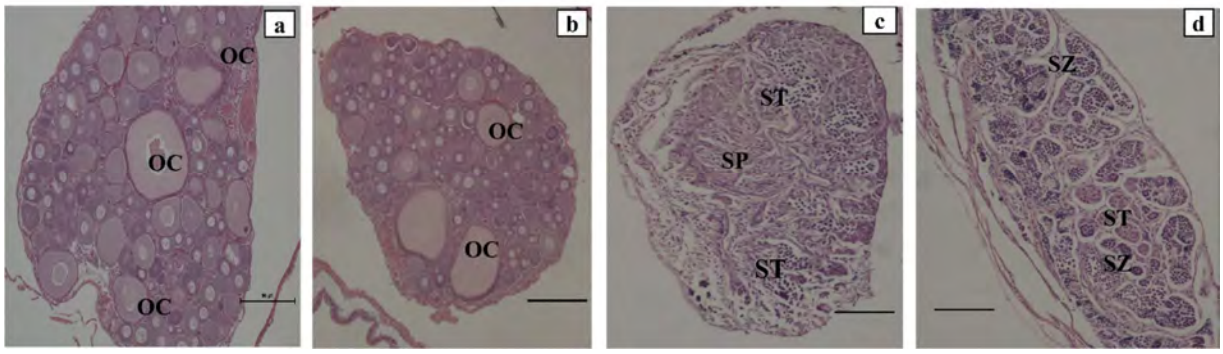


Figure 2.3: The histological features of Nile tilapia gonads at 114 day post hatching (dph): a) and b) oocyte (OC) in female individuals obtained from 1,280 mg PP kg^{-1} of diet and MT treatments, respectively; c) spermatocyte (SP) and spermatid (ST) observed in male fish obtained from 1,280 mg PP kg^{-1} of diet treatment; and d) testis containing spermatid (ST) and spermatozoa (SZ) in specimens from MT treatment. Scale bar (a – d): 100 μm.

2.3.3 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 2.3). Both PP and MT induced a significant shift from females to

males, hence deviating from the expected 1:1 sex ratio (Chi-square: $X^2 = 54.396$, df = 9, $P < 0.001$). A dose-dependent increase in male individuals was observed in Nile tilapia fed diets supplemented with PP. The dose of 1,280 mg PP kg^{-1} diet significantly altered the sex ratio from the expected 50:50 pattern, producing 80.0 ± 2.9 % males individuals compared to 50.8 ± 2.2 % in the non-treated (negative control) diet ($P < 0.05$). Regarding the proportion of masculinisation in PP treatments, the male individuals from tanks fed 1,280 mg PP kg^{-1} diet were higher than in the fish fed low and high concentrations (Table 2.3). Notably, the percentage of males produced by MT treatment was not significantly different from the male proportion in the fish fed diets supplemented with 1,280 mg PP kg^{-1} diet (Overall mean: 84.6 ± 2.6 %; $P > 0.05$).

Table 2.3: Fish sex ratios observed across the different treatments of the experiment. Means in a column with a different superscript are significantly different (Chi-square: $X^2 = 54.396$, df = 9, $P < 0.001$).

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: 80, 160, 320, 640, 1,280, 1,920, 2,560 and 3,200 mg 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).

2.3.4 Growth performance

Dietary inclusion of PP significantly augmented the growth of Nile tilapia than fish fed basal diet only (One-way ANOVA: $F_{(9, 20)} = 9.303$, $P < 0.001$; Table 2.4). 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 12^{th} and 14^{th} weeks in all treatments (Figure 2.4). 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 3,200 mg PP kg^{-1} of diet treatment was significantly higher than 8.63 ± 0.34 g. and 12.62 ± 0.78 g in control and MT- treatments, respectively (Tukey's HSD post-hoc: $P < 0.05$; Table 2.4). Notably, fish individuals fed diets treated with 1,280 mg PP kg^{-1} (with comparable levels of masculinisation to MT) were significantly heavier than fish fed diets supplemented with MT (Table 2.4). 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 (One-way ANOVA: $F_{(9, 20)}$, = 9.303, $P < 0.001$ for WG and One-way ANOVA: $F_{(9, 20)} = 14.196$, $P < 0.001$ for SGR; Table 2.4). In particular, fish fed diets supplemented with 1,280, 1,920, 2,560, and 3,200 mg PP kg^{-1} of diet displayed significantly higher weight gain and specific growth rate than those fed either CT or MT (Tukey's HSD post-hoc: $P < 0.05$).

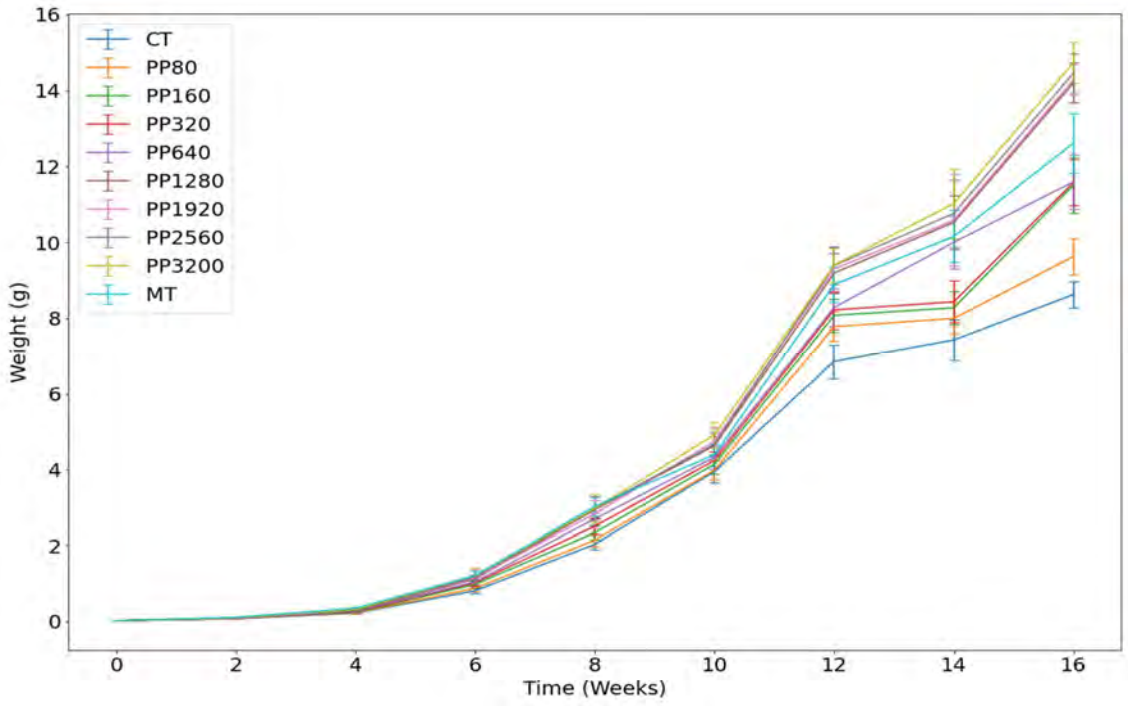


Figure 2.4: Wet weight of Nile tilapia raised on pine pollen (PP) based diets at concentrations of 80 to 3,200 mg PP kg^{-1} diet for PP80 to PP3200, respectively; 60 mg 17α-methyltestosterone kg^{-1} diet (MT); or a basal diet only (CT). Error bars represent the standard errors of three replicates for each treatment.

2.3.5 Apparent feed conversion ratio

The mean (± SE) AFCR 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; One-way ANOVA: $F_{(9, 20)} = 2.869$, $P = 0.024$; Table 2.4). In PP-treated groups, AFCR ranged from 1.03 ± 0.33 to 1.42 ± 0.06 and was not significantly different from one obtained in the MT-treated fish (Tukey's HSD post-hoc: $P > 0.05$).

2.3.6 Survival

The mean percentage survival (% ± SE) of Nile tilapia did not vary significantly amongst the treatments. The survival rate of the fish ranged between 72.50 ± 5.21 % and 75.69 ± 0.61 % (One-way ANOVA: $F_{(9, 20)} = 0.106$, P = 0 .999; Table 2.4).

Table 2.4: Final body weight (FBW), specific growth rate (SGR), daily weight gain (DWG), apparent feed conversion ratio (AFCR), and relative condition factor (Kn) of Nile tilapia across different treatments (values are mean ± standard error). Means with a different superscript in a column are significantly different (One-way ANOVA: P < 0.05).

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

Pine pollen (PP) concentrations: 80, 160, 320, 640, 1,280, 1,920, 2,560 and 3,200 mg kg^{-1} diet for PP80, PP160, PP320, PP640, PP1280, PP1920, PP2560 and PP3200 respectively; 60 mg 17α-methyltestosterone kg^{-1} diet (MT) and basal diet (CT).

2.3.7 Relative condition factor and length-weight relationship

The relative condition of Nile tilapia did not differ significantly amongst the treatments (One-way ANOVA: $F_{(9, 20)} = 0.493$, P = 0.923; Table 2.4) and ranged from 1.01 ± 0.01 to 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 (Figure 2.5). Positive allometric growth was observed across treatments, and the regression

coefficients (“b‟ values) were above 3 (3.066 to 3.110) in all groups. Further, MT, PP640, PP1280, PP1920, PP2560, and PP3200 treatments had larger fish individuals compared to the other treatments. However, the length-weight relationship of these individuals was consistent with one observed in smaller ones in the same and other treatments (Figure 2.5).

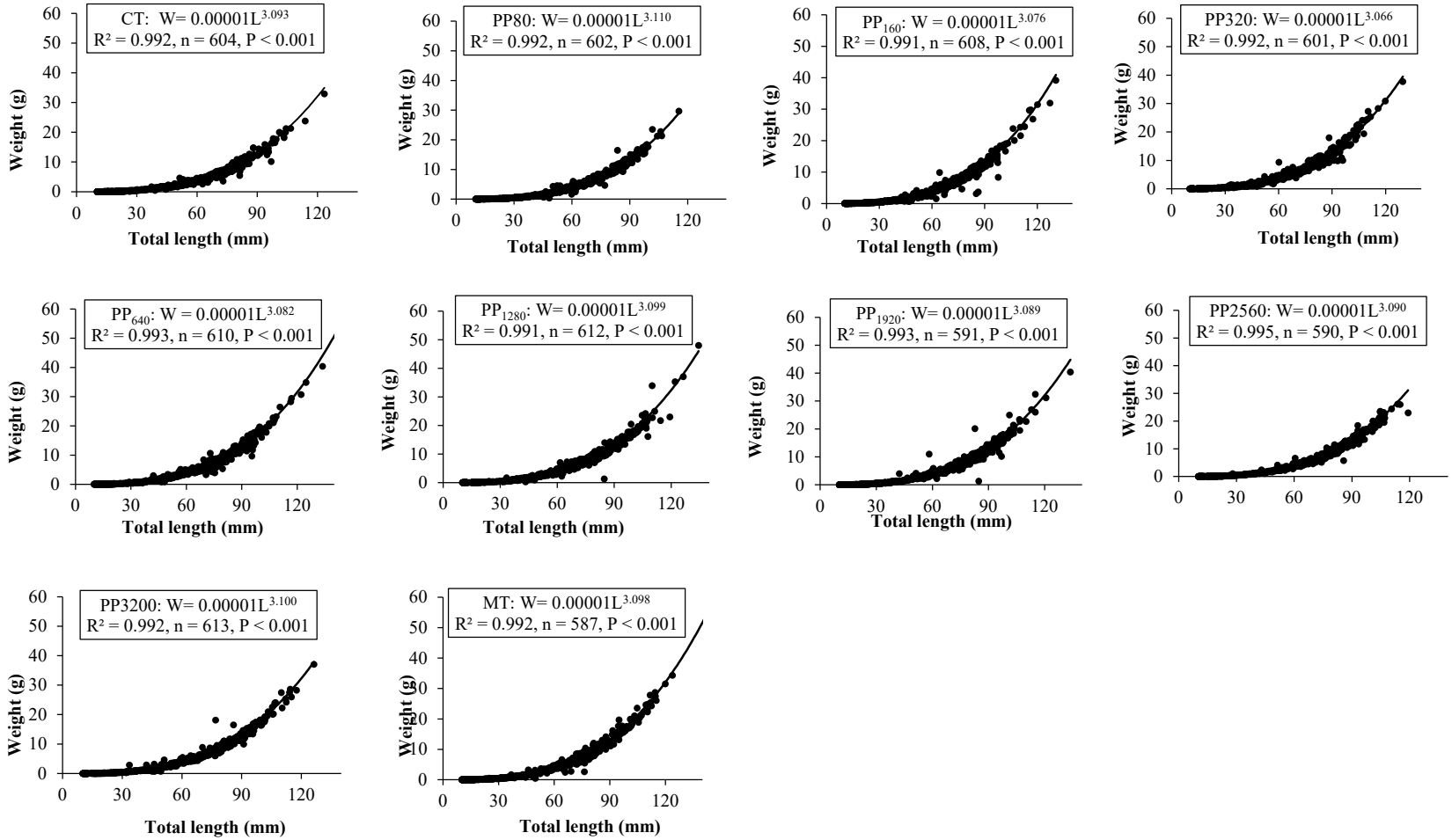


Figure 2.5: Relationship between weight (g) and total length (mm) of Nile tilapia fed diets supplemented with varying levels of pine pollen (PP): 80 to 3,200 mg PP kg^{-1} diet for PP80 to PP3200, respectively; 60 mg 17α-methyltestosterone kg^{-1} diet (MT); or a basal diet only (CT), for 28 days, and thereafter only basal diet for 84 days.

2.4 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 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, pine pollen (PP) is considered a potential substitute for MT in the production of all-male tilapia populations (Nian *et al.* 2017, Nieves 2017). Pine pollen is a yellowish powder harvested from the male reproductive parts of pine trees (*Pinus* spp.) such as *P. kesiya, P. nigra, P. silvestris* and *P. tabulaeformis* (Christenhusz *et al.* 2011). The pollen contains testosterone (T) as one of the major bio-active compounds (Saden-Krehula *et al.* 1971, 1979, Janeczko and Skoczowski 2005, Velasco *et al.* 2018), which is an androgenic steroid responsible for promoting the development of male reproductive characteristics in fish (Celik *et al.* 2011, Leet *et al.* 2011). Therefore, the inclusion of PP in fish diets is expected to alter the sex development process in favour of male individuals. In the present study, PP was incorporated into the diets of sexually undifferentiated Nile tilapia, and female-to-male sex change was investigated. The study utilised the early stages of sexual development, where the gonads are bi-potential and can be modified to follow a differentiation pathway oriented to either the ovary or testis by exogenous hormonal treatment (Ijiri *et al.* 2008, Baroiller *et al.* 2009, Budd *et al.* 2015). 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 the gender of choice, male individuals, was observed in addition to increased feed utilisation with corresponding superior growth performance.

In the present study, the proportion of male Nile tilapia individuals increased with the incorporation of PP in diets in a dose-dependent manner, up to 1,280 mg PP kg^{-1} of diet. Compared to the control group, dietary PP shifted the sex ratio from the expected 1:1 (male: female), producing a higher percentage of males. Comparable findings were obtained from previous studies, which 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). The results are attributed to the presence of phytoandrogens, mainly T, in PP powder (Saden-Krehula *et al.* 1971, 1979, Adenigba *et al.* 2017, Velasco *et al.* 2018, Tarkowska 2019), which induce androgenic activity in animals (Guiguen *et al.* 2010, Wang *et al.* 2017b, Gharaei *et al.* 2020). The study confirmed the presence of steroids: T and AED, as the 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 favour of androgens. (Baroiller *et al.* 1999, Leet *et al.* 2011, Golan and Levavi-Sivan 2014). Consequently, the development of male characteristics was stimulated while inhibiting feminisation (Golan and Levavi-Sivan 2014, Velasco *et al.* 2018). Similar results were obtained in earlier studies using root extracts from Puncture vine (*Tribulus terrestris)* seed and Shatavari (*Asparagus racemosus)* to masculinise Nile tilapia, where elevated levels of 11-ketotestosterone (11-KT) levels were observed in the gonads of the treated fish (Ghosal *et al.* 2021). Therefore, future studies should explore the changes in the levels of endogenous steroids to understand the role of PP in the masculinisation 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,

60 mg MT kg^{-1} diet yielded 95 – 100 % male individuals (El-Greisy and El-Gamal, 2012; Mehrim *et al.*, 2019), while 3,200 mg PP kg^{-1} diet produced an 89 % masculinisation rate of Nile tilapia (Nian *et al.* 2017). The discrepancy in results 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 the steroids to hepatic metabolism; hence, very low levels reach systemic circulation, reducing the androgenic potency. In the present study, the steroids in PP could have been more suspectable 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 (Phelps and Popma 2000, Gao *et al.* 2005). Besides, α-alkylation minimises the aromatisation of the MT to estrogens (Mor *et al.* 2001, Attardi *et al.* 2008, Fragkaki *et al.* 2009). 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 masculinising zebra fish (*Danio rerio*) with extracts from *T. terrestris* extract (Gharaei *et al.* 2020). Testosterone is more prone to aromatisation and hence is converted to estrogens, which bind to estrogen receptors, eliciting paradoxical feminisation effects. In the present study, higher doses of PP could have produced more T, which catalysed aromatisation as a substrate (Pawlowski *et al.* 2004, Attardi *et al.* 2008).

Regarding the effectiveness of PP in masculinising Nile tilapia, the gonads of fish treated with 1,280 mg PP kg^{-1} of diet were histologically examined since a maximum proportion of males was obtained based on the acetocarmine squash approach. Also, the gonads of the male fish from the MT were analysed, and the level of masculinisation was compared with the PP-treated fish. The testes of the obtained male individuals were fully differentiated with various stages of spermatogenesis, i.e., spermatocytes, spermatids, and spermatozoa. No ovarian tissues were observed in the individuals for both treatments, which suggests complete masculinisation. 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 masculinisation at a mature stage of spermatogenesis (Martinez Chavez *et al.* 2021), which is evidence of PP-induced female-to-male sex change in Nile tilapia. Since sex differentiation in Nile tilapia occurs between 21 and 35 dph (Nakamura *et al.* 1998, Ijiri *et al.* 2008, Kobayashi *et al.* 2013, Melo *et al.* 2019), in the present study, all the sex-inversed germ cells were fully differentiated, and the direction of gonadal development determined by 114 dph.

A suitable natural alternative to MT for producing the all-male fish population should also provide desirable growth effects. Therefore, the present study analysed the effects of PP on the growth performance of Nile tilapia. Overall, a positive effect on fish growth was observed in PP treatments. 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), which observed improved weight gain, SGR, and FCR in 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). The augmentation of fish growth by PP is attributed to the presence of anabolic and androgenic compounds, i.e., testosterone and androsterone, which stimulate growth by increasing the mass of the muscle (Turan and Akyurt 2005, Albano *et al.* 2021). Besides, the bioactive compounds in plant extracts are vital in promoting stress tolerance and boosting the immunity of the fish (van Doan *et al.* 2018, 2019, Elabd *et al.* 2022). For PP, the presence of antioxidants and polyphenolics could also have enhanced the growth promotion of fish in the present study (Baluran *et al.* 2018, Velasco *et al.* 2018, Baldove *et al.* 2019). Nile tilapia also exhibits sexual growth dimorphism in favour 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 the treatments. Notably, the higher growth performance in both MT and PP treatments coincided with the lower AFCR values. Since AFCR is an indicator of enhanced feed utilisation, with the fish effectively converting the consumed feed into body growth (Abaho *et al.* 2020), lower AFCR 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 (Froese 2006, Ighwela *et al.* 2011, Abaho *et*

al. 2020). 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. The obtained fish condition conforms to the positive allometric growth pattern ($b > 3$) observed among all treatment groups, with a positive relationship between the 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 a faster weight increase than body length, hence the fish becoming heavier as length increases (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. Although some plant extracts can result in deleterious effects on the physiological processes of fish (Ayotunde and Ofem 2008), no adverse effects were observed in Nile tilapia whose diets were supplemented with PP. As such, the better survival rates observed in PP-treated groups could be associated with the immunostimulatory effect of PP on fish (Baluran *et al.* 2018, Baldove *et al.* 2019). Besides, during the 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).

2.5 Conclusion

Dietary inclusion of PP has a masculinisation effect on Nile tilapia. Therefore, the extract can be used as an alternative to the MT in the sex inversion of Nile tilapia to control unwanted reproduction in the culture systems. Besides, PP offers better growth performance, survival rate, and feed utilisation, which are vital for the success of an aquaculture enterprise. However, further studies with all-female Nile tilapia progeny are required to precisely understand the effectiveness of PP in inducing masculinisation. Additionally, analysis of the functional mechanisms responsible for the androgenic potency of PP in Nile tilapia is paramount. Finally, haematological and biochemical studies to further understand the effect of PP on fish health are necessary before recommending the product for large-scale commercial application.

Since 1,280 mg PP kg^{-1} of diet resulted in the highest proportion of male individuals, subsequent studies of the thesis utilised the dose to understand how female-to-male sex change in Nile tilapia occurs after exposure to PP. The following chapter describes the molecular mechanism underlying PP-induced sex inversion of Nile tilapia.

CHAPTER 3

Effect of dietary pine pollen supplementation on the expression of sex-related genes in Nile tilapia (*Oreochromis niloticus*)

3.1 Introduction

Teleosts display a diverse array of sex determination mechanisms comprising genetic, environmental, and a combination of the two systems (Valenzuela *et al.* 2003, Ospina-Alvarez and Piferrer 2008, Capel 2017, Nagahama *et al.* 2021, Rajendiran *et al.* 2021). In genetic sex-determination systems, the master sex-determining genes activate the sex differentiation network. The sex determination genes include: a Y-linked duplicate of anti-müllerian hormone (*amhy*) in Nile tilapia (*Oreochromis niloticus*) (Li *et al.* 2015, Liu *et al.* 2022), a sexually dimorphic Y-chromosome gene (*sdY)* in rainbow trout (*Oncorhynchus mykiss*) (Yano *et al.* 2012), and anti-müllerian hormone receptor type II (*amhr2*) in tiger pufferfish (*Takifugu rubripes*) (Kamiya *et al.* 2012) Once the sex differentiation genetic network has been initiated, the endocrine environment is regulated, thereby directing the sexual fate of the bi-potential gonadal primordium (Devlin and Nagahama 2002, Rajendiran *et al.* 2021). Some of the genes involved in female sex differentiation are cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), forkhead box L2 (*foxl2*) and factor in the germline alpha (*figla*). For the male phenotypes, doublesex and mab-3 related transcription factor 1 (*dmrt1*), gonadal soma-derived factor (*gsdf*), SRY-box transcription factor 9 (*sox9*) and anti-müllerian hormone (*amh*) are responsible for testicular differentiation (Ijiri *et al.* 2008, Piferrer and Guiguen 2008, Wu *et al.* 2009, Eshel *et al.* 2014, Wang *et al.* 2019, Teng *et al.* 2020b, Nagahama *et al.* 2021, Liu *et al.* 2022).

Within the male and female promoting networks, the up or down-regulation of the genes mentioned above re-programs the process of sex differentiation (Li *et al.* 2013, Sun *et al.* 2018b, Lyu *et al.* 2019, Dai *et al.* 2021, Zhou *et al.* 2021). For instance, higher expression of *dmrt1* and *amh* represses *cyp19a1a* and *foxl2* to trigger testicular differentiation (Herpin and Schartl 2011a, Li *et al.* 2013, Pfennig *et al.* 2015, Sacchi *et al.* 2016, Chen *et al.* 2019). On the other hand, *foxl2* activates the expression *cyp19a1a*, which is responsible for estrogen production, hence inducing ovarian development (Wang *et al.* 2007, Jorgensen *et al.* 2008, Guiguen *et al.* 2010, Todd *et al.* 2016, Li *et al.* 2019). Notably, the sex differentiation process in teleosts is plastic, and the ultimate gonadal sex is influenced by other extrinsic factors such as hormones, temperature, pH, density, and photoperiod (Devlin and Nagahama 2002, Brown *et al.* 2014, Sun *et al.* 2018a, Hayasaka *et al.* 2019, Li *et al.* 2019, Imiuwa 2020, Yan *et al.* 2021, Zhou *et al.* 2021). Changes in the expression levels of sex genes are evident once the fish is exposed to exogenous estrogens prior to and during gonadal differentiation. The estrogens stimulate and inhibit female and male sex-related genes, respectively (Scholz and Gutzeit 2000, D'Cotta *et al.* 2001, Govoroun *et al.* 2001, Kobayashi *et al.* 2003, Bhandari *et al.* 2006, Schulz *et al.* 2007, Paul-Prasanth *et al.* 2013). In addition, the androgens inhibit aromatase activity, subsequently attenuating the production of estrogens while up-regulating the expression of male sex-specific genes (Kitano *et al.* 2000, Bhandari *et al.* 2006, Kobayashi *et al.* 2008, Golan and Levavi-Sivan 2014, Gennotte *et al.* 2015, Banh *et al.* 2017). As such, exogenous treatment of fish with androgenic steroids before and during gonadal differentiation promotes male sex development, while estrogens induce ovarian differentiation. This scenario is exploited to produce fish of the desired sex in aquaculture (Devlin and Nagahama 2002, Baroiller and D'Cotta 2019).

Nile tilapia is a gonochoristic teleost, which exhibits XY/XX sex-determining system (Penman and Piferrer 2008, Kobayashi and Nagahama 2009). The gonadal primordium of the fish is bi- potential during early sex development. Therefore, the gonad can develop into either testis or ovary, under the control of a combined effect of sex-specific genes and endogenous sex steroids (Kwon *et al.* 2001, Strussmann and Nakamura 2002, Ijiri *et al.* 2008, Kaneko *et al.* 2015, Liu *et al.* 2022, Wang *et al.* 2022). Notably, irrespective of the genetically determined sex, the natural gonadal differentiation network can be overridden by external factors, mainly temperature and hormones, which subsequently re-orient the sex development process (Poonlaphdecha *et al.* 2011, 2013, El-Sayed *et al.* 2012, Gennotte *et al.* 2014, Golan and Levavi-Sivan 2014, Chen *et al.* 2016, Sun *et al.* 2018a). Given the sex growth dimorphism displayed by Nile tilapia, all-male individuals grow faster than females and hence are commercially preferred (Chavez-Garcia *et al.* 2020). The gonadal bi-potentiality and sexual plasticity exhibited by tilapia are currently exploited to attenuate and stimulate female and male-promoting networks, respectively, to obtain only male populations. Androgens are utilised to inhibit estrogen synthesis, subsequently inducing female-to-male sex inversion (Srisakultiew and Kamonrat 2013, Golan and Levavi-Sivan 2014, Rahma *et al.* 2015, Junior *et al.* 2017, Abaho *et al.* 2022b). For example, once fish is exposed to the 17α-methyltestosterone (MT) hormone, prior to histological differentiation, the male and female sex genes are stimulated and repressed, respectively, and eventually induce masculinisation. Earlier studies observed a dominant expression of *dmrt1* and *amh*, accompanied by down-regulation of *foxl2* and *cy19a1a* in Nile tilapia exposed to MT (Kobayashi *et al.* 2008, Chen *et al.* 2016, Shi *et al.* 2017, Wang *et al.* 2022). Despite the benefits of MT, public concern over the use of the hormone continues to rise due to its potentially hazardous effects on humans and aquatic biodiversity. The steroid is carcinogenic, and hence continued exposure by fish hatchery personnel results in adverse health effects such as hepatotoxicity and fetotoxicity. Also, the

leakages of the hormone from uneaten or un-metabolized disrupt the endocrine and reproductive systems of non-target aquatic organisms (Rivero-Wendt *et al.* 2013, Yılmaz *et al.* 2013, Abo-Al-Ela 2018, Baroiller and D'Cotta 2019). As such, current research on sex control in tilapia suggests the use of bioactive compounds from plant extracts as safe and environmentally sustainable alternatives.

In the previous chapter and earlier studies (Nian *et al.* 2017, Nieves 2017, Abaho *et al.* 2022b, Aziz *et al.* 2022), pine pollen (PP) demonstrated the capacity to sex inverse Nile tilapia. Nonetheless, information on the molecular mechanism underlying the ability of PP to re-direct gonadal differentiation in fish is unknown. Since MT-induced gonadal differentiation involves alteration of the expression profiles of sex-specific genes, the present study postulated that the steroids in PP (Saden-Krehula *et al.* 1971, Velasco *et al.* 2018, Tarkowska 2019) follow a similar mechanism to modify sex differentiation process in Nile tilapia. The differential expression pattern of sex-related genes after treatment with a sex-inversing agent is an essential cue for the re-orientation of sex in fish. Therefore, to gain insights into the mechanism underlying PP-induced masculinisation, the present study investigated the temporal changes in the expression pattern of four genes previously suggested to be involved in the sex differentiation of Nile tilapia. Specifically, the expression profiles of *cyp19a1a*; *foxl2*; *amh,* and *dmrt1* were examined in all-female (XX) Nile tilapia fed a basal diet supplemented with 1,280 mg PP kg^{-1} since it induced maximum masculinisation of Nile tilapia in the previous experiment of Chapter 2. This was compared with all-female (XX) fish in the control groups fed: 1) only the basal diet (negative control) and 2) basal diet supplemented with 60 mg kg^{-1} MT (positive control).

3.2 Materials and methods

3.2.1 Identification of neo-male Nile tilapia

A sample of 100 sex-inversed Nile tilapia individuals, obtained from the previous experiment in Chapter 2, were anaesthetised using 20 mg of tricaine methanesulfonate (MS-222; Sigma-Aldrich), dissolved in 1 L of water. Caudal fins were clipped from 100 individuals using a sterilised blade (size 24) and capped into collection tubes filled with absolute ethanol. The samples were stored at -20 ºC for later DNA extraction.

Total genomic DNA was extracted at the molecular biology laboratory, Department of Environmental Management, Makerere University, using the DNeasy blood and tissue kit (Qiagen, Germany), according to the instructions of the manufacturer. The fin clips were thawed, sliced into small pieces using sterile surgical blades, and then transferred into a 1.5 mL microcentrifuge tube. Buffer ATL (180 µL) and proteinase K (20 µL) were added to each sample and vortexed thoroughly at maximum speed for 15 s. Complete lysis of the tissue was ensured by overnight incubation of the mixture at 56 ºC. To eliminate possible contamination with RNA, 4 µL RNase A was added, vortexed and incubated at room temperature for 2 min. Further purification was done following the manufacturer's protocol, and finally, the DNA was eluted with 150 µL of buffer AE. The quality of extracted DNA was checked by electrophoresis on 2 % agarose, and the gel image was visualised under a GeneFlash ultraviolet (UV) transilluminator (Syngene, USA). The DNA was stored at -20 ºC until use.

To identify the XX individuals (neo-males), the extracted DNA was subjected to a polymerase chain reaction (PCR) analysis for a Y-specific duplicate of the *amh* gene. The gene-specific primers for *amhy*: amhΔ-y-F: AAACCTCCTTCCTTTGTGAATGTC and amhΔ-y-R:

CGTGGCCACTCCCTCCACCC, were utilised. A 547-base pair (bp) Y-specific fragment amplification was targeted to screen only *amhy*-positive (XY or YY) fish (Li *et al.*, 2015). The *amhy* accession number was not included because the gene sequence was not yet submitted to the gene bank. All PCR reactions were performed in a Thermal Mastercycler (Eppendorf, PCR system 9700, Germany). In every reaction, a DNA-free template was included as a negative control. The reaction volume was 25 µL, constituting of 2 µL of DNA, 9.5 µL nuclease-free water, 0.5 µL each for the forward and reverse primers and 12.5 µL of OneTaq Quick-Load 2X Master Mix (New England BioLabs, USA). The PCR conditions consisted of initial denaturation at 94°C for 3 min, followed by a 33-cycle reaction at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and 72°C for 5 min. From each sample, the PCR product (5 µL) was electrophoresed at 100 V for 40 min on 2% agarose gel with gel red staining (Invitrogen, Carlsbad, CA, USA). The gel image was captured under the UV transilluminator, and the size of bands on the gel image was compared against GelPilot 100 bp plus DNA ladder (Qiagen, Germany). A binary matrix was used to score the samples based on the banding pattern on the gel images, whereby the presence or absence of a DNA fragment was recorded as 1 or 0, respectively. Samples without *amhy* amplification (amhy-) were denoted as XX, whereas those with *amhy* amplification (amhy+) were scored as XY or YY. The *amhy*-negative individuals were used in the subsequent experiment as neo-males, i.e., spermiating genetic female individuals.

3.2.2 Fish rearing

The neo-males (XX) identified in Section 3.2.1 above were used as broodstock. Fish conditioning was done following a similar approach as described in Chapter 2. Sexually mature neo-males were crossed with normal Nile tilapia females to produce all XX progeny. Mating

was performed at a ratio of three females to one neo-male in 60 m^3 concrete circular tanks under natural photoperiod and water temperature (27 – 30 ºC). After 14 days, eggs were collected from the mouths of Nile tilapia females and incubated at 27 ± 1.0 ºC in Zuger jars until hatching. Three days post-hatch (3 dph) fish (mean body weight: 0.025 ± 0.002 g) were collected randomly, stocked in nine continuously aerated experimental plastic tanks (each filled with 120 L of water), and connected in a flow-through system at a flow rate of 1.2 L min^{-1}. The experimental tanks, set in triplicate per treatment, were each stocked with 300 fish. To maintain water quality within the optimal range for the growth of Nile tilapia, culture water was monitored and regulated daily using a water quality digital meter (In-Situ SmarTROLL™ MP, USA). Water temperature and dissolved oxygen were maintained at 27 ± 0.8 ºC using thermostatic heating rods (Sera Aquarium heater thermostat, Germany) and 5.5 ± 0.20 mg L^{-1} by continuous aeration, respectively, and pH at 7.2 ± 0.30. Ammonia-nitrogen was monitored daily using a commercial freshwater aquaculture kit (LaMotte Company Ltd, USA) and kept below 0.2 mg L^{-1}. All uneaten food and other debris were removed from the culture unit daily, in the morning, before feeding.

3.2.3 Pine pollen and 17α-methyltestosterone hormone treatment

The pine pollen (PP) dose of 1,280 mg kg^{-1} of feed was utilised since it yielded the highest proportion of Nile tilapia male individuals, as established in Chapter 2. Likewise, a similar basal diet formulation in Chapter 2 was used in the experiment (Chapter 2, Table 2.1). Fish were fed the basal diet with no PP or MT (negative control), or the same basal diet treated with 1.28 g PP kg^{-1} of diet, or the same basal diet treated with 0.06 g MT kg^{-1} (positive control) from 3 to 30 dph (Figure 3.1). Fish in all treatments were subsequently fed only a basal diet from 31 to 120 dph. Throughout the experiment, the fish were fed to apparent satiation six times a

day during the daylight hours from 3 to 30 dph, and this was reduced to four times daily from 31 to 120 dph (Figure 3.1).

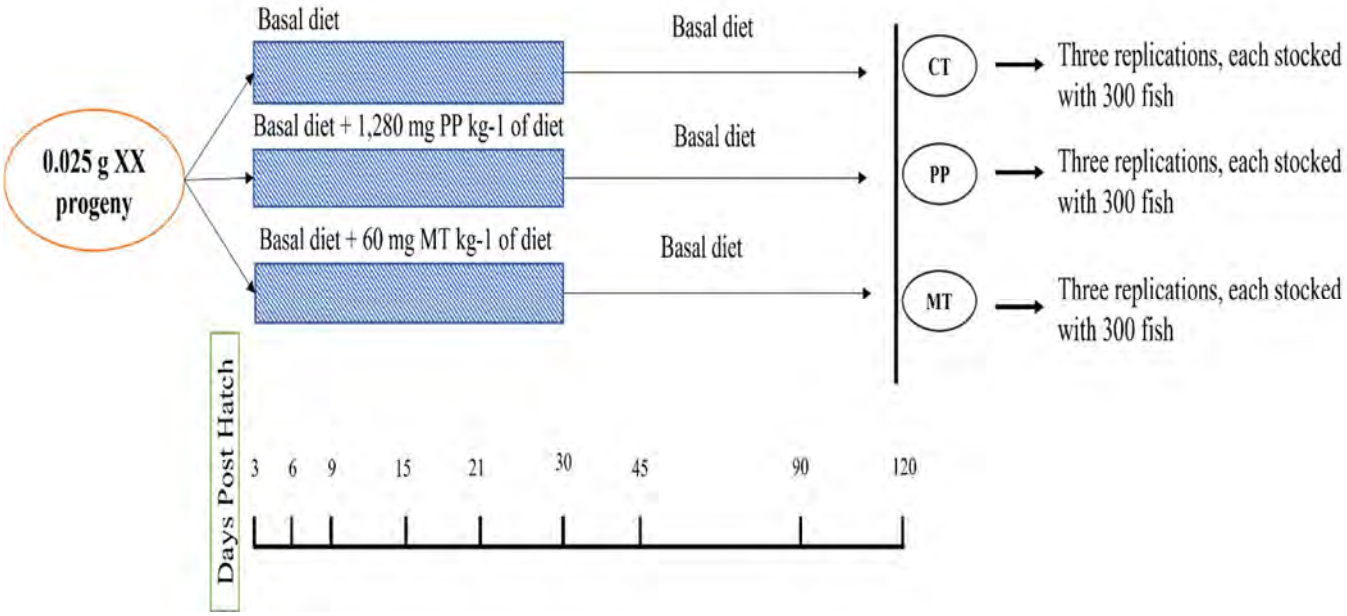


Figure 3.1: Schematic diagram of the experimental design illustrating the applied three treatments: CT (Control treatment; negative control), PP (pine pollen treatment; 1,280 mg PP kg^{-1} of diet) and MT (17α-methyl testosterone hormone; 60 mg MT kg^{-1} of diet).

3.2.4 Sampling

A random sample of two fish per replicate (n = 6 per treatment) was selected from the experimental tanks and euthanised with 250 mg L^{-1} of MS-222 (Sigma-Aldrich) for gonadal extraction. The samples were taken at 3, 6, 9, 15, 21, 30, 45, 90 and 120 dph (Figure 3.1). Between 3 to 21 dph, the fish were too small, and it was not possible accurately excise the gonads. As such, the heads, tails, and viscera of the samples were scraped with sterile surgical blades to obtain body trunks with gonads. From 30 to 120 dph, the gonads were dissected using fine forceps. During gonadal extraction, RNALater (Sigma-Aldrich, St. Louis, USA) was dropped onto the coelomic epithelium to stabilise the RNA. Both the trunks and gonads were immediately placed in 1.5 mL tubes containing RNALater to preserve RNA integrity. The samples were kept at -20 ºC until RNA extraction.

3.2.5 Gonadal phenotype

To verify the gonadal sex of the experimental fish, 90 randomly selected individuals from the remaining fish were euthanised for sexing at 120 dph. Gonads were extracted from the fish and fixed in Bouin's solution at room temperature before the histological examination. The fixed samples were dehydrated and embedded in paraffin, and the tissue blocks were cross sectioned at 4.0 μm. The obtained histological sections were stained with haematoxylin-eosin and visualised under a light microscope for sex identification. Sex phenotype was classified as testis, ovary, or ovotestis (presence of oocytes and testis), and thereafter, percentage masculinisation was calculated based on the number of males in each treatment group.

3.2.6 Molecular analysis

RNA extraction

Total RNA was extracted using Quick-RNA™ MiniPrep Plus Kit (Zymo Research, Tustin, CA, USA) following the manufacturer's instructions. Samples were rapidly thawed, removed from RNALater, and homogenised with sterilised pestles in a 1.5 mL tube. Proteinase K (15 μL) and PK digestion buffer (30 μL) were added to each sample, thoroughly mixed by vortexing and incubated at room temperature for 2 hours. Each sample was then centrifuged at 13,000 rpm for 30 s, and the resulting supernatant was slowly removed with a pipette and transferred to a new 1.5 mL sterile tube. The RNA lysis buffer was then added to each sample and vortex mixed. Thereafter, RNA purification was done following the protocol provided by the manufacturer. To eliminate possible genomic DNA contamination, 5 μL and 75 μL of DNase I and DNA digestion buffer, respectively, were added to the extracted RNA, vortexed and incubated at room temperature for 15 min. For each sample, RNA was eluted in 100 μL DNase/RNase-free water. The concentration and purity of the RNA were measured on a

NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, Massachusetts, USA) at absorbance wavelength ratios of 260/280 and 260/230, respectively. Further, the RNA integrity was assessed by electrophoresis at 100 V for 40 min on 2 % agarose gel, with ethidium bromide staining, along with a GeneRuler 1 kb Plus DNA ladder (ThermoFisher Scientific, USA). Visualisation of the gel was carried out using a GeneFlash UV transilluminator (Syngene, USA). An example of the extracted RNA is shown in the gel image below (Figure 3.2). The extracted RNA was stored at -70 ºC prior to cDNA synthesis.

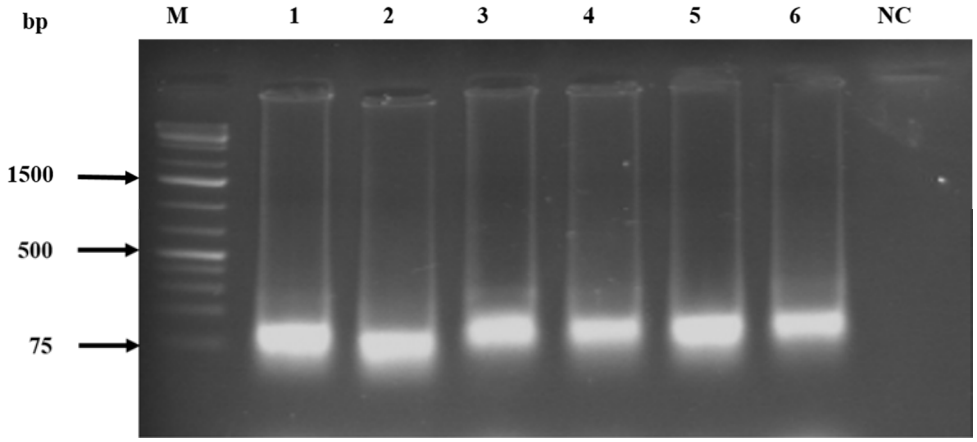


Figure 3.2*:* Representative gel electrophoresis image of RNA extracted from Nile tilapia using Quick-RNA™ MiniPrep Plus Kit (Zymo Research, Tustin, CA, USA). bp: base pair; M: GeneRuler 1 kb Plus DNA ladder; 1- 6: samples and NC: Negative control.

Complementary DNA (cDNA) synthesis

The RNA was reverse transcribed to cDNA using ProtoScript II First strand cDNA synthesis kit (New England Biolabs, USA). During cDNA synthesis, 2 µL of total RNA of each sample was made up to a volume of 8 µL with 2 µL and 4 µL of $d(T)_{23}$ VN (50 µM) and nuclease-free water, respectively. The mixture for each sample was incubated at 65 ºC for 5 min, briefly vortexed and promptly chilled on ice. For each sample, 12 µL of the reverse transcription mixture, containing 10 µL of ProtoScript 11 reaction mixture (2X) and 2 µL of ProtoScript 11

Enzyme mixture (10X), were added. The mixture (20 µL) was incubated at 42 ºC for 60 min, followed by heating at 80 ºC for 5 min using an Eppendorf® Mastercycler® Nexus Thermal Cycler (Sigma-Aldrich, Missouri, USA) to inactivate reverse transcriptase. The obtained cDNA was stored at -20 ºC until use in real-time polymerase chain reaction. For each sample, a negative control cDNA synthesis reaction without reverse transcriptase was performed to confirm no contamination with genomic DNA.

Real-time polymerase chain reaction analysis

The cDNA was subjected to real-time PCR analysis using a 96-well QuantStudio™ 7 Pro (A43055) real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Gene-specific primers for *cyp19a1a* and *foxl2* (Li *et al.* 2014), *amh* (Poonlaphdecha *et al.* 2011, 2013) and Primer 3 plus software-designed primers for *dmrt1* (Untergasser *et al.* 2012) were used. Beta-actin (*β-actin*; GenBank ID: XM_003443127) was used as the only internal control (reference gene) in the present study since the gene is stable and shows uniform mRNA expression in XX and XY Nile tilapia gonads (Yoshiura *et al.* 2003, Li *et al.* 2012, Sun *et al.* 2012; Tao *et al.* 2013). The nucleotide sequences of the primers for each of the analysed genes, as presented in Table 3.1 below, were synthesised by Eurofins (Eurofins Genomics, Vienna, Austria). Prior to real-time PCR analysis, the PCR conditions were optimised to an equal annealing temperature of 60 ºC using randomly selected representative cDNA samples. The efficiency of each primer set was assessed using the standard curve dilution method (mean threshold cycle [Ct] vs log cDNA dilution), melting curve analysis, and electrophoresis of the PCR product. Five serial dilutions of the pooled cDNA samples, each in triplicate, were used. The real-time PCR cycle consisted of initial denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. The Ct values were plotted against log10 of the five dilutions of cDNA to establish a standard curve. The slope value obtained from the curve was used to

calculate efficiency (E) from the equation, $E = [10^{(-1/slope)} -1]100$ %. Amplification efficiencies were: 103.8 %, 97.6 %, 107.2 %, 98.6 %, and 91.3 % for *β-actin*, *dmrt1*, *amh*, *cyp19a1a* and *foxl2*, respectively, and the linear standard curve (r^2) ranged from 0.984 to 0.994 for all genes. For each gene, PCR melting curves with a single melting peak and one clear band (comparable to the target size) on 2 % agarose gel electrophoresis, using a hyperLadder™ 50 bp DNA Ladder marker (Bioline, United Kingdom), were obtained.

Following the amplification of appropriate products, these were used for real-time PCR analysis. The samples were all analysed in duplicate, with reactions performed using Luna Universal qPCR Master mix (New England Biolabs, USA) following the instructions of the manufacturer. During real-time PCR analysis of both the target and reference genes, the reaction volume was 20 µL, comprising 2 µL of cDNA, 7 µL nuclease-free water, 0.5 µL (10 pmol) each for the forward and reverse primers and 10 µL of Luna Universal qPCR Mix. A negative control (non-template control-NTC) with no cDNA was also included in each real-time PCR. Throughout the study, no amplification was observed in the NTC, confirming no genomic DNA contamination. The amplification conditions for all PCR reactions were as follows: initial denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 5 s; 60 °C for 30 s; and 72 °C for 30 s. To confirm whether the real-time PCR amplified the intended target, the sizes of the products were electrophoresed on a 2 % agarose gel (125 V, 30 min), stained with ethidium bromide, along with 50 bp DNA size marker (Bioline, United Kingdom). The gel image was visualised with the Enduro™ GDS gel documentation system (Labnet International Inc, USA). A single clear band with the desired size was obtained for all genes. The expression of all genes was measured from cDNA belonging to the same fish per treatment to allow for comparisons amongst genes per treatment. Relative quantification of each target gene was determined following Pfaffl mathematical model (Pfaffl 2001), which takes into account

differences in reaction efficiencies. The data were normalised using *β-actin* as the reference gene.

Table 3.1: Primers and related information of the sex genes used in the study.

Gene	Primer Name	Primer sequence (5' to 3')	Size product (bp)	Tm	Accession number*
Amh	amh-F	AAGCAGCGCAAACATTAACA	169	53.2°C	EF512167
	amh-R	GTTCCAGTCCACAACCTCCA		59.4°C	
cyp19a1a	cyp19a1a- F	AGGCGATGAGTCCTGTAGGCTTAG	346	64.4°C	AF472620
	cyp19a1a-R	TTATTGTAGTAGTTGCTGGCTGTGC		61.3°C	
dmrt1	dmrt1-F	TGAGCCAGGACAAACAGAGT	210	59.9°C	AF203489.1
	dmrt1-R	CCATGACTCTCTGCCTCTCC		59.3°C	
foxl2	foxl2-F	TGGCAGAACAGCATCAGACACAAC	313	62.7°C	AY554172
	foxl2-R	TGTAGGACATCGGAGTGGGTGGCT		66.1°C	
β-actin	*β*-actin-F	GGCATCACACCTTCTACAACGA	332	60.3°C	XM_003443127
	β-actin-R	ACGCTCTGTCAGGATCTTCA		57.3°C	

amh: anti-müllerian hormone hormone; c*yp19a1a*: cytochrome P450, family 19, subfamily A, polypeptide 1a; *dmrt1*: doublesex and mab-3 related transcription factor 1; *foxl2*: forkhead box protein L2; *β-actin*: beta-actin; F: Forward; R: Reverse; bp: base pair; Tm: melting temperature; *GenBank (https://www.ncbi.nlm.nih.gov/).

3.2.7 Data analysis

Data were expressed as mean ± standard error (SE) for the number of samples of each treatment. The statistical differences in relative gene expression among treatments over the experimental period (days post-hatch) were analysed using repeated measures analysis of variance (RM-ANOVA). When significant interaction between the main effects was obtained, Tukey's HSD post-hoc test was used to compare treatment means at each time. Simple linear regression analysis was also performed to test for an increasing or a decreasing trend in the expression pattern of genes during the experimental period. In order to search for the relationship between the expression of *dmrt1*, *amh*, *cyp19a1a* and *foxl2* in all treatments, Spearman's rank correlation coefficients were calculated. The differences in masculinisation

among treatments were examined with One-way analysis of variance (One-way ANOVA). The differences between means or interactions between main effects were considered statistically significant at $P < 0.05$. All statistical analyses were performed with Statistica® statistical software package (TIBCO Software, Palo Alto, USA, version 14.0.1). The histograms were all generated using Origin 2022b software (OriginLab Corporation, USA).

3.3 Results

3.3.1 Nile tilapia neo-males

From the PCR-based genetic sex identification, 38 % of the screened individuals were *amhy*-negative. The samples had no *amhy* amplification, with the absence of the 547 bp DNA fragment (Figure 3.3). The obtained XX fish were used as neo-male broodstock to produce all-female progeny.

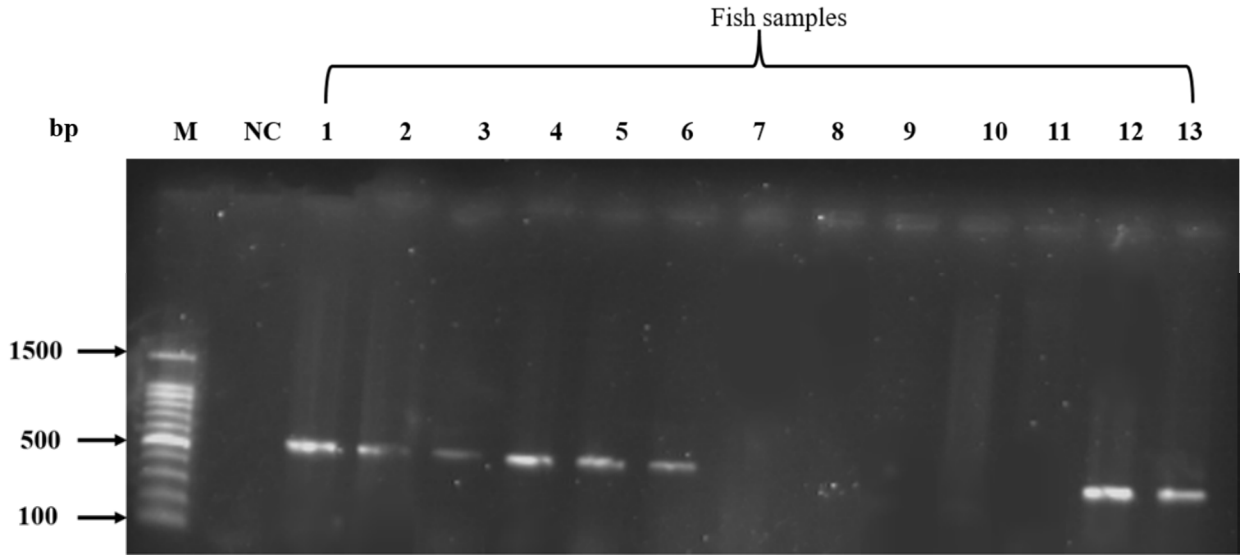


Figure 3.3: Representative results from the genetic identification of sex-inversed Nile tilapia individuals. Genetic screening was done on samples by genomic PCR analysis for *amhy* gene on tilapia Y-chromosome. Samples without the target DNA band are $amhy^-$ (XX neo-males; spermiating genetic females), while samples with bands are $amhy^+$ (XY or YY males because the assay did not distinguish heterozygotes from homozygotes). bp: base pair; M: GelPilot (100 bp) plus DNA ladder and NC: Negative control.

3.3.2 Sex identification

The gonadal phenotypes of fish from each treatment were histologically determined at 120 dph. Unlike the CT group, female-to-male sex change was observed in the PP and MT-treated fish (Figure 3.4).

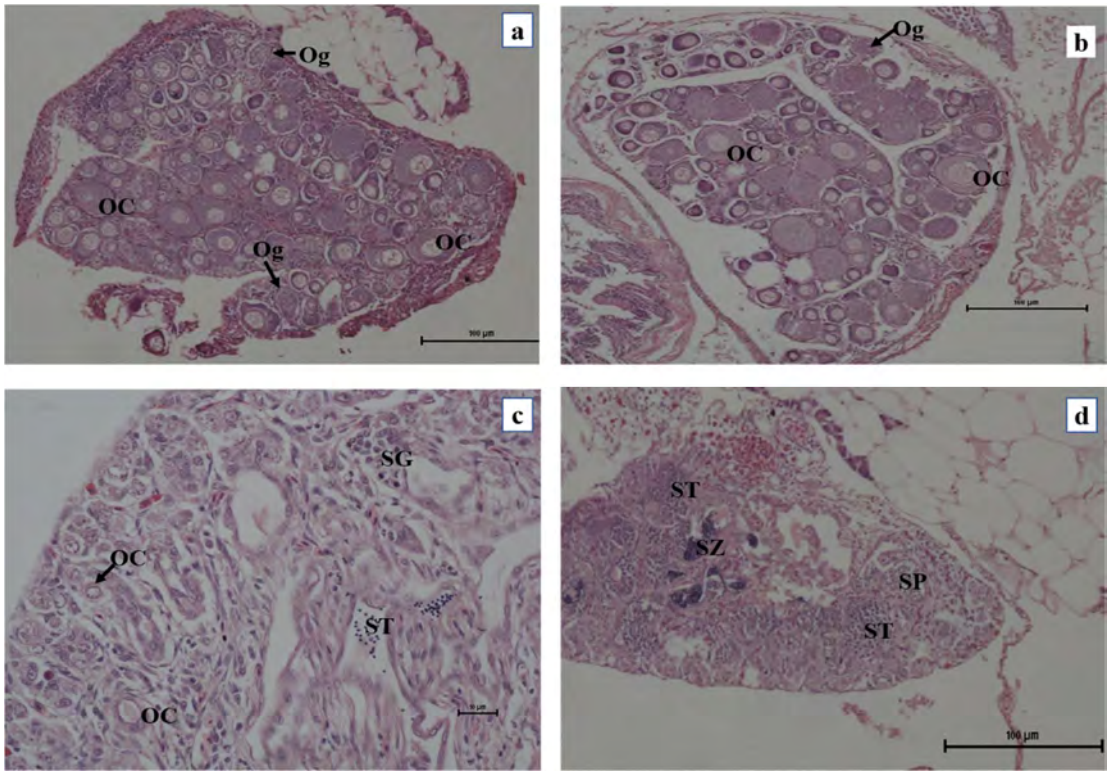


Figure 3.4: Gonad histological analysis of fish at 120 day post-hatch (dph). a: fish fed only a basal diet (CT treatment); b: fish that remained female after feeding on a basal diet supplemented with 1,280 mg pine pollen (PP) kg^{-1} (PP treatment); c: ovotestis fish obtained from the PP treatment after feeding on a basal diet supplemented with 1,280 mg pine pollen (PP) kg^{-1} (PP treatment); and d: male individuals obtained from PP and MT treatments, after feeding on a basal diet supplemented with 1,280 mg pine pollen (PP) kg^{-1} and 60 mg 17α-methyltestosterone (MT) kg^{-1}, respectively. Og: oogonia; OC: oocytes; SG: spermatogonia; SC: spermatocytes; ST: spermatid; and SZ: spermatozoa.

All the fish from the CT treatment remained females, while sex masculinisation was observed in PP and MT-treated fish (Table 3.2). Overall, the rate of female-to-male sex inversion with MT was significantly greater (97.8 ± 1.1 % males) compared to the 77.8 ± 2.9 % male

individuals obtained in the PP-treated group (One-way ANOVA: $F_{(1, 4)} = 40.5$, $P = 0.003$). Notably, 5.6 ± 1.1 % of the individuals in the PP-treated group had ovotestis; thus, only 15.6 ± 1.9 % remained un-masculinised (XX- females).

Table 3.2: Gonadal phenotype and percentage masculinisation of all-female (XX) Nile tilapia fed: basal diet only (CT) or the same basal diet + 1,280 mg of PP kg^{-1} of feed (PP) or the same basal diet + 60 mg of MT kg^{-1} of feed (MT).

Treatment	Number of fish examined	Number of ovaries	Number of testes	Number of ovo-testes	Masculinization (% ± S.E)
CT	90	90	0	0	0.0 ± 0.0
PP	90	15	70	5	77.8 ± 2.9
MT	90	2	88	0	97.8± 1.1

3.3.3 Expression of the target sex genes

All primers used in the study successfully amplified a single product of the expected size for each target gene: *cyp19a1a*, *foxl2*, *dmrt1* and *amh*, using cDNA templates derived from RNA extracted from the trunks and gonadal tissues of the fish (Figure 3.5).

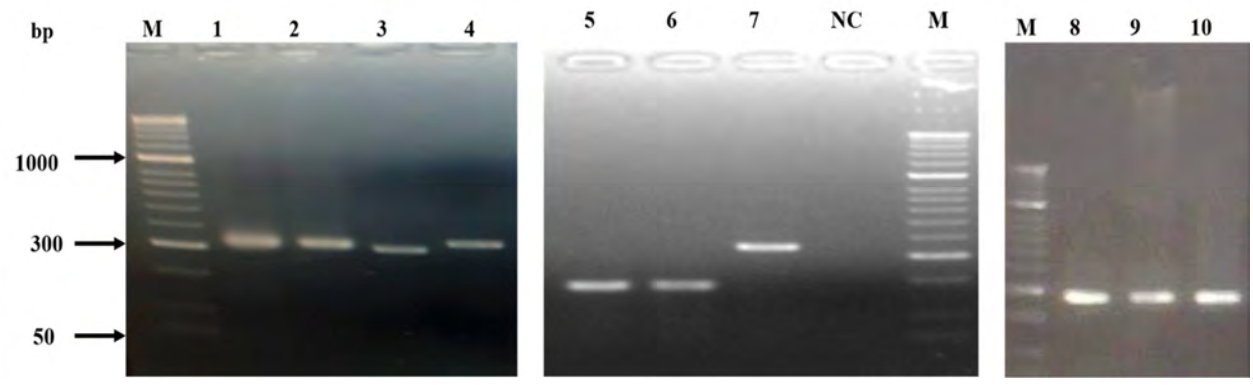


Figure 3.5: Gel electrophoresis image obtained while determining the specificity of primers prior to the real-time polymerase chain reaction. bp: base pair; M: marker (HyperLadder™ 50bp, Bioline); NC = negative control with no cDNA template; 1 – 2 and 7: beta-actin (*β -actin*); 3: forkhead box protein L2 (*foxl2*), 4: cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*); 5 – 6: anti-müllerian hormone (*amh*); and 8 –10: doublesex and mab-3 related transcription factor1 (*dmrt1)*.

Temporal gene expressions were analysed by real-time PCR in all-XX Nile tilapia individuals at different differentiation and development stages: 3, 6, 9, 15, 21, 30, 45, 90, and 120 days post-hatch (dph) of the gonads. Signals of all the examined genes: male sex genes (*dmrt1* and *amh*) and female-biased genes (*cyp19a1a* and *foxl2*), were detected as early as three days post-hatch, and thereafter, marked variations observed in the CT, PP and MT treatment groups (Figures 3.6 to 3.9). A sexually dimorphic expression pattern of the male and female genes was displayed among the experimental groups. The fish treated with either PP or MT had the highest expression of male sex genes (Figures 3.6 and 3.7), while expression levels of female genes in the same fish were low (Figures 3.8 and 3.9). The pattern was consistent with the proportion of male individuals in the two treatments, as described in Section 3.2 above. The MT-treated fish had the highest levels of *dmrt1* and *amh* and a larger percentage of male phenotypes than the PP-treated fish. In contrast, fish from the CT treatment remained females with elevated expression of *cyp19a1a* and *foxl2* and suppressed levels of *dmrt1* and *amh*. Moreover, a significant positive correlation between the expression of *dmrt1* and *amh* was obtained in all treatments, while *cyp19a1a* was strongly correlated to *foxl2*. A strong negative correlation was, however, observed between the expression levels of the male and female sex genes (Table 3.3).

Table 3.3: Spearman's rank correlation coefficients between the expression of *dmrt1*, *amh*, *cyp19a1a* and *foxl2* in Nile tilapia fed only basal diet or the same basal diet supplemented with 1,280 mg of pine pollen (PP) kg^{-1} or the same basal diet supplemented with 60 mg of 17α-methyltestosterone hormone (MT) kg^{-1} diet.

	dmrt1	***amh***	***cyp19a1a***	***foxl2***
dmrt1		r = 0.821** P < 0.001 n = 81	r = - 0.785** P < 0.001 n = 81	r = - 0.767** P < 0.001 n = 81
amh			r = - 0.828** P < 0.001 n = 81	r = - 0.837** P < 0.001 n = 81
cyp19a1a				r = 0.842** P < 0.001 n =81
foxl2				

n: number of observations; ** = correlation is significant at the 0.05 level; *dmrt1*: doublesex and mab-3 related transcription factor 1; *amh*: anti-müllerian hormone; *cyp19a1a*: cytochrome P450, family 19, subfamily A, polypeptide 1a and *foxl2*: forkhead box protein L2.

Expression analysis of male-specific genes

The expression of the *dmrt1* and *amh* was significantly influenced by the interaction between dietary treatment and time, with the expression changing differently over time among the treatments (RM-ANOVA; *dmrt1*: $F_{(16,\ 48)} = 4.985$, $P < 0.001$; Figure 3.6 and *amh*: $F_{(16,\ 48)} = 10.754$, $P < 0.001$; Figure 3.7). From 3 to 120 dph, an inverse expression pattern of the genes was observed in the CT fish (*dmrt1*: $y = 1.640 - 0.017x$, $F_{(1,\ 25)} = 12.844$, $r^2 = 0.198$, $P = 0.001$; Figure 3.6 and *amh*: $y = 1.713 - 0.013x$, $F_{(1,\ 25)} = 9.096$, $r^2 = 0.149$, $P = 0.004$; Figure 3.7). The genes were consistently down-regulated, with 8.3 and 3.4-fold decreases, respectively, from 3 to 30 dph. Further suppression of the transcripts was notable from 30 to 120 dph, at a 5.8 and 3.5-fold decrease for *dmrt1* and *amh*, respectively. However, a linear up-regulation of the same genes was displayed in the fish from PP (*dmrt1*: $y = 4.875 + 0.120x$, $F_{(1,\ 25)} = 27.194$, $r^2 = 0.343$, $P < 0.001$; Figure 3.6 and *amh*: $y = 3.190 + 0.137x$, $F_{(1,\ 25)} = 41.589$, $r^2 = 0.444$, $P < 0.001$; Figure 3.7) and MT (*dmrt1*: $y = 15.154 + 0.136x$,

$F_{(1,\ 25)}$ = 23.058, r^2 = 0.311, P < 0.001; Figure 3.6 and amh: y = 6.901 + 0.307x, $F_{(1,\ 25)}$ = 75.270, r^2 = 0.596, P < 0.001; Figure 3.7) treatments. A 12.1 and 3.4-fold increase in the expression levels of *dmrt1* in MT and PP-treated fish, respectively, was observed from 3 to 30 dph, compared to a 1.5 and 1.9-fold increase from 30 to 120 dph in the same treatments. Consistent results were evident for *amh*, with a 7.0 and 3.7-fold increase in expression in MT and PP groups, respectively, from 3 to 30 dph. Similarly, a 1.8-fold increase in the levels of the same gene was exhibited in the two treatments from 30 to 120 dph.

Significant differences in the expression of *dmrt1* among the treatments were noticeable by six days post-hatch. In both MT and PP-treated fish, similar expression levels of the gene were observed, which were significantly higher than in the fish from the CT treatment (Tukey's HSD post-hoc: P < 0.05; Figure 3.6). Notably, the transcript level was significantly elevated from 15 to 30 dph and 21 to 30 dph in the MT and PP-treated fish, respectively (Tukey's HSD post-hoc: P < 0.05; Figure 3.6). However, the expression levels of *dmrt1* were significantly higher in the fish from the MT treatment than in the PP-treated fish from 21 to 120 dph (Tukey's HSD post-hoc: P < 0.05; Figure 3.6).

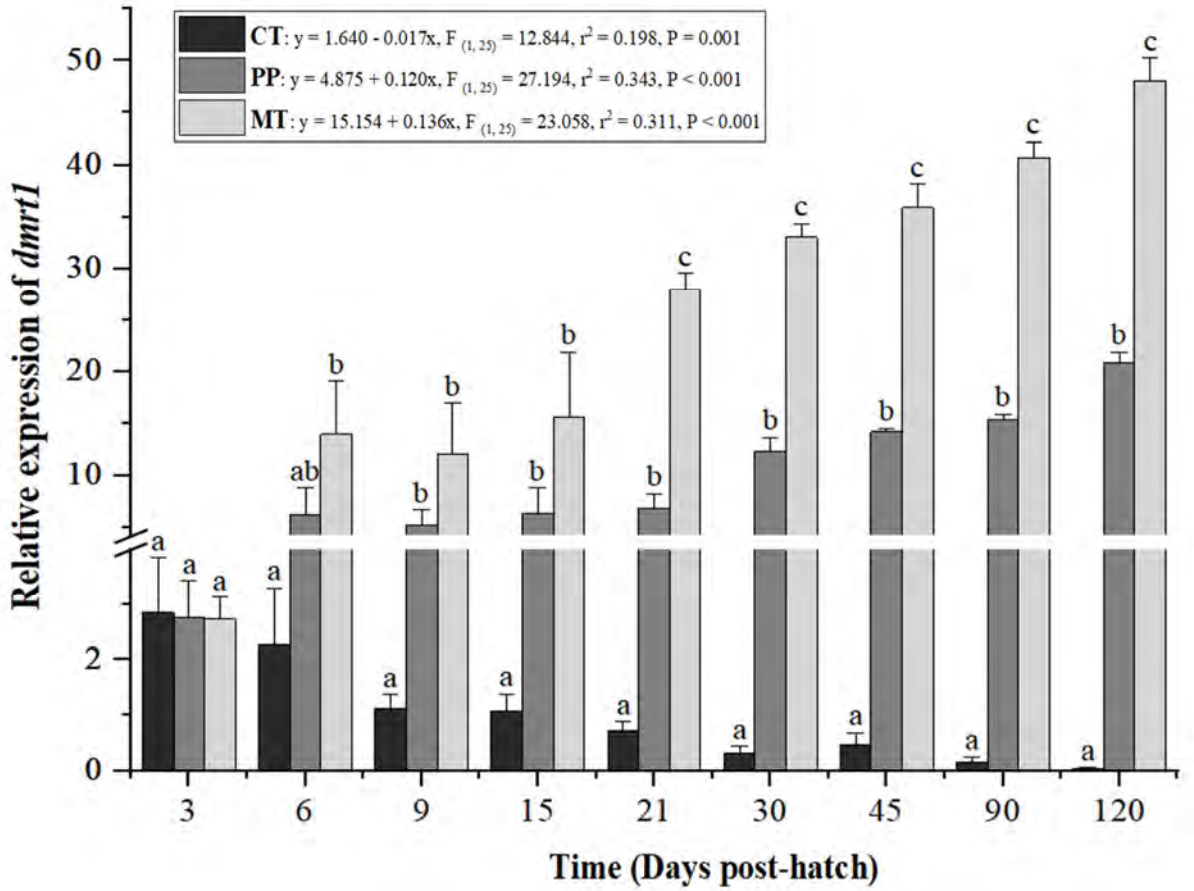


Figure 3.6: Mean (± standard error) relative expression of doublesex and mab-3 related transcription factor (*dmrt1*) in the trunks (3 to 21 dph) and gonads (30 to 120 dph) of Nile tilapia fed: 1) only basal diet (CT); 2) the same basal diet supplemented with 1,280 mg of PP kg^{-1} (PP); or 3) the same basal diet supplemented with only 60 mg of 17α-methyltestosterone hormone (MT) kg^{-1}. Each bar represents the mean of the normalised gene expression ratio of six individuals on each sampling day. Different superscript letters above the error bars indicate significant differences in gene expression among treatments in the same time interval (Tukey's HSD post-hoc, $P < 0.05$). The regression model equations indicate the change in the expression of *dmrt1* as a function of time for each treatment.

The expression levels of *amh* in the CT group and PP-treated fish were not significantly different, in the early developmental period, from 3 to 9 dph. However, significantly higher expression levels of the gene were observed in the PP treatment from 15 to 120 dph (Tukey's HSD post-hoc: $P < 0.05$; Figure 3.7). Compared to the fish from the CT and PP treatments, the MT-treated fish had higher expression of *amh* by 6 dph, and the transcript remained higher throughout the experiment (Tukey's HSD post-hoc: $P < 0.05$; Figure 3.7).

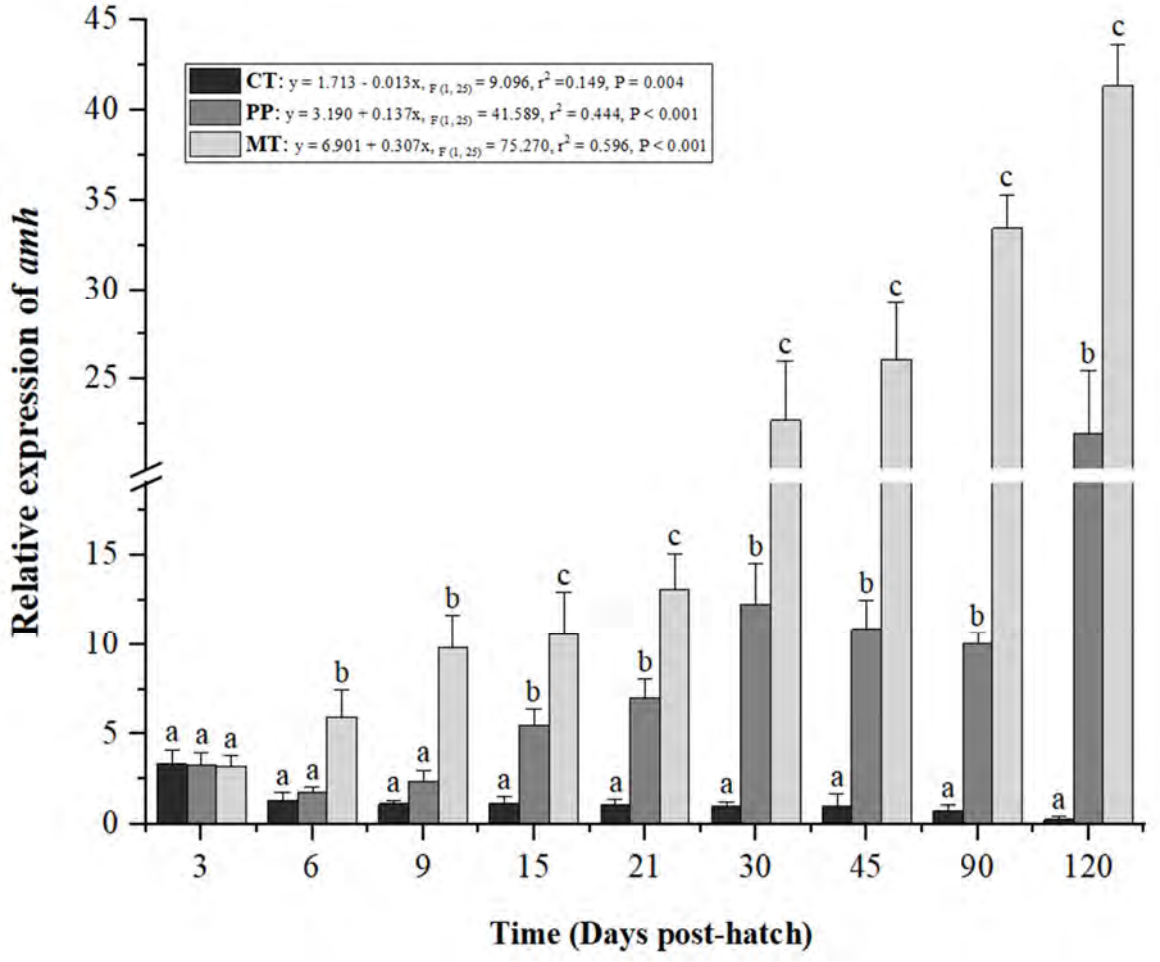


Figure 3.7: Mean (± standard error) relative expression of anti-müllerian hormone (*amh*) in the trunks (3 to 21 dph) and gonads (30 to 120 dph) of Nile tilapia. The fish were fed only a basal diet (CT), or the same basal diet supplemented with 1,280 mg of PP kg^{-1} (PP), or the same basal diet supplemented with only 60 mg of 17α-methyltestosterone hormone (MT) kg^{-1}. Each bar represents the mean of six individuals' normalised gene expression ratio on each sampling day. Different superscript letters above the error bars denote significant differences in gene expression among treatments in the same time interval (Tukey's HSD post-hoc, $P < 0.05$). The regression model equations describe the change in the expression of *amh* over time for each treatment.

Expression profiles of female-specific genes

The expression levels of *cyp19a1a* and *foxl2* were significantly influenced by the interaction between dietary treatment and time, with the expression changing differently over time among the treatments (RM-ANOVA; *cyp19a1a*: $F_{(16, 48)} = 4.330$, $P < 0.001$; Figure 3.8 and *foxl2*: $F_{(16, 48)} = 8.377$, $P < 0.001$; Figure 3.9). Overall, the genes were up-regulated in the non-treated fish (CT group) and down-regulated in PP and MT-treated fish throughout the experimental

period. The expression pattern of *cyp19a1a* and *foxl2* increased linearly in the fish from the CT treatment from 3 until 120 dph (*cyp19a1a*: $y = 3.502 + 0.085x$, $F_{(1, 25)} = 33.237$, $r^2 = 0.390$, $P < 0.001$; Figures 3.8 and *foxl2*: $y = 4.316 + 0.093x$, $F_{(1, 25)} = 30.609$, $r^2 = 0.371$, $P < 0.001$; Figure 3.9). A 4.0-fold increase in the expression of *cyp19a1a* was observed from 3 to 30 dph, compared to 1.8 from 30 to 120 dph. For *foxl2*, 2.3 and 1.8-fold increases were displayed during the same periods, respectively. However, a consistent decreasing pattern in the expression of the genes, which remained low throughout the study period, was observed in PP-treated fish (*cyp19a1a*: $y = 1.177 - 0.013x$, $F_{(1, 25)} = 17.347$, $r^2 = 0.250$, $P < 0.001$; Figure 3.8 and *foxl2*: $y = 1.586 - 0.018x$, $F_{(1, 25)} = 14.252$, $r^2 = 0.215$, $P < 0.001$; Figure 3.9). A similar trend in the expression of the same genes was observed in the MT-treated fish (*cyp19a1a*: $y = 0.582 - 0.007x$, $F_{(1, 25)} = 6.287$, $r^2 = 0.110$, $P < 0.001$; Figure 3.8 and *foxl2*: $y = 1.139 - 0.013x$, $F_{(1, 25)} = 5.804$, $r^2 = 0.102$, $P = 0.020$; Figure 3.9). A 33.5-fold decrease in the expression of *cyp19a1a* in the MT group from 3 to 30 dph, compared to a 3.7-fold decrease in the PP treatment during the same period (Figure 3.8). Likewise, *foxl2* was down-regulated by a 165.3 and 59.1-fold decrease in the MT and PP treatments during the same period (Figure 3.9). Furthermore, *cyp19a1a* was attenuated by PP between 30 to 120 dph, with a 25.6-fold decrease compared to 6.7 in the MT-treated fish. However, a 2.4-fold decrease in *folxl2* was observed in the two treatments from 31 to 120 dph.

No significant differences were observed in the expression levels of *cyp19a1a* in the fish from the CT and PP treatments from 3 to 9 dph. However, the transcript levels were significantly up-regulated in the fish from the CT treatment than in the PP-treated ones, from 15 to 120 dph (Tukey's HSD post-hoc: $P < 0.05$; Figure 3.8). In the MT-treated fish, *cyp19a1a* was significantly down-regulated and remained lower compared to the CT group from 6 to 120 dph (Tukey's HSD post-hoc: $P < 0.05$; Figure 3.8). Notably, the transcript expression was

significantly higher in the fish from PP treatment than in MT-treated ones from 6 to 45 dph (Tukey's HSD post-hoc: $P < 0.05$; Figure 3.8). Similar expression levels were, however, observed at 90 and 120 dph in both groups (Figure 3.8).

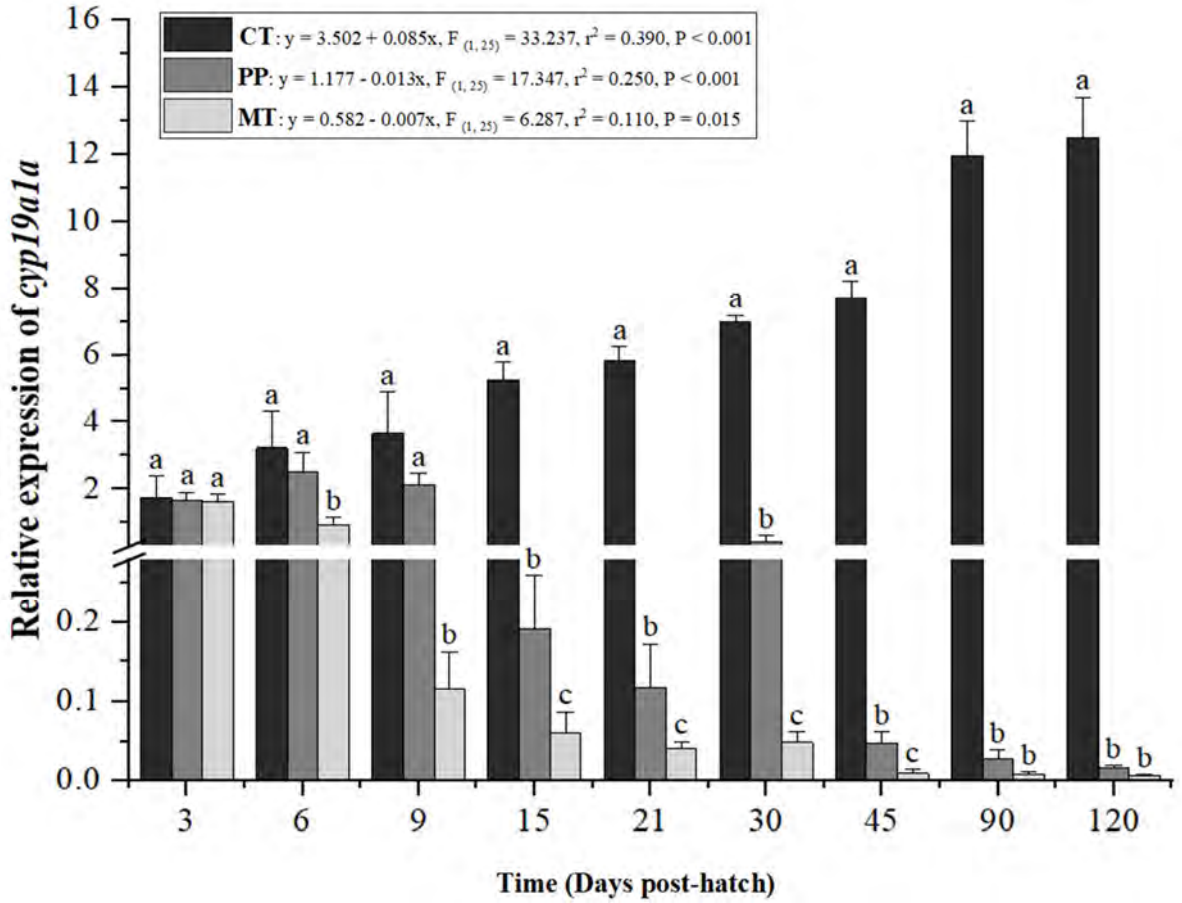


Figure 3.8: Mean (± standard error) relative expression of cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*) in the trunks (3 to 21 dph) and gonads (30 to 120 dph) of Nile tilapia. Fish were fed only a basal diet (CT), or the same basal diet supplemented with 1,280 mg of PP kg^{-1} (PP) or a basal diet complemented with 60 mg of 17α-methyltestosterone hormone (MT) kg^{-1} diet. Each bar represents the mean of the normalised gene expression ratio of six individuals on each sampling day. The different superscript letters above the error bars indicate significant differences among treatments ($P < 0.05$) in the same time interval (Tukey's HSD post-hoc, $P < 0.05$). The regression model equations describe the trend in the expression of *cyp19a1a* over time for each treatment.

The expression level of *foxl2* in the CT treatment was up-regulated throughout the experiment. However, the gene was down-regulated in the PP and MT-treated fish and remained significantly lower compared to the CT group from 9 to 120 dph (Tukey's HSD post-hoc:

P < 0.05; Figure 3.9). Notably, *foxl2* was significantly attenuated in the MT-treated fish than in the fish from the PP treatment from 21 and 30 dph (Tukey's HSD post-hoc: P < 0.05; Figure 3.9). However, from 45 to 120 dph, the gene was down-regulated in a similar manner by both PP and MT treatments (Figure 3.9).

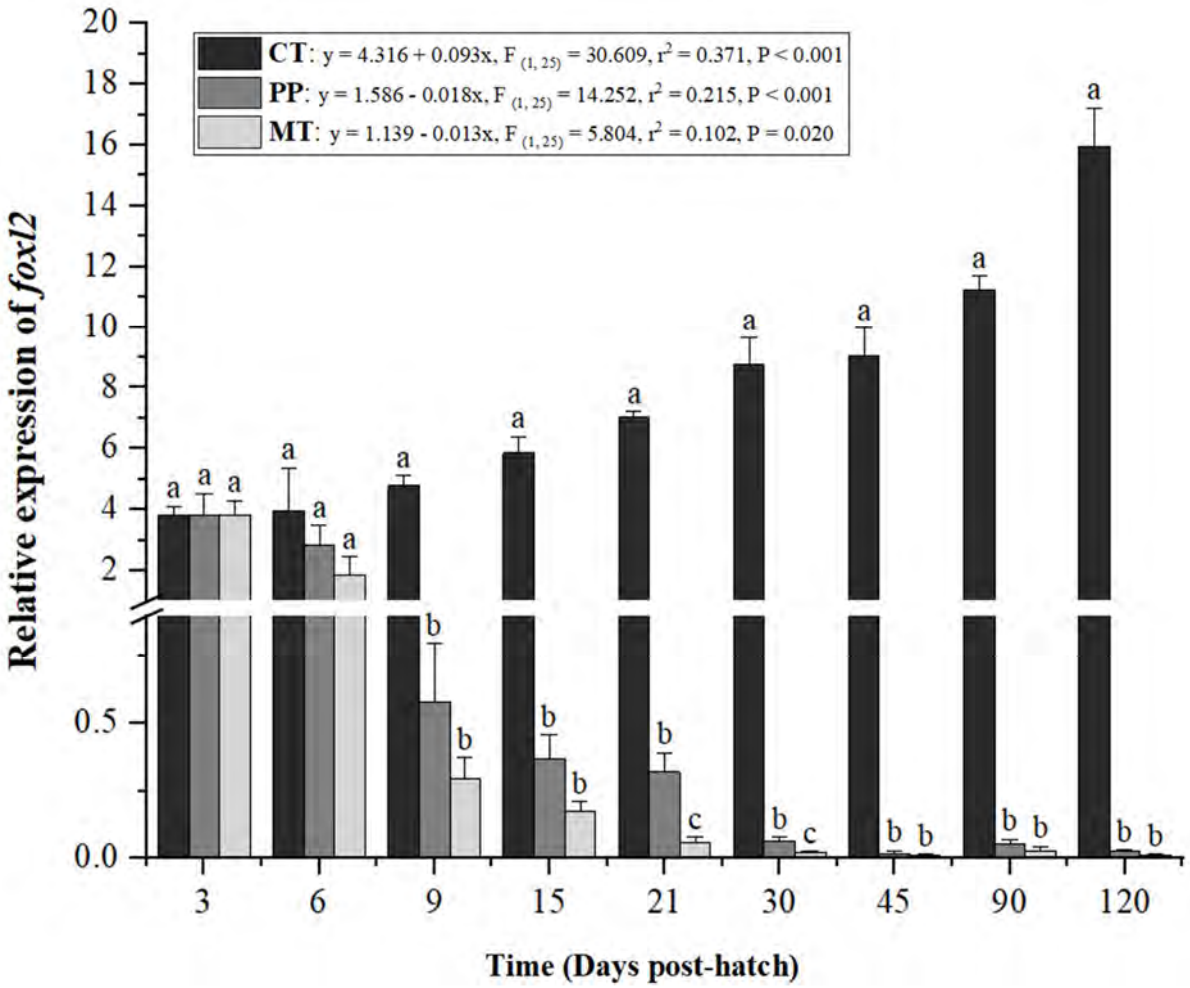


Figure 3.9: Mean (± standard error) relative expression of forkhead box protein L2 (*foxl2*) in the trunks (3 to 21 dph) and gonads (30 to 120 dph) of Nile tilapia. The fish were fed only a basal diet (CT), or the same basal diet supplemented with 1,280 mg of PP kg^{-1} (PP), or the same basal diet supplemented with only 60 mg of 17α-methyltestosterone hormone (MT) kg^{-1}. Each bar represents the mean of six individuals' normalised gene expression ratio on each sampling day. Different superscript letters above error bars indicate significant differences in the expression of the gene among treatments in the same time interval (Tukey's HSD post-hoc, P < 0.05). The regression model equations indicate the trend in the expression of *foxl2* over time for each treatment.

3.4 Discussion

In an effort to define the molecular mechanism underlying pine pollen (PP)-induced sex inversion, temporal expression patterns of specific sex genes were evaluated in PP-treated all-female Nile tilapia. The fish were treated with PP and MT from 3 to 30 dph, corresponding with the sensitive period for gonadal differentiation (Nakamura *et al.* 1998, Kobayashi *et al.* 2000, Ijiri *et al.* 2008). Besides, at 3 dph, the gonads are undifferentiated and bipotential, hence can develop into either ovaries or testes (Ijiri *et al.* 2008, Baroiller *et al.* 2009). In addition, differential expression of sex genes occurs during the early stages of gonadal development in Nile tilapia to program sex differentiation (Ijiri *et al.* 2008, Tao *et al.* 2018, Teng *et al.* 2021). Prior to and during gonadal differentiation, the male and female sex genes act antagonistically, and the prevailing network determines the ultimate sex of the fish (Ijiri *et al.* 2008, Poonlaphdecha *et al.* 2013, Pfennig *et al.* 2015, Tao *et al.* 2018, Lu *et al.* 2022). However, exogenous hormonal treatment interrupts the gene network and, eventually, the sex differentiation process (Pfennig *et al.* 2015, Todd *et al.* 2016, Dai *et al.* 2021). As such, the present study explored the effect of PP, in comparison with MT, on two male-biased genes: *dmrt1* and *amh*, and two female-related sex genes, *cyp19a1a* and *foxl2*. The genes were purposively selected since they are vital in gonadal differentiation and maintenance of phenotypic sex (Ijiri *et al.* 2008, Herpin and Schartl 2011b, Li *et al.* 2013, Lindeman *et al.* 2015, Lin *et al.* 2017, Zhang *et al.* 2017, Dai *et al.* 2021, Nagahama *et al.* 2021) In the early stages of gonadal development, from 3 to 21 dph, the experimental fish were small, and excision of tiny gonads was challenging. Hence, RNA was extracted from trunks containing gonads since the expression patterns of target genes in both gonads and trunks are not significantly different (Poonlaphdecha *et al.* 2013).

The expression of genes was low during the early stages of sex differentiation and increased or decreased based on the prevailing sex direction. The observation points to the role of genes in directing the process of gonadal differentiation and maintaining the gonadal phenotype (Nagahama 2005, Dai *et al.* 2021, Rajendiran *et al.* 2021). The developmental status of the gonad may also have contributed to the observed differences in gene expression (Ijiri *et al.* 2008). Moreover, previous studies reported consistent dimorphic expression of sex genes during the sex differentiation of Nile tilapia (Ijiri *et al.* 2008, Liu *et al.* 2022, Lu *et al.* 2022).

The expression of *dmrt1* and *amh* was higher in PP and MT-treated fish, while *cyp19a1a* and *foxl2* were elevated in the CT group. The up-regulation of male-specific genes and the down-regulated of female-related ones were consistent with the proportion of male individuals in the PP and MT treatments. Meanwhile, only females were present in the CT group, corresponding with higher levels of female-related genes. Therefore, *dmrt1* and *amh* were responsible for male sex development, while *cyp19a1a* and *foxl2* were crucial for female sex characteristics (Ijiri *et al.* 2008, Eshel *et al.* 2014, Teng *et al.* 2020b, Nagahama *et al.* 2021). The trends in gene expression are consistent with previous studies where the up-regulation of *dmrt1* and *amh* in Nile tilapia was male sex-specific, with a suppressive effect on female sex genes (Ijiri *et al.* 2008, Kobayashi *et al.* 2008, Eshel *et al.* 2014, Zhang *et al.* 2017, Tao *et al.* 2018). In addition, increased expression of male sex genes during gonadal differentiation of Nile tilapia induced masculinisation, while the suppression of the same genes triggered feminisation (Wang *et al.* 2010, Li *et al.* 2015, Dai *et al.* 2021, Lu *et al.* 2022). Besides, once *cyp19a1a* and *foxl2* are repressed or knocked out, female-to-male sex change occurs (Wang *et al.* 2007, Ijiri *et al.* 2008, Guiguen *et al.* 2010, Lau *et al.* 2016, Zhang *et al.* 2017).

Since the expression pattern of *dmrt1* and *amh* coincided with a higher proportion of males, the genes were needed to switch sex differentiation towards testes (Marchand *et al.* 2000,

Piferrer and Guiguen 2008, Matson *et al.* 2011, Liu *et al.* 2022). A sharp increase in the expression of *dmrt1* and *amh* from 15 to 30 dph in the PP and MT treatments in the present study corresponded with the sensitive period for testicular differentiation in Nile tilapia (Ijiri *et al.* 2008, Melo *et al.* 2019). Similarly, the significant elevation of *cyp19a1a* and *foxl2* in the CT treatment, which had only female individuals, suggests the pivotal role of the genes in ovarian differentiation and maintenance of the female phenotype (Wang *et al.* 2007, Ijiri *et al.* 2008, Guiguen *et al.* 2010, Zhou *et al.* 2021, Lu *et al.* 2022).

Compared to the MT treatment, an increasing trend of the male sex-specific genes was observed in the PP-treated fish. However, the expression levels of the genes were generally higher in the MT treatment. The high *dmrt1* and *amh* in the MT-treated fish could have arisen from the stable nature of MT. The hormone is less prone to aromatisation and hence more bioavailable to the fish, unlike the steroids in PP, particularly T (Schmit *et al.* 1980, Pawlowski *et al.* 2004, Attardi *et al.* 2008, Fragkaki *et al.* 2009). Thus, the effect of MT on the sex differentiation gene network, and the associated high masculinisation potency, is more significant compared to PP. Previous studies on zebra fish (*Danio rerio*) (Lee *et al.* 2017) and medaka (*Oryzias sakaizumii*) (Horie *et al.* 2016) also observed higher potency of MT with significant up-regulation of *dmrt1* and *amh*, accelerated spermatogenesis and masculinisation of the gonads. Similar results were obtained in XX progeny of mandarin fish (*Siniperca chuatsi*) (Zhu *et al.* 2022), triploid rainbow trout (*Oncorhynchus mykiss)* (Xu *et al.* 2021) and protogynous, orange-spotted grouper (*Epinephelus coioides*) (Wang *et al.* 2017a), following MT treatment. Also, since PP treatment was less effective compared to MT in inducing masculinization, some of the fish obtained for gene expression analysis, especially in the early stages of gonadal development, could have been still females. Therefore, a dilution effect

arising from ovarian RNA may have occurred, resulting in low expression of *dmrt1* and *amh* in PP-treated fish.

For the sexual fate of fish to be determined, an appropriate sex-specific gene network has to be initiated and the opposing sex-specific network repressed (Herpin and Schartl 2011b). The antagonistic behaviour among sex genes programs sex differentiation and gonadal development in fish. In the present study, an inverse relationship between male and female sex-specific genes was observed. The levels of *dmrt1* and *amh* were negatively correlated to *cyp19a1a* and *foxl2*, while at the same time, strong positive correlations were displayed between expression patterns of *dmrt1* and *amh* and for *cyp19a1a* and *foxl2*. The results point to an antagonistic interaction between male and female promoting pathways, where the prevailing network ultimately determines the direction of sex differentiation and gonadal development. For example, *dmrt1* interacts antagonistically with *foxl2* to suppress the expression of *cyp19a1a*, which in turn inhibits estrogen production, hence activating the male sex development (Wang *et al.* 2010, Li *et al.* 2013, Lindeman *et al.* 2015, Webster *et al.* 2017, Dai *et al.* 2021). In contrast, *foxl2* can also activate *cyp19a1a* by binding to the gene promoter region, as well as interacting with steroidogenic factor 1 (*sf1*), consequently triggering ovarian differentiation (Nagahama 2005, Wang *et al.* 2007, Yamaguchi *et al.* 2007, Guiguen *et al.* 2010, Kobayashi *et al.* 2013, Fan *et al.* 2019, Ortega-Recalde *et al.* 2020, Dai *et al.* 2021).

In the present study, female individuals were still present after exposure of all-female Nile tilapia progeny to PP, between 3 and 30 dph. Although sex change is more responsive to exogenous hormonal treatment in sexually undifferentiated tilapia, previous studies reported a second window for sex reversal, where already differentiated individuals undergo sex change

(Paul-Prasanth *et al.* 2013, Shi *et al.* 2017). An antagonism effect is reported to continue to exist between the male and female sex networks after gonadal differentiation, hence allowing for sexual plasticity (Gao *et al.* 2009, Takatsu *et al.* 2013, Sun *et al.* 2014, Shi *et al.* 2017, Li *et al.* 2019). The second window for sex change is designated as secondary sex reversal (Uchida *et al.* 2004, Iwamatsu *et al.* 2006, Komatsu *et al.* 2006). Earlier studies obtained higher proportions of male individuals after feeding Nile tilapia (Nian *et al.* 2017) and African catfish (*Clarias gariepinus*) (Adenigba *et al.* 2017) on PP-supplemented feeds for 56 and 72 days, respectively, confirming sex change beyond 30 dph. Therefore, future studies should extend the PP treatment of Nile tilapia to examine whether higher masculinisation results are achieved.

Androgens such as MT and T induce female-to-male sex inversion by suppressing aromatase activity while activating androgen nuclear receptors, hence down-regulating and up-regulating female and male sex genes, respectively (Mor *et al.* 2001, Kobayashi *et al.* 2003, Baron *et al.* 2007, Navarro-Martín *et al.* 2009, Wang *et al.* 2010, Golan and Levavi-Sivan 2014, Li *et al.* 2015, Zheng *et al.* 2016, Golshan *et al.* 2019). Both MT and PP followed a similar regulation pattern of the sex genes, implying a similar mechanism of sex inversion. The presence of steroids in PP could be responsible for the observed effect on the expression of genes (Abaho *et al.* 2022b). Like MT, the steroids in PP, such as T, may have inhibited estrogen synthesis by attenuating the expression of *cyp19a1a* and *foxl2* or acted via the androgen receptors to reinforce the expression of male sex genes, hence triggering female-to-male sex inversion. The *cyp19a1a* encodes the aromatase enzyme, which catalyses the conversion of androgens to estrogens to induce an estrogen environment (Nakamura *et al.* 2003, Guiguen *et al.* 2010, Li *et al.* 2019, Cai *et al.* 2022). Once the gene is suppressed, estrogen production, mainly 17β-estradiol (E2), is decreased. Consequently, the sex steroidal balance is disrupted, with excess androgens, which retards ovarian development and ultimately stimulates masculinisation

(Piferrer and Blázquez 2005, Bhandari *et al.* 2006, Guiguen *et al.* 2010, Siegfried 2010, Liu *et al.* 2012, Li *et al.* 2013, 2019, Wang *et al.* 2022). In contrast, increased expression of *cyp19a1a* and *foxl2* in the CT amplified E2 production, hence inhibiting male-specific genes from causing female phenotype differentiation and development (Masuyama *et al.* 2012, Jiang *et al.* 2016, Shi *et al.* 2017, Zhang *et al.* 2020, Zhou *et al.* 2021). Owing to the critical role of sex steroids, mainly 17β-Estradiol (E2) and 11-ketotestosterone (11-KT), in the sex differentiation of Nile tilapia, examining the relationship between changes in the expression of sex genes and the steroids after PP treatment, which was investigated in Chapter 4, is vital. The findings would provide further information on the role of PP in the sex inversion of Nile tilapia.

3.5 Conclusion

The present study provides the first description of the molecular mechanism underlying PP-induced sex inversion in Nile tilapia. This research provides preliminary information on how PP modifies the expression of sex genes responsible for phenotypic sex differentiation in Nile tilapia. Pine pollen follows a mechanism similar to MT in masculinising Nile tilapia by up-regulating and down-regulating the expression of male and female sex-related genes, respectively. Notably, the disruption in the expression of sex genes alters sex steroid levels in fish and consequently induces sex change. Therefore, studies on how PP modifies the endocrine environment of Nile tilapia during female-to-male sex inversion are crucial to corroborate the observed changes in gene expression. The findings will provide additional information towards understanding the role of PP in the sex masculinisation of fish. The following thesis chapter (Chapter 4) investigated the changes in sex steroid profiles and gonadal histology of Nile tilapia fed PP-supplemented diets.

CHAPTER 4

Sex steroid profiles and gonadal histology of Nile tilapia (*Oreochromis niloticus*) during pine pollen-induced masculinisation

4.1 Introduction

In teleosts, the sex steroids, mainly androgens and estrogens, are vital in various reproduction processes such as sex differentiation, sex change, gametogenesis, as well as maintenance of phenotypic sex (Yamamoto 1969, Nakamura *et al.* 1998, 2003, Devlin and Nagahama 2002, Nagahama *et al.* 2021, Zhou *et al.* 2021). The sex hormones are biosynthesised by steroid-producing cells prior to sex differentiation to facilitate the testicular or ovarian transformation of the undifferentiated gonad (Nakamura and Nagahama 1985, 1989, Hines *et al.* 1999, Strussmann and Nakamura 2002). As such, the changes in steroid-producing cells and concentrations of endogenous androgens and estrogens are evident before and during gonadal morphological differentiation (Yamamoto 1969, Feist *et al.* 1990, Nakamura *et al.* 1998, Hines *et al.* 1999).

The steroids are primarily biosynthesised in the leydig and theca cells of the testes and ovaries, respectively, as well as the non-gonadal tissues such as the liver and kidney (Rajakumar and Senthilkumaran 2020). During steroidogenesis, cholesterol is converted to pregnenolone and thereafter, various steroids through a series of gene actions (Golshan *et al.* 2019, Rajakumar and Senthilkumaran 2020, Meng *et al.* 2021, Sharma *et al.* 2022). One of the critical genes involved in the steroidogenic pathway is cytochrome P450 family 19 subfamily a polypeptide 1 (*cyp19a1a*), which encodes aromatase enzyme. The enzyme converts androgens into estrogens, i.e., testosterone to 17β-estradiol (Godwin *et al.* 2003, Guiguen *et al.* 2010, Chen *et al.* 2019,

Cai *et al.* 2022). Therefore, *cyp19a1a* is essential in the steroid synthesis cascade, especially in directing gonadal development in teleosts (Guiguen *et al.* 2010, Nakamoto *et al.* 2018, Li *et al.* 2019). The expression of *cyp19a1a* is regulated by the antagonism between forkhead box L2 (*foxl2)* together with doublesex and mab-3 related transcription factor 1 (*dmrt1)* and anti-müllerian hormone (*amh)* (Wang *et al.* 2007, 2010, Herpin and Schartl 2011b, Li *et al.* 2013, Sacchi *et al.* 2016). Therefore, *dmrt1* and *foxl2* also play a vital role in sex-specific steroid synthesis by regulating the production of either estrogens or androgens. In general, a shift in the expression of the sex genes alters the sex steroid profiles and, subsequently, the sex differentiation process (Li *et al.* 2013, Rajakumar and Senthilkumaran 2020, Bhat *et al.* 2021, Sharma *et al.* 2022).

Among the estrogens, 17β-estradiol (E2) is vital in the differentiation and maintenance of the ovaries of the fish. At the same time, androgen, 11-ketotestosterone (11-KT), plays a similar role in the testicular tissues (Borg 1994, Baroiller *et al.* 1999, Guiguen *et al.* 1999, Devlin and Nagahama 2002, Godwin *et al.* 2003, Jiang *et al.* 2016, Zhou *et al.* 2021). Testosterone (T), an androgen, acts as a precursor of 11-KT and E2. The hydroxylation of T by 11β-hydroxylase (*cyp11c1*) and 11β-hydroxysteroid dehydrogenase 2 (*hsd11b2*) produces 11-KT and yields E2 when aromatised (Wang and Orban 2007, Guiguen *et al.* 2010, Wang *et al.* 2017a). The conversion of androgens to estrogens is regulated by the activity of the aromatase enzyme, which is also controlled by the *cyp19a1a*. Therefore, the balance in bio-conversion rates of T to either 11-KT or E2 influences the process of differentiation and gonadal development. Male individuals usually exhibit higher levels of 11-KT, while substantial concentrations of E2 are displayed in females (Baroiller *et al.* 1999, Devlin and Nagahama 2002, Barannikova *et al.* 2004, Sandra and Norma 2010, Todd *et al.* 2016, Gennotte *et al.* 2017, Yarmohammadi *et al.* 2017).

Earlier research observed the disruption of the endogenous androgen-to-estrogen balance after exogenous treatment of fish with steroids before and during gonadal differentiation. The treatments altered phenotypic sex development, with no change to the genetic sex of the fish (Wang *et al.* 2010, 2022, Baroiller and D'Cotta 2016). In the production of all-male Nile tilapia (*Oreochromis niloticus*), androgens are frequently utilised to induce female-to-male sex change (Pandian and Kirankumar 2003, Srisakultiew and Kamonrat 2013, Baroiller and D'Cotta 2019, Teng *et al.* 2020a, Wang *et al.* 2022). Unlike in natural sex differentiation, during steroid-induced masculinisation of tilapia, the androgens stimulate testicular differentiation (Shi *et al.* 2012, Golan and Levavi-Sivan 2014). The androgenic activity of the steroids is promoted via interaction and activation of androgen receptors (Golan and Levavi-Sivan 2014, Zheng *et al.* 2016) or inhibition of the aromatase activity (Mor *et al.* 2001, Bhandari *et al.* 2006, Paul-Prasanth *et al.* 2013, Takatsu *et al.* 2013, Golan and Levavi-Sivan 2014, Lee *et al.* 2017). Androgen receptor interaction involves the synthetic androgen binding to and activation of an androgen receptor. The activated receptor functions as a transcription factor, which facilitates the expression of the target gene in response to the androgen, subsequently initiating masculinisation (Carson-Jurica *et al.* 1990, O'Malley and Tsai 1992, Golshan *et al.* 2019). This is facilitated by the synthetic androgens' structural and binding affinity similarities and the natural androgen receptor ligands, i.e., T and 11-KT (Pandian and Sheela 1995, Fragkaki *et al.* 2009). In contrast, aromatase inhibition averts the aromatisation of androgens to estrogens, thereby depleting estrogen levels in the differentiating gonad, eliciting female-to-male sex inversion (Guiguen *et al.* 1999, Uchida *et al.* 2004, Baron *et al.* 2007, Sun *et al.* 2014). Both mechanisms disrupt the androgen-to-estrogen balance, with decreased E2 production while amplifying androgens. Consequently, ovarian development is overridden, while the development of male sex characteristics is promoted (Takatsu *et al.* 2013, Todd *et al.* 2016, Li *et al.* 2019). As such, fish treatment with androgens enhances the levels

of endogenous male steroids, which act on the primordial germ cells to induce testicular development (Pandian and Sheela 1995, Andersen *et al.* 2003, Celik *et al.* 2011, Leet *et al.* 2011, Baroiller and D'Cotta 2019, Snake *et al.* 2020). However, the exposure of the fish to estrogens structures gonadal germ cells development to phenotypic females (Pandian and Sheela 1995, Baroiller and D'Cotta 2001, Devlin and Nagahama 2002, Alcántar-Vázquez *et al.* 2015, Marin-Ramirez *et al.* 2016).

In Chapter 2, the treatment of Nile tilapia with pine pollen (PP) induced sex masculinisation. Also, the male and female sex differentiation genes were down-regulated and up-regulated, respectively, after exposure of the fish to PP in Chapter 3. Notably, the sex-differentiating genes regulate the steroidogenic process of fish. Once the expression of the genes is altered, sex steroid production is interrupted, subsequently directing the development of an appropriate sex phenotype (Wang *et al.* 2010, 2022, Li *et al.* 2013, Shi *et al.* 2017). However, no study has investigated the effect of PP on sex steroids during and after the sex differentiation process in Nile tilapia. Therefore, to further understand the role of PP in masculinisation, changes in sex steroid profiles and gonadal histological were investigated in the current chapter. The study examined the changes in three primary sex steroids: T, 11-KT, and E2 in all-female fish fed a basal diet supplemented with PP from 3 to 30 days post-hatch (dph) and thereafter fed only basal diet to 120 dph. The widely utilised 17α-methyltestosterone hormone (MT) for producing all-male Nile tilapia was used as a positive control since the mechanism for sex inversion by the androgen is known (Golan and Levavi-Sivan 2014, Wang *et al.* 2017a, 2022).

4.2 Materials and methods

4.2.1 Experimental system, fish husbandry, and dietary treatments

The experimental data were collected from all-female Nile tilapia progeny, produced and reared in a similar production system and conditions, as described in Section 3.2.2, Chapter 3. The fish were subjected to the same dietary treatments, following the same feeding protocol (Section 3.2.3, Chapter 3). The dietary treatments included: 1) basal diet only (CT treatment), 2) the same basal diet supplemented with 1,280 mg PP kg^{-1} (PP treatment), and the same basal diet supplemented with 60 mg MT kg^{-1} (MT treatment).

4.2.2 Sampling protocol

Six randomly selected fish per age group, i.e., 3, 6, 9,15, 21, 30, 45, 90, and 120 dph (Section 3.2.3, Chapter 3), were sampled from each of three replicate tanks for each treatment (i.e., 18 fish per treatment, per sampling time). The samples were used to determine steroidal profiles and changes in the gonadal histology of the fish. Extracting gonads from fish on 3, 6, 9, 15, 21, and 30 dph was difficult because the samples were tiny. As such, body trunks containing the gonads were used after the incision of the head and tail. However, the gonads were harvested from the fish on 45, 90, and 120 dph. For histological analysis, the trunks and gonads were immersed in Bouin's solution and stored at room temperature. The samples for steroid analysis were stored at -30 ºC.

4.2.3 Histology

Sample preparation and processing

The histological samples were prepared and processed at the central diagnostic laboratory (CDL) at the College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Uganda. Before processing, the samples were removed from the fixative solution, cut, and loaded into tissue cassettes ready for processing. The cassettes were immersed in two changes of 50% ethanol to remove the remaining Bouin solution from the tissues.

Next, the samples were dehydrated by immersion in ascending ethanol series with varying concentrations from 70 %, 80 %, 90 %, 95 %, and finally, in three changes of absolute ethanol, each for one hour (Wang *et al.* 2021). After dehydration, the tissues were removed from alcohol and cleared in three xylene changes, each for one hour. The cleared tissues were impregnated with two changes of molten paraffin wax at a temperature of 56 ^{o}C for two hours in each bath of wax. Sample dehydration, clearing, and infiltration were done using an automated benchtop tissue processor (LEICA TP1020, Germany). The impregnated tissues were embedded in fresh paraffin wax using an embedding machine (SLEE Medical, Germany), left to cool, and cross-sectioned using a rotary microtome (LEICA RM2235, Germany) at 4.0 µm. The sections were floated on a water bath (Leica HI 1210, Germany) at 45 ^{o}C, picked up with a pre-coated slide with glycerine albumen, and labelled appropriately. The tissues were then dried overnight in a hot air oven at 53 ^{o}C. De-waxing the sections in two changes of xylene for two minutes each was conducted, followed by hydration in two changes of 100 %, 95 %, and 80 % ethanol and finally immersed in distilled water. Staining was then done using Mayer's haematoxylin for 15 min. The sections were blued and left under running tap water

for 15 min. Thereafter, counter-staining in 1 % eosin was done for two minutes, dehydrated in alcohol, and cleared in xylene for two minutes each. Finally, the cover slides were mounted onto slides using Dibutylphthalate Polystyrene Xylene (DPX) mounting medium.

Examination of slides

The stained sections were examined and classified as ovary, testis, or ovotestis under a light compound microscope (Nikon Eclipse c*i,* China). Using the NIS Elements BR 3.0 computer program (Driver Nikon Ds-U1, version 5.547), the images on the monitor were enlarged for analysis. Photomicrographs were taken using a digital camera (Nikon Digital Sight, Japan) mounted onto a microscope. The developmental stages of the gonad tissues were classified based on Bancroft's histological criteria (Suvarna *et al.* 2019).

4.2.4 Sex steroid extraction and assays

Sample preparation and steroid extraction

The preparation of samples for hormone extraction and analysis was carried out from the analytical biosciences laboratory at COVAB, Makerere University, Uganda. Freshly stored (- 30 °C) trunks and gonads were homogenised in a 1.5 mL tube using a pestle, one at a time to prevent cross-contamination. Then 0.5 mL double distilled water was added to each homogenised sample, which was thoroughly mixed and stored at -30 °C until hormone extraction.

Sex steroid extraction

The organic solvent extraction technique was utilised for both T and 11-KT (Rougeot *et al.* 2007). The sample (1 mL) was mixed with 4 mL of ethyl acetate and hexane solvent mixture and centrifuged (Eppendorf™ Centrifuge 5702, Germany) at 1500 revolutions per minute (rpm) for 30 min. The mixture was thereafter frozen on ice, and the ether solution pipetted into a clean tube. Ether extraction on the thawed aqueous phase was repeated for each sample. Each sample's top layer of ether solution was combined to achieve maximum extraction efficiency. The collected ether solution was dried for 30 min using a water bath at 50 ºC to form an ether residue. A mixture of pentane and 80 % methanol in water was added to the residue in a ratio of 1:1, vortexed for 10 min, and the solvent mixture was transferred to a separating funnel, stoppered with a glass stopper. The solvent mixture was allowed to stand overnight at -30 ºC to allow maximum dissolution of the steroids into pentane and thereafter collected in glass-capped vials. Methanol was evaporated, and the pentane layer was reconstituted in 15 mL of 70 % ethanol. The extracted steroids were dissolved in 0.5 mL of the ELISA buffer (Cayman Chemical Co., USA) and stored at -80 ºC until the assay was done.

Similarly, 17β-estradiol (E2) was extracted following the organic solvent principle (Rougeot *et al.* 2007). The homogenate of each sample was thoroughly mixed with absolute methanol in a ratio of 4:1, and the mixture incubated at room temperature for 10 min. The solvent mixture was centrifuged at 1500 rpm for 15 min, and the resulting supernatant was transferred to a clean tube and evaporated at 70 ºC using a water bath for 10 min. The extracted steroids were reconstituted with 300 µL ELISA buffer (Cayman Chemical Co., USA) and stored at -80 ºC until the assay was done.

Sex steroid assays

Before the assays, caution was taken to ensure no organic solvent was present in each sample. The levels of gonadal T, 11-KT, and E2 were measured using enzyme-linked immunosorbent assay (ELISA) kits; 582701, 582751, and 501890, respectively (Cayman Chemical Co., USA). For T, the cross-reactivities reported by the manufacturer were: 140 % with 19-nortestosterone, 100% with T, 27.4 % with 5α-dihydrotestosterone, 18.9 % with 5β-dihydrotestosterone, 4.7 % with methyl testosterone, 3.7 % with androstenedione, 2.2 % with 11-KT, 0.51 % with 5-androstenediol and < 5 % with all other steroids. The cross-reactivities with 11-KT were: 100 % with 11-KT, 2.9 % with adrenosterone and < 5 % with all other steroids, while E2 cross-reactivities are 100 % with E2, 2.5 % with methoxyestradiol, 2.3 % with estradiol 3-(β-D-Glucuronide), 1.38 % with estrone, 1.3 % with 2-hydroxyestradiol, 1% with estriol, 0.7 % with estradiol benzoate, 0.53 % with estradiol-3-sulfate and < 5 % with all other steroids.

The concentration of steroids was determined according to the manufacturer's instructions of the test kit, taking into account each steroid's unique analysis steps. In particular, the duration of the development stage and wavelength of each steroid hormone were considered. The reconstituted ELISA buffer for each steroid was added to the non-specific binding (NSB) and maximum binding (Bo) wells of the corresponding ELISA kit plates in quantities of 100 µL and 50 µL, respectively. Then 50 µL of the ELISA standards for T4, 11-KT, and E2 was pipetted to the standard wells (S1 to S8) of each kit for the specific steroid, in descending order, beginning with S8 wells. The samples (50 µL) for each steroid were added to corresponding sample wells in duplicate, following the loading plan (Table 4.1). The specific AChE tracer (50 µL) for each target steroid was then added per well of the plate, except for the total activity (TA) and blank (Blk) wells. Finally, the ELISA antiserum (50 µL) per target steroid was added to each well, except for TA, NSB, and Blk wells. Both plates were covered with an aluminium

foil, placed on an orbital shaker, and incubated for 120 min at room temperature. After incubation, the wells of the plates were emptied and rinsed five times with 200 µL of wash buffer for T and 11-KT, while 300 µL of wash buffer was used for E2. Two hundred microliters of the respective Ellman's reagent were added to each well, while 5 µL of the tracer was only added to the TA wells. The plates were again covered with an aluminium foil and placed on an orbital shaker to allow development for 90, 120, and 60 min for 11-KT, T4, and E2 plates, respectively. After development, the plate cover was removed, and the absorbance of each well was determined using a microplate reader (BioTek Epoch 2 microplate Reader, USA). Both T4 and 11-KT were read at 405 nm wavelength and 414 nm for E2.

Table 4.1: Loading plan for each enzyme-linked immunosorbent assay kit used during the assays for testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E2).

Blk	S1	S1	1	5	9	13	17	21	25	29	33
Blk	S2	S2	1	5	9	13	17	21	25	29	33
NSB	S3	S3	2	6	10	14	18	22	26	30	34
NSB	S4	S4	2	6	10	14	18	22	26	30	34
Bo	S5	S5	3	7	11	15	19	23	27	31	35
Bo	S6	S6	3	7	11	15	19	23	27	31	35
Bo	S7	S7	4	8	12	16	20	24	28	32	36
TA	S8	S8	4	8	12	16	20	24	28	32	36

Blk: Blank, NSB: Non-specific binding, Bo: Maximum binding, TA: Total activity, S1-S8: Standards and 1-36: Samples.

Calculation of steroid hormone concentrations using a standard curve

Prior to the graphical analysis, values for each well were obtained by subtracting the average absorbance readings of the blanks from all the other wells. The average absorbance values for NSB wells were then subtracted from the average absorbance readings of Bo wells to get the corrected maximum binding (Bo). Equally, the same NSB values were subtracted from the absorbance values of each sample and standards to obtain sample or standard bound (B). The

ratio (B/Bo) of each sample and standard bound to the corrected maximum binding was obtained and linearised using a logit transformation, i.e., logit (B/Bo) = *ln* [B/Bo / (1 - B/Bo)]

A calibration curve of logit (B/Bo) values against the concentrations of standards was plotted, and a linear regression fit performed to generate the slope and y-intercept, i.e., y = a*ln* (x) + b, where; *a*= the slope (gradient) and *b*= the y-intercept. The linear equation obtained from the standard curve plot was used to calculate the concentration of the target steroid per sample. Samples with % B/Bo values greater than 80 % or less than 20 % were re-assayed since they were out of the linear range of the standard curve. For each steroid, one assay was done, and the mean intra-assay coefficients of variation were: 6.12 ± 1.54 %, 6.25 ± 1.25 %, and 7.20 ± 1.43 % for T, 11-KT, and E2, respectively.

4.2.5 Data analysis

The mean concentration ± standard error (SE) for each steroid was calculated for the three treatments. The differences in the concentration of steroids among the treatments over the experimental period were analysed using repeated measures analysis of variance (RM-ANOVA). When significant interactions between the main effects were obtained, Tukey's HSD post-hoc test was used to compare treatment means at all experimental periods. The differences between means were considered significant at $P < 0.05$ significance. The changes in the concentration of sex steroids as a function of time were analysed using linear regression. All statistical analyses were performed with Statistica® software, version 14.0.1 (TIBCO Software, Palo Alto, USA). The histograms were all generated using Origin 2022b software (OriginLab Corporation, USA).

4.3 Results

4.3.1 Gonadal histology

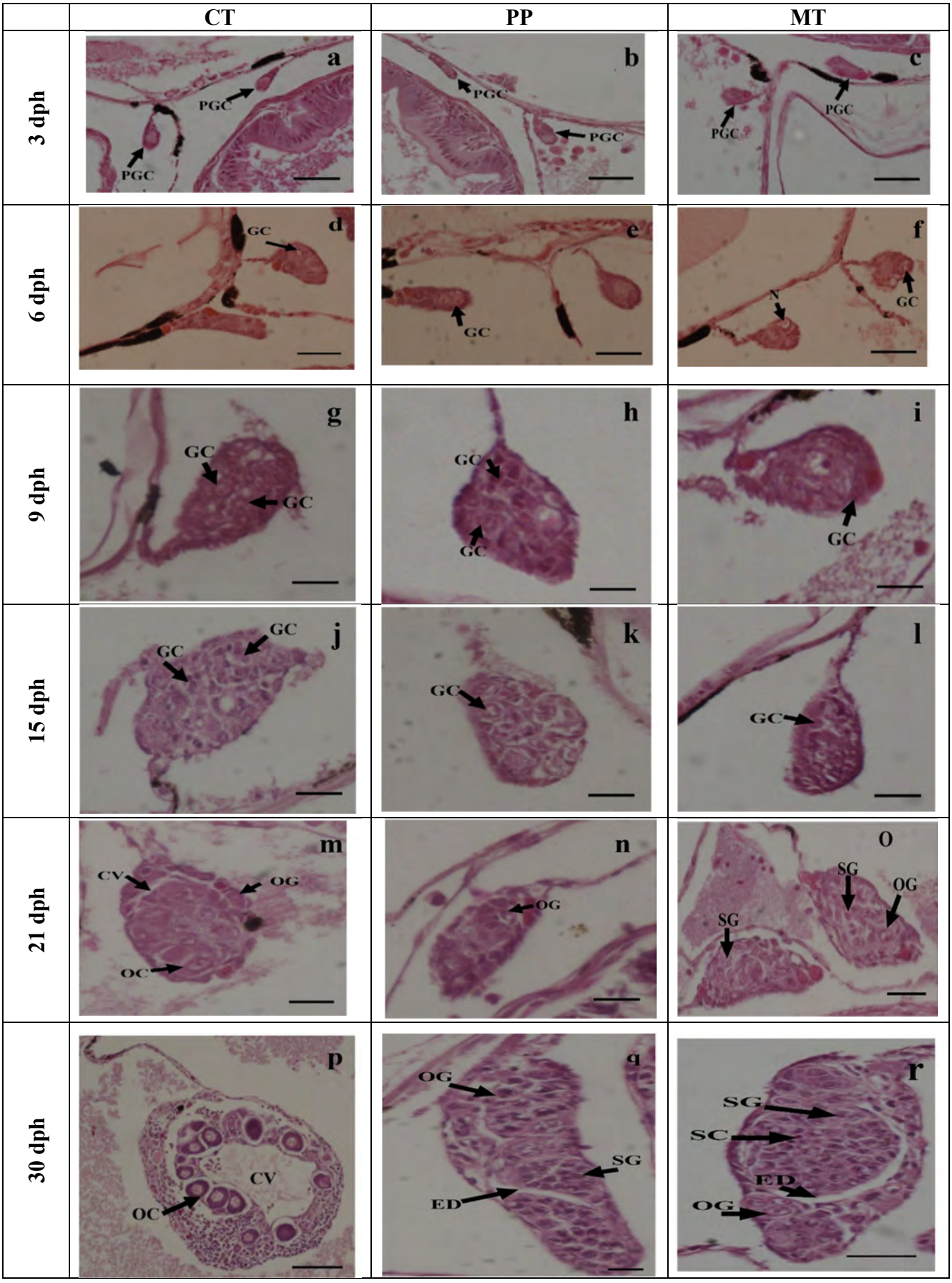
The histological changes in the gonads of all-female Nile tilapia were described from 3 to 120 dph (Figure 4.1). Before exposing fish to the treatments, at 3 dph, the gonads showed similar structures with no signs of differentiation, constituting primordial germ cells. In all treatments, the gonadal tissues appeared in the abdominal cavity as elongated thin filaments (Figures 4.1 a, b, and c). At 6 dph, no pronounced variations in histological features of the gonads were observed in all treatments. However, the paired arrangement of the gonads became more distinct (Figures 4.1 d, e, and f). The gonadal tissues were suspended into the coelomic cavity, extending caudally from the pectoral fins. Active multiplication of germ cells commenced at 9 dph (Figures 4.1 g, h, and i), and as a result, the cells were smaller compared to 3 and 6 dph. Enlargement of the gonads was also observed, consistent with increasing germ cell numbers. Furthermore, sexual dimorphism was displayed, where more germ cells were present in CT and PP treatments (CT: Figure 4.1 g and PP: Figure 4.1 h) than in the gonadal tissues obtained from the MT-treated fish. (Figure 4.1 i). Similarly, an increase in germ cell number and gonadal size was evident at 15 dph compared to the preceding stages. However, the PP and MT-treated fish showed a slow increase of germ cells compared to the CT treatment. As such, numerous germ cells were observed in the CT group than in the PP and MT treatments (Figures 4.1 j, k, and l).

The first signs of ovarian differentiation appeared in the CT treatment at 21 dph, with the initiation of ovarian cavity formation (Figure 4.1 m). The gonads had small, rounded oogonia with a spherical nucleus occupying the entire cell. The ovarian cavity formation appeared to begin from the anterior region of the gonad, proceeding to the caudal portion. Similarly,

female-to-male sex change commenced in the MT-treated fish at 21 dph, with the gonads undergoing ovarian-to-testicular differentiation (Figure 4.1 o). Although some ovarian tissues were still present in the gonads, the formation of spermatogonia was initiated. However, in the PP-treated fish, the gonads were still developing as ovaries by 21 dph, having only oogonial tissues. However, compared to the CT treatment, the gonads in the PP treatment were smaller, with fewer germ cells (Figure 4.1 n). At 30 dph, the structure of the ovary in the CT treatment was more precise, with a perfect construction of the ovarian cavity, showing normal ovarian differentiation. Also, an increase in oocyte number was observed (Figure 4.1 p). Female-to-male sex differentiation was also observed in PP-treated fish with the formation of the slit-like space for the formation of the efferent duct (Figure 4.1 q). The gonads were, however, still ovotestes, with both oocytes and spermatogonia. In the MT gonadal tissues, female-to-male sex differentiation was more prominent with lobular testicular tissues, although a few oocytes were still present in the gonads (Figure 4.1 r). The efferent duct became more distinct compared to 21 dph, and the proliferation of spermatocytes. At 45 dph, the gonads of fish from the CT comprised only fully developed oocytes, with increased ovarian volume (Figure 4.1 s). The gonads of PP-and MT-treated fish had sex inversed from females to males. In the PP treatment, the gonads constituted spermatogonia (Figure 4.1 t), while in the MT group, spermatogonia and spermatocytes were present (Figure 4.1 u). The appearance of testicular tissues followed the reduction in the germ cells in PP and MT treatments.

By 90 dph, sex differences were evident among the treatment groups. No sex inversion was observed in the CT treatment; hence, all fish possessed female gonadal tissues (Figure 4.1 v). The gonads from all the MT-treated fish were fully transformed into the testis, with definite testicular tissues at different stages of spermatogenesis. Spermatogonia, spermatocytes, and spermatids were present in the gonadal tissues (Figure 4.1 x). A similar scenario was observed

in the fish from the PP treatment (Figure 4.1 w), except for one individual, which was intersex, having both testis and ovarian features. The ovotestis individual comprised mainly spermatocytes and a few oocytes (Figure 4.2 a), while the non-feminised individuals in the same treatment had only ovarian tissues similar to the samples from the CT treatment. At the end of the experiment (120 dph), the gonadal cross-sections of the sampled fish showed the presence of the following phenotypes: a) all males with typical testes in MT treatment (Figure 4.1 z_2), b) 8 males (Figure 4.1 z_1) and 1 female (Figure 4.2 b) from the PP-treated fish, and c) all females with normal mature ovaries containing oocytes in the CT group (Figure 4.1 y). Notably, the testicular lobules of the PP-treated fish were dominated by spermatogonia and spermatids (Figure 4.1 z_1), compared to the males from the MT treatment whose gonads constituted mainly spermatids and spermatozoa (Figure 4.1 z_2). Generally, female-to-male sex inversion to males occurred between 21 and 45 dph in MT and PP-treated, while ovarian differentiation happened from 21 to 30 dph.


CT
PP
MT
3 dph
6 dph
9 dph
15 dph
21 dph
30 dph
a
b
c
d
e
f
g
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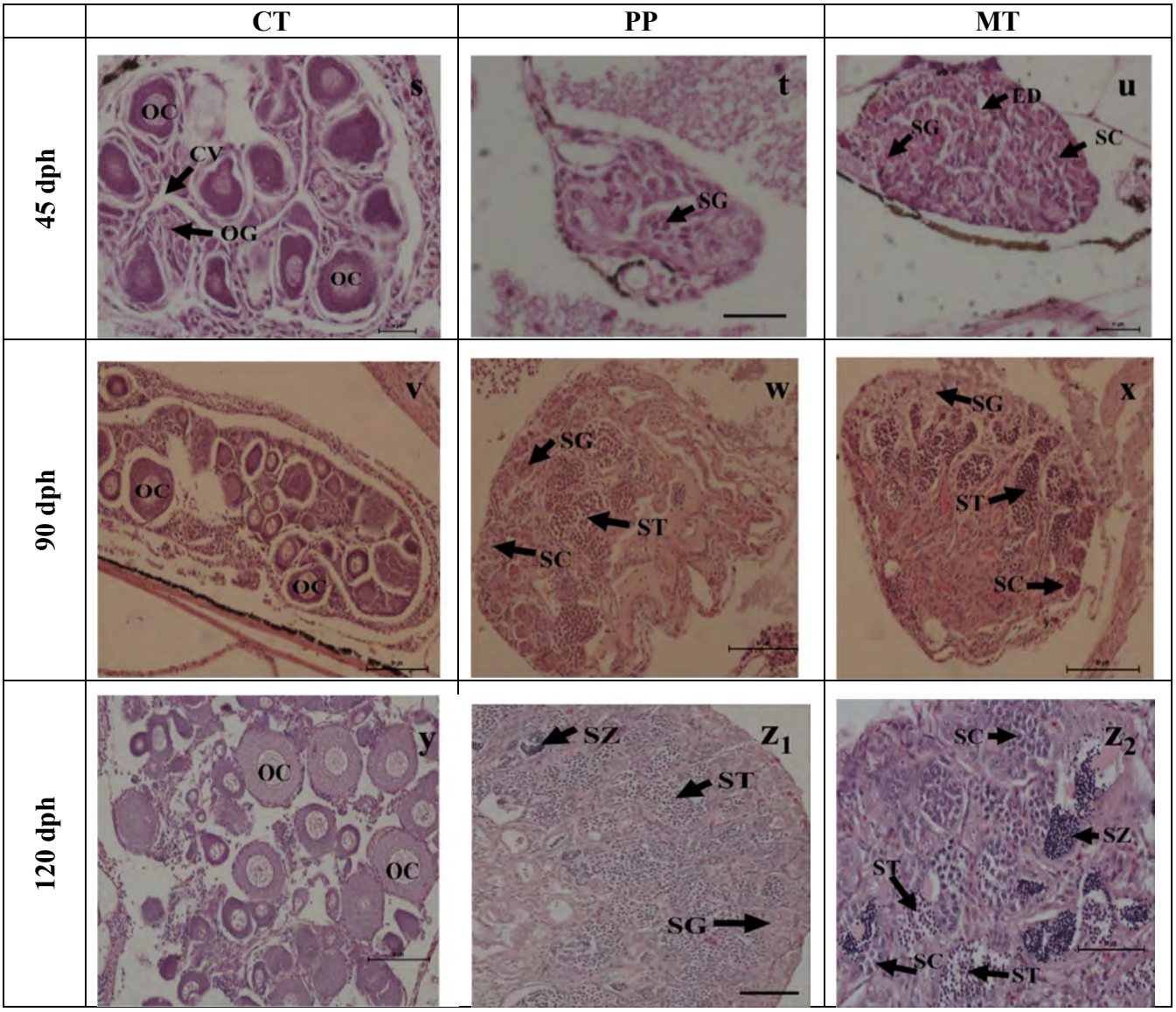

Figure 4.1: Changes in the gonadal histology of all-female Nile tilapia from 3 to 120 days post-hatch (dph). CT: fish fed only on basal diet from 3 to 120 dph; PP: fish fed a basal diet supplemented with 1,280 mg pine pollen kg^{-1} (PP) from 3 to 30 dph, and thereafter, the same basal diet from 31 to 120 dph; MT: fish fed the same basal diet supplemented with 60 mg of 17α-methyltestosterone (MT) kg^{-1} for 28 days from 3 dph, and thereafter a basal diet up to 120 dph. PGC: primordial germ cell; GC: germ cell; N: nucleolus; CV: ovarian cavity; OG: oogonia; OC: oocyte; ED: efferent duct; SG: spermatogonia; SC: spermatocyte; ST: spermatid; SZ: spermatozoa. Scale bar: a - u = 10 μm and v-z_2 = 50 μm.

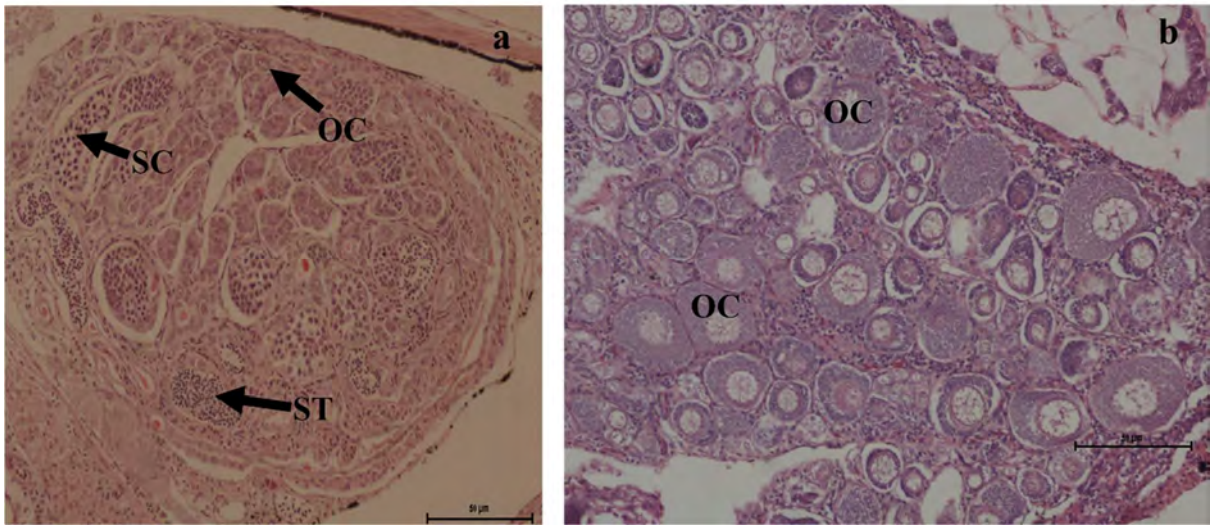

Figure 4.2: Gonadal histology of ovotestis and female individuals obtained from the pine pollen-treated fish at 90 (a) and 120 (b) days post-hatch, respectively. OC: oocyte; SC: spermatocyte; ST: spermatid. Scale bar: 50 μm.

4.3.2 Sex steroids

The concentrations of sex steroids from the gonadal tissues of Nile tilapia were variable among treatments throughout the experimental period (Figures 4.3 to 4.5). The E2 levels were generally higher in the gonads of fish obtained from the CT treatment. At the same time, E2 was consistently low in the PP and MT-treated fish (Figure 4.3). However, higher levels of 11-KT and T were largely displayed in the PP and MT treatments than for the CT group throughout the experimental period (11-KT: Figure 4.4 and T: Figure 4.5).

17β-estradiol

The concentrations of 17β-estradiol (E2) in Nile tilapia among the treatments were significantly influenced by an interaction between fish rearing period and dietary treatment (RM-ANOVA; E2: $F_{(16, 48)} = 5.338$, $P < 0.001$; Figure 4.3). The steroid displayed a significant positive relationship with time in the fish from the CT treatment ($y = 883.012 + 13.205x$, $F_{(1, 25)} = 25.584$, $r^2 = 0.506$, $P < 0.001$; Figure 4.3). However, a significant negative

relationship between E2 and time was observed in the fish from the PP ($y = 594.277 - 6.731x$, $F_{(1, 25)} = 13.830$, $r^2 = 0.349$, $P = 0.001$; Figure 4.3) and MT ($y = 445.609 - 5.156x$, $F_{(1, 25)} = 7.431$, $r^2 = 0.229$, $P = 0.012$; Figure 4.3) treatments. At the beginning of the experiment, no significant differences were observed in the concentrations of E2 among treatments (Tukey's HSD post-hoc: $P > 0.05$; Figure 4.3). From 3 to 6 dph, the steroid decreased in all treatments and thereafter, significantly increased in the fish from the CT group until 120 dph (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.3). However, E2 continuously decreased in the MT and PP treatments during the remaining experimental period, reaching the lowest levels at 120 dph (PP: 10.135 ± 2.168 pg mL^{-1} and MT: 8.956 ± 0.247 pg mL^{-1}; Figure 4.3). From 15 to 30 dph, the steroid was significantly depressed in the MT treatment compared to the PP-treated fish (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.3). However, E2 was also consistently suppressed in the PP-treated fish, reaching significantly similar levels as in MT treatment from 45 to 120 dph (Tukey's HSD post-hoc: $P > 0.05$). Within each treatment, a significant increment of E2 in CT group was observed from 15 to 45 dph, while the steroid significantly declined in the PP and MT-treated fish between 15 and 30 dph (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.3).

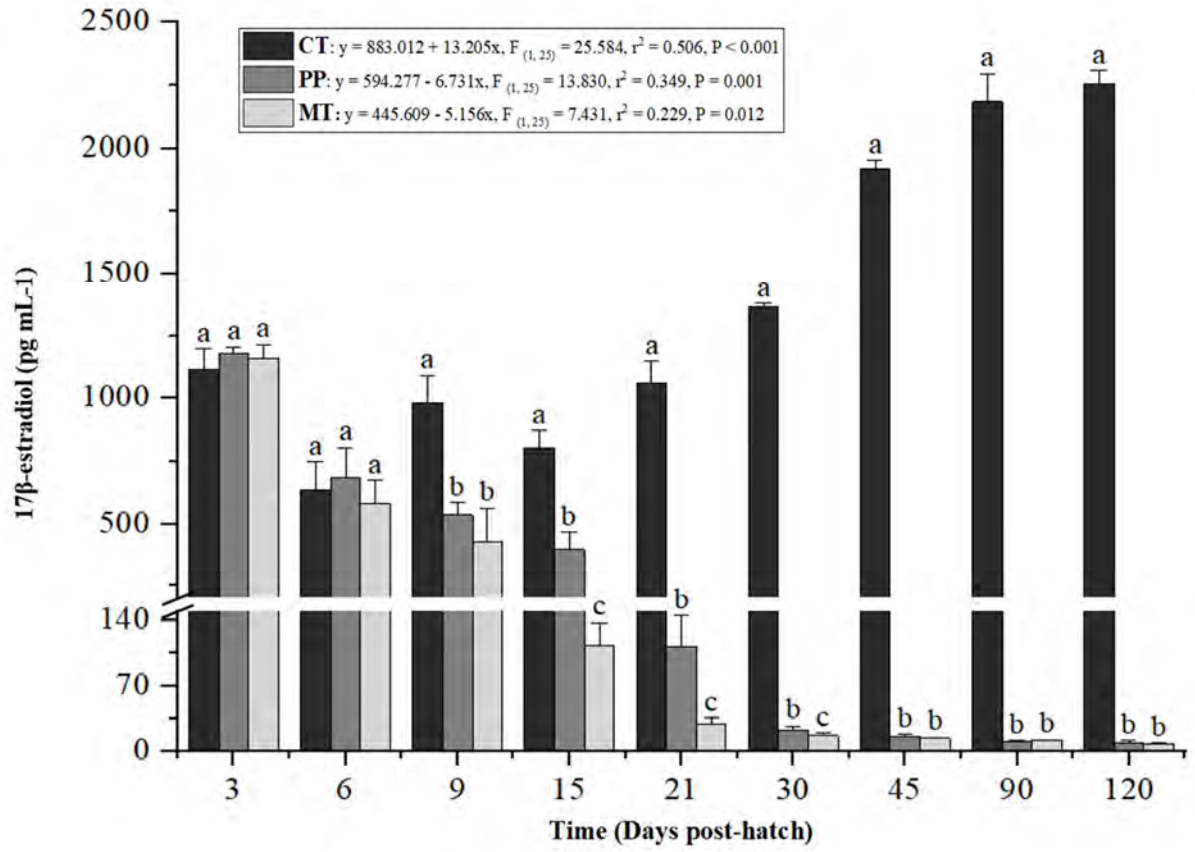

Figure 4.3: The changes in 17β-estradiol (E2) concentration (pg mL^{-1}) in gonadal tissues of Nile tilapia fed only basal diet (CT) or the same basal diet supplemented with 1,280 mg pine pollen kg^{-1} diet (PP) or the same basal diet supplemented with 60 mg 17α-methyltestosterone hormone kg^{-1} diet (MT). Data were expressed as the mean ± standard deviation (SE) for the nine fish samples from each treatment per sampling point. Different superscript letters above error bars indicate significant differences in the steroid concentration among treatments in the same time interval (Tukey's HSD post-hoc, $P < 0.05$). The regression model equations present the changes in E2 concentration as a function of time for each treatment.

11-ketotestosterone

The concentrations of 11-ketotestosterone (11-KT) among the treatments were significantly influenced by an interaction between the fish rearing period and dietary treatment (RM- ANOVA; 11-KT: $F_{(16, 48)} = 13.705$, $P < 0.001$; Figure 4.4). A significant positive relationship between the steroid concentration and time was observed in the PP ($y = 10.855 + 0.546x$, $F_{(1, 25)} = 92.133$, $r^2 = 0.787$, $P < 0.001$; Figure 4.4) and MT ($y = 19.036 + 0.743x$, $F_{(1, 25)} = 79.143$, $r^2 = 0.760$, $P < 0.001$; Figure 4.4) treatments up to 120 dph. However, no significant relationship between 11-KT and time was exhibited in the CT

treatment (Regression analysis; $F_{(1, 25)} = 0.309$, $P = 0.583$; Figure 4.4). The steroid has in the CT treatment, the steroid was significantly low throughout the experimental period and did not exceed 10.62 ± 2.27 pg mL^{-1}. In contrast, 11-KT levels steadily increased in PP and MT-treated fish, although the increment was faster in the MT treatment. The maximum steroidal levels were obtained in the MT-treated fish (93.72 ± 6.67 pg mL^{-1}) at 120 dph compared to 69.64 ± 7.14 pg mL^{-1} in the PP treatment.

In the initial stages of the experiment, i.e., from 3 to 9 dph, no significant differences were observed in 11-KT levels among the treatments (Tukey's HSD post-hoc: $P > 0.05$; Figure 4.4). However, by 15 dph, the MT-treated fish had significantly higher steroid levels than in the fish from the CT and PP treatments (Tukey's HSD post-hoc; $P < 0.05$; Figure 4.4). From 21 dph, a steady rise in 11-KT was observed in the PP treatment, and the steroid was significantly higher than in fish from the CT treatment until the end of the experiment (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.4). A faster increase of 11-KT was also observed in the MT-treated fish, with the steroid becoming significantly higher than in the fish from the PP group from 30 dph till the end of the experiment (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.4).

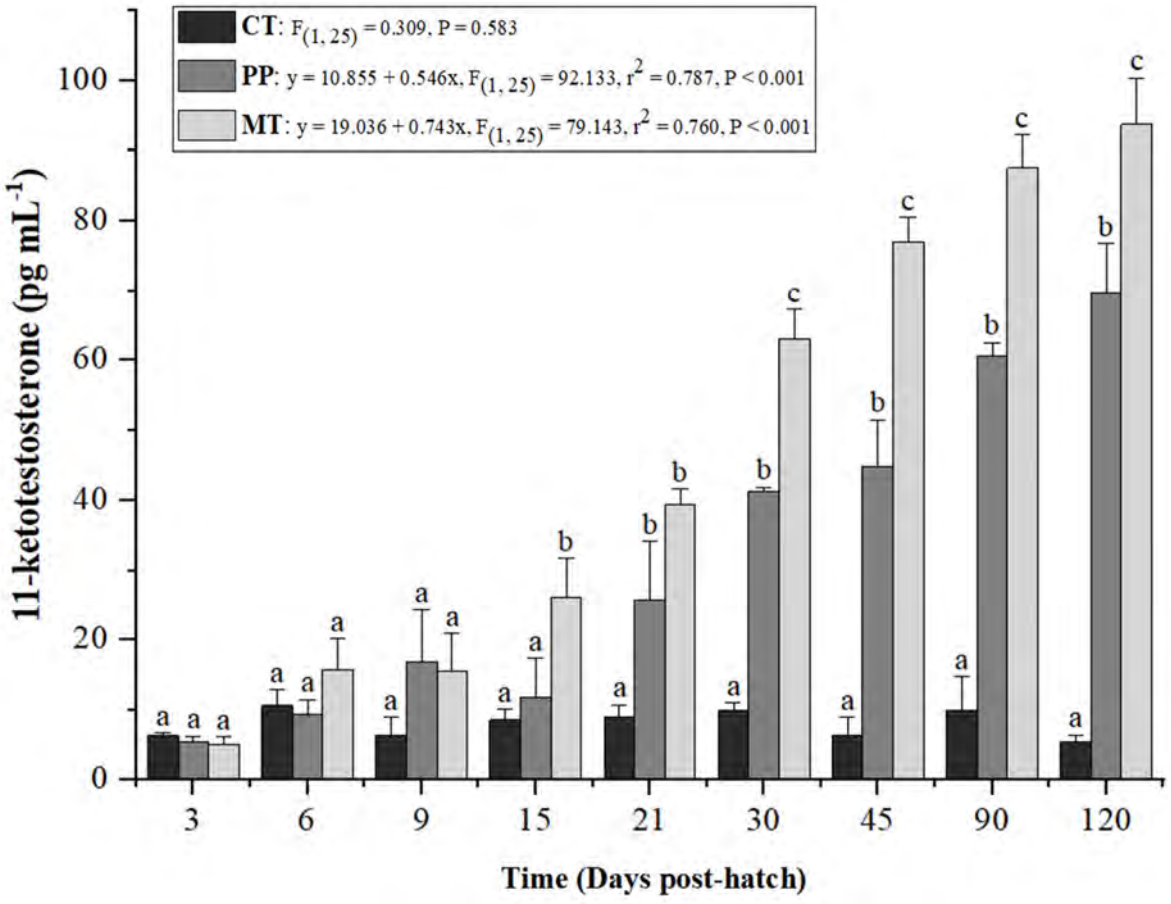

Figure 4.4: The 11-ketotestosterone (11-KT) concentration (pg mL^{-1}) in the gonadal tissues of Nile tilapia fed only a basal diet (CT) or the same basal diet supplemented with 1,280 mg pine pollen kg^{-1} diet (PP) or the same basal diet supplemented with 60 mg 17α-methyltestosterone hormone kg^{-1} diet (MT). Data were expressed as the mean ± standard deviation (SE) of the nine sampled individuals for each treatment per sampling point. The significant differences in the steroid concentration among treatments are indicated by different superscripts above error bars in the same time interval (Tukey's HSD post-hoc, $P < 0.05$). The regression model equations indicate the changes in 11-KT concentration as a function of time for each treatment.

Testosterone

The concentration of testosterone (T) in Nile tilapia among the treatments was influenced by a significant interaction between the fish rearing period and dietary treatment (RM-ANOVA; T: $F_{(16, 48)} = 2.636$, $P = 0.005$). In all the treatments, a significant positive relationship between the concentration of T and time was observed (CT: $y = 15.404 + 1.286x$, $F_{(1, 25)} = 75.291$, $r^2 = 0.751$, $P < 0.001$; PP: $y = 24.045 + 2.148x$, $F_{(1, 25)} = 71.893$, $r^2 = 0.742$, $P < 0.001$ and MT: $y = 106.551 + 2.984x$, $F_{(1, 25)} = 31.019$, $r^2 = 0.554$, $P < 0.001$; Figure 4.5). At the initial stage

of the experiment (3 dph), the levels of the steroid (T) did not significantly differ among the three treatments (Figure 4.5). Thereafter, T in the MT-treated fish increased and became significantly higher than in the CT and PP groups by 6 and 9 dph, respectively (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.5). Thereafter, the steroid in the MT treatment continuously increased and reached a maximum of 481.29 ± 23.42 pg m L^{-1} at 120 dph. The T concentration in the PP-treated fish also steadily increased from 30 dph and reached a significantly similar level as in the MT group from 45 to 90 dph (Tukey's HSD post-hoc: $P > 0.05$; Figure 4.5). However, at 120 dph, the same steroid became significantly higher in the MT-treated fish than in the ones exposed to PP (Tukey's HSD post-hoc: $P < 0.001$; Figure 4.5). In the CT treatment, the steroidal profile increased slowly from 6 dph and thus remained lower than in the MT-treated fish until 120 dph. The T level was similar in the PP and CT treatments, from 3 to 21 dph, and thereafter became significantly higher in the PP-treated fish until the end of the experiment (Tukey's HSD post-hoc: $P < 0.05$; Figure 4.5).

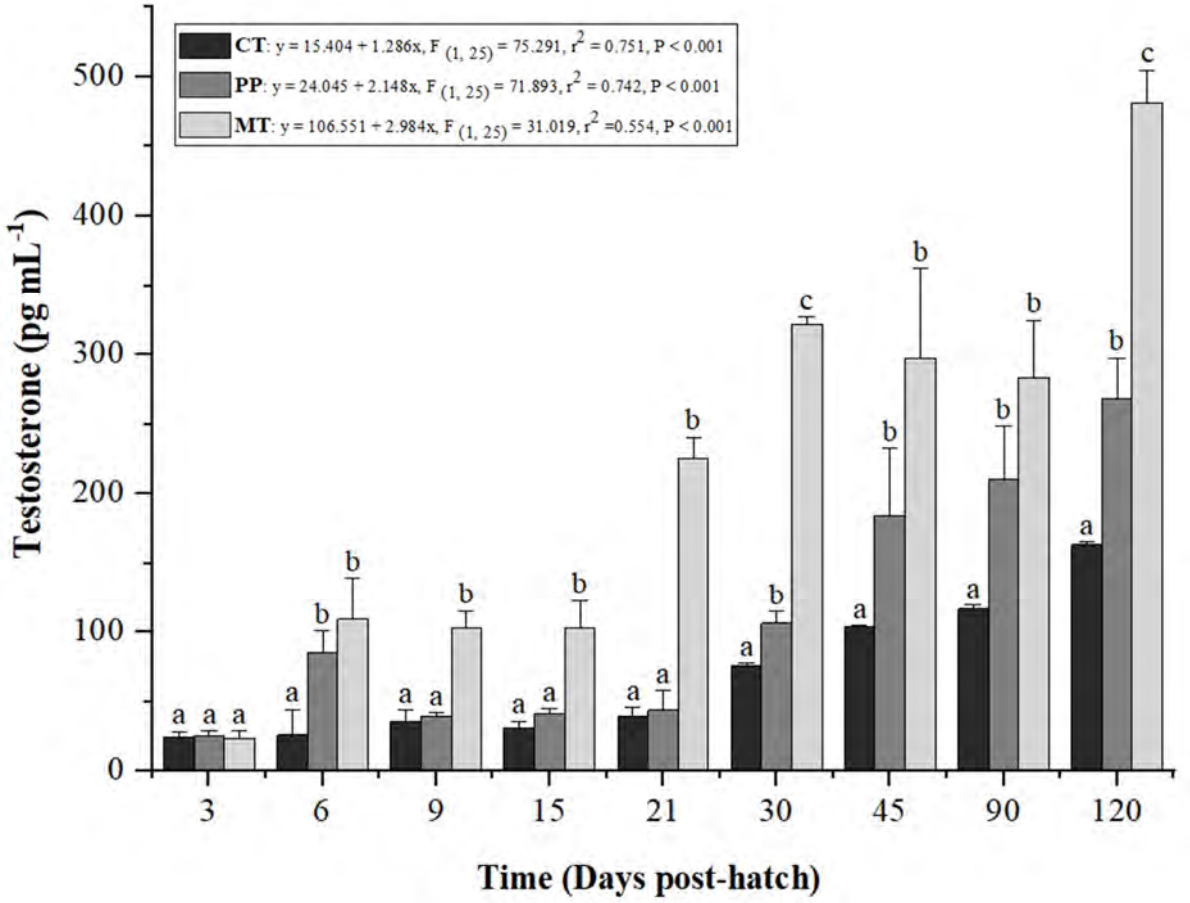

Figure 4.5: Testosterone concentration (pg mL^{-1}) of Nile tilapia fed only a basal diet (CT) or the same basal diet supplemented with 1,280 mg pine pollen kg^{-1} diet (PP) or the same basal diet supplemented with 60 mg 17α-methyltestosterone hormone kg^{-1} diet (MT). All data were expressed as the mean ± standard deviation (SE) for nine randomly sampled fish individuals per treatment at each sampling point. Different superscript letters above error bars indicate significant differences in the steroid concentration among treatments in the same time interval (Tukey's HSD post-hoc, $P < 0.05$). The regression model equations show the changes in the concentration of T over time for each treatment.

11-ketotestosterone to 17β-estradiol ratio

An increasing trend of the 11-ketotestosterone (11-KT) to 17β-estradiol (E2) ratio was observed in PP ($y = -0.458 + 0.064x$, $F_{(1,25)} = 456.990$, $r^2 = 0.979$, $P < 0.001$; Figure 4.6) and MT ($y = -0.261 + 0094x$, $F_{(1,25)} = 170.340$, $r^2 = 0.953$, $P < 0.001$; Figure 4.6) treatments. No significant relationship between the ratio and time was exhibited in the fish from the CT treatment (Regression analysis; $F_{(1,25)} = 1.099$, $P = 0.305$; Figure 4.6). Among the treatments, the 11-KT to E2 ratio was influenced by a significant interaction between dietary treatment and time (RM-ANOVA; $F_{(16, 48)} = 15.984$, $P < 0.001$; Figure 4.6). The ratio was higher in the PP

and MT-treated fish and lower in the CT fish for the entire period of the experiment. By 15 dph, the ratio was significantly higher in the MT-treated fish than in those from the CT treatment and remained so till 120 dph (Tukey's HSD post-hoc: $P < 0.05$). Similar findings were exhibited in the PP-treated fish from 30 to 120 dph (Tukey's HSD post-hoc: $P < 0.05$). Notably, a significant rise in the 11-KT/E2 ratio was displayed in both MT and PP treatments between 21 to 30 dph, from 0.324 ± 0.161 to 1.972 ± 0.372, and 1.450 ± 0.494 to 3.901 ± 0.895, respectively (Tukey's HSD post-hoc; MT: $P < 0.05$; Figure 4.6). Meanwhile, the ratio was significantly higher in the MT treatment than in the PP group from 21 dph until the end of the experiment (Tukey's HSD post-hoc; $P < 0.05$; Figure 4.6).

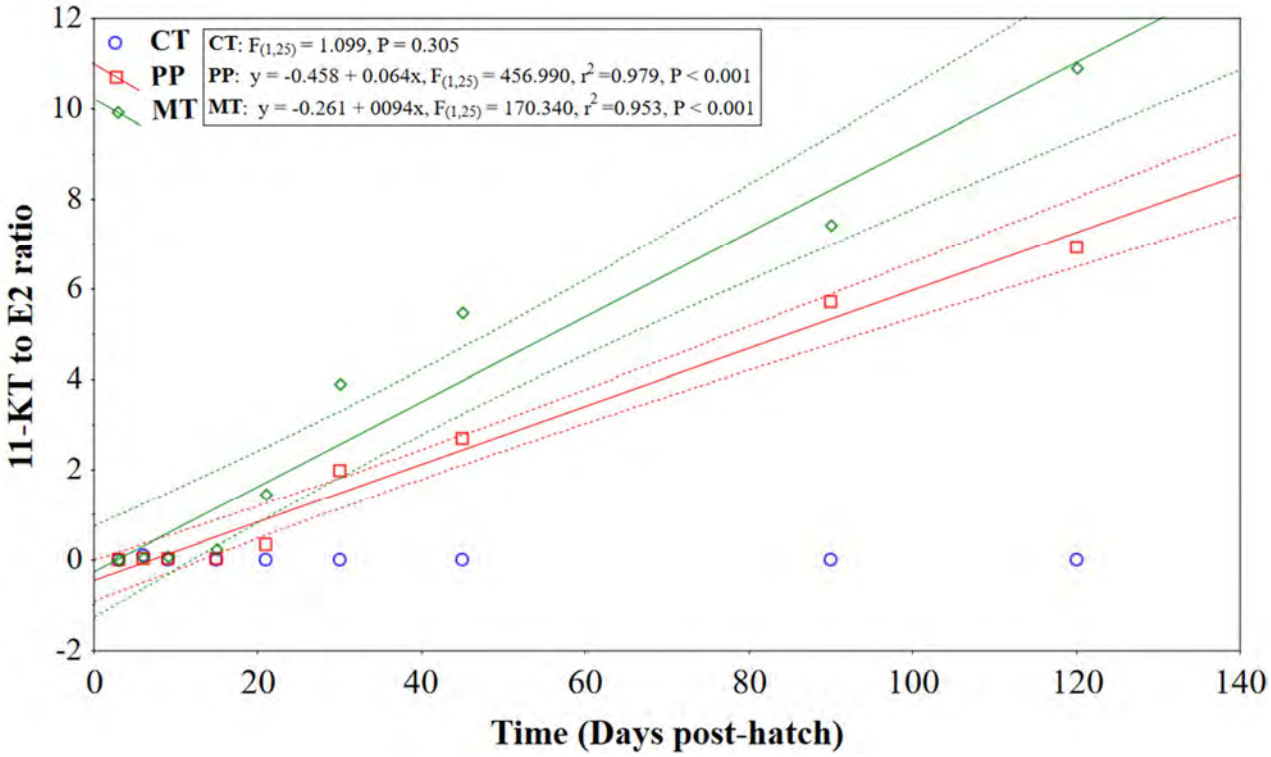

Figure 4.6: The change in the 11-ketotestosterone (11-KT) to 17β-estradiol (E2) ratio in the gonadal tissues of Nile tilapia over time. The fish were fed only on a basal diet (CT), or the same basal diet supplemented with 1,280 mg pine pollen kg^{-1} diet (PP) or the same basal diet supplemented with 60 mg 17α-methyltestosterone hormone kg^{-1} diet (MT). The dotted lines represent 95 % confidence bands.

4.4 Discussion

Bioactive compounds from plant extracts, such as saponins, flavonoids, alkaloids, and steroids, have been reported to induce female-to-male sex change in fish (Gabriel 2019, Khumpirapang *et al.* 2021, Abaho *et al.* 2022b). The phyto-compounds augment T and 11-KT, which are vital in the development of male sexual characteristics (Gharaei *et al.* 2020, Hassona *et al.* 2020, El-Kady *et al.* 2022). Therefore, various plant extracts are considered safe alternatives to synthetic hormones for sex control in aquaculture production systems (Abaho *et al.* 2022b). Amongst the phyto-extracts, pine pollen (PP) was found to induce masculinisation in Nile tilapia (Nian *et al.* 2017, Nieves 2017, Abaho *et al.* 2022a, Aziz *et al.* 2022; Chapter 2). The present study examined changes in the levels of endogenous sex steroids: T, 11-KT, and E2 and gonadal histology to understand how PP modifies the direction of sex differentiation in Nile tilapia.

Typically, testicular differentiation in fish is associated with a decrease in estrogen levels, followed by an increase in the concentration of androgens (Devlin and Nagahama 2002, Bhandari *et al.* 2003). Feeding Nile tilapia on diets supplemented with exogenous androgens results in a drop and rise in E2 and 11-KT, respectively, hence promoting masculinisation (Chen *et al.* 2016, Wang *et al.* 2022). The present study showed a general decrease in E2 levels, accompanied by an increase in T and 11-KT in the PP and MT treatments, where female-male gonadal transformation was observed. At the same time, the elevation of E2 and suppression of 11-KT were consistent with ovarian differentiation in all-female fish from the CT treatment. Therefore, the disruption of 11-KT and E2 levels was crucial in the gonadal differentiation of Nile tilapia, as was earlier reported (Paul-Prasanth *et al.* 2013, Sun *et al.* 2014, Chen *et al.* 2016, Wang *et al.* 2022). In non-treated fish, the increase of E2 and oocyte development implies a normal ovarian differentiation process. Besides, the concentration of

estrogen is naturally higher in females than males (Borg 1994, Baroiller *et al.* 1999, Guiguen *et al.* 1999, Devlin and Nagahama 2002, Godwin *et al.* 2003, Zhou *et al.* 2021).

The ovarian-to-testicular transformation in PP and MT-treated fish occurred between 21 to 45 dph, matching with the critical period for male sex differentiation in Nile tilapia (Ijiri *et al.* 2008, Kobayashi *et al.* 2013, Tao *et al.* 2018, Melo *et al.* 2019). Consistent with previous studies, gonadal differentiation involved the proliferation of testicular germ cells in PP and MT-treated fish, while oogonia and oocytes increased in the CT group (Nakamura and Nagahama 1985, 1989, Lu *et al.* 2022). Significant down-regulation of E2 and up-regulation of 11-KT also corresponded with the period for female-to-male sex inversion. The findings suggest estrogen repression and amplification of the androgens by PP and MT during the sensitive period for sex differentiation to induce masculinisation (Nakamura 1975, Kitano *et al.* 2000, Uchida *et al.* 2004, Bhandari *et al.* 2005, Iwamatsu *et al.* 2006, Komatsu *et al.* 2006, Thresher *et al.* 2011). Besides, E2 suppression in female tilapia triggered the differentiation of leydig cells, male-specific secondary sex characteristics, and sexual behaviour (Paul-Prasanth *et al.* 2013).

The *cyp19a1a*, which encodes aromatase enzyme, was down-regulated by PP and MT treatments in the previous chapter. Therefore, the attenuation of the estrogen in the PP and MT-treated fish in the present study could have been a consequence of suppressed aromatase activity. Exposure of fish to exogenous androgens down-regulates *cyp19a1a*, which consequently inhibits aromatase enzyme and, ultimately, E2 production (Fragkaki *et al.* 2009, Shi *et al.* 2012, Golan and Levavi-Sivan 2014). Similar results were obtained while utilising androgenic phytochemicals: saponins (Golan *et al.* 2008) and quassinoids (Low *et al.* 2013b,

2013a), where decreased E2 levels were due to the inhibition of estrogen synthesis by the aromatase enzyme. Furthermore, the down-regulation of *cyp19a1a* and inhibition of aromatase activity, followed by decreased and increased E2 and 11-KT levels, respectively, were observed during the masculinisation of Nile tilapia with MT, T, and Fadrozole (Paul-Prasanth *et al.* 2013, Golan and Levavi-Sivan 2014, Shi *et al.* 2017, Wang *et al.* 2022). Likewise, female-to-male sex change was accompanied by similar alterations in E2 and 11-KT for orange-spotted grouper (*Epinephelus coioides*) (Wu *et al.* 2015), red-spotted grouper (*Epinephelus akaara*) (Li *et al.* 2006) and longtooth grouper (*Epinephelus bruneu*) (Hur *et al.* 2012) after treatment with MT. Similarly, in honeycomb grouper (*Epinephelus merra*), T and 11–KT also increased, while E2 decreased after exposure of the fish to Fadrozole, an aromatase inhibitor (Bhandari *et al.* 2004, 2005).

Furthermore, the gonads of fish from the CT treatment remained ovary-like, with higher levels of E2, which also corresponded with the up-regulated levels of *cyp19a1a,* as was observed in Chapter 3. Therefore, the transformation of gonadal tissues to a male phenotype in PP and MT treatments resulted from decreased estrogen levels and a rise in the androgen concentration. Besides, a high 11-KT to E2 ratio was displayed in the PP and MT treatments, particularly between 21 to 45 dph, the period when ovarian and testicular differentiation was observed among the treatments. The ratio between 11-KT and E2 controls sex differentiation, where the excess steroid determines the direction of gonadal sex development, regardless of the genetically determined sex (Bogart 1987, Baroiller *et al.* 1999). Once fish is exposed to exogenous androgens and estrogens, the steroidal ratio is altered and, subsequently, the phenotypic sex. Therefore, the 11-KT to E2 ratio was key in controlling sex differentiation in Nile tilapia since notable differences were observed between the treatments,

Notably, the sex steroids in the current research were all detected among the treatments, as early as 3 dph, before the fish were exposed to PP and MT. The observation points to the maternal transfer of steroids, which consequently initiates the process of sex differentiation (Yeoh *et al.* 1996, Hines *et al.* 1999, Santi *et al.* 2019). In contrast, steroidogenesis may have already commenced by 3 dph since the steroid-producing cells are present in Nile tilapia before the onset of sexual differentiation (Nakamura and Nagahama 1989, Nakamura *et al.* 1993, Hines *et al.* 1999, D'Cotta *et al.* 2001). Besides, steroidogenic activity in the interrenal tissues has been reported to precede steroid synthesis in the gonads (Fitzpatrick *et al.* 1993). The tissues could also have been a source of steroids in the experimental fish in the present study. The sharp decrease of E2 from 3 to 6 dph in all the treatments could have been due to the metabolism of the initially derived maternal estrogen, during the early developmental stages, before and during yolk sac absorption. Previous studies also observed a decline in the levels of the same steroid in Nile tilapia (Rothbard *et al.* 1987, Hines *et al.* 1999), steelhead trout (*Oncorhynchus mykiss*) (Yeoh *et al.* 1996), and coho salmon (*Oncorhynchus kisutch*) (Feist *et al.* 1990), during embryonic and early development. In contrast, the gradual increase in T and 11-KT levels in PP and MT-treated fish during the same period, unlike the CT group, could have arisen from the accumulation of the androgens from the exogenous supply of diets supplemented with the PP and MT (Gennotte *et al.* 2017).

A significant drop in E2, which occurred earlier than the increase in 11-KT, was observed in the PP and MT-treated fish. Therefore, the decreased estrogen level could have triggered sex inversion to maleness, and the subsequent rise in androgens maintained the process. Similar findings were obtained in earlier studies, where the suppression of estrogen triggered testicular development in Nile tilapia (Guiguen *et al.* 1999, Paul-Prasanth *et al.* 2013, Chen *et al.* 2016). The general rise in 11-KT from PP and MT-treated fish, and E2 in the CT group, during the

experimental period, implies increased steroid activity during and after the onset of sex differentiation. Earlier studies also observed a positive relationship between steroidogenesis and the stage of gonadal differentiation in Nile tilapia (Rothbard *et al.* 1987) and coho salmon (Feist *et al.* 1990). Generally, the steroidogenic activity is low before sex differentiation, and as germ cells proliferate during gonadal development, a corresponding increase in the steroids is exhibited (Kobayashi *et al.* 2008). In the present study, 11-KT increased in PP and MT-treated fish and E2 in the fish from the CT group as gonadal differentiation proceeded. Since sex differentiation involves an increase in steroid-producing cells (Nakamura and Nagahama 1989, Kobayashi *et al.* 2008), the cells could have been responsible for the displayed steroidal patterns in either experimental group. Notably, the rise in T among the treatments is attributed to the role of the steroid as a biosynthetic precursor to E2 and 11-KT (Kobayashi *et al.* 1988, Jensen *et al.* 2001, Kagawa *et al.* 2003, Wang and Orban 2007, Guiguen *et al.* 2010, Wang *et al.* 2017a). The level of endogenous T was regulated in a similar manner after exposure of rare minnow (*Gobiocypris rarus*) and Atlantic cod (*Gadus morhua*) to MT (Zhong *et al.* 2005, Kortner and Arukwe 2007). The lower T levels in the CT group compared to MT and P-treated fish were presumed to be a result of sex difference since females usually have lower quantities of steroids than male individuals (Papadaki *et al.* 2005).

Generally, the disruption of the estrogen and androgen levels by PP and MT was similar. The result is attributed to the presence of steroids, mainly T, in the PP powder (Abaho *et al.* 2022a), which, too, have been reported to induce masculinisation in Nile tilapia (Shi *et al.* 2017). However, the lower levels of endogenous androgens obtained in the PP-treated fish compared to the MT treatment could be attributed to the low steroid quantities in PP. In addition, since T is less stable than MT, the phytoandrogen may have been lost due to aromatisation and gut catabolism (Phelps and Popma 2000, Pawlowski *et al.* 2004, Gao *et al.* 2005, Attardi *et al.*

2008). Subsequently, the potency of PP is reduced, resulting in low masculinisation, as was observed in the PP-treated fish compared to the ones from the MT treatment.

4.5 Conclusion

The research showed suppression of E2 levels and stimulation of the production of 11-KT once Nile tilapia is treated with PP. The results were consistent with the histological events in the gonads. Therefore, exposure of all-female fish to PP induced female-to-male sex change by depleting E2 production and amplifying 11-KT concentration. The findings were also in line with the effect of PP on the expression of female and male sex-related gene genes, as described in Chapter 3. However, to gain further detail on how PP modifies the sex ratio in Nile tilapia, it is worth exploring whether aromatase activity is inhibited, subsequently reducing estrogen production. Additionally, assessing whether PP-induced masculinisation is via interactions with androgen receptors is needed in further studies.

CHAPTER 5

General discussion, conclusions, and future research avenues

5.1 General discussion

Using natural products of plant origin in tilapia culture is gaining momentum as an alternative to potentially harmful synthetic hormones (Chakraborty *et al.* 2014, Reverter *et al.* 2017, Elumalai *et al.* 2020). Plant extracts are considered safe, nature-based alternatives to hormones and are crucial to meeting the increasing demand for organic food products (Leet *et al.* 2011). In tilapia aquaculture, different studies have proposed several phytoextracts as alternatives to MT in producing all-male individuals to control unwanted spawning (Gabriel 2019, Abaho *et al.* 2022b). The present thesis investigated the potential of pine pollen (PP) to masculinise Nile tilapia (*Oreochromis niloticus*) and the mechanism underlying the female-to-male sex change during gonadal differentiation. This chapter discusses the contribution of the research, as well as proposals for future studies.

5.1.1 Pine pollen induces female-to-male sex change in Nile tilapia

Phytochemicals such as steroids and flavonoids are associated with the sex inversion of fish (Miyahara *et al.* 2003, Gabriel *et al.* 2015, Tarigan *et al.* 2016, Kapinga *et al.* 2019, Abaho *et al.* 2022). The phyto-compounds inhibit estrogen production, consequently promoting masculinisation (Golan *et al.* 2008, Low *et al.* 2013b, 2013a). In the present study, steroids, which are vital in gonadal differentiation, were confirmed present in PP. Moreover, PP altered the process of sex differentiation in Nile tilapia in favour of male sex development, producing up to 80 % male individuals. The findings confirmed sex differentiation as a labile process in the fish, susceptible to extrinsic factors (Kobayashi *et al.* 2008, Nivelle *et al.* 2019). Since sex-

differentiating genes regulate steroid production, the changes in sex gene expression and steroidal profiles were used to examine the role of PP in female-to-male sex change induction (Shi *et al.* 2017, Wang *et al.* 2018, 2022, Nagahama *et al.* 2021). Sex inversion involved increased expression of male-specific genes (*dmrt1* and *amh*) and masculinising 11- ketotestoterone (11-KT) steroid. In contrast, suppression of the female-related genes (*cyp19a1a* and *foxl2*) and the feminising 17β-estradiol (E2) steroid was observed. The findings made a first contribution to answering the overarching question of the role of PP in female-to-male sex change. The research demonstrated the potential of PP to control prolific reproduction in tilapia culture facilities. Since pine trees are globally distributed, the findings will benefit tilapia hatchery operators, especially in developing countries, where access to MT is limited and a lack of appropriate equipment for safe hormone application (Gabriel *et al.* 2017). With the fast-growing use of MT in Africa, access to the European and United States tilapia markets, where synthetic hormones are prohibited, will be limited (Penman and McAndrew 2000, European Commission 2003). Therefore, adopting organic tilapia production using PP provides an opportunity to enter the new competitive markets.

5.1.2 Pine pollen feeding stimulates the growth of Nile tilapia

Maximising fish growth with minimal production costs and producing a safe product acceptable to consumers is desired in aquaculture. Fast weight gain translates to increased productivity, reduced production time, more culture cycles annually, reduced operational costs, and increased return on investment (Allen and Steeby 2011). Therefore, a growth-promoting diet is ideal for the aquaculture industry. Incorporating various plant extracts in fish diets has been reported to enhance growth (Gültepe *et al.* 2014, Reverter *et al.* 2017, Aanyu *et al.* 2018, Gabriel 2019, Tadese *et al.* 2022). The phytoextracts contain growth-promoting compounds and are healthier than synthetic ones, hence more acceptable to the public (Aanyu *et al.* 2018).

In the present research, PP stimulated higher somatic growth of Nile tilapia (Chapter 2). Besides inducing the highest proportion of male individuals, the dietary inclusion of 1,280 mg PP kg^{-1} resulted in a higher specific growth rate than fish fed only basal and MT- treated diets. The growth-promotion attribute of PP is attributed to the presence of phytochemicals, such as alkaloids, flavonoids, and steroids (Chakraborty *et al.* 2013). These phyto-compounds improved feed intake and utilisation, appetite, stress relief and immunostimulation, subsequently promoting fish growth (Chakraborty and Hancz 2011, Chakraborty *et al.* 2014, van Hai 2015, Tadese *et al.* 2022). Also, the presence of stimulants in PP makes the diets more attractive to the fish, hence minimising wastage (Nian *et al.* 2017). Therefore, Nile tilapia fed on PP-supplemented diets is expected to reach the market size earlier, contributing to increased profitability of the enterprise. As such, fish feed manufacturers can utilise PP as a growth-promoting feed additive. However, extending the feeding period of fish to marketable size is vital in future research to confirm whether the growth-promoting benefits of PP are sustained at all growth stages.

5.2 Future research avenues

While the research yielded promising results, more studies are still required to comprehensively understand PP-induced masculinisation. The expression of a few sex changes: *cyp19a1a*, *foxl2*, *dmrt1,* and *amh*, was explored in the present thesis. Future studies should incorporate other genes involved in the sex differentiation process of fish, such as germline alpha (*figla*), gonadal soma-derived factor (*gsdf*), and sry-related HMG box 9 (*sox9*). Similarly, the effect of PP on various steroidogenic enzymes, such as steroidogenic acute regulatory protein (*star*), 3β-hydroxysteroid dehydrogenase (*hsd3b1*), cytochrome P450 family 17 subfamily A member 1 (*Cyp17a1*), and cytochrome P450, family 11, subfamily C, polypeptide 1 (*cyp11c1*), should be examined. The findings would provide more detailed information on the mechanism

underlying PP-induced sex masculinisation and guide to fine-tune the utilisation of the product, especially increasing the proportion of male individuals from 80 % to close to 100 %.

Although PP attenuated the level of E2 in Nile tilapia, whether the observed effect is associated with changes in aromatase activity remains to be answered in future research. Aromatase converts androgens to estrogens, and the enzyme's activity is directly proportional to estrogen concentration, hence instrumental in ovarian differentiation (Guiguen *et al.* 2010). The down- regulation of *cyp19a1a* results in the inhibition of the aromatase enzyme followed by reduced estrogen levels, thereby eliciting female-to-male sex change after exogenous steroidal treatment (Li *et al.* 2006, Paul-Prasanth *et al.* 2013, Sun *et al.* 2014, Teng *et al.* 2020a). The bioactive compounds from plant extracts such as saponins (Golan *et al.* 2008) and quassinoids (Low *et al.* 2013a) were reported to inhibit the aromatase enzyme and thereafter stimulate spermatogenesis. Therefore, evaluating the aromatase inhibition potency of PP to correlate the changes in E2 with the enzyme's activity and *cyp19a1a* is worthwhile. The results would provide more information towards understanding the mechanism underlying PP-induced sex change.

The androgen receptors in gonadal tissues act directly on the pathway for inducing sex inversion in fish by exogenous androgens. For instance, synthetic MT directly interacts with androgen receptors to elicit masculinisation in Nile tilapia (Golan and Levavi-Sivan 2014). No such studies have been conducted to examine the effect of PP on androgen receptors. In the present thesis, the bioactive compounds in PP were hypothesised to interact with the androgen receptors to enhance the expression of male sex genes and subsequently induce

masculinisation. Future studies should, however, investigate androgen receptor agonism and changes in sex steroids in Nile tilapia after exposure to PP.

Despite low steroid levels in PP, considerable female-to-male sex change was obtained. Since the phytochemical analysis of PP showed the presence of flavonoids and alkaloids, in addition to steroids, the observed masculinisation could have resulted from the action of the multiple bioactive compounds in the extract. Synergistic and additive growth-promotion effects have been reported in fish fed different phyto-compounds (Aanyu *et al.* 2018, İnanan and Acar 2019, 2021, Raissy *et al.* 2022). Such a scenario has, however, not been previously reported in the sex inversion of fish. Moreover, flavonoids and alkaloids have also been implicated in the sex inversion of fish (Miyahara *et al.* 2003, Tarigan *et al.* 2016). Therefore, further research is required to assess the contribution of the other phytochemicals in PP to the masculinisation of female fish. The results would guide the use of appropriate extraction protocols that might increase the bioavailability of specific compounds.

Generally, sex change to either female or male individuals in teleosts is very effective during the labile period before sex differentiation has occurred. Hence, successful sex change is considered unattainable after gonadal differentiation. However, in some fish species, sexual plasticity is maintained after the sensitive period of sex differentiation. For example, degeneration of ovaries, accompanied by the appearance of testes, was observed in mature female zebrafish (*Danio rerio*) after exposure to an aromatase inhibitor (Takatsu *et al.* 2013). Likewise, retention of sexual plasticity beyond the critical period for gonadal differentiation was observed in Nile tilapia (Paul-Prasanth *et al.* 2013, Sun *et al.* 2014, Shi *et al.* 2017, Li *et al.* 2019). Therefore, female-to-male sex change occurs from an undifferentiated and

differentiated gonad, referred to as primary sex inversion and secondary sex reversal, respectively (Kwon 2000, Uchida *et al.* 2004, Iwamatsu *et al.* 2006, Komatsu *et al.* 2006, Paul- Prasanth *et al.* 2013). With reports of secondary sex reversal, future studies should extend the exposure period of Nile tilapia to PP beyond the labile period of 30 days post-hatch (dph) to assess whether masculinisation increases. Moreover, higher masculinisation levels were obtained when the same fish was fed PP for 56 days (Nian *et al.* 2017). During the extended exposure period of Nile tilapia to PP, genetic XY progeny should be included to provide more information on the extent of up-regulation of male sex genes in the sex-inversed XX female fish.

In the present research, the masculinisation rate of PP was low compared to previous studies. Better results from earlier studies could have resulted from higher phytochemical levels, although the phytochemical composition of the used PP was not reported. The concentration of active ingredients varies based on the plant species and growth stage (Akula and Ravishankar 2011), seasons and environmental parameters (Isah 2019), and geographical origin (Dinchev *et al.* 2008). Since pine trees are grown globally (Keeley 2012), pollen from different sources should be characterised and screened to recommend one with the highest quantity of steroids. Also, optimising the processing method, storage duration, and conditions is crucial to prevent or minimise the loss of the target bioactive compounds (Applegate *et al.* 2010, Yang *et al.* 2015).

In addition to sex masculinisation, PP enhanced the growth performance of experimental fish, which is attributed to the presence of growth-promoting compounds. Besides, the antioxidants and polyphenolics in PP are reported to increase immunity and stress resistance in fish

(Baldove *et al.* 2019). However, further investigation is required to quantify the immunostimulant compounds in PP. Further examining the effects of the extract on haematological and biochemical indices as well as the genes associated with growth and immunity, is vital. The generated information will guide the recommendation of the PP dose with no undesirable effects on the physiological processes of the fish.

Finally, hormonal leakages from tilapia hatcheries can interfere with the reproductive and endocrine functions of the non-target organisms in the aquatic ecosystems (Ramirez-Godinez *et al.* 2013, Rivero-Wendt *et al.* 2013, Thanasupsin *et al.* 2021). Therefore, the occurrence and fate of PP steroids in water released from the hatcheries to the surrounding aquatic ecosystems should be determined before the extracts are recommended for large-scale hatchery application.

5.3 Conclusion

The research confirmed PP as a masculinisation and growth-promoting plant product in the production of Nile tilapia. For the first time, information on the molecular mechanism, endocrine regulation, and gonadal histological dynamics involved in PP-induced sex inversion was documented. Female-to-male sex change followed an up-regulation of male sex genes with a corresponding increase in male sex steroids. Therefore, PP is a potentially safe and environmentally sustainable alternative to the harmful synthetic hormones in producing all-male Nile tilapia for controlling unwanted spawning in the production systems. Adopting PP in the aquaculture industry will play a crucial role in meeting the global demand for safe organic fish food products and subsequently contribute to sustainable aquaculture production.

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